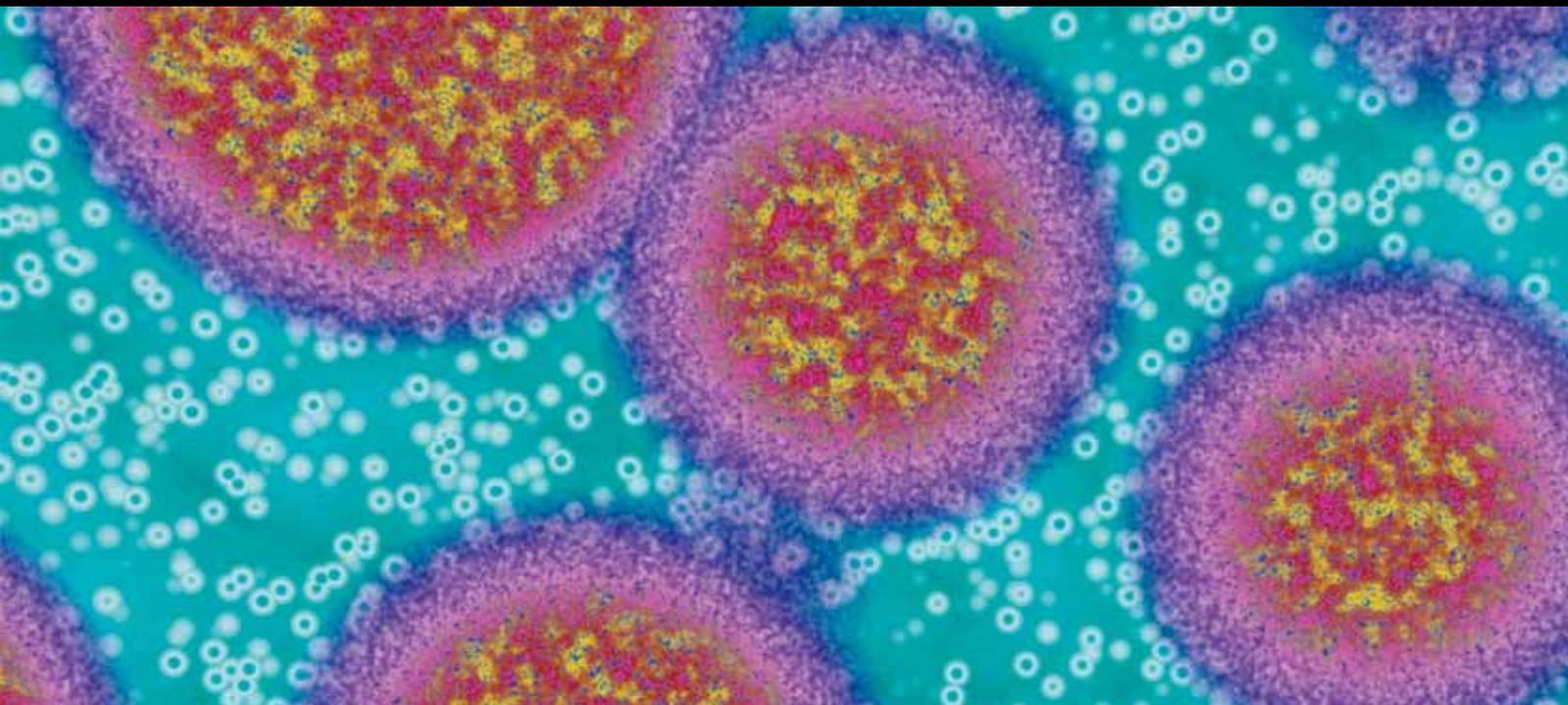


# REDOX STATUS AND BIOENERGETICS LIAISON IN CANCER AND NEURODEGENERATION

GUEST EDITORS: GIUSEPPE FILOMENI, JUAN P. BOLAÑOS,  
AND PIER GIORGIO MASTROBERARDINO





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Guest Editors: Giuseppe Filomeni, Juan P. Bolaños,  
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## Editorial

# Redox Status and Bioenergetics Liaison in Cancer and Neurodegeneration

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During the last decades, the involvement of redox reactions in each aspect of cellular physiology has emerged, rapidly steadied, and is currently assuming extensive connotations. From a mere pathological condition leading to widespread biomolecules damage and cell degeneration, (over)production of reactive oxygen and nitrogen species (ROS and RNS, resp.) is now also deemed to be among the early upstream events of signal transduction pathways governing cellular response. It is now well established that the thiol moiety of reactive cysteines is the main molecular switch selected by the evolution to transduce a redox signal. Even at physiological pH, the sulfhydryl group of these residues is present under thiolate form and primed to be modified by ROS and RNS in a reversible manner. S-nitrosylation, S-hydroxylation, and S-glutathionylation are, indeed, all oxidations that meet the conditions of specificity and reversibility required for a chemical modification (signal) being transduced in a biological event (response).

In this special issue, the double-faced role of nitroxidative stress (deleterious, or signal mediator) has been carefully considered, mostly in regards to the implications that it could have in neurodegeneration. The paper by R. P. Guttman and T. J. Powell underlines this aspect elucidating, in the first part, the biochemical basis of the high susceptibility of brain towards redox stress and providing, in the second one, a very clear overview of the possible sources, mechanisms, and targets of oxidative stress, namely cysteine-containing enzymes, which can be implicated in the onset of neurodegenerative diseases. Although it is still unclear

whether oxidative stress is the primary initiating event or a secondary effect related to other pathological conditions, its role in neurodegenerative diseases is confirmed by the evidence that the brain is particularly prone to ROS and RNS production and susceptible to their harmful effects. Its high metabolic rate and content of oxidizable molecules, (e.g., neurotransmitters), as well as the high concentration of redox-active metals (e.g., iron and copper) and the limiting levels of antioxidants, mainly glutathione (GSH), are features concurring to exacerbate oxidative challenges in the central nervous system (CNS). These aspects have been fully covered in a series of papers included in this special issue. In particular, R. B. Mounsey and P. Teismann dissect out in depth the contribution of iron accumulation in Parkinson's disease (PD) and debate the promising ongoing pharmacological approaches aimed at reversing this phenomenon, including the use of both synthetic and natural iron chelators. C. Consales et al. deal with the emerging lines of evidence linking electromagnetic fields (EMF) and neurodegenerative diseases onset. They discuss about the still not reconciled results reporting both neuroprotective and pro-oxidant role of EMF and critically assess methodological limitations that could affect pathophysiological relevance of any alteration found in EMF-exposed biological systems. The paper by W. Li et al. complements the information on the role of environmental factors in exacerbating pro-oxidant conditions in neurodegeneration, by providing a very interesting overview of the role of GSH in brain homeostasis and protection. Remarkably, besides the role of GSH in driving

cell signaling and redox state maintenance, the authors shed new light on its involvement in the control of neurovascular function. GSH-mediated protection against endothelial cell injury and promotion of postdamage cell proliferation in endothelial repair are discussed on the basis of the very recent lines of evidence reporting the involvement of GSH-dependent redox modification (e.g., S-glutathionylation) in cerebral microvascular biology and pathobiology, as well as in oxidative stress-associated neurovascular disorders, such as stroke and diabetes. The paper by L. Rossi et al. concludes this part of the special issue revisiting the current opinion regarding the role of oxidative stress in motor neuron diseases (MNDs). Interestingly the authors provide convincing evidence that increased oxidative stress, mitochondrial damage, and altered gene expression (due to epigenetic defects and RNA dysmetabolism) are common features of different MDNs that could underlie MDNs pathogenesis.

Amongst the environmental factors contributing to neurodegenerative diseases, chemicals and drugs affecting the mitochondrial electron transport chain (e.g., paraquat and rotenone) are frequently reported to induce apoptotic cell death. However, endogenous molecules have also been demonstrated to be detrimental if produced in excess, such as glutamate or certain cytokines. Recently, the macrophage migration inhibitory factor (MIF), a macrophage-produced cytokine, has been reported to be involved in disease pathogenesis. In this special issue, N. E. Savaskan provide an overview of our current knowledge about MIF action, especially regarding its potential role in brain disease and redox regulation in apoptosis. Neuronal apoptosis is an important mechanism contributing to the selective loss of cell populations in specific regions of the CNS. It can take place *via* the mitochondrial pathway, the main step of which is the release of the mitochondrial respiration mediator cytochrome *c* within the cytosol. On this topic, L. Berghella and E. Ferraro provide an original research paper dissecting out in depth the sequence of events of neuronal apoptosis. They provide evidence that cytosolic cytochrome *c* has no role in chromatin condensation and that it is not a primary cause of mitochondrial respiration impairment, which occurs long before its release. These results are in agreement with the current knowledge suggesting that the effects of apoptotic-associated dysfunction on mitochondrial respiration are not mere epiphenomena, but are rather crucial for proper execution of the apoptotic program. In particular, the massive production of ROS and the generation of pro-oxidant state favoring the activation of downstream apoptotic factors are gaining increasing importance. Indeed, mitochondria represent an important endogenous source of ROS that are physiologically generated at the mitochondrial electron transport chain. The sustained and long-lasting production of ROS is the principal cause of nitroxidative damage to biomolecules. It can produce DNA damage and, in the long term, it can give rise to established somatic mutations. These effects can contribute to neoplastic transformation and are detrimental to neuronal viability. In this special issue, D. De Zio et al. deeply address this topic, providing a detailed overview regarding the occurrence of oxidative DNA damage in neurons and how it is specifically counteracted by the

activity of DNA repair systems. The authors discuss how the unbalance between these processes results in neurodevelopment disorders and neurodegenerative diseases and focus on the role of the nuclear factor Ku70/80 as pivotal mediator of neuronal viability. Likewise, S. Gonfloni et al. discuss the role of the c-Abl tyrosine kinase, as its increased activation has been reported in several neurological disorders. They provide a paper in which they propose that c-Abl signaling contributes to modulate molecular events at the interface between oxidative stress, metabolism, and DNA damage, suggesting possible therapeutic strategies targeting c-Abl for the treatment of neurodegenerative diseases.

Besides being an important site for ROS production, the mitochondrial electron transport chain generates the proton motive force that is used for ATP synthesis by the  $F_0/F_1$  ATP synthase. The mitochondrial transmembrane potential ( $\Delta\Psi_m$ ) is a measure of the proton electrochemical gradient generated across the mitochondrial inner membrane and represents a good indicator of the energy status of the mitochondrion. Under certain pathophysiological conditions, such as hypoxia-ischemia,  $\Delta\Psi_m$  can be transiently preserved at the expense of ATP. This is brought about by the reverse reaction of  $F_0/F_1$  ATPase, which can hydrolyze glycolytically generated ATP to pump protons into the intermembrane space, thus maintaining the  $\Delta\Psi_m$ . In this special issue, D. Facenda and M. Campanella provide an up-to-date overview of mitochondrial energetics and the molecular characterization of  $F_0/F_1$  ATPase, highlighting its function and the regulatory events that trigger either the ATP-synthesizing or the ATP-hydrolyzing activities. The authors focus on the role of the inhibitory factor 1 (IF<sub>1</sub>), a polypeptide that responds to mitochondrial intermembrane space acidification by inhibiting the ATP-hydrolyzing activity of the  $F_0/F_1$  ATPase; this occurs by stabilizing its dimerization status *via* a molecular link between two  $F_1$  domains. This molecular rearrangement remodels the mitochondrial cristae structure and, consequently, regulates organelle morphology, thus involving IF<sub>1</sub> in neurodegeneration and cancer. Given that mitochondrial healthy state strongly impacts on the generation of intracellular nitroxidative stress conditions, chronic alterations of mitochondrial homeostasis are among the events concurring to the onset of several pathological states, namely cancer and neurodegenerative diseases. Nevertheless, it should be also reminded that mitochondria have evolved specific molecular quality control mechanisms to counteract the damaging effects of nitroxidative stress. In this special issue E. Desideri and L. M. Martins provide an overview of the current knowledge of mitochondrial quality control and discuss the pivotal role that PTEN-induced putative kinase 1 (PINK1) and the protease high-temperature-regulated A2 (HTRA2) might play in this context. In particular, they report evidence and speculate about the possibility that defects in their activity might contribute to PD onset.

From a metabolic perspective, neurons and cancer cells exhibit sharp differences. Neurons strictly depend on efficient OXPHOS for ATP production; here, mitochondrial respiration does not depend directly on glucose availability and is rather fueled by pyruvate derived from glia-provided lactate in a Cori's cycle-like manner. Glucose taken up

by neurons is instead mainly redirected to the pentose phosphate pathway, allowing the generation of NADPH to sustain the indispensable sulfhydryl reductive pathways and antioxidant response. By contrast, tumor cells synthesize ATP almost entirely by means of glycolysis even in normoxic conditions, when oxygen availability is not restricted (the “Warburg effect”). This condition represents a major change of the entire metabolic reprogramming typical of tumors and allows cancer cells to grow even under low oxygen tension, when tumor vascularization is still incomplete, and therefore, local vessels fail to supply adequate amount of oxygen. Upregulation of the glycolytic pathway is therefore a selective advantage to sustain the ATP demand required for tumor proliferation under hypoxic conditions. M. Fernandes de Oliveira et al. comprehensively review the mitochondrial biochemical pathways underlying bioenergetics, ATP production, and ROS generation in cancer. The authors also discuss the connection between mitochondrial dynamics and function, and how this is related to apoptotic cell response. In detail, they give emphasis to the role of ROS and RNS in the activation of redox-sensitive transcription factors and protein kinases in tumor cells, and how they can be manipulated to selectively kill them. Along this line, M. Soga et al. discuss recent data underlying the tight liaison between redox state alteration and mitogen-activated protein kinases (MAPKs) activation, namely the apoptosis signal-regulating kinase 1 (ASK1). Being the Ichijo’s laboratory among the pioneers to have characterized the role of thioredoxin (Trx) as the regulatory partner of ASK1, in this paper, the authors revisit the main steps of this regulation and provide the new lines of evidence arguing for the involvement of ASK1 in several pathological states.

As previously mentioned, metabolic reprogramming of tumor cells synergizes with nitroxidative stress to maintain and develop tumor aggressiveness. T. Fiaschi and P. Chiarugi deepen this concept in their paper and extend it to the entire tumor microenvironment that, together with stromal cells, enhances pro-oxidant conditions and concurs to the establishment of a more aggressive and chemoinensitive neoplastic phenotype. To corroborate the intrinsic connection between metabolism and tumorigenesis, S. Cardaci and M. R. Ciriolo discuss the causative role of mutations in three enzymes belonging to the tricarboxylic acid (TCA) cycle, succinate dehydrogenase (SDH), fumarate hydratase (FH), and isocitrate dehydrogenase (IDH). Together with K. Smolková and P. Ježek, who reviewed in detail the oncogenic potential of the mitochondrial isoform of IDH, these authors highlight how point mutations of these enzymes generate an hypoxia-like phenotype, affect cellular redox state, and interfere with DNA methylation dynamics, thereby revealing the main tumor-promoting effects of TCA cycle alterations. To complete this section, N. Berndt et al. develop a kinetic model aimed at predicting and quantifying mitochondrial OXPHOS efficiency and ROS production induced by the reduced activity of  $\alpha$ -ketoglutarate dehydrogenase (KGDH). This condition occurs in several neurodegenerative diseases, such as PD and Alzheimer’s disease (AD), and the results shown in this paper give strength to the hypothesis that

decreased KGDH activity plays a pivotal role in the energetic failure of neuronal cells during neurodevelopment and neurodegeneration. KGDH is a multienzymatic complex responsible for the conversion of  $\alpha$ -ketoglutarate into succinyl-CoA, a critical step in the TCA cycle. The TCA cycle is a central hub in metabolism; its functions are not restricted to energy conservation and involve afferent as well as efferent anabolic pathways.  $\alpha$ -ketoglutarate is an important intermediate for afferent processes because it is the catabolic product of several amino acids and is also generated by deamination or transamination of glutamate.  $\alpha$ -ketoglutarate is therefore essential for energy production from amino acid substrates. On the other hand, efferent pathways generate new amino acids from the sugars entering the TCA cycle, which are indispensable for cancer cells to synthesize new proteins and produce biomass. The levels of amino acids in the extracellular milieu are sensed by the multimolecular mTOR complex 1 (mTORC1) and transduced *via* well-known signaling axes that lead to normal cell growth or autophagy. Here, mTOR serves as master metabolic switch; when mTOR is active, it promotes anabolic metabolism repressing autophagy, and when it is inactive (e.g., during nutrient starvation) or inhibited (e.g., by rapamycin), cells switch to a catabolic mode. In the latter case, autophagy is activated to recycle proteins, whereas organelles and protein synthesis are repressed. Although autophagy is a homeostatic process for normal cells, it is also exploited by cancer cells to grow and survive under restrictive conditions such as nutrient deprivation, which occurs in the core of solid tumors. The paper by V. Banerji and S. B. Gibson deals with the emerging relationship between metabolic changes of tumor cells and autophagy, and how it contributes to increase cancer progression and chemoresistance. It has been reported that this feature depends on the increased expression of NF-E2-related factor-2 (Nrf2), a transcription factor that induces detoxifying and antioxidant systems, such as molecular chaperones and GSH-regenerating enzymes. Although Nrf2 activity is necessary for cell protection against nitroxidative stress, in several tumor histotypes, its basal activation is overinduced. Several somatic mutations have been demonstrated to destroy the interaction between Nrf2 and its physiological inhibitor, Kelch-like ECH-associated protein 1 (Keap1), thereby promoting the persistent activation of the Nrf2-mediated response and tumorigenesis. The paper by R. Brigelius-Flohé et al. discusses the dichotomous role of Nrf2 and highlights how Nrf2 activation results in the expression of several selenium-containing antioxidant enzymes (e.g., Trx reductase and GSH peroxidase) that contribute to enhance the deleterious aspects of Nrf2 in tumor progression.

Although mitochondria are the main intracellular producers of nitroxidative stress, it should be considered that they are also the primary targets of oxidative damage, a concept that has inspired the mitochondrial theory of aging. This aspect has been also taken into account in this special issue and deepened in virtue of its possible involvement in cancer and neurodegeneration. In this context, the paper of P. B. L. Pun and M. P. Murphy provides a very interesting overview on the novel role of intracellular glycation

reactions as oxidative modifications specifically affecting the mitochondrial compartment, and how these can contribute to disease pathology. P. Sarti et al. discuss about the double (cytoprotective or detrimental) role of nitric oxide (NO) within mitochondria and, in particular, they describe the inhibitory effect of modifications of cytochrome *c* oxidase at different sites. The authors report a very detailed biochemical dissection of the different NO-mediated modifications of the mitochondrial complex IV and elucidate the environmental conditions in which they can occur, alongside the pathophysiological effects they can produce. This aspect is also addressed by R. M. Santos et al., who debate about the *in vivo* implications of NO signaling, focusing on the molecular mechanisms affecting bioenergetics and neurological disorders. Among these mechanisms, S-nitrosylation regulates protein function and modulates cellular homeostasis. In this field, Lipton's laboratory has greatly contributed to our better understanding of the implication of S-nitrosylation in cellular physiology. Thus, in this special issue, M. W. Akhtar provide a straightforward overview of proteins found to undergo S-nitrosylation that are directly involved in PD and AD pathogenesis. This paper is complemented by that of G. Di Giacomo, who specifically covered the partially neglected role of denitrosylation processes in NO-mediated signaling pathways, mainly those catalyzed by S-nitrosoglutathione reductase (GSNOR). They report the most recent lines of evidence arguing for this enzyme being implicated in pathophysiology and discuss how it can impact on mitochondrial function, dynamics, and selective removal by autophagy (the so-called mitophagy). Mitochondria, indeed, are highly dynamic organelles; they can fuse to form an interconnected network or undergo fission to form fragmented units that can in turn fuse again. Alternatively, fragmented mitochondria can be engulfed in autophagosomal membranes for removal. These processes take place in response to different stimuli impinging on the fusion/fission machinery, mediated by a series of mitochondria-shaping proteins, such as the large GTPase family members mitofusins (Mfns) and optic atrophy 1 protein (Opa1), which underlie fusion events, and dynamin-related protein 1 (Drp1) required for mitochondrial fragmentation. The paper by M. Corrado et al. provides a detailed description of the molecular processes underlying mitochondrial dynamics. They also define how alterations in its correct occurrence can affect cellular viability and lead to pathological states, for example, cancer, neurodegeneration, and neuroinflammatory diseases.

In addition to the continuous change of size, shape and removal by mitophagy, mitochondria are also neosynthesized. This process—mitochondrial biogenesis—is fundamental to ensure the generation of new mitochondria to maintain a constant mass of the organelle within the cell. It has been recently proposed that mitochondrial biogenesis is subjected to redox regulation as well, and this leads to hypothesize a general regulatory mechanism in which the appropriate magnitude of mitochondrial biogenesis is finely tuned by the very same early mediators of mitophagy, namely ROS and RNS. This aspect is also addressed in this special issue by the paper of E. D. Yoboue and A. Devin, who dissect out in depth the redox responsive transcription

factors underlying mitochondrial biogenesis, such as the peroxisome proliferator-activated receptor gamma coactivator 1- $\alpha$  (PGC1- $\alpha$ ). D. Lettieri Barbato et al. deepen this concept and extend the “redox model” of mitochondrial biogenesis towards a general responsiveness to further conditions affecting redox state, including caloric restriction. The authors suggest that oxidative stress and caloric restriction share the same signaling pathways that converge on PGC1- $\alpha$  to promote the expression of mitochondrial genes and speculate how these factors can be manipulated by means of nutritional intervention to prevent neurodegeneration.

That mitochondria are not only the “power station” of the cells is now a well-established notion, which is progressively enriched by novel insights of previously unsuspected functions and possible interplays with other cellular compartments. Overall, these elements strengthen the concept that mitochondria contribute in several aspects to the progression of pathologies. One of the most recent structural connections to be functionally characterized is the one occurring between mitochondria and endoplasmic reticulum (ER). It takes place through specific ER structures, the so-called mitochondrial-associated membrane (MAM), whose finding drove research to unravel the molecular mechanisms underlying the cellular processes bridging the two organelles. In particular, in the last years, it is becoming clear how mitochondria and ER participate to lipid-mediated signaling, calcium homeostasis, and apoptosis. In regards to this, the research paper by S. Perez-Alvarez et al. identifies in the perturbation of calcium homeostasis, the mechanism of methadone-induced neuronal cell death, which underlies its negative impact on human cognition. In particular, the authors provide evidence that methadone induces delayed calcium deregulation (DCD) by compromising the mitochondrial network and its ability to uptake calcium in a respiratory-dependent way. These results strengthen the functional association between mitochondria and ER in cell death pathways but also provide the proof of principle that additional processes (e.g., metabolism, respiration) could be regulated in concert with the two compartments. Along this line, MAM has been indicated to contain chaperons (e.g., Grp78) and proteins directly involved in oxidative protein folding, such as Ero1- $\alpha$  and protein disulfide isomerase (PDI). This is significant in the light of the fact that defects in protein folding induce the unfolded protein response (UPR), which can ultimately lead to the intrinsic (mitochondrial-mediated) pathway of apoptosis. It has been also demonstrated that, *via* MAM, Ero1- $\alpha$  competes with the mitochondrial electron transport chain for oxygen availability and represents the main hydrogen peroxide producers within ER. Besides being crucial for protein folding, the high levels of pro-oxidant molecules within the ER are also required to reversibly regulate the function of proteins residing in this compartment. Indeed, redox regulation of ER-contained proteins and enzymes has been exhaustively reported. In this special issue, we have addressed this aspect and attempted to enlighten the involvement of the tight relationship among mitochondria, oxidative stress, and ER in disease onset. To this aim, we provide a series of papers aimed at deepening the current knowledge and reporting novel findings that

shed new light on this topic. Results reported by Y. Xiong et al. show that treatment of breast cancer cells with the NO donor PABA/NO induces S-glutathionylation of PDI, impairs its chaperone-like activity, and induces apoptosis *via* the activation of UPR. In particular, the authors provide evidence that the activation of cell death is associated with inability of the S-glutathionylated PDI to bind to the estrogen receptor  $\alpha$  (ER $\alpha$ ) with consequent inhibition of the expression of genes involved in cell proliferation. Nevertheless, if the impairment of PDI activity and the aberrant activation of UPR could be exploited for cancer cell killing, it has been copiously reported to be detrimental for neuronal viability. Indeed, the accumulation of unfolded protein and excessive UPR are among the causes triggering neuronal cell death in a large number of neurodegenerative diseases. In this context, E. Ferreiro et al. elucidate the association between pathological changes found in AD brain (e.g., protein inclusion) and mitochondrial dysfunction, and how oxidative stress resulting from this condition is intimately linked to ER-derived ROS production and UPR. The paper by F. Di Sano and M. Piacentini adds new insights to this aspect by focusing on the role of reticulons family, and of RTN-1C in particular, in different neuron pathologies. RTN-1C is localized on ER membranes and regulates ER structure and function; in their paper, the authors provide strong lines of evidence that this protein might be a promising target in the treatment of different pathologies.

In conclusion, the field of redox biology has grown beyond all expectations and now interests virtually every biological process. Perturbation in redox balance and signaling has indisputable relevance in the pathobiology of cancer and neurodegenerative diseases, and restoration of homeostasis constitutes an appealing therapeutic strategy. This special issue recapitulates the state of the art of redox biology in relation to these disorders and highlights the most critical priorities to achieve better understanding of pathogenesis and develop successful therapies. We are therefore confident that this collection will be highly informative for those in the field as well as for the broad scientific community.

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## Review Article

# Electromagnetic Fields, Oxidative Stress, and Neurodegeneration

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Electromagnetic fields (EMFs) originating both from both natural and manmade sources permeate our environment. As people are continuously exposed to EMFs in everyday life, it is a matter of great debate whether they can be harmful to human health. On the basis of two decades of epidemiological studies, an increased risk for childhood leukemia associated with Extremely Low Frequency fields has been consistently assessed, inducing the International Agency for Research on Cancer to insert them in the 2B section of carcinogens in 2001. EMFs interaction with biological systems may cause oxidative stress under certain circumstances. Since free radicals are essential for brain physiological processes and pathological degeneration, research focusing on the possible influence of the EMFs-driven oxidative stress is still in progress, especially in the light of recent studies suggesting that EMFs may contribute to the etiology of neurodegenerative disorders. This review synthesizes the emerging evidences about this topic, highlighting the wide data uncertainty that still characterizes the EMFs effect on oxidative stress modulation, as both pro-oxidant and neuroprotective effects have been documented. Care should be taken to avoid methodological limitations and to determine the patho-physiological relevance of any alteration found in EMFs-exposed biological system.

## 1. Introduction

Over the past several decades people have been constantly exposed to electric (E) and magnetic (H) fields from both industrial and domestic uses. The EMFs are produced not only for technological applications (e.g., power lines mobile phones), but they are now widely used also in medicine for diagnostic (e.g., magnetic resonance imaging (MRI) scanner and microwave imaging) and therapeutic purposes (e.g., radiofrequency and microwave ablation and hyperthermia) [1, 2].

The increased social and public interest in this subject, based on the epidemiological data associating the extra risk of amyotrophic lateral sclerosis (ALS), childhood leukemia, adult brain cancer, and miscarriage with the EMFs exposure of the power line radiation [3–9], prompted the World Health Organization (WHO) Report (2007) and WHO Environmental Health Criteria (EHC) Report (2007) to issue precautions against the ELF-EMFs [10, 11].

*1.1. EMFs Spectrum and Physical Interaction Quantities.* The EMFs coupling with biological systems depends on the

frequency range of the employed signals, as well as on their characteristics as amplitude, modulation, waveform and polarization [12]. Mainly three categories of EMFs signals can be identified. They are classified as static, electric and magnetic fields (as direct current, DC, 0 Hz), Extremely Low Frequency fields (ELF, between 1 Hz up to 100 kHz) and high frequency (HF) fields, in the band of the Radio Frequency fields (RF, 100 kHz–3 GHz), and of the microwaves (MW, above 3 GHz) [13, 14]. These radiations (with frequencies below 300 GHz) are all nonionizing ones (Figure 1).

The established regulations against health hazards [13, 14] are based on two key mechanisms of interaction with biological systems, one elicited by DC and ELF sources, and the other by RF and MW exposures. For DC and ELF exposures, the induced E-field (V/m) and current density (J, A/m<sup>2</sup>) are the main physical quantities to describe the EMF interaction. They can be generated by both external applied E-fields and variable H-fields, and their amplitudes have to be limited in order to avoid hazardous health effects (e.g., magnetophosphenes induction, cardiac fibrillation, muscle and nerve contraction, and fulguration) [12]. When RF and MW exposures are taken into account, the main

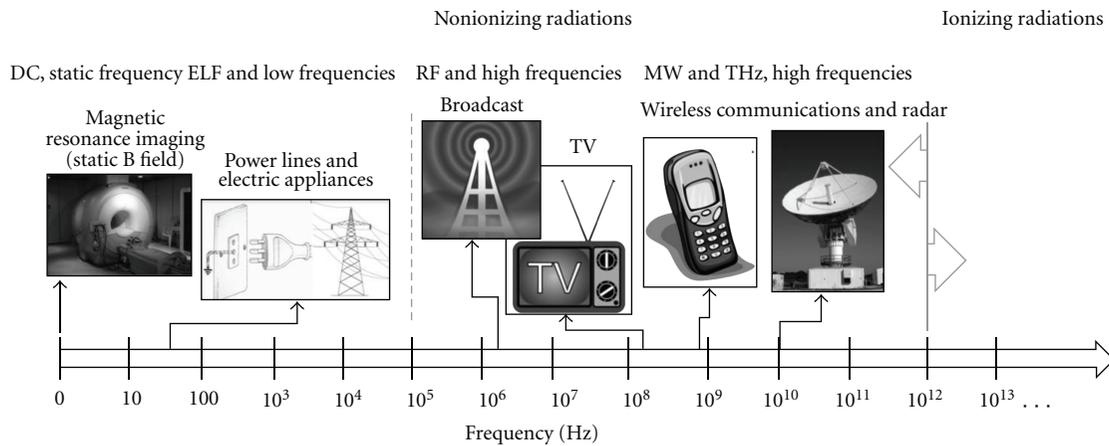


FIGURE 1: The whole electromagnetic spectrum, with partition between nonionizing and ionizing radiations, is reported. Main filed sources at the different frequencies are also sketched.

mechanism to be considered is the rise in temperature, as no charges movements are triggered at these frequencies. The heat effect is strictly dependent on both the water content of the biological target, the frequency, and intensity of the electromagnetic (EM) radiation. Therefore, for RF and MW exposure, the characteristic interaction quantity is the Specific Absorption Rate (SAR) [12], defined as the power (W) deposited by an EM radiation in a unitary mass (g) of the biological target, in a fixed time period(s), and it is measured in  $\text{Wkg}^{-1}$ .

**1.2. Interaction of the EMFs with the Biological Systems.** As EMFs are nonionizing, the search for conventional genotoxic mechanisms, as potentially responsible events underlying the interaction with the biological systems, have shown contradictory results. A convincing molecular mechanism, disclosing the link between human diseases and exposure to electromagnetic fields, is still lacking, although change in cell cycle, induction of cell death, modification of protein expression, and mainly oxidative stress have been proposed [15–18].

Metabolic processes which generate oxidants and antioxidants can be influenced by environmental factors, such as EMFs [19]. Increased EMFs exposure can modify the cellular balance by generating reactive oxygen species (ROS) [20–24]. Physical processes at atomic level are indeed the basis of reactions between biomolecules and EMFs, as the field can magnetically affect chemical bonds between adjacent atoms and alter the energy levels and spin orientation of electrons. Overproduction of ROS can damage cellular components, mainly lipids in membranes and nucleic acids. Moreover, ROS can harm cells by depleting enzymatic and/or nonenzymatic antioxidants triggering progressive dysfunction and eventually genotoxic events [25–27].

This redox-related mechanism has been mainly documented for the ELF-EMFs. Scaiano et al. [23] first proposed that ELF-EMFs exposure can stabilize free radicals in such a way as to increase their lifetime and permit a wider dispersion rather than their return to the basal level. This

might contribute to an increase in the activity and concentration of the free radicals, as also reported in the immune system, mainly mouse macrophages, human monocytes, and rat neutrophils [28–31]. Simkó et al. [31] in particular, demonstrated an increased phagocytic activity and an enhanced super oxide production in mouse macrophages after ELF exposure, in a dose-dependent manner. Besides, the inhibitory potential of chronic ELF-EMFs exposure on the availability of the pineal gland hormone melatonin, which physiologically acts as a radical scavenger, has been suggested as an additional pathway in the oxidative stress-driven interaction of ELF with the biological systems [32, 33]. ELF-EMFs might be therefore a stimulus to induce an “activated state” of the cells, such as in the phagocytic activity, which enhances the release of free radicals, and can eventually turn into a genotoxic event following chronic exposure. The suppression of the ELF-enhanced cell proliferation in the presence of radicals scavengers, as shown by Katsir and Parola in chick embryo fibroblasts [34], represents an another supportive finding for this proposed model of interaction between EMFs and biological systems via ROS generation.

The biological response induced by HF-EMFs, mainly RF exposure, may be instead explained by two distinct interaction mechanisms: thermal effects (that rely on the ability of RF fields to transfer their energy to biological matter, leading to an increase in average temperature through the vibration of atoms and molecules) and nonthermal effects [35, 36]. The latter only have been correlated to the generation of oxidative stress.

Nonthermal effects range from alterations in the permeability of the blood-brain barrier, to changes in encephalogram and blood pressure, although the matter is still controversial [37, 38]. The greatest mystery about these nonthermal effects is their lack of a theoretical basis, and, from an experimental point of view, a major problem in their definition is how to distinguish them from direct and indirect thermal effects. Oxidative stress has been proposed as the underlying mechanism responsible for this kind of RF effects, although the results are still controversial.

In this context, it has been proposed that RF-EMFs (875 MHz, 0.07 mW/cm<sup>2</sup>) generate extracellular ROS by stimulating cell membrane nicotinamide adenine dinucleotide (NADH) oxidase in Rat1 and HeLa cells *in vitro* [15]. ROS then activate metalloproteases on the outer surface of the cell, which cleave membrane-anchored progrowth factors and trigger the activation of p38 as well as the ERK (extracellular-signal-regulated kinase) mitogen-activated protein kinases (MAPKs) [15]. An enhanced production of ROS after combined exposure to RF radiation (930 MHz, SAR 1.5 W/kg<sup>-1</sup>) and iron ions was also reported in an experimental model of rat lymphocytes [39], and induced lipid peroxidation, accompanied by decreased activity of superoxide dismutase (SOD), myeloperoxidase (MPO) and glutathione peroxidase (GSH-Px) by RF exposure has been reported in various organs, such as rat kidney and guinea pigs liver [18, 40]. Moreover, in the latter animal model, treatment with epigallocatechin-gallate, the main active component of green tea, and N-acetyl cysteine, a glutathione (GSH) precursor, provided protection against oxidative stress-induced liver injury caused by RF-EMFs [40].

However, it should be noted that no significant ROS generation was measured in other human cell lines when exposed to 1800 MHz (0.5–2 W/kg<sup>-1</sup>, for 30–45 min) [41, 42], and no short term activation of ERKs was detected in auditory hair cells treated for 15 min with RF-EMFs (1763 MHz, SAR 20 W/kg<sup>-1</sup>) [43]. Hence, both the generality of activation of classical MAPKs cascade by RF-EMFs and the validity of the proposed ROS-mediated mechanism are still challenged. Differences in cell lines and experimental methods, used for both *in vitro* and *in vivo* exposure, might explain, in part, these still conflicting findings.

## 2. EMFs and Oxidative Stress in Brain

Free radicals are essential for physiological processes, especially in brain metabolism [44]. The brain consumes the highest amount of oxygen in the human body and, although most oxygen is converted into CO<sub>2</sub> and water, a small amount of O<sub>2</sub> forms ROS [45]. The high metabolic rate and the composition rich in polyunsaturated fatty acids which are ROS targets in brain, make this organ more sensitive to oxidative damage [46].

Here we aim at critically reviewing the scientific literature focused on the cross-talk between redox-driven biological systems and EMFs in brain and its pathologic degeneration.

**2.1. Criteria for Reference Selection.** This paper is an overview of the results arising from both the *in vitro* and *in vivo* studies that investigated whether the EMFs (both ELF and HF) exposure could affect the oxidative balance of cells in the central nervous system. The interest about this topic stems from the knowledge that oxidative stress is a hallmark of neurodegenerative diseases and the hypothetical influence of EMFs on the onset and/or progression of these pathologies is frequently debated.

The search was carried out by consulting both PubMed data base and the official reports concerning the biological effects of the EMFs at the following websites:

[http://efhran.polimi.it/docs/IMS-EFHRAN\\_09072010.pdf](http://efhran.polimi.it/docs/IMS-EFHRAN_09072010.pdf)

[http://ihcp.jrc.ec.europa.eu/our\\_activities/public-health/exposure\\_health\\_impact\\_met/emf-net/docs/reports/EMF%20NET%202.2\\_%20D4bis..pdf](http://ihcp.jrc.ec.europa.eu/our_activities/public-health/exposure_health_impact_met/emf-net/docs/reports/EMF%20NET%202.2_%20D4bis..pdf)

[http://ec.europa.eu/health/ph\\_risk/committees/04\\_scenihhr/docs/scenihhr\\_o\\_007.pdf](http://ec.europa.eu/health/ph_risk/committees/04_scenihhr/docs/scenihhr_o_007.pdf)

[http://www.hpa.org.uk/webw/HPAweb&HPAweb-Standard/HPAweb\\_C/1317133826368](http://www.hpa.org.uk/webw/HPAweb&HPAweb-Standard/HPAweb_C/1317133826368).

The PubMed search was conducted using combinations of the following search terms: (oxidative stress), (oxidative stress AND brain), (oxidative stress AND neurodegenerative disease) with (EMFs or ELF-EMFs or HF-EMFs). Publications about pulsed and/or static fields have not been considered. A new Pubmed search was then conducted for all authors previously identified, and the reference list of any additional papers examined. Papers have been classified considering the frequency of electromagnetic field analyzed, irrespective of the experimental models and conditions employed.

The whole search was last updated in May 2012.

All papers matching the above-mentioned criteria have been quoted and referenced throughout the paper, without assessing on the quality of methodology, even if a critical revision of the exposure methods and experimental conditions has been carried out in Section 4 of the present paper.

**2.2. ELF-EMFs and Brain Oxidative Stress.** The interaction between the ELF-EMFs and the biological systems directly implies the involvement of the oxidative stress, in particular by the radical pair mechanism, as the equilibrium of the elementary reaction producing a pair of radicals may be altered by the magnetic field [23, 47, 48]. Thus, ELF-EMFs may prolong the lifetime of free radicals and increase their concentration in living cells [20–27]. Although radical pair recombination has been well documented for different biological processes (such as several enzymatic activities or orientation ability of migratory birds) in response to environmental EMFs [49, 50], its role as candidate mechanism, underlying ELF ability to affect brain oxidative stress and disease, has not been detailed so far.

ELF-EMFs exposure (50 Hz, 0.1–1.0 mT) is reported to elicit redox and trophic response in rat cortical neurons [51], and to induce oxidative stress in mouse cerebellum [52] (Table 1). In accordance, ELF-EMFs increase free radicals content with consequent lipid oxidative damage in brains of mice and rats [53, 54]. A contributing factor to the ELF-EMF-induced oxidative stress may be zinc deficiency, as lipid peroxidation-induced in Sprague-Dawley rats by long term exposure to ELF-EMFs (50 Hz, 50 mG) can be ameliorated through systemic antioxidant zinc supplementation [55].

Oxidative stress further arises from a disequilibrium between the production of free radicals and the scavenging

TABLE 1: EMFs exposure and oxidative stress in brain.

Type of EMFs	EMFs exposure details	EMFs effect	Experimental model	Reference
ELF	50 Hz, 0.1–1.0 mT, 7 days	Prooxidant	Cortical neurons (Sprague-Dawley rat embryo)	Di Loreto et al. [51]
	60 Hz, 2.3 mT, 3 hours	Prooxidant	ICR Mouse cerebellum	Chu et al. [52]
	40 Hz, 7 mT, 30 min/day for 10 days	Prooxidant	Sprague-Dawley rat brain	Ciejka et al. [53]
	50 Hz, 0.5 mT, 7 days	Prooxidant	Wistar rat brain	Jelenković et al. [54]
	50 Hz, 50 mG, for 5 min/day for 6 months	Prooxidant	Sprague-Dawley rat brain	Bediz et al. [55]
	60 Hz, 12 G, 3 hours	Prooxidant	Balb/c mice brain	Lee et al. [56]
	50 Hz, 100 and 500 $\mu$ T, 2 hours/day for 10 months	Prooxidant	Sprague-Dawley rat brain	Akdag et al. [57]
	60 Hz, 2.4 mT, 2 hours	Prooxidant	Wistar rat brain	Martínez-Sámamo et al. [58]
	50 Hz, 0.1–1.0 mT, 10 days	Prooxidant	Sprague-Dawley rat brain	Falone et al. [60]
	60 Hz, 0.2–1.2 mT	No oxidative effect	ICR mouse brain	Kabuto et al. [61]
RF	900 MHz, SAR of 2 Wkg <sup>-1</sup> , 7 days	Prooxidant	Wistar rat brain	Ilhan et al. [63]
	890–915 MHz, SAR 0.95 Wkg <sup>-1</sup> , for 12 h/day for 30 days	Prooxidant	Guinea pig brain	Meral et al. [64]
	900 MHz, SAR of 1.5 Wkg <sup>-1</sup> , and 6 Wkg <sup>-1</sup> , 7 days	Prooxidant	Sprague-Dawley rat brain	Ammari et al. [65]
	1800 MHz, SAR of 2 Wkg <sup>-1</sup> , 24 hrs	Prooxidant	Primary cortical neuronal cultures (new-born SD rats)	Xu et al. [66]
	900 MHz, 0.02 mWcm <sup>-2</sup> , 30 min/day for 7 days	No oxidative effect	New Zealand rabbit brain	Irmak et al. [37]
	872 MHz, SAR of 5 Wkg <sup>-1</sup> , 1 hour and 24 hours	No oxidative effect	SHSY5Y and L929 cells	Höytö et al. [67]

capacity driven by various antioxidant compounds and enzymes, including catalase (CAT), glutathione (GSH), GSH-Px, and critically important in brain SOD [56]. All these antioxidant defense systems can be specifically deteriorated by the ELF-EMFs (60 Hz, 12 G, 3 hours), thus amplifying oxidative stress [56]. In particular, in an experimental model of rat brain, 50 Hz (100 and 500  $\mu$ T) exposure was reported to induce a severe toxic effect by impairing the catalase (CAT) antioxidant defense [57]. Also in combination to movement restriction, the chronic exposure to ELF-EMFs (60 Hz, 2.4 mT) was able to elicit both the impairment of CAT activity and a severe lipid peroxidation in brains of Wistar rats [58].

As an overall oxidative stress-based decline in physiologic functions and in resistance to stressors is an unavoidable consequence of aging [59], it has been also investigated whether the aging process per semight reduce resistance towards EMFs prooxidant attack. In this context, ELF-EMFs

exposure (50 Hz, 0.1–1.0 mT) was shown to significantly affect antioxidant enzymatic capacity in both young and aged rat brains [60], with aged rats exhibiting a remarkable fall of all the major antioxidative enzymatic activities, thus pointing to a greater age-dependent susceptibility to EMFs-dependent oxidative stress.

In this ELF-ROS-brain context, only one paper, to our knowledge, reported no effect following exposure of mice to ELF-EMFs (60 Hz, 0.2–1.2 mT) [61]. Kabuto et al. indeed demonstrated that no ROS generation nor lipid peroxidation could be detected in brain homogenates of exposed mice. Interestingly, they observed a slight decrease in oxidative damage in mice exposed to static field (2–4 mT).

**2.3. HF-EMFs and Brain Oxidative Stress.** Exposure to RF radiation (mainly from mobile phones) has been postulated to trigger a variety of neurological effects, including headaches, changes in sleep pattern, modification in

the neuronal electrical activity, and disturbance in the neurotransmitter release [62]. Although still controversial, increasing evidence indicates that oxidative stress may be involved in the adverse effects elicited by RF-EMFs in the nervous system (Table 1).

In favor of this hypothesis, Ilhan et al. [63] reported a marked oxidative damage in brain tissues of rats exposed to 900 MHz signal for GSM (Global System for Mobile communications) (SAR of  $2 \text{ Wkg}^{-1}$  in the brain) for 7 days. They first proved that RF-EMFs exposure of the brain in rats cause histopathological changes typical of brain injury, accompanied by oxidative stress, as biochemically revealed by increased levels of nitric oxide (NO), malondialdehyde (MDA), as well as xantine oxidase (XO), and adenosine deaminase (ADA) activities. Moreover, treatment with the antioxidant *Ginkgo biloba* extract, a potent free radical scavenger agent, significantly prevented oxidative damage and pathological alterations in brain tissues.

In a different experimental model of guinea pigs, Meral et al. [64] evaluated the effects of GSM signal (890–915 MHz EMF, SAR  $0.95 \text{ Wkg}^{-1}$ , for 12 h/day for 30 days) on the oxidative stress pathway, by assessing MDA, GSH, CAT and vitamin A, D<sub>3</sub>, and E (considered part of antioxidant defense systems of tissues) levels in both brain and blood. Authors reported an increase of MDA, and a decrease of both GSH and CAT levels in brains, without any modulation in vitamins concentration, thus suggesting that RF exposure could trigger depression of the antioxidant systems, due to increased lipid peroxidation and formation of free radicals.

Also in a model of rats brain, locally exposed to GSM-900 MHz signal by a head loop antenna (SAR of  $1.5 \text{ WKg}^{-1}$  and  $6 \text{ WKg}^{-1}$ ), the activity of the cytochrome oxidase, a specific redox-sensitive enzyme and marker of neuronal functional activity in brain, was found compromised, but only at the higher SAR used, and exclusively in specific brain areas, such as frontal cortex, posterior cortex, hippocampus, and septum [65].

In the context of the *in vitro* studies, Xu et al. [66] exposed primary cortical neuronal cultures to a 1800 MHz field (SAR of  $2 \text{ Wkg}^{-1}$ ) for 24 hrs. They reported a significant increase of ROS production, and demonstrated, for the first time, a reduction in the mitochondrial DNA copy numbers. Interestingly, these effects could be reverted by pretreating cultures with melatonin, a pineal neurohormone with known antioxidant capacity.

In contrast to these findings are the *in vivo* data reported by Irmak et al. [37]. They analyzed MDA, NO, ADA, XO, MPO, SOD, CAT, and GSH-Px levels in both brain and sera of RF-EMFs-exposed rabbits (900 MHz GSM signal, 2 W peak power, average power density  $0.02 \text{ mWcm}^{-2}$ , for 30 min/day). Although an elevated activity of SOD and a reduction of NO levels were observed in the sera of exposed animals, no change in any brain parameters of rabbits was reported. In accordance, exposure of the dopaminergic neuroblastoma cell line (SH-SY5Y) to GSM (SAR of  $5 \text{ WKg}^{-1}$  for 1 hr) triggered no effects on GSH levels, nor induced DNA fragmentation, even if a significant increase in lipid peroxidation was observed [67].

### 3. EMFs and Neurodegenerative Diseases

Physiological dysfunction by oxidative stress leads to pathogenic condition. It is well established that free radicals can interact with DNA, leading to mutation, and interfere with gene regulation to eventually promote carcinogenesis [68]. But an additional aspect of free radicals is their potentiality to affect neuropathological conditions such as Parkinson's disease (PD) and Alzheimer's disease (AD), the oxidative stress being a molecular hallmark of neurodegenerative diseases [69].

Despite the increasing interest in this field of research and the epidemiological data suggesting the potential association between EMFs and neurodegeneration, the experimental findings supporting this link are still controversial, and dependent on both the field frequency applied and the disease investigated, as here reviewed.

**3.1. EMFs Exposure and AD.** AD is the most common neurodegenerative disease, and is characterized by progressive loss of neurons, particularly in the cortex and hippocampus [70]. Oxidative damage has been implicated as a key mediator in the onset, progression and pathogenesis of AD. In particular, redox reactive metals, such as iron, are leading causes of redox-generated hydroxyl radicals, and can promote the synthesis of amyloid beta ( $A\beta$ ) precursor protein in an oxidative stress-mediated pathway [27, 71, 72].

Despite the knowledge of AD molecular basis, the etiology of Alzheimer's is poorly understood. Many environmental and lifestyle factors, together with age, family history of dementia, and apolipoprotein E  $\epsilon 4$  genotype have been hypothesized to increase the risk of developing AD [73]. Among the potential environmental factors, exposures to aluminium, solvents, pesticides, and lead and also EMFs (mainly ELF-EMFs) have been the most widely studied [74]. Several available epidemiological studies and meta-analysis data seem to suggest a potential association between occupational exposure to ELF-EMFs (typical of electric power installers and repairers, power plant operators, electricians, electric and electronic equipments repairer, telephone line technicians, welders, carpenters, and machinists) and AD onset [75–77], although their biological nexus remain unknown. Only suppositions have been proposed, involving melatonin and biosynthetic enzymes in the pineal gland,  $\text{Ca}^{2+}$  efflux in immune system cells and neurons, interference with the amyloidogenic process, and clearly oxidative stress [78–80]. Sobel and Davanipour [81] hypothesized that ELF-EMFs exposure might increase  $A\beta$  peripheral and brain production by modulating the  $\text{Ca}^{2+}$  channels. The proposed mechanism relied on the ability of the EMFs to increase the intracellular ion concentration levels, a molecular factor that positively correlates with the cleavage of the amyloid precursor protein to give the soluble  $A\beta$ . ELF would hence favor the production of  $A\beta$  secreted in the bloodstream.

A completely different scenario in the Alzheimer's response to EMFs has been recently proposed by Arendash et al. [82] (see Table 2). They first reported that long-term (7–9 months) RF-EMFs exposure, directly associated with cell phone use (918 MHz;  $0.25 \text{ WKg}^{-1}$ ), provide cognitive

TABLE 2: EMFs effects on oxidative stress and neurodegeneration: *in vitro* and *in vivo* experimental models.

Pathology	EMFs exposure details	EMFs effect	Experimental model	Reference
AD	RF: 918 MHz, SAR 0.25 WKg <sup>-1</sup> 7–9 months	Cognitive benefits No brain oxidative stress	Tg(A $\beta$ PPsw ) and non-Tg mice	Arendash et al. [82]
	RF: 918 MHz, SAR 0.25 and 1.05 WKg <sup>-1</sup> 1 hour/day for 1 month	Cognitive benefits Decreased mitochondria oxidative stress in Tg mice	Tg(A $\beta$ PPsw + PS1) and non-Tg mice	Dragicevic et al. [85]
	RF: 918 MHz, SAR 0.25 and 1.05 WKg <sup>-1</sup> 2 hour/day for 2 months	Cognitive benefits Decreased brain A $\beta$ deposition, No brain oxidative stress	Aged Tg(A $\beta$ PPsw + PS1) and non-Tg mice	Arendash et al. [84]
PD	RF: 900 MHz, SAR 0.25 WKg <sup>-1</sup> 24 hours	Down-regulation of $\alpha$ -synuclein No oxidative stress	Neuron-enriched mixed cortical cell culture from brains of rat embryos (Wistar rats)	Terro et al. [87]
ALS	ELF: 50 Hz, at 100 and 1000 T 2 hours/day, 5 days/week for 7 weeks	No effect	Tg (SOD1 <sup>G93A</sup> ) and non-Tg mice	Poullietier De Gannes et al. [88]
HD	ELF: 60 Hz, 0.7 mT, 2 hours in the morning + 2 hours in the afternoon, for 8 days	Neuroprotective Decreased oxidative stress	3NP-treated Wistar rats	Túnez et al. [89]
	ELF: 60 Hz, 0.7 mT, 2 hours in the morning + 2 hours in the afternoon, for 8 days	Neuroprotective Decreased GSH, GSH-Px, CAT levels	3NP-treated Wistar rats	Túnez et al. [90]
	ELF: 60 Hz, 0.7 mT, 21 days	Neuroprotective Decreased oxidative stress	3NP-treated Wistar rats	Tasset et al. [91]

benefits, disclosing a potential noninvasive, nonpharmacological therapeutic strategy against AD. Several earlier studies have already evaluated the EMFs exposure at cell phone frequencies (900 MHz) in normal rodents, showing no effects on cognitive performance, but the exposure involved a short-term period (7–14 days) [83]. In Arendash' paper, both cognitive-protective and cognitive-enhancing effects, associated to reduced brain A $\beta$  deposition and increased cerebral blood flow, were demonstrated in transgenic mice destined to develop AD over a long term exposure period, without increasing indices of oxidative stress in the brain.

Arendash and colleagues recently extended their earlier findings by evaluating the impact of long term RF-EMFs treatment given to very old (21–26 month old) APPsw (amyloid precursor protein) and APPsw + PS1 (presenilin) mice, both bearing much heavier brain A $\beta$  levels than the same animals used in their first publication. In these aged mice, with advanced A $\beta$  pathology, long term RF exposure further revealed a profound ability to reverse brain A $\beta$  deposition, to induce changes in the regional cerebral blood flow, and to provide selected cognitive benefits, all without induction of brain hyperthermia and without increase in brain oxidative stress [84].

It is worth noting that data from the same group attributed the long term-RF-dependent cognitive benefits to the enhancement of brain mitochondrial function of AD transgenic (Tg) animals [85]. They indeed reported that RF-EMFs treatment is able to reduce mitochondrial ROS generation and to enhance mitochondrial membrane

potential in both cerebral cortex and hippocampus, but not in the striatum or amygdale, selectively in AD Tg mice. These findings are in contrast with what is stated in the other two publications (where they reported no change in the indices of brain oxidative stress), and leaves open the question whether RF benefits in AD involve oxidative stress.

In accordance to a potentially neuroprotective function elicited by RF, Söderqvist et al. [86] reported increased serum concentrations of transthyretin (TTR), a molecule specifically sequestering A $\beta$  peptide, among long term users of wireless phone, in both a cross-sectional study of 313 subjects using mobile phones and cordless phone, and in a provocation study on 41 people exposed for 30 min to 890-MHz GSM signal (1.0 WKg<sup>-1</sup>), suggesting that TTR might be involved in the RF-mediated benefits in AD mice.

Further studies are needed to corroborate these findings, to elucidate the biological mechanism and to validate the therapeutic use of RF fields, if any. It must be pointed out that several other studies indicated an increased risk brain tumors in people with long-term use ( $\geq 10$  years) of mobile phones, taking into account which side of the head the handset has been mostly used [92], thus highlighting how this issue is still controversial and requiring further investigations.

**3.2. EMFs Exposure and PD.** PD is the second most common neurodegenerative disease, relying on the loss of dopaminergic neurons in the substantia nigra in association with the occurrence of intracytoplasmic neuronal inclusions (Lewy bodies) of  $\alpha$ -synuclein [93]. Oxidative stress, generated by

dopamine redox chemistry and by  $\alpha$ -synuclein mutation, is considered one of the pathogenic factors in PD [93]. The oxidative damage to lipids, protein, DNA, and elevated RNA oxidation have been observed in both postmortem substantia nigra tissue and cerebrospinal fluid from living PD patients [27].

Differently from AD epidemiology, there are poor epidemiological bases supporting an univocal association between PD and exposure to EMFs. A pilot study by Wechsler et al. [94] first suggested that PD may be induced by occupational exposure to EMF, although a too small number of subjects was included in the study. Subsequently, two retrospective cohort studies [95, 96] and a death certificate-based case-referent study [97] failed to find a convincing correlation between Parkinson's disease and occupational magnetic field exposure. The death certificate-based method only found modest risks for power plant operators and telephone installers and repairers [97]. In a study by Noonan et al. [98], welders, who are exposed to high levels of magnetic fields as well as to other potentially neurotoxic agents such as metals, accounted for some of the observed risk of PD, suggesting an association between welding and an increased risk to develop Parkinson's. Finally, a recent paper from Huss et al. [99], based on a cohort of 4.7 million people of the Swiss National Cohort, followed over the period 2000–2005, demonstrated no consistent association between mortality from Parkinson's disease and exposure to ELF power lines (220–380 kV, 50 Hz). Therefore, up to date, convincing epidemiological data supporting a correlation between PD and environmental/occupational EMFs exposure are still lacking.

Given the contradiction in epidemiological studies, *in vitro* and *in vivo* experimental findings disclosing the potential PD-EMFs correlation, are very sparse. To our knowledge, only a recently released paper attempted to investigate whether oxidative stress might be triggered by EMFs exposure and thus affect PD etiology and/or progression [87] (Table 2). Authors used a highly (80%) neuron-enriched mixed cortical cell culture from brains of rat embryos to study the impact of chronic (on the scale of the *in vitro* studies) exposure to GSM-900 MHz, at a low SAR ( $0.25 \text{ WKg}^{-1}$ ) [87]. Despite previous records, no ROS generation or oxidative damage were observed in the neuron-enriched experimental model following RF exposure, although authors reported the first evidence of an EMFs-mediated downregulation of the  $\alpha$ -synuclein, probably by promotion of its deubiquitination [87].

**3.3. EMFs Exposure and Amyotrophic Lateral Sclerosis.** Amyotrophic Lateral Sclerosis is a fatal neurodegenerative disorder characterized by progressive degeneration of motor neurons in the spinal cord, motor cortex, and brainstem. About 5–10% of ALS display familial inheritance, but in the majority of patients there is no inherited link. Both familial (fALS) and sporadic ALS (sALS) produce similar pathological symptoms [100]. At molecular level, a mutation in the gene encoding the antioxidant  $\text{Cu}^{2+}/\text{Zn}^{2+}$  SOD (SOD1) has been reported in about 20% of fALS patients [101],

still indicating the key role exerted by the oxidative stress in this neuropathological disorder [102]. In accordance, mitochondrial dysfunction may play a more significant role in the etiopathogenesis of this disorder than previously thought. The complex physiology of mitochondria and the alteration of their properties might confer an intrinsic susceptibility to long-lived, postmitotic motor neurons to energy deficit, calcium mishandling, and oxidative stress [103].

Although several hypotheses concerning the pathogenesis of the ALS have been generated, the etiology of the vast majority of cases is unknown. Electrical exposure has been cited as a possible environmental risk factor. Haynal and Regli were the first to raise the hypothesis that exposure to ELF-EMFs was linked to ALS in 1964 [6]. Since then, other epidemiological studies have positively correlated ALS death with occupational exposure to EMFs (electric utility workers), with relative risks ranging from 2 to 5, while only a few studies found little or no association [5, 95–97, 104–106]. A recent UK study found no risk increases in any job categories for motor neuron disease mortality among electricity generation and transmission workers compared to the general population [107]. Also Parlett et al. [108] did not provide any evidence for an association between magnetic field exposure and ALS mortality. After adjusting for age, sex, and education, they reported no increased risks of ALS mortality in relation to potential magnetic field exposure.

Thus, the evidence linking electrical occupations to an increased risk in ALS is remarkably consistent, but the evidence of an association with measured magnetic field levels is weaker. Lack of assessment of magnetic field exposure at the workplace and possible confounding by electric shocks, were the major limitations. Therefore, pending further well-designed epidemiological studies, there is still a need for confirmation of the correlation EMFs exposure-ALS from specifically designed laboratory experiments.

To our knowledge, the paper from De Gannes et al. [88] (see Table 2) is the only experimental study carried out in an animal model, in a controlled magnetic environment. Mutated SOD-1 mouse experimental model (Tg-SOD1<sup>G93A</sup>), which is currently the most accurate animal model for studying ALS, was employed to assess the possible effects of chronic exposure to ELF-EMFs (2 hours/day, 5 days/week for 7 weeks, to 50 Hz, at 100 and 1000  $\mu\text{T}$ ) on the development of this neurodegenerative disease. The exposure levels were chosen on the basis of the European recommendation setting limits of 100  $\mu\text{T}$  for public exposure and 500  $\mu\text{T}$  for workplace [88]. By monitoring body weight, motor function, and life span of mice over the exposure period, authors did not reveal any difference between exposed and control animals, providing no evidence of a link between ELF exposure and ALS in this oxidative stress-prone experimental model. Despite it being reported that the yield and nature of oxygen reactive species may be affected at magnetic field strength above 100  $\mu\text{T}$ , the reported lack of biological effect may reflect the fact the pathophysiology of the familial form, characterized by SOD-1 mutation, is probably different from the sporadic one, and does not proceed via oxidative stress at the dose/time chosen for the exposure. Whether longer

exposures or exposure of younger animals would affect the outcome is unknown and requires further investigation.

**3.4. EMFs and Huntington's Disease (HD).** Huntington's disease is an autosomal dominant, progressive neurodegenerative disorder characterized by an array of different psychiatric manifestations, cognitive decline, and choreiform movements. The underlying molecular genetic defect is an expanded trinucleotide (CAG)<sub>n</sub> repeat encoding a polyglutamine stretch in the N-terminus of the huntingtin protein. In most cases, HD is fully penetrant. Although huntingtin is ubiquitously expressed, the mutated gene leads to selective neuronal cell death in the striatum and cortex, even though the mechanisms by which it triggers neuronal dysfunction and degeneration are not fully understood. Impaired ubiquitin-proteasome activity, defective autophagy-lysosomal function, transcriptional dysregulation, apoptosis, mitochondrial, and metabolic dysfunction have been shown to play important roles in the pathogenesis of HD, as well as oxidative stress, like in other neuropathologies [91, 109, 110].

The potential correlation between EMFs exposure and HD pathogenesis is not sustained by epidemiological evidence. A few papers from a single research group attempted to disclose their connection in a mouse model of HD pathogenesis achieved by administering animals with the 3-nitropropionic acid (3NP). This toxin is a selective inhibitor of succinate dehydrogenase (SDH) in the complex II of the mitochondrial electron transport chain [111]. 3NP triggers energy impairment, cytotoxicity, oxidative stress, and, eventually, neuronal death. In addition, animals exhibit motor and cognitive changes similar to HD [112, 113]. Stimulation of rats with ELF-EMFs (60 Hz and 0.7 mT, 2 hours in the morning and 2 hours in the afternoon, for 8 days), given either before or after the 3NP administration, partially prevented or reversed the neurotoxin-induced oxidative stress. Besides, a reduction in cellular loss and an increase in SDH activity was also observed [89, 90] (see Table 2).

Further evidences by Tasset et al. [91] strengthened the hypothesis of a neuroprotective effect elicited by ELF-EMFs. In a rat model of 3NP-induced HD, behavior patterns as well as changes in neurotrophic factor, cell damage, and oxidative stress biomarker levels were monitored. Rats were given 3NP over four consecutive days (20 mg/kg body weight), whereas ELF-EMFs (60 Hz and 0.7 mT) were applied over 21 days, starting after the last injection of 3NP. If compared to control 3NP-treated animals, ELF-EMFs improved neurological scores, enhanced neurotrophic factor levels, and reduced both oxidative damage and neuronal loss. Moreover, exposure to electromagnetic fields alleviated 3NP-induced brain injury and prevented loss of neurons in rat striatum, thus showing considerable potential as a therapeutic tool.

Taken as a whole, these data support the hypothesis that magnetic stimulation in rats prompts an increase in neuron survival and/or in neuronal density; this would eventually lead to normalized functioning of the nervous system, evident in the recovery of behavior patterns similar to those of a healthy rat.

## 4. Comments and Perspectives

So far there is still no general agreement on the exact biological effect elicited by EMFs, on the physical mechanisms that may be behind their interaction with biological systems, or on the extent to which these effects may be harmful to humans. In particular ELF-EMFs, such as those generated by power lines, have been suggested to increase the risk of several human diseases, mainly neoplastic malignancies [7, 8, 114]. The International Agency for Research on Cancer (IARC) inserted ELF in the 2B section of the table of carcinogens ("possible") in 2001, and recently classified also the Radio Frequency (RF) fields as 2B [4, 115]. In addition, early studies seemed to indicate that ELF-EMFs could contribute to the etiology of neurodegenerative disorders, in particular of AD and ALS [6, 9, 74]. Hypotheses relating the EMFs to the neurodegenerative diseases are a relatively novel part of the EMF research area and, so far, only a modest number of studies have been performed if compared to cancer research field.

However, this area has quickly acquired attention because of implications in human health, occupational exposure, and aging, although, for a number of methodological reasons, the epidemiology of neurodegenerative diseases is more difficult to study than cancer. The most obvious difficulty is that neurological diseases are not recorded in registries in the same way as cancers, and that the mortality registries are less reliable as sources of cases. There are also lack of consensus on diagnostic criteria and difficulties in assessing time of disease onset. In addition, there is also a gender implication in epidemiological studies on neurodegeneration. Women display the higher incidence in pathologies such as the AD, but it is hard to base a study on their occupational exposure, as women have less often been employed especially in those work categories where the exposition to EMFs is high. Moreover, in occupational studies, distinguishing between exposure to EMFs and to chemical agents is often problematical, as workers are frequently exposed to a combination of both of these potentially neurotoxic factors. A notable weakness in neurodegenerative disease studies is case identification. In some studies, cases were identified in hospitals and controls among patients with other diseases in the same hospitals or among friends or relatives of cases. These studies are likely to have greater potential for selection bias than population-based studies, which, on the other hand, have often identified cases from mortality registries and thus have greater potential for disease misclassification. These and other difficulties are reflected in the literature, and the studies that have best avoided these limitations often suffer from small number.

Moreover, another important issue in the epidemiological studies, involving EMFs, is the exposure assessment, which is crucial to univocally link the appearance of the disease to the experienced exposure levels. In this case, the direct measure or numerical evaluation of the emitted EM field could be particularly hard and expensive, due to the elevated number of involved people and residential places (e.g., offices, houses, schools, or hospitals). So far, only a rough estimation of the dose has been possible, even based

on people interview asking for the most common exposure sources present in their daily-life environment. Therefore, a more careful approach seems to be necessary in arranging new epidemiological campaigns. For instance, it could be useful to provide personal dosimeters, able to record in real time the effective EMFs levels, together with the time and the exact position of the exposure.

In this paper, we have revisited the experimental *in vitro* and *in vivo* studies, focused on the impact of the EMFs-driven oxidative pathway of the brain (Tables 1 and 2), as the high metabolic rate and the lipid rich composition of nervous system make this organ particularly sensitive to oxidative damage in both physiological processes and pathological conditions, such as neurodegeneration [46]. Indeed, the *in vivo* and *in vitro* experiments are able to provide more controlled, repeatable, and defined exposure conditions with respect to the epidemiological investigations, necessary to assess the dose-relationship studies and to set the hypotheses of related action mechanisms.

In this context, oxidative damage appears to be a master regulator of the biological response to EMFs in different cellular systems, together with alterations of blood parameters, changes in cytokine profiles, and effects on the immune system, although no clear understanding of the underlying mechanisms has been uniformly documented [15–19].

**4.1. ELF-EMFs, Brain and Neurodegeneration.** ELF stimulation, given as both short- (minimum 3 hours) and long-term (up to 10 months) exposure, seems almost univocally to be able to trigger oxidative stress (Table 1). In both animal brain and *in vitro* rat cortical neurons cultures, ELF-EMFs are associated to oxidative stress, that arises both from field interaction with chemical bonds of biomolecules, thus giving ROS a higher concentration and activity [51–55], and from disequilibrium in the enzyme-dependent scavenging ability [56–58]. In this ELF-ROS-brain context, only one paper by Kabuto et al. reported no ROS and no peroxidation effects following exposure of mice to ELF-EMFs [61], but description of the exposure and dosimetric details is poor.

A big controversy in disclosing ELF-EMFs effects in brain arises in the context of neurodegenerative diseases (Table 2). Epidemiological studies correlate occupational exposure to ELF-EMFs and AD and ALS pathogenesis, while poor epidemiological evidences have linked them to the onset and/or progression of both PD and HD [6, 9, 74, 94–97].

In AD pathogenesis, experimental findings propose melatonin biosynthesis,  $\text{Ca}^{2+}$  efflux in immune system and neurons, interference with the amyloidogenic process, as potential cofactors of the ELF-mediated functions [78–81]. However, no univocal experimental findings by *in vitro* or *in vivo* studies have so far corroborated the hypothesis of the ELF-dependent oxidative stress as a key molecular regulator of the AD development.

In the ALS context, an attempt to assess a functional correlation between ELF and neurodegeneration has been carried out exclusively by De Gannes et al. [88], in an oxidative stress-prone experimental model of Tg (SOD1<sup>G93A</sup>) mice, at the moment the most accurate animal model for studying

this pathology. By precisely monitoring body weight, motor function, and life span, authors did not report any significant redox-related change in Tg-exposed mice, although exposure was carried out over a 7 weeks period. Whether a longer treatment or exposure of younger animals would affect the outcome is unknown, and definitely requires further investigations, also in additional experimental animal models that do not exclusively represent the ALS familial (mutated SOD) form.

In the research field of PD, although not described in this paper, it is worth mentioning the presence of different studies in favor of possible therapeutic potentials of the so-called transcranial magnetic field stimulation (TMFS) in the frequency range of the ELF [116]. TMFS is a relatively innovative technique applied to investigate corticospinal physiology and other properties of the primary motor cortex, such as excitability [117, 118]. Even though no involvement of oxidative stress has been so far reported, some records claim that TMFS is able to relieve patients from most parkinsonian symptoms, driving amelioration of the reaction and movement time, of the performance on the grooved pegboard test in patients whose dominant motor hand area was stimulated by a focal coil during testing [117]. These data may suggest a protective function of ELF, but TMFS is based on single- or paired-pulsed signal that cannot be properly considered as an ELF-EMF. Besides, there are no experimental data supporting clinical observations, and further animal studies may shed some light on the mechanisms involved and perhaps provide a stronger rationale for improvement of patients afflicted with PD treated with TMFS therapy.

Convincing experimental evidences, in support of a potential neuroprotective effect of ELF exposure, have been produced exclusively in HD animal models. Exposure to ELF-EMFs (administered as both short term treatment, for 8 days, and for long term exposure of 21 days) has been indeed reported to significantly prevent and reverse the oxidant effect induced by the neurotoxin 3NP [89–91]. It needs to be highlighted that all these set of experimental findings, carried out in the 3NP-treated Wistar rats, origins from the same research group. Besides, in the experimental procedures, the authors refer improperly to a transcranial magnetic stimulation (TMS) exposure, while TMS signals have completely different characteristics from those applied by Tùnez' group [89–91]. What they used is a simple sinusoidal ELF signal, while real TMS stimulation consists in a monophasic or biphasic pulse (e.g., a dumped cosine) provided to the biological sample in multiple trains at a repetition frequency of tens of Hz, as well described by Peterchev et al. [119].

Hence, it is now well accepted that ELF-EMFs influence the *in vitro* behavior of numerous cell types, and that these changes trigger diverse effects which may have positive or negative outcomes, depending on the cell type [120–122]. This phenomenon could partially explain the opposite results obtained in different *in vitro* studies, but does not give rise to any explanation for opposite findings in animal models upon ELF exposure in brain. It has been postulated that ELF stimulation can affect physiology of neurons by inducing

oxidative damage, lipid peroxidation, and neurotransmitter release. These data might suggest a possible prodegenerative effect of ELF, as the oxidative stress is clearly a hallmark of neurodegeneration. Unexpectedly, a completely different response is elicited if ELF stimulation is administered to neurons that are still compromised by an early event of neurodegeneration, and/or if applied over a long period. Like in other diseases, such as cancer, it is often a matter of balance between opposite stimuli, and a matter of when the external stress factor is hitting the cell, whether in early or late degenerative step.

In addition, it is worth to notice that an appropriate description of the ELF-EMFs homogeneity within the used exposure device, as well as temperature control, is lacking in the majority of the exposure configurations and protocols reviewed, in contrast to the requirements for controlled and high quality experiments in bioelectromagnetic reported by Kuster for low-frequency fields [123]. Moreover, at these frequencies, sham control is a crucial issue that needs to be carefully implemented. Normally, the exposure systems are turned off to obtain such a condition, while a more appropriate sham exposure should be represented by coil systems using separated strand cables wrapped in parallel directions. Only in this way, it is possible to reproduce exactly the same environmental conditions of the exposed case in term of vibrations and temperature variations.

**4.2. HF-EMFs, Brain, and Neurodegeneration.** The experimental evidences linking the field exposure to the oxidative stress in brain and neurodegeneration are controversial also in the context of the HF-EMFs. The influence of RF on biological systems, in particular the presence of biological effects on and risk to humans, has been a subject of intense debate for several decades. Recently, this debate intensified due to new applications of RF-EMFs in cordless stationary phones, wireless computer communication, and, most importantly, due to the exploding use of mobile phones. Since the quantum energy of RF-EMFs is extremely low compared to ionizing radiation, it is plausible that no conclusive and reproducible genotoxic effects, such as increased DNA damage or increased mutation rates, will be observed in response to RF-EMFs. Since interactions between RF-EMFs and certain molecules in biological systems form the basis for possible RF-EMFs-induced changes in these systems, it has been assumed that only the absorbed radiation from RF-EMFs can have effects in biological systems. Hence, the specific absorption rate should be a key measure for the induction of biological effects. Most of the RF-EMFs radiation absorbed is converted into increased thermal energy of the system [35], which is responsible for most effects observed in biological systems. Nevertheless, it is now well accepted that also low-level EMF exposure, which does not induce thermal effect, could carry a biological response. So, a major experimental problem is the definition of non-thermal effects and how to distinguish them from direct and indirect thermal effects [36–38].

One of the hypothesized targets for nonthermal effect of RF-EMFs is the oxidative stress, although experimental

*in vitro* and *in vivo* findings in brain are contradictory, ranging from prooxidant ability of GSM exposure observed in primary cortical neurons cultures and in animal model [63–66], to no-effect reported in SH-SY5Y (human neuroblastoma) and L929 (mouse fibroblasts) cell lines and in mice brain and sera [37, 67] (Table 2). This overall contradiction in neuronal parameters in response to RF definitely reflects the uncertainty in identifying the molecular effects driven by GSM, and in distinguishing between thermal and nonthermal ones.

The scenario in neurodegeneration response to RF stimulation has been recently revisited following data from Arendash and colleagues [82–84] (Table 2). They demonstrated for the first time that long term RF stimulation provides cognitive benefits to AD animals, disclosing a potential noninvasive, nonpharmacological therapeutic strategy against Alzheimer's. In accordance to a potential RF-driven neuroprotective effect (although exclusively supported by *in vitro* evidences), low SAR GSM-900 MHz exposure has been reported to downregulate the  $\alpha$ -synuclein in a highly (80%) neuron-enriched mixed cortical cell culture from brains of rat embryos [87], suggesting a hypothetic beneficial effect of these frequencies also in PD model (Table 2).

In the RF-induced neuroprotection of AD models, authors demonstrate that all the cognitive benefits occur without induction of brain hyperthermia and without increase in brain oxidative stress [82, 84]. Surprisingly, experimental data from the same group attributed the long term-RF-dependent effects to the enhancement of brain mitochondrial function of AD transgenic (Tg) animals [85], in terms of reduced mitochondrial ROS generation and enhanced mitochondrial membrane potential, in both cerebral cortex and hippocampus of AD Tg mice. These findings are in contrast to what is stated in the other two publications (where they reported no change in the indices of brain oxidative stress), and leaves whether GSM functions involve oxidative stress or not.

Moreover, major concerns remain on the exposure system employed by Arendash' group and on the dosimetric assessment performed to define the mentioned SAR levels. First, the provided SAR calculation does not specify if it is referred to the internal field levels (within mouse) or to the external ones. In this last case, the reported SAR values have no sense, as SAR is defined as the absorbed dose in the unitary mass of the biological target (a mouse in this case) within a certain time interval. Besides, it is not accurate to perform a SAR calculation that does not take into consideration the different conductivities and densities of the animal tissues. In this case, a sort of average value, for both conductivity and density, has been used, rendering the SAR estimation within the biological target extremely approximate. Also, no information about field homogeneity inside the exposure target is provided. This observation leads to the conclusion that the performed evaluation cannot be considered as a satisfactory dosimetry for the target. The methodology employed for field measurements should be clearly stated, and further EM simulations required to confirm the experimental SAR values, as well noted in Kuster and Schönborn 2000 [123]. Without a rigorous dosimetry

(local and mean SAR values obtained both experimentally and numerically, plus evaluation of the SAR homogeneity), the real delivered dose within mice remains unknown, consequently making unreliable and completely nonreplicable the obtained results.

On the basis of both Arendash' results, and other evidences that TTR can bind A $\beta$ , and thus protect against its deposition [124], Söderqvist et al. evaluated TTR levels in people exposed to GSM [86]. He describes an increase of TTR after GSM signal exposure, and argues that the hypothetic RF effect on AD could be TTR-mediated. A number of concerns arise with respect to the methodology chosen for the analysis. For cross-sectional study, people were asked to answer a postal questionnaire about use of mobile phones and cordless phones. This is a widely adopted solution in epidemiological studies on EM fields, leading to a series of mistakes related to the assessment of the exposure. Indeed, the information provided cannot be always complete and accurate. For provocational study, the EMFs exposure was performed at 890 MHz GSM signal for 30 min. A homogenous specific absorption rate (SAR 1g) of 1.0 Wkg<sup>-1</sup> to the temporal area was applied. However, authors do not specify how this SAR value was assessed. May be, numerical simulations were performed. In addition, the system used to deliver the EM fields close to human head is not described. Hence, it is difficult to effectively evaluate the dose and consequently to replicate the study.

Therefore, depending on the dose, the frequency, the exposure period, EMFs are reported to be either harmful or protective in neuronal response, suggesting even a possible application in medical therapy. Hence, so far no univocal interpretation of the EMFs effects in brain and neurodegeneration can be proposed, as epidemiological studies are difficult to be carried out, *in vitro* and *in vivo* models are heterogeneous, and laboratory exposure set-ups often present limitations without a proper dosimetry. The experimental conditions in the EMFs experiments, such as the induced field within the biological target, its frequency, as well as the impulse shape, and time of exposure, may affect biological response. Conflicting biological data might be thus attributable to differences in the frequency and intensity of the field, exposure time, heat generation, cell penetration, and experimental model considered. When RF exposure effects are investigated, it has to be considered that the biological samples modify the systems performances; hence, the features of the exposure devices have to be rigorously evaluated during their design steps and final characterization. As a consequence, the dosimetric assessment within the biological targets is of primary importance for well-controlled experiments [123]. In particular, Kuster and Schönborn [123] established that the required SAR homogeneity for high-quality investigations has to be of the order of 70%. This quantity should be assessed by using both experimental methodologies (e.g., EMF and SAR measurements) and numerical EM simulations, capable of describing precisely the biological target geometry and its electric properties, as well highlighted in different papers [125–128].

We would also like to stress that in a number of *in vitro* and *in vivo* studies performed at RF and MW frequencies, unacceptable exposure conditions for cell phones, in direct contact to the cell cultures or animals, have been employed [37, 82, 85, 129, 130]. This exposure conditions do not guarantee any control of the emitted power and thus of the SAR induced within the samples.

In the light of results reviewed here, we can conclude that there are no incontrovertible evidences of the role of EMFs in oxidative stress modulation. Hence, it is mandatory to proceed with intense research on this issue, paying particular attention to the choice of the appropriate biological model and well-controlled experimental conditions.

## Abbreviations

A $\beta$ :	Amyloid beta
AD:	Alzheimer's disease
ADA:	Adenosinedeaminase
ALS:	Amyotrophic lateral sclerosis
APP:	Amyloid precursor protein
CAT:	Catalase
DC:	Direct current
E-field:	Electric field
EHC:	Environmental health criteria
ELF:	Extremely low frequency
EM:	Electromagnetic
EMF:	Electromagnetic field
GSH:	Glutathione
GSH-Px:	Glutathione peroxidase
GSM:	Global system for mobile communications
HD:	Huntington's Disease
HF:	High frequency
H-field:	magnetic field
IARC:	International Agency for Research on Cancer
MDA:	Malondialdehyde
MPO:	Myeloperoxidase
MRI:	Magnetic resonance imaging
MW:	Microwave
3NP:	3-Nitropropionic
NO:	Nitric oxide
PD:	Parkinson's disease
PS1:	Presenilin
RF:	Radio frequency
ROS:	Reactive Oxygen species
SAR:	Specific absorption rate
SDH:	Succinate dehydrogenase
SOD:	Superoxide dismutase
Tg:	Transgenic
TMS:	Transcranial magnetic stimulation
TMFS:	Transcranial magnetic field stimulation
TTR:	Transthyretin
WHO:	World Health Organization
XO:	Xantine oxidase.

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## Review Article

# Brain Miffed by Macrophage Migration Inhibitory Factor

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Macrophage migration inhibitory factor (MIF) is a cytokine which also exhibits enzymatic properties like oxidoreductase and tautomerase. MIF plays a pivotal role in innate and acquired immunity as well as in the neuroendocrine axis. Since it is involved in the pathogenesis of acute and chronic inflammation, neoangiogenesis, and cancer, MIF and its signaling components are considered suitable targets for therapeutic intervention in several fields of medicine. In neurodegenerative and neurooncological diseases, MIF is a highly relevant, but still a hardly investigated mediator. MIF operates via intracellular protein-protein interaction as well as in CD74/CXCR2/CXCR4 receptor-mediated pathways to regulate essential cellular systems such as redox balance, HIF-1, and p53-mediated senescence and apoptosis as well as multiple signaling pathways. Acting as an endogenous glucocorticoid antagonist, MIF thus represents a relevant resistance gene in brain tumor therapies. Alongside this dual action, a functional homolog-annotated D-dopachrome tautomerase/MIF-2 has been uncovered utilizing the same cell surface receptor signaling cascade as MIF. Here we review MIF actions with respect to redox regulation in apoptosis and in tumor growth as well as its extracellular function with a focus on its potential role in brain diseases. We consider the possibility of MIF targeting in neurodegenerative processes and brain tumors by novel MIF-neutralizing approaches.

## 1. Introduction

Macrophage migration inhibitory factor was one of the first cytokines identified after interferon [1] and represents a key regulator of the immune system (MIF is historically also known as glycosylation-inhibiting factor, GIF) [2, 3]. MIF was initially described as a proinflammatory soluble factor derived from T cells under various conditions such as delayed-type hypersensitivity responses and inflammation guiding site-specific migration of immunocompetent cells [2, 4]. It soon became apparent that MIF possesses immunoregulatory effects and is even constitutively detectable in various body fluids and cells of the mammalian organism. MIF levels are higher at sites of inflammation, within immune and brain cells and various cancer cells (Figure 1). Later, MIF was shown to contribute to neuroendocrine

modulation, as a pituitary gland-derived hormone, inflammation, atherosclerosis, cancer development, and cancer progression [5–11]. MIF was first cloned from T cells in 1989, which revealed not only its primary sequence and conserved domains but also led to the discovery that MIF exhibits two catalytic centers, one for thiol-protein oxidoreductase activity and another one for tautomerase activity [12–14]. These findings fueled speculation that MIF was not only a cytokine, but a possible combination of enzyme and cytokine “cytozyme” [12, 13, 15, 16]. Hence, MIF’s conserved gene structure and structural homology with D-dopachrome tautomerase (DDT/MIF-2) aroused further speculation surrounding its proposed enzymatic actions and cytokine properties [17, 18]. This enigmatic property of MIF fostered the development of genetic approaches towards a better understanding of its biology in physiology and disease.

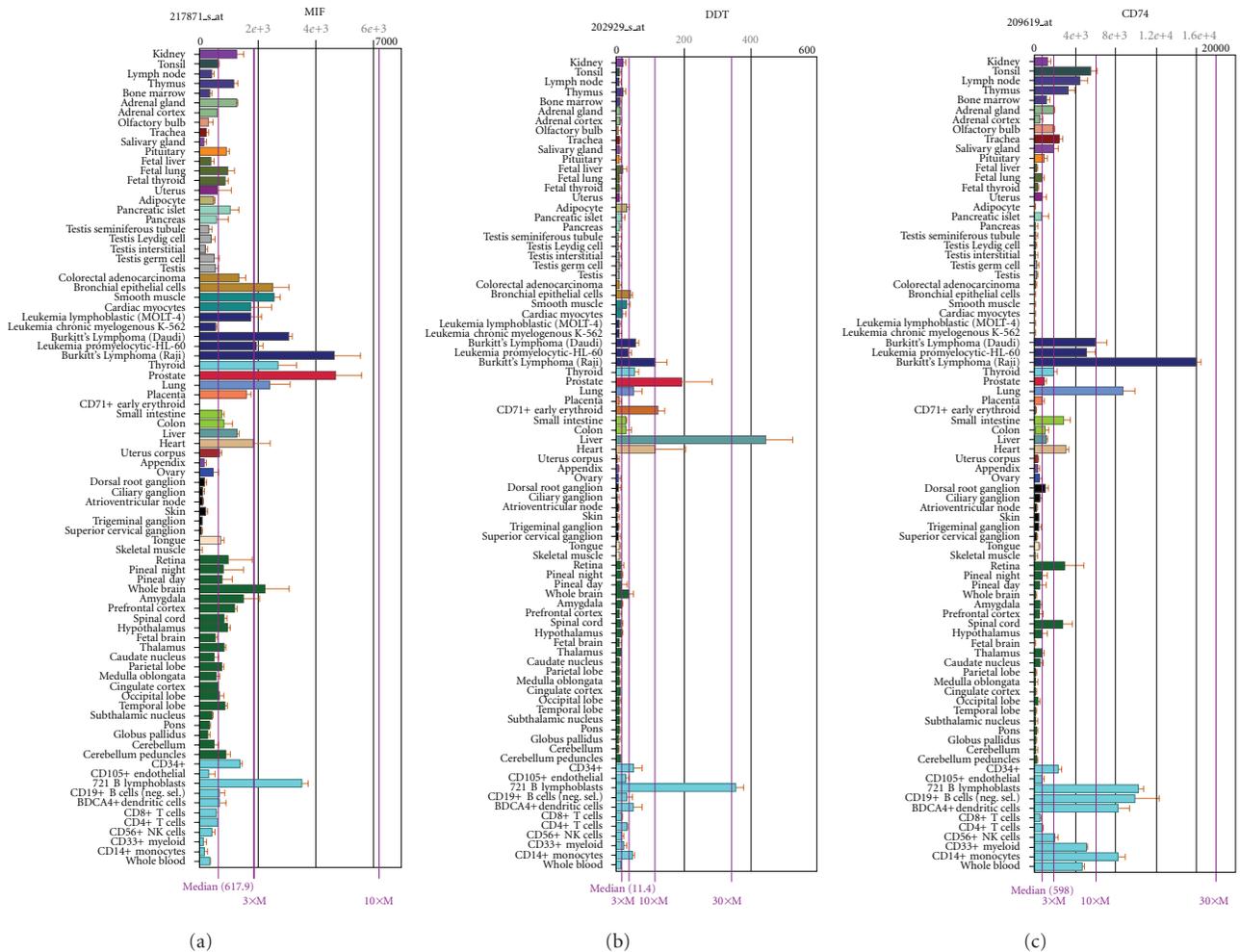


FIGURE 1: MIF, DDT, and CD74 distribution in human tissues. Comparative analysis of MIF, DDT (MIF-2), and its receptor CD74 expression in various human tissues. For human mRNA expression analysis, the BioGPS database (<http://biogps.gnf.org> profile graph) with the Affymetrix chip Human U133A was acquired. Note in particular the different expression values of MIF and DDT in brain tissue. For details on the Affymetrix chip analysis, see [37, 38].

To date, it is known that MIF induces pleiotropic functions in inflammation, malignant transformation, and endocrine and metabolic processes. In this paper, we focus on MIF-dependent signaling in redox regulation and brain cancer progression and discuss recent findings in MIF neurobiology.

## 2. MIF Structure and Function

The small and highly conserved protein MIF with an approximate molecular weight of 12.5 kDa (human MIF contains 115 aa) does not exhibit any similarities with known cytokines [12, 19, 20]. MIF protein does not require an N-terminal export-specific leader sequence for secretion as it is secreted via an alternative, nonclassical pathway.

However, MIF contains two conserved domain motifs (Figure 2). The CXXC domain motif (Cys-X-X-Cys at position 56–60) in the center of MIF has been shown to exhibit

catalytic activity [21–23]. It is a consensus sequence of proteins of the thiol-protein oxidoreductase superfamily, other members of which include thioredoxins, glutaredoxins, and peroxiredoxins [24, 25]. Common to this enzyme superfamily is that all members are involved in disulfide-mediated redox reactions and glutathione metabolism in which the CXXC domain takes center stage. In the case of MIF, the CXXC domain is potentially involved in forming MIF homodimers and trimers, the most likely active form of MIF [26–28]. Hence, the CXXC domain of MIF has been shown to exhibit low redox catalytic activity *in vitro* (compared to thioredoxin and glutaredoxins) and modulates cellular redox stress responses by elevating the intracellular glutathione (GSH) pool [14, 29–34]. In particular, reactive oxygen species (ROS) induce elevated MIF mRNA and protein expression in neurons, and MIF represents a negative regulator for angiotensin-II-induced chronotropic action

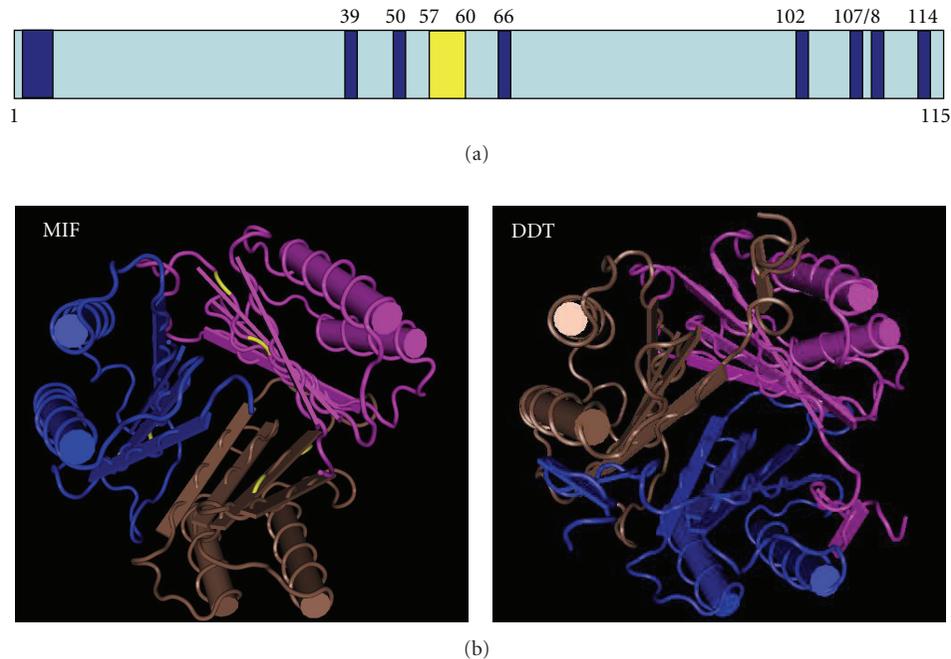


FIGURE 2: Structural homologies of MIF and DDT. (a) Primary structural scheme of the human MIF gene. Yellow region indicates the CXXC domain, the blue boxed domains indicate the proposed tautomerase/isomerase domains and clustered amino acids (Phe3, Val39, Gly50, Lys66, Asn102, Gly107, Trp108, Phe113, and Ala114) [18]. (b) Structural comparison of human MIF and DDT trimers. The catalytically important CXXC domain is shown in yellow.  $\beta$ -sheets are given as arrows, and  $\alpha$ -sheets are shown as columns. Data were obtained from the NCBI database (<http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?uid=89970>) based on the study of [28].

and firing in neurons [33, 35, 36]. In addition, MIF has been found to protect from oxidative stress in an ischemia/reperfusion cardiac lesion model [29, 34].

It is worthy to note that the CXXC domain in MIF seems to be essential in facilitating the inhibition of angiotensin II. Evidence for this comes from MIF peptide fragments containing the CXXC domain ( $\Delta$ MIF<sup>50–65</sup>) which mimic the wild-type MIF action whereas a mutant  $\Delta$ MIF<sup>50–65</sup> replacing the second cystine to serine (C60/S60) does not [36]. Redox stress is known to be elevated under conditions of hypoxia or malignant transformation. Hypoxia-inducible MIF elevation has been reported in head and neck cancer cells, pancreas, cervical carcinoma cell lines, and glial tumors [40–43]. Further studies revealed that MIF transcription is induced by hypoxia-inducible factor 1 $\alpha$  (HIF1 $\alpha$ ) and is physically linked to HIF1 $\alpha$  through COP9 signalosome subunit 5 (CSN5) interaction [42, 44, 45]. MIF can potentially inhibit apoptosis and p53-mediated growth arrest and its depletion impairs cell proliferation in cancer [6, 7, 46–48]. It was suggested that MIF's action of blocking apoptosis is dependent on its catalytic oxidoreductase activity. However, whether MIF deletion in tumors makes them prone to hypoxia and affects tumor vasculature *in vivo* remains to be thoroughly investigated. First studies already indicate that MIF expression and MIF signalling are associated with tumor angiogenesis [49–51].

The second enzymatic domain of MIF is its enigmatic tautomerase activity which has spurred intensive research

on the physiologic substrate and function. In an attempt to identify the enzyme responsible for converting the non-naturally occurring substrate L-dopachrome into dihydroxyindole carboxylic acid (a catalytic step important in biosynthesis of melanin), MIF was purified and subsequently identified by peptide sequencing from bovine lens tissue [13]. Further investigations of the structure of MIF revealed that the tautomerase/isomerase activity is located at the N-terminal portion with a conserved proline residue at position 2 [27, 28, 52–54]. The three-dimensional protein structure of MIF revealed striking similarities with D-dopachrome tautomerase (DDT/PPT2) although MIF shares solely ~30% amino acid sequence homology with DDT [28, 53] (Figure 2). These findings led to various enzymatic and mutational investigations identifying the N-terminal portion of MIF as essential for tautomerase activity [15, 16, 55]. However, since the finding of MIF's *in vitro* tautomerase activity investigations have focused on the identification of its physiological substrate and biological role which is still ongoing. Genetic studies in the meantime revealed that catalytically dead mutants still exert MIF-specific functions. Moreover, tautomerase-null MIF knock-in mice compensate the MIF gene deletion (MIF<sup>-/-</sup> or MIF null mutant) phenotype which leads to the argument that the tautomerase activity may be possibly dispensable *in vivo* [18, 56, 57]. MIF's highly conserved substrate pocket may represent a vestigial relict reflecting its ancestral origin in innate immunity and be dispensable at least for its function in

promoting cellular growth and tumorigenesis *in vivo* [11, 47, 58, 59]. However, the catalytically dead MIF mutant (P1G-MIF) shows reduced binding to some protein interaction partners, such as its cell surface receptor CD74 and the c-jun amino-terminal kinase activator Jab1/CSN5. This indicates that the N-terminal proline and the catalytic pocket may play a role in protein-protein interaction of MIF with its binding partners [57]. Noteworthy were findings reporting more pronounced phenotype and defects in CD74 knock-out mice (MIF receptor) than in solely MIF-deficient mice [59–64]. This led to the hypothesis that more MIF-like ligands acting on CD74 receptor may exist. The group of Bucala and colleagues recently identified D-dopachrome tautomerase (DDT) as a MIF-like cytokine with overlapping functions [65]. Neutralizing antibodies against DDT can protect mice from lethal endotoxic shock to a comparable extent as MIF neutralization, by reducing circulating TNF- $\alpha$ , IFN- $\gamma$ , IL12, and IL-1 $\beta$  [5, 60, 65–68]. It has subsequently been suggested to redefine DDT as MIF-2 due to their structural homologies and functional resemblance with data on DDT knock-out mice and combined neutralization studies to unravel this renaming.

### 3. MIF Distribution in the Brain

Distribution and microarray expression profiles (BioGPS analysis) of MIF, DDT, and their joint receptor CD74 already suggest spatial overlapping as well as ancillary functions (Figure 1). MIF is widely expressed in the body and shows high levels in lymphocytes, thyroid, prostate, placenta, and lungs. In the murine brain, MIF transcripts and protein are mainly present in the cortex, hippocampus, and pituitary gland [5, 69] and thus differ in distribution and expression level in comparison to DDT (Figure 3). In particular, MIF immunoreactivity has been found in neurons of the hippocampus within fiber structures and terminals such as the mossy fibers of the dentate gyrus and in dendrites of the hippocampal CA regions [69]. Furthermore, MIF is upregulated in neurons and in macrophages following intracranial LPS stimulation. Interestingly, MIF is also found in microglial cells, the resident macrophages of the brain as well as in cerebrospinal fluid (CSF), and shows elevation after experimental LPS treatment, too. Moreover, MIF pretreatment can reduce the number of invading microglial cells and macrophage into allogeneic fetal mesencephalic grafts in rodents [70]. However, this MIF treatment did not affect the outcome on graft function and survival leaving the potential of MIF as a neuroimmune modulator in Parkinson's disease open. It has recently been shown that MIF can promote the growth of neural progenitor cells *in vitro* [71], indicating already a growth-promoting effect in particular cell populations. Contrary to such growth promoting effect is one report on elevated MIF levels in the cerebrospinal fluid of Alzheimer patients and the beneficial effects of MIF inhibition after amyloid  $\beta$  protein-induced neurotoxicity *in vitro* [72]. As indicated above, MIF may function in a context-dependent manner with various effects on different neural and glial cells. The presence of MIF in

hippocampal structures which are prone to glucocorticoid-induced tissue damage has led to speculations of MIF and its association with glucocorticoid action under normal and pathophysiological processes.

### 4. MIF Signaling, Glucocorticoids, and Metabolism

MIF was one of the first cytokine-mediated activities derived from T cells described. It then became apparent that MIF is also expressed by monocytes/macrophages and signals in both an autocrine and paracrine manner [2, 4]. Gene-targeting experiments and neutralization approaches affirmed its upstream role in the inflammatory cascade promoting proinflammatory mediators such as TNF- $\alpha$ , IL-12, IL-1 $\beta$ , and PGE<sub>2</sub> [7, 59, 60]. MIF's role as an autocrine innate immune regulator has been exemplified by its "auto-loop" route through TNF- $\alpha$ , which in turn leads to further MIF secretion in macrophages [73]. Thus, it became apparent that MIF follows two signaling principles. First, MIF executes its biological function as a secreted molecule requiring specific receptor(s) at the cell surface of its target cells, that is, transcellular signaling. Secondly, MIF acts as an intracellular or autocrine signaling molecule with catalytic activity and specific binding partners due to its structural features (intracellular domains and mechanisms; see section above).

The identification of MIF's receptor-mediated signaling gave rise to a hub for the discovery of intracellular and extracellular interaction partners and functions [63, 74–79]. To date receptor-mediated MIF signaling has been identified through the cell surface receptor complexes CD74 (CD74/invariant chain—CD44 signaling complex), CXCR2, CXCR4, and CD74-CXCR2/4 [63, 74, 76, 80] (Figure 4). Especially the structural homology of the canonical CXCL8 ligand, a so called pseudo-(E)LR motif present in MIF and binding to CXCR2 and CXCR4 qualified MIF as a non-cognate chemokine ligand [27, 63, 81]. These receptors bind MIF to the surface of cells and mediate activation of extracellular-regulated mitogen-activated protein (ERK-MAP), phosphatidylinositol 3/protein kinase B (PI3K/AKT), and Src-tyrosine kinases through CD44, already indicating the presence of a link to oncogenic signaling utilized by cancer cells (Figure 4).

In particular, MIF impacts macrophage and lymphocyte functions and thereby regulates innate and acquired immunity [73, 82, 83]. In mice, MIF was cloned as an immunoregulatory peptide from the pituitary gland and was shown to specifically counteract glucocorticoid effects such as suppression of TNF- $\alpha$ , IL-8, and IL-1 $\beta$  secretion [5, 84, 85]. Moreover, MIF's impact on the innate immune system can be fatal in lethal endotoxic shock by counteracting the protective effects of glucocorticoids at various levels [86, 87]. Glucocorticoids and steroid analogues such as dexamethasone are widely used and are most effective anti-inflammatory drugs, acting through various mechanisms and recruiting downstream effectors such as NF $\kappa$ B, histone deacetylase 2 (HDAC2),  $\alpha$ 1 $\beta$ 1 integrin, and phospholipase

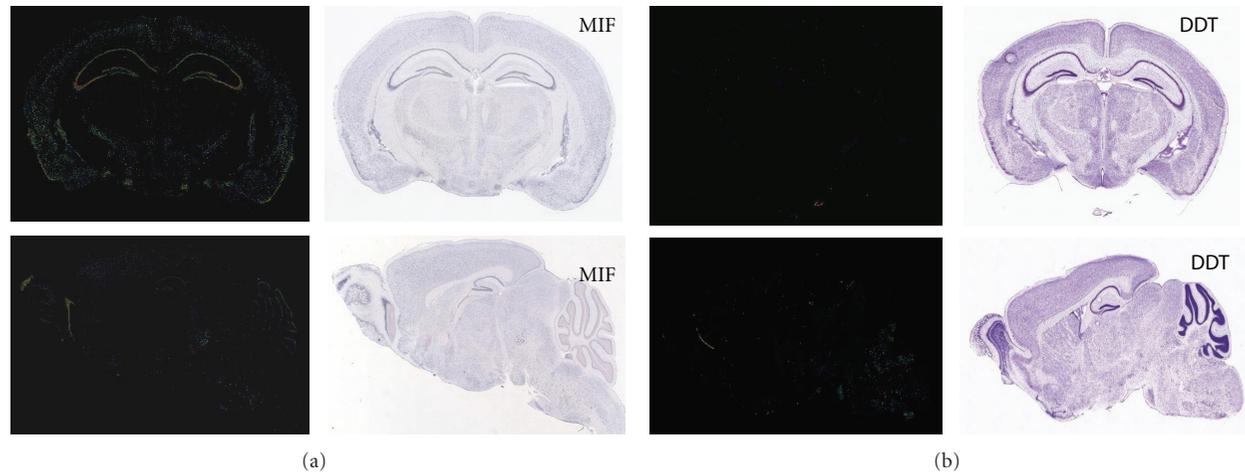


FIGURE 3: MIF and DDT distribution in the brain. Representative in situ hybridization images of MIF mRNA (a) and DDT mRNA distribution (b) in adult mouse brain (left) with consecutive counterstained brain section (Nissl stain, right). Upper panels of (a) and (b) represent coronal plane; lower panels show sagittal plane. Data were provided from the Allan Brain Atlas website (<http://www.brain-map.org/>), and the Brain Explorer 1.3 software was utilized for the visualization of gene expression [39].

A2 (PLA2) [88, 89]. In particular, glucocorticoids have been used for decades for the treatment of various neuroinflammatory, neurotrauma, and neurooncological disease conditions. One reason lies in that glucocorticoids are one of the most powerful classes of agents in reducing tumor-associated edema and tissue swelling and can thus reduce the incidence of fatal herniation in space occupying lesions to a certain extent. MIF in this pathway is therefore of clinical significance.

MIF counteracts glucocorticoid signalling by decreasing  $\text{I}\kappa\text{B}$  levels leading to  $\text{NF}\kappa\text{B}$  activation, upregulates PLA2, and downregulates MAP kinase phosphatase 1 [86, 87, 90]. The bell-shaped MIF regulation by glucocorticoids is worthy of note with increased MIF release from monocytes/macrophages at low physiological amounts of glucocorticoids and inhibited MIF release at high glucocorticoid concentrations [84, 91]. In this manner, MIF inhibition offers an alternative strategy for anti-inflammatory therapy in neuroinflammation such as multiple sclerosis and Guillain-Barré syndrome, although the effects of MIF on prescribed glucocorticoid analogues in patients require further consideration. Hence, MIF can control glucose catabolism in muscle cells by elevating the level of the key enzyme phosphofructo-2-kinase leading to lactate production [92]. MIF also modulates downstream AMP-activated protein kinase effects in cardiac cells such as the glucose transport function [93]. Whether MIF upregulates phosphofructo-2-kinase and glycolysis in brain tumor cells with subsequently increased lactate release has not yet been tested. Since the Warburg effect is one characteristic feature of malignant gliomas (i.e., primary brain tumors derived from glial and precursor cells), further investigation into the metabolic effects of MIF in brain tumor cells would be highly desirable.

## 5. MIF Links Inflammation with Cell Cycle Regulation

MIF has a central role as monocytes/macrophages in the global regulator of monocyte/macrophage-derived cytokines. It is an interesting finding that distinct thresholds of MIF affect monocytes/macrophages differentially. At low concentrations MIF induces the release of  $\text{TNF-}\alpha$ , IL-12, IL-1 $\beta$ , and  $\text{PGE}_2$  and, in a distinct difference from other “common” cytokines, involves MAPK, Akt, and PI3K activation and regulation of Jab1 and p53 [6, 7, 59, 94, 95]. In particular, the latter is involved in the resolution of inflammation by inducing p53-dependent, activation-induced cell death [6]. High and sustained MIF action, for instance, in chronic inflammation, also promotes the release of macrophage effector cytokines such as  $\text{TNF-}\alpha$ , IL-12, IL-1 $\beta$ , and  $\text{PGE}_2$ . On the other hand it also prevents cytoplasmic accumulation of the tumor suppressor gene p53, thus inhibiting apoptosis (Figure 4). This peculiarity of MIF caught the attention of the cancer research field. Bypassing p53-mediated growth arrest is an important feature of cancer cells and of a tumor promoting microenvironment. TP53, the human gene encoding the p53 protein, mutates at a high frequency (approx. 30%) in adult malignant gliomas and glioblastomas. The increased expression of MIF in malignant gliomas is of particular interest since MIF suppresses p53-dependent signaling and thereby enhances susceptibility to further oncogenic mutations. Hence, MIF interacts with Jab1/CSN5 and negatively regulates the cullin-1-containing ubiquitin E3 ligase complex with effects on p27- and E2F1-3-dependent cell cycle control [96, 97]. Conversely, loss of MIF in a p53-deficient background leads to uncoupled DNA damage checkpoint response, thereby aggravating tumorigenesis in  $\text{p53}^{-/-}/\text{MIF}^{-/-}$  mice [96]. It has recently

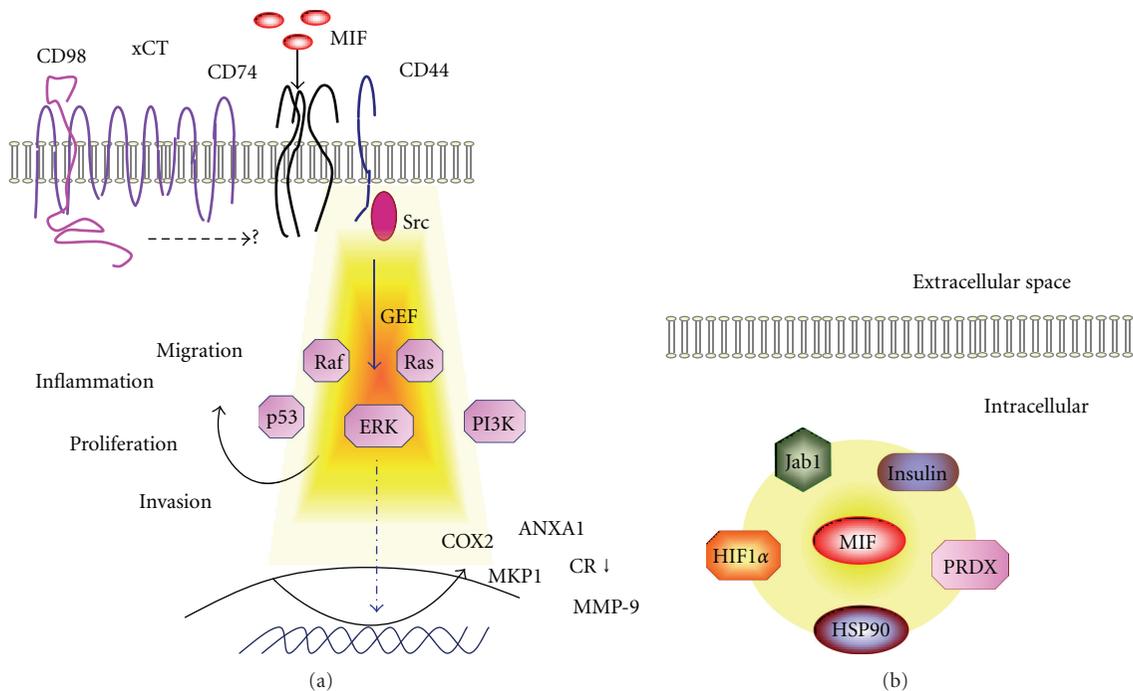


FIGURE 4: MIF receptor signalling and downstream effectors. (a) Schematic model of receptor-mediated MIF signalling involving CD74 and CXCRs. The involvement of the glutamate antiporter xCT (system  $x_c^-$ , xCT forms a heterodimer with CD98 as indicated) in CD74/CD44-dependent signalling is proposed, indicated by the dotted arrow. (b) MIF binding partners with link to brain cancer. Note that the indicated MIF-binding partners given in the scheme are far from complete. Abbreviations used: COX2, cyclooxygenase 2; ERK, extracellular signal-regulated kinases; GC, glucocorticoids; GR, glucocorticoid receptor; GEF, guanine exchange factor; HIF1 $\alpha$ , hypoxia-inducible factor-1; Jab1, Jun-activation domain-binding protein-1; MKP1, mitogen-activated protein kinase phosphatases; MMP-9, matrix metalloproteinase or type IV collagenase/gelatinase B; PRDX, peroxiredoxin; Src, sarcoma protooncogene.

been shown that the chaperone HSP90 stabilizes MIF for E3-ubiquitin-ligase-dependent proteasome degradation in various tumor cells, leading to increased MIF levels even under siRNA-mediated transcriptional silencing [98]. This regulatory protein stabilization feature secures persistent MIF action in cancer cells independent of transcriptional and translational levels.

## 6. MIF, Brain Tumors, Angiogenesis, and Tumor Microenvironment

MIF is produced by neuroendocrine and immune tissues and possesses several features that allow it to be classified as a neuroendocrine mediator [5, 99]. This cytokine has glucocorticoid-antagonist properties within the immune system and participates in the regulation of several endocrine circuits under physiological conditions. Further, initial *in vitro* studies indicate a growth-promoting activity of MIF on neural progenitor cells [71]. In this context, MIF controls the site-specific migration of the immunocompetent cells of the brain, the microglia. These cell entities are considered to be the resident macrophages of the brain and are involved in almost all pathophysiological mechanisms, including trauma, autoimmune and neuroinflammatory disease, and brain tumors. The precise role of these immunocompetent cells of the CNS in tumor progression is subject of much

controversy since its specific role is not yet completely understood. Immunological “escape mechanisms” could play a decisive role in tumor invasion and proliferation.

The association of MIF with the progression of malignant brain tumors places this cytokine in center stage [100, 101]. It is suggested that brain tumors secrete MIF to control the activity of accumulating tumor-promoting cells, which in turn might have inductive tumor-progressive as well as proangiogenic effects [58, 101]. Thus, based on its localization and functional features, MIF would be well in a position to execute important control of the tumor microenvironment. A conceptual framework has been sketched to reflect the metabolic and immune cell complexity of brain tumors in a simplified model classifying the tumor into three distinct zones (Figure 5). Although each border may depict a smooth shift into the next transition zone, Tumor Zone 1 (TZ1) consists of the main tumor—bulk, corresponding to contrast enhancing regions in clinical MRI settings. Here, MIF is mainly produced and secreted into the surrounding tissue. TZ2 represents the area of perifocal edema, which is characterized by its specific proangiogenic microenvironment and transitory glioma cells. Apart from these cells, there is a pronounced accumulation of microglial cells, which also infiltrate the TZ1. The TZ3 is the most challenging and intractable zone for therapeutic intervention, since this zone consists mainly of healthy brain parenchyma. However,

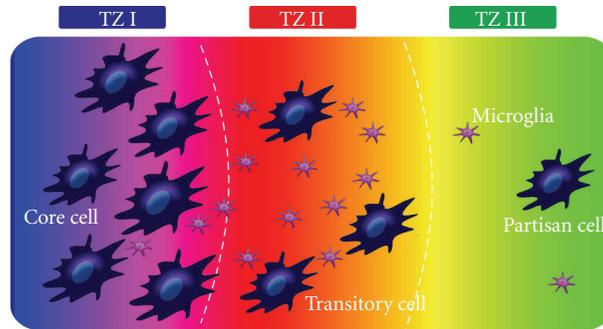


FIGURE 5: The brain tumor microenvironment, heterogeneous tumor zones and MIF actions. Conceptual framework depicting the metabolic and immune cell complexity of malignant brain tumors, (glioblastomas, GBM) is given as a simplified model classifying the tumor into three distinct tumor zones (TZ1–TZ3). Tumor Zone 1 (TZ1) consists of the main tumor—bulk and core glioma cells, corresponding to contrast enhancing regions in MRI images. MIF is mainly produced in TZ1 and secreted into the extracellular space. TZ2 represents the area of perifocal edema, which is characterized by its specific proangiogenic microenvironment and presence of transitory glioma cells. In addition, this tumor zone shows pronounced accumulation of microglial cells, which also infiltrate TZ1. TZ3 is the most awkward zone for therapeutic intervention, since this tumor zone consists mainly of healthy brain parenchyma. However, isolated glioma-initiating cells termed partisan cells colonize TZ3 and are most probably responsible for tumor recurrence following surgery. TZ2 is probably biologically most active, influencing TZ1 and TZ3 by tumor-derived metabolites impacting the immune system, angiogenesis, and cell fate.

isolated glioma-initiating cells termed partisan cells colonize TZ3 and are most probably responsible for tumor recurrence following surgery. The TZ2 is probably biologically most active, influencing TZ1 and TZ3 through tumor-derived metabolites impacting the immune system, angiogenesis, and cell fate. With regard to MIF, however, production and secretion of MIF occur in TZ1, while its receptors are mainly expressed by microglial cells in TZ2 and on glioma cells themselves. MIF could therefore act in a dual fashion both as an autocrine factor as well as a tumor-derived factor which influences the immune microenvironment (Figure 5). Another relevant aspect is that malignant gliomas secrete neurotoxic concentrations of the oncometabolite glutamate as a consequence of their metabolic alterations, and increased glutathione needs [102, 103]. Further, glutamate stimulates the migration and activation of microglial cells [104]. This aspect has not been given much attention from a neurooncological point of view. The metabolic cytokine crosstalk reveals its clinical implication: CD44 as coreceptor of CD74 is also a regulatory component of the glutamate transporter xCT controlling cancer redox state [105]. Additionally, as a specific surface cell receptor in mesenchymal stem cells, CD44 regulates the vascular architecture of highly vascularized tumors such as malignant gliomas through the activation of these stem cells, thereby playing a possible role in their progression. Nevertheless, it needs to be unambiguously demonstrated whether MIF is primarily effective in an autocrine or intracellular manner in malignant gliomas. Thus, further studies on this matter will be decisive for future MIF-neutralizing approaches. Two approaches are available in experimental and clinical studies for the therapeutic targeting of MIF. Firstly, MIF-neutralizing antibodies have been experimentally tested in a murine arthritis model and in rodent glomerulonephritis models with promising efficacy [106–108]. Along the same line, CD74-neutralizing antibodies have been applied to B-cell

malignancies, although comparable data of these approaches are missing. Additionally, soluble CD74 molecules have been isolated *in vitro*. Secondly, there are now effective, small-molecule MIF antagonists available, with ISO-1 being the most widely accepted one [78, 108]. Based on these findings further small compound library screenings and computational drug design studies are now underway. This approach will probably identify promising small-molecule MIF inhibitors in the future. Due to the lack of immunological responses, low-molecular-weight inhibitors are so far most promising for MIF-neutralizing approaches in humans.

Considering data from clinical studies as well, MIF expression also has predictive values, as patients with malignant gliomas and high MIF expression levels show worse prognosis and earlier tumor recurrence [109]. Interestingly, MIF abundance is associated with increased microvessels and elevated IL-8 expression. Moreover, the MIF receptor CD74 has been shown to contribute to temozolomide resistance [109, 110]. Taking all these facts into account, the underlying molecular mediators and metabolites and immunological crosstalk remain only partially understood despite the central role of dysregulated metabolism in brain tumors. A comprehensive understanding of the dynamics and hierarchy of MIF as a glioma-derived oncometabolite as well as immunological and vascular consequences is therefore critical in identifying effective drug targets in the development of multimodal managements of brain tumors. In order to achieve this target, a detailed analysis of MIF action in this disease with high unmet medical need appears mandatory. Future studies will show whether available MIF and CD74 receptor inhibitors could be efficiently used in our armamentarium against malignant brain tumors.

### Conflict of Interests

The authors declare no competing financial interests.

## Authors' Contribution

N. E. Savaskan and I. Y. Eytüpoğlu conceived and designed the paper and figures with contributions from G. Fingerle-Rowson and M. Buchfelder. All authors shaped the final paper.

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## Review Article

# Molecular Regulation of the Mitochondrial $F_1F_0$ -ATPsynthase: Physiological and Pathological Significance of the Inhibitory Factor 1 (IF<sub>1</sub>)

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In mammals, the mitochondrial  $F_1F_0$ -ATPsynthase sets out the energy homeostasis by producing the bulk of cellular ATP. As for every enzyme, the laws of thermodynamics command it; however, it is privileged to have a dedicated molecular regulator that controls its rotation. This is the so-called ATPase Inhibitory Factor 1 (IF<sub>1</sub>) that blocks its reversal to avoid the consumption of cellular ATP when the enzyme acts as an ATP hydrolase. Recent evidence has also demonstrated that IF<sub>1</sub> may control the alignment of the enzyme along the mitochondrial inner membrane, thus increasing the interest for the molecule. We conceived this review to outline the fundamental knowledge of the  $F_1F_0$ -ATPsynthase and link it to the molecular mechanisms by which IF<sub>1</sub> regulates its way of function, with the ultimate goal to highlight this as an important and possibly unique means to control this indispensable enzyme in both physiological and pathological settings.

## 1. Introduction

The  $F_1F_0$ -ATPsynthase is an H<sup>+</sup>-pumping ATPase evolutionary specialized in synthesizing ATP by using an H<sup>+</sup> gradient generated across a biological membrane. It is present in bacteria and intracellular organelles such as chloroplasts and mitochondria. In these, the enzyme is hosted within the inner membrane as part of the OXPHOS, where it couples the transport of H<sup>+</sup> from the intermembrane space into the matrix with the synthesis of ATP, guaranteeing the supply of energy to biological processes, since the majority of cellular ATP is generated by the mitochondrial  $F_1F_0$ -ATPsynthase.

The molecular structure, catalytic mechanism, and regulation of the mitochondrial  $F_1F_0$ -ATPsynthase were described by the seminal work of the Nobel Laureates Mitchell, Boyer and Walker, that revealed its complexity and the functional steps that drive the synthesis of ATP.

Besides its role as energy producer, the mitochondrial  $F_1F_0$ -ATPsynthase is also essential for the maintenance of the mitochondrial membrane potential ( $\Delta\Psi_m$ ) [1]—which

is crucial for the import of proteins into the organelle [2]—and cristae structure [3]. In addition, it is fundamental for an optimal supramolecular organization of the respiratory chain [4], and for regulating the mobilization of cytochrome *c* during apoptosis [5].

In animals and plants, the mitochondrial  $F_1F_0$ -ATPsynthase is molecularly regulated by an endogenous, nuclear-encoded polypeptide, the ATPase Inhibitory Factor 1 (IF<sub>1</sub>). IF<sub>1</sub> is primarily responsible for inhibiting the hydrolysis of ATP by the ATP synthase [6], an event that occurs when the electrochemical proton gradient across the mitochondrial inner membrane is lost (e.g., during hypoxic/ischaemic conditions), and the enzyme reverses to restore  $\Delta\Psi_m$  [7]. A number of studies have shown that IF<sub>1</sub> is also involved in the regulation of the oligomeric state of the  $F_1F_0$ -ATPsynthase, by facilitating the enzyme's dimerization via a molecular link between two F<sub>1</sub> domains [8]; for this reason, it is also implicated in the remodelling of cristae structure [9], and consequently in the regulation of mito-ultrastructure and morphology.

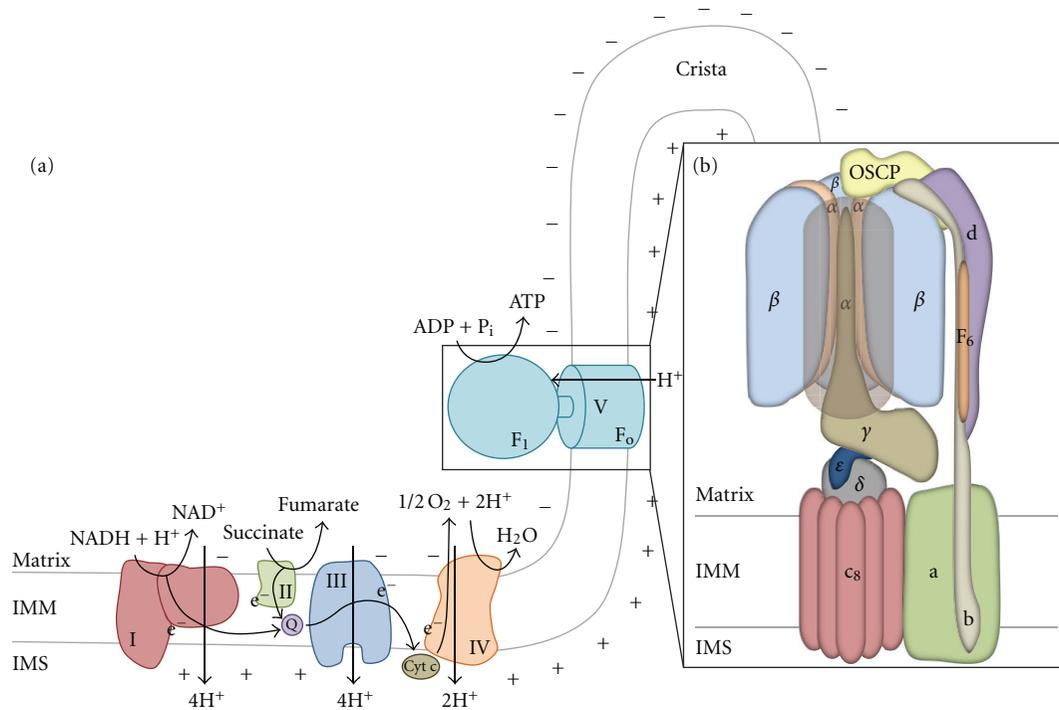


FIGURE 1: Oxidative phosphorylation and the mammalian  $F_1F_o$ -ATP synthase. (a) Scheme of the mitochondrial OXPHOS: it is composed of five complexes, which couple the generation of a proton motive force through the mitochondrial inner membrane (IMM) with ATP synthesis. The first four complexes form the electron-transport chain (ETC), which catalyses the oxidation of NADH and  $FADH_2$  to  $NAD^+$  and  $FAD$  respectively, with the associated reduction of molecular oxygen, to which electrons are transferred, to water. During the process, protons are translocated against a gradient in the intermembrane space by complexes I, III, and IV; the generation of a proton electrochemical potential ( $\Delta\mu_H^+$ ), also called proton motive force (pmf), is achieved, driving the ATP synthesis, which is catalyzed as the final step by the  $F_1F_o$ -ATP synthase (Complex V). The supramolecular organization of the respiratory chain, with the  $F_1F_o$ -ATP synthase localized to mitochondrial cristae, where a higher surface density of protons is realized, allows a better enzymatic performance of complex V. (b) Diagram of the structure of mammalian  $F_1F_o$ -ATP synthase. We can divide the enzymatic complex into 4 principal subdomains: a catalytic headpiece ( $\alpha_3\beta_3$ ), hosting the three catalytic sites for ATP synthesis (one in each  $\beta$  subunit), a proton channel ( $ac_8$ ) and two stalks, the central rotor ( $\gamma\delta\epsilon$ ) and the peripheral stator ( $bd(F_6)OSCP$ ) that link the first two subdomains together. While protons flow through the  $F_o$  channel from the intermembrane space into the matrix, a rotation of the stator inside the catalytic headpiece is induced, allowing a cyclic change in  $\beta$ -subunits conformation and the synthesis of ATP (N.B. Subunits A6L,  $e$ ,  $f$ , and  $g$  are omitted in the scheme).

The interest for this inhibitor, or regulator—as we like to consider it—stems from many reasons; among them, the evidence for a key role in pathologies is the most meaningful but the less explored.  $IF_1$  overexpression is reported in human carcinomas [10], differences in the ratio of expression between  $IF_1$  and the  $F_1F_o$ -ATP synthase are related to changes in cellular responses to ischaemia/reperfusion injury [11, 12], and its absence is recorded in a rare form of mitochondrial myopathy called Luft's disease [13, 14].

Despite this,  $IF_1$  seems underconsidered in pathologies whose etiology correlates with defective mitochondrial  $F_1F_o$ -ATP synthase. Here, we will explain why the interaction between  $IF_1$  and  $F_1F_o$ -ATP synthase is important, and why the quality of cellular bioenergetics depends on it.

## 2. Molecular Structure and Catalysis of the $F_1F_o$ -ATP synthase

The mitochondrial  $F_1F_o$ -ATP synthase is the smallest rotary motor in nature. It is a multisubunit complex ( $\sim 5,000$

aminoacid residues, with a mass of  $\sim 600$  kDa) consisting of an intrinsic membrane domain,  $F_o$  ( $\sim 1,500$  aminoacids), and a globular catalytic domain,  $F_1$  ( $\sim 3,500$  aminoacids), which extends into the mitochondrial matrix (Figure 1). In mammals, the enzyme contains 15 different subunits, nine of which form the  $F_o$  domain ( $a$ , A6L,  $b$ ,  $c$ ,  $d$ ,  $f$ ,  $F_6$ ,  $e$ , and  $g$ ), while the  $F_1$  domain is instead composed of only six ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and OSCP) [15–17]. The Inhibitory Factor 1 ( $IF_1$ ) is often regarded as the 16th subunit, although we will learn in this review that the protein is far more correctly defined as its endogenous regulator.

The enzyme could be ideally divided into 4 principal subdomains: the catalytic headpiece ( $\alpha_3\beta_3$ ), hosting the three catalytic sites for ATP synthesis (one in each  $\beta$ -subunit), the  $H^+$  channel ( $ac_{8-15}$ ) and two stalks, the central rotor ( $\gamma\delta\epsilon$ ) and the peripheral stator ( $bd(F_6)OSCP$ ), that link the first two subdomains together. The remaining minor subunits  $e$ ,  $f$ ,  $g$ , and A6L are associated with the proton channel; in particular, subunits  $e$  and  $g$  are involved in the dimerization of the complex [18] (a scheme of the structure of the ATP synthase is reported in Figure 1).

The crystal structure of the mitochondrial  $F_1$ -ATP synthase, extracted from bovine heart mitochondria, was revealed at the beginning of the Nineties [19, 20]. In this structure,  $F_1$  appears like a flattened sphere of 80 Å in height and 100 Å in width, with three  $\alpha$ - and three  $\beta$ -subunits arranged alternately forming a cylinder around the coiled-coil structure of the  $\gamma$ -subunit. Sequences of  $\alpha$ - and  $\beta$ -subunits are 20% identical [20], and this homology is reflected in their similar folds. All  $\alpha$ -subunits have almost the same conformation, while the three  $\beta$ -subunits adopt three different tertiary structures and are in three diverse nucleotide-bound states: the first, named  $\beta_{TP}$ , hosts the binding site for ATP; the second, called  $\beta_{DP}$ , binds with high-affinity ADP and  $P_i$ ; the third,  $\beta_E$ , does not efficiently bind any nucleotide. This asymmetry in the conformation and nucleotide occupancy of  $\beta$ -subunits supports the binding change mechanism of catalysis theorized by Boyer in the early 1970s, and fully developed in 1993 [21, 22]. According to this model, the three catalytic sites can be in three different conformations at any given time.  $\beta_{TP}$  and  $\beta_{DP}$  have, respectively, a “tight” (T) and a “loose” (L) form, in which ADP and  $P_i$  are converted into ATP, while the “open” conformation (O), adopted by  $\beta_E$ , permits the release of the newly formed ATP and the acceptance of another molecule of ADP. ADP +  $P_i$  are theorized to enter into an O  $\beta$ -subunit, which then assumes an L conformation allowing the synthesis of ATP. After the synthesis, subunit  $\beta$  is in the T conformation, before assuming the O conformation in order to release ATP. The sequential interconversion between these different conformations, driven by the rotation of the central  $\gamma$ -subunit relative to the  $(\alpha\beta)_3$  subcomplex, enables the catalysis.

Unfortunately, there is less structural information on the  $F_o$  domain of ATP synthase than the  $F_1$  complex. In the ring, subunits  $c$ , whose structure was determined by both NMR spectroscopy [23, 24] and X-ray crystallography [25], folds as a hairpin composed by two transmembrane  $\alpha$ -helices linked by a polar loop; part of the ring is in contact with subunit  $a$ , formed presumably by five transmembrane  $\alpha$ -helices and containing two proton half-channels that do not span the membrane [26].

To briefly describe the functioning of the  $F_1F_o$ -ATP synthase and its rotational catalysis, we must divide it into two parts: a central “rotor” ( $F_1\gamma\epsilon$ - $F_o c_{10}$ ) and a surrounding “stator” ( $F_1\alpha_3\beta_3\delta$ - $F_o ab_2$ ). The driving force for the rotation is generated by the electron-transport chain (ETC) and is based on the magnitude of the proton electrochemical potential across the inner membrane; this gradient allows  $H^+$  to flow through the  $F_o$  domain (the  $c$ -ring) causing the rotation of the whole “rotor.” The precession of the  $\gamma$ -subunit, which contacts only one  $\beta$ -subunit at a time, induces cyclic changes in the “stator” so that ATP can be synthesized.

The rotational mechanism is extremely complex and depends on the structures of subunits  $a$  and  $c$ ; the latter contain, in the middle of their C-terminal  $\alpha$ -helix, a critical aminoacid residue, D61, which can either be in a protonated (lipophilic) or unprotonated (hydrophilic) form. Protonation and deprotonation of this aspartic acid residue is at the basis of the  $c$ -ring rotation dictating the membrane

affinity of the single subunits. This occurs only in an aqueous environment and is realized when subunit  $c$  is in contact with subunit  $a$ , and the aspartate residue is hosted in one of the proton half-channels (see [27] for further details). In respiring mitochondria, the  $H^+$  motive force ensures the entrance of  $H^+$  residing in the intermembrane space into the cytosolic half-channel. The  $[H^+]$  is more than 25 times higher on the cytosolic side than on the matrix side, and the  $\Delta\Psi_m$  of 140–180 mV increases the  $[H^+]$  near the mouth of the cytosolic half-channel, thus resulting in protonation of the enclosed aspartate. Once the key residue is protonated, the  $c$ -ring can rotate clockwise (looking from the intermembrane space side), and the D61 of a new subunit  $c$  enters the matrix half-channel of subunit  $a$ , releasing a  $H^+$  into the mitochondrial matrix. Thus, the difference in  $[H^+]$  and potential on the two sides of the membrane leads to different probabilities of protonation through the two half-channels, which yields directional rotational motion. The coupling between the rotation of the  $c$ -ring and the conformational changes in the  $(\alpha\beta)_3$  barrel is guaranteed by the tight link of the ring with the central stalk; while the  $c$ -ring rotates, the  $\gamma$ -subunit turns inside the  $\alpha_3\beta_3$ -hexamer, whose rotation is blocked by the presence of the peripheral stalk. Thus, the proton-gradient-driven rotation of the  $c$ -ring drives the rotation of the  $\gamma$ -subunit, which in turn promotes the synthesis of ATP through the binding-change mechanism. A highly conserved acidic cluster sequence in the C-terminal helical domain of the  $\beta$ -subunit (the DELSEED motif) is thought to be essential for ATP synthesis by coupling catalysis and rotation [28]. After the binding of an adenosine nucleotide in the catalytic site, the C-terminus is lifted up to the nearly immobile N-terminal part of the protein, and the  $\beta$ -DELSEED sequence is moved in contact with the  $\gamma$ -subunit, probably allowing the coupling of the  $\gamma$ -subunit torque with the rotation of the  $F_1$  domain (this hypothesis is still controversial [29]).

Each 360° rotation of the  $\gamma$ -subunit leads to the synthesis and release of three molecules of ATP. The number of subunits in the  $c$ -ring, which ranges from 8 (in bovine and probably in all vertebrates and invertebrates  $F_o$  domain [30]) to 15 (in *Spirulina platensis* [31]), determines the number of protons that are required to generate a molecule of ATP.

### 3. Localization and Regulation of the Enzyme

Mitochondria are the sites where cellular energy is most abundantly produced, due to the constant activity of the mitochondrial  $F_1F_o$ -ATP synthase. Although publications have suggested that it may also be localized on the plasma membrane [32, 33], we shall be exclusively discussing and referring to that embedded in the mitochondrial inner membrane.

As the  $F_1F_o$ -ATP synthase is a reversible nanomotor, it can also hydrolyze ATP by translocating  $H^+$  from the matrix into the intermembrane space (an event that vigorously occurs when the enzyme is in isolation [34]). It does so to maintain the mitochondrial membrane potential ( $\Delta\Psi_m$ ) at a suboptimal level during deenergized conditions that occur

when respiration is impaired by defects in the activity of the ETC, or when the mitochondrial inner membrane is leaky due to alterations in its structural integrity [35]. The  $\Delta\Psi_m$  is not only important for ATP production, but also for mitochondrial protein import and assembly [2]. Disruption of the  $\Delta\Psi_m$  is therefore implicated in various apoptotic phenomena (see [36]), being its maintenance crucial for cell viability.

The reversal of the  $F_1F_0$ -ATP synthase is avoidable in eukaryotes and the enzyme must be controlled to prevent futile hydrolysis of ATP when the transmembrane proton electrochemical gradient collapses. Only facultative anaerobic bacteria employ this method for generating a vital proton electrochemical gradient in the absence of oxygen [37].

When reversal of the  $F_1F_0$ -ATP synthase does occur, the depletion of cellular ATP can be more or less severe depending on the energy requests of the tissue, but in organs with high ATP demand, like brain or skeletal muscle, or in case of augmented ATP request, cellular demise is tangibly accelerated.

Apart from pathological states, repression or upregulation of ATP synthesis normally occurs in physiological conditions when intracellular ATP levels are, respectively, sufficiently high or too low. It has been calculated that, in eukaryotic cells, the rate of ATP utilization changes by a factor of 5–10 [38], such as during exercise and/or acclimatization.

Several are the mechanisms by which the activity of the  $F_1F_0$ -ATP synthase is regulated: (a) *transcriptional factors* [39, 40]; (b) *translational control* [41–43]; (c) *modulation of the electron transport chain or the citric acid cycle* [44, 45]; (d) *ADP inhibition* [37] and (e) *regulatory proteins*, such as  $IF_1$ . Although recent evidence has also suggested that the oncoprotein Bcl-XL interacts with the  $F_1F_0$ -ATP synthase [46, 47],  $IF_1$  is the only molecular regulator of the enzyme characterized both biochemically and functionally. Nonetheless, other proteins which directly interact with the enzyme have been identified, such as factor B [48], essential for ATP synthesis and implicated in the regulation of the  $F_1F_0$ -ATP synthase oligomerization, CaBI [49], which may upregulate the enzyme in response to increased cytoplasmic  $Ca^{2+}$ , and finally S100A1 [50], which has been found to enhance the enzymatic performance in cardiac muscle.

#### 4. The Inhibitory Factor 1 ( $IF_1$ )

$IF_1$  was discovered in 1963 by Pullman and Monroy [6] in mitochondria from bovine hearts (a schematic representation of the structure of bovine  $IF_1$  is reported in Figure 2(a)). To date,  $IF_1$  homologues have been isolated from other mammals (e.g., rat [51] and human [52]), yeast (*Saccharomyces cerevisiae* [53] and *Candida utilis* [54]) and plants [55].  $IF_1$  is a small, basic, heat-stable protein of approximately 10 kDa (in mammals the mature form of the polypeptide is composed of a number of amino acid residues which ranges from 81 in human, chimpanzee, dog and mouse, to 84 in cow). It is predominantly compartmentalized inside the mitochondrial matrix (Figure 2(b)), although studies have proposed that  $IF_1$  is also present in the cytosol and on the plasma membrane [56], as well as secreted into

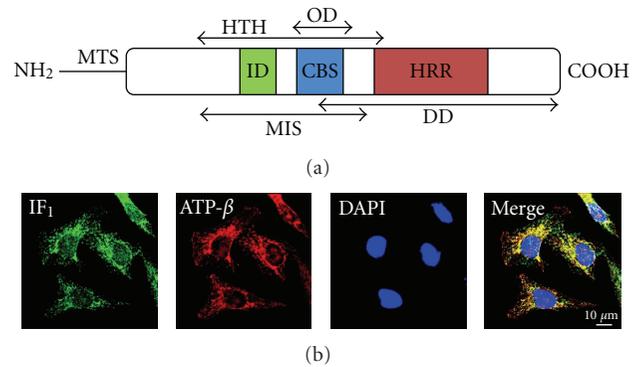


FIGURE 2:  $IF_1$ : structure and intracellular localization. (a) Schematic representation of bovine  $IF_1$ . The mature protein is composed of 84 residues and is  $\alpha$ -helical along most of its length; an amine-terminal presequence of 25 aminoacids represents the mitochondrial targeting sequence (MTS) required for the trafficking of  $IF_1$  into the mitochondrial matrix. In complex,  $IF_1$  shows an ordered *N*-terminal region, which adopts a helix-turn-helix structure (HTH: residues 14–50) and is flanked by two disordered regions. The inhibitory domain (ID) is located at the *N*-terminus and is part of the minimal inhibitory sequence (MIS: residues 14–47) necessary for a correct interaction with the  $F_1$  domain of the ATP synthase. A calmodulin-binding site (CBS: residues 33–42) have been identified at positions 33–42, followed by a histidine-rich region (HRR: residues 48–70) which is implicated in the pH-sensing mechanism and hence in the dimerization. The dimerization of  $IF_1$  depends on the C-terminal region, which hosts the dimerization domain (DD: residues 37–84), while the oligomerization domain (OD: residues 32–44) is located in the *N*-terminal region of the protein, so that after oligomerization the inhibitory domain is hidden and the protein inactivated. (b) Immunocytochemical localization of  $IF_1$  in HeLa cells: the preferential mitochondrial matrix compartmentalization of the protein is shown by its colocalization with the ATP synthase. Cells were costained with anti- $IF_1$  and anti- $F_1F_0$ -ATP synthase  $\beta$  chain antibodies, while DAPI was used for nuclear counterstaining.

the extracellular environment, where it is implicated in the modulation of the activity of endothelial cells [32]. Intriguingly, in this very extramitochondrial localization, a role for hepatic HDL-cholesterol and triglyceride metabolism was also proposed [32, 57].

$IF_1$  interacts with the catalytic subunit of the  $F_1F_0$ -ATP synthase, inhibiting the hydrolysis of ATP under conditions that favour the reversion of the enzyme activity (Figure 3(b)). The regulatory protein is therefore an indispensable component to protect the cell from ATP depletion-driven damage and demise.

$IF_1$  completely inhibits, through a noncompetitive mechanism, the ATP-hydrolyzing activity of the  $F_1F_0$ -ATP synthase without affecting the synthesis of ATP during oxidative phosphorylation, although a few studies argue differently on this [58, 59]; nevertheless,  $IF_1$  is reported to be largely active only at low pH [60], hence in conditions of ATP hydrolysis. The inhibitory protein binds to the soluble  $F_1$  domain in a 1 : 1 stoichiometry in the presence of  $Mg^{2+}$  and ATP [61]. This is important for maintaining cellular ATP,

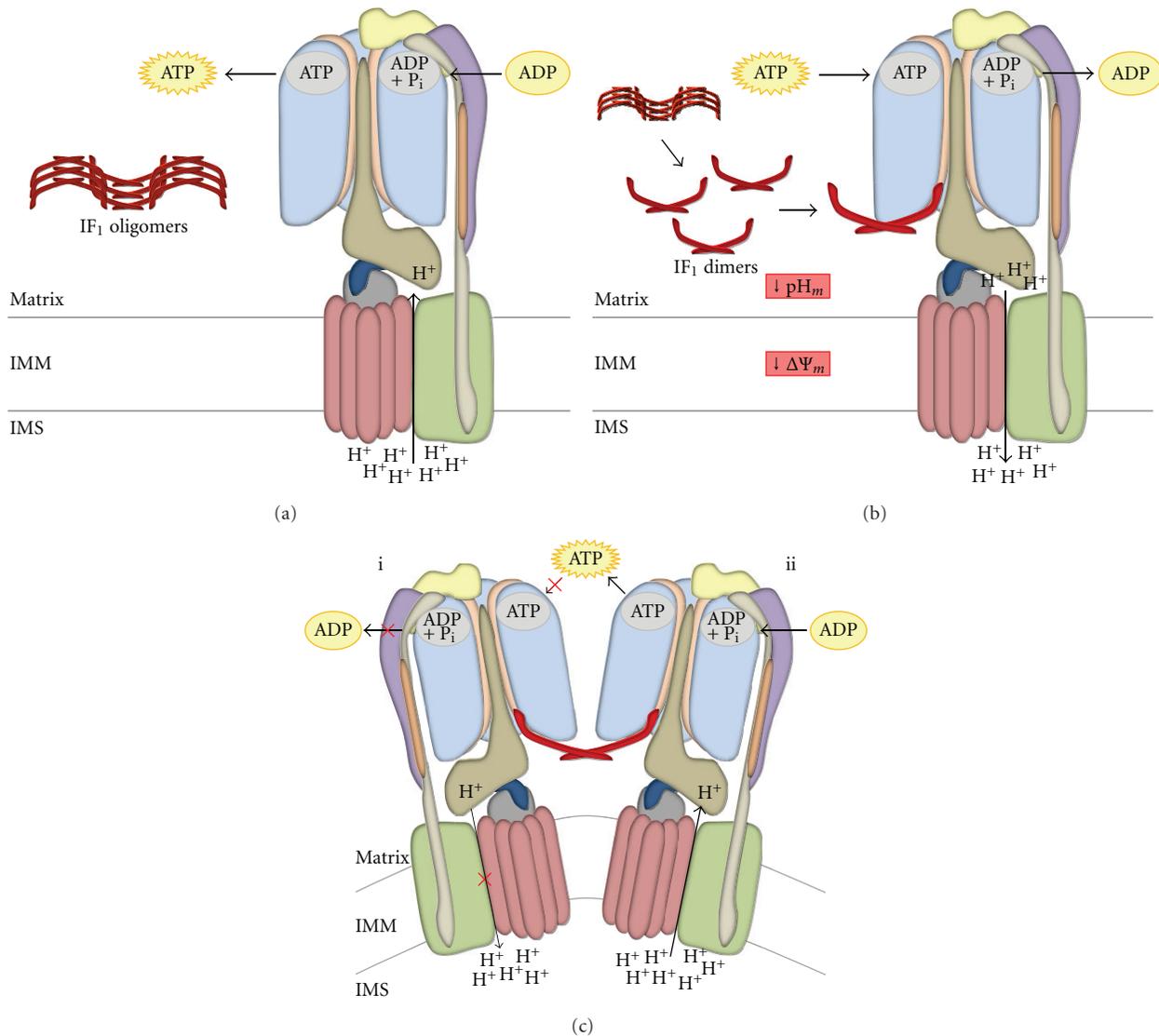


FIGURE 3: Interaction of IF<sub>1</sub> with the F<sub>1</sub>F<sub>0</sub>-ATP synthase. When mitochondria are in normal “energized” conditions (a), the F<sub>1</sub>F<sub>0</sub>-ATP synthase can sustain physiological levels of ATP synthesis thanks to the presence of sufficient mitochondrial inner membrane potential; in this situation, the matrix pH is slightly basic, and IF<sub>1</sub> is predominantly present in its inactive, oligomeric form. When the electrochemical H<sup>+</sup> gradient is lost, the F<sub>1</sub>F<sub>0</sub>-ATP synthase starts hydrolysing the ATP imported from the cytosol to pump H<sup>+</sup> back into the intermembrane space (b), restoring ΔΨ<sub>m</sub>. The augmented [H<sup>+</sup>] in the matrix causes a fall in pH that induces the disruption of IF<sub>1</sub> oligomers and the release of free active dimers. The binding of IF<sub>1</sub> dimers at the interface between α- and β-subunits of the F<sub>1</sub> domain is responsible for the selective inhibition of ATP hydrolysis (c-i), while its synthesis is not affected (c-ii). Active IF<sub>1</sub> is able to interact with two F<sub>1</sub> domains at the same time, inducing the dimerization of the F<sub>1</sub>F<sub>0</sub>-ATP synthase (c), with subsequent increased enzymatic performance and cristae formation.

by preventing its hydrolysis, when the H<sup>+</sup> electrochemical gradient across the mitochondrial inner membrane is lost (e.g., during hypoxic/ischaemic conditions) and the enzyme reverses its activity to transiently restore ΔΨ<sub>m</sub> [58, 59].

IF<sub>1</sub> is encoded by the *ATPIF1* gene, localized at chromosome 1, and is synthesized as a propolypeptide (106 residues in humans [52]) harbouring a highly conserved N-terminal presequence of 25 amino acids (identified by the comparison between the amino acid sequences deduced from the cDNA and the purified protein) presumably important for the mitochondrial targeting of the protein [61]. The mature

polypeptide (81–84 aa in mammals) is significantly conserved among various species. The human protein exhibits 67–74% of sequence homology with other known mammalian IF<sub>1</sub> inhibitors, and also partial homology with the yeast inhibitor Inh1p [52]. Interestingly, there is a strong correlation between the high sequence conservation and function, as IF<sub>1</sub> from one species is able to inhibit the F<sub>1</sub>F<sub>0</sub>-ATP synthase from another, including yeast [51, 62, 63]. Conversely, yeast IF<sub>1</sub> is not able to inhibit the animal F<sub>1</sub> domain because its activity is stabilized by accessory proteins which have no homologues in animals [64].

The most conserved segment of the inhibitory factor is the region that interacts with the  $F_1F_0$ -ATP synthase, comprising amino acid residues 20–50 (90% identity between the human and bovine sequences, 80% between bovine and rodent [61]), and a lot of research into understanding the minimal inhibitory sequence of  $IF_1$  has focused on this sequence. Van Raaij and coworkers investigated this [65], by measuring the activity of several truncated forms of the protein. The intact and truncated forms assayed for inhibition of  $F_1F_0$ -ATP synthase using  $IF_1$ -depleted submitochondrial particles revealed that the minimal inhibitory sequence consists of residues 14–47.

In 2001, Ichikawa and colleagues [66] showed by amino acid replacement that in yeast five residues (F17, R20, R22, E25 and F28) are essential for the inhibitory activity of the protein. Two years later it was brought up that six homologous residues of bovine  $IF_1$  (F22, R25, E26, Q27, E30, and Y33), which form a cluster on the surface of the  $\alpha$ -helix [67], represent the inhibitory site of the protein [68].

## 5. $IF_1$ : Molecular Structure and Conformational Changes

The molecular structure of  $IF_1$  was initially characterized by Cabezon et al. in 2001 [67] (2.2 Å resolution X-ray crystallographic analysis). The protein is  $\alpha$ -helical along most of its length ( $\sim 90$  Å [69]) and is active as a dimer at low pH ( $<6.7$ ). The dimerization of two  $IF_1$  monomers involves their C-terminal regions (residues 37–84 [70]), which form an antiparallel double-stranded coiled-coil unit stabilized by complementary hydrophobic interactions between the two helices, involving residues 49–81 [67]. Within the dimer, the two minimal inhibitory sequences are at the opposite ends (the dimer shows an end-end distance of at least 130 Å, while the distance between the two inhibitory regions is 62 Å) and can react with two  $F_1$  domains simultaneously (Figure 3(c)). When the pH is above neutrality (7.0–8.0), the dimers can assemble inactive tetramers or higher oligomers by forming antiparallel coiled-coils in the N-terminal regions (residues 32–44 are involved [70]). Every protomer of the dimer can participate in two coiled-coil units with two different helices, binding two dimers simultaneously. The formation of the oligomers masks the inhibitory sequences of the dimers, so that  $IF_1$  cannot bind the  $F_1F_0$ -ATP synthase (Figure 3(a)). Dimers and oligomers are in equilibrium at pH 6.5 [70].

Mammalian  $IF_1$  contains five highly conserved histidines (at positions 48, 49, 55, 56 and 70) that, if chemically modified or replaced, lead to a complete loss of the pH-susceptible activity of the protein without affecting its inhibitory capacity [60, 71]. This histidine-rich region (residues 48–70) is involved in the pH sensing mechanism of bovine  $IF_1$ , and undergoes conformational changes depending on acidity or alkalinity of the environment [61]. Critical for the pH-dependent interconversion between the two aggregation states of the polypeptide is the histidine 49 [70, 71].

It was observed that replacement of this residue with a different amino acid induces full activation of  $IF_1$  at pH 8, and abolishes the ability of the dimers to form oligomers

[67, 71]. The five histidines seem to be important for the pH-regulated decrease in activity between pH 6.7 and 8.0, even if they may not represent the only mechanism responsible for such regulation. In fact, a pH-dependent activity was also observed in the  $IF_1$  22–46 peptide [72] and detected in a 12-residue segment from 32 to 43 [70]; moreover, H49 is not conserved in yeast, suggesting a diverse pH-sensitivity of the protein [73].

Apart from controlling the oligomerization of the polypeptide and consequently the availability of its inhibitory site, the pH itself was proposed to represent the switch between inactive and active forms by controlling the helical content and the flexibility of the whole protein [74]. At low pH ( $\sim 6.7$ ), the helical content seems to be lower, so that the N-terminal region is less ordered and, instead of forming a coiled-coil unit with two other dimers, assumes the correct conformation for binding the  $F_1$  complex. However, this theory is controversial since pH was thought to simply act by modulating the electrostatic interactions between the polar residue-enriched N-terminal regions of the dimers, as presented by Cabezon et al. [67]. Recently, Ando and Ichikawa [73] discovered that pH could effectively change the conformation of the active site by acting on a highly conserved glutamate residue, E26 in bovine  $IF_1$  or E21 in yeast  $IF_1$  (H49 is not conserved in yeast and, as a consequence, cannot represent the only pH sensor residue of the protein). The mechanism of pH-dependency mediated by glutamate regulates only the inhibitory activity of the  $F_1$ -binding site and not the aggregation state of the polypeptide.

Very little is known about the transcriptional and post-transcriptional regulations of  $IF_1$ , despite speculations on a possible contribution by the hypoxia-inducible factor 1- $\alpha$  (HIF1- $\alpha$ ) [75] and evidence for a downregulation mediated by the Immediate early response gene X-1 (IEX-1) [76].

## 6. $IF_1$ in Complex with the $F_1F_0$ -ATP synthase: Outcome on Mito-Ultrastructure

The molecular crystal structure of bovine  $IF_1:F_1$ -ATPase complex is available [69, 77]. In the complex,  $IF_1$  shows an ordered N-terminal region and a disordered C-terminal part. The former adopts a helix-turn-helix structure, in which the two  $\alpha$ -helices extend between residues 14–18 and 21–50 and are linked by a turn from residues 19–20; residues 4–37 are directly involved in binding the  $F_1$  domain. Residues 4–18, which are disordered in the dimer, are instead resolvable after binding.

In the bound form, the inhibitory sequences of the two protomers in the dimer become closer (their distance is shortened from 62 Å to 31 Å). This is possibly due to the flexibility of the C-terminal coiled coil region, which probably has a greater curvature in the complex. The long helix at the N-terminal region of  $IF_1$  is inserted almost completely into the  $F_1$  domain; only residues 47–50 lie outside of it. This points to the central axis of the  $\gamma$ -subunit and forms a  $\sim 45^\circ$  angle with it, heading from the external surface towards the central cavity.

The contacts between the bovine inhibitor and the inhibited  $F_1$  domain are essentially located at the interface between the  $\alpha_{DP-}$  and  $\beta_{DP-}$  subunits, even though  $IF_1$  also contacts small portions of the  $\gamma-$ ,  $\alpha_E-$  and  $\beta_{TP-}$  subunits [77]. Although the inhibitory sequence is comprised of residues 14 to 47, this is not the only region that interacts with the  $F_1F_0$ -ATP synthase. In fact, as demonstrated by the time-dependent loss of inhibition seen by van Raaij and co-workers [65], residues 1–13 and 48–56 are important for stabilizing the structure (the first peptide interacts directly with the  $F_1$  domain, while the second probably contributes to stabilize only the  $IF_1$  dimer).

With different approaches, Cabezon et al. [77] and Ichikawa et al. [66] found that a cluster of six residues, F22, R25, E26, Q27, E30 and Y33 in bovine  $IF_1$ , is essential for the inhibitory activity of the peptide. In the crystal structure of the  $IF_1:F_1$  complex, the three core residues of the cluster, F22, E26 and E30, interact with three highly conserved residues of  $\beta_{DP}$  ( $\beta R408$ ,  $\beta R412$ , and  $\beta E454$ ) which are essential for the enzymatic catalysis and regulation of the  $F_1F_0$ -ATP synthase [78]. E30 directly interacts with the  $\beta$ -subunit of  $F_1$  and seems to be essential for the  $F_1$ - $IF_1$  interaction (Figure 3). Interestingly, one of the three residues, E26, is also implicated in the pH sensing mechanism of  $IF_1$ . In 2008, Ando and Ichikawa [73] discovered the key role of the glutamate residue in the pH-dependent activity of the inhibitory protein. They proposed that it was the high pH, by inducing the dissociation of the carboxyl group of E26, to affect the conformation or direction of the side chain of the neighboring residue E30, thus destabilizing the interaction between  $IF_1$  and the  $F_1$  domain.

As proposed by Walker and co-workers [69],  $IF_1$  seems to inhibit the hydrolysis of ATP that occurs in mitochondria under hypoxic conditions by initially binding to the  $\alpha_E/\beta_E$ -interface of the  $F_1$  domain, an event that appears to require the presence of ATP in the active site. It was also suggested that the binding of ATP induces a conformational change in the  $\beta_E$ -subunit, which creates the binding site for  $IF_1$  [77].

Garcia and colleagues provided the first compelling evidences for a role of  $IF_1$  in promoting the dimerization of the  $F_1F_0$ -ATP synthase, and for its involvement in the biogenesis of mitochondrial cristae [79]. Moreover, they also showed that, by increasing the expression of  $IF_1$  in rat liver or AS-30D hepatoma mitochondria, a rise in the dimer/monomer ratio of the  $F_1F_0$ -ATP synthase (correlated with an increase in the enzyme activity) is obtained, while the removal of the inhibitory protein from rat liver or bovine heart mitochondria resulted in a reduction of that ratio [8]. The dimerization of the enzyme is essential for a correct biogenesis of mitochondrial cristae; in fact, it represents a prerequisite for the generation of larger oligomers with a ribbon-like structure that promotes curvature and growth of tubular cristae membranes [80].

In a recent study, we demonstrated the pivotal role of  $IF_1$  in cell physiology through promotion of the  $F_1F_0$ -ATP synthase dimerization. Briefly, we showed that  $IF_1$  overexpression efficiently increase the activity and the ratio of dimeric to monomeric forms of the  $F_1F_0$ -ATP synthase, with augmented cristae number, mitochondrial membrane

stability, and mitochondrial volume [81], thus ensuring a correct mitochondrial inner structure. This is a phenomenon of secure relevance for apoptosis.

## 7. $IF_1$ in Cell Pathology: from Limitation of the Mitochondrial ATP Consumption to Anaemia

Decreased  $\Delta\Psi_m$  induces the reversion of the  $F_1F_0$ -ATP synthase, which starts hydrolysing ATP in the attempt to restore the  $H^+$  gradient through the mitochondrial inner membrane, transforming mitochondria in ATP consumers (Figure 3(b)). This condition associates with ischaemia, in which the interruption of tissue blood flow causes a reduction of cell oxygenation (hypoxia) inhibiting mitochondrial respiration. Clear evidence for the reversal of the  $F_1F_0$ -ATP synthase during ischaemia can be obtained by using an ETC inhibitor (e.g., rotenone, which acts on complex I, or NaCN, which inhibits complex IV) and concomitantly adding oligomycin, an antibiotic that blocks the  $F_1F_0$ -ATP synthase. These cells will experience a reduced depletion of ATP compared to those bathed with the ETC inhibitor but without oligomycin.

The negative effect of the reversal of the  $F_1F_0$ -ATP synthase is coupled to the reversal of the adenosine nucleotide translocator (ANT) [82], an IMM transmembrane complex that, in physiological conditions, mediates the exchange of cytosolic ADP and mitochondrial matrix ATP (utilizing the different gradients between the two compartments). Reverse activities of both  $F_1F_0$ -ATP synthase and ANT transform mitochondria from ATP producers to ATP consumers, leading to massive cytosolic ATP depletion in hypoxic cells following ischaemia.

Reduced intracellular ATP level is flanked by elevated cytoplasmic  $H^+$ ,  $Na^+$ , and  $Ca^{2+}$  concentrations, inducing osmotic loading and mitochondrial/endoplasmic reticulum injury so that, during ischaemia, death of hypoxic cells by necrosis easily occurs.

Early reperfusion minimizes the extent of cellular damage, salvaging cells within ischaemic regions from necrosis, but it can also cause lethal injury to cells with severe ischaemia-induced metabolic derangements (reviewed in [83]). In the latter case, reperfusion alters the activity of plasma membrane transporters (e.g., abolishing acidosis-mediated inhibition of the  $Na^+$ - $Ca^{2+}$  exchanger and inducing the activation of calpain, which disturbs  $Na^+$ - $K^+$  pump function), thus leading to massive influx of  $Ca^{2+}$  into the cytosol and mitochondrial  $Ca^{2+}$  overload. At the same time, resupply of oxygen to mitochondria restores ATP production but also induces a rise in reactive oxygen species (ROS) production. Both mitochondrial  $Ca^{2+}$  overload and augmented ROS levels represent the prelude to the opening of the mitochondrial permeability transition pore (mPTP) and cell death.

The entity of the reversal of the  $F_1F_0$ -ATP synthase and its action as an  $H^+$  motive ATPase during oxygen deprivation were shown by Jennings et al. in 1991 [7]. Studying the changes in ATP depletion and anaerobic glycolysis in totally ischaemic dog heart after inhibiting the  $F_1F_0$ -ATP synthase

via oligomycin, the same authors have evaluated that about 35% of ATP utilization during the first 90 minutes of total ischaemia in the dog heart is due to the reversion of the enzyme activity.

Oligomycin-mediated inhibition slows down ATP depletion during ischaemia. Rouslin and colleagues [11] proved that the antibiotic has a very small and transient effect on mitochondrial function when used in fast heart-rate animals, like rats, if compared to slow heart-rate species, like larger mammals are. The same authors have subsequently shown that this diversity depends on the different  $F_1F_0$ -ATPsynthase:  $IF_1$  ratios, with a diverse ability to inhibit mitochondrial-driven consumption of ATP when needed [84].

The estimation was that  $IF_1$  reduces the ATP-hydrolysing activity of  $F_1F_0$ -ATPsynthase during ischaemic conditions by up to 70–80% (in slow heart-rate species) [85], thus preventing cellular damage due to ischaemic conditions and delaying cell death when oxygen and glucose are limited. Upon reperfusion, the binding of  $IF_1$  to the  $F_1F_0$ -ATPsynthase is quickly reversed [86], so that sublethal ischaemic episodes could be followed by a relatively rapid recovery of intracellular ATP.

Although this model is challenged by later evidence showing that in rat heart, during ischaemic preconditioning, mitochondrial ATP hydrolysis is inhibited probably as a consequence of the binding of  $IF_1$  [87], the variations in ratio between the enzyme and its controller among animal species are still a fascinating possibility. Nonetheless, this ratio differs per se among organs and cell types of the same organ [12].

By modulating the expression of  $IF_1$  in human (HeLa) and murine (C2C12) cells, we demonstrated that, when  $IF_1$  is overexpressed, cells show a decrease in ATP consumption [81]. Thus, variations in  $IF_1$  expression could influence cellular or tissue resistance to ischaemic injury in different species or cell types.

**7.1. Central Nervous System.** Notably,  $IF_1$  expression is elevated in highly oxidative cells, like neurons and kidney proximal tubules [12], which are highly susceptible to mitochondria deregulations. In the central nervous system, for example, neurons and astrocytes show a great difference in the  $IF_1 : F_1\beta$ -subunit ratio, which is  $\sim 1.45$  in the former and  $\sim 0.8$  in the latter; as a consequence, the inhibition of respiration with NaCN causes a progressive loss of  $\Delta\Psi_m$  in neurons, while in astrocytes the proton gradient is maintained at a new steady state [81]. Thus, higher levels of  $IF_1$  could be advantageous in cells highly depending on oxidative phosphorylation by preventing ATP depletion and quick cellular damage during ischaemia.

**7.2. Preconditioning.** A final interesting aspect is the highly probable involvement of  $IF_1$  in the ischaemic preconditioning mechanism. This phenomenon, which is characterized by the acquirement of a strong resistance to ischaemia in tissue undergoing brief, repeated periods of sublethal ischaemia, is commonly observed in heart, skeletal muscle and brain [61]. It is described as a slowing of energy metabolism with a decreased rate of ATP depletion during ischaemia

[88].  $IF_1$  is proposed to take part in this process after the observation that rat heart preconditioning associates with the inhibition of mitochondrial ATP hydrolysis during ischaemia [87]. This was later confirmed by Penna et al., who showed that decrease in the enzyme's activity after ischemic preconditioning correlates with an augmented binding of  $IF_1$  [89]. Moreover, a consensus exists that the opening of mitochondrial ATP-sensitive  $K^+$  channels plays a central role in cell protection during ischemic preconditioning, causing mitochondrial deenergisation and acidification owing to  $H^+/K^+$  exchange [86]; both effects are likely to promote the binding of  $IF_1$  to the ATP synthase.

**7.3. Cancer.** The importance of mitochondrial metabolism in cancer cells is underlined by the frequently observed, close interaction of glycolytic enzymes with mitochondria. This creates a mutually sustaining relationship between glycolysis, which represents the primary metabolic pathway for tumours sustenance [90], and oxidative phosphorylation.

Regarding the  $F_1F_0$ -ATPsynthase endogenous regulator,  $IF_1$ , its overexpression has been observed in many human carcinomas (including lung, colon, breast, and cervix carcinomas [10], Ehrlich ascites carcinoma [91], Zajdela hepatoma and Yoshida sarcoma [92]), but little is still known about the associated effects, and the few theories that have been put forward are highly controversial. Increased expression of the protein is associated with a higher binding efficiency to the  $F_1F_0$ -ATPsynthase [93], suggesting a greater protection of cancer cells against energy dissipation upon  $F_1F_0$ -ATPsynthase reversal. This was theorized by Chernyak et al. [91], although more recent work has cast doubt on this hypothesis, revealing a relationship between the inhibitor overexpression in human carcinomas and an increase in both  $\Delta\Psi_m$  and glycolytic rate [10].

The protein may therefore be involved in protecting tumour cells from cytosolic ATP depletion and excessive reactive oxygen species (ROS) production (the majority of tumours have little or no vascularization, so that cancerous cells grow in a hypoxic environment). Over and above that, to guarantee cell viability, mitochondria should not become ATP consumers. ATP depletion, ROS imbalance, low cytosolic pH and oxidation of NAD(P)H facilitate the opening of the mitochondrial permeability transition pore (mPTP) and the activation of the intrinsic apoptotic pathway [94]. Numerous studies have also demonstrated that transient hyperpolarization of the mitochondrial membrane can lead to cell apoptosis [38, 95]. By preventing ROS production and IMM hyperpolarization,  $IF_1$  could also protect cancer cells from ROS-mediated apoptosis.  $IF_1$  induces the dimerization of the  $F_1F_0$ -ATPsynthase [8], which might play an essential role in preventing both mitochondrial network fragmentation and cytochrome *c* release from mitochondrial cristae, thus inhibiting the activation of the intrinsic apoptotic pathway. Our previous studies seem to support these hypotheses [81], and, future and focused studies will shed light on this (Tan et al., *under review*). Moreover, an original recent work by Cuezva and co-workers has elegantly demonstrated that  $IF_1$  is protective against chemotherapy

and supports cell proliferation of cancerous cells via the NfκB pathway [96]. Although this work focuses principally on the postmitochondrial effects of IF<sub>1</sub>, it is anyway a compelling evidence for a contribution to neoplastic degeneration and resistance to apoptosis. A starting point to unravel how mitochondrial structure and function are primed by IF<sub>1</sub> overexpression, and to understand to what extent this dictates cellular transformation.

**7.4. Luft's Disease.** To date, the absence of IF<sub>1</sub> has been correlated with only one human pathological condition of neuronal origin: a mitochondrial myopathy called Luft's disease, characterized by nonthyroidal hypermetabolism and densely packed mitochondrial cristae (it is one of the rarest of the mitochondrial diseases, with only two reported cases). Basal ATPase activity in one of the two patients was seven times higher than normal [13], and no IF<sub>1</sub> activity was detected in fibroblasts cultured from the skeletal muscle [14]; however, no mutations in the *ATPIF1* gene were identified, and the genetic cause of the disease remains obscure.

**7.5. Anaemia.** Despite what discussed above, we have very recently collected evidences for a deficiency in the *ATPIF1* gene associated with a form of hypochromic anaemia (Shah et al., *under review*). The mechanism we propose is related with the secondary effect that the absence of IF<sub>1</sub>, and the consequent lack in its inhibitory activity on the F<sub>1</sub>F<sub>0</sub>-ATPsynthase reversion, has on  $\Delta\Psi_m$  and matrix pH. It is known that erythroids' differentiation is triggered by a decrease in  $\Delta\Psi_m$ , that is responsible for a critical redistribution of intracellular Ca<sup>2+</sup> and a transient activation of caspases [97]. Anyway, we found that the increase in mitochondrial matrix pH, which is observed in zebrafish models and murine cells carrying the mutated form of the *ATPIF1* gene, is causally linked to a decrease in ferrochelatase activity, which leads to defects in the incorporation of <sup>59</sup>Fe into protoporphyrin IX to generate the hemoglobin prosthetic group heme.

Such a remarkable finding puts IF<sub>1</sub> amongst the regulators of heme biosynthesis, not only describing a new mechanism for sideroblastic anaemia, but also confirming the involvement of the inhibitory protein in human pathologies related to mitochondrial disorders.

## 8. Conclusions

The F<sub>1</sub>F<sub>0</sub>-ATPsynthase is a wonderful machinery, with the unique capacity of producing and consuming energy, if necessary, to preserve the integrity of the organelle to which it belongs. A precise and sustainable way to regulate its activity is therefore paramount, and the Inhibitory Factor 1, a protein encoded by the nuclear DNA, represents the molecule deputed to do so. In the face of a well-defined biochemistry, its role in cell physiology and mitochondrial anatomy has been only recently discovered, posing the protein at the crossroad between dynamics and energy balance. This, together with growing evidence for a contribution to cell and tissue

pathology, leads to novel ways to investigate and thoroughly address IF<sub>1</sub> functional biology.

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## Review Article

# Redox Regulation of Protein Function via Cysteine S-Nitrosylation and Its Relevance to Neurodegenerative Diseases

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Debilitating neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD), can be attributed to neuronal cell damage in specific brain regions. An important hallmark of these diseases is increased oxidative and nitrosative stress that occurs via overproduction of highly reactive free radicals known as reactive oxygen species (ROS) and reactive nitrogen species (RNS). These molecules are normally removed by cellular antioxidant systems. Under physiological conditions, ROS/RNS are present at low levels, mediating several neurotrophic and neuroprotective signaling pathways. In contrast, under pathological conditions, there is a pronounced increase in ROS/RNS generation, impairing normal neurological function. Nitric oxide (NO) is one such molecule that functions as a signaling agent under physiological conditions but causes nitrosative stress under pathological conditions due to its enhanced production. As first reported by our group and colleagues, the toxic effects of NO can be in part attributed to thiol S-nitrosylation, a posttranslational modification of cysteine residues on specific proteins. Here, we review several reports appearing over the past decade showing that S-nitrosylation of an increasing number of proteins compromises important cellular functions, including mitochondrial dynamics, endoplasmic reticulum (ER) protein folding, and signal transduction, thereby promoting synaptic damage, cell death, and neurodegeneration.

## 1. Introduction

A delicate balance in redox state exists in cells, in large part because of production of ROS/RNS and the antioxidant systems that detoxify them. This homeostatic redox balance maintains a relatively low concentration of ROS/RNS. Under physiological conditions, ROS/RNS can activate specific signaling pathways required for diverse cellular functions, including cell growth and immune responses [1]. However, increased ROS/RNS production or decreased antioxidant capacity can lead to perturbation of the redox balance, causing oxidative/nitrosative stress [2] (Figure 1). We and others have demonstrated that sustained oxidative/nitrosative stress elicits counterattack mechanisms, including activation of transcriptional pathways that activate (i) endogenous antioxidant phase 2 enzymes (the Keap1/Nrf2 cascade) and (ii) chaperones for refolding misfolded proteins (heat-shock proteins of the Hsp90/HSF1 cascade). These transcription

pathways can be activated directly by ROS/RNS or by electrophilic compounds generated in response to oxidation [3–6]. For example, upon reaction of an electrophile with Keap1, Nrf2 dissociates from the Keap1/Nrf2 complex in the cytoplasm and translocates into the nucleus to initiate transcription of phase 2 antioxidant genes [7–9]. HSF1 activates transcription of heat shock proteins to combat protein misfolding due to stress [10, 11]. If oxidant counteraction mechanisms, including activation of the Keap1/Nrf2 and Hsp90/HSF1 pathways, fail to combat ROS/RNS-related stress, cell injury, and death ensues (Figure 1). Synaptic loss and neuronal cell death due to excessive oxidative/nitrosative stress have been widely implicated in neurodegenerative disorders, including Alzheimer's disease (AD) and Parkinson's disease (PD).

ROS and RNS are highly reactive molecules or free radicals. For instance, free radical nitric oxide (NO) possesses an unpaired electron in its outer pi molecular orbital. Due

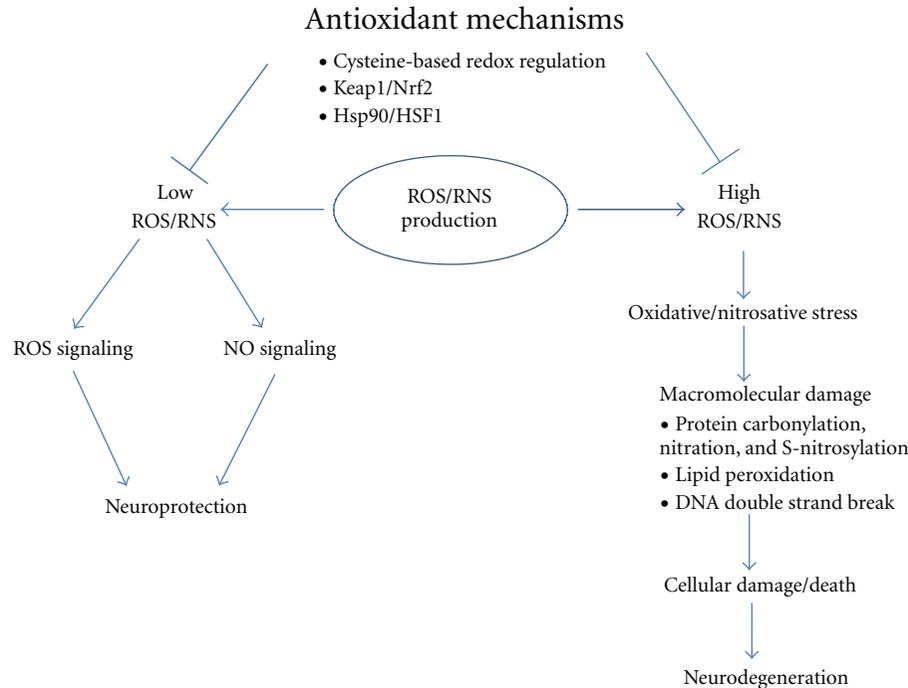


FIGURE 1: Imbalance in oxidant production and antioxidant mechanisms contributes to neurodegeneration. Under physiological conditions, antioxidant mechanisms such as cysteine-based redox regulation (Prx, Grx, Trx, glutathione (GSH), etc.), as well as transcriptional pathways represented by Keap1/Nrf2 and Hsp90/HSF1, maintain low concentrations of ROS/RNS in the neurons. These low levels of oxidants activate specific signaling pathways that subservise normal cell signaling and in fact may be neuroprotective in nature. On the other hand, under pathological situations, including AD and PD, there is a decrease in antioxidant mechanisms and increased oxidant production, effectively creating high levels of ROS/RNS. Oxidative/nitrosative stress generated in this manner can contribute to cell damage and results in neurodegeneration.

to this nature, ROS and RNS can react somewhat indiscriminately with all classes of biological macromolecules (e.g., protein, lipid, DNA) and cause cellular damage (Figure 1). In this paper, we will specifically address the effect of nitrosative stress triggered by NO species that react to form protein S-nitrosothiols. It should be noted, however, that NO signaling can result in other types of posttranslational modifications, such as protein tyrosine nitration and S-glutathionylation, as well as reaction with heme, for example, to activate soluble guanylate cyclase to form cGMP [12].

## 2. Nitric Oxide Production and Signaling

Cellular production of NO from L-arginine is catalyzed by a family of enzymes known as NO synthases (NOSs). The NOS family consists of endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) [13], and all three NOS subtypes are expressed in the mammalian brain. For instance,  $\text{Ca}^{2+}$ -dependent nNOS catalyzes production of NO predominantly in neurons, whereas  $\text{Ca}^{2+}$ -independent iNOS is primarily (but not exclusively) involved in NO production within microglia and astrocytes [14].

Many excitatory synapses contain N-methyl-D-aspartate-type glutamate receptor- (NMDAR-) operated channels. Activation of these channels results in  $\text{Ca}^{2+}$  influx, triggering NO production by nNOS [15]. NO can undergo a number of reactions under normal physiological conditions. For

example, NO reacts with soluble guanylate cyclase to produce cyclic GMP (cGMP) [16]. The second messenger cGMP then activates cyclic guanylate kinases (cGKs) [17, 18]. Once activated, cGKs can phosphorylate various physiological substrates in neurons, thereby controlling various important processes, including synaptic transmission and synaptic plasticity [14, 18] (Figure 2). An even more prominent physiological reaction of NO involves the posttranslational modification of S-nitrosylation or transfer of an NO group to a critical cysteine sulfhydryl to regulate protein function [19], in some sense analogous to phosphorylation of tyrosine, threonine, or serine residues. However, under pathological conditions, hyperactivation of NMDARs (often located at extrasynaptic or perisynaptic sites) causes massive  $\text{Ca}^{2+}$  influx and overproduction of NO [19–21]. Nitrosative stress due to NO overproduction compromises cellular signaling via aberrant protein S-nitrosylation and tyrosine nitration, which can contribute to neuronal cell injury or death [12] (Figure 2). In contrast, it should be noted that several of the proteins that are S-nitrosylated under physiological conditions, for example, the NMDAR itself and GOSPEL foster cell survival [22, 23].

## 3. Protein S-Nitrosylation

As alluded to above, protein S-nitrosylation is a reversible posttranslational modification whereby an NO group is

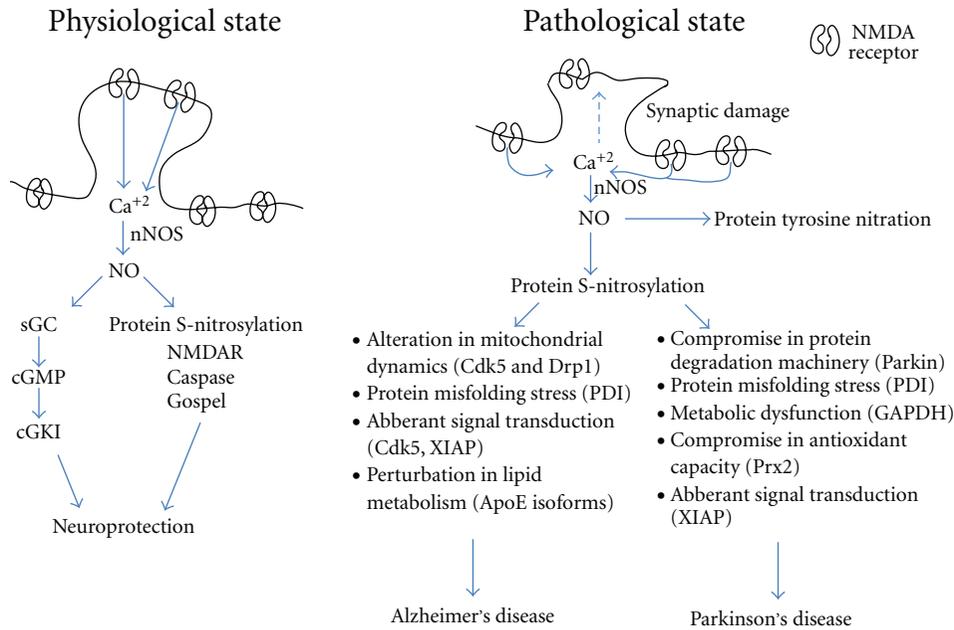


FIGURE 2: Nitric oxide signaling in neuroprotection and neurodegeneration. Synaptic activity results in NMDAR channel openings, allowing  $\text{Ca}^{2+}$  entry that can activate nNOS to generate NO in neurons. Under physiological conditions, low levels of NO are produced in neurons by synaptic activity to activate neuroprotective signaling pathways involving cGMP-CGKI or S-nitrosylation of several critical proteins (NMDARs, Gspcl, etc.). Under pathological conditions, excessive  $\text{Ca}^{2+}$  enters primarily through extrasynaptic NMDARs, generating large concentrations of NO. The nitrosative stress thus generated contributes to synaptic damage and neuronal loss, in part by fostering aberrant protein S-nitrosylation. Various cellular processes, including mitochondrial dynamics, protein folding, lipid metabolism, protein degradation, and signal transduction pathways can be perturbed by aberrant protein S-nitrosylation. Compromise in one or several of these cell processes can contribute to neurodegeneration. It should be noted, however, that a number of additional pathways, not related to NO, that are triggered by synaptic activity can also contribute to neuroprotection, while a number of pathways affected by extrasynaptic NMDAR activity appear to be involved in neuronal cell injury and death [99].

covalently attached to a cysteine thiol group (or more properly, a thiolate anion,  $-\text{S}^-$ ) to form an S-nitroso derivative (R-SNO). Thus, we refer to S-nitrosylated proteins as SNO-proteins. Importantly, not all the cysteines in a protein can be S-nitrosylated. Cysteines that are surrounded by a particular amino-acid motif are likely candidates for this modification. This “SNO motif” is a consensus grouping of amino acids that consists of nucleophilic residues (generally an acid and a base), which may result from protein tertiary or even quaternary structure [24]. A specific modification by S-nitrosylation typically affects protein activity (either activating or inhibiting), thus mediating NO signaling pathways [23, 25, 26]. Proteins also can be denitrosylated (although transnitrosylation may also be involved) reportedly by redox-sensitive enzymes such as the thioredoxin (Trx) and S-nitroso-glutathione reductase systems, protein disulfide isomerase (PDI), and alcohol dehydrogenase (ADH) class III, which are now referred to as denitrosylases [27].

#### 4. Implications of Protein S-Nitrosylation for Neurodegeneration

As discussed earlier, NO is produced in normal physiological conditions but does not induce nitrosative stress at low concentrations. However, under pathological neurodegenerative

situations, NO production is highly increased, activating harmful signaling pathways, in large part due to aberrant protein S-nitrosylation. In this section, we will review several of the effects of protein S-nitrosylation in neurodegenerative diseases, including AD and PD.

**4.1. Alzheimer's Disease.** AD is one of, if not the most, common forms of dementia, resulting in progressive decline of intellectual and social abilities that cause problems in day-to-day life. AD is a neurodegenerative disorder as there is a tremendous amount of cell injury and loss in various parts of the brain, including the hippocampus and neocortex [28]. One of the important observations in AD pathogenesis is that synaptic deficits precede cellular death and correlate well with decline in intellectual function [29, 30]. Most AD cases (>95%) are sporadic, meaning that the majority of AD onset is not associated with obvious genetic mutations. Since AD mainly occurs in elderly people over 60 years old, age is one of the predisposing factors for the disease. One important hypothesis of aging and diseases of aging is the free radical theory, which states that an organism accumulates oxidative damage over time due to decreases in antioxidant systems and overproduction of free radicals [31, 32]. Consistent with this theory, several studies have clearly shown that AD brains exhibit increased oxidative/nitrosative stress [33]. We have

found that several proteins critical to neuronal survival are S-nitrosylated and, in some cases, further oxidized in AD, thereby disrupting the normal activity of the protein and contributing to disease pathogenesis, as described below.

**4.1.1. S-Nitrosylation of Protein Disulfide Isomerase (PDI).** Once polypeptides are synthesized on ribosomes, those destined to be secreted are translocated to the endoplasmic reticulum (ER) for proper folding and disulfide bond formation. PDI is one of the enzymes that catalyze correct disulfide bond formation through a series of thiol-disulfide exchange reactions [34, 35]. In the absence of proper disulfide bond formation, proteins misfold and aggregate in the ER, resulting in ER stress [36]. If ER stress persists, it can result in cell death [37]. Several lines of research have implicated a role for ER stress in AD pathophysiology [38]. Our laboratory discovered that PDI is S-nitrosylated in human AD brain compared to control brain. S-nitrosylation of PDI facilitates further oxidation of cysteine residues to sulfenic (–SOH), sulfinic (–SO<sub>2</sub>H), and sulfonic (–SO<sub>3</sub>H) acid PDI derivatives. These redox modifications compromise PDI chaperone/protein folding function, leading to protein misfolding and ER stress [26]. These results highlighted the role of nitrosative stress and SNO-PDI in neuronal cell injury and death in AD.

**4.1.2. S-Nitrosylation of Dynamin Related Protein 1 (Drp1).** Neurons, and particularly their synaptic connections, require a tremendous amount of energy due to their high metabolic activity. Mitochondria, being the powerhouses of the cell, generate the vast majority of this energy. Recent reports suggest that to meet energy demand in an efficient manner, mitochondrial dynamics, consisting of fission and fusion events to generate new mitochondria, have to be carefully regulated [39]. Perturbation of mitochondrial dynamics can have deleterious effects on neuronal function and survival [40, 41]. Studies from our laboratory have shown that aberrant S-nitrosylation of Drp1 (a protein required for mitochondrial fission) hyperactivates Drp1, and, in turn, causes a dramatic increase in mitochondrial fission. We demonstrated that the altered mitochondrial dynamics due to S-nitrosylated Drp1 (SNO-Drp1) contributes to synaptic loss in neurons and subsequent neuronal cell death. In addition, SNO-Drp1 levels are significantly increased in postmortem sporadic human AD patient brains compared to controls [42]. Hence, this study clearly implicated the pathophysiological role of SNO-Drp1 in AD pathophysiology.

**4.1.3. Transnitrosylation of Cdk5 to Drp1.** Molecular signaling pathways are central to cellular physiology and function. Several signaling molecules have been implicated in AD pathophysiology [43]. One such molecule is cyclin-dependent kinase 5 (Cdk5), the activity of which has been shown to be altered in AD [44]. Cdk5 in neurons does not function as a cell cycle regulator, yet it exerts control over various aspects of neuronal function including cell survival, neuronal migration, dendritic spine density, and synaptic plasticity [45–47]. In a recently published article

from our laboratory, Qu et al. showed that Cdk5, in addition to being a kinase, is also a nitrosylase, capable of S-nitrosylating other targets involved in both AD and PD. Initially, we found that Cdk5 itself could be S-nitrosylated in an A $\beta$ - and NMDAR-dependant manner in neurons due to generation of NO by these insults [25]. Furthermore, we showed that S-nitrosylation of Cdk5 results in activation and contributes to A $\beta$ -induced dendritic spine loss, representing a decrease in synapses, the only pathological correlate to clinical dementia in AD. Moreover, SNO-Cdk5 levels are significantly increased in postmortem sporadic AD patient brains compared to age matched control brains. Importantly, SNO-Cdk5 then appears to contribute to synaptic failure by acting as an endogenous nitrosylase for Drp1, transferring the NO group from Cdk5 to Drp1 to form SNO-Drp1. This study revealed a role for protein S-nitrosylation of Cdk5 in aberrant cell signaling and links this nitrosylase activity to neuronal damage in AD [25].

**4.1.4. S-Nitrosylation of ApoE.** Apolipoprotein E (ApoE) represents a major risk factor locus for late onset Alzheimer's disease [48]. The different isoforms of ApoE vary at their cysteine residues, which are potential sites for S-nitrosylation, as our group had observed a number of years ago. A recent study showed that all ApoE isoforms can bind nNOS and that ApoE2 and ApoE3 can be found in the S-nitrosylated state in human hippocampal lysates [49]. S-Nitrosylation of ApoE isoforms has been suggested to cause loss of binding to low density lipoprotein (LDL) receptors. Thus, S-nitrosylation of ApoE may affect lipid metabolism, which is postulated to affect the progression of AD.

The above-mentioned studies highlight some of the roles of S-nitrosylated proteins and how they can alter diverse cellular functions, including mitochondrial dynamics and synapse loss, ER protein folding, signal transduction pathways, and lipid metabolism, thereby affecting the progression of AD (Figure 2). In addition to these pathways, we suspect that there are many more pathways altered by protein S-nitrosylation in AD pathophysiology.

**4.2. Parkinson's Disease.** PD is second only to AD in the prevalence of neurodegenerative disorders. It affects approximately 1% of people over 65 years of age [50] and is characterized by motor sequencing impairment and often has a component of dementia. Although there are some symptomatic treatments for patients suffering from PD, currently there is no successful therapy to prevent progression or restore function. The histopathological aspects of PD include the loss of dopaminergic neurons, primarily in the substantia nigra *pars compacta*, often with the simultaneous presence of intracellular inclusions called Lewy bodies. Lewy bodies are mainly distributed in the substantia nigra, neocortex, basal forebrain nuclei, and hippocampus [51, 52]. Despite the fact that some familial cases have been identified, more than 95% of PD cases are reported as sporadic, some of which appear to be correlated with exposures to agricultural pesticides, herbicides, fungicides, heavy metals, or neurotoxins [53, 54], although this epidemiological information has remained

contentious in some circles. We and others have shown that several of these environmental factors induce the generation of potentially toxic ROS/RNS species within neuronal cells [55]. Interestingly, dopaminergic neurons are especially vulnerable to oxidative/nitrosative stress, perhaps partly because of the oxidizing nature of dopamine. These observations have raised the hypothesis that in sporadic PD cases, oxidative or nitrosative stress contributes to PD pathogenesis via altering the function of PD-associated proteins. In several cases, the same gene product that is encoded in hereditary cases of PD may be affected by environmental factors to mimic the more rare genetic form or increase the susceptibility or severity of the hereditary phenotype, as highlighted below.

**4.2.1. S-Nitrosylation of Parkin.** As an example, mutations in the parkin gene are known to cause many cases of the autosomal-recessive juvenile Parkinsonism and some rare cases of adult-onset PD [56–59]. The parkin gene encodes an ubiquitin E3 ligase that targets many proteins for proteasomal degradation and also has a neuroprotective role in PD-related apoptotic events [56, 60]. Mutations in the parkin gene result in the disturbances in parkin-mediated protein ubiquitination [61–63], which leads to the accumulation of potentially neurotoxic protein aggregates of parkin substrates with consequent dysfunction of the ubiquitin-proteasome system degradative pathway [61, 64, 65]. Interestingly, recent reports suggest that, independently from its ubiquitin-ligase role, parkin also functions as a transcriptional repressor of p53 to protect dopaminergic neurons from PD-related stress [66, 67].

In addition to these rare mutations, several environmental toxins that trigger oxidative/nitrosative stress are believed to affect the enzymatic activity of parkin protein. For instance, certain pesticides, herbicides, and fungicides that generate ROS and RNS and have been linked epidemiologically to PD can cause alterations in parkin solubility, inducing its aggregation and compromising its protective function. Parkin has multiple cysteine residues in its RING domain and elsewhere [66, 68], which can react with NO to form SNO-parkin. This S-nitrosylation reaction compromises parkin's neuroprotective function. Our group reported that S-nitrosylation of parkin initially increases E3 ligase activity, but with additional time this activity is inhibited. This dysfunctional E3 ligase activity is associated with abnormal protein aggregation resembling Lewy bodies, thus contributing to the parkinsonian phenotype [68]. Moreover, S-nitrosylation of parkin has also been found by our group and others in a mouse MPTP model of PD and in brains of human patients with Lewy body disease (LBD) and PD [68, 69]. These findings support our notion that posttranslational changes to PD-related proteins via S-nitrosylation or other oxidation reactions may well contribute to the etiology of sporadic PD.

**4.2.2. S-Nitrosylation of Peroxiredoxin.** Our group and colleagues have also demonstrated that S-nitrosylation of another protein, peroxiredoxin 2 (Prx2), may be related

to PD pathogenesis. Prxs are a highly abundant family of antioxidant enzymes that reduce intracellular peroxides by redox reactions [70–72]. Among the Prx enzymes, Prx2 is the most abundant in the mammalian brain and neurons. The active site cysteine residues in Prx2 reduce peroxides to H<sub>2</sub>O, thus forming a sulfenic acid (–SOH) derivative of Prx2. Subsequently, the oxidized Prx2 cysteine(s) can either form an intermolecular disulfide bond (–S–S–) with another Prx2 molecule, undergo reduction/regeneration back to free sulfhydryl (–SH) by thioredoxin (Trx), or be further oxidized (termed hyperoxidation) to produce a sulfinic (–SO<sub>2</sub>H) or sulfonic (–SO<sub>3</sub>H) acid derivative.

In several neurodegenerative diseases linked to oxidative/nitrosative stress, Prx2 levels are increased [73, 74], which may represent an attempt of the cell to counteract oxidative/nitrosative insult during neurodegeneration. We and others recently reported that Prx2 activity can be regulated *in vitro* and in cell-based systems by NO through S-nitrosylation of redox-active cysteine residues, which would prevent the reaction of this protein with peroxides, thus preventing the neuroprotective action of Prx2 [75, 76]. In human samples of PD brains and cell-based models of PD, S-nitrosylation of Prx2 has been found to be increased compared to control samples [75]. Since SNO-Prx2 cannot react with peroxide because the active cysteines are already nitrosylated, the normal redox cycle to detoxify ROS is disrupted, inducing oxidative stress that can contribute to neuronal cell death.

**4.2.3. S-Nitrosylation of XIAP.** The protein X-linked inhibitor of apoptosis (XIAP) has also been found to be S-nitrosylated in several neurodegenerative disorders, including Alzheimer's, Parkinson's, and Huntington's diseases (HD), by our laboratory and others. Inhibitors of apoptosis (IAPs) are a family of proteins that regulate cell survival through binding to caspases to repress their catalytic activity [77, 78]. XIAP is the most commonly expressed and the most potent endogenous caspase inhibitor among the IAPs. XIAP has three copies of the baculovirus IAP repeat (BIR) domain and one RING domain at the C terminal. Biochemical and structural studies demonstrated that BIR domains confer the anticaspase activity [79], whereas the RING domain can act as an E3 ubiquitin ligase in the proteasome system [80–84].

Recent studies show a significant increase of S-nitrosylated XIAP in both cell-based and animal models of PD as well as in human brain samples from PD, AD, and HD patients [85, 86]. Our detailed experiments have identified that the RING domains of XIAP can react with NO by S-nitrosylation [85], although very high, nonphysiological concentrations of NO can also induce S-nitrosylation of the BIR domain [86]. S-Nitrosylation of XIAP at the RING domain inhibits its E3 ligase and antiapoptotic activity. Furthermore, we demonstrated recently that S-nitrosylated caspases can transfer their NO group to XIAP in a process called transnitrosylation. This reaction inhibits XIAP ubiquitin E3 ligase activity on caspases, thus effectively enhancing

caspase activity and thus promoting proapoptotic signaling [85].

**4.2.4. S-Nitrosylation of GAPDH.** Solomon Snyder's group has shown that the important metabolic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can be S-nitrosylated to form SNO-GAPDH. SNO-GAPDH manifests a loss of enzymatic activity [87]. More importantly, S-nitrosylated GAPDH efficiently binds Siah1 protein and then translocates to the nucleus. In the nucleus, this protein complex activates ubiquitination and thus degradation of several nuclear proteins, including nuclear receptor corepressor (N-COR); this process contributes to cell death [87]. These studies suggest that S-nitrosylation of GAPDH, on one hand, compromises its metabolic enzymatic activity, but, on the other hand, in conjunction with Siah1, forms an important signaling complex to promote cell death and neurodegeneration.

**4.2.5. S-Nitrosylation of PDI.** As discussed above, protein misfolding and ER stress can be precipitated by S-nitrosylation of PDI, thereby potentially contributing to neuronal injury in AD. Our laboratory has also shown this scenario to be true in PD models. For example, when SH-SY5Y dopaminergic cells were treated with rotenone, a pesticide implicated in the pathogenesis of PD, we observed an increase in SNO-PDI levels concomitant with a decrease in PDI chaperone activity. Additionally, we found dramatically increased levels of SNO-PDI in human postmortem PD brains compared to controls [26]. Since ER stress due to protein misfolding is thought to contribute to the neurodegenerative process in PD [88], our finding of a substantial degree of SNO-PDI in PD brains has both pathogenic and therapeutic implications.

**4.2.6. S-Nitrosylation of DJ-1.** Deletions or point mutations in the protein DJ-1 (PARK7) have been shown to be responsible for an early-onset, autosomal-recessive form of PD [89]. Interestingly, DJ-1-mediated signaling pathways have also been implicated in the much more common sporadic form of PD. It has been postulated that the increase in DJ-1 expression observed in cells undergoing nitrosative stress induced by the herbicide, paraquat, represents an attempt to protect the cells [90]. Consistent with this notion, DJ-1 knockdown makes neuronal-like cells more susceptible to peroxide-, MPP<sup>+</sup>-, and 6-hydroxydopamine-induced cell death [91, 92]. Additionally, DJ-1 deficient flies [93–96] or mice [97] are more vulnerable to environmental neurotoxins associated with dopaminergic degeneration. Sequence analysis of protein DJ-1 reveals three potentially redox-active cysteine residues, two of which (Cys46 and Cys53) appear to be susceptible to S-nitrosylation *in vitro* and in cell-based systems [98]. Our group has also observed S-nitrosylation of a critical redox-active cysteine in the crystal structure of DJ-1. These findings and others suggest that posttranslational modifications of DJ-1, including protein S-nitrosylation, can disrupt the antioxidant action of DJ-1 in dopaminergic neurons, rendering them more susceptible to damage in

sporadic PD. However, the elucidation of additional effects of SNO-DJ-1 in PD will require further investigation.

In summary, a number of studies suggest that nitrosative stress contributes to PD pathogenesis by altering neuroprotective proteins such as parkin, Prx2, PDI, GAPDH, and XIAP (Figure 2). These findings indicate that aberrant S-nitrosylation reactions may play an important role in this neurodegenerative disorder, providing additional insight into nitrosative mechanisms of PD pathogenesis as well as potential novel targets for the treatment of PD.

## 5. Conclusions

Nitric oxide signaling can be both beneficial and harmful to the nervous system depending on (i) the concentration of NO and (ii) the cell signaling pathways affected by various levels of NO. Physiological levels of NO activate both cGMP-cGKI and S-nitrosylation pathways responsible for various physiological processes, including those affecting synaptic transmission and plasticity. In contrast, high levels of NO compromise cellular functions by a variety of posttranslational modifications including aberrant S-nitrosylation reactions that would not normally occur in the presence of physiological levels of NO. In this paper, we have discussed data accumulated over the past several years that highlight the importance of protein S-nitrosylation in perturbing vital cell functions, including mitochondrial dynamics, protein folding, ubiquitination, synaptic transmission, and signal transduction pathways. Alteration of one or several of these events contributes to neuronal cell death and the development of neurodegenerative disorders (Figure 2). Although we have discussed the role of S-nitrosylation of several proteins here, including Drp1, PDI, GAPDH, ApoE, parkin, XIAP, Prx2, and DJ-1 in AD and PD, this list is by no means complete. Proteome-wide studies have already found hundreds, if not thousands, of proteins that are S-nitrosylated [99]. Future studies will unravel the role of S-nitrosylation of additional proteins in various cellular cascades and its implications for the pathogenesis and treatment of neurodegenerative disorders.

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## Review Article

# Established Principles and Emerging Concepts on the Interplay between Mitochondrial Physiology and S-(De)nitrosylation: Implications in Cancer and Neurodegeneration

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S-nitrosylation is a posttranslational modification of cysteine residues that has been frequently indicated as potential molecular mechanism governing cell response upon redox unbalance downstream of nitric oxide (over)production. In the last years, increased levels of S-nitrosothiols (SNOs) have been tightly associated with the onset of nitroxidative stress-based pathologies (e.g., cancer and neurodegeneration), conditions in which alterations of mitochondrial homeostasis and activation of cellular processes dependent on it have been reported as well. In this paper we aim at summarizing the current knowledge of mitochondria-related proteins undergoing S-nitrosylation and how this redox modification might impact on mitochondrial functions, whose impairment has been correlated to tumorigenesis and neuronal cell death. In particular, emphasis will be given to the possible, but still neglected implication of denitrosylation reactions in the modulation of mitochondrial SNOs and how they can affect mitochondrion-related cellular process, such as oxidative phosphorylation, mitochondrial dynamics, and mitophagy.

## 1. Introduction

Nitric oxide (NO) is a gaseous and membrane diffusible radical molecule generated by the NADPH-dependent enzyme NO synthase (NOS) from L-arginine and oxygen [1, 2]. Three are the major isoforms of NOS that have been so far identified, namely, neuronal and endothelial NOS (nNOS or NOS1 and eNOS or NOS3, resp.), which are constitutively active, and the cytokine-inducible NOS (iNOS or NOS2), mainly expressed in immune system to face host attack [3, 4]. The biochemical characterization of NO as new signaling molecule, as well as its implication in cardiovascular function earned Furchgott, Ignarro, and Murad the Nobel prize in Physiology or Medicine in 1998. In particular, they provided the most consistent lines of evidence that NO activates guanylyl cyclase by a direct binding to heme iron (Fe-nitrosylation) and induces cGMP-mediated signaling [5], thus regulating blood vessel tone [6], immune response [7], neurotransmission [8], and many other organic functions.

NO can also react with other oxygen-derived radical and nonradical species (ROS), thus generating more dangerous reactive nitrogen species (RNS, e.g., peroxynitrite, ONOO<sup>-</sup>), which target proteins and irreversibly affect their structure and function, a phenomenon commonly known as nitrosative (or nitroxidative) stress [9]. Tyrosine nitration is one of the modifications occurring under conditions of NO overproduction and mostly depends on the reaction with ONOO<sup>-</sup> [10]. It consists of a covalent addition of a nitro group (-NO<sub>2</sub>) to one of the two equivalent orthocarbons of the aromatic ring in tyrosine residues [11]. Although there are indications arguing for the existence of a denitrase activity, this has been not well characterized yet, and tyrosine nitration is still considered an irreversible modification of proteins subjected to massive nitroxidative stress. Indeed, elevated levels of tyrosine-nitrated proteins are reported in several neurodegenerative diseases and are commonly used as pathological markers of nitrosative stress [12–14].

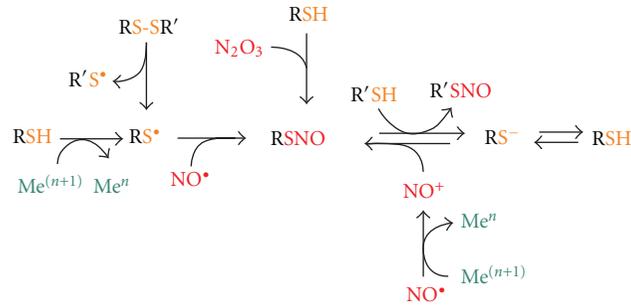


FIGURE 1: Mechanisms of *S*-nitrosylation. Cysteines of low molecular weight (e.g., GSH) and protein sulfhydryls (both termed as RSH) can undergo *S*-nitrosylation, thus generating *S*-nitrosothiols (RSNO), by different reactions involving different NO groups and different thiol substrates. RSNO can be formed upon the encountering of NO• with a thiyl radical (RS•), with the latter species deriving from an RSH upon metal-catalyzed oxidation or upon homolytic scission of a disulfide bridge (RS-SR') (on the left). However, as SH• is a rare and chemically unstable species, it is plausible to consider that the majority of cellular RSNO generates from the thiolate form of the cysteine (RS<sup>-</sup>) that can result from sulfur deprotonation even at physiological pH. Either as RS<sup>-</sup>, or directly as RSH, cysteine sulfhydryl can undergo nitrosylation by reacting with NO-derived dinitrogen trioxide (N<sub>2</sub>O<sub>3</sub>), or directly with nitrosonium ion (NO<sup>+</sup>) generated upon metal-catalyzed oxidation of NO•. The net transfer of NO<sup>+</sup> from an RSNO to an R'S<sup>-</sup> (transnitrosylation) also occurs inside the cells and represents a further reaction to produce *S*-nitrosylated adducts (on the right).

**1.1. *S*-Nitrosylation.** Besides these deleterious and pathological effects, NO and other RNS can also concur to modulate signal transduction upon certain stimuli by means of other mechanisms that lead to transient protein modification. The main chemical reaction underlying this mechanism is the *S*-nitrosylation (or *S*-nitrosation) of cysteine residues [15] (Figure 1). It consists on the covalent addition of an NO moiety to a reactive sulfhydryl, which results in the formation of an *S*-nitrosothiol derivative (SNO). SNOs generation depends on several factors, such as the environmental hydrophobicity conditions, the net charge and hindrance of the microenvironment in which reactive cysteines are embedded, and the presence of oxygen. NO can directly produce SNO if thiol residue, which is going to be modified, is present under the form of thiyl radical (-S•) (Figure 1). Nevertheless, this is a rare and unstable species; therefore, it is reasonable that the large amount of cellular SNOs generates from the reaction of thiols (present or not as thiolate anion, -S<sup>-</sup>) with the NO-derived species dinitrogen trioxide (N<sub>2</sub>O<sub>3</sub>) or, directly, with nitrosonium ion (NO<sup>+</sup>). The NO<sup>+</sup> group is directly transferable between different SNOs, by means of a process known as transnitrosation or transnitrosylation [16] (Figure 1). Due to its feature of specificity and reversibility, *S*-nitrosylation of reactive cysteines is a prototype mechanism of redox-based signaling [17].

**1.2. Thiol-Based Redox Modifications and Denitrosylating Enzymes.** Similarly to cysteine sulfenate derivative (-SOH, see Figure 2), SNOs are relatively unstable adducts that can undergo exchange reactions with reduced glutathione (GSH) to generate more stable *S*-glutathionylated (-SSG) species, or, as demonstrated for matrix metalloproteinases, be further oxidized to sulfinic (-SO<sub>2</sub>H) or sulfonic (-SO<sub>3</sub>H) derivatives [18]. On the other hand, SNOs can be reduced back to sulfhydryl state by denitrosylation reactions [19]. More properly, SNO to SH conversion takes place by

means of transnitrosylation reactions with a further cellular thiol moiety, the most representative of which are the low-molecular-weight antioxidant glutathione (GSH) and dithiol-containing oxidoreductases (Figure 2). Among this class of enzymes, thioredoxins (Trxs) are the best characterized examples of denitrosylases [20, 21], although other proteins, such as protein disulfide isomerase and glutathione-*S*-transferase  $\pi$ , have been suggested to act in the same way [19]. Trx-mediated reduction of SNOs leaves the NO moiety free being released intracellularly as nitroxyl (HNO) or NO, and Trx-contained dithiol being oxidized to disulfide bridge, which can be fully reduced to sulfhydryl state by the NADPH-dependent activity of the selenoprotein Trx reductase (TrxR) (Figure 2). This mechanism of denitrosylation has been largely described to influence the levels of protein SNOs; however, low-molecular-weight SNOs, such as *S*-nitrosoglutathione (GSNO), can also undergo the same reaction [19]. Nevertheless, a direct NADH-dependent GSNO targeting enzyme, named GSNO reductase (GSNOR), has been discovered one decade ago and found to deeply impact on protein SNOs levels as well [22]. Due to mere chemical transnitrosylation reactions, indeed, the redox couples GSH/GSNO and protein-SH/protein-SNOs are in a dynamic equilibrium (Figure 2) therefore, by directly reducing GSNO, GSNOR indirectly decreases the concentration of protein SNOs. Actually, GSNOR is not properly a “new” enzyme, as it was one of the first enzymes to be discovered and characterized as the class III alcohol dehydrogenase (ADH III) or GSH-dependent formaldehyde dehydrogenase. However, in 1998, Jensen and coworkers found that GSNO is the elective substrate of ADH III, as the specific dehydrogenase activity was about the 6% of the GSNO reducing one [23]. Although both act as “SNO-scavenging” enzymes, Trx and GSNOR produce different side effects, which could differently affect cellular redox homeostasis. Indeed, whereas Trx-mediated denitrosylation leaves NO moiety being still reactive and



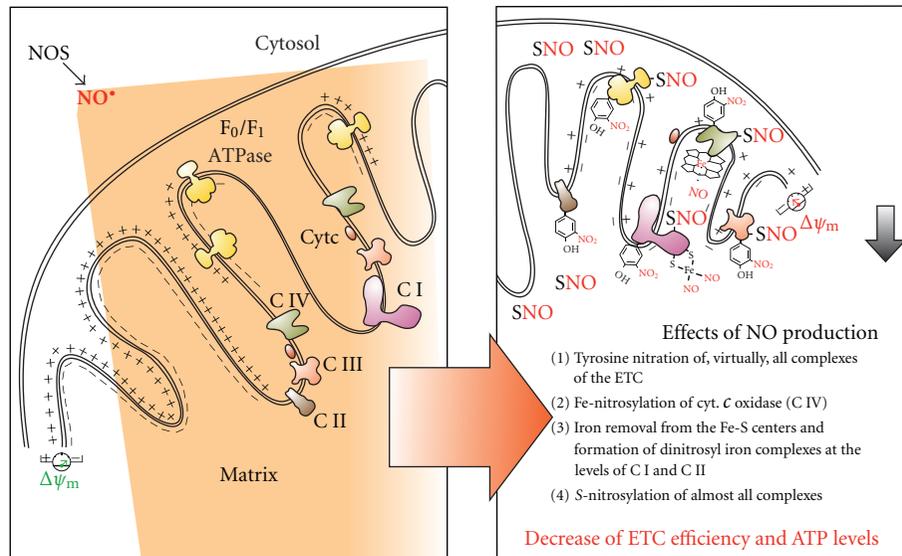


FIGURE 3: Effects of NO and nitrosative stress on mitochondrial electron transfer chain. NO, namely, the fraction produced in the cytosol by NOS, can cross cell membranes (e.g., the mitochondrial outer and inner membranes) and reversibly or irreversibly modifies mitochondrial complexes of the electron transfer chain (ETC). Specifically, nitration of all complexes, nitrosylation of Fe-heme-containing cytochrome *c* oxidase (C IV), generation of dinitrosyl iron complexes of Fe-S centers (e.g., those present in the Complex I), and S-nitrosylation of Complex I, III, IV, F<sub>0</sub>/F<sub>1</sub> ATPase, as well as other unspecified mitochondrial proteins are shown. These modifications negatively affect ETC efficiency, and ATP production and decrease mitochondrial transmembrane potential ( $\Delta\Psi_m$ ), which represents a crucial event upstream of several mitochondrial functions, such as mitochondrial dynamics, mitophagy, and apoptosis.

ultimately used for the tetravalent reduction of molecular oxygen at the level of cytochrome *c* oxidase. Concomitantly, ATP is synthesized by the F<sub>0</sub>/F<sub>1</sub> ATP synthase exploiting the electrochemical proton gradient generated at the inner mitochondrial membrane. NO and RNS have been copiously reported to negatively affect mitochondrial respiration rate by inhibiting the activity of proteins implicated in this process, such as, virtually, all complexes of the electron transfer chain [24–26] (Figure 3). This inhibitory effect ranges from reversible to irreversible, up to be harmful for the entire mitochondrial compartment in dependence of (i) the concentration of NO and (ii) the RNS being engaged in the reactions. It is worthwhile noting, in fact, that mitochondria are the principal source of superoxide anion that can react at the diffusion-limited rate with NO to generate ONOO<sup>-</sup>. Therefore, the possibility that tyrosine nitration reactions could occur in metabolically active mitochondria is quite high. Indeed, all complexes have been demonstrated to undergo tyrosine nitration upon endogenous production of ONOO<sup>-</sup> or after its administration [27].

NO itself, at physiological low (nanomolar) concentrations, can bind with high affinity to free Fe<sup>2+</sup> or Fe<sup>2+</sup> within any heme-containing protein with a free ligand position, such as cytochrome *c* oxidase, thus determining its inhibition [28] (Figure 3). In particular, NO reversibly binds to Fe<sup>2+</sup> cytochrome *a*<sub>3</sub> forming a nitrosyl-heme complex, condition that allows NO increasing the apparent K<sub>m</sub> of cytochrome *c* oxidase for oxygen [29]. In such a way, even low physiological levels of NO can cause significant inhibition of respiration and potentially make it very sensitive to oxygen tension [30]. Since the reversible NO-mediated inhibition of cytochrome

*c* oxidase occurs at nanomolar levels NO and in competition with oxygen [31], NO is considered a potential physiological regulator of respiration [32]. It is worthwhile noting that, besides competitive binding to Fe-heme, which remains the elective target of NO, and the main modification responsible for its inhibitory effects on mitochondrial respiration, NO has been reported to inhibit Complex IV activity also by binding the copper binuclear center of cytochrome *c* oxidase in a noncompetitive manner [33].

Similarly, NO can impact on mitochondrial respiration by reacting directly with iron of the iron-sulfur (Fe-S) centers of Complexes I and II, as well as aconitase (Figure 3) [25]. In this way, NO can damage iron-sulfur centers by removing iron (to form dinitrosyl iron complexes) and/or oxidize the iron-bound cysteine residues to disulfide or SNO. The formation of SNO derivatives can also occur on cysteine residues that are not engaged in the formation of iron-sulfur centers. Although in theory all complexes contain putative nitrosylable cysteines [25], the only evidence indicating how S-nitrosylation affects mitochondrial respiration deals with studies on Complexes I, IV, and ATPase (Figure 3). As in the case of other proteins, S-nitrosylation of mitochondrial complexes generally induces inhibition of protein function, thus reducing electron transfer and ATP production efficiency [25]. Particularly for what Complex I concerns, no comprehensive mechanism or specific cysteine residue undergoing S-nitrosylation has been reported so far, unless that the inhibition, which occurs at the 75 kDa subunit, is light-sensitive and reversed by reducing agents [32, 34, 35]. Studies of cardioprotection by GSNO also indicated that GSNO-preconditioned cardiomyocytes have a significant increase

of *S*-nitrosylated  $F_1$  ATPase,  $\alpha 1$  subunit, which causes a dose-dependent decrease of its activity [36]. In a more detailed manner, Zhang and colleagues found that, in lung endothelial cells, NO induces the selective *S*-nitrosylation of Cys<sup>196</sup> and Cys<sup>200</sup> residues of the mitochondrial Complex IV, subunit II, thereby allowing, also in this case, a transient inhibition of oxygen reduction [37].

ATP generation is coupled with the extrusion of  $H^+$  from the mitochondrial matrix to the inner-membrane space, thus generating the proton motive force, which is used to drive the synthesis of ATP and other energy-requiring mitochondrial activities [38]. Proton motive force and the mitochondrial membrane potential ( $\Delta\Psi_m$ ) are then tightly related, so that  $\Delta\Psi_m$  represents a good indicator of the energy status of the mitochondrion and of the cellular homeostasis in general. The majority of the reports dealing with NO effects on mitochondrial homeostasis indicate that pathophysiological conditions in which NO is generated at high rate are tightly associated with mitochondrial membrane depolarization [26] (Figure 3). This event underlies several processes, such as mitochondrial dynamics, apoptosis, and autophagy.

**2.2. Mitochondrial Dynamics.** Mitochondria are in constant movement within cells, with fusion/fission events routinely taking place in order to allow physiological organelle turnover [39], to maximize mitochondrial efficiency [40], to regulate  $Ca^{2+}$  signaling/homeostasis and apoptotic response [41, 42], and to adapt ATP production to cellular energy demand [43]. Mitochondrial size, number, and mass are modulated by a variety of physiological stimuli. More than 1000 genes and ~20% of cellular proteins are involved in this process [42], and a complex regulatory network coordinates mitochondrial dynamics. Moreover, chemical species endogenously produced by the cell, such as NO, RNS, and ROS seem to play a key role in this process.

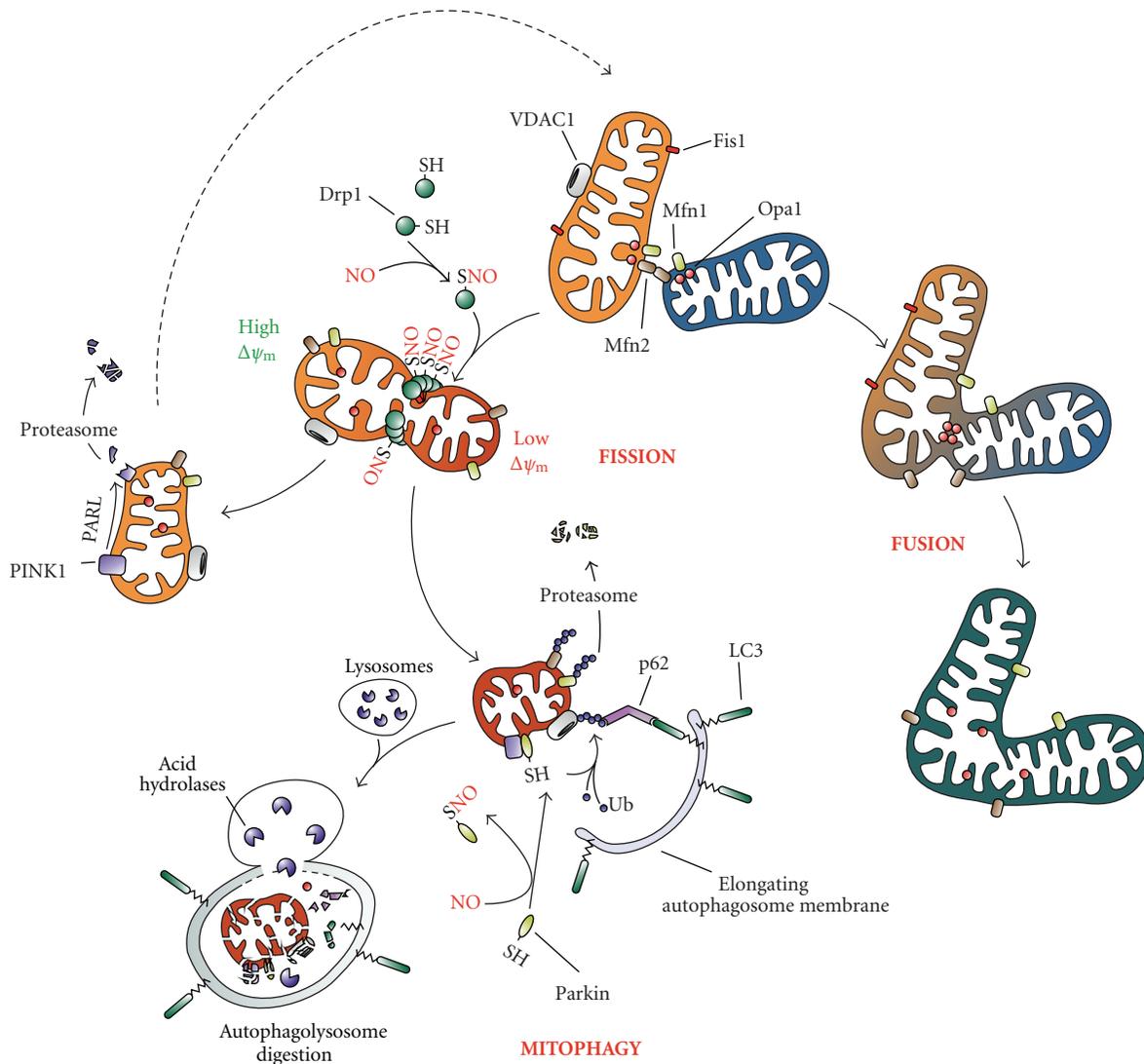
Mitochondrial fission contributes to the elimination of damaged mitochondrial fragments through mitochondrial autophagy (mitophagy) [44], whereas mitochondrial fusion facilitates the exchange of mitochondrial DNA (mtDNA) and metabolites needed for the maintenance of functional mitochondria [45] (Figure 4). Both events are controlled by four members of large GTPases: mitofusin 1 and 2 (Mfn1 and Mfn2), optic atrophy 1 (Opa1), and dynamin-related protein1 (Drp1), which are conserved from yeast to mammals, indicating that the fundamental mechanisms controlling mitochondrial dynamics have been maintained during evolution. Mfn1, Mfn2, and Opa1, act in concert to regulate mitochondrial fusion and cristae organization and localize in the outer and inner mitochondrial membrane [46], respectively, whereas Drp1 is a cytosolic protein, whose main function—that is induced upon translocation on the outer mitochondrial membrane—is to regulate mitochondrial fission [47] (Figure 4).

Mitochondrial fusion involves the tethering of two adjacent mitochondria followed by merging, or fusion, of the inner and outer mitochondrial membranes. Efficient mitochondrial fusion is important for cell viability as cells defective for fusion events display reduced cell growth, decreased  $\Delta\Psi_m$ , and defective respiration [48]. In particular, studies on

knockout mice have demonstrated the importance of Mfn1 and Mfn2 for mitochondrial fusion, as loss of both proteins leads to excessive mitochondrial fragmentation [49]. While Mfns are important for fusion of the outer mitochondrial membrane, Opa1 is pivotal for the fusion of inner mitochondrial membranes. Opa1 is a dynamin-related protein located on the mitochondrial inner membrane, and its ablation deeply impairs mitochondrial fusion [50]. Evidence also suggests that Opa1 has an important role in maintaining mitochondrial cristae structure, as loss of this protein results in disorganization of cristae and widening of cristae junctions [51].

During fission events, cytosol-distributed Drp1 localizes at the mitochondrial surface by means of Fis1, an integral outer mitochondrial membrane protein that interacts with Drp1 and functions as an exquisite mitochondrial Drp1 receptor [52]. Cells lacking Fis1 exhibit elongated mitochondria and a senescence-related phenotype, which lends the intriguing hypothesis that mitochondrial fission may counteract cellular senescence [53]. The putative relationship between mitochondrial dynamics and cell proliferation has been also reinforced by the identification that cell-cycle-dependent kinases phosphorylate and, thereby, modulate Drp1 activity [54].

Among the aforementioned large class of GTPases, Drp1 is the sole so far identified to be regulated by posttranslational modifications influencing its translocation onto the outer mitochondrial membrane and to induce mitochondrial fragmentation. For example, phosphorylation of several serine residues has been reported to modulate Drp1 activity [55], and the role (activating or inhibitory) of some of them still remains an issue of debate. However, it is well established that Cdk1/cyclin B-mediated phosphorylation of Ser<sup>616</sup> activates Drp1 fission activity [56], whereas phosphorylation of Ser<sup>637</sup> by cAMP-dependent protein kinase (PKA) is inhibitory [57]. In this regard, the calcium-dependent phosphatase calcineurin has been demonstrated to catalyze dephosphorylation of the same residue and to restore mitochondrial fragmentation process [58]. Sumoylation and *S*-nitrosylation have been reported to positively regulate Drp1-mediated mitochondrial fission as well. In particular, Cys<sup>644</sup> has been identified to sense nitrosative stress. In accordance to Cho and coworkers [59], indeed, SNO-Drp1 translocates onto mitochondria and undergoes polymerization, which represents a structural modification stimulating GTP hydrolysis and allowing mitochondria to be fragmented (Figure 4). Consistent with these lines of evidence, C644A substitution of Drp1 abrogates fission events. In regard to these findings and their involvement in AD pathogenesis, the group of Bossy-Wetzel raised some concerns [60]. Indeed, though confirming that SNO-Drp1 represents a mitochondria-localized modification of the protein mainly present in postmortem brains from AD patients, the authors refuse that *S*-nitrosylation positively affects its enzymatic activity, leaving this issue still questionable. Interestingly, a significant amount of Opa1 was found to be *S*-nitrosylated in AD brain as well [60]; however, no implication for this modification in the regulation of mitochondrial dynamics has been never hypothesized.



**FIGURE 4:** Effects of NO on mitochondrial dynamics and mitophagy. Mitochondrial network is dynamically regulated by fusion/fission events. Fusion between adjacent mitochondria (on the right) relies on the activity of Mfn1, Mfn2, and Opa1 which act in concert to mediate the merge of the outer and inner membrane, respectively. Although the presence of S-nitrosylated Opa1 has been observed, no role for this modification has been still proposed, such as phosphorylation (not shown in the figure) and S-nitrosylation. Conversely, Drp1 has been reported to undergo several posttranslational modifications which modulate its fission activity (on the left), such as phosphorylation (not shown in the figure) and S-nitrosylation. Once S-nitrosylated and driven by mitochondria depolarization (low  $\Delta\Psi_m$ ), Drp1 is recruited onto the outer mitochondrial membrane by means of the recognition of its anchor protein Fis1. There, SNO-Drp1 multimerizes and acts to tighten the target organelle in order to share the depolarized portion from the healthy part. Although there is the possibility for a fragmented mitochondrion to refuse by means of Mfns and Opa1-mediated activity, frequently a depolarized organelle is targeted for its selective removal by autophagy (mitophagy). PINK1, which is normally degraded by PARL, is stabilized and recruits Parkin onto the outer membrane of an impaired mitochondrion and, in turn, catalyzes the covalent addition of an ubiquitin (Ub) tail to several protein targets. Ubiquitinated Mfns are extracted from the membrane and degraded *via* the proteasome in order to inhibit refusion processes, whereas ubiquitination of VDAC1 is required for mitochondria to be recognized and embedded by p62/LC3-bound autophagosome and ultimately degraded by lysosome-contained acid hydrolases. Parkin can undergo S-nitrosylation-mediated inactivation of its ubiquitin E3 ligase activity, thereby inhibiting mitophagy and disbalancing fusion/fission dynamics.

**2.3. Mitophagy.** Autophagy is a self-degradation process activated by the cells under several pathophysiological conditions, such as nutrient deprivation, infection, development, and stressful conditions in general. It includes the chaperone mediated autophagy (CMA), microautophagy, and macroautophagy that are highly conserved degradation pathways

for bulk cellular components [61, 62]. Macroautophagy (hereafter referred as to autophagy) is morphologically characterized by the formation of double-membrane autophagosomes, which sequester impaired or unwanted cellular components and deliver them to lysosomes for degradation and recycling of building blocks [62]. The mechanism of

mitochondrial sequestration and delivery to lysosomes for degradation falls into this class and is commonly termed mitophagy [63, 64]. The elimination of mitochondria is a critical process as dysfunctional mitochondria produce higher amount of ROS which can be harmful for cellular biomolecules [65, 66]. However, under certain physiological conditions (e.g., erythroid differentiation, or starvation), mitophagy can also eliminate functional mitochondria [67, 68]. Mitochondrial depolarization is a hallmark of damaged mitochondria, and data from the recent literature argue for this being a prerequisite for mitophagy [69] (Figure 4). Two are the main proteins that are involved in targeting mitochondria to the selective removal by autophagy and whose mutations are associated with inherited forms of Parkinson's disease (PD): the PTEN-induced putative kinase 1 (PINK1) and the multifunctional ubiquitin E3 ligase Parkin.

**2.3.1. PINK1/Parkin System.** Once synthesized, PINK1 is imported within mitochondria where undergoes cleavage catalyzed by the protease presenilin-associated rhomboid-like protein (PARL) in the mitochondrial inner membrane and then rapidly removed by a proteasome-dependent pathway [70] (Figure 4). Upon mitochondrial depolarization, PINK1 processing by PARL is inhibited, thereby leading to full-length PINK1 accumulation in the mitochondrial outer membrane, probably facing the cytosol [70, 71]. PINK1 stabilization is the driving event which leads to the recruitment of Parkin to mitochondria. In particular, mitochondrial-located Parkin promotes ubiquitylation of several protein substrates that are essential for the correct autophagosome targeting of mitochondria [70]. Indeed, once modified by ubiquitylation, a number of proteins (e.g., the voltage dependent anion channel 1, VDAC1) are recognized and bound by the ubiquitin-binding adaptor protein p62/SQSTM1 (p62), that concomitantly binds the autophagosome-located microtubule-associated protein light chain 3 (LC3) [72]. This "bridge-like" function of p62 lets fragmented mitochondria being correctly encompassed within the autophagosome without any possibility to re-fuse with the healthy mitochondrial network (Figure 4). This inhibition is guaranteed by the Parkin-mediated ubiquitylation of Mfn1 and Mfn2 that is a prerequisite for the extraction of both proteins from mitochondrial outer membrane through the catalytic activity of the AAA-type ATPase p97 and their subsequent degradation via the proteasome [73, 74] (Figure 4).

S-Nitrosylation is a well-established mechanism through which the ubiquitin E3 ligase activity of Parkin can be regulated [75] (Figure 4). At least five cysteine residues have been suggested to be potentially S-nitrosylated, thereby inhibiting Parkin activity [75]; however, for none of these the capability to undergo S-nitrosylation has been unequivocally reported. Very recently, Meng and coworkers have demonstrated that the formation of a sulfonic acid derivative at Cys<sup>253</sup> induces Parkin aggregation and its incapability to translocate to mitochondria upon H<sub>2</sub>O<sub>2</sub> overproduction, such as that occurring in PD-like conditions [76]. Although sulfonylation is an irreversible modification of the protein, it can be speculated that Cys<sup>253</sup> could be particularly susceptible to oxidation by ROS, as well as by RNS, and

that it could also react with NO, thus reversibly generating inactive SNO adducts of Parkin. Whatever is the residue involved in the generation of Parkin-SNO derivative, it is worthwhile mentioning that, thus modified, Parkin is no longer able to exert protective (antiapoptotic) effects in neuronal cell systems challenged with mitochondrial toxins or proteasome inhibitors. On the basis of what previously reported, it looks likely to hypothesize that S-nitrosylation of Parkin could negatively affect cell viability by impairing mitochondrial mitophagy. However, no direct evidence that Parkin-mediated protection of neuronal cells relies upon its capability to correctly induce mitochondrial degradation has been provided yet.

**2.3.2. HDAC6.** It has been recently reported that, alongside p62, the class II histone deacetylase 6 (HDAC6) is even required for Parkin-mediated mitophagy and for perinuclear transport of depolarized mitochondria [77]. HDAC6 contains a nuclear exclusion signal and a cytoplasmic retention signal making it a cytoplasmic enzyme, whose main function is to catalyze tubulin deacetylation [78] and to play key regulatory roles in microtubule dynamics [79] and motor protein motility [80]. Although S-nitrosylation has been reported impairing the activity of cytosolic HDACs [81], no study has been performed aimed at comprehending whether it specifically targets HDAC6. Interestingly, HDAC6 is the main class II HDAC member reported to reside in the cytoplasm [82]; therefore, it lets presume that, effectively, HDAC6 could undergo S-nitrosylation. Nevertheless, direct evidence demonstrating the presence of its nitrosylated form is still lacking.

**2.3.3. DJ-1.** Another protein that deserves to be mentioned in this context and whose mutations have been associated with the genetic forms of Parkinson's disease (PD) is the redox-sensitive chaperone DJ-1 [83]. Although its physical and functional association with PINK1 and Parkin is still controversial, it has been clearly arising that DJ-1 plays a crucial role in the correct fusion/fission events and processes targeting mitochondria for mitophagy, as DJ-1-null cell systems show significant alteration in both these processes [84, 85]. DJ-1 has been proposed to be active as dimer and preserves mitochondria from oxidative damage as it can directly react with ROS and RNS by means of reactive cysteine sulfhydryls. In particular, three cysteine residues have been identified to be redox-sensitive, with the Cys<sup>106</sup> undergoing sulfi(o)nylation, and Cys<sup>46</sup> and Cys<sup>53</sup> being modified by S-nitrosylation. So far, no definitive role for these modifications has been provided; however, sulfinylation of Cys<sup>106</sup> seems to be protective for mitochondria against prooxidant conditions [86], such as those occurring upon PD toxins administration. It has been also recently demonstrated that sulfinylated Cys<sup>106</sup> plays a crucial role in cell survival against UV radiations, as it interacts with and stabilizes the antiapoptotic protein Bcl-X<sub>L</sub>, thereby preventing its degradation *via* the proteasome system [87]. On the contrary, since its first characterization [88], S-nitrosylation of DJ-1 has been implicated to allow the correct dimerization of the protein. In this regard, Cys<sup>46</sup>, but not

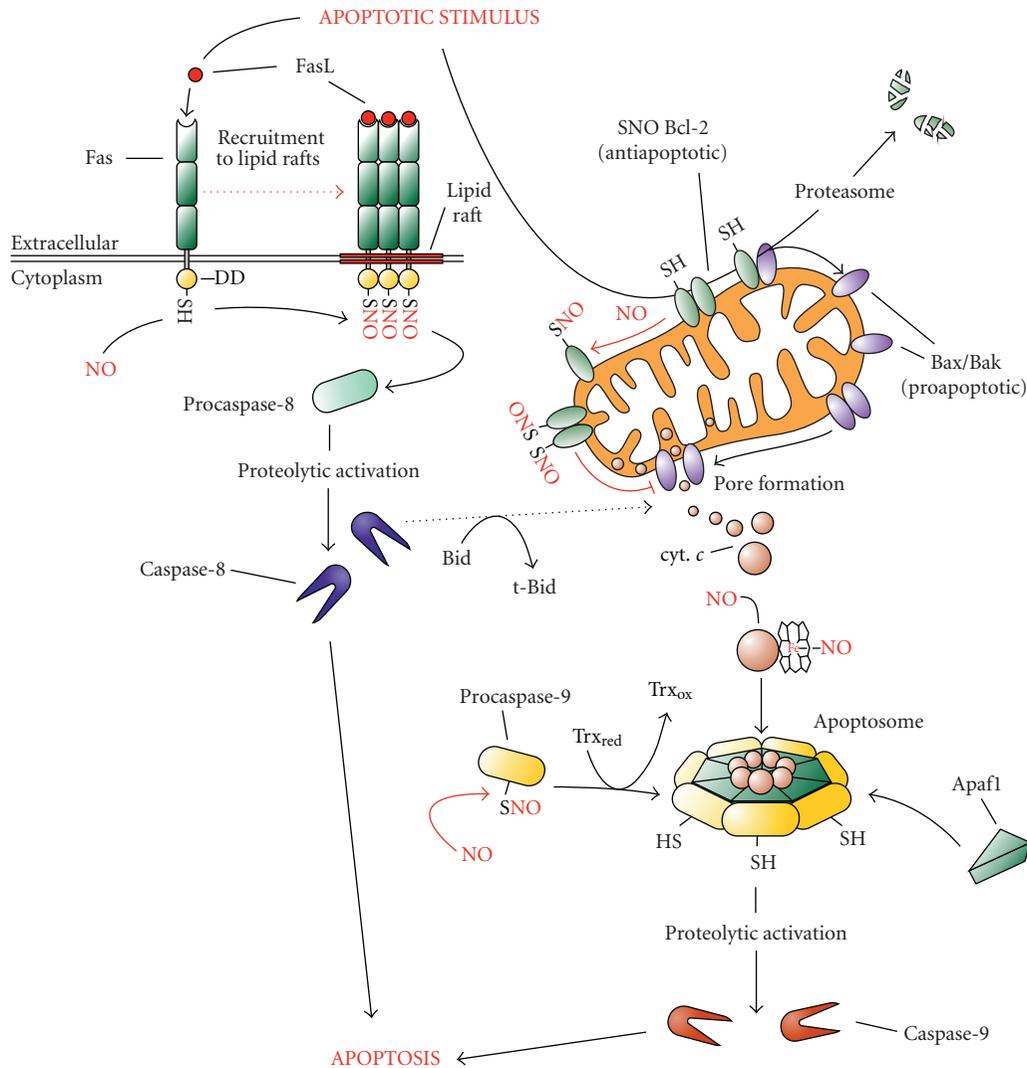


FIGURE 5: Effects of NO and nitrosative stress on apoptosis. NO-mediated effect on cell viability and death has been carefully characterized in the last years. For example, Bcl-2 has been reported to be S-nitrosylated and thus modified to be stabilized and not degraded by the Ubiquitin/proteasome system. Cytochrome *c* has been also indicated to undergo S-nitrosylation in order to bind Apaf1 and procaspase-9 and promote the assembling of the apoptosome. Zymogen procaspase-9, and the executioner pro-caspase3, remain in a quiescent (inactive) form since they are S-nitrosylated in their catalytic cysteine residue in order to avoid unwanted activation of death program. Upon apoptotic stimulus, Trxs are able to denitrosylate caspases, thereby allowing their proteolytic activation and the progression of the apoptotic events downstream it. Recently, it has been also highlighted that the recruitment of the death receptor Fas to lipid rafts of plasma membrane upon binding to its ligand (FasL) is enhanced by S-nitrosylation of Cys<sup>304</sup> of its cytoplasmic domain (DD, death domain). In this case S-nitrosylation positively affects the execution of apoptosis that takes place directly *via* the caspase-8-initiated extrinsic route or can synergize with the mitochondrial pathway through the proteolytic activation of the proapoptotic protein Bid in its truncated form (t-Bid).

Cys<sup>53</sup>—which is even nitrosylated—seems to assist DJ-1 dimerization, as C46A substitution is the sole mutation that completely abrogates the formation of DJ-1 dimers [88].

**2.4. Apoptosis.** Apoptosis is a mode of programmed cell death that is crucial for mammalian development and whose deregulation may contribute to the development of neurodegenerative disorders and cancer [89]. Cells are routinely exposed to various stimuli that can be interpreted either as good or harmful and that determine whether downstream pathways should be transduced towards life or death. In

several apoptotic pathways, such a choice is made at the level of mitochondria. These organelles are permeabilized by the proapoptotic proteins of the Bcl-2 family (e.g., Bax and Bak), that are generally antagonized by the antiapoptotic members of the same family (e.g., Bcl-2 itself, Bcl-X<sub>L</sub>), which mainly lead to the release of cytochrome *c* into the cytosol where it concurs to caspase activation and degradation of the entire cellular content (Figure 5) [90].

NO generated from NO donors, or synthesized by NOS, has been copiously demonstrated to induce cell death *via* apoptosis in a variety of different cell types; however, other

pieces of evidence argue for NO being a protective molecule against proapoptotic stimuli [91]. The evidence to be, at the same time, pro- and antiapoptotic was found to depend on the concentration of NO employed, with nanomolar range inducing Akt phosphorylation and hypoxia inducible factor (HIF)-1 $\alpha$  stabilization (prosurvival pathways), whereas micromolar levels triggering phosphorylation of p53 and the induction of apoptosis downstream of it [91]. This double feature confers to NO the name of “Janus-faced” molecule. Besides these effects which rely on the role of nitrosative stress as upstream inducer of signaling cascade, NO-mediated S-nitrosylation events have been reported to directly modulate a number of proteins involved in apoptotic response. Among them, cytochrome *c* should be undoubtedly mentioned, although it does not undergo S-nitrosylation. Indeed, NO binds the protein on its heme iron, in a way resembling the heme nitrosylation of cytochrome *c* oxidase, and this modification has been reported to occur during apoptosis and to positively influence the induction of cell death [92] (Figure 5). The release of cytochrome *c* from mitochondria is a crucial step in apoptosis, and, as above mentioned, it depends on the outer mitochondrial membrane amount of proapoptotic *versus* antiapoptotic members of the Bcl-2 superfamily. Regarding this, issue it should be reminded that Bcl-2 has been found to undergo S-nitrosylation at the level of Cys<sup>158</sup> and Cys<sup>229</sup> [93]. These modifications, that are not related to Bcl-2 phosphorylation, have been indicated to be crucial to stabilize the protein and to inhibit its degradation *via* the proteasome system, acting, in such a way, as an antiapoptotic event [93] (Figure 5). Generally, S-nitrosylation reactions are considered inhibitory of apoptotic cell demise. Indeed, many positive regulators of the apoptotic process, such as the L-type Ca<sup>2+</sup> channel [25, 94] and the mitochondrial permeability transition pore components cyclophilin D [25, 95], ANT and VDAC [25, 96, 97] have been reported to undergo S-nitrosylation as protective mechanism against apoptosis. Although the list of proapoptotic members belonging to this redox-sensing class of proteins can be widely extended, S-nitrosylation of caspases remains the prototype of how this posttranslational modification can impact on the apoptotic signal [20, 98]. That S-nitrosylation was inhibitory for caspase proteolytic activity is a concept that goes back to the late 90s, where a number of publications showed that NO donors were able to inhibit apoptosis due to the occurrence of S-nitrosylation of cysteine-based enzymes involved in the execution of programmed cell death, such as caspase-3 and tissue transglutaminase [99], caspase-1 [100], and almost all caspases [101]. Afterwards, when Mannick and colleagues found that the sole mitochondrial subpopulation of caspase-9 and caspase-3, but not the cytosolic counterpart, were S-nitrosylated [98], the role of S-nitrosylation in the apoptotic context became clear. Mitochondria-generated NO leaves mitochondrial-located caspases in a quiescent state to inhibit unwanted activations of apoptosis but allows their induction whenever they are released in the cytosol downstream of an apoptotic stimulus. Accordingly, it was concomitantly found that Fas-induced apoptosis needs cytosolic caspases denitrosylation to proceed [102] (Figure 5). Although not directly involving

mitochondria, a novel regulatory pathway that regulates apoptosis and that depends on S-nitrosylation has been reported to occur on the cytoplasmic domain of the death receptor Fas. In particular, Leon-Bollotte and coworkers demonstrated that both Cys<sup>199</sup> and Cys<sup>304</sup> of Fas intracellular portion undergo S-nitrosylation upon treatment with the NO donor glyceryl trinitrate, or the NOS activating molecule monophosphoryl lipid A, with the former thiol residue being indispensable for Fas recruitment to lipid drafts and activation of downstream apoptotic signal (Figure 5) [103].

### 3. Role and Mediators of Denitrosylation Process in Mitochondrial Homeostasis

NO can cross cell membranes. Therefore, once produced by NOS, it can freely pass mitochondrial membranes and act inside this organelle (Figure 3). In addition, some lines of evidence argue for the existence of a mitochondrial-sited isoform of NOS (mtNOS), that can directly regulate mitochondrial respiration and functions [104]. However, this aspect of NO biology remains still controversial as several studies let to hypothesize that the presence of any mitochondrial-associated NOS activity could be, merely, the consequence of experimental artifacts linked to mitochondrial purification [105]. In particular, this suspect takes cue from several observations indicating that mtNOS and nNOS are the same enzyme. Indeed, no canonical mitochondrial localization sequence, which could allow to discriminate between the cytosolic and the mitochondrial form of nNOS, has been never found. Apart from the possibility to be or not generated by a mitochondrial form of NOS, it should be reminded that, under hypoxic conditions, NO can be generated within the mitochondria without any NOS-dependent catalysis, but through the cytochrome *c* oxidase-mediated reduction of nitrite (NO<sub>2</sub><sup>-</sup>) back to NO [106]. This body of evidence, though leaving questionable the precise site of production of mitochondrial NO (inside or outside the organelle), provides an indication about the high exposure/susceptibility of mitochondria towards nitrosative stress conditions. To have a general idea of how a mitochondria can suffer nitrosative stress, it should be taken into consideration that they are furnished by a large amount of mitochondrial-sited antioxidant and denitrosylating enzymes, which play a key role in modulating NO effect. Indeed, their scavenging activity counteracts the noxious effects of NO and decreases the effects of S-nitrosylation. Of note, the equilibrium between the opposite function of NO sources and systems aimed at mitigating NO effects is made more complex if one takes into account that many members of antioxidants enzymes and denitrosylases (e.g., glutathione reductase, glutathione peroxidase, peroxiredoxins, Trx, glutaredoxin 1) undergo thiol S-nitrosylation (or oxidation) that commonly results in the inhibition of their activity [107]. Letting this issue be omitted and focusing only on the contribution of denitrosylation reactions, it is worthwhile reminding that both the denitrosylating enzymes GSNOR and Trx1 have never been found to localize inside or to be associated with mitochondria; therefore, in theory, they cannot directly modulate mitochondrial SNOs levels. Moreover, no direct evidence has been provided yet in support of

the sole mitochondrial form of thioredoxin (Trx2) being able to reduce S-nitrosylated complexes, or other mitochondrial proteins, and restoring electron transfer chain efficiency. Therefore, a question spontaneously arising is “what is the main denitrosylating enzyme implicated in the modulation of mitochondrial SNOs levels?” Recent observations arguing for a protective role of mitochondrial glutaredoxin 2 (Grx2) in *in vitro* models of neurodegeneration have been reported [108, 109]. As it is insensitive to S-nitrosylation [110] and has been demonstrated to catalyze reduction reactions of several S-glutathionylated mitochondrial proteins [109], a putative implication of Grx2 in mitochondrial denitrosylation reactions should be considered. Besides the putative roles of Trx2 and Grx2 as mitochondrial denitrosylating enzymes, it should be taken into account that GSH is present at high concentrations within the cell and that it can translocate in/out the mitochondrial membranes through the facilitative dicarboxylate transporters (DCTs). Given the capability of GSH to take part to transnitrosylation reactions with protein-SNOs, GSH is reasonably included among the principal candidates for denitrosylation of mitochondrial protein-SNOs. When GSNO is formed upon reaction of GSH with mitochondrial SNOs, it can be extruded in the cytosol by means of DCTs and there reduced by GSNOR, making this cytosolic enzyme the most reliable player of the complete reduction of mitochondrial S-nitrosylated proteome. This hypothesis is reinforced if one considers the large contribute that free GSH provides in denitrosylation of protein-SNOs from spinal cord challenged by exogenous supplementation of NO by means of transnitrosylation reactions [111].

On the basis of these assumptions, we can speculate that GSNOR deficiency or mutation could be predictive of mitochondrial morbidity towards nitrosative stress. Data from the recent literature demonstrate that GSNOR deficiency severely impacts on different aspects of mammalian physiology. For example, it (i) protects from heart failure and asthma [112, 113]; (ii) decreases vascular resistance [22, 114]; (iii) worsens septic shock conditions [114]; (iv) increases angiogenesis and protect against myocardial injury [115]; (v) compromises lymphocyte development [116]; (vi) weakens DNA damage response [117, 118]; however, no indication about whether some of these effects depend on S-nitrosylation-induced mitochondrial impairment has been provided so far. What is certain is that the last three effects of the above-mentioned list, alongside the observation that GSNOR is the sole alcohol dehydrogenase expressed in adult rat, mouse, and human brain [119], argue for a strong implication of GSNOR, and S-nitrosylation disbalance, in tumorigenesis and neurodegeneration.

#### **4. Redox State and Energetic Metabolism in Cancer and Neuronal Cells: Role of Mitochondria and Possible Modulation by S-Nitrosylation**

The mitochondrial theory of aging is based on the hypothesis of a vicious cycle, in which somatic mutations of mtDNA, such as those caused by ROS and RNS overproduction [120],

generate respiratory chain dysfunction, thus enhancing the production of further DNA-damaging events. Therefore, chronic alterations of mitochondrial homeostasis, such as those impairing the removal of damaged (e.g., radical-producing) mitochondria, are a major event in the onset of several pathological states. Cancer and neurodegeneration, which are included in the list of “nitroxidative stress-based” diseases, are the two sides of the same molecular dysfunction. Indeed, if from one hand nitroxidative conditions can be deleterious for cell survival, as demonstrated by the massive cell death phenomena of neuronal populations observed during neurodegenerative processes, from the other one, they can induce mutagenesis and trigger limitless replication, condition occurring upon neoplastic transformation [121–123]. From a mere metabolic point of view, the maintenance of vital mitochondria and efficient oxidative phosphorylation is, in theory, a prerequisite much more critical for survival of neurons than for cancer cells. Indeed, tumor cells obtain ATP almost entirely by means of glycolysis even in normoxic conditions (the so-called Warburg effect or aerobic glycolysis), which represents a major change of the entire metabolic reprogramming typical of tumors [124, 125].

*4.1. Role of S-Nitrosylation of HIF-1 in Tumor Metabolic Changes.* Cancer cells have developed the aptitude to grow under low oxygen tension in order to face up the inability of local vessels to supply adequate amount of oxygen. Therefore, the upregulation of glycolytic pathway is a selective advantage to sustain ATP demand needed for tumor proliferation under hypoxic conditions. One of the major regulators of this metabolic change is HIF-1, a heterodimeric transcription factor composed of an oxygen-sensing  $\alpha$  subunit and a constitutively expressed  $\beta$  subunit [126]. In normoxic conditions HIF-1 $\alpha$  undergoes rapid proteasomal degradation elicited by prolyl-hydroxylases- (PHDs-) mediated hydroxylation, which lets HIF-1 $\alpha$  being recognized for the subsequent ubiquitylation [127, 128]. Even under normoxic conditions, NO positively affects HIF-1 stabilization by indirectly inhibiting PHDs activity [129]; however, it has been also indicated that NO can directly impact on HIF-1 $\alpha$  subunit by means of S-nitrosylation reactions on specific cysteines, thereby enhancing its stability and gene transactivating capacity [130]. In particular, Li and collaborators demonstrated that a specific S-nitrosylation event of HIF-1 $\alpha$  on Cys<sup>533</sup> inhibits its degradation as this modification stabilizes the protein, thereby determining the overall activity of HIF-1 [131]. Moreover, it has been also found that Cys<sup>800</sup> located at the C-terminal activation domain can undergo S-nitrosylation and, thus modified, facilitates HIF-1 binding to its co-activator p300/CREB, thereby allowing the activation of HIF-1-mediated gene transcription. HIF-1 activation causes the induction of pyruvate dehydrogenase kinase 1 which shunts pyruvate away from mitochondria and concomitantly triggers mitophagy by means of the alternative pathway that relies upon the induction of Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) [132, 133]. Overall, these metabolic rearrangements lead to a reduction in mitochondrial mass and to an enhancement of the glycolytic flux, therefore reinforcing the hypothesis that

S-nitrosylation of HIF-1 $\alpha$  might be involved in metabolic changes occurring in cell malignant transformation.

**4.2. Nrf2/Keap1 System in Cancer and Neuronal Cells.** By contrast, neurons are “addicted” to ATP synthesized by mitochondria, whose efficiency does not depend upon glucose availability, but are continuously fueled by pyruvate deriving from glia-provided lactate in a Cori’s cycle-like manner [134, 135]. Indeed, glucose taken up by neurons is mainly redirected to the pentose phosphate pathway allowing the generation of NADPH to sustain sulfhydryl reductive pathways (e.g., denitrosylation) and antioxidant response in general, which are indispensable for neuron survival [135, 136]. Indeed, in normal conditions, ROS levels are finely controlled by the transcriptional induction of many antioxidant systems which are predominantly regulated by the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) [137]. In several tumor histotypes, a basal activation of this transcription factor is induced, and several somatic mutations have been demonstrated to destroy the interaction between Nrf2 and its physiological inhibitor Kelch-like ECH-associated protein 1 (Keap1), thereby promoting the persistent activation of the Nrf2-mediated antioxidant/detoxifying response and tumorigenesis [138, 139]. At the same time, several observations indicate that the activation of Nrf2 by nitroxidative insults is protective against conditions recapitulating neurodegenerative diseases. Recently, it has been shown that S-nitrosylation of Keap1—that, in some cases, can resolve in disulfide bridge formation [140]—in neuronal cells induces a persistent activation of Nrf2 signaling by allowing the dissociation of Nrf2-Keap1 heterodimer [141, 142].

**4.3. NOS and GSNOR in Tumorigenesis.** On the basis of these pieces of evidence, NO produced at moderately high rate, alongside with S-nitrosylation-induced impairment of respiratory chain, can be more dangerous for neurons than for cancer cells, where, conversely, it can promote tumor survival and malignancy by inducing further mutagenic events. In regard to this, all NOS isoforms have been detected in tumor cells from a wide range of isolates [143, 144]. Ambs and coworkers also demonstrated that an NO-mediated upregulation of vascular endothelial growth factor is related to increased xenograft vascularisation, indicating that NO generated by NOS promotes blood vessel formation, thereby enhancing the ability of tumor to indefinitely grow [145, 146]. iNOS has been also found being expressed in hepatocellular carcinoma (HCC) and is often increased in the hepatocytes of patients with chronic hepatitis and alcoholic cirrhosis that predispose to HCC [147–149]. In line with this assumption, it has been very recently demonstrated that GSNOR-KO mice, which are no longer able to denitrosylate SNOs *via* GSNOR activity, spontaneously develop HCC [117, 118, 150]. Wei and collaborators demonstrated that the mechanism underlying hepatocyte transformation involves the inactivation of the DNA repair system [118]. Particularly, GSNOR deficiency, or somatic loss-of-function mutations (e.g., deletion) in GSNOR gene, which have been found being associated with many cases of hepatic cirrhosis and

chronic hepatitis B or C, induces S-nitrosylation of the DNA repair system member O<sup>6</sup>-alkylguanine-DNA alkyltransferase (AGT). Thus modified, AGT is degraded via the proteasome, thereby failing DNA damage being repaired and allowing mutations being established [118].

## 5. S-Nitrosylation and Cellular Quality Control Efficiency in Cancer and Neurodegeneration

NO, S-nitrosylation, and mitochondrial defects have long been regarded as contributors of the neurodegenerative processes [59, 151–153]; indeed, it has been demonstrated that PD, Alzheimers disease (AD), amyotrophic lateral sclerosis (ALS), and Huntingtons disease (HD) are characterized by an increase in nitrosative stress in neurons [154–156]. S-nitrosylation of Parkin, protein disulfide isomerase (PDI), peroxiredoxin 2 (Prx2), X-linked inhibitor of apoptosis (XIAP), and Drp1 has been implicated in stress-induced neuronal death [59, 157–159] and has been also observed in brains from patients with neurological disorders. Of note, these proteins are key players in the regulation of mitochondrial dynamics/autophagy (Parkin, Drp1), antioxidant and antiapoptotic response (Prx2, XIAP), correct protein folding (PDI), which represent the processes reported to be widely affected in neurodegenerative diseases. In particular, S-nitrosylation of PDI catalytic cysteines has been demonstrated to inhibit its enzymatic activity [160], thereby leading to the accumulation of polyubiquitylated proteins and, in turn, to endoplasmic reticulum stress [161]. Also, both peroxidatic and resolving cysteines of Prx2 (Cys<sup>51</sup> and Cys<sup>172</sup>, resp.), as well as cysteines of XIAP located in its BIR domain, have been demonstrated to undergo S-nitrosylation. This posttranslational modification transiently inhibits isomerase and chaperone-like activities of Prx2 [158], as well as antiapoptotic function of XIAP, although it leaves unaltered its ubiquitin E3 ligase activity [159].

Taking into account the above-mentioned observations, it clearly arises that the efficiency of the systems deputed to cellular quality control is crucial for the homeostasis of cellular physiology [162–165]. DNA repair system, autophagic/mitophagic machinery, and the ubiquitin/proteasome system (UPS) ensure the maintenance of a correct equipment of biomolecules and organelles, whereas apoptosis guarantees the final elimination of cells whose vital functions are definitely compromised. A number of proteins and enzymes involved in DNA repair, autophagy, ubiquitylation and protein degradation, as well as in apoptosis are continuously subjected to nitroxidative modification (e.g., S-nitrosylation) which can compromise their correct activity. However, whereas loss-of-function modification of DNA repair systems and apoptotic proteins have much more severe repercussions on neoplastic transformation, the alterations of autophagy and UPS operation are harmful mostly for neuronal cell survival.

**5.1. Modulatory Role of NO and S-Nitrosylation in Autophagy: Relevance in Cancer and Neurodegeneration.** Autophagy and the UPS act synergistically to hydrolyze damaged proteins;

actually, autophagy is regarded as a backup system to complement proteasomal degradation when it is overwhelmed or incapable of dealing with specific aggregated substrates [166]. This aspect gains increasingly value in neuronal homeostasis in which the presence of protein aggregates has been frequently associated with the etiology of the disease, and autophagy is being defined as antiapoptotic and anti-neurodegenerative process [167]. Remarkably, aggregates of proteins involved in neurodegenerative disease, such as  $\alpha$ -synuclein or mutant huntingtin, have been identified as substrates for autophagy [168, 169]. The recent observation that NO, reasonably via S-nitrosylation, inhibits autophagy and that NOS inhibition enhances the clearance of autophagic substrates and reduces neurodegeneration in HD models [170] reinforces the hypothesis that nitrosative stress-mediated protein aggregation in neurodegenerative disorders may be, in part, due to the inhibition of autophagy. The reason according to which a correct autophagic flux is necessary to preserve neuronal viability does not exclude that it could be also implicated in tumorigenesis. Nevertheless, this issue is still controversial. Indeed, a growing body of evidence argues for autophagy being a crucial process in oncogenesis and in tumor progression [171–173]. The still uncharacterized autophagic mechanism leading to suppress tumorigenesis could depend on (i) the removal of nitrooxidatively damaged biomolecules; (ii) the degradation of specific organelles or proteins essential for cell growth [174–177]. By contrast, many observations show that autophagy activation enables tumor long-term survival (i.e., when apoptosis is defective) keeping cancer cells alive when limited angiogenesis leads to nutrient deprivation and hypoxia [178, 179]. From this perspective, one would expect that increased autophagy could promote solid tumors growth. It should be reminded that autophagy can suppress tumorigenesis by means of the elimination of p62 [180]. As previously described, p62 is an adaptor protein that targets damaged mitochondria and ubiquitylated proteins and that drives the correct autophagosome membrane formation being, at last, degraded during the process. Autophagy defects produce p62 accumulation, thereby causing persistent Nrf2 activation. In this regard, Komatsu and colleagues demonstrated that autophagy defects leading to p62 accumulation may induce nuclear translocation of Nrf2 through the interaction between Keap1 and p62 [181]. This novel regulation of Nrf2 provides a convincing evidence allowing to speculate that increased NO levels and S-nitrosylated proteins, by inhibiting autophagic flux, could function as protooncogenic also by impacting on such a mechanism.

*5.2. Effects of an Altered Mitophagy in Neurodegeneration: Focus on Parkin and Drp1.* Besides its role in damaged proteins removal, autophagy is principally implicated in the elimination of large cellular portions and organelles, such as in the case of mitophagy [182]. Mitochondrial dysfunction is a specific feature of almost all neurodegenerative diseases; however, the connection between mitophagy and neuropathology has been predominantly explored with respect to PD [183–185]. Several reports indicate that defective mitophagy, *via* a lack of mitochondria targeting due to mutated Parkin,

may be to blame for much of the pathological phenotypes observed in PD [186]. As above described, S-nitrosylation inhibits Parkin ubiquitin E3 ligase activity and its protective function [187], resembling in such a way the alteration typical of PD-associated mutations [75, 187]. We above described that Parkin interacts with and polyubiquitinates Mfn1 and Mfn2, thereby promoting their degradation via the proteasome [188]. Accordingly, PD-related mutations in Parkin attenuate the occurrence of these processes and lead to excessive mitochondrial fusion. Based on these findings, Glauser and colleagues suggest that a close relationship between Parkin and mitochondrial dynamics exists and that mutations in the protein affect mitochondrial fission/fusion event, thus inducing neuronal death [188]. The demonstration that Mfns are ubiquitylated by Parkin provides a support for a link between Parkin ubiquitin E3 ligase activity and mitochondrial dynamics. An intriguing possibility is that PD-associated mutations that impair the Parkin-mediated ubiquitylation and degradation of Mfns may result in excessive mitochondrial fusion and impaired mitophagy, leading to an accumulation of damaged or dysfunctional mitochondria. The disruption of this dynamic equilibrium may herald cell injury or death and contribute to neurodegenerative disorders, as well as tumor development. Conversely, neurons from postmortem human AD brains [189] show excessive fission that results in abnormally small mitochondria with fragmented cristae [189, 190]. This phenotype has been indicated to be dependent on S-nitrosylation of Drp1 at Cys<sup>644</sup> and excessive activation of its fission activity [59]. Accordingly, the exposure to oligomeric amyloid  $\beta$  ( $A\beta$ ) peptide of cell culture models of AD has been reported to induce mitochondrial fragmentation as observed upon NO donors administration [191–194]. This phenotype is associated with synaptic damage and apoptotic cell death, thereby suggesting that the S-nitrosylation of Drp1 contributes to AD pathogenesis. Thus, denitrosylation of Drp1, such as by inducing GSNOR activity, may represent a potential new therapeutic target for protecting neurons and their synapses in sporadic AD.

## 6. Concluding Remarks

In this paper we have focused on the possible consequences of NO bioactivity, especially S-nitrosylation, on mitochondrial homeostasis, and we have reported how they can impact on cancer and neurodegenerative diseases. We have shown that many proteins involved in respiration, mitochondrial turnover, and apoptosis are subjected to S-nitrosylation, thereby modulating cellular response and the correct occurrence of several cellular functions. Whereas the implication of S-nitrosylation in apoptosis has been copiously investigated, research dealing with the role of redox-mediated posttranslational modifications in mitochondrial dynamics and mitophagy is still at the beginning phases. The comprehension of how redox mechanisms govern these phenomena will deserve deep investigations in the future in order to propose new pharmacological approaches able to interfere with S-nitrosylation state, and that can be useful as valuable tools for the treatment of diverse pathological

conditions related to defect in mitochondrial dynamics and mitophagy. In regard to this aspect, great efforts in the last years have been profused by researchers to understand the involvement of the sole NO production in the onset of the diseases, without taking into account that SNOs levels can be modulated even by tuning denitrosylase activity. For instance, the observation that iNOS<sup>-/-</sup> mice develop intestinal tumors led to substantiate the idea that iNOS was implicated in the macrophage-mediated tumor killing process [195]. In accordance, a growing body of evidence pointed out that NO-releasing drugs killed tumor cells [196–198]. However, the generation of NOS transfectants resulted in promotion of tumor growth, rather than killing, suggesting that, being NO a Janus-faced molecule, a precise modulation of its production rate is very hard to set up, and that even minor inaccuracies in setting the stage for clinical approaches could result in opposite effects [199, 200]. In line with these observations and consistent with the current opinion that NO is also, or rather principally, a molecule indispensable for cell viability and correct physiology, the putative use of the pan NOS inhibitor, N(G)-nitro-L-arginine methyl ester (L-NAME), has been reported to display a plethora of side effects that hamper its employment for the cure of aberrant S-nitrosylation-associated diseases [201]. At the mitochondrial level, for example, a reduction of NO levels, due to an inhibition of its production, profoundly affects the efficiency of oxidative phosphorylation and this could result in alterations of mitochondrial morphology and dynamics [202]. Results of the last years also argue for this relationship being biunivocal, as abnormal mitochondrial dynamics is strictly related to metabolic alterations [203]. Such an intimate interplay among processes involved in different aspects of mitochondrial homeostasis underlies the multiple mitochondrial phenotype observed in several neurodegenerative diseases. For example, patients with early stage AD, whose brains are distinguished by the presence of abnormal or fragmented mitochondria [189, 191], regularly exhibit declining mitochondrial energy metabolism and ATP production, which may subsequently cause synaptic loss and neuronal damage [203–205].

On the basis of what previously mentioned and taking into account that many proteins regulating mitochondrial dynamics and removal by autophagy are affected in their function by S-nitrosylation, it becomes clear that S-nitrosylation of this class of proteins could also impact on mitochondrial respiration and metabolism [206]. Indeed, Drp1 has been shown to be instrumental for sustaining mitochondrial ATP synthesis, as mitochondrial bioenergetics in Drp1-depleted cells is profoundly impaired [206, 207]. *Vice versa*, pharmacological inhibition of respiratory chain Complex I alters the organization of the mitochondrial network, which is paralleled by decreases in the mitochondrial membrane potential and an increased ROS production [206, 208]. In addition, the increased ROS production occurring under hyperglycemic condition requires dynamic changes in the morphology of mitochondria, with fragmentation being a necessary event to increase high-glucose-induced respiration and, in turn, to generate ROS [207]. Interestingly, cells expressing a dominant-negative mutant form of Drp1

show mitochondria that retain their tubular form and do not exhibit any increased respiration, hyperpolarization, or ROS production.

Altogether, these indications argue for regulation of mitochondrial morphology being intimately associated with the metabolic function of the organelle [207]; however, the molecular nature of this link remains still unknown. Animal models in which the denitrosylase activity is genetically impaired (e.g., GSNOR-KO mice) have been very recently employed to characterize the involvement of aberrant S-nitrosylation in liver cancer development. Nevertheless, the finding that AGT S-nitrosylation is a driving event in HCC does not exclude that other mechanism(s) could be operative for neoplastic transformation. Indeed, AGT-KO mice do not recapitulate GSNOR-KO phenotype, as they do not necessarily develop HCC [117]. On the basis of what has been described in this paper, it is reasonable to hypothesize that other factors related to mitochondrial homeostasis maintenance may have a role in the etiopathogenesis of liver cancer. It is desirable that, in the next future, transgenic mouse models of impaired denitrosylation, alongside with synthetic inhibitors of denitrosylating enzymes, namely, GSNOR, which have been recently designed [209–211] and yielded for the treatment of asthma, will be used to dissect how denitrosylation reactions, mainly those occurring within the mitochondria, are involved in the onset of several diseases. The results originating from these studies will provide the proof-of-principle of how, and whether, S-nitrosothiols targeting, *via* denitrosylase inhibition, could be a promising tool for the treatment of cancer and neurodegeneration.

## List of Abbreviations

AD:	Alzheimers disease
ADH III:	Class III alcohol dehydrogenase
AGT:	O <sup>6</sup> -alkylguanine-DNA alkyltransferase
ALS:	Amyotrophic lateral sclerosis
PKA:	cAMP-dependent protein kinase
CMA:	Chaperone mediated autophagy
DCTs:	Dicarboxylate transporters
Drp1:	Dynamin-related protein1
Grx:	Glutaredoxin
GSH:	Reduced glutathione
GSNO:	S-Nitrosoglutathione
GSNOR:	S-Nitrosoglutathione reductase
GSSG:	Glutathione disulfide
HCC:	Hepatocellular carcinoma
HD:	Huntingtons disease
HDAC:	Histone deacetylase
HIF1:	Hypoxia inducible factor 1
Keap1:	Kelch-like ECH-associated protein 1
L-NAME:	N(G)-Nitro-L-arginine methyl ester
Mfn:	Mitofusin
NO:	Nitric oxide
NOS:	Nitric oxide synthase
Nrf2:	Nuclear factor (erythroid-derived 2)-like 2
ONOO <sup>-</sup> :	Peroxynitrite
Opa1:	Optic atrophy 1
PARL:	Presenilin-associated rhomboid-like protein

PD: Parkinson's disease  
 PDI: Protein disulfide isomerase  
 PHDs: Prolyl hydroxylases  
 PINK1: PTEN-induced putative kinase 1  
 Prx2: Peroxiredoxin 2  
 RNS: Reactive nitrogen species  
 ROS: Reactive oxygen species  
 SNOs: S-Nitrosothiols  
 Trx: Thioredoxin  
 TrxR: Thioredoxin reductase  
 UPS: Ubiquitin/proteasome system  
 VDAC1: Voltage dependent anion channel 1  
 XIAP: X-linked inhibitor of apoptosis.

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## Review Article

# TCA Cycle Defects and Cancer: When Metabolism Tunes Redox State

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Inborn defects of the tricarboxylic acid (TCA) cycle enzymes have been known for more than twenty years. Until recently, only recessive mutations were described which, although resulted in severe multisystem syndromes, did not predispose to cancer onset. In the last ten years, a causal role in carcinogenesis has been documented for inherited and acquired alterations in three TCA cycle enzymes, succinate dehydrogenase (SDH), fumarate hydratase (FH), and isocitrate dehydrogenase (IDH), pointing towards metabolic alterations as the underlying hallmark of cancer. This paper summarizes the neoplastic alterations of the TCA cycle enzymes focusing on the generation of pseudohypoxic phenotype and the alteration of epigenetic homeostasis as the main tumor-promoting effects of the TCA cycle affecting defects. Moreover, we debate on the ability of these mutations to affect cellular redox state and to promote carcinogenesis by impacting on redox biology.

## 1. Introduction

Cancer cells differ from normal ones due to a plethora of oncogenes-driven biochemical changes designed to sustain an high rate of growth and proliferation [1]. The first tumor-specific alteration in metabolism was reported at the beginning of the 20th century by Warburg [2]. His observations demonstrated that cancer cell metabolism relies on an increased glycolytic flux maintained even in the presence of oxygen (“aerobic glycolysis” or “Warburg effect”), without an associated increase in oxidative phosphorylation rate. The switch from respiration to glycolysis has usually been considered a consequence, rather than a cause, of cancer. However, in the last decade, the discovery that inherited and acquired alterations in some enzymes of tricarboxylic acid (TCA) cycle have a causal role in carcinogenesis has changed this viewpoint, pointing towards altered metabolism as the underlying hallmark of neoplastic transformation. These alterations consist of germline defects in genes encoding subunits of SDH and FH, as well as somatic mutations in coding sequence for IDH. Together with metabolomics studies documenting the alteration of HIF-dependent signaling pathway

and epigenetic dynamics as main tumor-promoting effects of these mutations, a mounting body of evidence also supports how alterations in the TCA cycle enzymes may favor tumorigenesis by impacting on cellular redox state. Therefore, in this paper, we summarize the prooncogenic defects in the TCA cycle enzymes discussing their involvement in the tuning of redox environment and the engagement of redox-dependent tumorigenic signaling.

## 2. Fundamentals of the TCA Cycle

The TCA cycle is a core pathway for the metabolism of sugars, lipids, and amino acids [3]. It is usually presented in a naive perspective of a cyclic mitochondrial route constantly oxidizing the acetyl moiety of acetyl-coenzyme A to CO<sub>2</sub>, generating NADH and FADH<sub>2</sub>, whose electrons fuel the mitochondrial respiratory chain for ATP generation. The TCA cycle begins with the condensation of acetyl-CoA with oxaloacetate to form citrate, catalyzed by citrate synthase. Citrate can be exported to the cytoplasm, where it is used as precursor for lipid biosynthesis or remains in the mitochondria, where it is converted to isocitrate by aconitase.

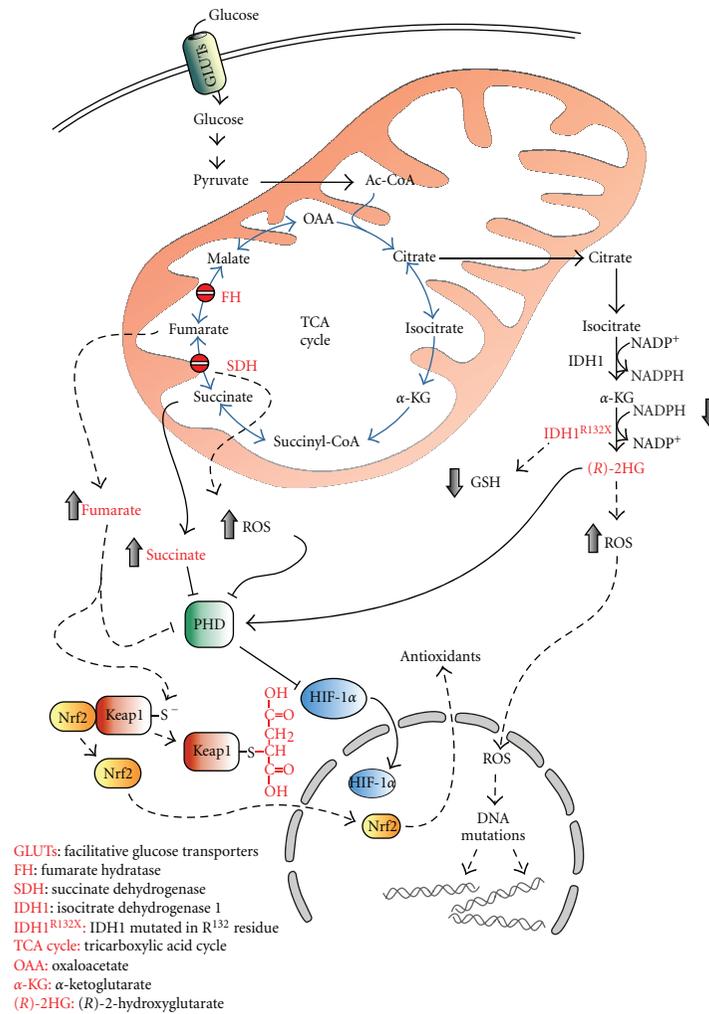


FIGURE 1: Redox alterations induced by TCA cycle defects. Redox alterations induced by mutations in SDH, FH, and IDH are shown. Loss of function of SDH increases ROS levels leading to DNA mutations and HIF-1 $\alpha$  stabilization. IDH1 and IDH2 (not shown) mutations decrease GSH and NADPH levels. (R)-2-HG, produced by oncogenic mutations in IDH1 and IDH2, triggers ROS accumulation. Defects in FH stimulate nuclear translocation of Nrf2 and the transcription of antioxidant enzymes through the succination of Keap1. Enzymes and metabolites involved in tumor formation and redox alterations are in red. Blue arrows indicate TCA cycle reactions. Dotted arrows indicate pathways modulating cell redox state.

In the next step,  $\alpha$ -ketoglutarate ( $\alpha$ -KG), formed by the oxidative decarboxylation of isocitrate catalyzed by IDH, is converted to succinyl-CoA by a further decarboxylation by the  $\alpha$ -KG dehydrogenase complex. Succinyl-CoA is then transformed to succinate by the succinyl-CoA synthetase. Fumarate, produced by succinate oxidation catalyzed by the SDH complex, is hydrated to malate by FH. Oxidation of malate, catalyzed by malate dehydrogenase, finally regenerates oxaloacetate, thus ensuring the completion of the cycle (Figure 1). On the mere biochemical viewpoint, the TCA cycle in nontumor cells has been divided into two stages: (i) decarboxylating, in which citrate is converted to succinyl-CoA releasing two CO<sub>2</sub> molecules; (ii) reductive, which comprises the successive oxidations of succinate to oxaloacetate. Interestingly, emerging findings from the last year support the hypothesis that, in several cell systems

such as (i) cancer cells containing mutations in complex I or complex III of the electron transport chain (ETC), (ii) patient-derived renal carcinoma cells with mutations in FH, (iii) cells with normal mitochondria subjected to acute pharmacological ETC inhibition, as well as (iv) tumor cells exposed to hypoxia, the first stage of the cycle can proceed in the opposite direction through the reductive carboxylation of  $\alpha$ -KG to form citrate. This allows cells to produce acetyl-coenzyme A to support *de novo* lipogenesis and their viability [4–6]. Although in physiological and resting conditions mitochondria are necessary and sufficient to perform the cycle, isoforms of some of its enzymes have been also found in the cytosol. This ensures a dual compartmentalization (cytosolic and mitochondrial) of reactions and metabolites which, being free to diffuse through the outer and the inner mitochondrial membranes by channels and active carriers,

respectively, allows the cycle to respond to environmental and developmental signals, thus sustaining anabolic reactions as well as fueling the ATP-producing machinery. The TCA cycle is also a major pathway for interconversion of metabolites arising from transamination and deamination of amino acids and provides the substrates for amino acids synthesis by transamination, as well as for gluconeogenesis and fatty acid synthesis. Regulation of the TCA cycle depends primarily on a supply of oxidized cofactors: in tissues where its primary role is energy production, a respiratory control mediated by respiratory chain and oxidative phosphorylation is operative. This activity relies on availability of  $\text{NAD}^+$  and ADP, which in turn depends on the rate of utilization of ATP in chemical and physical work.

### 3. Genetic Defects in the TCA Cycle

Genetic defects affecting the TCA cycle enzymes have been known for more than two decades. Until recently, only recessive mutations were documented whose clinical consequences were similar to alterations in the electron transport chain (ETC) and oxidative phosphorylation [7]. These defects were associated with multisystem disorders and severe neurological damage, but no cancer predisposition, as a result of very considerably impaired ATP formation in the central nervous system. In the last ten years, dominant defects associated with oncogenesis were described in cytoplasmic and mitochondrial isoforms of three nuclear-encoded enzymes, SDH, FH, and IDH, allowing to investigate the extrametabolic roles of the TCA cycle metabolites and their signaling to tumor formation.

**3.1. Succinate Dehydrogenase.** The SDH complex (also known as succinate:ubiquinone oxidoreductase or mitochondrial complex II) is a highly conserved heterotetrameric tumor suppressor, composed by two catalytic subunits (SDHA and SDHB), which protrude into the mitochondrial matrix, and two hydrophobic subunits (SDHC and SDHD), which anchor the catalytic components to the inner mitochondrial membrane and provide the binding site for the ubiquinone, as well [8]. All the subunits are encoded by nuclear genome and, unlike most of the TCA cycle enzymes, have no cytosolic counterparts. SDH catalyzes the oxidation of succinate to fumarate in the TCA cycle with the simultaneously reduction of ubiquinone to ubiquinol in the ETC. A decade ago, mutations in SDHB, SDHC, and SDHD subunits were identified in patients with hereditary paragangliomas (hPGLs) and pheochromocytomas (PCCs), a rare neuroendocrine neoplasm of the chromaffin tissue of the adrenal medulla or derived from the parasympathetic tissue of the head and neck paraganglioma, respectively [9–12]. More recently, mutations in SDHA and the SDH assembly factor 2 (SDHAF2), required for flavination of SDH [13, 14], have been associated with hPGL/PCC syndrome [15]. The genetic defects in the SDH genes predisposing to the hPGL as well as PCC are heterozygous germline mutations, inducing the inactivation of the protein and the neoplastic transformation develops as result of loss of heterozygosity, caused by the complete loss of the enzyme

function by a second mutagenic hit (usually deletion) [16]. In addition to hPGL and PCC, a number of other neoplasms have been associated with mutations in SDH genes, including gastrointestinal stromal tumors, renal cell cancers, thyroid tumors, neuroblastomas, and testicular seminoma [8].

**3.2. Fumarate Hydratase.** FH is homotetrameric TCA cycle enzyme which catalyzes the stereospecific and reversible hydration of fumarate to L-malate. Homozygous FH deficiencies result in fumaric aciduria [17], characterized by early onset of severe encephalopathy and psychomotor retardation; on the contrary, heterozygous FH mutations predispose to multiple cutaneous and uterine leiomyomas (MCUL), as well as to hereditary leiomyomatosis and renal cell cancer (HLRCC) [18, 19]. In particular, the kidney tumors in HLRCC, whose morphological spectrum include papillary type II, tubulopapilar, tubular, collecting duct, and clear cell carcinoma, are particularly aggressive. Growing evidence suggests that *FH* mutations may also be involved in the pathogenesis of breast, bladder, as well as Leydig cell tumors [20, 21]. The most common types of tumor predisposing genetic defects are missense mutations (57%), followed by frameshift and nonsense mutations (27%), as well as large-scale deletions, insertions, and duplications [22]. Like SDH, enzymatic activity of FH is completely absent in HLRCC as result of the loss of the wild-type allele in the transformed cell.

**3.3. Isocitrate Dehydrogenase.** IDH is a member of the  $\beta$ -decarboxylating dehydrogenase family of enzymes and catalyzes the oxidative decarboxylation of isocitrate to produce 2-oxoglutarate ( $\alpha$ -KG) and  $\text{CO}_2$  in the TCA cycle. Nuclear genome encodes three isoforms of IDH: IDH1 and IDH2 are  $\text{NADP}^+$ -dependent homodimers, whereas IDH3 is a  $\text{NAD}^+$ -reliant heterotetrameric enzyme. Whereas IDH1 is found into cytoplasm and peroxisomes, IDH2 and IDH3 are exclusively localized into the mitochondrial matrix, and, although all three isoforms are able to decarboxylate isocitrate, IDH3 is the main form of IDH functioning in the TCA cycle under physiological conditions whereas IDH1 and IDH2 are mainly involved in the reductive glutamine metabolism, under hypoxia and ETC alterations [4, 5, 23]. Though it plays a central role in energy production, to date there have been no reports of cancer-associated mutations in any of the IDH3 subunits. Conversely, genomewide mutation analyses and high-throughput deep sequencing revealed the presence of mutations in either IDH1 or its mitochondrial counterpart IDH2 in 70% of grade II–III gliomas and secondary glioblastomas [24, 25]. Since these initial reports, mutations in IDH1 and IDH2 have been identified in 16–17% of patients with acute myeloid leukemia, in 20% of angioimmunoblastic T-cell lymphomas [26], and spotted in a variety of other malignancies at lower frequencies [27, 28] such as B-acute lymphoblastic leukemias, thyroid, colorectal, and prostate cancer [29, 30]. Unlike *SDH* and *FH* mutations in hPGL and HLRCC, respectively, IDH1 and IDH2 mutations are somatic and monoallelic. Moreover, whereas mutations in *SDH* and *FH* occur throughout the gene, the majority of IDH mutations

identified in gliomas and AML are changes in the amino acid residues R132 in IDH1 and either R172 or R140 in IDH2 [31]. As result of these alterations, mutated IDHs are unable to efficiently catalyze the oxidative decarboxylation of isocitrate and acquire a neomorphic catalytic activity that allows a NADPH-dependent reduction of  $\alpha$ -KG into the oncometabolite (*R*)-2-hydroxyglutaric acid ((*R*)-2HG) [31, 32].

#### 4. Mechanisms of Tumorigenesis Caused by the TCA Cycle Defects

The finding that many tumors arising from mutations in both *SDH* and *FH* genes are characterized by hypoxic features has suggested that the activation of the hypoxia-inducible transcription factor-1 $\alpha$  (HIF-1 $\alpha$ ) could play a supportive role in the tumorigenic processes induced by TCA cycle dysfunctions. Indeed, HIF-1 $\alpha$  is known to coordinate the biochemical reprogramming of cancer cells aimed to sustain their growth and proliferation as well as tumor vascularization [33–35]. The causal link between TCA cycle dysfunction and HIF-1 $\alpha$  activation was initially suggested by Selak and coworkers demonstrating that the accumulation of succinate in SDH-deficient cells causes the inhibition of prolyl 4-hydroxylases (PHDs), a negative regulators of the stability of the  $\alpha$  subunit of HIF [36]. The PHDs are members of the superfamily of  $\alpha$ -KG-dependent hydroxylases, which couple the hydroxylation of the substrates with the oxidation of  $\alpha$ -KG to succinate in reactions that are dependent on O<sub>2</sub> and Fe<sup>2+</sup> [37]. In normoxic conditions, PHDs hydroxylate two proline residues in the oxygen-dependent degradation domain of HIF-1 $\alpha$ , allowing it to be polyubiquitinated and degraded *via* proteasome. The accumulated succinate in SDH-deficient or SDH-inactive cells impairs PHDs activity leading to HIF-1 $\alpha$  stabilization under normoxic conditions (pseudohypoxia) [36]. Similarly to succinate, also fumarate, which accumulates in tumors harboring loss of FH function, has been demonstrated to be potent inhibitors of PHDs [38]. Interestingly, fumarate-mediated stabilization of HIF was observed to induce the upregulation of several HIF-target genes, including those that stimulate cell growth and angiogenesis, allowing to hypothesize pseudohypoxia response as a plausible mechanism for HLRCC onset [38]. Despite that this large body of evidence showed a direct link between HIF-1 $\alpha$  expression and tumorigenesis, recent findings have raised some questions about the protumorigenic role of pseudohypoxic adaptation in all types of tumors arising from TCA cycle defects. The first question was raised from the study of Adam and colleagues. They demonstrated that neither the presence of HIF nor the absence of PHDs is required for hyperplastic renal cysts formation (typical hallmark of HLRCC) in a kidney-specific *Fh1* (the ortholog of human FH) knockout mice that recapitulates many features of the human disease [39], suggesting that alternative oncogenic actions of fumarate could be responsible for HLRCC generation (see next paragraph). In addition to this study, depicting HIF as a sort of “bystander player” in the onset of tumors harboring FH mutations, another report indicated this transcription factor as a tumor-suppressor

protein in tumors carrying IDH1/2 mutations. Indeed, as demonstrated by Koivunen and colleagues, contrarily to succinate and fumarate, (*R*)-2HG stimulates PHDs activity, driving, in such a way, HIF-1 $\alpha$  for proteasome-mediated degradation [40]. Moreover, they pointed out that HIF-1 $\alpha$  downregulation enhances the proliferation of human astrocytes and promotes their transformation, providing a justification for exploring PHDs inhibition as a potential treatment strategy for tumors harboring IDH1/2 mutations [40].

As member of the  $\alpha$ -KG-dependent hydroxylases, PHDs catalyze the hydroxylation of a wide range of substrates, besides HIF-1 $\alpha$  [37]. Therefore, the reduced hydroxylation of PHD targets may contribute to tumorigenesis regardless of HIF-1 $\alpha$  activity and the acquisition of a hypoxic signature. For instance, it has been proposed that SDH deficiency could impair PHD-dependent programmed cell death of neurons, therefore setting the stage for neoplastic transformation of neuronal cells. This hypothesis finds support in the recent studies demonstrating that the proapoptotic activity of the prolyl hydroxylase EglN3 requires a functional SDH, being feedback inhibited by succinate [41, 42]. Since EglN3 is required during development to allow the programmed cell death of some sympathetic neuronal precursor cells, its inhibition, elicited by the elevation of succinate levels, could play a role in the pathogenesis of tumors arising from a defective developmental apoptosis, such as pheochromocytomas.

Highlighting the HIF-1 $\alpha$ -independent tumorigenic mechanisms, growing body of evidence clearly places the alteration of TCA flux upstream of the epigenetic dynamics as well. Histone methylation is an important epigenetic modification which has been demonstrated to regulate gene expression by modifying chromatin structure and, thereby, fine-tuning the binding of transcription factors [43, 44]. One of the most studied enzymes regulating histone methylation signature are the Jumonji C-terminal domain (JmjC) family of histone demethylases [45]. As they remove the methyl groups on the arginine and lysine residues of histones after performing an  $\alpha$ -KG- and oxygen-dependent hydroxylation, they have been included in the  $\alpha$ -KG-dependent hydroxylases family. It was shown that succinate accumulation, in SDH-deficient cells, negatively affects the activity of many members of such class of histone demethylase. For instance, succinate-mediated JMJD3 inhibition leads to changes in the methylation mark of histone H3 on arginine [46]. Furthermore, in a yeast model of paraganglioma, the histone demethylase, Jhd1, was found to be inhibited by succinate accumulation in an  $\alpha$ -KG-competitive manner [47]. Similarly, recent studies demonstrate that, besides SDH alterations, also IDH1/2 defects are associated with hypermethylated phenotype. Indeed, in cells harboring *IDH1/2* mutations, intracellular (*R*)-2-HG levels can reach the value of 10 mM. These concentrations promote the competitive inhibition of the  $\alpha$ -KG-dependent histone N<sup>ε</sup>-lysine demethylase JMJD2A, and the ten-eleven translocation (TET) family of 5-methylcytosine (5mC) hydroxylases, a class of protein mediating the  $\alpha$ -KG-dependent removal of methyl mark from 5-methylcytosines, resulting in an enhanced histone

and DNA methylation, respectively [48, 49]. Interestingly, although fumarate is able to inhibit HIF-regulating PHDs similarly to succinate, no evidence attesting its putative capability to mirror its cognate metabolite succinate in affecting histone methylation has been documented so far. In addition, as TET enzymes are members of the  $\alpha$ -KG-dependent hydroxylases family, a putative ability of both succinate and fumarate in their inhibition can be reasonably argued. On the basis of the ability of the epigenetic alterations to affect lineage-specific differentiation and to result in the activation of oncogenes or silencing of tumor suppressors [44, 50, 51], the competitive inhibition of histone and DNA demethylases elicited by defects in fluxes of TCA cycle metabolites may drive tumorigenesis by promoting cell transformation and uncontrolled proliferation.

### 5. Redox-Dependent Tumorigenic Alterations Elicited by the TCA Cycle Defects

Apart from the mere metabolic viewpoint, compelling evidence suggests that the reactive oxygen species (ROS), produced by a deregulated mitochondrial functioning, might trigger the oncogenic signal or, at least, participate in the progression of tumors characterized by defects in the TCA cycle enzymes (Figure 1). This assumption finds support in the observation that, compared with their normal counterparts, many types of cancer cells have increased levels of ROS generated by a defective mitochondrial electron-transport chain [52–54]. By exploiting their chemical reactivity with biomolecules, such as nucleic acids, ROS are known to induce several types of DNA damages, including depurination and depyrimidination, single- and double-stranded DNA breaks, base and sugar modifications, and DNA-protein crosslinks. In such a way, permanent modifications of DNA, resulting from sustained prooxidant conditions, drive the mutagenic events underlying carcinogenesis.

The observation that specific SDHC mutant (*mev-1*) of the *C. elegans* nematode was able to generate superoxide  $O_2^{\bullet}$  [55, 56] suggested the possibility that ROS could have a causal role in the pathogenesis of tumors bearing defects in the TCA cycle. This hypothesis was further strengthened by the evidence that mouse fibroblasts transfected with a murine equivalent of the *mev-1* mutant were featured by a sustained ROS production and a significantly higher DNA mutation frequency than wild-type counterparts [57]. Although these lines of evidence supported the mutagenic role of ROS generated by defective SDH complex, no detectable DNA damages, despite an increased production of ROS and protein oxidation, was described in a *S. cerevisiae* strain lacking Sdh2 (the yeast ortholog of mammalian SDHB) [47]. To link the prooxidant state elicited by SDH dysfunctions to tumorigenesis, Guzy and colleagues proposed that the ROS could play a supportive role in the oncogenic process by contributing to the activation of HIF-1 $\alpha$  [58]. Indeed, relying on a previously characterized role of respiratory chain-derived ROS as signals for HIF-1 $\alpha$  stabilization under hypoxia [59, 60], it has been shown that cells expressing mutant SDHB, but not mutant SDHA, are

characterized by significant mitochondrial ROS production required, together with succinate, for a complete inactivation of PHDs and HIF-1 $\alpha$  stabilization [58]. Therefore, these results reinforce the role of ROS as amplifier of the pseudo-hypoxic response, observed in all cells carrying SDH defects, providing a biochemical rationale for the severity of SDHB mutations which are usually associated with aggressive PCC.

The capabilities of the TCA cycle defects in the tuning of cellular redox state have been supported by the evidence that also oncogenic mutations in IDH1/2 genes are associated with the oxidation of intracellular milieu. Normally, in aerobic organisms, the control of cellular redox state is ensured by the balance between the prooxidant species, mainly produced by mitochondria, NADPH oxidases or as byproduct of the intermediate metabolism, and their clearance through the synergistic action of the antioxidant enzymes and the thiol-containing antioxidants. Among the latter, the tripeptide glutathione (GSH) plays a pivotal role in determining the steady-state value of the intracellular redox potential. Indeed, its intracellular abundance (1–10 mM) allows GSH to participate, as electron donor, in the enzymatic reduction of hydrogen peroxide and lipid peroxides and in the generation of reversible S-glutathionylated adducts with protein thiols, preventing them to undergo irreversible forms of oxidation [61]. The capability of IDH mutations to induce oxidative intracellular conditions is linked to a decrease in GSH levels, observed both in IDH1-R132H—and IDH2-R172K—expressing glioma cells with respect to their *wt* counterparts [62]. GSH is synthesized in two ATP-dependent steps: (i) synthesis of  $\gamma$ -glutamylcysteine, from L-glutamate and cysteine *via* the rate-limiting enzyme glutamate-cysteine ligase (GCL); (ii) addition of glycine to the C-terminal of  $\gamma$ -glutamylcysteine *via* the enzyme glutathione synthetase. Intracellular glutamate, required for the first reaction of GSH biosynthesis, is mainly produced by the oxidative deamination of glutamine catalyzed by the enzyme glutaminase [63]. As IDH1/2 mutant cells are characterized by lower levels of glutamate with respect to their *wt* matching parts [62], it is possible that oncogenic defects in IDH result in impaired GSH synthesis due to a lower glutamate availability, thus phenocopying the prooxidant conditions observed in glutaminase deficient cells [64]. The dampened glutamate levels could be the result of an enhanced  $\alpha$ -KG demand of IDH1/2 mutant cells allowing the biosynthesis of the oncometabolite (R)-2-HG. This assumption is supported by the evidence that treatment of glioma cells with (R)-2-HG does not deplete neither glutamate nor glutathione levels [62], suggesting that many metabolic changes observed in IDH-mutated cells are not due to the direct action of (R)-2-HG but a consequence of its oncogenic production. The involvement of IDH1/2 mutations in the generation of prooxidant conditions is not only related to the alteration of intracellular GSH content. Indeed, the oxidative decarboxylation of isocitrate, which is impaired in all mutants of IDH1 and IDH2 proteins, is coupled to a reduced ability to generate NADPH. Moreover, the failure to sustain intracellular NADPH production is associated with an increased NADPH oxidation, necessary to allow the reductive biosynthesis of (R)-2-HG [31, 32, 65]. As GSH and the thiol-based

antioxidant protein thioredoxin require NADPH as a source of reducing equivalents for their own regeneration [61], the altered equilibrium of NADP<sup>+</sup>/NADPH elicited by IDH1/2 mutations could contribute to the shift of the intracellular redox state towards more oxidizing conditions. Although, these lines of evidence bring about the ability of mutant IDH1/2 to elicit prooxidant conditions independently on the direct action of (*R*)-2-HG on human redox metabolome, it has been proposed that this oncometabolite could contribute itself to oxidize intracellular environment. Indeed, some reports demonstrate its ability to induce oxidative damages in cerebral cortex of young rats [66] and to elicit ROS generation through the stimulation of NMDA receptor [67]. Although these findings support prooxidant capability of (*R*)-2-HG, to date no striking evidence has been provided attesting its mutagenic role.

Whereas accumulating pieces of evidence support the capability of oncogenic mutations in SDH as well as IDH genes to oxidize intracellular milieu, conflicting findings do not allow for defining a clear role of FH deficiency in cellular redox state modulation. The most convincing evidence showing the capability of FH-deficient cells to promote intracellular ROS accumulation comes from the work of Sudarshan and colleagues [68]. This study demonstrated that inactivating mutations of *FH* in an HLRCC-derived cell line result in glucose-induced NADPH oxidases-mediated generation of O<sub>2</sub><sup>•-</sup> and ROS-dependent HIF-1 $\alpha$  stabilization. On the contrary, O'Flaherty and colleagues provided clear evidence that accumulation of fumarate, due to the absence of a functional FH, is the sole mechanism responsible for the inhibition of HIF-1 $\alpha$  prolyl hydroxylation, independently on defect in mitochondrial oxidative metabolism [69]. Indeed, the complete correction of HIF-1 $\alpha$  pathway activation in Fh1<sup>-/-</sup> MEFs by extra-mitochondrial FH expression suggests that, at least in tumors harboring FH defects, neither impaired mitochondrial function nor the consequent dependence of energy metabolism on glycolysis contributes significantly to HIF-1 $\alpha$  engagement. The most substantial pieces of evidence, depicting the elevation of fumarate levels as a condition linked to the reduction of intracellular redox state, came from two recent studies demonstrating that FH loss results in the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) [39, 70], the pivotal transcription factor responsible for the induction of the antioxidant-responsive-element- (ARE-) driven genes, which codify for phase II detoxification enzymes and antioxidant proteins such as glutathione S-transferases and GCL [71]. Both studies demonstrated that reconstitution of FH-deficient cells with wild-type FH or an extra-mitochondrial FH decreased fumarate levels and restored Nrf2 regulation [39, 70]. In addition, elevation of intracellular fumarate content by a membrane-permeable fumarate ester was found sufficient to induce Nrf2 and its orchestrated antioxidant program [70]. According to the current view, in resting conditions, Nrf2 is retained in the cytoplasm through its interaction with Keap1 which prevents its nuclear translocation and rules its ubiquitin-proteasome-mediated turnover, as well. However, in the presence of electrophiles as well as during redox unbalance, Keap1 is

modified at several reactive cysteine residues, resulting in Nrf2 stabilization and the activation of the protective gene expression program [71, 72]. In line with this accepted model, both groups revealed by mass spectroscopy analyses that fumarate was able to succinate several cysteine residues previously shown to be electrophile targets, including Cys<sup>151</sup> and Cys<sup>288</sup>, thereby providing a mechanistic explanation of the fumarate-induced Nrf2 activation [39, 70]. Although ROS can promote carcinogenesis by inducing oxidative damages to DNA, a recent outstanding study demonstrates that oncogene-induced Nrf2 activation promotes tumorigenesis by lowering ROS levels and conferring a more reduced intracellular environment [73]. Therefore, on the basis of this evidence, it is possible to hypothesize that the fumarate-mediated activation of the Nrf2-antioxidant pathway might drive the oncogenic signal for tumors characterized by defects in the FH enzyme. Although this assumption has not been demonstrated yet, the observation that heme oxygenase 1, one of the best defined target genes of Nrf2, is upregulated in FH-deficient cells allowing their survival [74] supports the putative causal role of Keap1 succination in the onset of tumors carrying FH defects. Furthermore, mounting bodies of evidence show that Nrf2 and its downstream genes are overexpressed in many cancer cell lines and human cancer tissues conferring them advantage for survival and growth as well as acquired chemoresistance [75, 76]. Therefore, it is possible to speculate that besides driving renal tumorigenesis, fumarate-induced succination of Nrf2 could contribute to the reduced sensitivity of particularly aggressive and recurrent forms of kidney cancer, such as HLRCC [77], to many chemotherapeutic approaches. The enhanced activation of Nrf2 observed both by Pollard and Furge groups contributes to explain the results obtained by Raimundo and coworkers in nontumor cells [78]. Indeed, they documented that FH-deficient diploid human fibroblasts are characterized by a highly reduced redox state with increased GSH levels, as result of increased expression of the GSH biosynthetic enzyme GCL. As highly reducing environment has been shown to stimulate cell proliferation [79], it is possible to hypothesize that the reduced redox state elicited by FH mutations could favor the doublings of stem-cell-like populations promoting thus the initial event of tumor formation. This assumption finds support in the observation that lower ROS levels have been found in many cancer stem cells with respect to the nontumorigenic counterparts, allowing them to maintain a high proliferative status and to prevent their differentiation [80].

## 6. Concluding Remarks

The direct involvement of TCA cycle enzymes in tumor formation has been arousing from a decade. In tumors associated with defects of SDH, FH, and IDH enzymes, the underlying mechanisms of tumorigenesis involve the accumulation of metabolites (succinate, fumarate, and (*R*)-2-HG) that convey oncogenic signals (oncometabolites). Large amount of evidence points towards the generation of pseudohypoxic phenotype and the alteration of epigenetic homeostasis as the main cancer-promoting effects of

the TCA cycle affecting mutations. Besides inhibiting the  $\alpha$ -KG-dependent hydroxylases, mounting body of evidence supports the ability of these oncometabolites to alter cellular redox state in precancerous as well as transformed cells. Therefore, alternatively or concomitantly to the generation of pseudohypoxic phenotype and the alteration of epigenetic dynamics, the oncometabolites-induced engagement of redox-dependent signaling pathways could contribute both to the neoplastic transformation of healthy cells as well as to the progression of malignancies characterized by germline mutations in SDH and FH and of somatic defects in IDH. These emerging findings reveal a dynamic interaction between the genetic profile, the metabolic status, and the redox tuning of the cell. Moreover, the different impact of oncogenic mutations of the TCA cycle on cellular redox state could contribute to explain the differences in the clinical phenotype and outcome of their associated tumors, opening new perspectives in the comprehension of the molecular mechanisms of oncogenesis and therapeutic targeting of these neoplastic alterations.

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## Review Article

# Caloric Restriction and the Nutrient-Sensing PGC-1 $\alpha$ in Mitochondrial Homeostasis: New Perspectives in Neurodegeneration

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Mitochondrial activity progressively declines during ageing and in many neurodegenerative diseases. Caloric restriction (CR) has been suggested as a dietary intervention that is able to postpone the detrimental aspects of aging as it ameliorates mitochondrial performance. This effect is partially due to increased mitochondrial biogenesis. The nutrient-sensing PGC-1 $\alpha$  is a transcriptional coactivator that promotes the expression of mitochondrial genes and is induced by CR. It is believed that many of the mitochondrial and metabolic benefits of CR are due to increased PGC-1 $\alpha$  activity. The increase of PGC-1 $\alpha$  is also positively linked to neuroprotection and its decrement has been involved in the pathogenesis of many neurodegenerative diseases. This paper aims to summarize the current knowledge about the role of PGC-1 $\alpha$  in neuronal homeostasis and the beneficial effects of CR on mitochondrial biogenesis and function. We also discuss how PGC-1 $\alpha$ -governed pathways could be used as target for nutritional intervention to prevent neurodegeneration.

## 1. Introduction

Mitochondria are dynamic organelles fundamental for cell life. The central roles of mitochondria in metabolism place them at the center stage of global energy modulation. Indeed, these organelles are best known for producing ATP via oxidative phosphorylation (OXPHOS). In the matrix, tricarboxylic acid cycle (TCA) generates reduced carriers (NADH and FADH<sub>2</sub>), which donate electrons to the inner membrane-localized electron transport chain (ETC). In this way electrons derived from metabolites flow through the ETC generating intermembrane proton gradient (mitochondrial membrane potential), which fundamental for the activity of rotary turbine-like ATP synthase producing ATP from ADP [1].

Mitochondria contain a circular genome, mitochondrial DNA (mtDNA), which has been reduced during evolution

through gene transfer to the nucleus. Even if mitochondria are characterized by an own genome encoding for 13 subunits of ETC, they strongly necessitate of many other nuclear encoded proteins (about 1000–1500) for the execution of their function [2]. Therefore, it is clear that mitochondria are not autonomous entities but strongly dependent on nuclear genome. Indeed, mitochondrial homeostasis is assured by a well-functioning bidirectional network of mitochondrial-nuclear communications (MN-C). MN-C control many cell activities including mitochondrial biogenesis, an intricate biological process consisting in the growth and division of preexisting mitochondria that requires the replication of the mtDNA and the synthesis and import of proteins and lipids to the existing mitochondria [3]. Mitochondrial biogenesis is substantially driven by the nuclear genome, where an interconnected network of transcription factors regulates the expression of mitochondrial proteins including those that

control replication and transcription of mtDNA. All these processes have to be finely regulated and coordinated in response to a wide range of physiological cues that affect cellular energetic need (e.g., temperature, physical activity, and nutrient availability).  $\text{NAD}^+$  and AMP are considered key second messengers for nuclear-mitochondrial communications and hence for mitochondrial metabolic adaptation and mitochondrial biogenesis. They orchestrate an integrated physiological response through the specific activation of molecular effectors including transcription factors, cofactors, nuclear receptors, and kinases. Important cellular sensors of metabolic status enrolled into mitochondrial-nuclear axis are: (i) the AMP-activated protein kinase (AMPK), (ii) the  $\text{NAD}^+$ -dependent deacetylase sirtuin 1 (SIRT1), and (iii) the peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 $\alpha$ ). AMPK is activated by an increase in AMP:ATP ratio and increased ADP concentrations, both of which are linked to an energetic drop [4–6]. SIRT1 responds to elevated levels of  $\text{NAD}^+$  that occur upon starvation and acts in synergy with AMPK in regulating mitochondrial mass, nutrient oxidation, and ATP production to fit cells needs via the transcriptional coactivator PGC-1 $\alpha$  [6, 7]. Mitochondria also represent the principal source of reactive oxygen species (ROS), which are considered important second messengers orchestrating MN-C. Many reports have showed that ROS can enhance the transcription of genes responsible for the growth of many cellular types [8]. In particular, in cancer cells the high proliferation rate is associated with an increased oxidative intracellular environment due to dysbalance of mitochondrial ROS production [9]. A key role in the MN-C is also played by nitric oxide (NO), a small signaling molecule that in brain functions as a neurotransmitter and neuromodulator, exerting a regulatory effect on neuronal function. Even though the production of NO by mitochondria is still a debated matter of research [10], it is now clear that this second messenger is able to dictate the shuttling and/or activation of redox-sensitive transcription factors to the nucleus. In doing so, similarly to ROS, NO redirects gene expression for adaptation of cells to stress or commitment to death. Physiological concentration of NO may efficiently buffer cell death induced by antiproliferative agents dampening the activity of the mitochondrial apoptotic cascade governed by the JNK/c-Jun signaling pathway [11]. On the contrary high concentration of NO can inhibit cell proliferation via the activation of p53 and/or induce caspase-independent apoptosis mediated by the shuttling of AIF from the mitochondria to the nucleus [12, 13]. The uncontrolled increase of ROS/NO flux is considered a contributing pathogenic factor in the ageing process and in many human diseases [14, 15]. The oxidative/nitrosative stress caused by the increase of mitochondrial ROS as well as upregulation of NO synthases are commonly observed in many neurodegenerative diseases including Parkinson's disease (PD) [16]. It is worth noting that also physiological flux of NO can become detrimental for neuronal cells in condition of limited antioxidant availability. In particular, we have showed that the decline of glutathione (GSH), the most abundant nonenzymatic thiol antioxidant, is the primary cause of endogenous NO neurotoxicity, that is, characterized

by a prominent protein damage via S-nitrosylation and nitration [13, 17]. In line with this evidence, also the decrease of enzymatic antioxidant for example, SOD1, result in a ROS-mediated damaging effect on cellular targets including mitochondria specifically in cells of neuronal origin [18, 19]. Therefore, the decline of antioxidant levels, generally occurring during physiological ageing, may be likely considered a key factor triggering NO/ROS-mediated neuronal death in ageing and neurodegenerative-related diseases.

Among cellular systems, neurons are exquisitely dependent upon mitochondria to support their high energy-demanding functions [20]. The intricate role of mitochondria in maintaining neuronal metabolism emerges from the causal relationship between their impairment and several neurological disorders. In neurons, mitochondria supply substantial amounts of ATP as well as TCA intermediates that serve as the building blocks for synthesis of neurotransmitters [21, 22]. For instance it has been shown that pyruvate carboxylase deficiency strongly alters brain amino acid content affecting the release of gamma-aminobutyric acid (GABA) and glutamate neurotransmitters [23]. Mitochondria-mediated lipid synthesis is also critical for neuronal function, as defects in lipoic acid synthase cause severe neonatal onset of epilepsy [24]. Mitochondrial dysfunction has been extensively claimed to have a contributing factor also in other neurological disorders, for example, amyotrophic lateral sclerosis (ALS) and Alzheimer disease's (AD). In AD, alterations in enzymes involved in oxidative phosphorylation have been reported in hippocampal neurons. In PD, mutations in proteins related to mitochondria have been identified and mitochondrial DNA mutations have been found in neurons of the *substantia nigra*. In ALS, the activity of some mitochondrial respiratory-chain enzymes is generally affected and mitochondrial-resident cell death proteins result altered. It is recently emerging that also alteration of mitochondrial quality control may have a leading role in the neurodegenerative process. Indeed, altered mitochondrial biogenesis and/or mitochondrial clearance of damaged mitochondria through autophagy (mitophagy) have been detected in degenerating neurons.

A growing body of evidence suggests that restricting calorie intake has a strong beneficial effect on neuronal function. In particular, caloric restriction (CR) is now widely considered a dietary regimen that may delay brain senescence and prevents neurodegeneration. It has been suggested that many of the beneficial effects of CR not only derive from its ability to regulate  $\text{NAD}^+$  and AMP cellular levels but also to modulate ROS and NO production ultimately leading to efficient mitochondrial activity [25]. Interestingly, NO and ROS may regulate via a redox-dependent mechanism PGC-1 $\alpha$  both at expression and activity level [14, 25–27].

Here, we review the current knowledge and recent data related to the role of PGC-1 $\alpha$  and CR in neurodegeneration and illustrate the potential underlying signaling pathways characterizing the MN-C. We also discuss how such pathways might serve as targets for nutritional intervention to prevent ageing and age-related neurodegenerative diseases.

## 2. PGC-1 $\alpha$ in the Orchestration of Mitochondrial-Nuclear Communication

A key factor in the MN-C is the transcriptional coactivator PGC-1 $\alpha$ . Like all transcriptional coactivators, PGC-1 $\alpha$  modulates the activity of several transcription factors without directly binding to DNA and without having any known intrinsic histone deacetylase or other enzymatic activities. The activity of PGC-1 $\alpha$  only consists in mediating the interaction between transcription factors and RNA polymerases so influencing gene expression [28]. PGC-1 $\alpha$  was originally identified as a transcriptional regulator of PPAR $\gamma$  capable of stimulating UCP-1 expression in brown adipose tissue [29]. Subsequently, PGC-1 $\alpha$  has been shown to coactivate a number of nuclear receptors, including PPARs, HNF4 $\alpha$ , GR, and ERR $\alpha$ , in addition to transcription factors such as NRF-1, MEF2C, FOXO1, and YY1 [28, 30]. This wide variety of binding partners empowers PGC-1 $\alpha$  to induce context-specific coordinated sets of gene expression that are tailored to the immediate physical demands of the organism, including energetic failure (nutrient limitations/physical exercise) and oxidative stress.

Recent studies indicate that regulation of cellular oxidative capacity through enhancing mitochondrial biogenesis may be beneficial for neuronal recovery and survival in human neurological disorders. Indeed, PGC-1 $\alpha$  protein is very abundant in neural progenitor cells and newly generated neurons in the embryonic and early postnatal brain, suggesting a role for PGC-1 $\alpha$  in the dynamic processes of neuroplasticity occurring during this time period [31]. In mitochondrial biogenetic pathways, PGC-1 $\alpha$  is able to induce the expression and activation of NRF1 and 2 transcriptional factors implicated in the induction of mitochondrial genes including TFAM, which in turn coordinates the replication and the expression of genes located on mtDNA [32]. Consistent with its role as a master regulator of mitochondrial biogenesis, PGC1 $\alpha$  expression closely correlates with mitochondrial content and is induced upon different stimuli eliciting a shift from glycolytic to oxidative metabolism. In particular, PGC-1 $\alpha$  induces thermogenesis in brown adipose tissue, fiber-type switching in skeletal muscle, and fatty acid  $\beta$ -oxidation, along with gluconeogenesis, in the liver [33]. More recently, it was discovered that in the intestine PGC-1 $\alpha$  governed the intestinal epithelium cell fate by promoting enterocytes differentiation and apoptosis by virtue of its capacity to enhance mitochondrial respiration and consequently ROS production [34]. Moreover, PGC-1 $\alpha$  in neuronal cells is able to induce mitochondrial biogenesis and elevate basal respiration [35]. Several lines of evidence link PGC-1 $\alpha$  alteration and neurodegeneration and it has been reported that PGC-1 $\alpha$  is capable of improving or rescuing mitochondrial dysfunctions implicated in the pathogenesis of several age-related diseases including neurodegenerative diseases [36–38]. Very recently, it was demonstrated that PGC-1 $\alpha$  is downregulated in postmortem brains of PD patients. This event is due to decreased E3 ligase activity of mutated Parkin impairing the proteasome-mediated degradation of a major transcriptional repressor of PGC-1 $\alpha$  gene promoter (i.e., parkin-interacting substrate, PARIS) [38].

In a clinical context, being oxidative stress and mitochondrial dysfunction typical hallmarks of neurodegeneration, PGC-1 $\alpha$  could be a useful tool to counteract or to limit the development of neurological disorders. Coherently with this hypothesis, PGC-1 $\alpha$  null mice are prone to the development of neurodegeneration under treatment with PD-related drugs and upregulation of PGC-1 $\alpha$  in mouse model of neurodegeneration ameliorates disease's symptoms [37, 39, 40]. Similarly, PGC-1 $\alpha$  deficiency leads to behavior abnormalities, which are associated with axonal degeneration in the brain, especially in the striatum, a key region controlling body movements. The molecular basis of this axonal degeneration is not completely understood, even though impaired energy homeostasis and ROS dysmetabolism due to defect in mitochondrial function are likely the most prominent causes. A pathogenetic hypothesis could be that in neurodegeneration there is an inability of PGC-1 $\alpha$  in sensing mitochondrial-produced ROS causing a break in MN-C. It was demonstrated that the expression of this coactivator could be induced by ROS and this event is able to stimulate redox transduction pathways culminating in the expression of antioxidant genes (e.g., GPx1 and MnSOD) [37]. So, PGC-1 $\alpha$ , promoting mitochondrial biogenesis, which unavoidably increases ROS level through the ETC, is also able to induce antioxidants expression, thus maintaining normal redox status in response to changes in oxidative capacity. For this reason, PGC-1 $\alpha$ -way of action has been defined as “a clean energy program” [41]. Besides modulating PGC-1 $\alpha$  expression, mitochondrial ROS are also able to modulate PGC-1 $\alpha$  activity [37, 42]. In particular, it has been found that ROS-induced p38 mitogen-activated protein kinase (p38 MAPK) cascade culminates in the phosphorylation of PGC-1 $\alpha$  at three residues (T262, S265, and T298) leading to PGC-1 $\alpha$  increased stability and half-life. It is interesting that these phosphorylations occur in a region previously shown to play an important regulatory role in PGC-1 $\alpha$  binding to transcription factors including many nuclear receptors and NRF-1. The docking of these transcription factors causes a conformational change that accelerates the binding of other transcriptional effector proteins into this complex, including CBP/p300 and SRC-1 resulting in a more efficient PGC-1 $\alpha$ -mediated stress resistance [43]. Anderson and colleagues have shown a subcellular redistribution of PGC-1 $\alpha$  upon oxidative stress. After the treatment of cells with an oxidative insult, GSK3 $\beta$  is activated and phosphorylates PGC-1 $\alpha$  leading to its intranuclear digestion by the proteasome system. This regulatory pathway allows that the activation and degradation of PGC-1 $\alpha$  can be independently controlled. Moreover, this study demonstrated that PGC-1 $\alpha$  represents a good candidate for the regulation of cellular metabolic activity through the coordination of MN-C upon stress conditions [44]. In this scenario the comprehension of the molecular mechanisms that control cellular fate through the mitochondria-nucleus network could be useful to explore new strategies and pharmacological treatments for neurodegenerative diseases characterized by ROS imbalance.

Also other mitochondrial mediators can modulate PGC-1 $\alpha$  activity thus affecting its ability to promote compensatory responses under stress conditions. Indeed, PGC-1 $\alpha$  activity

can be finely tuned in response to different metabolic situations and the metabolic sensors AMPK and SIRT1 have been described to directly affect PGC-1 $\alpha$  activity through phosphorylation and deacetylation, respectively [45]. These sensors would act as integrative nodes in MN-C determining which transcriptional responses will be operative in order to adapt to the environmental conditions.

SIRT1 is one of the mammalian homologues of yeast, the Sir2 protein, and the founding member of the sirtuin gene family and mediates NAD<sup>+</sup>-dependent deacetylation of specific target substrates. As the cellular redox balance of NAD<sup>+</sup> and NADH is highly related to mitochondrial activity, it has been postulated that SIRT1 could act as a sensor that directly connects metabolic perturbations with transcriptional outputs, as it was initially characterized as a histone deacetylase [46]. Although our understanding of mammalian SIRT1 biology is still surprisingly weak, there is compelling evidence implicating it as a major factor controlling metabolic homeostasis. First of all, during the last decade, a number of reports have shown that SIRT1 is not just a histone deacetylase. In fact, SIRT1 can directly interact and regulate the activity of coregulators, including PGC-1 $\alpha$ . In particular, it has been shown that SIRT1-mediated PGC-1 $\alpha$  deacetylation enhances its transcriptional activation, and mutation of the acetylation sites to arginine, which mimics the deacetylated state, markedly increases basal PGC-1 $\alpha$  transcriptional activity [28]. In relation to this aspect, several evidences demonstrate that activation or overexpression of SIRT1 could be used to compensate neuronal mitochondrial loss [47]. On the contrary, energetic state linked to an increased NADH/NAD<sup>+</sup> ratio are able to promote an hyperacetylation of PGC-1 $\alpha$  mediated by general control nonderepressible 5 (GCN5) and steroid receptor coactivator 3 (SRC-3), thus diminishing PGC-1 $\alpha$  transcriptional activity. Moreover, when PGC-1 $\alpha$  is deacetylated by SIRT1 and sequestered into the nucleus, an effect on transcriptional activity of PGC-1 $\alpha$  mitochondrial target genes (nuclear genes encoded for component of ETC) is observed.

AMPK constitutes a molecular hub for cellular metabolic control, common to all eukaryotic cells. Numerous reports have established how AMPK responds to changes in the AMP/ATP ratio as a measure of cellular energy levels. AMPK activation is highly relevant for the transcriptional adaptation to physiological situations of energy demand. Mice expressing a dominant-negative form of AMPK cannot increase mitochondrial biogenesis in response to energy deprivation in skeletal muscle [48, 49]. On the other hand, in mice overexpressing an activated form of the AMPK, the expression of genes controlling mitochondrial activity is induced [50]. These results strongly confirm a potential role of AMPK in the MN-C. In fact, AMPK constitutes a major regulator of basal mitochondrial gene expression as well as mitochondrial gene expression upon energy stress. Interestingly, there is a strong overlap in the genes transcriptionally regulated by AMPK and those by PGC-1 $\alpha$ , hence suggesting that PGC-1 $\alpha$  might be an important mediator of AMPK-induced gene expression. Supporting this hypothesis, AMPK activation leads to increased PGC-1 $\alpha$  expression and AMPK requires PGC-1 $\alpha$  activity to modulate the expression

of several key players in mitochondrial process [51, 52]. However, a closer link has been provided by findings showing that AMPK can directly interact and phosphorylate PGC-1 $\alpha$ . It has been revealed that AMPK activation (phosphorylation on ser 172) seems to increase transcriptional activity of PGC-1 $\alpha$ , even though the reasons why, where, and how that happens are still elusive. On the opposite mechanisms, diet rich in energy, which is associated with an increased ATP/AMP ratio, reduces AMPK phospho-activation and this event is associated with a decreased activation of PGC-1 $\alpha$  [52]. In relation to the fact that neurons are highly metabolically active, and have poor capacity for nutrient storage, the role of AMPK in the development, function, and maintenance of the nervous system has only recently gained attention.

### 3. MN-C during Caloric Restriction: Implication in Neurodegenerative Diseases

Nutrient stress is generally considered from the standpoint of how cells detect and respond to an insufficient supply of nutrients to meet their bioenergetic needs. However, cells also experience stress as a result of nutrient excess, during which there is an increased ROS production combined with augmented ATP and NADH levels [53]. Therefore, it is possible that the resulting energetic overload can generate cell responses correlated with pathophysiological conditions. Sparks and coworkers [54] have shown that a high-fat diet (HFD) was linked to down-regulation of PGC-1 $\alpha$  protein and mRNA, as well as expression of genes encoding proteins of complexes I, II, III, and IV of the ETC [55]. These changes were recapitulated and amplified in a murine model of HFD, along with decreases in PGC-1 $\alpha$  and cytochrome *c* protein. A hypothetical model by which nutrient excess, and in particular a chronic nutrient excess, can induce mitochondrial dysfunction could be related to an engulfment in MN-C pathways. In particular, the increase in ATP and NADH induces a reduction in AMPK activation and SIRT1 deacetylating potential, thus negatively modulating PGC-1 $\alpha$  activity. This status, in concomitance to a persistence of high ROS levels could induce a switch from cell resistance to cell damage, promoting a degenerative process especially in neurons. By far the greatest risk factor for neurodegenerative disease is ageing, and the age-dependence of such pathologies has led to experiments investigating the therapeutic effects of CR on neurodegeneration. CR is usually defined as a moderate reduction, generally 20–40%, in caloric intake compared with *ad libitum* feeding, without compromising the basic nutritional needs [56, 57]. Several studies have reported the inverse relationship between caloric intake and risk of neurodegeneration [57], fitting well within the context of the CR paradigm, one of the most robust in gerontology [58]. In Rhesus monkeys, CR lowered the incidence of aging-related death, diabetes, cancer, cardiovascular disease, and brain atrophy [59]. A significant increase in survival in CR monkeys as well as attenuation of the age-related declines in brain volume in selected regions was found. Thus, in specie closely related to humans, CR has shown

promise as an intervention that could retard brain aging and neurodegeneration.

Although the mechanisms by which CR performs these effects are still not well understood, a possible role for MN-C could be envisaged [45, 60]. As aforementioned, brain strongly depends upon the functions of mitochondria, which can dialog with nucleus through some metabolic intermediaries (ATP and NAD<sup>+</sup>) and ROS. In the energetic context, neurons are highly specialized cells requiring large amounts of ATP and about 90% of this metabolite generated in the brain is synthesized in mitochondria *via* oxidative phosphorylation [61]. In neurons it has been demonstrated that CR-mediated AMPK activation brings a strong neuroprotective function by regulating mitochondrial biogenesis [62, 63]. In particular, a potential mechanism, by which CR is able to require AMPK-mediated mitochondrial biogenesis, could be linked to a metabolic shift from carbohydrate to fat utilization, which produces less ATP by the ETC [64]. Thus, it is possible that the observed mitochondrial biogenesis during CR compensates for the reduction in ATP production (increased AMP/ATP ratio)/reducing equivalent when fat is used as an energy source. In a molecular context, the mechanisms by which the activated AMPK regulates mitochondrial biogenesis are beginning to be elucidated. However, by using *in vivo* models it is emerging that nuclear PGC-1 $\alpha$  could be a crucial mediator of AMPK action on mitochondrial-nuclear loop [65]. Interestingly, even in human subjects engaged in CR or CR plus exercise it has been demonstrated an AMPK-mediated increase in PGC-1 $\alpha$  levels [66]. Similarly, a cell culture model of CR, in which cells are cultured with serum from calorie-restricted humans or rats, exhibited an increased PGC-1 $\alpha$  expression with a parallel enhancement in mitochondrial efficiency [67]. However, the beneficial effects of CR on neuronal function may also rely on the intervention of other mediators of MN-C; that is, NAD<sup>+</sup> [64]. In light of this, CR may be able to retain SIRT1 activity due to the recent evidence demonstrating that NAD<sup>+</sup> levels in liver increase with fasting [68], and that changes in the NAD<sup>+</sup>/NAM-ratio *in vivo* critically influence cellular responses to CR in mammals by modulating SIRT1 activities [69]. The SIRT1 deacetylation reaction consumes NAD<sup>+</sup> with consequent release of 2'-O-acetyl-(ADP) ribose NAM [70], which in turn inhibits SIRT1 by a negative loop. Thus, the recycling of NAM back to NAD<sup>+</sup> by the enzyme nicotinamide phosphoribosyltransferase is absolutely crucial for the maintenance of cellular NAD<sup>+</sup> and for maintaining SIRT1 functions [71]. An important major target for SIRT1 deacetylation activity is PGC-1 $\alpha$ , which has been shown to be deacetylated in a model of neurodegeneration and in the brain in response to resveratrol treatment. This natural compound is believed to exert beneficial effects that nicely mimics those related to CR. The way of action of resveratrol on cell metabolism is a still-debated matter. The most fascinating theory is that resveratrol can increase the affinity of SIRT1 for its nuclear acetylated substrates or augment NAD<sup>+</sup>/NADH ratio [72]. The resulting PGC-1 $\alpha$  activation by resveratrol represents a signaling pathway promoting the slowdown of the neurodegenerative process [73]. This neuroprotective action is very likely because PGC-1 $\alpha$  induces

mitochondrial activity, being neurodegeneration linked to mitochondrial failures. However, other SIRT1-independent beneficial action of resveratrol could be hypothesized. Indeed, resveratrol is able to selectively inhibit the activity of MEK1/2, responsible for the activation of the down-stream ERK1/2 [8]. Autophagic cell death seems to be a contributing factor in the neuronal loss observed in neurodegeneration and this event is mediated by ERK1/2-governed signaling pathways [74, 75]. Therefore, besides the SIRT1-mediated activation of PGC-1 $\alpha$ , it is plausible to postulate that the beneficial effect of resveratrol on neuronal homeostasis could be partially due to the inhibition of ERK1/2-mediated autophagic cell death.

In the study of Anderson et al. [44], PGC-1 $\alpha$  translocates to the nucleus during CR and here it is activated by SIRT1 and subsequently phosphorylated by GSK3 $\beta$ , priming it for ubiquitination and nuclear degradation. The net result is a transient PGC-1 $\alpha$  activation enabling a rapid transcriptional stress response that is quickly shuttled off by degradation. However, under chronic stress conditions such as prolonged CR, GSK3 $\beta$  does not phosphorylate PGC-1 $\alpha$ , so that PGC-1 $\alpha$  is perpetually active in the nucleus, facilitating a sustained increase in transcription of genes involved in mitochondrial function [44].

Recently, Fusco et al. [76] have reported that the effects of CR on neuronal plasticity, memory, and social behavior are abolished in mice lacking CREB, a molecular mediator which can be implicated in MN-C. It was shown that CREB deficiency drastically reduces the expression of SIRT1 and the induction of genes relevant to neuronal metabolism and survival in the cortex and hippocampus of dietary-restricted animals. Biochemical studies reveal a complex interplay between CREB and SIRT1: CREB directly regulates the transcription of SIRT1 in neuronal cells by binding to SIRT1 chromatin; SIRT1, in turn, is recruited by CREB to DNA and promotes CREB-dependent expression of the target gene PGC-1 $\alpha$ . Accordingly, expression of these CREB targets is markedly reduced in the brain of SIRT1 KO mice that are, like CREB-deficient mice, poorly responsive to CR [76]. The beneficial effect of CR on lifespan is likely linked to the increase of NO production. Specifically, it was demonstrated that CR increases endothelial NOS (eNOS) and neuronal NOS (nNOS) [25, 76]. The raise of NO levels is the mediator of mitochondrial biogenesis that is paralleled by the increased oxygen consumption, ATP production, and an enhanced expression of SIRT1. Only few reports indicate that CR may induce increased GSH levels or dampen the effects of ageing on GSH decrease [77, 78]. Controversially, we demonstrated that GSH is an efficient physiological buffer of NO reducing its bioavailability [13]. Unpublished data from our laboratory indicate that nutrient limitation by fasting is instead associated with decreased level of GSH in brain, which may potentiate the beneficial action of NO. With this in mind, the effective increased level of brain GSH during CR certainly merits further investigation.

ROS can be generated by a variety of enzymes of oxidative metabolism among which the ETC is the most significant source. But during CR, how might the biogenesis of mitochondria correlate with a reduction of ROS? An

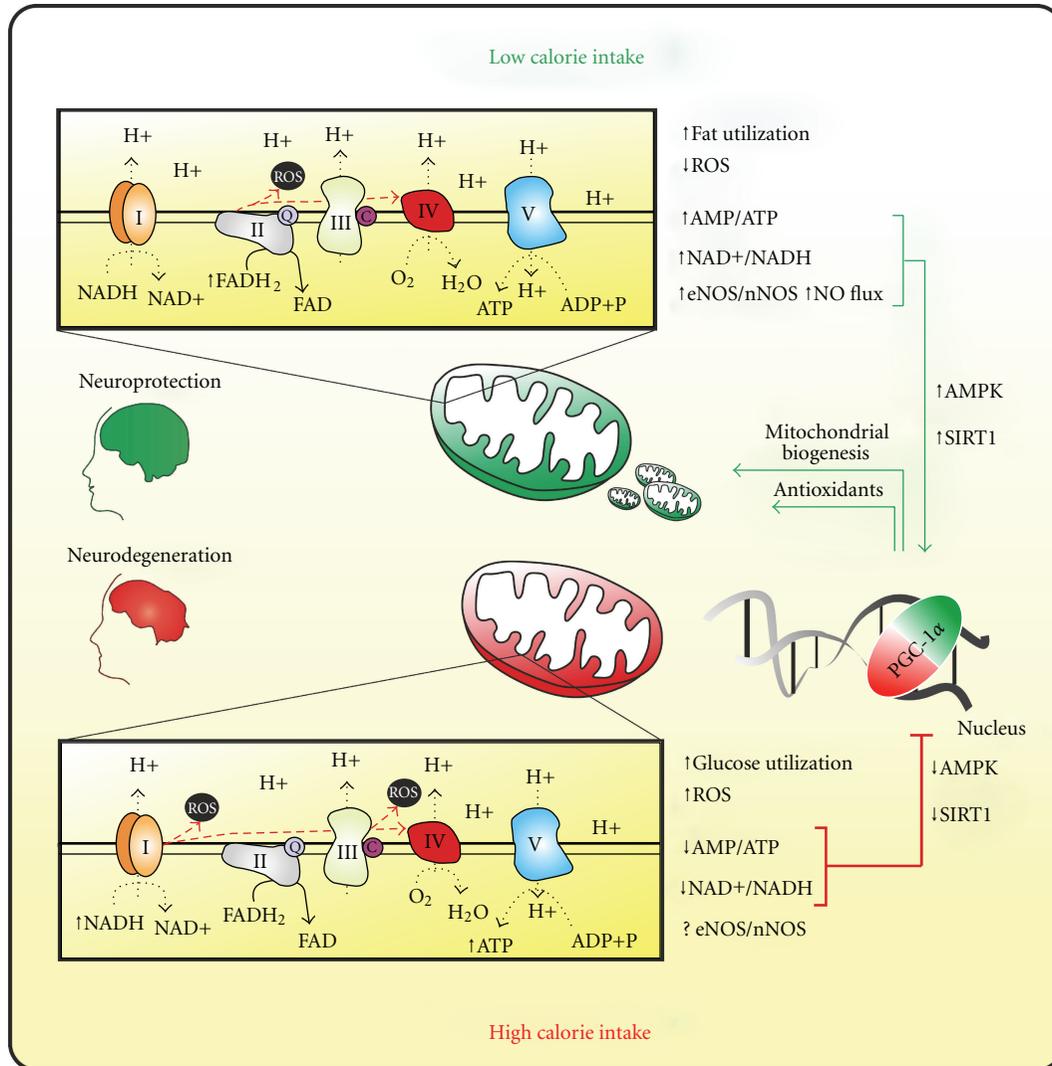


FIGURE 1: Schematic model of the effect of calorie intake on PGC-1 $\alpha$ -dependent mitochondrial activity and its implication in neuronal health.

important factor reducing ROS is the increase of antioxidant levels that often associates with CR and that is mediated by PGC-1 $\alpha$  as discussed above [41]. Another potential mechanism could be that, when fat is used as energy source, the frequency with which electrons enter the ETC bypassing complex I is increased. Given that complex I is one of the primary source of ROS, this could justify ROS reduction during CR. A schematic model of how the rate of caloric intake can modulate mitochondrial biogenesis and affect neuronal homeostasis is reported in Figure 1.

#### 4. Recent Advances in PGC-1 $\alpha$ Biology: The Extranuclear Forms

For long time PGC-1 $\alpha$  has been prevalently considered a nuclear-resident protein prevalently acting on nuclear gene expression. It has been recently described that PGC-1 $\alpha$  can have an extra-nuclear localization. Chang and colleagues

have identified a novel, biologically active 270 amino acid isoforms of PGC-1 $\alpha$  (NT-PGC-1 $\alpha$ ) produced by alternative splicing. In contrast to full-length PGC-1 $\alpha$  (~90 kDa), which is relatively short-lived (~2.3 h) due to rapid targeting to ubiquitin/proteasome-mediated proteolysis in the nucleus, NT-PGC-1 $\alpha$  (~38 kDa) is more stable and predominantly sequestered in the cytoplasm. Upon increase of cAMP levels, that is, during cold exposure of brown adipose cells, NT-PGC-1 $\alpha$  efficiently accumulates within nuclei and its recruitment to the promoters of PGC-1 $\alpha$  target genes is strongly enhanced. Moreover, the phosphorylation of NT-PGC-1 $\alpha$  by PKA inhibits the nuclear export and a greater proportion of NT-PGC-1 $\alpha$  remains into nucleus [79], where it mediates the transcription of many genes that are both distinct from and common to those that are regulated by PGC-1 $\alpha$ . These data suggest that the expression of NT-PGC-1 $\alpha$  instead of full-length PGC-1 $\alpha$  would be important for a more efficient and complete induction of a set of genes that

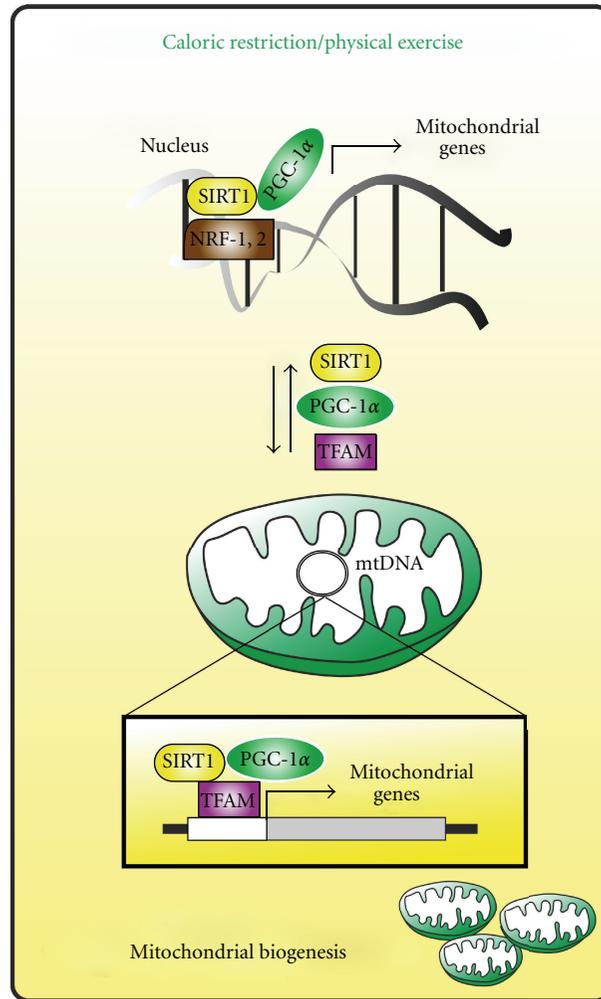


FIGURE 2: Hypothetical role of extra-nuclear forms of PGC-1 $\alpha$  and SIRT1 in mitochondrial biogenesis. Upon energetic stress conditions (e.g., physical exercise, caloric restriction) PGC-1 $\alpha$  and SIRT1 are implicated in the expression of mitochondrial genes at the nuclear level. Concomitantly, PGC-1 $\alpha$  and SIRT1 might migrate into mitochondrial matrix wherein, by interacting with TFAM, mediate the transcription of mtDNA-encoded mitochondrial genes, thus inducing mitochondrial biogenesis.

are transcriptionally regulated by PGC-1 $\alpha$  isoforms [79, 80], thus assuring a prolonged induction of PGC-1 $\alpha$ -regulated genes. Unfortunately the presence and the role of NT-PGC-1 $\alpha$  in other cell types including neurons are unascertained yet. Given that PGC-1 $\alpha$  deregulation has been found as the cause of neuronal death, the role of NT-PGC-1 $\alpha$  in neurodegenerative diseases needs to be clarified.

The extra-nuclear localization of full length of PGC-1 $\alpha$  was also demonstrated in our laboratory [81]. Specifically, we showed that PGC-1 $\alpha$  localizes together with SIRT1 within mitochondria, wherein they indirectly associate with the regulatory D-loop region of mtDNA. Such region is the site of initiation of mtDNA replication and transcription and here they interact with TFAM (which specifically binds to D-loop). On the basis of these findings we suggested that SIRT1 and PGC-1 $\alpha$  likely participate in the transcription/replication of mtDNA. We postulated that SIRT1 could activate TFAM and PGC-1 $\alpha$  by deacetylation, and PGC-1 $\alpha$  in turn coactivates TFAM. With this in mind the group of

Safdar successively showed that upon acute energy demands; that is, endurance physical exercise, PGC-1 $\alpha$  is increased and localizes both into nuclear and mitochondrial fractions of skeletal muscle cells [82]. With regard to mitochondrial PGC-1 $\alpha$ , they demonstrated that an increased interaction of PGC-1 $\alpha$  with TFAM was operative, resulting in enhanced TFAM coactivation and more efficient mtDNA transcription. This mitochondrial event is accompanied by the augmented activity of the nuclear PGC-1 $\alpha$  allowing the concomitant transcription of nuclear-encoded mitochondrial genes. Therefore, altogether these recent findings put PGC-1 $\alpha$  at the center stage of MN-C being able to contemporarily promote the transcription of nuclear and mitochondrial-encoded genes upon specific metabolic needs such as during physical exercise and CR (Figure 2). Notwithstanding, the mitochondrial import of PGC-1 $\alpha$ , as well as of SIRT1, remains an unsolved and intricate question. Indeed, PGC-1 $\alpha$  and SIRT1 do not contain the canonical mitochondrial targeting signal needed for the mitochondrial import [83].

However, data from proteomic analyses indicate that nearly half of the nuclear-encoded proteins residing within mitochondria may be imported by exploiting cryptic internal signals [84] and this could be the case of PGC-1 $\alpha$  and SIRT1. The precise stimuli dictating the mitochondrial redistribution of PGC-1 $\alpha$  and SIRT1 is still unknown and will be of great interest for future studies. Given that chronic physical exercise is associated with oxidative stress and a substantial decrease and oxidation of GSH [85], a role of ROS and/or NO could be postulated as determinants of such redistribution. For instance these second messengers could regulate nuclear and mitochondrial shuttling of PGC-1 $\alpha$  by posttranslational modifications comparable to those necessary for its full activation. Similar pathways managing the nuclear-mitochondrial shuttling of PGC-1 $\alpha$  could be operative during CR. Actually, PGC-1 $\alpha$  activators that is, AMPK, p38MAPK, and SIRT1 can be modulated by NO/ROS signaling as well as by CR [86–89].

## 5. Conclusion and Perspectives

Mitochondria and nuclei are interconnected through a communicative loop. Their cross-talk operates broadly at two levels: one mechanism involves cellular responses to changes in the functional state of the mitochondria itself, where mitochondrial mediators are able to modulate a set of nuclear transcription factors, or coactivators, impinging the expression of both nuclear and mitochondrial proteins fundamental in maintaining cell metabolism. In the opposite route, several nuclear-encoded proteins can “talk” with mitochondria inducing adaptive responses. Mitochondrial-nuclear reciprocal signaling is a central question that needs to be answered to understand the molecular mechanisms, assuring mitochondrial activities and functions. Considering the negative impact of mitochondrial dysfunction on cell function and human health, the comprehension of the molecular mechanisms underlying MN-C continues to be a fundamental issue and matter of research.

The dysfunction of mitochondrial metabolism is the main contributing factor in the etiology/progression of many neurodegenerative diseases. It is emerging that altered bioenergetics is triggered by a deregulation of mitochondrial genes expression. Impairment of SIRT1 and/or PGC-1 $\alpha$  activity takes center place in such deregulation being fundamental regulators of mitochondrial metabolism and ROS homeostasis at transcriptional level. The possible direct contribution of altered SIRT1 and/or PGC-1 $\alpha$  activity in neurodegenerative diseases has been demonstrated by many *in vivo* and *in vitro* experimental models. Pharmacological or genetic manipulation of SIRT1 and PGC-1 $\alpha$  prevents neurodegeneration in animal and cellular models of ALS, AD, and PD [90, 91]. Interestingly, PGC-1 $\alpha$  has been directly linked to AD and PD pathogenesis, since it is significantly decreased in postmortem brains [38, 92].

The discovery of the involvement of SIRT1 and/or PGC-1 $\alpha$  in neurodegenerative diseases supports the use of therapies targeting the cellular metabolism and mitochondrial biogenetic pathway at SIRT1/PGC-1 $\alpha$  level. CR could be a

promising nutritional strategy ultimately leading to PGC-1 $\alpha$ -mediated mitochondrial biogenesis [73] and life-span extension. Consistent data corroborates the existence of cytoplasmic/mitochondrial forms of PGC-1 $\alpha$  and SIRT1, in which deregulation could be causative of neurodegenerative diseases characterized by mitochondria dysfunction. However, at least in recent studies, this issue remains underexplored. It is plausible to hypothesize that mitochondrial impairment in neurodegenerative disorders could be due not only to a defective levels/activities of PGC-1 $\alpha$  and SIRT1 at the nuclear but also at the mitochondrial level, with a significant negative impact on mtDNA transactions (replication and expression), mitochondrial number, and metabolic function. This can be due to the downregulated basal level of these proteins or to a deficit in the mitochondrial import mechanisms. In this regard, similarly to physical exercise, energetic failure caused by CR may have a functional role not only in promoting PGC-1 $\alpha$  and SIRT1 activity at the nuclear genome but also in enhancing mtDNA transcription, thus, likely resulting in a more efficient mitochondrial biogenesis and increased lifespan.

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## Review Article

# Targeting Metabolism and Autophagy in the Context of Haematologic Malignancies

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Autophagy is a cellular process that maintains the homeostasis of the normal cell. It not only allows for cell survival in times of metabolic stress with nutrient recycling but also is able to lead to cell death when required. During malignant transformation the cell is able to proliferate and survive. This is due to altered cell metabolism and the presence of altered genetic changes that maintain the cell survival. Metabolism was considered an innocent bystander that was a consequence of the increased nutrient requirement for the survival and proliferation of haematological malignancies. The interdependency of metabolism and cellular mechanisms such as autophagy are becoming more evident and important. This interdependence contributes to increased cancer progression and drug resistance. In this paper we aim to discuss autophagy, how it pertains to metabolism in the context of hematologic malignancies, and the implications for therapy.

## 1. Introduction

Autophagy was first described in the 1960s but its importance in various physiological conditions in addition to the basic molecular understanding of autophagy has only come into focus in the last decade. The word autophagy is derived from Greek: *auto*, meaning “self” and *phagy*, “to eat.” This term was coined due to the process by which cellular components are degraded through the lysosomal enzymatic pathway providing a cell with essential amino acids, nucleotides, and fatty acids that enable production of the elements required for energy and macromolecule production [1, 2]. Normal cells engage in autophagy as a means to survive disruptions in nutrient and growth factor availability. It also serves to eliminate damaged organelles and proteins to prevent accumulation. This prevents them from becoming toxic to the cell. If autophagy is prolonged to a point where normal cell function is compromised, cells undergo

cell death either through apoptosis or by autophagy itself. One of the main inducers of autophagy is metabolic stress, and understanding the relationship between autophagy and metabolism could lead to better therapeutic strategies in treatment of haematological malignancies.

## 2. Regulation of Autophagy

Autophagy is characterized by cytoplasmic constituents sequestered into double-membraned vacuoles called autophagosomes. Autophagosomes then fuse with lysosomes (autolysosomes). Autolysosomes degrade cellular components releasing required nutrients to the cell. The regulation of autophagosome and autolysosome structures requires both positive and negative signaling pathways. The discovery in yeast of autophagy-related genes (ATGs) has provided greater understanding of these signaling pathways involved in autophagosome formation [3, 4]. The initial signal to form

autophagosomes is by the class III phosphatidylinositol (PI) 3 kinase complex consisting of Beclin1/Atg6, p150hVSp35, and class III PI3K (Vps34). This complex is required for formation of the preautophagosome structures [5]. Binding of ATG14, UVRAG (protein product of ultraviolet radiation resistant gene), and AMBRA1 (activating molecule of Beclin1-regulated autophagy) to the PI3K-III complex further increases the formation of autophagosomes allowing cells to regulate the amount of autophagy. AMBRA1 has also been shown to be a target of ULK1 [6]. ULK, TOR, FIP, Atg13, and AMPK represent molecules in the autophagy signaling network. The formation of the Beclin1 complex is important to autophagosome formation. This process is negatively regulated by binding of Bcl-2 family members such as Bcl-x<sub>L</sub> to Beclin1 preventing Beclin1 binding to the PI3K-III complex and thereby reducing autophagy [5, 7].

Following PI3K-III complex induction of preautophagosome structures, a series of ATG proteins build autophagosomes using an ubiquitin-like mechanism. There are two ubiquitin-like mechanisms used in autophagosome formation [8]. The first reaction is the ubiquitin-like protein ATG12 forming a conjugation to ATG5 via the E1 like protein Atg7 and E2-like protein ATG10 or ATG3. ATG16 then binds to the complex and integrates into the autophagosome membrane. The second reaction is the formation of the autophagosome membrane by ubiquitin-like protein LC3 (ATG8) conjugation with phosphatidyl ethanolamine (PE). This is regulated by ATG4 cysteine protease cleavage of LC3 at the C-terminus that facilitates lipidation of LC3 and generating LC3-PE conjugates. When both LC3-PE conjugates and Atg5-Atg12-Atg16 protein complex are localized to the autophagosome, the formation of autophagosomes is complete [8–10]. This process is regulated by acetylation of ATG-3 by histone acetyltransferase Esa-1 suggesting that protein acetylation regulates autophagy [11].

Autophagosome formation is negatively regulated by the mammalian target of rapamycin (mTOR) pathway, a nutrient-sensing kinase pathway. Under growth conditions, the mTOR pathway regulates cell growth and survival but under nutrient starvation conditions, the mTOR pathway is inhibited allowing for induction of autophagy [5]. There are two different mTOR complexes: mTORC1 and mTORC2 [12]. mTORC1 complex contains mTOR and regulatory associated protein of mTOR (Raptor). The mTORC2 complex contains mTOR and rapamycin insensitive companion of mTOR (Rictor) [12, 13]. In nutrient rich conditions, the PI3K/AKT signaling pathway activates mTOR allowing formation of mTOR complexes and suppresses autophagy. This is through the mTORC1 where it binds to ULK1/2 (orthologue of yeast ATG1), mATG13, FIP200, and Atg101. Upon formation of this complex, mTOR phosphorylates ULK1 and ATG13 preventing ULK1 activation thereby blocking autophagosome formation. In nutrient limiting conditions, the LBK/AMPK pathway is activated blocking mTOR activation. This is achieved by preventing mTORC1 binding to ULK complexes. This leads to ULK1 phosphorylation of ATG13 and FIP200 and autophagosome formation [12–14]. Alternatively, growth factor deprivation leads to activation of glycogen synthesis kinase-3 (GSK-3) that phosphorylates

an acetyltransferase TIP60 which in-turn acetylates and activates ULK1 [15]. This leads to autophagy. Besides mTOR signaling, rubicon is also a negative regulator of autophagy and the normal maturation of the autophagosome. It thus serves as a brake in the autophagy process [16].

Autolysosomes are formed when autophagosomes and the lysosomes fuse. This allows the degradation of autophagosomal cargo. The lysosome proteins LAMP1 and LAMP2 are found in autolysosomes and are involved in degradation. In addition, the presence of cargo receptors or chaperone proteins, such as p62/SQSTM1 and NBR1, are responsible for sequestration of the ubiquitinated proteins into autophagosomes and autolysosomes [17]. Interactions between these autophagic adapters and the autophagosomal marker protein LC3, are required for efficient selective autophagy. The best characterized is p62/SQSTM1 that is responsible for delivering ubiquitinated proteins to autolysosomes for degradation [18]. Inefficient autophagic degradation of p62 leads to accumulation of ubiquitinated aggregates. This process is inhibited by reactivation of the mTOR pathway that causes the conversion of autolysosomes back to lysosomes. Thus, autophagy is a tightly regulated process that breaks down cellular constituents in response to cellular stress.

### 3. Autophagy and Normal Haematopoiesis

In the normal development of the erythrocyte, the reticulocyte is enucleated but retains its organelles. Through the process of autophagy, it then loses its organelles to become a circulating red blood cell [19]. Nix, also known as BNIP3L, has been demonstrated to play a role in this process through regulation of mitochondrial clearance [20]. Chemical differentiation of the K562 CML cell line also demonstrates a role for autophagy in megakaryocyte differentiation [21]. In lymphocytes autophagy proteins Atg5, Beclin1, and LC3 are upregulated in early thymocyte development and T-cell activation but downregulated in the mature CD4+ and CD8+ T cells [22]. Loss of autophagy gene Atg5 is important for B-cell survival during development. Loss of this gene leads to inefficient B cell development characterized by increased cell death [23]. Autophagy is also known to regulate haematopoietic stem cells (HSCs) that are critical for normal haematopoiesis [24–26]. Recent studies showed that autophagic gene ATG7 is an essential regulator of adult HSCs maintenance since haematopoietic stem and progenitor cell lacking ATG7 expression have increased proliferation and DNA damage [27, 28]. This confirms that autophagy is an important regulator of early development, homeostasis, and maintenance of haematopoiesis.

### 4. Autophagy in Haematologic Malignancies

The role of autophagy in haematologic malignancies is controversial [29, 30]. Autophagy has been shown to be either tumor promoting or tumor suppressive. Studies demonstrating a tumorigenic role of autophagy suggesting cancer cells can adapt and thrive to harsh environmental

conditions such as low nutrients, growth factor deprivation, and metabolic stress because of autophagy [31–33]. This is due to the ability of autophagy to protect against apoptotic signaling through the degradation of damaged mitochondria, aggregated proteins, and pathogens within a cell [30, 34, 35]. However, this distinct role of autophagy during tumor progression is cancer type specific depending on the developmental context and stage of cancer [30, 34, 35]. In normal haematopoiesis, autophagy regulates homeostasis. However, when this balance is disturbed the initiation of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) ensues [36, 37]. In addition, autophagy plays a role in cell survival in haematologic malignancies. This is illustrated by development of resistance to therapy such as chronic myelogenous leukemia resistance to imatinib [38].

Besides the role of autophagy in tumour progression, there is also evidence that supports a tumor suppressive role of autophagy. Beclin1, an autophagy gene, is found to be haploinsufficient tumour suppressor in mice and is mono-allelically deleted in human breast, ovarian, and other tumors but not in haematologic malignancies [39, 40]. p53 and PTEN are the most commonly mutated tumor suppressor genes and regulate autophagy in haematologic malignancies [41]. Through increased expression of autophagy genes such as DRAM, p53 increases autophagy where mutant p53 fails to increase autophagy gene expression and contributes to cell survival. Indeed, altered expression of autophagic genes Beclin1 or LC3 are considered prognostic markers in many tumours including non-Hodgkin lymphoma [42–44]. PTEN inhibits the PI3K/AKT pathway leading to decreased activation of the mTOR signaling pathway (Figure 1) and increased autophagy [45]. In contrast, mutant PTEN suppresses autophagy levels [46]. Autophagy can also function to promote apoptosis or induce cell death. Autophagic cell death has been demonstrated under various conditions in hematologic malignancies but the mechanisms that govern autophagy leading to tumour suppressive roles being unknown.

## 5. Autophagy and Metabolism

Autophagy is negatively regulated by growth factors, amino acids, and glucose signals leading to the nutrient responsive mTOR-signaling pathway [47] (Figure 1). Autophagy is regulated by adenosine monophosphate kinase (AMPK) via the mammalian target of rapamycin (mTORC1) pathway. AMPK senses changes in lipids and glucose to function as a metabolic sensor. It restores energy balance in the adenosine monophosphate (AMP) and adenosine triphosphate (ATP) ratio by the LKB1-AMPK activation. In AML the LKB1/AMPK pathway plays a tumor suppressor role through repression of mTOR-dependent mRNA translation [37]. Similarly, tumour necrosis inducing apoptosis ligand (TRAIL) is involved in apoptosis via intrinsic and extrinsic pathways. However, certain blood cancers such as chronic lymphocytic leukemia (CLL) are resistant to TRAIL-induced apoptosis. This could be due in part to TRAIL-induced cytoprotective autophagy. Thus, targeting autophagy genes

such as Beclin1 and Atg-5 enables TRAIL induced apoptosis [48, 49]. Autophagy may play a role in the progression of low-risk MDS to AML by protecting the cells from extensive reactive oxygen species (ROS) induce damage from altered metabolism [37].

ROS play an important role in regulating metabolism and autophagy. ROS consist of unpaired electrons molecules such as superoxide ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $OH^-$ ), nitric oxide ( $\cdot NO$ ), peroxyxynitrite ( $ONOO^-$ ), and nitrogen dioxide radical ( $\cdot NO_2$ ) [50–52]. Although ROS is formed from normal metabolism mainly from the mitochondria (Figure 1) and plays an important role in cell signaling and homeostasis leading to cell survival, ROS levels can increase causing irreversible oxidative damage leading to impaired metabolism and cell death [51, 53–55]. It has been documented that many chemotherapeutic agents raise levels of intracellular ROS [54, 55]. The essential *role of mitochondria* in generation of ROS and regulating tumorigenesis is implicated in many cancers including hematologic malignancies [36, 56]. Metabolic and oxidative stress also increases autophagy and blockage of ROS production or use of free radical scavengers inhibits autophagy. The mechanism of ROS induced autophagy is unclear but several possible mechanisms have been proposed. The cysteine protease Atg4 could be oxidized on a cysteine residue located near the active site, critical for its regulation. Atg4 regulates the reversible conjugation of Atg8 (LC3 in mammals) to the autophagosomal membrane, required for autophagosome formation [57]. Starvation-induced oxidative inactivation of ATG4 promotes lipidation of ATG8, facilitating autophagosome formation [57]. ROS accumulation could also be caused by selective autophagic degradation of catalase. Catalase degradation subsequently caused further ROS accumulation [58]. Other potential mechanisms for ROS regulation of autophagy could be through activation of transcription factor activity, leading to altered gene expression [59]. Indeed, autophagy genes are up-regulated in response to oxidative stress in yeast, and ROS induce Beclin1 and ATG-7 expression in different cancer cells. We have demonstrated that mitochondria are an important source of ROS leading to autophagy since oxidative phosphorylation inhibitors could induced autophagy mediated by ROS [51]. ROS induced autophagy has been shown to lead to cytoprotection and autophagic cell death. It was demonstrated that histone deacetylase inhibitor, SAHA induced autophagy and increased ROS leading to a cellular prosurvival mechanism in Jurkat T-cells [60]. In addition, FTY720, an immunosuppressive drug, induced cytoprotective autophagy in ALL [61]. In contrast, many chemotherapeutic drugs induce oxidative stress causing autophagic cell death. For example, increases in ROS, autophagosome formation and cell death have been detected upon Brevinin-2R treatment in Jurkat and BJAB (B-cell lymphoma) cells [62]. Another study showed that natural compound eupalinin-induced autophagic cell death through increased ROS in human leukemia cells [63, 64]. Taken together, increased cellular ROS production by therapeutic drug initiates a stress response leading to either cell survival or cell death.

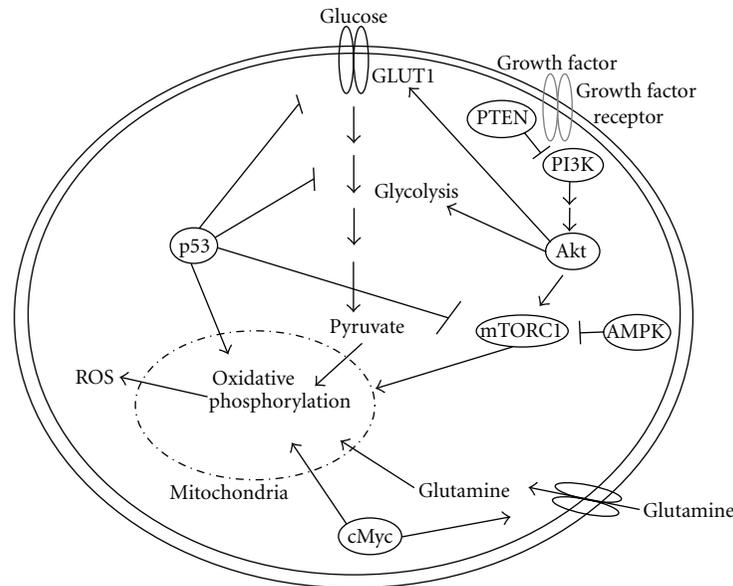


FIGURE 1: Metabolic signaling regulation. Glucose uptake in cells is regulated by GLUT1 transporters that increase glycolysis and oxidative phosphorylation. This is enhanced by cMyc regulation of glutamine uptake in cells. Growth factors influence metabolism through activation of the PI3K/AKT/mTOR signaling pathway contribute to increased glycolysis and oxidative phosphorylation. This is inhibited through PTEN. Under glucose limiting conditions, AMPK is activated inhibiting the mTOR signaling pathway. In addition, p53 activation inhibits glycolysis, and the mTOR pathway but increases oxidative phosphorylation. ROS increases through inefficient oxidative phosphorylation at the mitochondria.

Both oncogene activation and tumor suppressor gene loss can all lead to deregulation of metabolic pathways such as glycolysis, pentose phosphate pathway, and lipid and energy metabolism. Cancer growth is dependent on functional mitochondria that are using glutamine as their major source of fuel for the citric acid cycle and the generation of NADPH and lipid synthesis [65]. The oncoprotein MYC activation is common in haematologic malignancies such as Burkitt's lymphoma and AML. MYC upregulates glutamine transporters (Figure 1) and glutaminolysis, which increase ammonia production and autophagy protecting the cells from apoptosis [66, 67]. NF $\kappa$ B activation is common in a variety of B-cell neoplasm including diffuse large B-cell lymphoma. Sommermann et al. showed that inhibition of NF $\kappa$ B-induced cell death via the PI3K pathway and GLUT1 by restricting glucose transport [68]. To this end, it has been demonstrated that autophagy inhibitors in combination with NF $\kappa$ B induce a "metabolic crisis" and cell death [68]. Activating mutations in the oncogene Ras induce autophagy possibly through a novel AKT1-GLI3-VMP1 pathway [69]. This is essential for overcoming metabolic stress by impaired acetyl-CoA production leading to survival and tumor growth [70]. Overall, this demonstrates the interdependence of oncogene-mediated metabolic pathways and autophagy in response to cellular stress and cancer progression.

Besides oncogenes, tumour suppressors also regulate autophagy. Otto Warburg first observed that cancer cells undergo aerobic glycolysis due to lack of mitochondrial oxidative phosphorylation. The tumor suppressor, p53, positively regulates oxidative phosphorylation via synthesis

of cytochrome c oxidase (COX-2) and downregulates glycolysis via transcription of TP53-induced glycolysis and apoptosis regulator (TIGAR) [71] (Figure 1). Loss of p53 enhances aerobic glycolysis resulting in more aggressive cancer phenotypes. p53 is often lost in cancer thus maybe an important genetic change contributing to the "Warburg effect." p53 is known as a regulator of apoptosis, however its role in coordination of nutrient utilization in order to preserve cell survival is equally important. TIGAR is a direct transcriptional target of p53 and alters cellular use of glucose. TIGAR shares sequence homology with the bisphosphatase domain of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase and dephosphorylates fructose 2,6-bisphosphate reducing the levels of this metabolite. In addition, TIGAR suppresses ROS levels and autophagy. In the glycolytic pathway 6-Phosphofructo-1-kinase (PFK-1) converts fructose 6 phosphate to fructose 1,6-bisphosphate. This in turn activates PFK-1 mediated by TIGAR and leads to inhibition of glycolysis. p53 also modulates another enzyme later in the glycolytic pathway, phosphoglycerate mutase (PGM). Wild type p53 downregulates PGM whereas p53 mutation increases its activity and leads to glycolytic flux. In addition to regulation of glycolytic enzymes via TIGAR and PGM, p53 is important in the regulation of glucose transport. p53 can also down regulate glucose transporter expression leading to a reduction in intracellular glucose. Glucose transporter 1 (GLUT1) and glucose transporter 4 (GLUT4) are directly repressed at the gene promoter by p53 (Figure 1). This is important in autophagy since autophagy is activated by metabolic stress

(glucose deficit) leading to degradation and recycling of cellular substrates that support metabolism and promote survival and tumor growth. p53 acts as a key regulator element autophagy through regulation of glycolytic pathway and hence metabolic stress.

The role of p53 in regulating autophagy through metabolism is complicated. It regulates through cellular location and by transcriptional dependent and independent mechanisms. Nuclear localization of p53 enables activation of AMPK which then leads to autophagy. A fine balance between nuclear and cytoplasmic p53 is responsible for autophagy homeostasis [72]. Nuclear p53 induces autophagy (Figure 2) through upregulation of mTOR pathway regulators. Under metabolic stress, basal p53 expression regulates multiple detoxifying pathways such as upregulation of antioxidant targets such as GPX1, MnSOD, ALDH4, and TPP53INP1 [73–77]. In addition, p53 target genes, *sestrin1* and *sestrin2*, have been identified as a connection between p53 activation and mTORC1 activity [73, 78]. p53 exerts the antioxidant effect via inducing Sestrin expression in response to DNA damage and oxidative stress which leads to inhibition of mTORC1 activity and autophagy. Sestrins inhibit mTORC1 activity by interacting with mTOR pathway suppressors AMPK, TSC1, and TSC2 [78]. In contrast, cytoplasmic p53 inhibits autophagy mediated by activation of mTOR downstream signaling [79] (Figure 2). In addition, the mTOR pathway activates MDM2, the major ubiquitin ligase that reduces nuclear p53 expression [80]. Cytoplasmic p53 also binds to high mobility group box 1 (HMGB1) preventing formation the HMGB1/Beclin 1 complex, and inhibiting autophagy [81]. Beclin1 also controls the protein stabilities of ubiquitin-specific peptidases, USP10 and USP13, by regulating their deubiquitinating activities. Since USP10 mediates the deubiquitination of p53, regulating deubiquitination activity of USP10 and USP13 by Beclin1 provides a mechanism for Beclin1 to control the levels of p53 [82]. Moreover, p53 inhibition was found to promote cell survival in response to glucose starvation through autophagy [83]. All these results suggest that the autophagy induced by p53 deletion in tumors provide a survival advantage to malignant cells in response to unfavorable conditions. Taken together, p53 signaling regulates autophagy in response to metabolic stresses.

All these oncogenes and tumor suppressors play important roles in development and progression of hematological malignancies. Metabolic alterations are also a common feature in hematological malignancies. Thus, it is reasonable to suggest that these alterations regulated autophagy in hematological malignancy contributing to tumor survival and suppression. There are, however, many unanswered questions. What autophagy supplied substrates are essential for sustain metabolism? What affect do changes in metabolism and upstream signaling pathways have on autophagy in normal hematological stem cells or other immune cells? Nevertheless, targeting of autophagy regulatory pathways could provide treatments for hematological malignancies through either blocking or inducing autophagy.

## 6. Targeting Autophagy and Metabolic Deregulation in Hematological Malignancies

Chemotherapy or radiotherapy can both induce autophagy as a protective mechanism and lead to therapy resistance directly via mTOR inhibition and others indirectly by cytotoxic stress [84]. It may also cause chemoresistance by interfering with ROS activation that is the mechanism by which many chemotherapeutic agents function [84] (Figure 2). Inhibition of the proteasome induces autophagy and may pose reason for concern and resistance to therapy [85]. Hydroxychloroquine and chloroquine are known inhibitors of autophagy. They are also known antimalarials and thus clinically relevant compounds [86] (Figure 2). These agents have shown efficacy in targeting p53 loss induced autophagy and Myc induced autophagy in pre-clinical models [87, 88]. Thus, the rational combination of an autophagy inhibitor chloroquine is being tested in clinical trial with bortezomib, a proteasome inhibitor, and cyclophosphamide in relapsed refractory multiple myeloma in a nonrandomized open label phase II clinical trial to determine if the combinatorial effects have clinical efficacy (<http://clinicaltrials.gov/ct2/show/NCT01438177>).

Many anticancer agents induce cell death through autophagy in hematologic malignancies instead of through cell survival by altering metabolism (Figure 2). For instance, arsenic trioxide ( $As_2O_3$ ) a potent antimetabolite exhibited potent antitumor effects through autophagic cell death in leukemic cell lines and primary leukemic progenitors from acute myelogenous leukemia (AML) patients [89–91]. Moreover, arsenic trioxide-induced autophagy through inhibiting the mTOR pathway contributes to degradation of the PML/RARA fusion protein in acute promyelocytic leukemia (APL) [91, 92]. In addition, mTOR inhibitor NVP-BEZ235 treatment in T-ALL cells caused suppressing PI3K/Akt/mTOR signaling and induced autophagic cell death [93]. mTOR inhibitor RAD001 (Everolimus), also induced cell death by inducing autophagy in an *in vivo* model of childhood ALL [94, 95]. Resveratrol (RSV) is another attractive agent that induces autophagic cell death by inhibiting the AMPK/mTOR pathway in CML cells [96, 97] Histone deacetylase inhibitors are another class of agents that can be used to target autophagy. Although currently approved for the use of cutaneous T-cell lymphomas, suberoylanilide hydroxamic acid (SAHA) has been found to have activity in imatinib refractory CML. In addition, there is evidence to suggest that chloroquine maybe synergistic with SAHA in this clinical scenario [98, 99]. Sphingolipids can also induce autophagy leading to increased apoptosis in leukemias and changes in sphingolipid metabolism have been observed in hematological malignancies [100, 101]. Thus, targeting metabolic signaling pathways leading to autophagy could be an effective treatment of malignant hematologic disorders.

Finally, metformin, a biguanide, used to treat diabetes has been suggested as a potential anticancer drug. Metformin is a known LKB-1/AMPK activator (Figure 2). In melanoma, metformin was found to induce autophagy by increased expression of Beclin1, and accumulation of LC-3 secondary to mTOR inhibition leading to cell death [102]. Similar

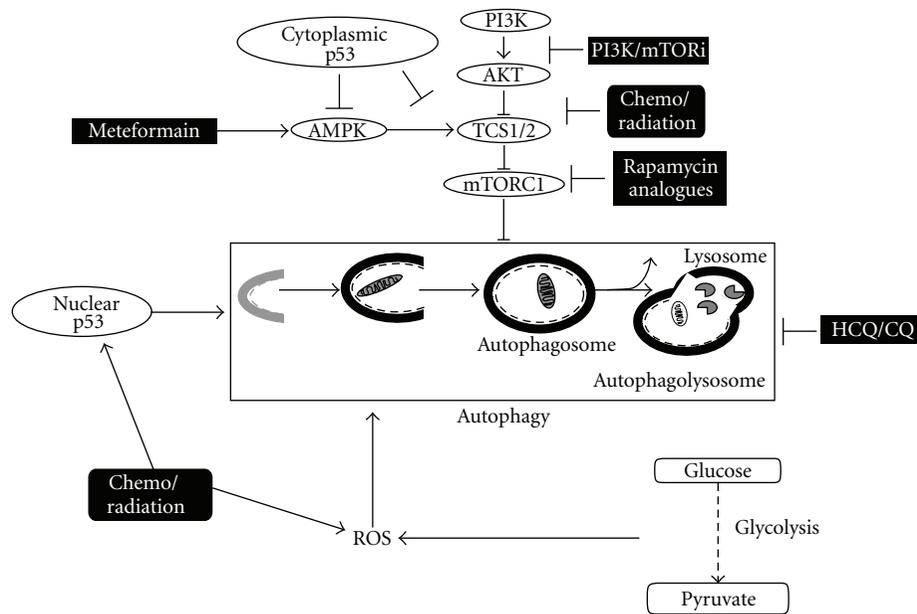


FIGURE 2: Signaling pathways regulating autophagy and their inhibitors. Autophagy is a catabolic process that results in the autophagosomal-lysosomal degradation of bulk cytoplasmic contents. The kinase mTOR is a critical regulator of autophagy induction, with activated mTOR (PI3K/Akt) suppressing autophagy. AMPK-signaling negatively regulates mTOR signaling therefore promoting autophagy. ROS stress is an important inducer and regulator of autophagy generated by reduced oxidative phosphorylation and increased glycolysis. Nuclear p53 induces autophagy through transcriptional regulation of multiple genes whereas cytoplasmic p53 inhibits autophagy by blocking the mTOR pathway. Autophagy inhibitors chloroquine (HCQ/CQ) and metformin are under clinical investigation. Many chemotherapy/radiation therapies induce autophagy through ROS, inhibition of the mTOR pathway or nuclear p53 whereas PI3K/mTOR inhibitors or rapamycin analogues specifically block the mTOR signaling pathway leading to autophagy.

effects have recently been described in lymphoma [103]. Metformin-induced activation of AMPK and inhibition of mTOR is AKT independent manner [103]. This lead to attenuated cell growth via induction of autophagy. The effect was evident in combination with doxorubicin versus single agent therapy and was reversed by autophagic inhibitor 3-methyladenine [103]. In T-ALL, metformin was found to have a significant antileukemic effect [104]. Metformin induced autophagy as evidenced by electron microscopy and increase in the LC3-II protein possibly contributing to cell death.

The major issue remains in hematological malignancies therapy as to whether induced or inhibited autophagy. The context of metabolism in cancer cells might be the key to this question and will govern the development of innovative metabolic therapies for hematological malignancies in the future.

## 7. Conclusion

The role of autophagy in cancer is multifaceted and its implication in metabolism is no different. This being said we are making headway in its understanding; however, there is more research required to understand the interactions between these currently distinct entities that are now merging in the pathogenesis of cancer. In hematologic malignancies it plays a role in pathogenesis, homeostasis, survival, and even cell death. An emerging role for metabolism has shed light

on the interconnection between metabolism and autophagy. Metabolism's effect on autophagy is still ambiguous; it may lead to cell survival or cell death. Clinical evidence does support a role for metformin as an anticancer agent. It is also being looked at in the context of cancer prevention. In leukemia, it may be a realistic thought to use emerging technologies for metabolic profiling and treat patients in a personalized manner. The question that remains unanswered is whether to inhibit or activate autophagy as a treatment of hematological malignancies.

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## Review Article

# Redox Regulation of Cysteine-Dependent Enzymes in Neurodegeneration

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Evidence of increased oxidative stress has been found in various neurodegenerative diseases and conditions. While it is unclear whether oxidative stress is a cause or effect, protein, lipid, and DNA have all been found to be susceptible to oxidant-induced modifications that alter their function. Results of clinical trials based on the oxidative-stress theory have been mixed, though data continues to indicate that prevention of high levels of oxidative stress is beneficial for health and increases longevity. Due to the highly reactive nature of the sulfhydryl group, the focus of this paper is on the impact of oxidative stress on cysteine-dependent enzymes and how oxidative stress may contribute to neurological dysfunction through this selected group of proteins.

## 1. Introduction

It is clear that while oxygen is essential for life in order to produce chemical energy in the form of ATP, paradoxically, the byproduct of its metabolism generates multiple reactive oxygen species (ROS) that are associated with cellular toxicity. Specifically, in regards to neurodegeneration, there is substantial evidence that ROS are a major component of diseases including Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis [1–4]. While clinical trials aimed at decreasing the burden of oxidative stress have not clearly demonstrated effectiveness, genetic research has found that high levels of antioxidant enzymes prolong life and decrease pathology. In addition, animal models have also indicated that oxidative stress is an important and consistent characteristic of many forms of neurodegeneration.

One particular group of proteins that appear to be intimately involved in the neurodegenerative processes is the cysteine-dependent proteins. This group includes various proteases, antioxidant enzymes, kinases, phosphatases, and other types of enzymes as well as other nonenzymatic proteins such as those that use cysteine as a structural component rather than as part of a catalytic site. More research will be needed to firmly establish the extent

to which oxidative stress is causal in these diseases, but based on current understanding, therapies to reverse the oxidant-induced modifications of proteins, lipids or, DNA are expected to be beneficial. This paper will highlight some selected, yet significant cysteine-dependent enzymatic systems that rely on a proper redox environment for their activity and provide evidence for their redox control in neurodegenerative disease. Potential relationships to cancers will also be discussed.

## 2. Redox Sensitivity of Cysteine

The amino acid cysteine is highly sensitive to redox state. This is largely due to the reactivity of anionic sulfur to various oxidizing agents that can form multiple types of oxidized species (see Figure 1). However, not all cysteines are equally sensitive, and such sensitivity has been utilized throughout evolution to provide protection against oxidative stress.

A close examination of the variety of physiologically occurring antioxidant systems, that use cysteine as a major component of their antioxidant activity or as part of a redox “sensor,” clearly demonstrates the sensitivity and evolutionary significance of cysteine as part of a protein's

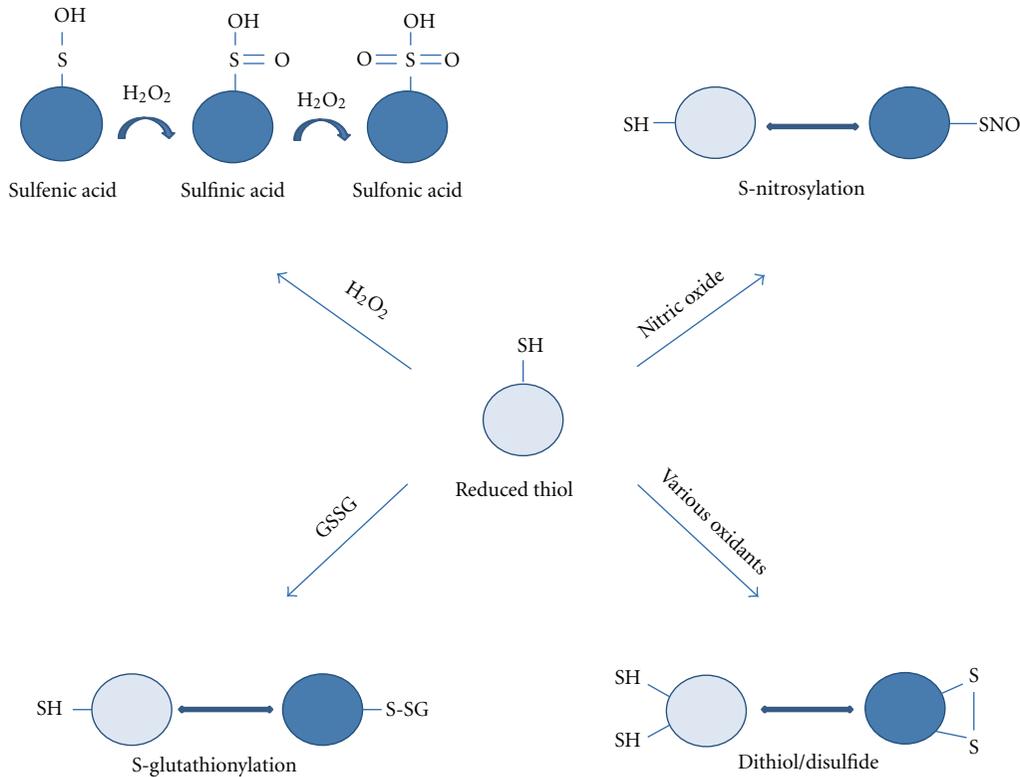


FIGURE 1: Diagrammatic representation of major oxidation states of cysteine that have been found *in vivo*. Circles represent a protein that contains a cysteine within its primary structure. In its most reduced state, the sulfur group of cysteine is found in the form of -SH. The sulfur can become modified in a number of ways including S-nitrosylated by nitric oxide or S-glutathionylated by glutathione, which are being increasingly recognized for their importance in regulated many cysteine-containing enzymes. In addition, the sulfur group can be oxidized to sulfenic, sulfinic, and sulfonic acids or it may form an intra- or inter-molecular disulfide bond.

active center [5]. For example, glutathione (GSH) consists of glutamate, glycine, and cysteine and is the major antioxidant found in brain. It is found at millimolar levels and is a major determinant of intracellular redox conditions. Cysteine itself has been shown to be the major extracellular antioxidant. Further examples of cysteines' critical role in redox balance can be found in other enzymatic systems including the multiple enzymes involved in the maintenance of peroxiredoxins, glutaredoxins, and thioredoxins among others. The natural role of cysteines as redox sensors is further observed by the observation that throughout evolution, cysteines are found in transcriptional regulators that are modulated by oxidative stress such as oxyR and Nrf2/Keap [6].

Due to the varying microenvironments that exist for cysteine within a given protein structure, cysteines are not equally reactive. For example, as discussed further below, the Parkinson's disease-linked protein, DJ-1 cysteine at position 106, appears to be highly sensitive to oxidative attack, while two other cysteines within its structure are not as easily modified [7]. Such apparent specificity of cysteines within the same protein is also observed among many other proteins [8, 9]. In terms of the macroenvironment, cysteine-dependent enzymes require a reducing environment for activity, which is the condition maintained in the cytoplasm

in contrast to the extracellular space that is oxidizing. However, the lysosomal compartment is variable, and changes in redox state have been shown to modulate enzymatic activities located within it. For example, cathepsin activity was found to be altered through redox state as detected by a change in cleavage pattern produced under varying redox conditions [10]. Thus, the location of the cysteine within the overall tertiary/quaternary structure as well as its macroenvironment (e.g., intracellular versus extracellular or organelle) plays major roles in the extent to which a cysteine is stabilized in the anionic transition state, thereby affecting its reactivity to a change in the redox state.

### 3. Sources of Oxidants in Brain

Environmental toxins are thought to be a significant contributor to neuronal-related disorders including AD and particularly PD. These include a variety of naturally occurring and synthetic compounds, which results in the production of reactive species through well-characterized chemical pathways including the Fenton reaction and others [11]. In addition to direct chemical means, many of these environmental molecules, such as rotenone or paraquat, target mitochondria and disrupt the efficient production

of energy, leading to abnormal increases in free radical production such as superoxide [12]. The identification of these toxins and their mechanisms of action is the subject of extensive research with a major emphasis on how their toxicity relates to the production of free radicals.

Besides environmental toxins, there are also important cellular sources of oxidants localized within cytosolic and mitochondrial compartments. Cellular sources include NADPH oxidases, which are enzymes associated with both signal transduction and the killing of foreign organisms through the production of superoxide. Monoamine oxidase (MAO) located at the mitochondrial surface is also a source of hydrogen peroxide. Due to MAO-B's role in the metabolism of dopamine, MAO activity is linked to PD in part due to the production of reactive oxygen species resulting from MAO-mediated metabolism of dopamine [13].

Endogenous superoxide production is strongly associated with the mitochondria and can occur within the matrix, the intermembrane space, and at the outer membrane of mitochondria. For example, reactive species are formed as part of electron transport including complex I (NADH-ubiquinone oxidoreductase). Complex I is considered to be an important source of free radical generation [14, 15] and does so during either forward electron flow or reverse electron transport [16]. Though debate exists about the mechanisms involved (one-site versus two-site model), the importance of superoxide and hydrogen peroxide formation through the various mitochondrial pathways should not be underestimated as oxidants produced through the mitochondria are considered highly relevant to aging and neurodegeneration.

#### 4. Oxidative Stress in Neurodegenerative Disease

Over the last few decades it has become increasingly clear that the human brain is more sensitive to various forms of oxidative attack damage compared to other organs in the body. This is due in large part to the high metabolic activity found in brain and the seemingly limited capacity for the repair of damage to neurons as a result of injury. Many types of oxidizing molecules have been observed in the human brain, and their presence is associated with selective damage to brain regions linked with neurodegenerative disease. While it is uncertain as to the extent in which the increase in reactive species causes the visible pathological hallmarks, the formation of reactive oxygen, nitrogen, or sulfur species is nevertheless generally recapitulated in animal models of each disease, strongly suggesting a potential causal link. Observed biomarkers of increased oxidative stress include 4-hydroxynonenal, thiobarbituric acid-reactive substances, free fatty acid release, and acrolein formation for lipid peroxidation; 8-hydroxy-2-deoxyguanosine for DNA; protein carbonyls, 3-nitrotyrosine, and glutathionylation for proteins. In regards to proteins, along with cysteine, multiple aminoacids are found to be modified including lysine, methionine, histidine, and others.

*4.1. Alzheimer's Disease.* AD is an age-associated progressive neurodegenerative disease that affects behavior, cognition, and memory and is characterized by two major pathological hallmarks: extracellular plaques composed primarily of A $\beta$  and intracellular inclusions of tau protein known as tangles. It currently has no known cause or cure and remains the most common form of irreversible dementia affecting approximately 20 million people worldwide. Oxidative damage is one of the earliest detectable changes observed in both genetic and sporadic forms of Alzheimer's disease [17].

While there are several theories about the source of the various oxidizing molecules, A $\beta$  has been a prime candidate. Indeed, treatment of various model systems with different A $\beta$  forms typically results in increased oxidative stress. Recent work has shown that extracellular A $\beta$  treatment results in atypical redox effects in astrocytes compared to treatment with other oxidizing molecules, suggesting that A $\beta$  possesses unique oxidizing properties [18]. In addition to the potential for A $\beta$  to stimulate increased oxidative stress, there is also evidence that major antioxidant systems such as superoxide dismutase, catalase, and others have decreased activity associated with AD progression [19].

*4.1.1. Examples of Oxidized Enzymes in AD.* Peroxiredoxins (Prxs) are a family of peroxidases that reduce peroxyinitrate and a variety of other hydroperoxides. They use a redox-sensitive cysteines within their active site reducing the peroxide substrates either through the formation of an intramolecular disulfide bond or oxidation to sulfinic acid or sulfonic acid [20]. Proteomic studies for subjects with early AD found that Prx-2 was oxidized in a brain region containing significant AD-related pathology compared to age-matched controls [21]. In another study by Cumming and colleagues [22], it was not only shown that Prx-2 was more oxidized in AD brains, but also treatment of cultured primary neurons with A $\beta$  resulted in Prx oxidation that was reversible by addition of a cysteine-specific antioxidant, N-acetylcysteine. In addition, Fang and colleagues found that Prx-2 was S-nitrosylated at active-site cysteines Cys 51 and Cys 172 [23].

Protein disulfide isomerase (PDI) is a multifunctional enzyme with several family members. These enzymes include chaperone activity mediated by catalyzing the reduction, oxidation, and isomerization of protein disulfides to maintain proper protein folding. PDI redox activity is based on the presence of two thioredoxin-like motifs (CXXC) (human PDI: Cys 36/39 and Cys380/383). It has been found to be oxidized in AD and colocalizes with neurofibrillary tangles [24]. Though no changes in the amounts of PDI have been noted in AD brain, Uehara and colleagues [25] did show that PDI was S-nitrosylated at multiple cysteines in AD brain and that such oxidation resulted in enzyme inactivation. Since PDI is important for the folding of proteins by catalyzing cysteine-disulfide exchange, its inactivation increased the levels of misfolded of proteins, leading to the activation of the unfolded protein response.

Calpains are calcium and cysteine-dependent endoproteases whose active-sites are sensitive to oxidative inactivation. In addition to AD, calpains play a role in multiple

disease states, including cancer [26, 27]. As a putative physiological regulator of key proteins associated with AD such as amyloid precursor protein and tau among others, understanding calpains' potential dysregulation by redox status is important. Calpain's active site cysteine (Cys105) was found to be oxidized, *in vitro* and in cultured cells only in the presence of calcium [28, 29]. Presumably, this is because the active site is otherwise inaccessible to oxidative attack when the enzyme is in an inactive conformation. Evidence suggests that calpain-like enzymatic activity is also inhibited in brain regions of AD associated with high pathology [30].

**4.2. Parkinson's Disease.** Parkinson's disease is the second most common neurodegenerative disease characterized by loss of dopaminergic neurons, glutathione depletion, oxidative stress, and the formation of intracellular inclusions of alpha-synuclein called Lewy bodies. Similar to AD, the vast majority of PD cases are sporadic with only 5–10% of cases due to genetic causes [31]. Much of what we understand has been gained through the use of animal models of PD that involve the administration of exogenous compounds such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), rotenone, and paraquat. Investigation of these compounds has strongly linked them to mitochondrial dysfunction and the abnormal production of free radicals, which generally reflects what is observed in the human disease. In the course of these studies, several highly relevant cysteine-dependent enzymes known to contribute to PD have been observed to be modified at key cysteine residues by these reactive species.

As discussed above, PDI has also been found to be oxidized in samples of PD brain [25]. The potential impact of PDI in PD brain is evidenced by experiments suggesting that PDI plays a role in the folding of both synphilin-1 and alpha-synuclein [25, 32], two proteins closely linked to PD. Other studies have also found links between PDI oxidation and PD [33, 34].

DJ-1 activity is also altered by oxidative stress. DJ-1 is a 20 kDa that has multiple putative activities as a protease and an antioxidant among others [35]. DJ-1 is strongly associated with PD because mutations in DJ-1 result in autosomal recessive early-onset form of Parkinson's disease. DJ-1 contains three cysteine residues that each has been evaluated in response to oxidative stress. Based upon multiple studies, it is clear that only Cys-106 oxidation is an important regulatory component for DJ-1 activity. For example, Waak and colleagues [36] found that the formation of a mixed disulfide, created under oxidizing conditions between DJ-1 and apoptosis signal-regulating kinase 1 (ASK1), contributes to DJ-1's neuroprotective effects. Such protective effects are predicted to be lost in cases that may occur with aging or exposure to oxidative toxins such as those used in animal models of PD including MPTP or rotenone.

Another example of a cysteine-containing enzyme that is modified in PD is parkin. Parkin is an ubiquitin E3 ligase that serves to ubiquitinate a series of proteins and contains multiple cysteines that are required for full activity. Mutation of parkin is responsible for early-onset autosomal recessive juvenile Parkinsonism. Several studies have convincingly demonstrated that parkin is S-nitrosylated in cases of PD

as well as in model systems [37, 38]. Such oxidation inhibits parkin's ubiquitin E3 ligase activity and therefore prevents proper ubiquitination of its substrates leading to accumulation of misfolded proteins. In addition, it also appears to be sensitive to covalent modification by dopamine itself [39].

Tyrosine hydroxylase (TH) is the initial and rate-limiting step in the biosynthesis of the dopamine (DA) and norepinephrine. This enzyme contains seven cysteines some of which have been found to be important for full TH activity. Kuhn and colleagues [40] found that 4-5 cysteines were modified by quinone derivatives of DOPA, dopamine, and N-acetyldopamine that were prevented by various thiol-reducing agents. Further, they found that such oxidations resulted in inhibition of TH enzymatic activity. Other evidence of TH redox sensitivity comes from Sadidi et al., [41] who found that peroxynitrite and nitrogen dioxide both inhibited TH through nitration of cysteines or through S-thiolation in the presence of GSH or cysteine. Additional discussion of redox regulation of TH can be found in a recent, excellent review [42].

**4.3. Amyotrophic Lateral Sclerosis.** More commonly known as Lou Gehrig's disease, ALS is the most common degenerative disease of the motor neuron system that results in the death of motor neurons, causing muscle weakness and eventually death. Despite an annual incidence rate of one-to-two cases per 100,000, the etiology of the disease remains largely unknown [43]. Although multiple theories have been presented, research focusing on neurotoxicity has revealed excessive entry of glutamate into the neurons damages cell metabolism, resulting in pathologic changes [43]. It has been offered that ALS develops when vulnerable persons are exposed to a neurotoxin at times of strenuous physical activity [44].

**4.3.1. Examples of Oxidized Enzymes in ALS.** Mutations in Cu/Zn superoxide dismutase gene (SOD1) are associated with familial amyotrophic lateral sclerosis. Recent work has found that oxidative modification of SOD1 results in the formation of an epitope consistent with misfolding of SOD1 that is observed in ALS [45]. Subsequently, Redler and colleagues [46] evaluated the effects of specific modification of Cys-111 on this conformation change and found oxidation of Cys-111 via glutathionylation (see Figure 1) resulted in the destabilization of the SOD1 dimer. This destabilization increases the potential for unfolding of the monomer and subsequent aggregation, leading to loss of SOD1 activity and promoting cell death.

## 5. Other Cysteine-Dependent Enzymes Affected by Oxidative Stress

Beyond cases clearly associated with specific disease pathology, there are other physiologically regulated or pathologically modified cysteine-dependent enzymes that are equally important to consider and have been suggested to play a role in neurodegeneration. For example, Janus kinase 2 (JAK2)

TABLE 1: Examples of cysteine-dependent enzymes that use cysteine within their catalytic site within the various domains as delineated by the enzyme commission categories.

Class 1	Class 2	Class 3	Class 4	Class 5	Class 6
Oxidoreductases	Transferases	Hydrolases	Lyases	Isomerases	Ligases
Protein-disulfide reductase	Mercaptopyruvate sulfurtransferase	PTEN	MerB*	Protein disulfide isomerase	Parkin
Peroxiredoxin	Akt	Ubiquitinyl hydrolase 1	LuxS*	GluRS*	
Glyceraldehyde-3-phosphate dehydrogenase	Janus kinase 2	Histone deacetylase			
SOD1	Sulfurtransferase	PTP1B			
ALDH1L1	Epidermal growth factor receptor				
Tyrosine hydroxylase					

\*For some enzymes above, data suggests that they are oxidized and that the cysteine is essential for activity but may or may not be considered part of the catalytic site in all species.

Note: Other enzymes are not listed here although they depend upon cysteine for activity; often such cysteines are linked to a structural requirement such as a disulfide bond rather than as part of a catalytic domain.

is part of the JAK2/signal transducers and activators of Transcription pathway that plays a role in synaptic plasticity, cell proliferation, migration, and apoptosis. JAK2 contains a pair of cysteine residues (Cys866 and Cys917) that act as a redox-sensitive switch for its activity [47] and was shown to be inactivated by treatment of human BE (2)-C neuroblastoma cells with rotenone, a chemical used to model PD in animals [48].

Members of the caspase family are also found to be regulated by redox state. Caspases are involved in the initiation and execution of certain forms of programmed cell death and are therefore linked to multiple neurodegenerative conditions. Several studies have confirmed that members of this group can be oxidized at their active-site cysteine through S-nitrosylation, resulting in enzyme inhibition [49–51]. However, there are other examples in which nitric oxide (NO) may activate these caspases [52]. Such discrepancies are likely due to duration and dose of NO as well as other indirect effects of NO on other activation mechanisms [53].

Phosphatase and tensin homolog (PTEN) dephosphorylates phosphatidylinositol (3,4,5)-trisphosphate (PIP<sub>3</sub>) to phosphatidylinositol (4,5)-bisphosphate (PIP<sub>2</sub>), serving to antagonize the kinase activity of phosphatidylinositide-3-kinase. As part of this pathway that includes the Akt cascade, PTEN activity is relevant to apoptosis. Numajiri and colleagues [54] reported that S-nitrosylation of PTEN at Cys-83 inhibited PTEN activity resulting in increased AKT activity downstream, promoting cell survival. Interestingly, they also found that at higher levels of NO, AKT itself could be S-nitrosylated and therefore inhibited, resulting in a pro-apoptotic environment [54]. Finally, the active-site cysteine of PTEN, Cys124, has also been found to be oxidized in the presence of high concentrations of hydrogen peroxide [55].

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme that also has other recently discovered roles including participating in apoptosis. Cumming and Schubert [56] showed that GAPDH is sensitive to oxidative stress in affected brain regions of AD. They reported an increase in GAPDH intermolecular disulfide formation that

was reversed by addition of the cysteine-specific reducing agent, dithiothreitol (DTT), that included the active-site Cys149. Treatment of cultured neuronal and neuronal-like cells with A $\beta$  also resulted in GAPDH oxidation in addition to nuclear translocation and aggregation that may contribute to apoptosis [56]. As GAPDH's role is more fully elucidated, its oxidation is likely to be discovered to reach beyond what is presently known. See Table 1 for examples of cysteine-dependent enzymes that have been found to be regulated by redox state within the various categories of enzymes.

## 6. Cysteine-Dependent Enzymes and Their Link to Cancer

Given the linkage between age and cancer, there are likely important connections between redox regulation of the enzymes associated with neurodegeneration discussed above and tumor formation. Indeed, there are many examples of cysteine-dependent enzymes playing important roles in the various aspects of cancer progression including the impact of cancer therapies on these enzymes. The following is a brief summary of examples of this overlap.

Wang and colleagues [57] found in their model using MCF-7 human breast cancer cells that became resistant to radiation, that Prx2 is upregulated and may be a contributing factor to resistance to radiation. They hypothesized that this may be due to the antioxidant function of Prx2, resulting in attenuating radiation-induced oxidative stress effects. Goplen and colleagues [58] found that PDI is highly expressed during glioma invasion and that treatment with bacitracin, or a monoclonal antibody to PDI, inhibited tumor migration and invasion. Calpain-2 has been shown to play a role in calcium-dependent glioblastoma invasion, but not migration, which may be related to calpain-2's function in invadopodial dynamics mediated by its regulation of matrix metalloproteinase 2 [59, 60]. DJ-1 appears to be upregulated in multiple forms of cancer and is considered a ras-dependent oncogene [61, 62]. DJ-1 upregulation, as with Prx2, is likely due to its antioxidant properties and

the protective effects it would convey upon tumor cells. Alterations of parkin, observed in multiple cancer types, with genetic or other causes of decreased parkin levels are linked to increased tumorigenesis. As recently reviewed, and epidemiological studies suggest, parkin, along with DJ-1 and other genetically linked proteins are under investigation with respect to increasing risk of melanoma in PD [63–67]. SOD1, by virtue of its potent antioxidant activity, has been identified as a potential drug target to induce cell death in certain cancers. Somwar and colleagues [68], using a lung adenocarcinoma cell line, found that inhibition of SOD-1 led to increased apoptosis in these cells. Finally, Joshi and colleagues [69] treated mice with adriamycin, a chemotherapeutic agent, resulting in increased oxidation of Prx1, a cysteine-dependent peroxiredoxin, in brain.

From these data, two observations can be made. First, there are multiple cysteine-dependent enzymes that are sensitive to oxidative stress linked to tumor formation or migration. Second, the oxidizing effects of chemotherapeutic agents such as adriamycin should be considered when evaluating the potential effects of these compounds in terms of both their therapeutic and pathological effects.

## 7. Summary

Clearly, the redox regulation of cysteine-dependent enzymes is an important area of study. This is particularly evident in neurodegenerative conditions due to their strong association with increases in oxidative stress. Many of these same enzymes are also associated with tumorigenesis, invasion, or migration.

The selected enzymes for this paper appear not only sensitive to oxidation, but also key players in the underlying pathologies and in some cases, genetic causes of disease. It would appear that while nature has taken advantage of the reactivity of the sulfur group within cysteine to help regulate the response to oxidative stress, it also leaves these enzymes vulnerable to chronic conditions that promote prolonged exposure to an oxidizing environment. Thus, as our antioxidant defenses decline over time and cellular exposure to oxidizing conditions is increased, either through metabolic activity of the mitochondria, or by exposure to oxidizing environmental agents, this subset of cysteine-dependent enzymes become increasingly inhibited. Such inhibition is expected to contribute to and promote neurodegeneration, with variable effects on cancer. This paper has only highlighted some of the significant cysteine-dependent enzymes that have been shown to be related to neurodegenerative diseases and not all of the tremendous efforts of the many researchers that have contributed have been referenced here.

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## Review Article

# The Chemical Interplay between Nitric Oxide and Mitochondrial Cytochrome *c* Oxidase: Reactions, Effectors and Pathophysiology

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Nitric oxide (NO) reacts with Complex I and cytochrome *c* oxidase (CcOX, Complex IV), inducing detrimental or cytoprotective effects. Two alternative reaction pathways (PWs) have been described whereby NO reacts with CcOX, producing either a relatively labile nitrite-bound derivative (CcOX-NO<sub>2</sub><sup>-</sup>, PW1) or a more stable nitrosyl-derivative (CcOX-NO, PW2). The two derivatives are both inhibited, displaying different persistency and O<sub>2</sub> competitiveness. In the mitochondrion, during turnover with O<sub>2</sub>, one pathway prevails over the other one depending on NO, cytochrome *c*<sup>2+</sup> and O<sub>2</sub> concentration. High cytochrome *c*<sup>2+</sup>, and low O<sub>2</sub> proved to be crucial in favoring CcOX nitrosylation, whereas under-*standard* cell-culture conditions formation of the nitrite derivative prevails. All together, these findings suggest that NO can modulate physiologically the mitochondrial respiratory/OXPHOS efficiency, eventually being converted to nitrite by CcOX, without cell detrimental effects. It is worthy to point out that nitrite, far from being a simple oxidation byproduct, represents a source of NO particularly important in view of the NO cell homeostasis, the NO production depends on the NO synthases whose activity is controlled by different stimuli/effectors; relevant to its bioavailability, NO is also produced by recycling cell/body nitrite. Bioenergetic parameters, such as mitochondrial ΔΨ, lactate, and ATP production, have been assayed in several cell lines, in the presence of endogenous or exogenous NO and the evidence collected suggests a crucial interplay between CcOX and NO with important energetic implications.

## 1. Introduction

It is nowadays established that nitrogen monoxide (NO), nitric oxide in the literature, inhibits mitochondrial respiration. The inhibition is induced by the reaction of NO with some of the complexes of the respiratory chain, according to mechanisms studied over more than 20 years. The reaction of NO with Complex III is sluggish [1], whereas the reaction of NO with Complex I and Complex IV, that is, cytochrome *c* oxidase (CcOX), is rapid and to a large extent reversible. Both reactions lead to formation of derivatives responsible of the mitochondrial nitrosative stress observed in different pathophysiological conditions, including main neurodegenerations [2–6]. The functional groups of the mitochondrial complexes reacting with NO include the metals at the catalytic active site of CcOX, namely, the Fe and Cu ions of the

heme *a*<sub>3</sub>-Cu<sub>B</sub> site [7, 8]. The inhibition of Complex I results from the reversible S-nitrosation of Cys39 exposed on the surface of the ND3 subunit [9, 10]. The functional effects on cell respiration depend on the complex targeted by NO and on type of reaction. Inhibition of both Complex I and CcOX is mostly reversible, becoming irreversible, however, depending on duration of the exposure to NO and on its concentration [10, 11]. The onset of NO inhibition on Complex I is slow (minutes [10]), whereas on CcOX is very fast (milliseconds to seconds [12]). In this paper the attention is focused on the interactions between NO and CcOX. The balance between the concentrations of cytochrome *c*<sup>2+</sup> and O<sub>2</sub> proved to be critical in inducing different CcOX inhibition patterns, spanning from a finely tuned *control* to a severe, almost irreversible enzyme inactivation [13]. The interplay between CcOX and NO is based on the inhibition

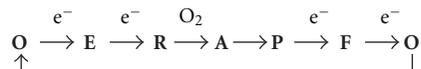
exerted by NO on the enzyme that, in turn, actively controls the NO concentration at the mitochondrial site [14].

The redox active site of CcOX contains one heme  $a_3$  and one  $\text{Cu}_B$  tightly coupled in the so-called binuclear site, where the  $\text{O}_2$  and NO chemistry as well as the reaction with common ligands occur. The active site receives electrons intra-molecularly from the reduced heme  $a$  and  $\text{Cu}_A$ , forming together the electron accepting pole of CcOX, maintained physiologically reduced by cytochrome  $c$ . Also relevant to the reaction of NO with CcOX, the availability in the mitochondrion of reduced cytochrome  $c$  depends on the relative rate at which it is reduced by Complex III and oxidized by  $\text{O}_2$  via CcOX. It is also worth mentioning that the absolute cytochrome  $c$  concentration may vary in different cell lines and tissues [15]. The rate of reaction of CcOX with  $\text{O}_2$  is close to diffusion limited ( $k \approx 1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$  [16, 17]), whereas the reaction with cytochrome  $c$  is slower,  $k \approx 1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ , the actual rate constant value being dependent on pH and ionic strength [18]. During turnover, the reduction level of the CcOX redox sites, and particularly of the metals in the active site, depends on (i) the actual concentration of reduced cytochrome  $c$  and  $\text{O}_2$  (weighted for their relative  $K_M$  values) at the redox competent sites and (ii) the internal electron transfer rate from the electron accepting pole (heme  $a$ - $\text{Cu}_A$ ), where cytochrome  $c$  reacts, to the active (heme  $a_3$ - $\text{Cu}_B$ ) site, where the  $\text{O}_2$  reaction takes place. At saturating concentration of the physiological substrates, the rate limiting step in the CcOX catalytic cycle is the internal electron transfer [19–21]. Over and above the description of the reaction mechanisms, the aim of this work is to stress the idea that CcOX uses both  $\text{O}_2$  and NO as physiological substrates [5, 14, 22, 23] and to review the experimental evidence pointing to a central role of the NO interplay with CcOX in cell bioenergetics.

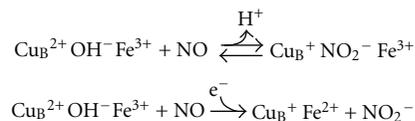
## 2. CcOX Binds Reversibly or Oxidizes NO to Nitrite at the Active Site Where $\text{O}_2$ Binds

In order to better understand the reciprocal interactions between CcOX and NO, it may help summarizing the intermediates populated by CcOX during turnover with physiological substrates. During the catalytic cycle the fully oxidized (**O**) heme  $a_3$ - $\text{Cu}_B$  site accepts a first electron from  $\text{Cu}_A$ /heme  $a$ , leading to formation of a partially, single-electron, reduced (**E**) species; subsequently, a second electron is transferred to the active site, and the fully reduced (**R**) species is formed. Once in the **R** state,  $\text{O}_2$  binds rapidly generating the short-lived (microseconds, at  $20^\circ\text{C}$ ) compound **A**, in which  $\text{O}_2$  is complexed to heme  $a_3^{2+}$  [24]. Electrons are rapidly delivered to bound  $\text{O}_2$ , and Compound **A** converts to a nominal peroxy (**P**) complex with both heme  $a_3$  and  $\text{Cu}_B$  oxidized; actually, the experimental evidence suggests that the O–O (peroxy) bond in this **P** species is already cleaved off, showing heme  $a_3$  in the ferryl ( $\text{Fe}^{4+}=\text{O}$ ) form and a tyrosine residue in a radical state [25, 26]. By accepting a third electron, **P** decays quickly into a canonical ferryl (**F**) intermediate [27], that eventually converts back to the fully oxidized **O** state upon arrival of one last electron from  $\text{Cu}_A$ /heme  $a$ . The sequential steps and the

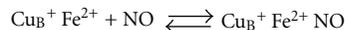
intermediates populated during a single turnover are indicated, starting and ending with the fully oxidized **O** species:



Since first proposed as a unified picture based on experiments carried out using purified CcOX [28], the enzyme adducts formed upon reacting with NO have been spectroscopically identified as a nitrosyl-derivative (heme  $a_3^{2+}$ -NO) or as a nitrite-bound (heme  $a_3^{3+}$ - $\text{NO}_2^-$ ) derivative, or a mixture of these two species, depending on the steady-state fractional accumulation of all the intermediates [29]. It is worthy to notice that the Fe and Cu ions in the active site undergo redox changes only upon reacting in the oxidized state (i.e.,  $\text{Fe}^{3+}$ ,  $\text{Fe}^{4+}$ ,  $\text{Cu}^{2+}$ ) with NO. During the reaction NO is oxidized to  $\text{NO}_2^-$ , that is released in the medium; the whole event is identified as pathway 1 (PW1):



Otherwise, if the active site is partially or fully reduced, an affinity-driven NO binding to these metals takes place; the whole event is identified as pathway 2 (PW2) and occurs without further redox events:



NO is very reactive towards the fully reduced **R** binuclear site. It binds to heme  $a_3^{2+}$  at a rate similar to that of  $\text{O}_2$ , that is,  $k = 0.4 - 1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$  [16, 17], yielding the high affinity  $\text{Fe}^{2+}$  nitrosyl adduct, whose accumulation is observable directly by spectroscopy or indirectly by NO amperometry [30, 31], when the fully reduced CcOX in detergent solution is mixed with NO. Interestingly, in the presence of NO, all circumstances favoring the electron donation to the catalytic site of CcOX or slowing down its oxidation by  $\text{O}_2$  as during hypoxia (i.e., when the  $[\text{O}_2] \leq K_{M,\text{O}_2}$  of CcOX) proved to favor CcOX nitrosylation [32]. Figure 1 shows schematically how accumulation of the turnover intermediates correlates with the build up of the nitrosylated ( $\text{Cu}_B^+ \text{Fe}^{2+} \text{NO}$ ) or the nitrite-bound ( $\text{Cu}_B^+ \text{NO}_2^- \text{Fe}^{3+}$ ) species.

It is worth mentioning that, contrary to a few bacterial oxidases [34–36], mitochondrial CcOX cannot reduce to  $\text{N}_2\text{O}$  the NO bound at reduced heme  $a_3$  [30]. This implies that the functional recovery of the enzyme after NO binding necessarily lags behind the thermal dissociation of NO from the active site. The dissociation reaction is relatively slow ( $k_{\text{off}} = 3.9 \times 10^{-3} \text{ s}^{-1}$  at  $20^\circ\text{C}$ ) and photosensitive [28]. Photosensitivity has been widely used by Sarti and coworkers to gain insight, through amperometric measurements, into the mechanism of CcOX inhibition by NO in mitochondria or whole cells [37], that is, under conditions unfavorable to spectroscopy. Since the fully reduced binuclear site reacts eagerly with both  $\text{O}_2$  and NO, the inhibition of CcOX via

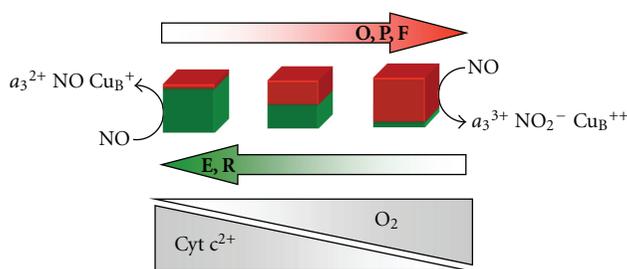


FIGURE 1: Dual-pathway model for the interaction of NO with mitochondrial cytochrome oxidase. The nature of the interaction between NO and CcOX depends on the catalytic intermediates targeted, and these are differently populated at different concentrations of  $O_2$  and reduced cytochrome  $c$ . The oxidized intermediates O, P, F (see text) are overall more populated with increasing  $O_2$  availability, and/or decreasing the concentration of reduced cytochrome  $c$  in the mitochondrion: these intermediates react with NO generating a nitrite-inhibited CcOX. The reduced species E and R (see text) buildup, instead, upon decreasing  $O_2$  and/or increasing the concentration of reduced cytochrome  $c$ : upon reacting with NO, these intermediates generate a heme  $a_3^{2+}$ -NO complex, in competition with oxygen.

formation of a nitrosyl adduct is expected to occur in competition with  $O_2$ , that is, according to PW2. Consistently, the  $O_2$  competition is more clearly observed when the concentration of the reducing substrates favors the reduction of the enzyme [29, 32]. In any case, the inhibition of the nitrosylated CcOX is reverted at the rate of the NO thermal dissociation from reduced heme  $a_3$  [28]. It is worth noticing that, although the NO dissociation process is mechanistically independent of  $O_2$  concentration, bulk  $O_2$  shortens the duration of inhibition by oxidizing free NO in solution, thus hampering NO rebinding to CcOX.

### 3. The Fully- and Half-Reduced Binuclear Site

The ability of the single electron reduced E species to bind NO was investigated using the K354M mutant of the *Paracoccus denitrificans* CcOX [38]. In this mutant the internal electron transfer from the electron accepting pole to the active site is severely impaired, so that the full reduction of the active site and its reaction with  $O_2$  is achieved very slowly, that is, within several minutes. Under these conditions the electron transferred intramolecularly from heme  $a/Cu_A$  resides on either heme  $a_3$  or  $Cu_B$ , and the resulting E species can not react with  $O_2$ . Interestingly, however, E reacts promptly with NO generating the nitrosyl derivative. Thus, one can conclude that, unlike  $O_2$ , NO binds to the binuclear active site even before its complete reduction [12, 31]. Whether the reaction with E plays a role in the mechanism of CcOX inhibition by NO during turnover is still unclear, since it has been also suggested that at steady-state the reaction of NO with E is not required to account for fast inhibition [32, 39]. Regardless of whether the reaction of NO with either E or R is predominant, it seems feasible to conclude that all conditions leading to reduction of the binuclear site in the presence of NO favor nitrosylation of the enzyme.

### 4. The Role of $Cu_B$ in the Reaction with NO

The reaction of NO with  $Cu_B$  in the fully oxidized CcOX to form nitrite was first reported by Brudvig and coworkers in the early 80s [40]. Later on this reaction was reinvestigated by Cooper et al. [41] and Giuffrè et al. [42], using a *pulsed (fast)* preparation of CcOX. The pulsing procedure that *in vitro* consists in preliminary reduction-reoxidation of CcOX [43], removes chloride from the oxidized active site of the enzyme thereby allowing fast reaction with NO [42]; indeed, CcOX is expectedly in the pulsed state *in vivo* where CcOX turnover takes place continuously. During the reaction with the oxidized  $Cu_B$  ( $k = 2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  at  $20^\circ\text{C}$ ), NO is transiently oxidized to nitrosonium ion ( $NO^+$ ), which is subsequently hydroxylated (or hydrated) to nitrite/nitrous acid.

Thus, after the reaction, the enzyme displays nitrite bound to ferric heme  $a_3$  and is inhibited. The affinity of nitrite for the reduced heme  $a_3$ , however, is much lower than the affinity for the oxidized active site. The intramolecular electron transfer to heme  $a_3$ - $Cu_B$ , therefore, causes the prompt dissociation of nitrite and the subsequent full restoration of activity [29, 44]. Relevant to possible pathological effects of CcOX inhibition by NO, it is worthy to notice that the nitrite dissociation upon reduction of heme  $a_3$  ( $k \sim 6 \times 10^{-2} \text{ s}^{-1}$  at  $\text{pH} = 7.3$ ,  $T = 20^\circ\text{C}$  [29]) is approximately one order of magnitude faster than the NO-dissociation from the nitrosylated site, accounting also for the observed production of nitrite by isolated mitochondria [45, 46].

It has been proposed that nitrite formation could follow an alternative route via reaction with  $O_2$  of the NO bound to the fully reduced CcOX [46]. According to this proposal, a superoxide anion ( $O_2^-$ ) forms by the reaction of  $O_2$  with reduced  $Cu_B$  and reacts with NO bound to reduced heme  $a_3$  to yield peroxynitrite; peroxynitrite is reduced in turn by the enzyme to nitrite, which is finally released in the bulk. The hypothesis, though feasible and intriguing, was not confirmed by independent experiments specifically designed to investigate the kinetics and the products of the reaction of fully reduced nitrosylated CcOX with  $O_2$  [50]. Using myoglobin as an optical probe for free NO, the NO bound to reduced heme  $a_3$  was shown to be displaced by excess  $O_2$  at the low rate of thermal dissociation, to be eventually released in the bulk as such, and not as nitrite [50]. The NO dissociation from the heme iron takes minutes, also when assayed in mitochondria or intact cells, at  $37^\circ\text{C}$  and in the dark, that is, under conditions common *in vivo* in internal organs and tissues. The slow recovery of function of the nitrosylated CcOX is compatible with a more severe state of inhibition characteristic of PW2.

The role of  $Cu_B$  in the CcOX-mediated oxidation of NO to nitrite was also addressed in experiments carried out using the *E. coli* cytochrome *bd*. This oxidase lacks  $Cu_B$  and, consistently, reacts with NO much more slowly ( $k = 1.5 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$  at  $20^\circ\text{C}$ ) than mitochondrial CcOX, without forming nitrite [51]. Interestingly, the NO dissociation from the  $Cu_B$ -lacking cytochrome *bd* oxidase (from *E. coli*) is much faster [52, 53], pointing to a specific property of heme *d* [54] and/or to a role of  $Cu_B$  also in the NO dissociation

TABLE 1: Cytochrome *c* oxidase versus NO—kinetic and thermodynamic parameters.

CcOX intermediate	CcOX adduct formed	$K_I$ (nM)	$k_{on}$ ( $M^{-1} s^{-1}$ ) ( $T = 20^\circ C$ )	$k_{off}$ ( $s^{-1}$ ) ( $T = 20^\circ C$ )	O <sub>2</sub> -competition
<b>E, R</b>	Nitrosylated CcOX-NO	0.2 [32]	$0.4-1 \times 10^8$ [16, 17]	$3.9 \times 10^{-3}$ [28]	yes
<b>O, P, F</b>	Nitrite bound CcOX-NO <sub>2</sub> <sup>-</sup>	20 [32]	$\sim 1 \times 10^5$ ( <b>O, P</b> ) $\sim 1 \times 10^4$ ( <b>F</b> ) [29, 58]	$6.0 \times 10^{-2}$ [29]	no

from the active site. As a matter of fact, this peculiarity was suggested to confer to cytochrome *bd*-expressing bacteria a higher resistance to nitrosative stress [53, 55, 56], a hypothesis supported by *in vitro* studies on *E. coli* deletion mutants of each of the two alternative respiratory oxidases (cytochrome *bd* and cytochrome *bo*<sub>3</sub>) [55].

### 5. Cells Respiring in the Presence of NO and Using Endogenous Substrates

The respiration of cells grown under *standard* conditions, that is, in the presence of (unlimited) O<sub>2</sub> and endogenous reducing substrates, is inhibited by NO but without detectable accumulation of nitrosylated CcOX [37, 57]. As a matter of fact, these standard culture conditions favor the overall accumulation of the CcOX intermediates **P**, **F** and **O** [29, 41, 42, 58]; these are the species responsible for the NO oxidation to nitrite. Consistently, upon rapid and efficient scavenging of bulk NO, respiration is promptly recovered. It is worthy to point out that nitrite, far from being a simple oxidation byproduct, represents a source of NO particularly important in view of the NO cell homeostasis [59–62]. When the oxygen tension decreases in tissues, not only respiration but also the production of NO by nitric oxide synthases (NOSs) is severely impaired, as the NOS uses O<sub>2</sub> as cosubstrate [63]. Anoxia, however, induces tissue acidification, which promotes the reduction of nitrite to NO, compensating for impairment of the NOS-dependent NO production [59, 60, 64]. Consistently, and apparently important for a cardiovascular response, low doses of nitrite ( $\sim 50$  nM) administered to ischemic, heart-arrested mice, early during resuscitation procedures, were shown to significantly improve survival of the treated animals compared to controls [61].

The CcOX NO-inhibition pathway prevailing in mitochondria under given metabolic conditions might be responsible for pathological responses of cells and tissues [57]. Compelling experimental evidence has been collected suggesting that the O<sub>2</sub>-uncompetitive nitrite inhibition pathway (PW1) prevails under conditions of low electron flux through the respiratory chain and high O<sub>2</sub>, whereas the O<sub>2</sub>-competitive nitrosyl pathway (PW2) takes over as the electron flux increases and O<sub>2</sub> concentration decreases [32, 37].

The main features of the two pathways can be summarized as follows:

- (i) both reactions lead to the rapid accumulation of a CcOX inhibited species, characterized by different stability,  $K_I$ , and O<sub>2</sub> competitiveness (Table 1);
- (ii) one pathway prevails over the other one depending on the fractional accumulation of the NO-targeted

CcOX turnover intermediates [28, 29], whose distribution depends in turn on the *in situ* availability of O<sub>2</sub> and reduced cytochrome *c*; the concentration of the latter ultimately depends on its absolute concentration and on the electron flow level through the respiratory chain;

- (iii) PW1 prevails under basal mitochondrial metabolic conditions;
- (iv) PW2 prevails under conditions favoring the accumulation of **E** and **R**, that is, when the concentration of cytochrome *c*<sup>2+</sup> at the CcOX site increases and/or the O<sub>2</sub> tension decreases;
- (v) the accumulation of CcOX-NO or CcOX-NO<sub>2</sub><sup>-</sup> affects differently the mitochondrial bioavailability of NO: the nitrosyl-derivative releases NO in the medium as such, that is, still reactive, whereas the nitrite-derivative releases nitrite to be further oxidized to nitrate, eliminated or rereduced to NO.

The NO concentration level in the cell varies depending on the relative rate of its production, and degradation or scavenging. Unless exogenously supplemented to the cells (NO-donors), the enzymatic endogenous NO production is controlled via the activation/inhibition of the cell NO-synthases. Alternatively, as mentioned above, NO is generated by the protein-bound or free metal ions (Fe<sup>2+</sup>, Cu<sup>+</sup>) catalyzed reduction of NO<sub>2</sub><sup>-</sup>, a reaction that commonly occurs in solution, at acidic pH [59, 60]. The NO bioavailability can be lowered, therefore, by specific cell-permeable NO-synthase inhibitors or by NO scavengers, such as heme-proteins or reduced glutathione [65].

As pointed out by Cooper and Giuliivi [5], when the NOS activity is inhibited, one may expect the O<sub>2</sub> consumption by respiring mitochondria to increase. This event, however, has been often but not always observed [5], probably owing to the activation of alternative NO-releasing systems, such as nitrosoglutathione and S-nitrosated protein thiols, or the NO<sub>2</sub><sup>-</sup> reduction, all active regardless of the presence of NOS inhibitors.

### 6. Effectors and Pathophysiology

Over the years, the enzymatic NO release has been induced in cultured cells, tissues, and organs, either using effectors able to activate cell Ca<sup>2+</sup> fluxes [66], thus stimulating the constitutive NOS, or by enhancing the expression of the inducible isoform of NOS (iNOS) [67]. Morphine is the prototype of a family of drugs used in analgesia and cancer pain treatment [68, 69]. Relevant to the NO chemistry,

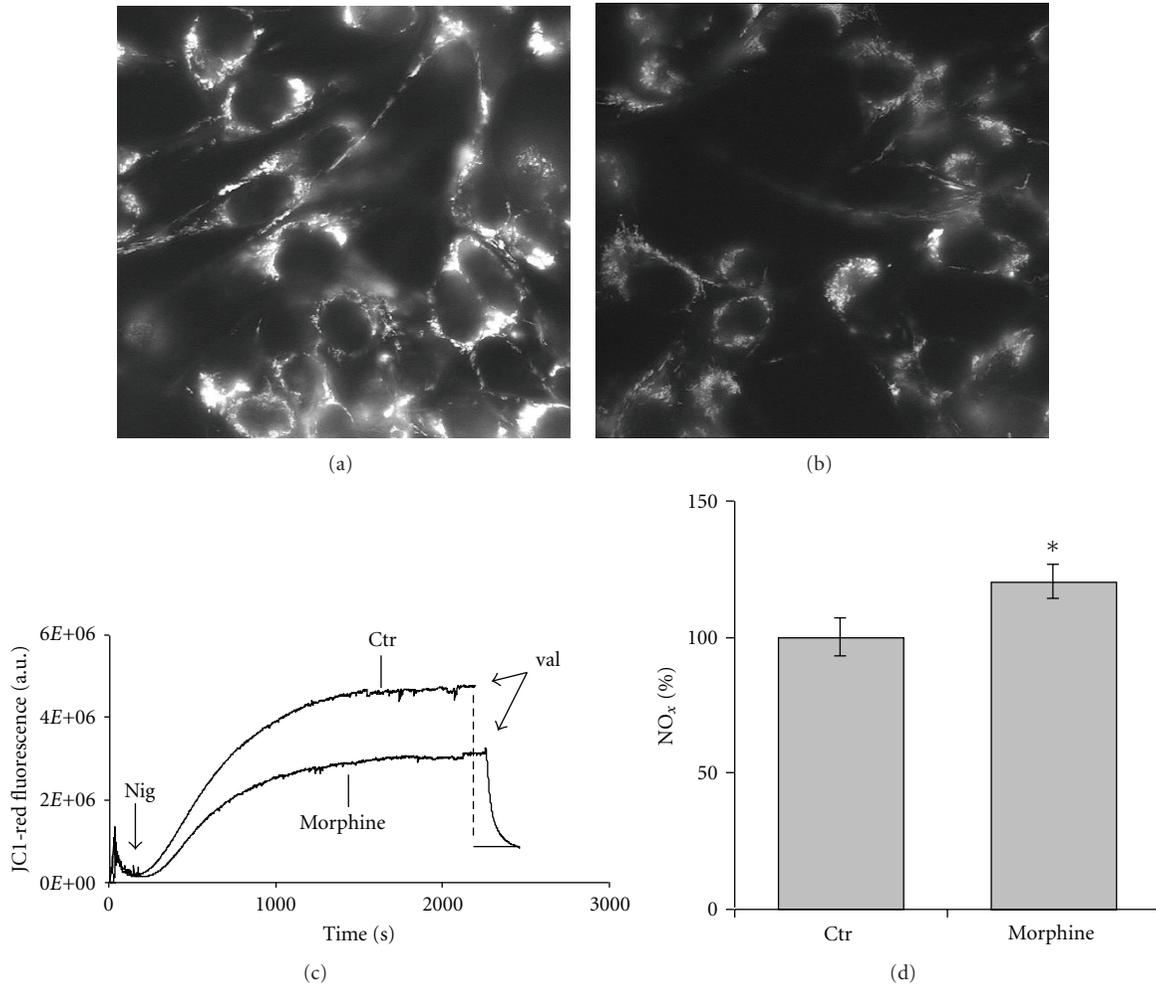


FIGURE 2: Morphine-induced mitochondrial membrane potential and NO<sub>x</sub> changes in Glioma cells. *Fluorescence microscopy*: control cells (a), 20 nM morphine incubated for 24 h (b). Mitochondrial membrane potential ( $\Delta\Psi$ ) was probed using Rhodamine123; the dye is electrophoretically accumulated by the cell mitochondria. *Bulk fluorescence* (c). Control (ctr) versus morphine-treated (morphine) cells, assayed in air-equilibrated medium and in the presence of 2  $\mu$ M ouabain and 0.4  $\mu$ M JC-1; after signal stabilization, 0.6  $\mu$ M nigericin is added and fluorescence changes followed over time. Addition of valinomycin abolishes the membrane potential. Excitation and emission wavelength, 575 nm and 590 nm, respectively. *Nitrite accumulation* (d). The release of NO<sub>x</sub> (nitrite and nitrate) in the medium and during incubation with morphine was assessed spectrophotometrically by the Griess reaction; results expressed as percentage of control cells (ctr). \* $P < 0.05$ . Modified from [33].

morphine activates the opioid and the N-methyl-D-aspartate receptors of neuronal cells, triggering Ca<sup>2+</sup> fluxes and NO release [70, 71]. In 2004, Mastronicola et al. [33] confirmed that the persistence of nanomolar morphine in the cell culture of glioma cells was able to induce the accumulation of nitrite/nitrate in the medium. Interestingly, the cell mitochondria displayed a membrane potential drop off, as probed by a significant decrease of the intramitochondrial JC-1 red-aggregates, whose accumulation requires high mitochondrial  $\Delta\Psi$  values (Figure 2) [72]. Thus, over the same time scale of a cell Ca<sup>2+</sup> transient (seconds to minutes) the NOS activation can affect the mitochondrial potential [33]. More recently, Arese et al. [48] have shown a transient inhibition of the mitochondrial respiratory chain in human adult low calcium

temperature (HaCaT) cells, maintained in a standard culture medium, in the presence of nanomolar (or less) melatonin. After a few hours incubation compatible with a receptor-mediated process [73], and with a timecourse compatible with the circadian melatonin biorhythm, the basal mRNA expression level of the neuronal NOS (nNOS) in the cells was raised by a factor of  $\sim 4$  (Figure 3(a)), returning, thereafter, to basal level [48]. As shown in the same figure, within the same time scale, the authors observed that: (i) the production of nitrite and nitrate (NO<sub>x</sub>) was increased (Figure 3(b)) and (ii) the mitochondrial membrane potential was decreased (Figure 3(c)). Consistently, the ATP<sub>OXPHOS</sub> production was also decreased and an increase of glycolytic ATP and lactate was detected [48]. Taken together, all these findings suggest

that mediated by the melatonin receptors, NO is released and CcOX is reversibly inhibited, with significant bioenergetic consequences. Since cells are not likely facing conditions compatible with the accumulation of CcOX intermediates **E** or **R**, we can infer that inhibition has occurred via PW1. Interestingly, therefore, under physiological conditions, within the limits of a cell culture, a few hours exposure to hormonal-like concentrations of melatonin is able to exert some inhibition on mitochondrial OXPHOS and to raise the  $\text{ATP}_{\text{glycolytic}}/\text{ATP}_{\text{OXPHOS}}$  ratio by a factor of  $\sim 2$  (Figure 3(d)) as expected on the basis of a compensatory physiological Warburg effect [74]. All together these findings suggest that physiological concentrations of melatonin may play a mitochondrial role and interestingly in a circadian context. Indeed, the hypothesis that the melatonin-driven shift towards glycolysis might have a physiological role in the chemistry of the night rest, though attractive, is presently fully speculative, and remains to be investigated.

Based on the effects of melatonin and on the information collected about the NO inhibition of purified CcOX or mitochondria [75, 76], it is also tempting to speculate on how the mitochondrial state can affect the response to NO, particularly under conditions compatible with a limited, and transient raise of NO concentration. It is worthy to consider that isolated state 3 mitochondria proved to be inhibited by NO more effectively than state 4 mitochondria [75, 76]. This suggests that the sensitivity to NO inhibition increases with the electron flux level of the respiratory chain, and particularly with the turnover rate of CcOX; under these conditions the CcOX inhibition is oxygen competitive [32]. In state 3 mitochondria, therefore, and in the presence of suitable amounts of reduced cytochrome *c*, the fractional accumulation of the reduced (**E** and **R**) CcOX species is expected to increase; these species are promptly nitrosylated in the presence of NO. At low turnover rate, as in state 4, the oxidized catalytic intermediates (**O**, **P** and **F**) are expected to be more populated [29], and the NO inhibition predominantly occurs following PW1. Both in state 3 and state 4, if the NO concentration is low (e.g., subnanomolar), the fraction of CcOX inhibited is limited, and the depression of respiration is almost insignificant [77, 78], a finding consistent with an excess capacity of CcOX [79, 80]. When NO persists in the cell environment, as during a prolonged incubation with even low (nM) concentration of NO, and particularly if the turnover rate of CcOX is increased, a substantial inhibition of the respiratory chain is predictable and synthesis of  $\text{ATP}_{\text{OXPHOS}}$  decreases [81]. Under these conditions, glycolysis likely takes place to compensate for ATP loss [82].

## 7. How Does the NO/CcOX Interplay Turn into Pathology

As just mentioned, the transient inhibition of mitochondrial OXPHOS may induce a physiological, compensatory activation of glycolysis [74]. This original observation by Warburg was recently repropounded by Almeida et al. [83], to rationalize the energetic changes of astrocytes and neurons

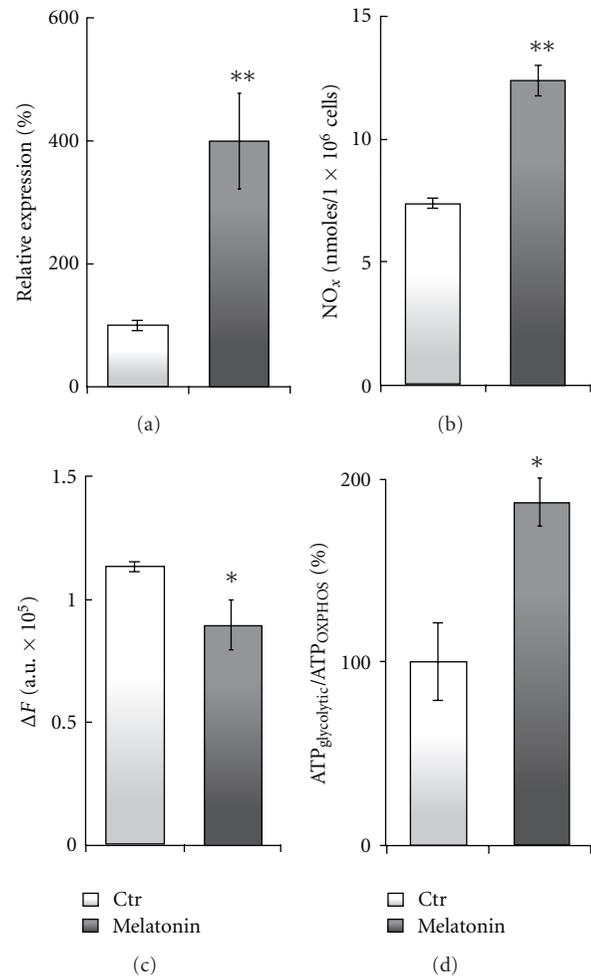


FIGURE 3: Melatonin-induced changes of the nNOS mRNA expression in HaCaT cells: effect on NO<sub>x</sub> production and mitochondrial membrane potential. (a)—Real-time PCR quantification of nNOS mRNA ( $\beta$ -actin gene used for normalization). (b)—Fluorometric determination of the NO<sub>x</sub> release in the cell culture medium. (c)—Mitochondrial membrane potential evaluated as the fluorescence difference,  $\Delta F$ , from the maximal (*plateau*) to the minimal level reached after addition of valinomycin (see also Figure 2(c)). (d)—Contribution of oxidative phosphorylation and glycolysis to ATP production, directly evaluated according to [47]. The  $\text{ATP}_{\text{glycolytic}}/\text{ATP}_{\text{OXPHOS}}$  ratio is indicative of the ability of a given cell line to compensate with glycolysis an OXPHOS impairment (so-called, Warburg effect) [48, 49]. The release of NO induced by melatonin almost doubles the glycolytic contribution to ATP synthesis in HaCaT cells. Cells were incubated with 1 nM melatonin for 6 h. (a), (b): \*\* $P < 0,01$  versus CTR; (c), (d): \* $P < 0,05$  versus CTR. Modified from [48].

inhibited by NO. In this respect, it is worth considering that neurons, astrocytes, lymphoid, keratinocytes cells, and in general different cell lines may possess a different glycolytic compensatory capacity of coping with OXPHOS NO-inhibition [48, 57, 83]. All the evidence so far collected shows that under standard cell culture conditions, a pulse of NO

leads to the accumulation of the CcOX-NO<sub>2</sub><sup>-</sup> derivative [37], which is able to immediately and fully recover its function, provided that free NO is scavenged in the mitochondrial environment. On the contrary, when CcOX nitrosylation is induced by (artificially) rising the electron flux level at the CcOX site or by allowing the cells to respire towards hypoxia ( $[O_2] \leq K_{M,O_2}$ ), the respiratory chain remains inhibited for longer times at the CcOX site [28, 29, 32, 84]. It is worth recalling that indeed everything else being equal, the functional recovery of CcOX-NO is approximately 10–20 times slower than recovery of CcOX-NO<sub>2</sub><sup>-</sup>. Thus, at least in a first approximation, it is feasible to propose that, compared to conditions promoting the formation of the CcOX-nitrite adduct, conditions favoring CcOX nitrosylation are expectedly more dangerous for cells, since causing a 10–20 times longer inhibition of the mitochondrial respiratory chain. One may indeed speculate that the compensatory glycolytic ATP synthesis might become insufficient, when CcOX is maintained nitrosylated for longer times.

In 2008 Masci et al. [57] characterized the mitochondria NO inhibition pattern of cells collected from patients affected by Ataxia Telangiectasia (AT). This is a multisystemic genetic human disorder characterized by a conjunctival telangiectasia and by a cerebellar degeneration leading to progressive ataxia [85, 86]. The disease is caused by mutations of the AT-mutated gene (ATM), coding for a nuclear 350 kDa protein that controls cell cycle and DNA damage repair [87–89]. AT patients are characterised by a genetic instability and vulnerability to radiation-induced oxidative stress [90–94]. Compared to control cells, AT cells display a defective reactive oxygen species (ROS) scavenging capacity [95, 96], with a decreased bioavailability of reduced glutathione [96].

Relevant to a possible pathological implication of the NO mitochondrial inhibition, AT patients show a bioenergetic deficiency [97]. The mitochondrial functional characterization, and the NO inhibition pattern of lymphoid cells collected from AT patients, proved to be significantly altered. Based on the rate of respiration recovery from inhibition, under otherwise identical conditions of substrates availability (O<sub>2</sub> and reductants), the CcOX in AT cells underwent nitrosylation to a substantially higher extent than in control cells [57]. As expected, based on the higher stability of the nitrosyl-adduct compared to the nitrite-adduct, after NO inhibition and subsequent removal of free NO, recovery of respiration of AT cells is slow, occurring at the rate of the NO displacement from the reduced CcOX active site, whereas control cells recover almost immediately (Figures 4(a) and 4(b)). As a matter of fact the inhibition of AT cells respiration was promptly removed upon shedding light on the cells (photosensitivity of the nitrosyl-adduct!). This peculiarity of AT cells has been correlated to their 1.7 fold higher concentration of mitochondrial cytochrome *c* compared to control cells (Figure 4(c)) [57]. The whole picture is consistent with the hypothesis that in AT cells, showing a lower ATP<sub>glycolytic</sub>/ATP<sub>OXPHOS</sub> ratio compared to control cells (Figure 4(d)), the formation of **E** and **R** and thus CcOX nitrosylation is favored owing to the higher availability of reduced cytochrome *c* [29, 32].

## 8. The Dark Side of the Interplay between NO and CcOX

In conclusion, regardless of the pathway leading to inhibition of CcOX, in the presence of NO, mitochondrial OXPHOS is impaired to some extent. Impairment is due to the slow displacement of NO from the active site or to the involvement of the site in the NO oxidation to nitrite. The evidence so far collected suggests that, if NO remains available in the mitochondrial environment, the mitochondrial membrane potential decreases, and glycolysis begins to contribute significantly to ATP synthesis. Thus, it seems crucial that cells responding to NO pulses are endowed with an efficient glycolytic machinery able to compensate for the decreased aerobic ATP production [82, 83].

Finally, let us consider for the sake of the argument a chronic hypoxia induced by an impaired microcirculation, for instance in the brain. Under these conditions common to many age-related neurodegenerations, one might expect an increased NO release to enhance the blood flow in response to hypoxia. In this already pathological scenario, however, the blood flow and thus O<sub>2</sub> concentration may not increase significantly, owing to the vessel sclerosis; neurons could rather become hypoxic and in the presence of an increased NO concentration. These are the circumstances favouring PW2 (CcOX nitrosylation), even more so if the respiratory chain concentration of reducing substrates is still large enough. Under these conditions and in the absence of a suitable glycolytic compensation, the ATP levels could decrease dramatically, leading to cell death.

## Abbreviations

CcOX:	Cytochrome <i>c</i> oxidase
CcOX-NO:	Nitrosyl cytochrome <i>c</i> oxidase derivative
CcOX-NO <sub>2</sub> <sup>-</sup> :	Nitrite-bound cytochrome <i>c</i> oxidase
PW1:	NO reaction pathway leading to nitrite-bound CcOX
PW2:	NO reaction pathway leading to nitrosyl CcOX
OXPHOS:	Oxidative phosphorylation
ΔΨ:	Membrane electrical potential difference
<b>O</b> :	Fully oxidized CcOX
<b>E</b> :	CcOX with single-electron reduced heme <i>a</i> <sub>3</sub> -Cu <sub>B</sub>
<b>R</b> :	CcOX with fully reduced heme <i>a</i> <sub>3</sub> -Cu <sub>B</sub>
<b>A</b> :	CcOX with ferrous oxygenated heme <i>a</i> <sub>3</sub>
<b>P</b> :	“Peroxy” CcOX intermediate
<b>F</b> :	“Ferryl” CcOX intermediate
NOS:	Nitric oxide synthase
nNOS:	Neuronal NOS
NO <sub>x</sub> :	Nitrite-nitrate
AT:	Ataxia Telangiectasia

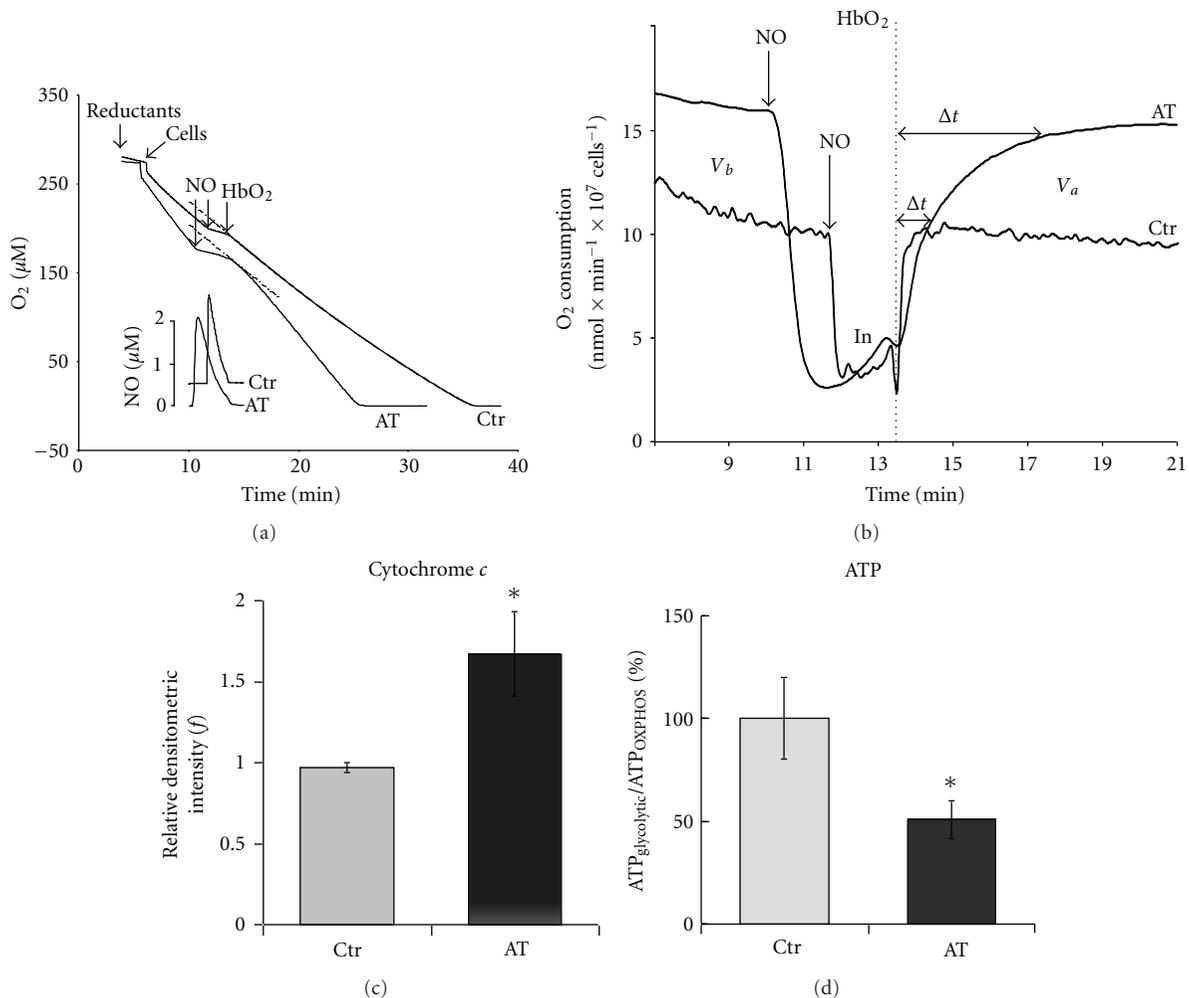


FIGURE 4: Oxygen consumption of Ataxia Telangiectasia (AT) cells: the inhibitory effect of NO. (a)—O<sub>2</sub> consumption profiles of AT and control lymphoblastoid cells, recorded in the dark and in the presence of excess ascorbate and tetramethyl-p-phenylenediamine (TMPD). Inhibition of respiration was induced by adding a single bolus of pure NO gas solution (see lower NO profiles). In order to assess the fraction of residual inhibited CcOX-NO, the instantaneous rate was measured at 45 s after HbO<sub>2</sub> addition. (b)—First derivative plots (integration time  $t = 2$  s). Rate of O<sub>2</sub> consumption before addition of NO ( $V_b$ ), and after addition of oxygenated hemoglobin, HbO<sub>2</sub> ( $V_a$ ), that is, in the absence of free NO. *In*: inhibited state (in the presence of free NO). The  $\Delta t$  value is the time necessary for complete recovery of activity after addition of HbO<sub>2</sub>.  $T = 25$  C. (c)—Cytochrome *c* immunoblot. Cell-lysate (30 μg/well) of AT patients and controls (ctr). (d)—Relative contribution of OXPHOS and glycolysis to ATP production in AT and control cells. Modified from [57].

HaCaT: Human adult low calcium temperature, that is, keratinocytes cell line  
HbO<sub>2</sub>: Oxygenated haemoglobin  
State 3 respiration: Induced by ADP, causing a burst of O<sub>2</sub> consumption and ATP synthesis and relaxing into the slower State 4 respiration after ADP consumption.

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## Review Article

# Mitochondrial Dynamics in Cancer and Neurodegenerative and Neuroinflammatory Diseases

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Mitochondria are key organelles in the cell, hosting essential functions, from biosynthetic and metabolic pathways, to oxidative phosphorylation and ATP production, from calcium buffering to red-ox homeostasis and apoptotic signalling pathways. Mitochondria are also dynamic organelles, continuously fusing and dividing, and their localization, size and trafficking are finely regulated. Moreover, in recent decades, alterations in mitochondrial function and dynamics have been implicated in an increasing number of diseases. In this review, we focus on the relationship clarified hitherto between mitochondrial dynamics and cancer, neurodegenerative and neuroinflammatory diseases.

## 1. Introduction

In eukaryotic cells, the role of mitochondria is pivotal both in providing essential molecules and signals for life and in amplifying signals of death. In regard to the cell life, mitochondria produce most of the ATP necessary to the cell through oxidative phosphorylation, and they are involved, among the others, in TCA cycle, fatty acid metabolism, hemesynthesis, and gluconeogenesis. As regards the cell death, mitochondria are involved in  $\text{Ca}^{2+}$  and red-ox homeostasis, which are dysregulated during cell death, and they release proapoptotic proteins, such as cytochrome *c*, SMAC/DIABLO, AIF, Endo G, and Omi/HTRA2, after mitochondrial membrane permeabilization and cristae remodeling [1–3].

Moreover, mitochondria are highly dynamic organelles that can fuse and divide, forming an interconnected network or fragmented units inside the cell, according to different stimuli impinging on the fusion/fission machinery, represented by the mitochondria shaping proteins: MFN1, MFN2, OPA1, regulators of fusion, and DRP1, FIS1, MFF, and MIEF1, which modulate fission [4] (see Figure 1(a)).

*1.1. Mitochondrial Fusion.* MFN1 and MFN2, two dynamin-related GTPases, are the main regulators of mitochondrial fusion at the level of outer mitochondrial membrane (OMM). They can interact, forming homo- and heterodimers; after conformational changes led by the hydrolysis of GTP, they force the OMM to fuse [5]. Interestingly, MFN2 is also responsible for ER/mitochondria tethering with an important implication in  $\text{Ca}^{2+}$  homeostasis and signalling [6]. OPA1, another dynamin-related GTPase, is located in the inner mitochondrial membrane (IMM) where, together with MFN1, it plays a role in controlling fusion at this level [7]. OPA1 has also a role in controlling cell-death; in fact, heterocomplexes between proteolytic processed or unprocessed forms of OPA1 regulate the width of cristae junctions and the subsequent release of cytochrome *c*, which then interacts with APAF1 and caspase 9 forming the apoptosome, whose activation results in the amplification of cell death signals [8, 9]. Other proteins have been linked to mitochondrial fusion, such as LETM1 [10], the Phospholipase D (PLD) [11], and Prohibitins (Phb) [12], the latter necessary for OPA1 processing.

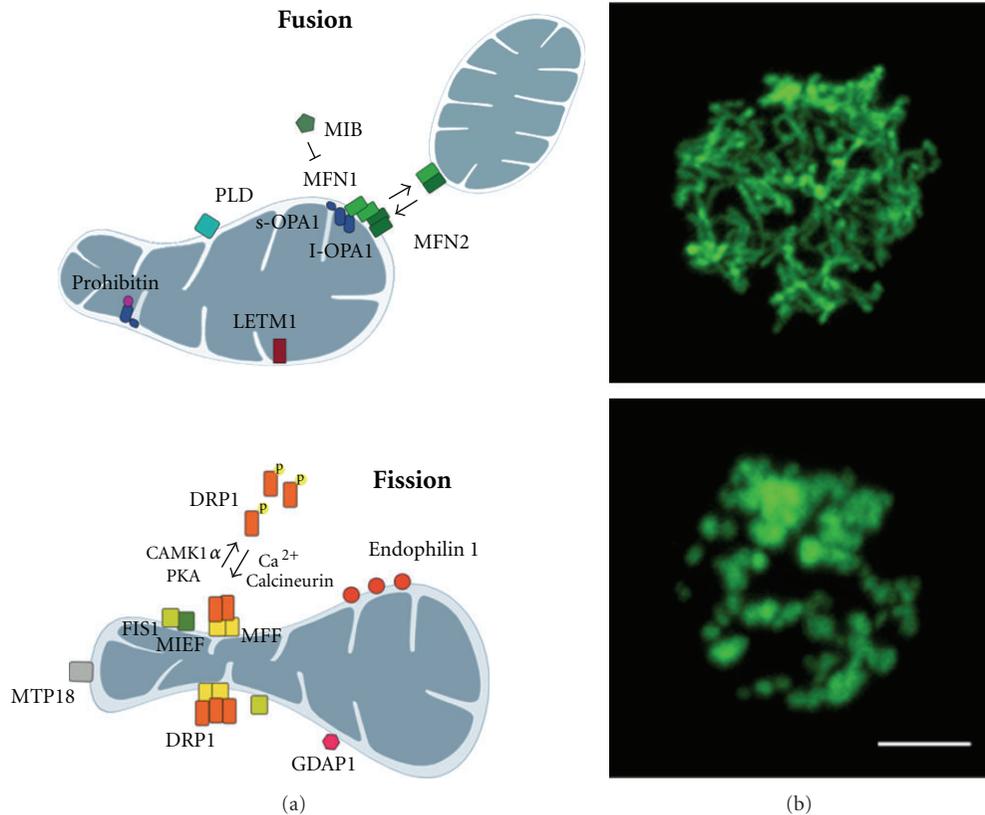


FIGURE 1: The dynamic nature of mitochondrial shape. (a) Main proteins involved in mitochondrial shape changes are depicted. In fused unopposed condition, DRP1 is phosphorylated and sequestered in the cytoplasm. Once dephosphorylated, it is recruited to the OMM where it oligomerizes and interacts with FIS1, MFF, or MIEF inducing constriction of membranes and, eventually, fission of mitochondria. MFNs homo- and heterooligomerization on the OMM and oligomerization between long and short isoform of Opa1 on the IMM control fusion of mitochondrial membranes. Additional proteins affecting mitochondrial shape are also shown. (b) Mitochondrial morphology in Jurkat cells overexpressing yellow fluorescent protein targeted to mitochondria. The upper panel shows a network of elongated and interconnected mitochondria. In the lower panel, mitochondria appear fragmented (Scale bar: 5  $\mu\text{m}$ ).

In summary, the pleiotropic mitofusins (MFN1 and MFN2) and OPA1 are the main regulators of the mitochondria fusion machinery. Although steps forward have been made, some points of this mechanism still remain to be clarified; in particular, how the IMM fuses and how its fusion is coordinated with events of the OMM fusion.

**1.2. Mitochondrial Fission.** The fission machinery is based on DRP1, FIS1, MFF, and MIEF1. DRP1 is a large GTPase protein [13], which in conditions of unopposed fusion is cytosolic and after dephosphorylation by calcineurin is recruited on mitochondria [14], where it oligomerizes and interacts with its putative adaptors on the OMM (FIS1, MFF, and MIEF1), forming ring-shaped structures and inducing mitochondrial constriction and fission [15]. Endophilin B1 [16], MTP18 [17], MIB [18], and GDAP1 [19] have been described, moreover, among the fission components.

The scenario of mitochondrial fission is becoming more complex, taking into account the different roles proposed for MFF, MIEF, and FIS1. MFF has recently been shown to be an adaptor of DRP1 on the OMM [20], whereas the binding of MIEF to DRP1 on the OMM inhibits the GTPase function of DRP1 and its profission activity [21]. On the other hand,

FIS1, that initially was considered the only DRP1 receptor at the OMM, has now been proposed to exert its profission role by interacting and sequestering MIEF, thus allowing DRP1 to mediate the constriction and fission of the membrane [21].

It has also been suggested that mitochondrial fission events predominantly occur at the contact sites between ER and mitochondria, specifically where some ER tubules cross over and wrap around mitochondria. Interestingly, DRP1 and MFF localize at these contact sites [22]. New studies that will address the biochemical mechanism, by which the ER participates in mitochondrial division, placing this observation in the overall picture of mitochondrial division, will be of great interest in the field.

In summary, the fission machinery depends on the activation and translocation of DRP1 to the OMM, where it interacts with some other components, not clearly defined, that take part to the modulation of mitochondrial fragmentation.

**1.3. More than Just Morphology: The Intimate Connection with Physiological Functions.** Changes in mitochondrial morphology have been related to alterations in mitochondrial function, transport, location, and quality control. Mitochondrial

TABLE 1: Mitochondrial dynamics and cancer.

Pathology	Proteins involved (expression level)	Mitochondrial phenotype	Mechanisms of pathophysiology involving mitochondria
Different types of tumors	MFN1, MFN2, OPA1 ↓↓ DRP1, FIS1 ↑↑	Fragmentation.	Inhibition of TCA cycle and oxidative phosphorylation, mitochondrial membrane permeabilization; fission accompanied by ROS production, polarization and chemotaxis of lymphocytes and tumoral cells [42–45].

damage, for example, induces fission, which, in turn, allows mitochondria to be engulfed by the autophagosomes and then degraded [23].

Autophagy represents a cellular self-degradation process involved in the degradation of bulk cytoplasmic components, proteins, or entire organelles in basal or nutrient depleted conditions. This process is also described as macroautophagy and is different from the selective autophagy. The latter is responsible for selective degradation of damaged and dysfunctional organelles, and, thus, it represents the quality control system for mitochondria [23]. In case of damage, mitochondria undergo fragmentation, and PARKIN is recruited to the organelles in a PINK1-dependent manner, allowing their engulfment into the autophagosome and their selective degradation (mitophagy) [24, 25]. On the other hand, when macroautophagy is induced, mitochondria elongate, being so spared by autophagosomes to ensure the major energy supply required by the cell in starving condition [26]. Besides their role in mitochondrial quality control, PINK1 and PARKIN have also a role in regulating mitochondrial dynamics. Moreover, BNIP3, a BH3-only member of the Bcl-2 family, enlarges the number of proteins crosstalking between autophagy and mitochondrial dynamics [27]. Its misregulation has implications in the development of muscular atrophy [28]. The existence of a crosstalk between autophagy and the mitochondrial dynamics machinery, as well as with the apoptotic process, opens new questions and is in need of further investigations.

To reinforce the idea of an intimate connection between mitochondrial dynamics and function, recently published data have revealed that *in vivo* genetic ablation of fusion (*Opa1* knock-out mice [29, 30], *Mfn1/Mfn2* double knock-out mice [5]), or fission (*Drp1* knock-out mice [31]) proteins results in early embryonic lethality. Other data reveal mutations or abnormal regulation of mitochondria shaping proteins in many pathological conditions, as we will see below.

## 2. Cancer

According to the classification of the hallmarks of cancer by Hanahan and Weinberg [32], a cell needs a multistep process to become tumoral and, later on, to develop metastasis. Mitochondria are crucially positioned for establishing resistance to cell death and sustaining proliferative signalling. Their role is essential for the metabolic shift to glycolysis (the so-called Warburg effect), common in tumoral cells. Increasing evidence shows the involvement of mitochondrial dynamics in cancer development (see Table 1).

**2.1. Escaping Cell Death and Regulating Mitochondrial Morphology: A Role for the Bcl-2 Family Proteins.** Escaping death signals is one of the first characteristics of a tumoral cell. Bcl-2 family proteins play an important role in balancing life and death signals [33] converging on mitochondria and, at the same time, in regulating changes in mitochondrial morphology. Generally, prosurvival signals are associated with elongated mitochondria, while cell death is usually accompanied by mitochondrial fragmentation. BCL-2 is a tumoral marker overexpressed in many lymphomas contributing to resistance to cell death [34–36]. CED-9, the homolog of BCL-2 in *C. elegans*, is able to interact with MFN2-inducing mitochondrial fusion [37]. BCL-X<sub>L</sub> promotes instead fission stimulating the DRP1 GTPase activity [38]. The proapoptotic BAK and BAX stabilize DRP1 on mitochondria promoting fission [39], indeed, *Bak*<sup>-/-</sup>*Bax*<sup>-/-</sup> cells have an elongated mitochondrial network [40]. Interestingly, BAX colocalizes with DRP1 and MFN2 at sites of fission so promoting mitochondrial membrane permeabilization [39]. Finally, consistent with their proapoptotic role, NOXA and PUMA trigger DRP1-dependent mitochondrial fragmentation [41].

**2.2. Metabolic State and Mitochondrial Shape Changes.** Another feature of tumoral cells is the already mentioned shift from the production of ATP by oxidative phosphorylation to a glycolytic phenotype despite the presence of oxygen [32]. It is not yet clear if this metabolic modification is an adaptation to a hypoxic microenvironment or the result of defects in OXPHOS respiration. Nevertheless, the existence of a double relationship between mitochondrial morphology and metabolic state is, however, increasingly evident. In OXPHOS cells, mitochondria appear elongated (State III); in glycolytic cells, they have a more fragmented phenotype (state IV) [46, 47]. The molecular mechanism underlying this phenomenon involves fusion proteins: reduced levels of MFN2, MFN1, or OPA1 results in the inhibition of TCA cycle, the decrease of oxidative phosphorylation, and the increase of glycolysis and lactic fermentation [42, 43]. Moreover, in a tumoral mass, the cellular response to hypoxia triggers mitochondrial elongation, dependent on HIF1; this, in turn, increases the resistance to apoptotic stimuli [48]. On the other hand, an efficient oxidative phosphorylation, and the consequent optimal mitochondrial membrane potential, ( $\Delta\psi$ ), is necessary for mitochondrial fusion [49, 50].

**2.3. Cell-Cycle Regulation: When Cell Division Means Mitochondrial Fragmentation.** It has been shown that mitochondria undergo fragmentation during the S and M phase of

the cell cycle to allow a limitation of the mutation rate during DNA replication, through a temporary decrease of oxidative phosphorylation (which is the main source of ROS). Such fragmentation is also necessary for an equal segregation of mitochondria between the daughter cells [51, 52]. This process is mainly regulated by CDK1/Cyclin B complex, which phosphorylates DRP1 at the beginning of the S phase, resulting in DRP1 recruitment on mitochondria and subsequent mitochondrial fragmentation [53]. Thus, in normal conditions, the fragmentation of mitochondria, required during cell division, is a DRP1- and CDK1-dependent process, but in many tumors the cell cycle is dysregulated, and CDK1 activity becomes altered. It remains to be investigated whether this has an effect also on the morphology of mitochondria.

**2.4. ROS Production and Mitochondrial Fragmentation.** To continue this overview, ROS can be considered as both initiator factors of the tumor (inducing genome and mtDNA mutations) and enhancing factors giving a higher rate of proliferation to the cells [54, 55]. ROS production is mainly attributed to mitochondria, at the level of respiratory chain, and, in case of mitochondrial fragmentation, it is enhanced. Significantly, ionizing radiation is accompanied by ROS production and mitochondrial fragmentation in a DRP1-dependent way, so contributing to genome instability and carcinogenesis [44].

Interestingly, clinical studies of lung adenocarcinoma reveal a role for DRP1, independent of the mitochondrial morphology. In this tumor, DRP1 is overexpressed, but is sequestered in the nucleus by hHR23A, so avoiding its localization on mitochondria and conferring resistance to cisplatin [56]. A central role for mitochondrial dynamics also emerges in other studies. IL-6 dependent cancer cachexia is characterized by MFNs mRNA reduction and FIS1 mRNA upregulation [57]. FIS1 is also upregulated in some subtypes of human malignant melanoma [58].

In a more general way, cancer cells share characteristics with stem cells, in particular regarding mitochondrial morphology, localization, function, and mtDNA content [59]. Of note, in embryonic stem cells, there is a growth factor *erv-1*-like (GERF)-dependent DRP1 downregulation, which leads to mitochondrial elongation and an enhanced cell viability [60].

Finally, in lymphocytes mitochondria fission and relocation to the uropod are necessary for the polarization and chemotaxis of these cells [45], so unravelling a possible role for mitochondrial morphology also in metastasis formation, where the acquisition of migratory capability represents the main feature of a metastatic cell phenotype.

### 3. Neurodegenerative Diseases

**3.1. Beyond the Morphology: Physiological Mechanisms Affected by Altered Mitochondrial Dynamics in Neurons.** Before starting the examination of different pathologies directly related to mitochondrial dynamics failure or imbalance (see Table 2), we would like to give an overview of some of

the possible mechanisms whereby mitochondrial dynamics alterations can lead to neurodegeneration: aberrant mitochondrial trafficking, altered interorganellar communication and impaired mitochondrial quality control [61].

**3.1.1. Mitochondrial Trafficking.** Neurons, especially motor neurons, are characterized by long axons up to more than one meter at the end of which synapses exert their role in cellular-cellular communication. The resulting importance of mitochondrial anterograde transports to the synapses (to ensure ATP production necessary for neurotransmitters vesicles to be discarded [62]) and of retrograde movement to the soma of the cell are both clearly evident. Mitochondria rely on dynein/dynactin motor for the anterograde movement, on kinesin motor for the retrograde one, and on MIRO and MILTON as an additional mitochondrial linker and regulatory proteins [63]. Thus, a defect in the cellular motors, or in the mitochondria compartment to be loaded as cargo, could result in a mitochondrial deficit at synapses and in neurodegeneration. There is no direct correlation described so far in patients although, in some sporadic cases of Alzheimer disease (AD), trafficking alteration has been observed due to mutation in *Kinesin1* [64]. That said, increasing data are emerging in experimental models. Anterograde and retrograde trafficking is altered in Amyotrophic lateral sclerosis (ALS) mouse models in which SOD1 [65, 66], guanin-nucleotide exchange factor (GEF) and TAR DNA-binding protein 43 (TDP-43) are mutated [67, 68]. Noteworthy, a role for mitochondrial trafficking impairment has been demonstrated in pathologies not only affecting long axon neurons but also short cortex and hippocampal ones (this is the case of Alzheimer disease—AD—models) [64, 69, 70]. Similar observations come from works in a Huntington's disease (HD) mouse model, in which mutated *Htt* (the gene of HUNTINGTIN protein) is able to block mitochondrial movement [71] and causes a redistribution of kinesin and dynein in primary cortical neurons [72]; in Parkinson disease (PD) cellular and mouse models where PINK1 has been shown to interact with MIRO and MILTON [73], as well as with  $\alpha$ -SYNUCLEIN, LRRK2, and PARKIN, to disrupt the microtubule network in the cell [74–76].

**3.1.2. Mitochondria-Associated Membranes and  $Ca^{2+}$  Homeostasis.** In recent decades, a functional role for mitochondria-ER interactions and  $Ca^{2+}$ -signalling implications has emerged [6]. The sites where these two organelles interact are defined as mitochondria-associated membranes (or MAMs), and MFN2 activity is pivotal in ER-mitochondria tethering and MAM formation. MAMs have a role in regulating calcium crosstalk between ER and mitochondria, so avoiding  $Ca^{2+}$  overload in mitochondria in physiological condition and revealing, thus, an unexpected connection with mitochondrial trafficking. In fact, MIRO is a calcium-binding protein; it has been proposed that only in calcium unbound state (low local  $Ca^{2+}$  concentration) is it able to interact with MILTON, so allowing movement of mitochondria [101].

TABLE 2: Mitochondrial dynamics and neurodegenerative diseases.

Pathology	Proteins involved (expression level and/or mutation)	Mitochondrial phenotype	Mechanisms of pathophysiology involving mitochondria
Alzheimer disease	MFN1, MFN2, OPA1 ↓↓ DRP1, FIS1 ↑↑ KINESIN mutation	Fragmentation, disruption of cristae structure, reduction in number of mitochondria in dendrites, impaired mitochondrial trafficking, defects in KGDH complex, PDH complex and COX.	$\beta$ amyloid accumulation and interaction with DRP1, enhanced CDK1 activity, altered interaction between mitochondria and Kinesin motor complex in cerebral cortex [77–79].
Huntington's disease	MFN1, MFN2, OPA1 ↓↓ DRP1, FIS1 ↑↑ HTT mutation	Fragmentation; impaired mitochondrial trafficking, defects in SDH (complex II) and Aconitase.	HTT interaction with DRP1, increased calcineurin and DRP1 activity, redistribution of kinesin and dynein motor complexes in striatal neurons [80–82].
Parkinson disease	Parkin mutation or ↓↓ Pink1 mutation or ↓↓ DJ-1 mutation DRP1 ↓↓ MFN2 $\alpha$ -synuclein mutation LRRK2 mutation	Fragmentation, impaired mitochondrial trafficking.	Altered interaction between mitochondria and motor complexes, impaired mitophagy of damaged mitochondria in <i>substantia nigra</i> [74, 75, 83–86].
Amiotrophic lateral sclerosis	SOD mutation GEFmutation TDP-43 mutation	Fragmentation, disruption of cristae structure with expansion of IMS, impaired mitochondrial trafficking, complex I dysfunctions.	Toxicity associated to the formation of aggregates of mutant SOD, in subsarcolemmal region of muscles and anterior horn neurons of lumbar spinal cord [87–93].
Autosomal dominant optic atrophy	OPA1 mutation	Fragmentation, complex I dysfunctions.	Major sensitivity to death stimuli in retinal ganglion cells and optic nerve [94–98].
Charcot Marie Tooth Type 2	MFN1 mutation GDAP1mutation	Fragmentation (MFN1 mut) or elongation (GDAP1 mut).	MFN1: probably alteration in ER-mitochondria tethering and Calcium signalling [99]; GDAP1: altered localization of GDAP1 [100].

Thus, an alteration in  $\text{Ca}^{2+}$  homeostasis, due to abnormal ER-Mito crosstalk, results in impaired mitochondrial movement and consequently in neurodegeneration [101]. Moreover, Amyloid  $\beta$ , a constituent of extracellular neurite plaques in AD, is abundant in MAMs, contributing to interorganellar dysfunctions [102]. Altered MAM organization has been proposed also for spinocerebellar ataxias (SCA), due to mutation in the regulatory subunit of Protein phosphatase 2A, PPP2R2B [103], and for PD, due to mutation in the subunit 2b of phospholipase A2 (iPLAS2b). The latter is important in ER-mitochondria crosstalk during apoptosis mediated by ER stress [104].

**3.1.3. Mitochondrial Quality Control.** The role of mitochondrial quality control is becoming increasingly prominent in the explanation of neurodegenerative diseases such as Parkinson disease (PD) and others. A general mechanism of mitochondrial quality control relies on the PINK1/PARKIN

pathway, deeply studied *in vitro*. Briefly, loss of  $\Delta\psi$  induces stabilization of PINK1 on the OMM and allows PARKIN recruitment on mitochondria. This, in turn, leads to ubiquitination of mitochondrial substrates and their interaction with p62 and LC3 so as to induce the engulfment of mitochondria inside the autophagosome [24, 25]. MFNs, for example, are ubiquitinated in a PARKIN-dependent manner [105] and then degraded by proteasome [106]. Others showed that DRP1 stability is also regulated by PARKIN [107].

**3.2. Focus on the Pathologies.** Coming back to the pathologies, in this paragraph, we will focus on the links between some of them and the mitochondrial dynamics.

**3.2.1. Alzheimer Disease.** The main clinical feature of Alzheimer disease (AD) is the accumulation of extracellular

deposits of amyloid  $\beta$  ( $A\beta$ ) plaques and tau-containing intracellular neurofibrillary tangles in the brain, these leading to progressive neuronal death. From a morphological point of view, neurons expressing amyloid protein precursor (APP), or Amyloid  $\beta$ , show abnormal levels of mitochondrial shaping proteins with downregulation of MFNs and OPA1 and upregulation of DRP1 and FIS1 [69, 70, 108, 109]. Amyloid  $\beta$  interacts with DRP1 [77], promoting mitochondrial fission in a DRP1 S-nitrosilation-dependent manner [110, 111]. Tissues from patients affected by AD show mitochondria with disrupted cristae structure [112] and reduction of the number of mitochondria in dendrites [69]. Interestingly, although cell-cycle-coupled events are rare in postmitotic cells, the activity of CDK1 and CDK5 is enhanced in AD. CDK5 phosphorylates tau [78], while a high level of phosphorylated DRP1 at Serine 616 appears to be dependent on both CDK1 and protein kinase C  $\delta$  (PKC  $\delta$ ) [79], as it has been shown in rat primary neurons.

**3.2.2. Huntington's Disease.** A mitochondrial connection is emerging also in Huntington's disease (HD); mitochondrial succinate dehydrogenase (SDH, complex II), aconitase defects [80], and mtDNA damage [113] have been reported in *in vivo* models of HD. In addition, 3-nitropropionic acid, an irreversible inhibitor of complex II, has been shown to induce mitochondrial fragmentation and HD-like symptoms in rats and mice [81]. Of note is that primary striatal neurons from HD mouse models reveal mitochondrial fragmentation [114] with an alteration of mitochondrial shaping proteins in the brain (DRP1 and FIS1 upregulation, OPA1 and MFN1 downregulation) [115]. Mutant HUNTINGTIN is able to bind DRP1, increasing its GTPase activity and inducing mitochondria fragmentation both in mice and in human brains [82]. This phenotype is rescued by MFNs or DRP1-K38A-dominant negative overexpression and by the use of two DRP1 inhibitors, mdivi1 or miR-499 [116–120].

**3.2.3. Parkinson Disease.** Independent studies identified  $\alpha$ -synuclein, *Pink1*, *Parkin*, *DJ-1*, and *Leucine-rich repeat kinase 2* (*LRRK2*) as commonly mutated genes in Parkinsonism.  $\alpha$ -SYNUCLEIN and LRRK2 have been proposed as playing a role in microtubule organization and, thus, in mitochondrial trafficking [74, 75]. PINK1 and PARKIN are key proteins in mitochondrial quality control [24, 25], as we discussed above. Interestingly, opposite to what has been observed in drosophila [121, 122], downregulation of PINK1 or PARKIN by siRNA in neuroblastoma cells leads to mitochondrial fragmentation [83]. Also in this case, this fragmentation is rescued by genetically forcing the mitochondria morphology equilibrium towards fusion, or by treatment with the calcineurin inhibitor FK506 [84, 85]. The recruitment of PARKIN to mitochondria has been nicely investigated in different models; generally, it has been shown that upon mitochondrial membrane depolarization PARKIN is recruited to mitochondria both in primary and cultured cell models [25], but this mechanism is also inhibited in primary neurons [86]. The apparent discrepancy in the results among different experimental models could be explained, at least in

part, by the observation that those cell models rely on different bioenergetic systems. It has been shown, in fact, that, in primary rat neurons, which largely depend on mitochondrial respiration to produce ATP, or in nonneuronal cells forced to mitochondrial respiration, PARKIN fails to translocate to mitochondria after membrane depolarization in contrast to what is observed in cells relying on glycolytic production of ATP [86]. This suggests that additional regulatory and/or protective mechanisms against mitochondrial damage have to be investigated in neurons. We should also consider that, in all these studies, loss of  $\Delta\psi$  is obtained by treating cells with high concentrations (or for long terms) of the protonophores CCCP or FCCP. A common challenge in the next future will be the identification and the use of more physiological stimuli to induce mitochondrial damage, and mitophagy, to better mimic the *in vivo* mechanism of pathophysiology of neurodegenerative diseases. By this way, it will be possible to clarify some of the discrepancies remaining in the field such as, for example, the differences observed so far for the PINK1/PARKIN functional interaction in various cell lines; or considering the loss of  $\Delta\psi$  (artificially induced and forced in all the *in vitro* experiments performed until now) as the only event triggering mitophagy, another point highly debated and controversial to date.

A mouse model of *DJ-1* knockout presents mitochondrial fragmentation [123], increased ROS production, and reduced respiration rates accompanied by basal autophagy impairment [124]. Complex I dysfunctions are common in PD, and its inhibition, by rotenone or 6-hydroxydopamine (6-OHDA) treatment, results in a DRP1-dependent mitochondrial fragmentation in neurons [125, 126], so suggesting another link between bioenergetic dysfunctions and altered mitochondrial dynamics in neurodegenerative diseases.

**3.2.4. Amyotrophic Lateral Sclerosis.** In the 20% of patients affected by an autosomal dominant form of amyotrophic lateral sclerosis (ALS), a gain of function mutation in SOD1 has been detected [87]. Defects of complex IV activity and mtDNA rearrangement have been also reported in patients affected by ALS [127, 128]. Abnormal aggregation of mitochondria is common in the subsarcolemmal region of muscles and in the anterior horn neurons of the lumbar spinal cord [88, 89]. At ultrastructural levels, in the case of ALS, mitochondria show disorganized cristae with expansion of intermembrane space (IMS) [90, 91]. Of note, the overexpression of a mutated form of SOD1 in ALS (SOD1-G93A) induces fragmentation of mitochondria in NSC-34 motoneuronal-like cells [92]. Moreover, motor neurons from mice overexpressing SOD1-G93A show impaired mitochondrial fusion both in axons and in the cell body with impaired retrograde axonal transport and reduction of frequency and speed of the movement [93].

**3.2.5. Autosomal Dominant Optic Atrophy.** *Opa1* mutations are responsible for the autosomal dominant optic atrophy (ADOA) [94, 95]. Contrary effects regarding oxidative phosphorylation impairment related to this disease have been published. Phosphorus magnetic resonance spectroscopy

and luciferin/luciferase assays of muscle cells from ADOA patients showed a reduced ATP production mainly due to reduced complex I activity [129, 130]. On the other hand, in other studies, no energetic impairment was observed in lymphoblasts and muscle cells from ADOA patients [96, 97]. Whatever the case, consistent with the role of OPA1 in regulating mitochondrial dynamics, mitochondrial fragmentation is a common feature of ADOA with a severity score of the pathology directly proportional to the level of fragmentation observed [97, 98, 130]. Interestingly, fibroblasts from ADOA patients also reveal a major sensitivity to death stimuli [98], in line with the antiapoptotic role of OPA1 described above [8].

**3.2.6. Other Neuropathies and Neurological Disorders.** Among other neuropathies Leber hereditary optic neuropathy (LHON) has, as a primary cause, mutations in mtDNA [131]. Recently, a possible link with mitochondrial dynamics has been presented in a genome-wide linkage scan study, in which a mutation in *Parl* (the mitochondrial protease responsible for OPA1 cleavage, [132]) has been associated with LHON [133].

MFN2 is involved in the most common form of the autosomal dominant axonal Charcot-Marie-Tooth (CMT2A) disease [99]. Moreover, ganglioside-induced differentiation-associated protein 1 (GDAP1), whose mutations cause an autosomal recessive form of CMT, appears to be related to mitochondrial fission in mammalian cells [100].

In addition, in a mouse model of apoptosis-inducing factor (AIF) deficiency, MFN1 levels are decreased in the cerebellum and are accompanied by death of Purkinje cells [134]. This phenomenon is generally observed in many neurological diseases such as autism, HD, AD, multiple system atrophy, and epilepsy [135]. Consistently, also in *Mfn2* knock-out systems, death of Purkinje cells has been observed, confirming a role for MFNs in protecting against lack of mtDNA and dysfunction of mitochondria in the cerebellum [136].

In the last decade, a link between neurological and lymphatic aspects has emerged in schizophrenia [137]. Recently, a study in schizophrenia-derived lymphoblastoids revealed altered oxidative phosphorylation at level of complex I and clustering of mitochondria in a limited area of the cell, with a reduction in OPA1 expression levels [138].

To conclude, neurons rely on mitochondrial distribution, function, and dynamics to allow synapses and dendrites formation, energy supply, and quality control. The main properties of neurons constitute risk factors themselves if we consider the effects of mitochondrial dysfunctions. First, they are cells highly demanded in energy; second, they have long processes connecting the soma to synapses and dendritic spines; third, they are long-lived postmitotic cells. Although knowledge is increasing about mitochondrial role in neurodegenerative diseases, much remains to be elucidated, in particular, why different subpopulations of neurons are more vulnerable to mitochondrial fusion/fission imbalance and dysfunctions.

## 4. Neuroinflammatory and Autoimmune Diseases

Little is known about the link between mitochondrial dynamics and neuroinflammatory or autoimmune diseases. In this section we present correlations described to date with multiple sclerosis (MS) and type I diabetes, as examples for this category of diseases (see Table 3). We then introduce some general outcomes about mitochondrial dynamics and T-cell compartment with the potential for opening up new perspectives regarding cellular mechanisms and clinical therapies for many pathologies.

**4.1. Optic Neuritis and Multiple Sclerosis.** Optic neuritis (ON) is a neuropathy characterized by demyelination of the optic nerve; it can be present by itself or as part of MS. Multiple sclerosis is an autoimmune disorder characterized by chronic demyelination of the central nervous system (CNS). The pathogenesis of MS is thought to involve self-antigen-reactive T lymphocytes that have the capacity to invade the CNS and to promote tissue damage.

It is not rare to find mtDNA mutations and mitochondrial abnormalities in patients affected by ON [143]. For instance, in a case of *OPA1* mutation (S646L) ADOA has been shown to be associated with MS. This mutation leads to reduction of respiratory rates with lower ATP production [139], which is implicated in demyelination of axons in MS [140]. Interestingly, it has been shown that symptoms of autoimmune disorders, including MS, improve during pregnancy due, at least in part, to the expression of embryo-derived preimplantation factor (PIF). This protein is able to reduce neuroinflammation and to promote neural repair in the experimental autoimmune encephalomyelitis (EAE) model of MS, through a general decrease in proinflammatory cytokine and chemokine secretion, and a downregulation of proapoptotic factors and of activating and migrating proteins such as OPA1 [144].

**4.2. Type I Diabetes.** Type I diabetes is an autoimmune disorder caused by autoimmune elimination of insulin-producing  $\beta$  cells in the pancreas, clinically leading to increased glucose in blood and urine. Coronary endothelial cells from diabetic mice are characterized by fragmented mitochondria with a downregulation of OPA1 and an upregulation of DRP1 [141]. In addition, Prohibitin (Phb) has been shown to have a protective role in  $\beta$  cells [142]. Interestingly, *phb*-genetic ablation results in aberrant mitochondrial cristae structure and an increased apoptosis, dependent on increased proteolytic processing of OPA1 [15]. Moreover, it has been shown that the embryo-derived preimplantation factor (PIF) also prevents type I diabetes in mouse models of this disease [145].

**4.3. Mitochondrial Morphology and T-Cell Function.** The importance of mitochondrial localization and activity in T cell function is well established. T cells are activated at the so-called “immunological synapse” between a T cell and an antigen-presenting cell (APC) [146]. Mitochondria usually

TABLE 3: Mitochondrial dynamics and neuroinflammatory and autoimmune diseases.

Pathology	Proteins involved (expression level and/or mutation)	Mitochondrial phenotype	Mechanisms of pathophysiology involving mitochondria
Multiple sclerosis	OPA1 mutation	Fragmentation.	Reduction of respiratory rates with lower ATP production [139, 140].
Type 1 diabetes	OPA1 ↓↓ DRP1 ↑↑ PHBs mutation	Fragmentation, disruption of cristae structure.	Alteration in OPA1 processing in $\beta$ -cells in the pancreas and coronary endothelial cells from diabetic animals [141, 142].

fragment and relocalize at the immunological synapse in close proximity to the plasma membrane to buffer  $\text{Ca}^{2+}$  entrance and to avoid calcium-dependent T-cell inactivation [147]. Moreover, upon activation, T cells migrate to the site of inflammation towards a chemoattractant gradient. Our group reported that mitochondria allow lymphocyte migration by relocating and accumulating at the uropod (the posterior area of an activated T cell) where they can provide the necessary energy to class II myosin proteins, these being the major cellular motors. Interestingly, this reorganization needs mitochondrial fission while a forced fusion inhibits both mitochondrial relocalization and lymphocyte migration [45]. In recent years, the polarization of mitochondria towards cell-cell surface has also been shown to occur between natural killer (NK) and tumoral cells [148, 149].

Mitochondrial fission, driven by FIS1 and DRP1, also contributes to the immune system tolerance and the tumor-immune escape in a galectin-1 (GAL-1)-dependent manner. GAL-1 sensitizes resting and activated T cells to FAS-mediated cell death program, which is characterized by mitochondrial dysfunctions, membrane potential alteration, mitochondrial fission, and cytochrome *c* release [150].

It still remains to be elucidated whether or not mitochondrial dynamics play a crucial role in other important physiological processes in T cells.

Finally, CD47 can trigger cell death in a B lymphocyte leukemia. Also this apoptotic pathway is characterized by DRP1 translocation to mitochondria, which depends on chymotrypsin-like proteases,  $\Delta\psi$  loss and ROS production [151]. This last observation is an indication of a possible role of mitochondrial dynamics in general in the whole immune system, more than only in the T-cell physiology and pathophysiology.

## 5. Conclusion

The observations here presented suggest a prominent role for mitochondrial dynamics in a plethora of pathways from cell proliferation to resistance to apoptosis, from cell-energetic requirements to quality control mechanisms, from homeostasis to activation and movement of immune system cells. However our observations also suggest the potential for many new findings to come. The aim of future studies would, therefore, be to extend our knowledge of basic mechanisms underlying pathologies, and their relationship with mitochondrial morphology alterations. This, in turn, could make it possible to draw up novel strategies and

treatments to improve the prognosis of an increasing number of patients.

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## Review Article

# Pathological Significance of Mitochondrial Glycation

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Glycation, the nonenzymatic glycosylation of biomolecules, is commonly observed in diabetes and ageing. Reactive dicarbonyl species such as methylglyoxal and glyoxal are thought to be major physiological precursors of glycation. Because these dicarbonyls tend to be formed intracellularly, the levels of advanced glycation end products on cellular proteins are higher than on extracellular ones. The formation of glycation adducts within cells can have severe functional consequences such as inhibition of protein activity and promotion of DNA mutations. Although several lines of evidence suggest that there are specific mitochondrial targets of glycation, and mitochondrial dysfunction itself has been implicated in disease and ageing, it is unclear if glycation of biomolecules specifically within mitochondria induces dysfunction and contributes to disease pathology. We discuss here the possibility that mitochondrial glycation contributes to disease, focussing on diabetes, ageing, cancer, and neurodegeneration, and highlight the current limitations in our understanding of the pathological significance of mitochondrial glycation.

## 1. Introduction

Glycation is a common feature of diabetic complications [1, 2] and ageing pathologies [3, 4]. This nonenzymatic glycosylation process stems from multiple reactions between reducing sugars or sugar derivatives and amino groups on proteins, lipids and nucleotides. Glycation involves multiple oxidative and nonoxidative reactions, collectively termed the Maillard reaction, eventually generating advanced glycation end products (AGEs) [5]. In clinical and experimental models of diabetes and ageing, the levels of intra- and extracellular AGEs have been found to increase relative to healthy or young controls [3, 6–10]. Such correlative data suggests that glycation can potentially contribute to disease progression and ageing pathology. However, whether there is a causal relationship between glycation and pathology is uncertain and the mechanistic details are unclear.

That the formation of AGEs can have significant functional consequences further supports this hypothesis. Glycation affects all major classes of biomolecules (Figure 1), with damage levels estimated at 0.1–1% of lysine and arginine residues on proteins, 1 in  $10^7$  nucleotides on DNA and 0.1% of basic phospholipids [11]. The consequences of such glycation damage can be severe. The formation and

accumulation of protein AGEs, for instance, can result in biochemical dysfunction. For example, some AGEs like MOLD (methylglyoxal-derived lysine dimers) and pentosidine form protein crosslinks, altering protein structure, generally causing proteins to become more resistant to proteolysis [12]. Protein structure may also be modified as a result of charge neutralization when arginine and lysine residues are glycated [13]. Consequently, structural integrity of proteins becomes compromised. For instance, glycated collagen is stiff and nonelastic relative to the non-glycated protein [14, 15]. The change in charge distribution could also promote protein aggregation, for instance, of lens crystallins, leading to cataract formation in diabetes and in old age [16, 17]. Changes in protein conformation could also influence its function, as would glycation of amino acid residues at sites for substrate binding and allosteric regulation on enzymes. It has been shown, for instance, that methylglyoxal-induced glycation of lys 126 and arg 463 in glutamate dehydrogenase isolated from bovine liver affects its ability to bind its substrate and allosteric activator (adenosine diphosphate (ADP)), respectively, resulting in a decrease in glutamate dehydrogenase activity [18]. Similarly, glycation of extracellular matrix (ECM) proteins affects cell-ECM interactions. The reaction of methylglyoxal with arginine



residues on the RGD and GFOGER motifs in the integrin-binding sites of collagen, for instance, resulted in endothelial cell detachment by interfering with cell-ECM interactions [7].

Membrane interactions [19] may also be affected by the formation of lipid glycation adducts which increase membrane fluidity [20]. Lipids possessing a free amino group such as phosphatidylethanolamine are susceptible to glycation, whereas those without, for example phosphatidylcholine, are not glycated [21]. It is thought that lipid glycation may promote lipid peroxidation, resulting in oxidative damage [22–24]. For example, *in vitro* incubation of human low-density lipoprotein (LDL) with 200 mM glucose for up to 12 days increased levels of both glycation and lipid oxidation products, which appears to support the hypothesis that lipid glycation causes, or at least enhances, lipid peroxidation [22]. However, since lipid glycation and peroxidation can both occur in parallel, it is difficult to distinguish one from the other as being the primary initiating event versus a secondary downstream event, particularly *in vivo* [22].

Likewise, glycation of DNA can have multiple effects such as strand breaks, unwinding of the double helix, mutations and formation of DNA-protein and nucleotide-nucleotide cross-links [25–30]. The extent of modification appears to be dependent on the glycating agent used. For example, methylglyoxal induced 10-fold more DNA-protein crosslinks than did glyoxal when incubated *in vitro* with DNA and DNA polymerase I derived from *Escherichia coli* [31]. Such DNA-DNA polymerase crosslinks could stall replication and subsequently promote frameshift mutations [31]. The steric hindrance imposed by DNA glycation adducts may also impair transcription by preventing transcription factors from binding [32]. That is, DNA glycation may not only influence genome integrity but could also alter gene expression.

Besides direct glycation damage to biomolecules, AGEs, especially extracellular AGEs, could also contribute to disease pathology by binding to cell surface receptors such as the receptor for AGEs (RAGE), thereby activating the proinflammatory NF- $\kappa$ B pathway and downstream signalling molecules such as p21, MAP kinases, and JNK [33, 34].

The formation of AGEs arises from reactions between reducing sugars (e.g., glucose) or sugar derivatives (e.g.  $\alpha,\beta$ -dicarbonyls) and amino groups on biological macromolecules (Figure 2). Glycation reactions can be broadly classified as early and late stages of glycation. During early glycation, the carbonyl group of acyclic glucose reacts with amino groups, then undergoes dehydration to form a Schiff base, which subsequently undergoes further rearrangement to form a more stable Amadori product, fructosamine [35] (Figure 2). Alternatively, the Schiff base may undergo spontaneous degradation to form reactive  $\alpha,\beta$ -dicarbonyl species such as methylglyoxal and glyoxal [36] (Figure 3(a)). These dicarbonyls may also be formed from glucose breakdown via the Wolff pathway [37] (Figure 3(a)), triose phosphate fragmentation [38] (Figure 3(b)), acetone [39] and threonine metabolism [40] (Figures 3(c) and 3(d)), lipid peroxidation [41], and fructose-3-phosphate decomposition [42]. Downstream of Amadori product formation, further oxidative and nonoxidative modifications may occur,

generating AGEs [5] (Figure 2). Where oxidative processes are involved, the term glycooxidation has also been used [43]. AGEs can also be formed by the direct modification of amino groups by  $\alpha,\beta$ -dicarbonyls (Figure 2).

Although glycation is traditionally thought of as a reaction between sugars and amino groups, other functional groups on macromolecules, for example, protein thiols (-SH), may also react in analogous reactions [44]. Similar to glycation of lysine and arginine residues on proteins, glycation of thiol groups can modulate enzyme activity. For example, glyceraldehyde-3-phosphate dehydrogenase can be inhibited by methylglyoxal-mediated loss of thiol groups at its active site [44]. Glycation of creatine kinase by glyoxal also led to enzyme inactivation [45]. Although thiol modification by  $\alpha,\beta$ -dicarbonyls could be damaging to protein function, low molecular weight thiols such as N-acetylcysteine and GSH have been suggested as therapeutic agents for the removal of  $\alpha,\beta$ -dicarbonyls before they can react with proteins [45, 46].

Despite the common association of glycation with glucose, this reducing sugar is not itself highly reactive. Rather, it is reactive  $\alpha,\beta$ -dicarbonyl species such as methylglyoxal and glyoxal, which are up to 50,000-fold more reactive than glucose, that are thought to be the major physiological precursors of glycation damage [47]. Because these dicarbonyls are mostly produced intracellularly through processes such as triose phosphate fragmentation [38] and lipid peroxidation [41], there is a greater likelihood for cellular proteins to be glycated relative to plasma proteins. Therefore, levels of AGEs tend to be higher in cellular proteins than in plasma proteins. For instance, levels of the methylglyoxal/arginine-derived hydroimidazolone, MG-H1 [ $N^{\delta}$ -(5-hydro-5-methyl-4-imidazolone-2-yl)ornithine], were found to be 1.22 mmol/mol arginine in human blood cells and 0.92 mmol/mol arginine in plasma proteins [48]. Similarly, levels of another AGE, CML ( $N^{\epsilon}$ -carboxymethyl-lysine), was at least three-fold higher in cellular proteins (0.068–0.233 mmol/mol lysine) than in plasma proteins (0.021 mmol/mol lysine) [48].

To minimize glycation damage by reactive  $\alpha,\beta$ -dicarbonyls, there exist various enzyme systems that catalytically remove these species (Figure 4). One of the most important of these defences is the glyoxalase enzyme system which functions primarily to remove methylglyoxal and, to a lesser extent, other compounds such as glyoxal [49, 50]. Using glutathione (GSH) as a cofactor, glyoxalase I catalyzes the formation of S-2-hydroxyacylglutathione from methylglyoxal or glyoxal. Glyoxalase II then converts this intermediate compound into an  $\alpha$ -hydroxyacid (either D-lactate from methylglyoxal or glycolate from glyoxal), regenerating GSH in the process. Other enzymes involved in the detoxification of  $\alpha,\beta$ -dicarbonyls include aldehyde dehydrogenases which oxidize methylglyoxal and glyoxal to pyruvate and glyoxylate, respectively [51], and aldo-keto reductases/aldose reductases which reduce them to form alcohols (e.g., methylglyoxal to acetol and lactaldehyde, glyoxal to glycoaldehyde and ethylene glycol) [52].

Under normal physiological conditions, these anti-glycation defences are sufficient to prevent significant glycation

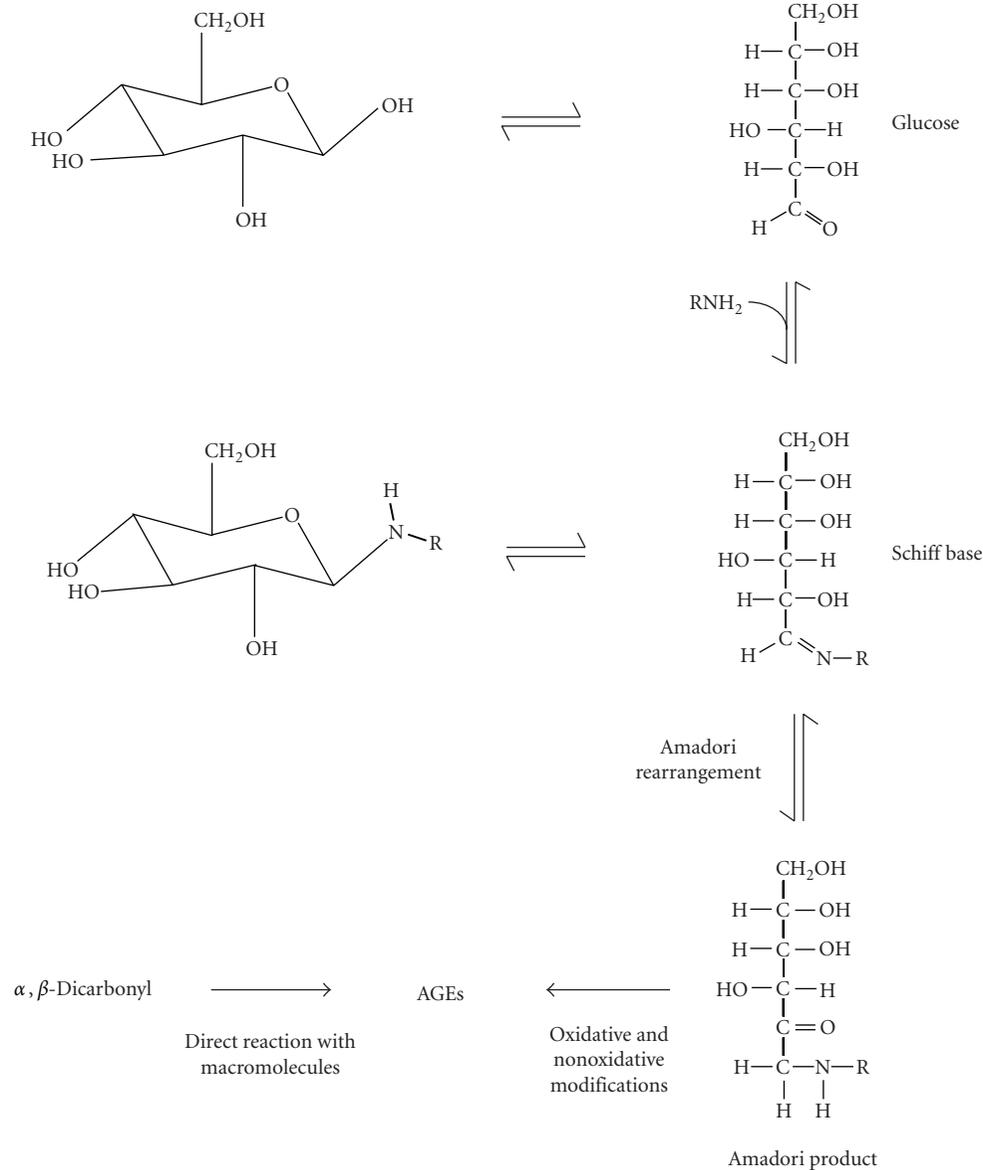


FIGURE 2: Formation of AGEs in physiological systems. In early glycation, glucose reacts reversibly with amine groups on macromolecules to form a Schiff base and subsequently an Amadori product. This can undergo further oxidative and nonoxidative modifications to form AGEs. Alternatively, AGEs may also be formed by the direct reaction of reactive  $\alpha, \beta$ -dicarbonyls with macromolecules.

damage. Any damage that does occur is dealt with by damage repair and removal systems. For example, proteasomes and lysosomes prevent the accumulation of glycosylated proteins [53]; the nucleotide excision repair system removes glycosylated nucleotides [54]; lipid turnover helps clear glycosylated lipids [19]. When there is an imbalance between the production of glycation precursors such as  $\alpha, \beta$ -dicarbonyls and the removal of these species such that the former is favoured, carbonyl stress occurs [55] and glycation damage accumulates.

Because  $\alpha, \beta$ -dicarbonyls are the major precursors of AGEs and their production is largely intracellular, it is likely that there would be significant functional consequences of AGEs formation and accumulation at the organellar level. In this review, we focus on glycation in mitochondria and its

association with mitochondrial dysfunction in disease and ageing.

## 2. Glycation in Mitochondria: Involvement in Diabetes and Ageing

The levels of both intra- and extracellular AGEs have been found to increase in diabetic or old animals relative to healthy or young controls [3, 6–10]. Indeed, a glycation adduct of glucose to haemoglobin,  $\text{HbA}_{1c}$ , is used clinically as an indicator of glycaemic control [56, 57], and levels of glycation adducts like CML and pentosidine have been suggested as prognostic tools in diabetics [58]. The accumulation of AGEs arises from an increase in their production and/or a decrease

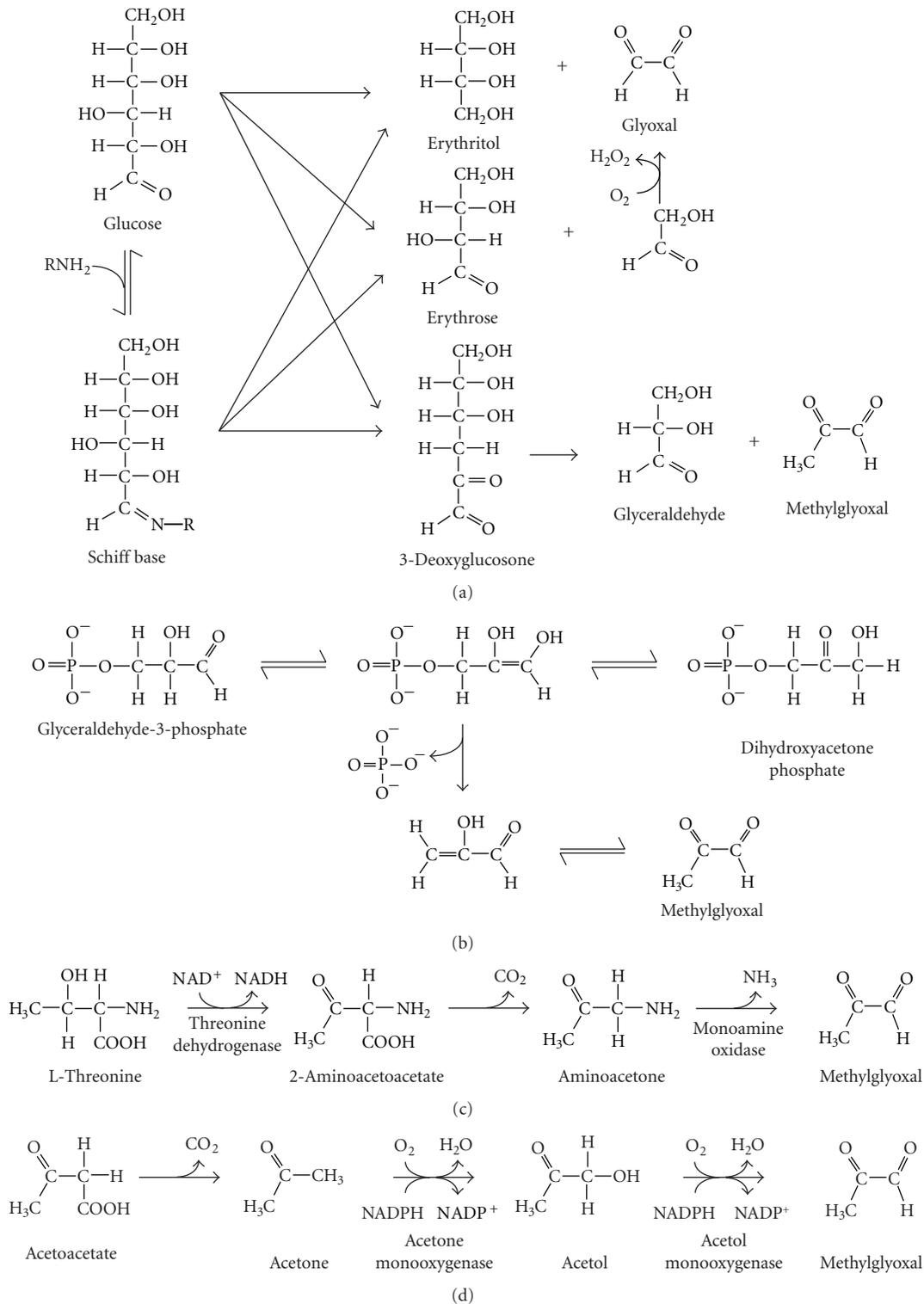


FIGURE 3: Formation of methylglyoxal and glyoxal in biological systems. (a) The acyclic form of glucose may undergo autooxidation via the Wolff pathway to form glyoxal or break down to 3-deoxyglucosone which in turn degrades to glyceraldehyde and methylglyoxal. Glucose may also react with amine groups on proteins to form a Schiff base which can undergo similar breakdown via the Namiki pathway to also generate glyoxal and methylglyoxal. (b) The major pathway by which methylglyoxal is formed in cells involves the breakdown of the triose phosphates, glyceraldehyde-3-phosphate, and dihydroxyacetone phosphate, via phosphate elimination from an ene-diol intermediate. (c) Methylglyoxal may also be generated during threonine catabolism. This involves oxidation of threonine by threonine dehydrogenase to 2-aminoacetoacetate, followed by spontaneous decarboxylation to aminoacetone. Monoamine oxidase then catalyses the conversion of aminoacetone to methylglyoxal. (d) Another pathway by which methylglyoxal is produced is from acetoacetate metabolism. This reaction proceeds via acetone and acetol and is catalysed by acetone monooxygenase and acetol monooxygenase.

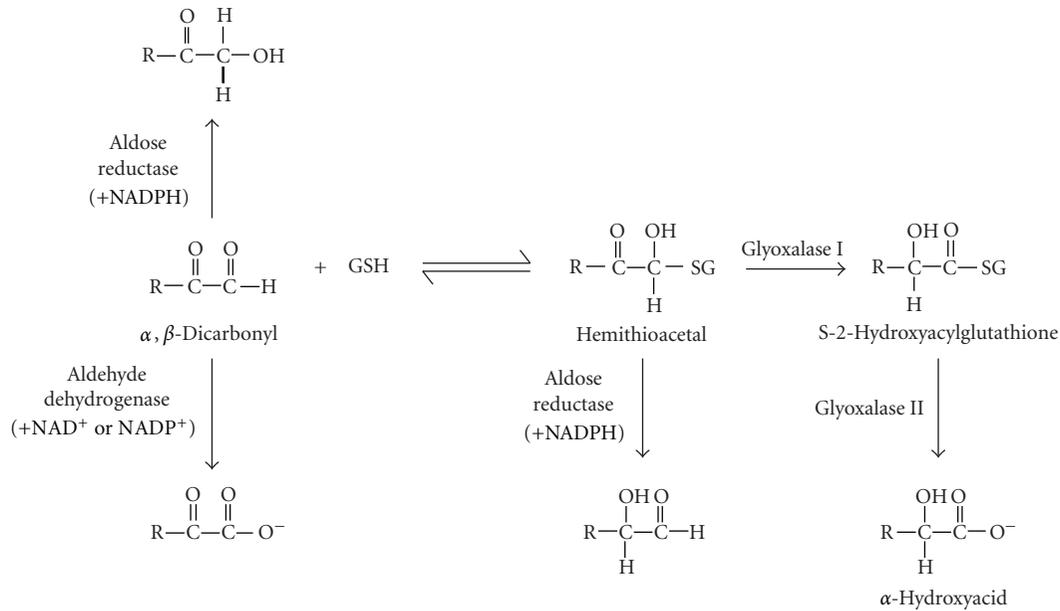


FIGURE 4: Physiological defences against  $\alpha,\beta$ -dicarbonyl precursors of glycation.  $\alpha,\beta$ -dicarbonyls like methylglyoxal (R:CH<sub>3</sub>) and glyoxal (R:H) can be removed by aldose reductase-catalysed reduction to the corresponding alcohol or by oxidation to pyruvate and glycolate, respectively, catalysed by aldehyde dehydrogenase. Reactive  $\alpha,\beta$ -dicarbonyls may also combine reversibly with reduced glutathione to form a hemithioacetal which can then be reduced by aldose reductases, forming lactaldehyde and glycoaldehyde from methylglyoxal and glyoxal, respectively. Alternatively, the hemithioacetal may undergo a two-step conversion to an  $\alpha$ -hydroxyacid, catalysed by glyoxalase I and glyoxalase II. The glyoxalase enzyme system represents the major pathway by which methylglyoxal is removed.

in their removal. For instance, low protein turnover is responsible for the accumulation of extracellular AGEs on collagen with age [59]; in diabetics, plasma levels of HbA<sub>1c</sub> increase as a result of high levels of blood glucose which forms an adduct to the N-terminal valine on the  $\beta$ -chain of haemoglobin [56, 57].

Within cells, the levels of AGEs also increase with age or in diabetes, and this is likely to reflect changes in intracellular  $\alpha,\beta$ -dicarbonyl levels (Figure 5). In the case of diabetes, the levels of intracellular AGEs rise extremely quickly compared to that of extracellular AGEs and are thought to be a result of the intracellular formation of the reactive dicarbonyl species, methylglyoxal and glyoxal [60]. According to the unifying hypothesis proposed by Brownlee [1], mitochondrial superoxide production increases during hyperglycaemia, resulting in DNA damage and poly(ADP-ribose) polymerase (PARP) activation. ADP-ribose polymers generated by PARP may then bind glyceraldehyde-3-phosphate (G3P) dehydrogenase, inhibiting it. Consequently, there is a build-up of glycolytic intermediates upstream of this enzyme. Among these intermediates are the triose phosphates, G3P and dihydroxyacetone phosphate (DHAP), which break down to form methylglyoxal [38]. In addition, oxidative stress resulting from hyperglycaemia promotes lipid peroxidation [61, 62], another source of a major  $\alpha,\beta$ -dicarbonyl species, glyoxal [41]. As such, there is increased formation of reactive dicarbonyls in diabetes. With respect to ageing, decreases in glyoxalase I activity have been reported in *Caenorhabditis*

*elegans* [3] and rats [63]. The less efficient removal of  $\alpha,\beta$ -dicarbonyls consequently leads to elevation of the levels of AGEs in older animals [3].

Once formed within the cell, these reactive dicarbonyl species may then diffuse across membranes [47, 64] and access mitochondrial targets (Figure 5). It is estimated that there is 1-2 mmol CML/mol lysine in rat heart mitochondria [65], 0.5-1 mmol CML/mol lysine in rat liver mitochondria [66] and 0.7-1 mmol CML/mol lysine in rat brain mitochondria [67]. Corresponding values for carboxyethyl-lysine (CEL) are 0.5, 0.2-0.5, and 0.1-0.2 mmol CEL/mol lysine [65-67]. It is striking that levels of CML and CEL in mitochondria from the three tissues (heart, liver, and brain) are kept within narrow range (<2 mmol AGE/mol lysine). This suggests that excessive glycation of mitochondrial proteins is highly damaging and that levels of mitochondrial AGEs have to be minimized to prevent mitochondrial dysfunction. Likewise, mitochondrial DNA (mtDNA) [68] and lipids [69] are also targets for glycation. Indeed, measurements of CEdG [N<sup>2</sup>-(1-carboxyethyl)guanidine], a nucleotide glycation adduct, were found up to three-fold more CEdG in mtDNA of cultured fibroblasts than in nuclear DNA [68], suggesting that mtDNA is more vulnerable to glycation than nuclear DNA due to the absence of protective histones in the former [70]. The presence of glycated phosphatidylethanolamine in mitochondrial membranes has also prompted suggestions that mitochondrial lipid glycation could affect electron transport chain activity and cause mitochondrial dysfunction [69].

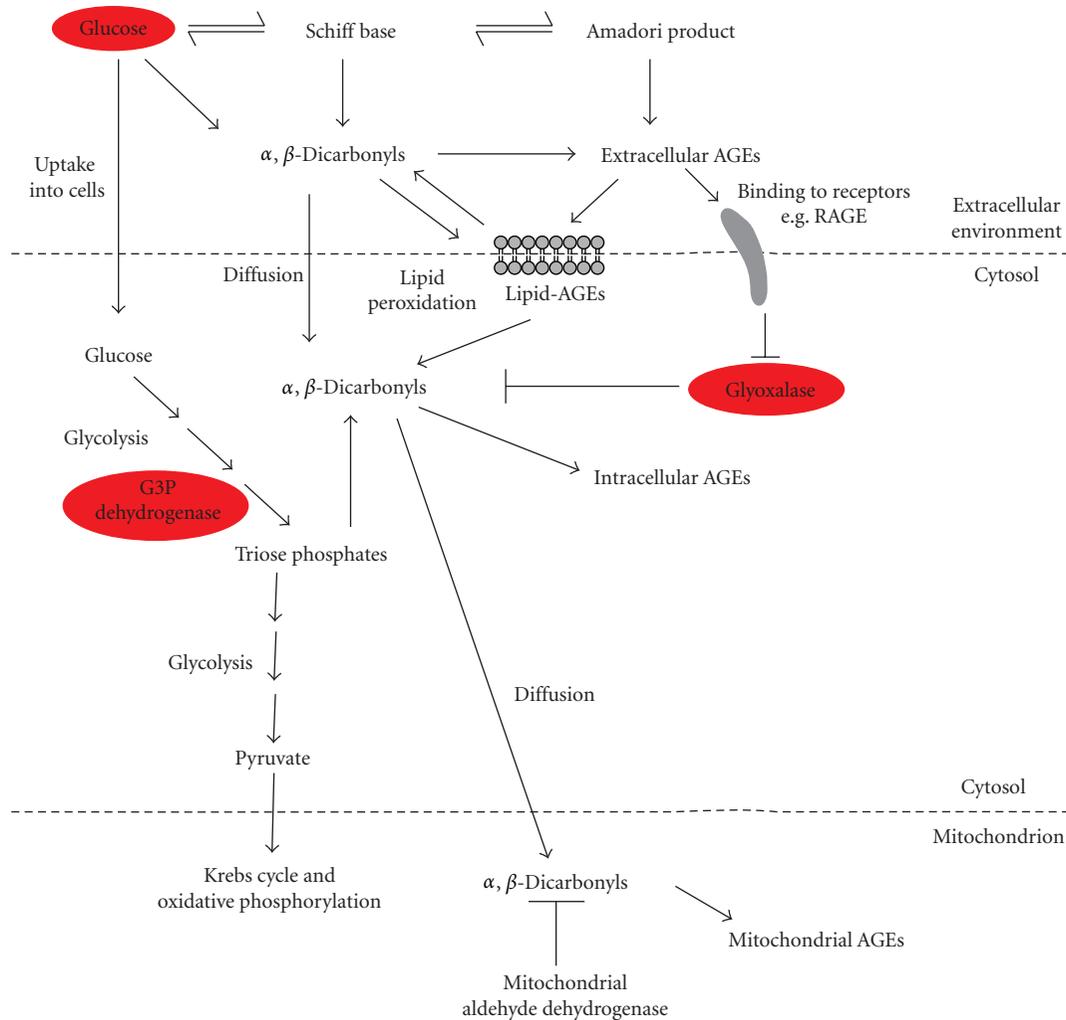


FIGURE 5: Intra- versus extracellular glycation— involvement in diabetes and ageing. Glycation can take place in the extracellular environment or within cells in the cytosol and in organelles like mitochondria. Extracellular AGEs may arise from oxidative and nonoxidative modifications of the Amadori product or from direct reaction of  $\alpha, \beta$ -dicarbonyls with proteins. Extracellular AGEs may bind to cell surface receptors such as RAGE, thereby activating cell signalling pathways. The formation of lipid-AGEs on the cell surface membrane can further generate reactive  $\alpha, \beta$ -dicarbonyls such as glyoxal. These reactive carbonyl species can diffuse through lipid membranes and enter cells where they react with cellular biomolecules to form intracellular AGEs. They can also diffuse further into mitochondria and similarly cause glycation damage within these organelles. Intracellular glycation may also arise from  $\alpha, \beta$ -dicarbonyls produced during the breakdown of triose phosphates generated during glycolysis. Normally, glycation damage is kept under control by defences such as the glyoxalase system and aldehyde dehydrogenases. However, in diabetes, glycation damage increases due to elevated formation of  $\alpha, \beta$ -dicarbonyls, arising from high glucose levels and consequent inhibition of the glycolytic enzyme, G3P dehydrogenase, both of which lead to a rise in triose phosphate levels. These in turn break down nonenzymatically to form methylglyoxal, a major precursor of glycation. In addition, activation of RAGE is associated with decreased glyoxalase I expression, which is expected to further raise methylglyoxal levels by preventing its removal. During ageing, glycation damage also increases, but mainly as a result of a loss of glyoxalase activity with age.

Intriguingly, specific mitochondrial protein targets of glycation have been identified in kidneys of streptozotocin-induced diabetic rats [71] and in livers of aged rats [18]. Increasing the removal of  $\alpha, \beta$ -dicarbonyls either by administering the prototypical dicarbonyl scavenger, aminoguanidine, or by overexpression of glyoxalase I decreased both glycation and oxidative damage, restored complex III activity, and improved respiration in experimental models of diabetes [71, 72]. Likewise, increasing glyoxalase I expression in nematodes decreased mitochondrial levels of methylglyoxal

and AGEs, and extended lifespan, while inhibiting the enzyme increased methylglyoxal levels and reduced lifespan [3]. These results suggest that glycation of mitochondrial proteins could account, at least in part, for the mitochondrial dysfunction and oxidative damage observed in hyperglycaemia and ageing. Separately, changes in the expression of mitochondrial proteins have been observed in Schwann cells grown under hyperglycaemic conditions [73] and in Akita mice, a mouse model of type 1 diabetes [74]. While the molecular mechanisms inducing these changes have not

been elucidated, one study in cultured endothelial cells found that glycation could cause epigenetic changes in expression of nuclear-encoded genes [75], highlighting the potential for glycation to do likewise in mitochondrial genes, thereby prompting the “remodelling of the mitochondrial proteome” as is observed in Akita mice [74].

The hypothesis that glycation of mitochondrial targets profoundly influences mitochondrial function is supported by studies in which exogenous dicarbonyls were administered and mitochondrial parameters studied. For example, methylglyoxal treatment of isolated mitochondria from rat kidney [76] and from several carcinoma cell lines [77–79] decreased oxygen consumption by mitochondria. Similarly, treatment of cultured cells with methylglyoxal or glyoxal decreased mitochondrial membrane potential reduced the activities of the respiratory chain complexes, reduced ATP synthesis, and increased reactive oxygen species (ROS) levels [80–83]. While these studies all point towards a glycation-induced mitochondrial dysfunction, the physiological relevance of such experiments is uncertain. Some have suggested that the use of millimolar concentrations of dicarbonyls in such experiments is physiologically irrelevant [47, 84], especially since cellular levels of methylglyoxal have been estimated to be in the low micromolar range. Others, however, have argued that these values, being measures of steady state concentrations of dicarbonyls, do not accurately reflect the actual dicarbonyl flux within cells where  $\alpha,\beta$ -dicarbonyls are produced and that millimolar concentrations are reasonable estimates of the actual flux *in vivo* [85]. That only a very small proportion of exogenous dicarbonyl becomes incorporated into cells has also been used to justify the use of high concentrations of exogenous dicarbonyls [83, 86]. It should also be noted that in treating cultured cells with exogenous methylglyoxal or glyoxal, extracellular, cytosolic and mitochondrial levels of these dicarbonyls and their associated AGEs are all increased. Therefore it is unclear whether any mitochondrial dysfunction observed is a direct result of the glycation of mitochondrial targets or if it is a downstream consequence following the glycation of extracellular or cytosolic targets especially since incubation of cells with exogenous AGEs can similarly induce mitochondrial dysfunction [87–89].

### 3. Mitochondrial Glycation in Cancer and Neurodegeneration: A Hypothesis

As Experimental uncertainties notwithstanding, the issue of mitochondrial glycation and its links to disease is scientifically and clinically interesting. Should mitochondrial glycation be a major cause of disease, then a broad strategy of limiting glycation damage in mitochondria may ameliorate disease initiation and progression across multiple clinical conditions. Such a strategy would be akin to that of targeting antioxidants to mitochondria for the treatment of conditions as disparate as Parkinson’s disease, diabetes and ischaemia-reperfusion injury [90]. In this context, it is intriguing to consider whether mitochondrial glycation could contribute to conditions apart from diabetes, and

ageing. We discuss here the possibility of this hypothesis in cancer and neurodegeneration.

**3.1. Cancer.** The Warburg phenotype in which aerobic tumour cells are largely dependent on glycolysis, rather than oxidative phosphorylation, for their energy supply has been well-described in many cancer cell lines [91]. Since triose phosphate intermediates of the glycolytic pathway are an important source of the major glycation precursor, methylglyoxal [38], the levels of methylglyoxal and consequently glycation should be increased in tumour cells. It appears that the type and distribution of such glycation damage varies between tumour types, and not all tumours necessarily display equal extents of glycation [92]. In addition, overexpression of glyoxalase I and glyoxalase II has been correlated with multidrug resistance in tumours [93]. The upregulation of the glyoxalase enzyme system is thought to allow tumour cell growth by counteracting the rise in methylglyoxal production [93]. However, tumour cells with high glyoxalase I expression have higher levels of DNA glycation adducts than those with relatively lower expression [94] suggesting that glyoxalase I expression increases to try to counteract methylglyoxal-induced cytotoxicity [94]. It is therefore not surprising that glyoxalase I inhibitors are being evaluated for use in cancer chemotherapy [95] as they render multidrug-resistant tumours vulnerable to apoptosis [96]. Glyoxalase I inhibitors may potentiate apoptosis by increasing DNA glycation that results in PARP activation which depletes cellular nicotinamide adenine dinucleotide (NAD<sup>+</sup>) and thereby inhibits G3P dehydrogenase, leading to further increases in methylglyoxal formation [97]. Methylglyoxal also induces cytotoxicity in a variety of other ways such as promoting the mitochondrial permeability transition pore, activation of protein kinase C-delta [93] and inhibition of STAT3-associated signaling [98].

As in diabetes and ageing, mitochondria are profoundly affected in cancers; the upregulation of glycolysis in tumour cells is accompanied by mitochondrial dysfunction and decreased oxidative phosphorylation [99, 100]. For example, a recent review [101] argues that such mitochondrial dysfunction is critical for tumour growth, citing a strong correlation between tumour progression and increased mitochondrial DNA (mtDNA) mutations. Although the role of mitochondrial glycation in cancers has not been explored, it is possible that elevated glycolysis in cancer cells leads to increased glycation and mutation of mtDNA [75]. Thus up-regulation of glycolysis in tumour cells may contribute to increased mitochondrial damage and thereby establish a vicious cycle that enhances the Warburg effect.

**3.2. Neurodegeneration.** As triose phosphates are a major source of methylglyoxal [38], any disturbance in triose phosphate metabolism will influence methylglyoxal formation. For example, triose phosphate isomerase (TPI) deficiency results in an inherited neurological disorder [102]. This occurs because TPI catalyzes the interconversion of DHAP and G3P, and its inhibition results in an accumulation of DHAP which breaks down to methylglyoxal

[103]. Methylglyoxal-induced protein and nucleotide glycation might result in paralysis and neurodegeneration as is observed in flies expressing a mutant form of TPI [104]. Nitrotyrosinated TPI has been detected in brains from mouse models of, and human patients with, Alzheimer's disease [105], and this nitration of TPI decreases its enzyme activity and increases methylglyoxal production [105]. Tau aggregation and neurofibrillary tangle formation can also be promoted by methylglyoxal [106, 107]. These suggest that methylglyoxal and glycation can contribute to the progression of neurodegeneration, and this is supported by observations that glyoxalase I expression increases in the brains of early and middle stage Alzheimer's patients [108], indicating an attempt to scavenge excessive  $\alpha,\beta$ -dicarbonyls. That type II diabetics are at 2–2.5 fold greater risk of developing dementia also lends weight to a contribution of glycation in neurodegenerative diseases [109]. It has also been proposed that intra- and extracellular AGEs contribute to neurodegeneration by two main pathways, the former by promoting protein aggregation and thus inhibiting their proper function, and the latter by accumulating on senile plaques and inducing oxidative stress and inflammation [110].

The role of mitochondrial dysfunction in neurological disorders has been extensively reviewed [111–113]. While mitochondrial glycation was not specifically investigated in these studies, it may be that glycation damage of proteins involved in oxidative phosphorylation and of mtDNA may contribute to the mitochondrial dysfunction observed. Specific protein targets of carbonylation, oxidation, and nitration have been identified in mitochondria in neurodegenerative disorders [114, 115]. Since glycation and oxidative damage are closely correlated, with both types of damage markers often increasing in parallel with ageing and disease [22–24], it is plausible that glycation of specific mitochondrial targets also occurs. A recent study in a mouse model of Alzheimer's disease further found that mitochondrial dysfunction precedes the presentation of any neurodegenerative pathology [116]. Particularly striking was the observation in the same study of increased glycolysis and decreased oxidative phosphorylation in neurons from these mice, features reminiscent of the Warburg phenotype of tumour cells. The many similarities between the conditions discussed above—diabetes, ageing, and cancer—with neurodegeneration suggest a potential for mitochondrial glycation to contribute to these conditions.

#### 4. Conclusion

In the four conditions discussed above—hyperglycaemia, ageing, cancer, and neurodegeneration—there is increased production of glycation precursors, namely, reactive  $\alpha,\beta$ -dicarbonyls such as methylglyoxal and glyoxal, leading to elevated molecular glycation damage as evidenced by the rise in levels of AGEs. However, despite the correlation between glycation, mitochondrial dysfunction, and disease, the relative importance of mitochondrial glycation as opposed to extracellular or cytosolic glycation is still unclear. This

is in part because it is the whole cell levels of  $\alpha,\beta$ -dicarbonyls that are altered by experimental manipulation with aminoguanidine or glyoxalase I overexpression, and also because treatment of cell cultures with exogenous methylglyoxal or glyoxal raises both extra- and intracellular dicarbonyl levels. As such, the effects of any changes are not isolated to mitochondria. Besides, the amount of exogenous dicarbonyl that is physiologically relevant is a further point of contention. Unfortunately, it is technically difficult to accurately measure dicarbonyl levels within mitochondria in cells and *in vivo* as existing methods are prone to artefacts arising from variations in mitochondria isolation, sample preparation, and derivatization [84, 117, 118]. There is also a lack of experimental tools for manipulating dicarbonyl levels within mitochondria alone without modifying cytosolic and extracellular levels. Therefore, it is difficult to isolate the effects of glycation observed in mitochondria from more general consequences on the whole cell. Consequently, to divorce the contribution of mitochondrial glycation from cytosolic or extracellular glycation to disease is experimentally challenging. Nonetheless, the prospect of mitochondrial glycation contributing as a common damaging agent across a broad spectrum of diseases is an intriguing possibility and is also a novel potential therapeutic target.

#### Abbreviations

ADP:	Adenosine diphosphate
AGEs:	Advanced glycation end products
ATP:	Adenosine triphosphate
CEdG:	N <sup>2</sup> -(1-carboxyethylguanidine)
CEL:	Carboxyethyl-lysine
CML:	N <sup>ε</sup> -carboxymethyl-lysine
DHAP:	Dihydroxyacetone phosphate
ECM:	Extracellular matrix
G3P:	Glyceraldehyde-3-phosphate
GSH:	Reduced glutathione
LDL:	Low-density lipoprotein
MG-H1:	[N <sup>δ</sup> -(5-hydro-5-methyl-4-imidazolone-2-yl)ornithine]
MOLD:	Methylglyoxal-derived lysine dimers
mtDNA:	Mitochondrial DNA
NAD <sup>+</sup> :	Nicotinamide adenine dinucleotide
PARP:	Poly(ADP-ribose) polymerase
RAGE:	Receptor for AGEs
ROS:	Reactive oxygen species
TPI:	Triose phosphate isomerase.

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## Review Article

# Oxidative Stress, DNA Damage, and c-Abl Signaling: At the Crossroad in Neurodegenerative Diseases?

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The c-Abl tyrosine kinase is implicated in diverse cellular activities including growth factor signaling, cell adhesion, oxidative stress, and DNA damage response. Studies in mouse models have shown that the kinases of the c-Abl family play a role in the development of the central nervous system. Recent reports show that aberrant c-Abl activation causes neuroinflammation and neuronal loss in the forebrain of transgenic adult mice. In line with these observations, an increased c-Abl activation is reported in human neurodegenerative pathologies, such as Parkinson's, and Alzheimer's diseases. This suggests that aberrant nonspecific posttranslational modifications induced by c-Abl may contribute to fuel the recurrent phenotypes/features linked to neurodegenerative disorders, such as an impaired mitochondrial function, oxidative stress, and accumulation of protein aggregates. Herein, we review some reports on c-Abl function in neuronal cells and we propose that modulation of different aspects of c-Abl signaling may contribute to mediate the molecular events at the interface between stress signaling, metabolic regulation, and DNA damage. Finally, we propose that this may have an impact in the development of new therapeutic strategies.

## 1. Introduction

A broad range of pathological disorders is linked to oxidative stress, including carcinogenesis and several age-dependent disorders (i.e., as neurodegenerative diseases). Oxidative stress is defined as an imbalance in which the production of reactive oxygen species (ROS) overcomes the antioxidative cell defence system. Oxidative stress can be induced by exogenous and endogenous sources. For instance, hydrogen peroxide and chemotherapeutic reagents are exogenous sources of ROS, whereas mitochondrial energy metabolism is considered a major source for the production of ROS within the cell [1]. ROS can directly react with macromolecules, such as DNA, lipids, and proteins. Oxidative DNA lesions, if unrepaired, can induce mutations and deletions in both nuclear and mitochondrial genomes [2] and chromosomal

abnormalities. Cells are also very sensitive to lipid peroxidation [3] and most amino acid residues in a protein can be oxidized by ROS. Often these modifications impair protein function [4]. Antioxidant defences are built in a complex network of nonenzymatic and enzymatic components of the cell. This network has been extensively reviewed [5, 6]. In short, Glutathione (GSH) is a nonenzymatic antioxidant, which acts in the cellular thiol/disulfide system, with the ratio of GSH to GSSH (glutathione disulphide) mirroring the redox status of the cell. On the other hand, enzymatic antioxidants include superoxide dismutases SODs, catalase, peroxiredoxins (PRxs), and glutathione peroxidases (GPx). The toxicity of ROS is only one facet of their action. ROS are also produced at low level within the cell, where they can play an important role in the redox-dependent regulation of signaling [7]. Hence, ROS are implicated

in several cellular processes, including cell proliferation, cell cycle arrest, and programmed cell death [8]. Cellular responses to DNA damage or oxidative stress are critical for survival, and the direct link between ROS and oxidative DNA damage indicates the interplay of ROS signaling with the DNA damage response (DDR) [9]. Evidence indicates the involvement of the phosphatidylinositol-3-kinases- (PI3K-) related kinases, Ataxia telangiectasia mutated (ATM), DNA-dependent protein kinase catalytic subunit (DNA-PKcs), and ATM- and Rad-3 related (ATR) in oxidative DNA lesion repair and signaling response [10]. This finding together with the emerging role of c-Abl in the DDR [11] and in oxidative DNA damage [12] seems to point out a role for these DDR kinases as “sensors” for redox signaling. In particular, herein we discuss how an aberrant (nonspecific) c-Abl signaling may contribute to maintain high levels of ROS that in turn can damage organelles, mitochondria, and DNA, with these effects ending towards neuronal degeneration.

## 2. ROS and c-Abl Signaling

Oxidative stress contributes to the pathogenesis of a large number of human disorders. No doubt that a better understanding of the controlled production (and of regulatory targets) of ROS should provide the rationale for novel therapeutic treatments [13]. ROS signaling is reversible, tightly controlled through a regulatory network. This network results from a concerted assembly of protein complexes, built through protein interactions mediated by interaction modules and posttranslational modifications in the binding partners. Protein modularity and the reversible nature of posttranslational modifications allow the dynamic assembly of local temporary signaling circuits regulated by feedback controls. The strength and the duration of redox signaling are regulated *via* the oxidative modifications of the kinases and phosphatases that in turn control the activity of enzymes involved in antioxidant activities and vice versa. Oxidant level modulates c-Abl activity [14, 15]. In turn, c-Abl can interact (and regulate) with several enzymes implicated in controlling the redox state of the cell. One of them, the catalase is an immediate effector of the antioxidant cellular defense by converting  $H_2O_2$  to  $H_2O$  and  $O_2$  in the peroxisomes. c-Abl and the product of the c-Abl-related gene (Arg) target catalase on the two residues Y321 and Y386 leading to its ubiquitination and to a consequent proteasomal-dependent degradation of the enzyme [16]. Similarly, c-Abl-deficient cells display a higher level of expression of the antioxidant protein peroxiredoxin I (Prx1) [17]. Prx1 is considered a physiological inhibitor of c-Abl. Prx1 interacts with the SH3 domain of c-Abl and inhibits its catalytic activity [18]. Depending on the oxidative level in the cell, glutathione peroxidase1 can be phosphorylated on Tyr-96 and activated by c-Abl/Arg [19]. In short, c-Abl activation has mostly a negative effect on enzymes involved in the antioxidant defence, with rare exceptions. Moreover, c-abl, as a component of redox regulatory circuits, can be modified by S-glutathionylation, with this reversible modification leading to downregulation of its kinase activity [20].

## 3. c-Abl Signaling in Neurodegenerative Disease

Oxidative stress, accumulation of protein aggregates, and damaged mitochondria are common hallmarks of neurological diseases. Aberrant c-Abl activation is linked to many neuronal disorders as recently reviewed by Schlatterer and coworkers [21]. In the brain, c-Abl activation can be monitored by specific antibodies, which target phosphorylated residues present only in the active conformation of the kinase. Staining with these phosphoantibodies indicates that c-Abl colocalized with granulovacuolar degeneration (GVD) in brains of human Alzheimer (AD) patients. Moreover, c-Abl phosphorylated at T735, a site required for binding 14-3-3 in the cytosol [22], colocalized with amyloid plaques, neurofibrillary tangles (NFTs), and GVD in the entorhinal cortex and hippocampus and brain of AD patients [21, 23]. Tau phosphorylation mediated by c-Abl is detected in NFTs in Alzheimer disease [21, 24, 25]. Oxidative stress activates c-Abl in neuronal cells [26] and amyloid  $\beta$  results in increased expression of c-Abl and p73 [27]. Amyloid- $\beta$  ( $A\beta$ ) fibrils in primary neurons induce the c-Abl/p73 proapoptotic signaling, while STI571, a pharmacological c-Abl inhibitor, prevents Amyloid  $\beta$ -dependent toxicity [26]. The c-Abl/p73 proapoptotic pathway is also targeted in the cerebellum of Niemann-Pick type C (NPC) mice [28]. Niemann-Pick type C (NPC) is a neurodegenerative disorder characterized by intralysosomal accumulation of cholesterol leading to neuronal loss. Pharmacological inhibition of c-Abl with STI571 rescues Purkinje neurons, reduces general cell apoptosis in the cerebellum, improves neurological symptoms, and increases the survival of NPC mice [29]. Evidence indicates that c-Abl binding with p73 is induced by ROS, with NAC (the oxidant scavenger N-acetylcysteine) treatment reducing the c-Abl/p73 activation as well as the levels of apoptosis in NPC neurons [28].

Recent findings indicate that some effects of c-Abl induced by glucose metabolism might be mediated through p53 phosphorylation. In fact, c-Abl is involved in high glucose-induced apoptosis in embryonic E12.5 cortical neural progenitor cells (NPCs) derived from mice brain. Once more again, inhibition of c-Abl by ST571 reduced apoptosis in NPCs by preventing the nuclear protein accumulation of p53 in response to high glucose [30]. Moreover, administration of reactive oxygen species scavengers impairs the accumulation of c-Abl and p53 leading to a decreased NPCs apoptosis. In human neuroblastoma (SH-SY5Y) cells, c-Abl targets cyclin-dependent kinase 5 (Cdk5) on tyrosine residue Y15 in response to oxidative stress by hydrogen peroxide. In turn, Cdk5 can modulate p53 levels and p53 activity. Hence, both c-Abl and Cdk5 cooperatively mediate p53 transcriptional activation resulting in neuronal death [31]. A recent study also indicates that hyperglycemia-induced apoptosis of NPCs is mediated by a PKC $\delta$ - (Protein-Kinase C-delta-) dependent mechanism [32]. Tyrosine phosphorylation of PKC $\delta$  by c-Abl is important for the translocation of the PKC $\delta$ -Abl complex from the cytoplasm to the nucleus. Downregulation of PKC $\delta$  (or c-Abl) or inhibition of c-Abl

by STI571 can decrease this translocation, impairing p53 accumulation in the nucleus of NPCs [32].

A redox imbalance is apparently a predominant feature of brains of individuals with Parkinson's disease (PD). Evidence derived from postmortem studies indicates an increased oxidation of lipids, proteins and DNA, a severe decrease in GSH concentration, and an accumulation of SOD2 (see [33] and references therein). Oxidative DNA damage occurs to a higher extent in Parkinson's disease individuals compared with age-matched controls [34]. Brains of Parkinson's patients are also enriched in autophagosome-like structures reminiscent of autophagic stress. Interestingly, inherited forms of Parkinson's disease are associated with loss-of-function mutations in genes encoding proteins that target the mitochondria and modulate autophagy, including the E3 ubiquitin ligase parkin (see [33] and references therein). c-Abl phosphorylates parkin on Y143 and inhibits parkin's ubiquitin E3 ligase activity and its protective function. Conversely, STI-571 treatment prevents the phosphorylation of parkin, maintaining it in a catalytically active state. Interestingly, the protective effect of STI-571 is not observed in parkin-deficient cells. Conditional knockout of c-Abl also prevents the phosphorylation of parkin, the accumulation of its substrates, and results in neurotoxicity in response to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) intoxication [35]. Briefly, STI-571 prevents tyrosine phosphorylation of parkin and restores its E3 ligase activity and cytoprotective function both *in vitro* and *in vivo*. Compelling evidence indicates that tyrosine phosphorylation of parkin by c-Abl is a major posttranslational modification that leads to loss of parkin function and disease progression in sporadic PD [36]. Moreover, a selective inhibition of c-Abl offers new therapeutic strategies for blocking PD progression [35, 36]. Another level of c-Abl-dependent-regulation impinges on the activation of PKC $\delta$ . In cell culture models of PD, oxidative stress activates PKC $\delta$  through a caspase-3-dependent proteolytic cleavage inducing apoptotic cell death [37, 38]. Interestingly proteolytic activation of PKC $\delta$  is regulated through phosphorylation of its tyrosine residues [39]. Evidence regarding a functional interaction between PKC $\delta$  and c-Abl has been provided following oxidative stress response [14]. c-Abl phosphorylates PKC $\delta$  on tyrosine 311, with this modification contributing to the apoptotic effect of hydrogen peroxide [40]. On the other hand, ST571 can block PKC $\delta$  activation protecting cells from apoptosis [41]. Moreover, Xiao et al. identified c-Abl as a novel upstream activator of the protein kinase MST1 (mammalian Ste-20 like kinase1) that plays an essential role in oxidative-stress-induced neuronal cell death. Upon phosphorylation of MST1 at Y433 by c-Abl, authors demonstrated activation of FOXO3 that leads eventually to neuronal cell death. The latter mechanism is inhibited either by STI571 or c-Abl knockdown [42].

In short, this combined evidence stresses the physiological relevance of the interface between c-Abl signaling and redox state, metabolic regulation and DNA damage response mediated by transcription factors, such as FOXO-3 or members of the p53 family.

The dynamic of each signal-transduction path seems to be governed by a small set of recurring c-Abl-mediated

regulatory circuits, that depending on their subcellular localization and response duration may result in neuronal death (Figure 1). Of note, inactivation of c-Abl by STI571 can have a protective effect and can reduce neuronal loss.

#### 4. Autophagy, Mitochondria, and Oxidative Stress: Cross Talk with c-Abl Signaling

Protein aggregation and organelle dysfunction are peculiar hallmarks of many late-onset neurodegenerative disorders. Mitochondrial damage and dysfunction is indeed linked to neurodegeneration in a variety of animal models [33]. Clearance of misfolded proteins and damaged organelles may be considered an effective recovery strategy for stressed neuronal cells [43]. Autophagy is a lysosome-dependent pathway involved in the turnover of proteins and intracellular organelles. It is becoming increasingly evident that induction of a certain level of autophagy may exert a neuroprotective function, while its inappropriate or defective activation may result in neuronal cell loss in most neurodegenerative diseases [44]. Abnormal autophagosomes are frequently observed in selective neuronal populations afflicted in common neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis. However, whether accumulation of autophagosomes plays a protective role or rather contributes to neuronal cell death is still a controversial issue [44, 45]. Despite this uncertainty, an accurate titration of autophagy should favor a neuroprotective response. In particular, if it is strictly modulated through an efficient concerted action of the complex autophagy machinery. ROS can induce autophagy [46]. In addition, inhibition, depletion, or knock-out of the c-Abl family kinases, c-Abl and Arg, resulted in a dramatic reduction in the intracellular activities of the lysosomal glycosidases alpha-galactosidase, alpha-mannosidase, and neuraminidase. Inhibition of c-Abl kinases also reduced the processing of the precursor forms of cathepsin D and cathepsin L to their mature, lysosomal forms, leading to an impaired turnover of long-lived cytosolic proteins and accumulation of autophagosomes [46, 47]. Together all these findings suggest a positive role for c-Abl kinases in the regulation of autophagy with important implications for therapies [46].

In conclusion, many observations indicate that c-Abl activity is increased in human neurodegenerative diseases (Alzheimer, Parkinson, and tauopathies). However, where c-Abl meets the cascade of events underlying neurodegenerative disorders remains still elusive. A plausible scenario implies the involvement of c-Abl on multiple interconnected pathways eventually acting as an arbiter of neuronal survival and death decisions, most likely playing with autophagy, metabolic regulation and DNA damage signaling response. In adult mouse models, aberrant c-Abl activation causes neurodegeneration and neuroinflammation in forebrain neurons, thus implying c-Abl as a possible target for therapeutic treatments [21]. Several reports have shown that c-Abl plays distinct roles based upon its subcellular localization. Is the achievement of a certain/specific relocalization of c-Abl required for the development of the neuronal disease?

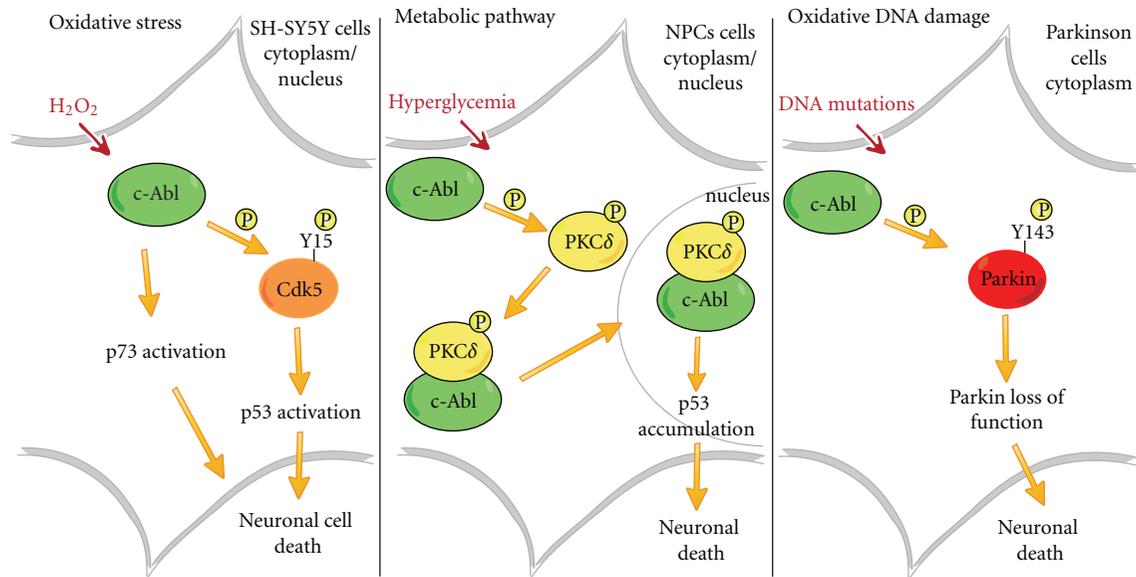


FIGURE 1: The figure illustrates the involvement of c-Abl in many cellular stress pathways. Oxidative stress, hyperglycemia, and DNA damage response induce c-Abl activation. In human neuroblastoma (SH-SY5Y cells), c-Abl targets p53, promoting neuronal death in response to hydrogen peroxide. In addition, c-Abl can also phosphorylate Cdk5 and in tandem with Cdk5 can mediate p53 activation, promoting neuronal death. Hyperglycemia-induced apoptosis of NPCs is mediated by the translocation of the PKC $\delta$ -Abl complex to the nucleus. This translocation impacts on p53 activation leading to neuronal death. Oxidative DNA damage in Parkinson disorder is associated with increased c-Abl activity. c-Abl mediates tyrosine phosphorylation of Parkin and inhibits parkin's ubiquitin E3 ligase activity.

The interplay between cytoplasmic, nuclear and mitochondrial localization of c-Abl is an important aspect for oxidative stress-induced apoptosis. In concert with this, c-Abl catalytic outcomes are strictly associated with its subcellular localization. TTK, also known as PYT (Phosphotyrosine Picked Threonine Kinase), the human homolog of MSP1 (Monopolar Spindle 1), regulates nuclear targeting of c-Abl through the 14-3-3-coupled phosphorylation site. Nihira et al. demonstrated that TTK-dependent phosphorylation of c-Abl on Y735 is required for the cytoplasmic sequestration/localization of kinase. TTK/Msp1 deficiency enhances the oxidative-stress-induced apoptosis while favoring the nuclear accumulation of c-Abl [48].

c-Abl co-localizes with the endoplasmic reticulum (ER)-associated protein grp78 [49]. Subcellular fractionation studies indicate that over 20% of c-Abl is detectable in the ER. Induction of ER stress with the calcium ionophore A23187, brefeldin A, or tunicamycin is linked to translocation of the ER-associated c-Abl fraction to mitochondria. In concert with targeting of c-Abl to mitochondria, cytochrome c is released in response to ER stress through a c-Abl-dependent mechanism. In c-Abl-deficient cells, ER-stress-induced apoptosis is attenuated thus implying the involvement of c-Abl in signaling from the ER to mitochondria [49]. Kumar et al. indicated that in response to oxidative stress, cytoplasmic c-Abl moves to mitochondria, where it mediates mitochondrial dysfunction and cell death. Moreover, targeting of c-Abl to mitochondria is also dependent on activation of PKC $\delta$  and relies on c-Abl catalytic activity. In the response to hydrogen peroxide, pharmacological inhibition of c-Abl with STI571 decreases c-Abl targeting to mitochondria and

attenuates mitochondrial dysfunction and cell death [50, 51]. Downregulation of c-Abl or PKC $\delta$  impaired PARP cleavage, suggesting that both PKC $\delta$  and c-Abl can induce apoptosis through the mitochondrial pathway in the absence of p53, p73, and FAS upon genotoxic stress [52].

Taken together all these observations suggest that c-Abl activation can exert a positive role both in the intrinsic and extrinsic apoptotic signaling pathways.

## 5. Perspectives

Signaling networks are composed of multiple layers of interacting proteins. Activation of most cell signaling circuits is modulated by feedback control, and disease conditions are often caused by the loss of the feedback control. A comprehensive understanding of the complexities of the signaling network is required to design therapies that are effective without inducing off-target consequences [53]. In neurodegenerative disorders, the duration and the spatial organization of signaling complexes can cause a system failure ending in neuronal loss. Evidence compiled above indicates that c-Abl activation could act as an arbiter of neuronal cell fate under various stress conditions. Subcellular localization of c-Abl can play an important role to modulate activation and assembly of signaling networks. Pharmacological inhibition of the catalytic activity can prevent targeting of c-Abl to mitochondria and the consequent programmed cell death. In the nucleus, c-Abl signaling modulates oxidative-stress-induced transcription resulting in neuronal death. In this scenario, a new therapeutic strategy for degenerative neurological diseases may be based on the possibility to

rewire the network characterizing the pathological states, by restoring a feedback control *via* inhibition of c-Abl signaling. Several types of inhibitors have been designed to target with high selectivity the c-Abl kinase by different mechanisms [54, 55]. Allosteric inhibitors repress the catalytic activity by binding to a site far from the kinase-active site. Allosteric binding does not prevent the binding of ATP-competitive inhibitors such as STI571. Experimental data provide evidence that both types of inhibitors can work in synergy to inhibit aberrant activation of Bcr-Abl [55, 56]. Insufficient or excessive inhibitor doses not only may be inefficacious but may also have adverse effects. In addition, targeting of c-Abl to different cellular compartments is linked to the catalytic domain conformation. A recent report indicates that binding of STI571 to the catalytic domain can restore the nuclear import of the Bcr-Abl mutant, suggesting that the auto-inhibited conformation of c-Abl is required for nuclear translocation [57]. Interestingly, an allosteric inhibitor, GNF-2, induces a translocation of myristoylated c-Abl to the endoplasmic reticulum, competing with the intramolecular engagement of the NH<sub>2</sub>-terminal myristate for binding to the c-Abl kinase myristate-binding pocket [58]. A priority is now the identification of effective combination therapies for native conformations of c-Abl kinases, allowing the reactivation of appropriate regulation circuits in aged neurons. As mentioned, administration of reactive oxygen species scavengers prevents the accumulation of c-Abl and p53 leading to a decreased apoptosis of NPCs. In line with this, treatment with curcumin, an activator of the antioxidant Nfr2 [59] pathway can ameliorate the neurological symptoms and survival of Niemann-Pick type C mice [60]. This suggests the possibility to develop combined targeted therapies of antioxidants in tandem with c-Abl kinase inhibitors [42]. Despite the technical hurdles, rewiring of cell signaling networks *via* inhibition of a single node, such as c-Abl, may prove an effective therapeutic strategy.

## Abbreviations

c-Abl:	Abelson tyrosine kinase
ROS:	Reactive oxygen species
GSH:	Glutathione
GSSH:	Glutathione disulphide
SOD:	Superoxide dismutases
PRxs:	Peroxiredoxins
GPx:	Peroxidases
DDR:	DNA damage response
ATM:	Ataxia telangiectasia mutated
DNAPKcs:	DNA-dependent protein kinase catalytic subunit
ATR:	Rad-3 related
Arg:	c-Abl-related gene
GVD:	Granulovacuolar degeneration
AD:	Alzheimer disease
NTFs:	Neurofibrillary tangles
A $\beta$ :	Amyloid- $\beta$ fibrils
STI571:	Imatinib mesylate
NPC:	Niemann-Pick type C
NAC:	N-acetylcysteine

NPCs:	E12.5 cortical neural progenitor cells
Cdk5:	Cyclin-dependent kinase 5
PKC $\delta$ :	Protein Kinase C-delta
PD:	Parkinson's disease
MPTP:	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MST1:	Mammalian Ste20-like kinase1
FOXO3:	Forkhead box O3
TTK/PYT/Msp1:	(Phosphotyrosine-Picked Threonine Kinase), the human homolog of MSP1 (Monopolar Spindle 1)
ER:	Endoplasmic reticulum
PARP:	Poly (ADP-ribose) polymerase
Bcr-Abl:	Oncogenic fusion protein (breakpoint cluster region).

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## Review Article

# Glutathione in Cerebral Microvascular Endothelial Biology and Pathobiology: Implications for Brain Homeostasis

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The integrity of the vascular endothelium of the blood-brain barrier (BBB) is central to cerebrovascular homeostasis. Given the function of the BBB as a physical and metabolic barrier that buffers the systemic environment, oxidative damage to the endothelial monolayer will have significant deleterious impact on the metabolic, immunological, and neurological functions of the brain. Glutathione (GSH) is a ubiquitous major thiol within mammalian cells that plays important roles in antioxidant defense, oxidation-reduction reactions in metabolic pathways, and redox signaling. The existence of distinct GSH pools within the subcellular organelles supports an elegant mode for independent redox regulation of metabolic processes, including those that control cell fate. GSH-dependent homeostatic control of neurovascular function is relatively unexplored. Significantly, GSH regulation of two aspects of endothelial function is paramount to barrier preservation, namely, GSH protection against oxidative endothelial cell injury and GSH control of postdamage cell proliferation in endothelial repair and/or wound healing. This paper highlights our current insights and hypotheses into the role of GSH in cerebral microvascular biology and pathobiology with special focus on endothelial GSH and vascular integrity, oxidative disruption of endothelial barrier function, GSH regulation of endothelial cell proliferation, and the pathological implications of GSH disruption in oxidative stress-associated neurovascular disorders, such as diabetes and stroke.

## 1. Glutathione and Neurovascular Homeostasis

*1.1. Function of the Blood-Brain Barrier.* Central to neurovascular homeostasis is the function of the blood-brain barrier (BBB). The BBB is a highly regulated interface between the systemic circulation and brain parenchyma and is comprised of a monolayer of brain capillary endothelial cells on the blood side and perivascular cells on the brain side of microvessels. The BBB functions to protect the parenchymal cells from fluctuations in plasma composition, such as during exercise and following meals, and against circulating neurotransmitters or xenobiotics capable of disrupting neural function [1]. In this regard, the BBB acts as a mechanical barrier; brain capillaries are ~50–100 times tighter than peripheral microvessels, a property that is attributed to intercellular tight junctions between neighboring endothelial cells that restrict the paracellular diffusion of hydrophilic

solutes. Only small molecules such as oxygen and CO<sub>2</sub> can freely diffuse across the lipid membranes of the endothelium.

On the luminal and abluminal membranes, specific transport systems regulate the transcellular traffic of small hydrophilic molecules, such as GLUT-1 and L-system carrier 1 in the transport of glucose or leucine, respectively, thereby providing a selective “transport barrier” that facilitates nutrient entry [2]. The highly expressed P-glycoprotein transporter on endothelial luminal surface protects the brain from xenobiotics and the potentially toxic neurometabolite, glutamate. In addition, an enrichment of endothelial degradative enzymes serves as an enzymatic barrier. Examples include ectoenzymes such as peptidases and nucleotidases, which metabolize peptides and ATP, respectively, and the intracellular enzymes monoamine oxidase and cytochrome P450 1A and 2B which inactivate blood-borne neuroactive compounds. Moreover, the cerebral endothelium exhibits

specific systems for receptor-mediated and adsorptive endocytosis that allow for the transfer of specific peptides and lipoproteins to the brain [2]. Such multiple functions of the BBB regulate the brain microenvironment and maintain parenchymal homeostasis.

**1.2. Glutathione Redox System and Cellular Function.** The glutathione/glutathione disulfide (GSH/GSSG) couple is the most abundant thiol redox system that plays a key role in the maintenance of the redox environment in cells [3, 4]. Under physiological conditions, intracellular GSH homeostasis depends on *de novo* GSH synthesis, GSH redox cycling, and transmembrane GSH transport. Cellular GSH exists mainly in the reduced form with GSSG constituting less than 10% of the total GSH pool. The biological functions of GSH are attributed to its unique  $\gamma$ -glutamyl bond between the glutamate and cysteine residues and to the presence of a free thiol group. Reduced GSH is synthesized in the cytosol in two steps from its constituent amino acids (glutamate, cysteine, glycine) catalyzed by  $\gamma$ -glutamyl cysteine ligase (GCL) and GSH synthase [5]. GCL catalyzes the formation of  $\gamma$ -glutamylcysteine, the first and rate-limiting reaction in GSH synthesis, and enzyme function is controlled by GSH feedback inhibition or by transcriptional upregulation of enzyme subunits (Section 1.4). An important aspect of cellular GSH homeostasis is that increased GSH oxidation is generally followed by increases in the total pool size, notably through enhanced *de novo* GSH synthesis.

The versatility of GSH in contributing to a myriad of cellular functions is notable in its role in detoxication reactions (e.g., hydroperoxide and xenobiotic catabolism), regulation of amino acid transport into cells, maintenance of native three-dimensional protein structure in biosynthetic/metabolic processes (e.g., prostaglandins D2 and E2 synthesis), serving as a cofactor for enzyme systems (e.g., glyoxalase I), and redox signaling. Thiol-disulfide exchanges and protein S-glutathiolation are mechanisms by which GSH modulates the oxidative modification of redox active cysteines within proteins and thereby regulates the activity of a variety of enzyme functions, including those controlling proliferation, differentiation, or apoptosis [6, 7].

**1.2.1. Subcellular Distribution of GSH.** Intracellular GSH is differentially distributed among the various subcellular compartments of cytosol, mitochondria, endoplasmic reticulum, and nucleus wherein distinct redox pools are formed [8, 9]. Cytosolic GSH is highly reduced, and under physiological conditions cytosolic GSH concentrations are between 1 and 11 mM with the GSH to GSSG ratio maintained in excess of 10 to 1 depending on cell types [10]. The redox state of a cell is generally represented by the ratio of GSH to GSSG given the large GSH pool size. Quantitatively, the cytosolic pool accounts for >70% of the total cellular GSH, while the nuclear and mitochondrial compartments comprise 10% to 30% of the total cellular GSH, respectively [11]. The uniqueness of the nuclear and mitochondrial GSH pools is evidenced by the differences in compartmental GSH turnover rate and sensitivity to chemical depletion [9].

Specifically, the distinct characteristic of the nuclear GSH redox state is consistent with its physiological role in the nucleus, significantly during cell cycle [12] (Section 3.2 below). Indeed, increased nuclear-to-cytosol GSH distribution is a crucial factor in cell proliferation wherein elevated nuclear GSH maintains the functional integrity of the nucleus in gene transcription [13].

While the biological importance of metabolically unique GSH compartments in redox regulation of various endothelial cell functions [14, 15] is yet to be fully defined, it can be readily appreciated that such independent GSH pools would afford an elegant mechanism for specific control of redox-sensitive metabolic processes, the failure of which will have significant implications for endothelial pathobiology. The reader is referred to previous excellent reviews for a full discussion of redox compartmentation and its integration in redox signaling [3, 8, 15].

**1.2.2. GSH in Cellular ROS and Redox Signaling.** One of the undesired consequences for an organism living in an aerobic environment is an increased potential for oxidative damage by reactive oxygen species (ROS). However, the ability to thrive within such an aerobic environment also implies an evolved capability to handle ROS-mediated tissue damage [16]. The major intracellular sources of ROS, namely, superoxide anion ( $O_2^{\bullet-}$ ), hydrogen peroxide ( $H_2O_2$ ), or hydroxyl radical ( $HO^{\bullet}$ ), are derived from mitochondrial respiration, arachidonic acid pathway, and activities of cellular oxidases, such as cytochrome P450, glucose oxidase, amino acid oxidases, xanthine oxidase, NADH/NADPH oxidases, or NO synthases [17, 18]. ROS derived from xenobiotic metabolism or UV/ $\gamma$ -radiation are examples of exogenous sources. Elevated ROS levels are damaging to cellular macromolecules like proteins, lipids, and DNA and will induce a state of oxidative stress and redox imbalance [8]. Central to maintaining intracellular redox balance is GSH-dependent ROS elimination that includes GSH peroxidase-catalyzed hydroperoxide metabolism, GSSG reductase-catalyzed, NADPH-dependent GSH regeneration, or GSH S-transferase-catalyzed xenobiotic detoxication [19].

The recognition that ROS can serve as important mediators of cell signaling and that signal transduction may be mediated by ROS-induced GSH redox imbalance is major conceptual breakthrough in our understanding of GSH-dependent redox signaling [20, 21]. Significantly, low ROS levels participate in the signaling of proliferation, senescence, and apoptosis. For instance,  $H_2O_2$ -targeted proteins containing redox sensitive cysteine residues (P-SH) can result in the formation of reversible sulfenic (P-SOH) as well as irreversible sulfinic (P-SO<sub>2</sub>H), and sulfonic (P-SO<sub>3</sub>H) acid derivatives [22, 23]. The protein sulfenic acid derivative can further react with nitric oxide (NO) to yield nitrosothiol (P-SNO) or with another P-SH to form a disulfide bond (P-SS-P) [22, 23]. The latter posttranslational modification, termed S-glutathiolation (also known as S-glutathiolation), refers to the formation of a mixed disulfide between the cysteine of GSH and a cysteine moiety of a protein [6]. Reversible protein cysteine oxidation and protein

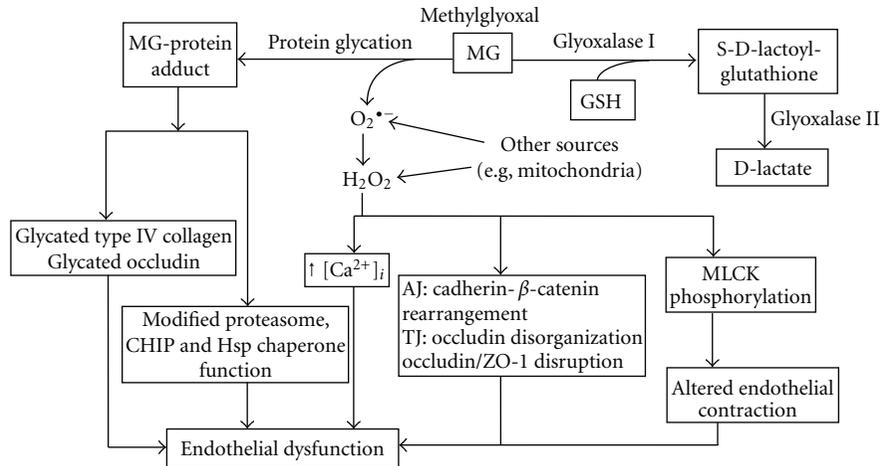


FIGURE 1: Mechanisms of MG-mediated endothelial barrier dysfunction and its protection by GSH. MG-induced endothelial barrier dysfunction can be caused by MG-protein crosslinking (glycation) resulting in the formation of MG-protein adducts, such as tight junction occludin and basement membrane type IV collagen. MG-protein glycation can also modify the proteasomal and chaperone functions. ROS generated during protein glycation can further mediate barrier dysfunction through various pathways: (a) increased intracellular  $[Ca^{2+}]_i$ , (b) direct disruption of adherens junction and tight junction, or (c) phosphorylation of myosin light chain kinase and altered endothelial cell contraction. Protection of barrier integrity is mediated by GSH, which functions as a cofactor in glyoxalase I-catalyzed metabolism of MG. MG: methylglyoxal, GSH: reduced glutathione,  $O_2^{\bullet-}$ : superoxide anion,  $H_2O_2$ : hydrogen peroxide, ROS: reactive oxygen species, AJ: adherens junction, TJ: tight junction, MLCK: myosin light chain kinase.

mixed disulfide formation are catalyzed by the thioredoxin (Trx) and glutaredoxin (Grx) family of redox proteins [6]. This GSH-protein cysteine interaction protects against irreversible protein thiol oxidation and is an important redox mechanism in regulating protein function at low or modest levels of ROS [6]. ROS-dependent protein cysteine oxidation has been implicated in the redox regulation of a wide range of protein functions including enzyme activity, protein expression and abundance, subcellular protein localization, and interaction with other molecular partners in controlling new patterns of cell signaling and gene expression. Viewed simply, control of protein functions by reversible S-glutathiolation/deglutathiolation is akin to that of phosphorylation/dephosphorylation.

**1.3. Endothelial GSH and S-Glutathiolation in the Control of Vascular Integrity.** GSH exerts profound effects on vascular endothelial function, which include endothelial barrier permeability [24], cell apoptosis [25], chemotaxis, angiogenesis [26, 27], constitutive and agonist-induced adhesion molecule expression [28], leukocyte-endothelial adhesion response [29], and endothelial dependent vasodilation [28, 30]. The modulatory effects of GSH are accomplished through the scavenging of ROS [31], an important second messenger in many endothelial functions. For instance, GSH was shown to attenuate  $H_2O_2$ -induced decrease in transendothelial electrical resistance via negative regulation of the activation of p38 MAP kinase [24]. In other roles, reduced GSH acts as a substrate for the detoxication enzymes, GSH peroxidase, and GSH S-transferase. Our recent studies showed that GSH served as a cofactor in glyoxalase 1-catalyzed detoxication of methylglyoxal and prevented carbonyl stress-induced brain endothelial barrier dysfunction (Figure 1).

A large body of evidence supports a role for S-glutathiolation in redox regulation of vascular function, ranging from cell signaling, apoptosis, protein folding, to cytoskeletal reorganization. In hypertensive vessels, the thiolation of endothelial nitric oxide synthase (eNOS) is pivotal in the redox control of vascular tone. The bioactive nitric oxide (NO) molecule plays a crucial role in normal endothelial function, including modulation of vascular dilator tone, inhibition of platelet activation, inhibition of leukocyte adhesion and migration, and inhibition of smooth muscle cell migration and proliferation [32]. Therefore, altered NO production, such as during oxidative stress, would compromise vascular homeostasis. Oxidative stress has been shown to mediate S-glutathiolation of eNOS that was associated with decreased NOS activity, attenuated NO production, increased  $O_2^{\bullet-}$  generation, and impaired endothelium-dependent vasodilation, dysregulated processes that were restored by thiol-specific reducing agents [33]. As for cell signaling, oxidants have been shown to trigger direct S-glutathiolation of p21ras at Cys<sup>118</sup>, which activated p21ras and mediated downstream phosphorylation of ERK and AKT in both endothelial and smooth muscle cells [34, 35]. Similarly, oxidant-induced insulin resistance was mediated through S-glutathiolation of p21ras and ERK-dependent inhibition of insulin signaling [36]. During diamide-induced oxidative stress, activation of endothelial  $Ca^{2+}$  signaling was associated with S-glutathiolation of the inositol-1,4,5-trisphosphate (IP3) receptor (IP3R) and the plasmalemmal  $Ca^{2+}$ -ATPase pump, which promoted  $Ca^{2+}$  release from IP3-sensitive internal  $Ca^{2+}$  stores and elevated basal  $[Ca^{2+}]_i$  in the absence of extracellular  $Ca^{2+}$  [37].

Current evidence implicates the involvement of S-glutathiolation/deglutathiolation in apoptotic signaling. In

TNF- $\alpha$ -mediated apoptosis, Grx-catalyzed deglutathiolation of procaspase-3 induced caspase-3 activation [38]. In Fas-mediated apoptosis, Fas thiolation following caspase-dependent Grx1 degradation resulted in the activation of caspases-8 and -3 [39]. Molecular chaperones are an interesting class of proteins that are readily S-glutathiolated wherein thiolated proteins exhibited potentiation of chaperone activities, such as the correct folding of newly synthesized polypeptides [6]. The activities of several S-glutathiolated members of the glucose-related protein (GRP) family of proteins including GRP78, heat shock protein 60 (Hsp60), heat shock cognate 71-kDa protein, and Hsp90 were similarly increased by S-glutathiolation in diamide-treated endothelial cells [40]. Remarkably, even endothelial cytoskeletal reorganization can be modulated by protein S-glutathiolation, notably that of actin and tubulin. Under physiological conditions, S-glutathiolated actin (at Cys<sup>374</sup>) inhibited F-actin polymerization, which was reversed by EGF via actin deglutathiolation [41], consistent with a dynamic role of actin assembly/disassembly in the biological process of cell division and cell growth. Notably, actin-glutathiolation also occurred under conditions of oxidative stress [42]; in this instance, intracellular actin disassembly or disrupted actin-junctional protein interactions would likely mediate the loss of endothelial barrier function. A role for S-glutathiolated annexin A2-actin interaction is currently unknown. Similarly, while S-glutathiolation of endothelial  $\beta$ -tubulin has been reported [40], the biological importance of this modification in endothelial barrier function remains to be defined.

**1.4. GSH Regulation: Transcriptional Control of GCLc and GCLm Expression.** GCL-catalyzed *de novo* synthesis is central to the preservation of tissue GSH balance, particularly during oxidative stress. GCL is a heterodimeric protein composed of catalytic (GCLc) and modifier (GCLm) subunits. The GCLc subunit alone possesses all of the catalytic activities of the enzyme; however, heterodimerization with the GCLm subunit increases GCL activity ( $V_{max}$  and  $K_{cat}$ ), substrate affinity ( $K_m$ ) for glutamate and ATP, and the  $K_i$  for GSH feedback inhibition [43]. Metabolic regulation of GCL is mediated by protein phosphorylation at serine and threonine moieties, which inhibits enzyme activity and transcriptional control of GCL function is through the expression of the catalytic and modulatory subunits.

**1.4.1. Regulation of GCL Catalytic (GCLc) and Modifier (GCLm) Subunits.** The promoters of GCLc and GCLm subunits share common elements and coordinate transactivation results in overall increase in subunit expression. Key mediators of GCL expression are the redox sensitive transcription factors, nuclear factor kappa B (NF- $\kappa$ B), Sp-1, activator protein-1 and -2 (AP-1, AP-2), and nuclear factor E2-related factor 2 (Nrf2) [44]. The promoter of the human *GCLc* gene contains consensus binding sites for AP-1, NF- $\kappa$ B, Nrf2, and for the antioxidant response (ARE) or electrophile responsive (EpRE) elements [44]. A proximal AP-1 element was crucial for the transcription of GCLc induced

by oxidative stress [45] while NF- $\kappa$ B was essential in TNF $\alpha$ -mediated increase in GCLc transcription either directly or indirectly via transactivation of AP-1 sites through induction of C-Jun expression [43]. Among the four AREs in the human GCLc promoter, ARE4 was important in the constitutive expression of hepatic GCLc induced by  $\beta$ -naphthoflavone ( $\beta$ -NF) or cytochrome P450 2E1 [46, 47]. In macrophages, elevated GCLc expression caused by homocysteine was mediated by ARE4 and the MERK-ERK1/2 kinase pathway [48]. Involvement of the PI3 kinase pathway was also described in adrenomedullin-induced transcriptional activation of the GCLc promoter [49]. Recent studies from our laboratory demonstrated a role for Nrf2 in the constitutive and insulin-induced endothelial GCLc expression [50]. Insulin-induced GCLc promoter activation was ARE4 dependent [51]. Significantly, the increase in GCL activity and GSH synthesis via insulin signaling and activation of the PI3K/Akt/mTOR/Nrf2/GCLc pathway prevented hyperglycemia-induced endothelial apoptosis [52]. Interestingly, rat GCLc promoter exhibited only three AREs in the 5'-flanking region, of which ARE3 was involved in Nrf2-dependent expression of GCLc [53], suggesting species differences in ARE requirements for GCLc activation.

Constitutive or induced posttranslational phosphorylation of GCLc further contributes to GCL control. In contrast to insulin and hydrocortisone, which induced GCLc gene expression [17], stress hormones such as glucagon and phenylephrine caused GCLc phosphorylation through activating the protein kinases, PKA, PKC, or Ca<sup>2+</sup>-calmodulin kinase [54, 55]. Notably, GCLc phosphorylation decreased GCLc activity.

The transcriptional regulation of GCLm is poorly understood. Current evidence shows that the human GCLm promoter also contained an ARE site that mediated Nrf2-dependent GCLm upregulation induced by  $\beta$ -NF and lipid peroxidation products [56, 57]. In rat liver, an ARE element similarly mediated the basal and TNF $\alpha$ -induced of the GCLm promoter activity [58]. Additionally, the rat GCLm promoter has an AP-1 consensus site for constitutive and tert-butylhydroquinone-induced GCLm expression. For reasons yet unclear, NF $\kappa$ B-dependent GCLm expression appeared to be linked to AP-1 activation within the GCLc promoter [43], suggesting possible cross-talk between the two promoters in subunit expression.

## 2. Oxidative Challenge and Endothelial Barrier Dysfunction

**2.1. Influence of Reactive Oxygen Species (ROS).** It is abundantly clear that oxidative stress induced by ROS such as O<sub>2</sub><sup>•-</sup>, HO<sup>•</sup>, or H<sub>2</sub>O<sub>2</sub> can elicit endothelial barrier dysfunction. Moreover, oxidative stress also increased intracellular endothelial calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>) [59, 60]; in pulmonary artery endothelial cells, the blockade of Ca<sup>2+</sup> entry abolished oxidative stress-induced solute permeability [61], indicating that oxidative stress was linked to elevated [Ca<sup>2+</sup>]<sub>i</sub>, an important modulator of endothelial permeability (Figure 1). In addition, oxidants like H<sub>2</sub>O<sub>2</sub> were shown to

increase the phosphorylation of myosin light chain kinase [62], suggesting that ROS can alter endothelial contraction and contribute to endothelial barrier dysfunction (Figure 1). This means that oxidant modulation of the cytoskeletal architecture of the endothelial monolayer could be central to the loss of barrier integrity. Moreover, increased ROS concentrations can decrease NO bioavailability through chemical inactivation to form the powerful oxidizing agent, peroxynitrite [63]. Tetrahydrobiopterin (BH4), a critical cofactor for eNOS function, is a crucial target for oxidation by peroxynitrite [64]. Significantly, BH4 oxidation and depletion were shown to induce eNOS uncoupling, a process that was associated with increased  $O_2^{\bullet-}$  generation and decreased NO production. In this regard, uncoupled eNOS is akin to a dysfunctional  $O_2^{\bullet-}$  generating enzyme that could contribute to endothelial oxidative stress and vascular dysfunction. The uncoupling of eNOS has been demonstrated *in vitro* and in hypertensive rat (SHR) models of cardiovascular pathophysiology, such as angiotensin-II-induced hypertension and diabetes [65].

Control of paracellular permeability in the endothelium is a function of the intercellular endothelial adherens junctions (AJ) and tight junctions (TJ), a complex structure comprised of specific junctional proteins. The cadherins,  $\alpha$ -catenin, and  $\beta$ -catenin proteins are components of the AJ, while the transmembrane proteins, occludin, claudin, junction adhesion molecule, and the cytoplasmic accessory zonula occludin (ZO-1, -2, and -3) proteins comprised the TJ [66].  $H_2O_2$ -induced barrier disruption has been shown to occur through rearrangement of endothelial cadherin and  $\beta$ -catenin and the disruption of  $\beta$ -catenin/cytoskeletal association [67], but the signaling events are unresolved. However, activation of ERK1/ERK2 signaling and occludin phosphorylation were shown to mediate the disorganization of occludin and the disruption of occludin-ZO-1 interactions on endothelial cell surfaces [68]. ROS activation of signaling pathways, such as PKC, may further regulate the phosphorylation state of other AJ and TJ proteins. In this regard, a reversal of thrombin-induced loss of the cadherin junctional proteins,  $\rho$ -catenin,  $\alpha$ -catenin, and p120, by PKC inhibitor has been described [69].

**2.2. Role of Carbonyl Stress.** Carbonyl stress is the result of enhanced reactive carbonyl species (RCS) production and decreased carbonyl-scavenging capability, leading to tissue accumulation of reactive dicarbonyl species, such as methylglyoxal (MG). MG is produced from cellular glycolytic intermediates and can induce carbonyl stress through irreversible reaction with free arginine residues of proteins to form advanced MG-glycated end product (AGE) [70] (Figure 1). The generation of protein carbonyls or protein-glycated products could be a major problem in diabetic neurovascular pathology. An MG-derived argpyrimidine adduct has been detected in human lens and kidney and in atherosclerotic lesions of diabetic patients [71–73], and argpyrimidine-modified heat shock protein 27 (Hsp 27) was shown to alter diabetic endothelial cell function [74]. Moreover, diabetes-associated hyperglycemia and MG-induced

modification of the corepressor *mSin3A* gene were linked to elevated angiotensin-2 transcription in microvascular endothelial cells [75]. Other evidence revealed that the modification of 20S proteasome by MG decreased proteasomal chymotrypsin-like activity and impaired the CHIP and chaperone-dependent quality control of the protein [76], leading to the accumulation of toxic aggregates and endothelial cell death. Further, MG-induced glycation of vascular basement membrane type IV collagen yielded hotspots of arginine-derived hydroimidazolone residues at RGD and GFOGER integrin-binding sites, causing endothelial cell detachment, anoikis, and inhibition of angiogenesis [77].

The crosslinking of MG and amino acids was shown to yield the  $O_2^{\bullet-}$  radical anion [78] that can be quenched by  $O_2^{\bullet-}$  scavenger and membrane-permeable catalase [79]. Significantly, MG-derived ROS has important implications for vascular and endothelial function. It is noteworthy that MG-induced mitochondrial  $O_2^{\bullet-}$  generation stimulated eNOS activity [79], while MG-mediated eNOS phosphorylation (at ser<sup>1777</sup>) attenuated endothelial NO production [80], suggesting that carbonyl stress modulation of endogenous endothelial NO production is a complex process. In rat carotid arterial endothelium MG was found to augment AT1R-induced NADPH oxidase-derived mediated  $O_2^{\bullet-}$  and  $H_2O_2$  production, which increased Ang II-dependent vascular contraction [81]. Similarly, MG-derived ROS mediated the oxidative and hyperglycemic stress-induced impairment of endothelium-dependent vasorelaxation. This oxidative stress response was attenuated by the overexpression of glyoxalase I which promoted MG degradation [82]. In recent studies, we found that MG-occludin glycation induced barrier dysfunction of human brain microvascular endothelial cells; surprisingly, MG-dependent endogenous ROS generation did not contribute majorly to barrier dysfunction. Our study further revealed that the endothelial GSH status is a determinant of barrier integrity by facilitating glyoxalase I-catalyzed MG metabolism and thereby decreasing the availability of free MG (Li and Aw, unpublished data, Figure 1). Moreover, GSH depletion significantly promoted MG-induced endothelial oxidative stress and cell apoptosis [25, 83].

Altered cell morphology, aberrant cytoskeletal rearrangement, and ZO-1 loss were notable biological consequences of glyoxal, another sugar-derived aldehyde product. Additionally, glyoxal also elicited mitochondrial dysfunction, inhibition of DNA and cell replication, and cell cytotoxicity through protein carbonyl formation [84]. Collectively, these findings underscore the wide-ranging cellular effects of carbonyl stress on vascular endothelial function.

### 3. Endothelial Repair through Proliferation and Growth

**3.1. Biology of Cell Cycle Control.** Cell cycle control is crucial for proper postdamage endothelial repair and growth. The mammalian cell cycle is characterized by a quiescent  $G_0$  phase of nondividing cells followed by cell entry into the cell cycle at  $G_1$  and progression through the S,  $G_2$ , and M phases

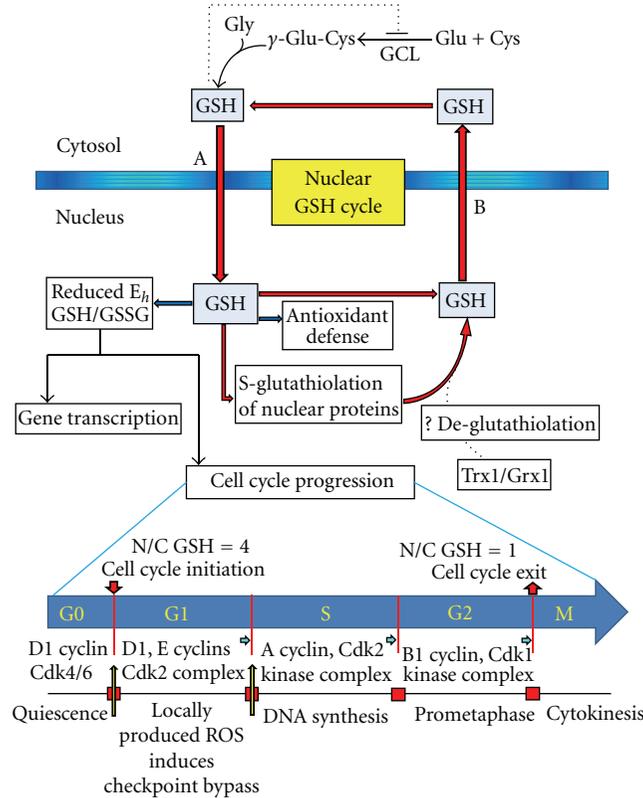


FIGURE 2: Nuclear glutathione cycle and associated redox changes during cell cycle progression. A nuclear GSH cycle is established during cell cycle progression that involves the dynamic partitioning of cellular GSH between the nuclear and cytosolic compartments. Cell entry into the cycle in early  $G_1$  is associated with sequestration of GSH into the nucleus (A). At this stage of cell cycle initiation, the nuclear-to-cytosol (n/c) GSH ratio approximates 4. The transient decrease in cytosolic GSH releases feed-back inhibitory effect of GSH on GCL activity and triggers *de novo* GSH synthesis, a process that continues until the feedback control is reestablished. Sequestered intranuclear GSH exists in the reduced form or bound to nuclear proteins, which together changes the GSH/GSSG redox potential ( $E_h$ ) in favor of gene transcription and cell cycle-associated DNA synthesis/replication. Free GSH functions in antioxidant defense that protects against oxidative DNA damage during DNA replication. As yet unclear, free GSH may be regenerated via deglutathiolation of thiolated nuclear proteins, likely catalyzed by Trx1 and/or Grx1. The dissolution of the nuclear envelope in the prometaphase and cytokinesis (cell cycle exit) induces nuclear-to-cytosol GSH export (B) resulting in equal GSH distribution (n/c = 1) in the two compartments in the newly divided cells. Redox-dependent activation of regulatory checkpoints governs cell exit from quiescence (cyclin D1 and associated Cdk4), entry into and progression through cell cycle (cyclin E1-Cdk2, cyclin A-Cdk2 kinase complexes), and final exit from cell cycle (cyclin B1-Cdk1 kinase complex) (blue arrows). Additionally the checkpoints at the  $G_0/G_1$  or  $G_1$ -to-S transitions can be bypassed by locally generated ROS (yellow arrows). GSH: glutathione, GSSG: glutathione disulfide, GCL:  $\gamma$ -glutamate cysteine ligase; n/c: nuclear-to-cytosol, Trx1: thioredoxin1, Grx1: glutaredoxin 1, and Cdk: cyclin-dependent kinase.

in response to environmental or cellular cues that overcome the biological constraint of a mitotic block [20]. DNA replication takes place during the S phase, and accurate replication commits cell progression into the M phase while aberrant DNA replication induces transient  $G_2$  arrest that allows for DNA repair [85]. Failure of DNA repair initiates cell cycle withdrawal and permanent senescence. Cell progression through the cell division cycle is governed by regulatory checkpoints controlled by specific serine/threonine cyclin-dependent kinases (CDKs) and their respective cyclin subunits. Specifically, the checkpoints for cell transitions from  $G_0/G_1$  to S, late  $G_1$  to early S, S to  $G_2$ , and  $G_2$  to M are, respectively, regulated by D-type cyclin D1, D2 and associated with CDK4-6, cyclin E1/CDK2 complex, cyclin A/CDK2 kinase complex, and cyclin B1/CDK1

kinase complex in association with Cdc25 phosphatase [86] (Figure 2).

**3.2. Glutathione and Cell Cycle Regulation.** The progression of cells through the cell cycle has been linked to dynamic changes in the intracellular redox environment particularly that of the GSH/GSSG redox couple from a more oxidized state prior to cell cycle initiation to a more reduced state throughout cell cycle until cell cycle exit after prometaphase and cytokinesis (Figure 2). Specifically, studies have documented that cell exit from the quiescent stage at  $G_0$ /early  $G_1$  and entry into cell cycle was characterized by a relatively more oxidizing milieu [87, 88] than that during progression from  $G_1$  through S to  $G_2/M$  [87, 89]. The redox status of cysteine residues of cell cycle regulatory

proteins and their functions were highly sensitive to the intracellular redox environment, which is impacted by cellular production and/or removal of ROS [86]. For example, in actively dividing cells, redox-dependent activation of specific cyclin/CDKs complexes by locally produced ROS allowed for checkpoint bypass at the  $G_1$  restriction point or at late  $G_1$  to S transition [7, 14, 90]. Similarly, growth-factor-mediated ROS production and redox regulation of p16, p27, and cyclin D1, which drove terminally differentiated cells into cell cycle [91, 92], governed the reentry of quiescent cells into the cell cycle [91, 92]. E2F, pRB, MAP kinase, Cdc25 phosphatase, and cyclin are other important cell cycle proteins shown to undergo redox changes and/or modifications during cell cycle progression [90, 93–95].

A role for ROS in mitogenic signaling is underscored by the finding that treatment of serum-starved cells with the thiol antioxidant, N-acetylcysteine (NAC), elicited cell cycle arrest at  $G_1$ , a delay of  $G_0$  to  $G_1$  progression that correlated with defective redox control [92]. Interestingly, during exponential growth of cultured mouse embryonic fibroblasts, NAC treatment arrested cells at the  $G_1$  to S transition but allowed cell transit through the S,  $G_2$ , and M phases [96], indicating that redox control at the early event at  $G_1$  governed cell progression from  $G_1$  to S. An increase in MnSOD activity was implicated in NAC-induced inhibition of  $G_1$  to S entry [90]. Collectively, these studies illustrate the importance of ROS in mitogenic signaling during cell cycle, a redox process that appears to be coordinated through defined cellular mechanisms for ROS generation and elimination. A reduced intracellular redox environment protected genomic DNA from oxidative damage upon breakdown of the nuclear envelope [89] and was therefore essential to enhance DNA synthesis during cell transition from  $G_1$  to  $G_2$ /M. Early accumulation of soluble thiols at the mitotic spindle was observed during mitosis in sea urchin eggs [97]. Similarly a graduation of low to high GSH content was associated with the transition of Chinese hamster ovary fibroblasts through  $G_1$  to S to  $G_2$ /M [89], consistent with a well-defined dynamics of redox changes in the intracellular environment during cell cycle.

Intracellular redox homeostasis is maintained by the thiol/disulfide redox systems of GSH/GSSG, thioredoxin (Trx/TrSS), and cysteine (Cys/CySS). The product of reducing potential and reducing capacity of the redox couples determined the cellular redox environment, which in most cells are largely governed by that of the GSH/GSSG couple [4]. Indeed, the cellular GSH/GSSG redox status provides a good quantitative indicator of the intracellular redox state, often expressed as the redox potential,  $E_h$ . Under physiological conditions,  $E_h$  for GSH/GSSG, as calculated by the Nernst equation, is between  $-260$  mV and  $-200$  mV [15]. Notably, a change in GSH/GSSG  $E_h$  from a reduced value of  $\sim 260$  mV to an oxidized value of  $-170$  mV was associated with phenotypic cell transition from proliferation to growth arrest and apoptosis [15]. As discussed in Section 1.2.1, specificity of redox signaling and independent redox regulation of the functions of single proteins or protein sets are in part attributed to the existence of distinct compartments of GSH within the subcellular organelles.

Recent evidence suggests that the dynamic cytosol-to-nuclear GSH distribution was a crucial factor in cell cycle progression in that nuclear GSH accumulation provided an intranuclear redox environment that enabled proper regulation of redox signaling events during the various stages of the cell cycle [13]. A novel concept of a nuclear GSH cycle that operated during cell cycle has been proposed [98] as illustrated in Figure 2. According to this hypothesis, GSH was recruited and sequestered into nucleus in early  $G_1$  phase, likely through a Bcl-2-dependent import mechanism [99]. Increased cytosolic-to-nuclear GSH translocation transiently caused GSH imbalance within the cytosol that initiated *de novo* GSH synthesis, resulting in progressive increases in the total cytosolic GSH pool. Cell transition through  $G_2$ /M and the dissolution of the nuclear envelope during mitosis enabled the reequilibration of the cytosolic and nuclear GSH pools, and this return to a pre-cell cycle nuclear-to-cytosolic GSH ratio of 1 to 1 was maintained in non-proliferating cells at  $G_0$ / $G_1$ . It was further proposed that it was the transient decrease in cytosolic GSH that promoted early  $G_1$  signaling. Moreover, the increased GSH presence in the nucleus during the S phase coincided with the activation of DNA replication as evidenced by elevated S-glutathiolation of histones, telomerase, and polyADP ribose polymerase [13, 100]. Additionally, DNA synthesis and replication could be further facilitated by GSH-dependent reorganization of the nuclear matrix and chromatin structure [101]. The details of GSH control of cell cycle checkpoints during endothelial cell proliferation are sketchy and are the subjects of current investigation in our laboratory.

**3.3. Glutathione Disruption and Implications for Endothelial Growth and Repair.** As an organ that is highly dependent on oxidative metabolism for its energy needs, the brain is susceptible to tissue GSH imbalance and oxidative damage mediated by increased formation of free radical species and lipid peroxidation [102, 103]. Given the location of the BBB at the interface between brain parenchyma and systemic blood, the endothelial monolayer is easily exposed to the oxidizing conditions of elevated ROS or RCS associated with various pathological states (Section 4 below). Additionally, an often decreased tissue or systemic GSH level under these diseased states would enhance oxidative damage to the vascular endothelium and the consequent loss of vascular integrity will have important implications for cerebral homeostasis. The enzyme  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT) is regarded as a marker of BBB integrity in the mammalian brain. It is noteworthy that  $\gamma$ -GT levels were lowest in the more primitive regions of the brain and highest in the more specialized regions of the brain [104], the reason of which is yet unknown. Importantly, within the brain, the microvesicular fractions exhibited significantly higher  $\gamma$ -GT activity than the neuronal or glial fractions, consistent with a microvesicular localization of the enzyme [104]. However,  $\gamma$ -GT activity in type I cells (“cobblestone” phenotype) increased 10–12-fold after glial stimulation, indicating a role for type I cells in BBB function as well [105]. Equally notable was the finding that membrane-associated  $\gamma$ -GT

activity in the endothelium of capillaries was higher than that in larger vessels in the brain, implying that cerebral small vessel endothelial monolayers will likely be more sensitive to fluctuations in the plasma GSH levels in terms of both susceptibility to injury and efficiency of repair. Furthermore, given that  $\gamma$ -GT can catalyze the metabolism of not only GSH but also S-nitrosoglutathione (GSNO), cerebral microvascular  $\gamma$ -GT function could be pivotal in mediating the bioactivity of GSNO and/or NO (Section 4.2).

The findings that GSH levels in endothelial cells in culture increased during the lag phase, were elevated during the initial exponential growth phase, and then fell as cells become confluent [106] suggest that systemic GSH interruption would alter endothelial growth. Our recent studies in human microvascular endothelial cells showed that inhibition of GSH synthesis and GSH depletion elicited a delayed S-to-G<sub>2</sub> transition reflected in a lengthening of the cell cycle S-phase resident time (Busu and Aw, unpublished), in agreement with previous observations [107]. Significantly, cellular GSH depletion was largely confined to the cytosolic pool while the nuclear GSH compartment remained relatively unchanged. Somewhat surprisingly, delayed S-to-G<sub>2</sub> transition remained evident for 6 h despite the restoration of cytosolic GSH synthetic capacity and near normalization of basal cellular GSH levels (Busu and Aw, unpublished), consistent with a significant time lag between restored cellular redox balance and recovery of normal cell cycle activity. The reason for this temporal dissociation is unclear and is currently under investigation in our laboratory. What is clear, however, is that through perturbing cell cycle events, a disruption in cellular GSH such as that occurring during oxidative or carbonyl stress could delay endothelial proliferation and tissue repair following oxidative damage to the endothelium, a deleterious scenario for brain function in cerebrovascular and neurodegenerative disorders.

#### 4. Pathological Implications of Impaired Glutathione in Neurovascular Disease States

**4.1. Neurovascular Pathology of Diabetes.** Increased BBB permeability has been demonstrated in patients with type II diabetes [108] and in the streptozotocin- (STZ-) induced type I diabetic experimental rat model [109]. Elevated activities of plasma metalloproteinases 2 and 9 were implicated in the loss of tight junctional proteins (occludin, claudin-5, ZO-1, and JAM-1) and BBB failure [110]. Interestingly, the receptor for AGE (RAGE) was upregulated during diabetes [111], suggesting that increased plasma-to-cellular MG uptake and enhanced GSH-dependent intracellular MG catabolism could provide a means to attenuate the elevated systemic MG levels associated with the diabetic state. BBB disruption was notable during diabetic ketoacidosis wherein neurovascular inflammation, accompanying CCL-2 chemokine expression, NF- $\kappa$ B activation, and nitrotyrosine formation were likely contributors to the attenuated BBB integrity and increased barrier permeability [112]. In STZ-induced diabetic rats, BBB function was improved by the administration of growth

hormone and insulin [113, 114]. Our recent studies demonstrated that insulin-mediated protection of human microvascular endothelial cells against MG-induced apoptosis was the result of increased intracellular GSH through activation of the insulin-PI3K/Akt/mTOR/Nrf2/GCLc signaling pathway [50, 52].

It is well known that diabetes is associated with hyperglycemia, elevated oxidative and carbonyl stress, and low tissue and plasma levels of GSH [115–120], conditions that complicate the diabetic state, which would lead to further exacerbation of GSH loss. Thus, mechanisms that promote neurovascular GSH status or those that attenuate oxidative and/or carbonyl stress could preserve endothelial barrier function. A viable approach could involve activation of insulin signaling to maintain cellular GSH balance and support GSH-dependent attenuation of oxidative or carbonyl stress mediated by ROS or MG [25, 51, 52, 121]. Furthermore, increasing GSH protection of redox sensitive thiols of membrane proteins, including those of the AJ or TJ, could preserve the functional integrity of the endothelium. The question of whether acute or chronic GSH therapy would be effective in abrogating systemic hyperglycemia-linked oxidative and carbonyl stress and mitigate diabetes-associated BBB dysfunction remains an open question that warrants further investigation.

**4.2. Microvascular Dysfunction in Stroke.** Stroke is a cerebrovascular disorder wherein a blood clot or interrupted blood flow to a region of the brain leads to a rapid loss of brain function. Significantly, a lack or delayed flux of oxygen and glucose to the brain will result in neuronal death and brain damage. Clinical studies have shown that subjects at risk for stroke exhibited low tissue GSH levels and decreased GSH-to-GSSG ratio and that the restoration of normal cerebral GSH balance could be as long as 72 h after the ischemic insult [122, 123]. Importantly, acute ischemic stroke was associated with elevated oxidative stress, a major contributor to immediate and delayed ischemic brain injury and changes in the parenchymal GSH redox status [124, 125]. An increase in free radical production during acute cerebral ischemia can arise from multiple sources including stimulation of N-methyl-D-aspartate receptors [126], mitochondrial dysfunction [127], activation of neuronal NO synthase (NOS) [128, 129], autooxidation of catecholamines, and metabolism of free fatty acids [130]. The activation and migration of inflammatory cells, such as neutrophils, further contributed to O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> generation [130].

The restoration of endothelial integrity after thrombotic or hemorrhagic stroke is crucial to preserving BBB function and neurovascular homeostasis. The proliferation of endothelial cells adjacent to the lesion or injury site is a pivotal step. Given the role of GSH in cell proliferation (Section 3), maintaining cellular GSH balance is therefore essential for postdamage endothelial repair and wound healing. S-nitrosoglutathione (GSNO) is an important physiological metabolite produced by the reaction of NO with GSH [131] that is involved in NO storage and release through the function of  $\gamma$ -GT [132]. The affinity of  $\gamma$ -GT

for GSNO ( $K_m$  of 0.4 mM) was comparable to other  $\gamma$ -glutamyl substrates [132], suggesting a physiological role for  $\gamma$ -GT-in GSNO metabolism. Whether high micromolar concentrations of GSNO are achievable in cells remains uncertain. However, recent studies demonstrated that, at least in plasma, GSNO levels are likely to be higher than previously reported due to the presence of exogenous  $\gamma$ -GT [133], further underscoring the significance of the enzyme in modulating GSNO levels and bioactivity.

Reportedly, GSNO functions in cellular signaling [134, 135] and protection of the central nervous system (CNS) against excitotoxicity, inflammation, and ROS [136, 137]. Notably, GSNO protection against peroxynitrite-induced oxidative stress is severalfold more potent than GSH [138]. GSNO-mediated CNS protection against inflammation appeared to be through suppressing iNOS induction and promoting eNOS expression, and maintaining cerebral blood flow [139]. The anti-inflammatory activity of GSNO in downregulating iNOS was mediated by inhibition of NF- $\kappa$ B activation and decreased expression of ICAM-1 and ED-1. Additionally, the expression of ZO-1 and occludin at endothelial tight junctions was enhanced by GSNO treatment [140]. Unlike conventional NO donors that mediate quick NO release, GSNO elicits slow NO release that was implicated in neurovascular protection against ischemia reperfusion [141]. In addition to  $\gamma$ -GT, S-nitrosoglutathione reductase (GSNOR), which catalyzes the reduction of GSNO, has been shown to be an important regulator of the endogenous GSNO levels and NO bioactivity. The pathophysiological role of GSNOR in SNO signaling and NO bioactivity in the regulation of vascular tone is incompletely understood; recent evidence suggests that GSNOR regulates airway SNO levels in cell signaling [142] and protects against nitrosative stress and cancer risk in human lung [143]. This notwithstanding, it remains unclear whether therapeutic strategies involving exogenous GSH and/or NO supplementation during neurovascular inflammatory conditions, such as stroke, would be clinically efficacious in the short term in attenuating the oxidative burden and protecting the BBB, or in the long term in reducing brain edema and tissue damage.

## 5. Summary and Perspective

The integral function of the microvascular endothelium underpins cerebrovascular homeostasis. ROS- and/or RCS-induced endothelial dysregulation is an underlying concern in barrier failure, and, as such, much research has focused on the use of antioxidants as a strategy to attenuate oxidative or carbonyl stress and restore monolayer function. The finding that GSH, a major cellular antioxidant, is able to afford cytoprotection supports the notion that antioxidant therapy is important in endothelial barrier preservation. In past years, more recent conceptual advances in redox cell biology have uncovered a fundamental role of GSH in signal transduction and redox signaling in cellular functions. Moreover, the finding that distinct pools of GSH exist in subcellular organelles that allow for independent redox regulation has revolutionized our thinking of GSH-dependent redox

mechanisms in controlling metabolic processes. One such biological process is that of cell proliferation. In the context of enhanced endothelial proliferation and self-repair surrounding lesion sites in response to systemic cues, for example, growth factors, little is known of a role for GSH. The dynamics of cytosol-to-nuclear GSH distribution appears to be pivotal in governing cell cycle responses. The notion that cell proliferation and growth can be a relevant biological process for monolayer repair/restitution following endothelial injury in much the same way as epithelial cell restitution/proliferation restores postinjured epithelium suggests exciting new avenues for future research in endothelial biology. Importantly, an understanding of GSH control of endothelial cell proliferative potential under different oxidizing conditions and plasma GSH levels will expand our perspective for future development of therapeutic strategies. Targeting endothelial restoration after oxidative insult and tissue damage is likely to be clinically relevant to the neurovascular disorders of diabetes and stroke and additionally could have broader implications for neurodegenerative and neurological disorders as well.

## Abbreviations

AGE:	advanced glycated end product
AJ:	Adherens junctions
AP-1:	Activator protein-1
AP-2:	Activator protein-2
ARE:	Antioxidant response element
BBB:	Blood-brain barrier
CDK:	Cyclin-dependent kinases
CNS:	Central nervous system
Cys:	Cysteine
CySS:	Cystine
$E_h$ :	Redox potential
eNOS:	Endothelial nitric oxide synthase
EpRE:	Electrophile responsive element
GCL:	$\gamma$ -glutamyl cysteine ligase
GCLc:	GCL catalytic subunit
GCLm:	GCL modifier subunit
GRP:	Glucose-related protein
Grx:	Glutaredoxin
GSH:	Glutathione
GSNO:	S-nitrosoglutathione
GSNOR:	S-nitrosoglutathione reductase
GSSG:	Glutathione disulfide
H <sub>2</sub> O <sub>2</sub> :	Hydrogen peroxide
Hsp27:	Heat shock protein 27
Hsp60:	Heat shock protein 60
IP3:	Inositol-1,4,5-trisphosphate
IP3R:	IP3 receptor
MG:	Methylglyoxal
NAC:	N-acetylcysteine
NF- $\kappa$ B:	Nuclear factor kappa B
NO:	Nitric oxide
NOS:	NO synthase
Nrf2:	Nuclear factor E2-related factor 2
O <sub>2</sub> <sup>•-</sup> :	Superoxide anion radical
RCS:	Reactive carbonyl species

ROS: Reactive oxygen species  
 STZ: Streptozotocin  
 TJ: Tight junctions  
 Trx: Thioredoxin  
 ZO: Zonula occluding protein  
 $\beta$ -NF:  $\beta$ -naphthoflavone.

## Authors' Contribution

W. Li and C. Busu, contributed equally.

## Conflict of Interests

The authors declare no conflict of interests.

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## Research Article

# Pharmacological Characterization of the Mechanisms Involved in Delayed Calcium Deregulation in SH-SY5Y Cells Challenged with Methadone

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Previously, we have shown that SH-SY5Y cells exposed to high concentrations of methadone died due to a necrotic-like cell death mechanism related to delayed calcium deregulation (DCD). In this study, we show that, in terms of their Ca<sup>2+</sup> responses to 0.5 mM methadone, SH-SY5Y cells can be pooled into four different groups. In a broad pharmacological survey, the relevance of different Ca<sup>2+</sup>-related mechanisms on methadone-induced DCD was investigated including extracellular calcium, L-type Ca<sup>2+</sup> channels,  $\mu$ -opioid receptor, mitochondrial inner membrane potential, mitochondrial ATP synthesis, mitochondrial Ca<sup>2+</sup>/2Na<sup>+</sup>-exchanger, reactive oxygen species, and mitochondrial permeability transition. Only those compounds targeting mitochondria such as oligomycin, FCCP, CGP 37157, and cyclosporine A were able to amend methadone-induced Ca<sup>2+</sup> dyshomeostasis suggesting that methadone induces DCD by modulating the ability of mitochondria to handle Ca<sup>2+</sup>. Consistently, mitochondria became dramatically shorter and rounder in the presence of methadone. Furthermore, analysis of oxygen uptake by isolated rat liver mitochondria suggested that methadone affected mitochondrial Ca<sup>2+</sup> uptake in a respiratory substrate-dependent way. We conclude that methadone causes failure of intracellular Ca<sup>2+</sup> homeostasis, and this effect is associated with morphological and functional changes of mitochondria. Likely, this mechanism contributes to degenerative side effects associated with methadone treatment.

## 1. Introduction

Methadone (D,L-methadone hydrochloride) is frequently used in different therapies including opioid addiction [1], long-lasting analgesics in cancer and neuropathic pain syndromes [1–3]. However, numerous reports indicate a negative impact on human cognition by chronic exposure to opioid drugs. Patients subjected to methadone maintenance programs show impaired cognitive abilities in aspects such

as psychomotor performance, information processing, attention, problem solving, memory, decision making, reaction time, and emotional facial expression recognition [4–10].

Changes in the cytosolic free-calcium concentration ([Ca<sup>2+</sup>]<sub>cyt</sub>) are involved in control of a large number of cellular and physiological processes including neuronal excitability, synaptic plasticity, and gene transcription [11, 12]. However, the physiological Ca<sup>2+</sup> signal can switch to a death signal when the [Ca<sup>2+</sup>]<sub>cyt</sub> increases dramatically.

For example, excitotoxic high glutamate concentrations result in an initial transient increase in  $[Ca^{2+}]_{cyt}$  that is followed by a delayed, irreversible rise in  $[Ca^{2+}]_{cyt}$  known as delayed calcium deregulation (DCD). Although several steps preceding DCD remain to be clarified, there is evidence that DCD is the irreversible end point of a sequence involving mitochondrial  $Ca^{2+}$  overloading. DCD precedes and reliably predicts the necrotic death of cultured neurons [13].

Mitochondria are important for cellular  $Ca^{2+}$  homeostasis. They buffer variations in  $Ca^{2+}$  concentrations by taking up  $Ca^{2+}$  when and where  $[Ca^{2+}]_{cyt}$  levels are passing a threshold level above which the mitochondrial  $Ca^{2+}$  uniporter is activated, and slowly release  $Ca^{2+}$  back to the cytosol when  $[Ca^{2+}]_{cyt}$  drop below this point [14]. Mitochondrial  $Ca^{2+}$  overload, if large and sustained enough, may contribute to mitochondria permeability transition pore (MPTP) formation and ultimately lead to cell death [11, 15]. Because, mitochondria may accumulate a considerable amount of  $Ca^{2+}$  during neurotoxic exposure, a possibility is that DCD may represent the final consequence of mitochondrial  $Ca^{2+}$  overload. MPTP is a large, proteinaceous,  $Ca^{2+}$ -activated, proton- and ADP-inhibited voltage-dependent pore. It spans the inner and outer mitochondrial membrane allowing the passage of ions and substrates less than 1.5 kDa. Characteristically, opening of the MPTP is inhibited by cyclosporin A [16, 17].

SH-SY5Y cells are considered a suitable model for investigating opioid-mediated responses in neurons. These cells express both  $\mu$ - and  $\delta$ -opioid receptors [18]. In previous studies, we showed that SH-SY5Y cells exposed to high concentrations of methadone (0.5 mM) died through a necrotic-like cell death mechanism and that methadone may induce changes in the  $[Ca^{2+}]_{cyt}$  [19, 20]. However, the underlying mechanisms causing alterations of the  $[Ca^{2+}]_{cyt}$  in SH-SY5Y cells in the presence of methadone remained unknown. Therefore, the aim of the present study was to investigate those mechanisms. A clear understanding of the factors that mediate this phenomenon might help to resolve the mechanisms that promote neuronal cell death during methadone-induced cognitive damage.

## 2. Material and Methods

**2.1. Cells Cultures.** SH-SY5Y cells (ATCC) were plated at a density of  $5.3 \times 10^4$  cells/cm<sup>2</sup> on  $\mu$ -Dish 35 mm High IbiTreat (ibidi GmbH, Martinsried, München, Germany) as previously reported [21].

**2.2. Calcium Measurements.** Changes in  $[Ca^{2+}]_{cyt}$  in SH-SY5Y cells were measured by loading cells with the calcium probe Fura2/AM and using an inverted fluorescence microscope (Nikon Eclipse TE2000-S) as described elsewhere [22]. Cells were perfused with a medium containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 2.5 mM CaCl<sub>2</sub>, 10 mM Hepes, and 11 mM Glucose, pH 7.35. Ratios of fluorescence emission excited at 340 and 380 nm were captured every 5 seconds [22]. Methadone or other compounds were added from 1000x stock solutions to reach the appropriate final

concentrations. The effects of selected compounds were tested on  $[Ca^{2+}]_{cyt}$  in the absence and the presence of 0.5 mM methadone in different sets of cells. Cells were pooled according to the dynamics of the rises in  $[Ca^{2+}]_{cyt}$ .

**2.3. Mitochondrial Morphology.** To directly visualize mitochondrial morphology changes in intact cells overexpressing pDsRed2-mito plasmid (Clontech Laboratories, Inc. Mountain View, CA, USA) a Leica SP2 confocal microscope (63 × 1.4 NA objective) was used. For transfection of cells the reagent Lipofectamine was used (Invitrogen, Carlsbad, CA, USA) [23].

**2.4. Mitochondria Isolation.** Rat liver mitochondria were isolated in MSH/EDTA and MSH media containing 210 mM mannitol, 70 mM sucrose, 5 mM Hepes, with or without 1 mM EDTA, pH 7.4, by differential centrifugation according to the standard procedure [24]. Mitochondrial protein concentration was measured using the Micro BCA Protein Reagent Kit. The mitochondrial suspensions were kept on ice and immediately used for measurements of oxygen-uptake rate.

**2.5. Mitochondrial Oxygen Uptake.** The rate of oxygen uptake of isolated rat liver mitochondria was measured at 37°C in a water-thermostated incubation chamber with a computer-controlled Clark-type O<sub>2</sub> electrode (Oxygraph, Hansatech, UK) in 0.5 ml incubation buffer (145 mM KCl, 30 mM Hepes, 5 mM KH<sub>2</sub>PO<sub>4</sub>, 3 mM MgCl<sub>2</sub>, 0.1 mM EGTA, 0.1% defatted BSA, pH 7.4). The respiratory substrates used were complex I- or complex II-linked, 2.5 mM glutamate/2.5 mM malate or 5 mM succinate in the presence of 2  $\mu$ M rotenone. The following additions were applied: 250  $\mu$ M ADP, 200  $\mu$ M CaCl<sub>2</sub>, 0.4  $\mu$ M FCCP, and 0.5 mM methadone. For estimating mitochondrial  $Ca^{2+}$  uptake in isolated mitochondria a  $Ca^{2+}$  index was calculated, which denotes the ratio of oxygen-uptake rate triggered by addition of 200  $\mu$ M  $Ca^{2+}$  to previous oxygen uptake rate.

## 3. Results and Discussion

In this study, we have investigated the mechanisms involved in methadone-induced rises in  $[Ca^{2+}]_{cyt}$  in SH-SY5Y cells. Consistent with previous observations from our laboratory [19] and other data [25], methadone induced a rise in  $[Ca^{2+}]_{cyt}$  in most of the SH-SY5Y cells. However, the effect of methadone differed considerably across cells. Analysis of the dynamics of the  $[Ca^{2+}]_{cyt}$  recordings in the absence and the presence of methadone suggested that four different types of calcium recordings can be observed in SH-SY5Y cells. Figure 1(a) shows typical examples of recordings. For the first group of cells no rise in  $[Ca^{2+}]_{cyt}$  was observed during the entire period (30 min) of measurements (type 1). A second group of cells was unable to regulate  $[Ca^{2+}]_{cyt}$  homeostasis shortly after the addition of methadone (type 2) and  $[Ca^{2+}]_{cyt}$  increased continuously. A third group displayed  $[Ca^{2+}]_{cyt}$  deregulation with a delay of 15–20 minutes (type 3). Finally, the fourth group was able to control  $[Ca^{2+}]_{cyt}$  after an initial increase (type 4). Groups

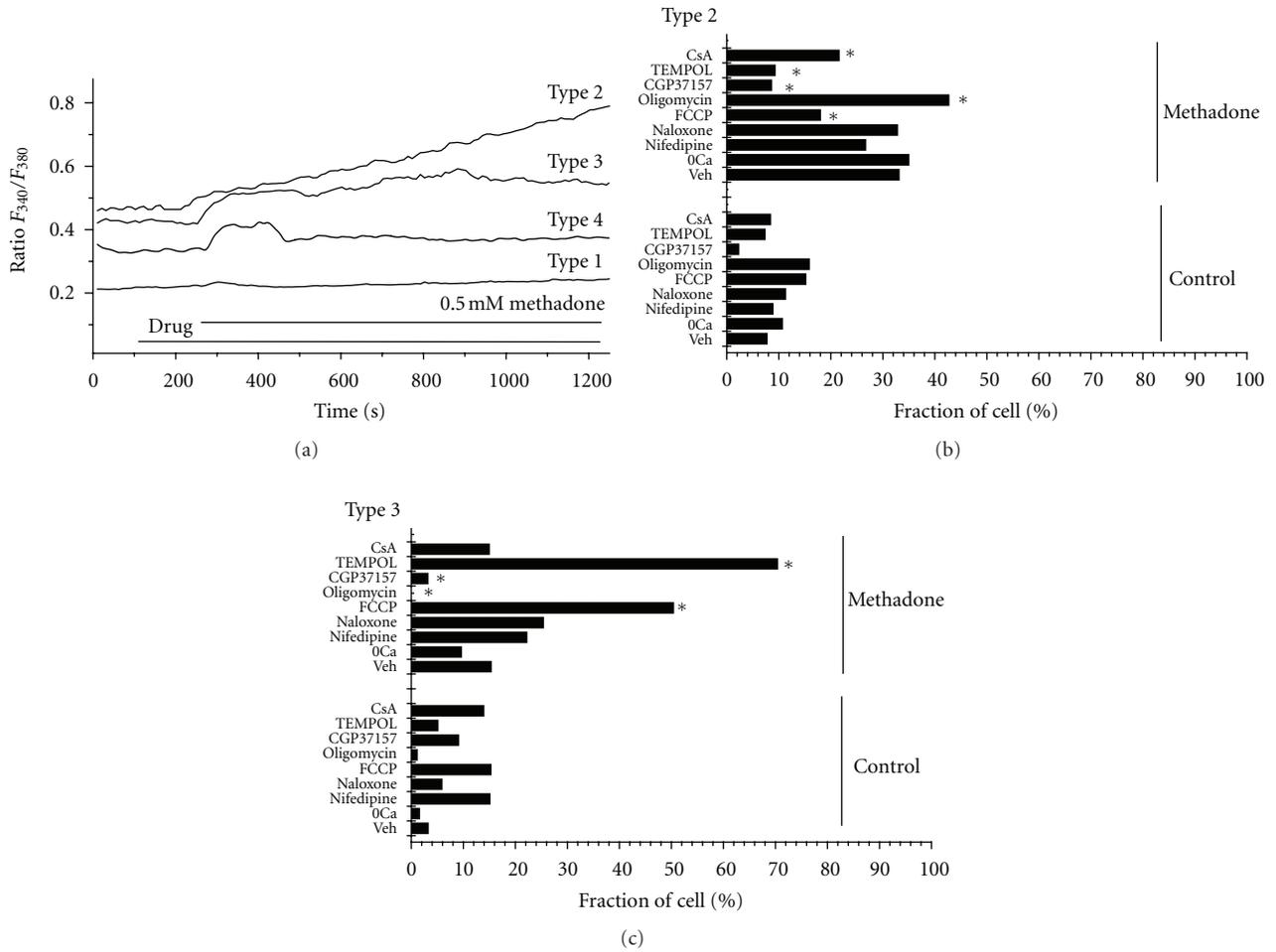


FIGURE 1: Methadone induces Ca<sup>2+</sup> dyshomeostasis. (a). Effects of 0.5 mM methadone on [Ca<sup>2+</sup>]<sub>cyt</sub> in SH-SY5Y cells. Representative recordings of the four different types of response including no [Ca<sup>2+</sup>]<sub>cyt</sub> increase (type 1), early [Ca<sup>2+</sup>]<sub>cyt</sub> sustained increase (type 2), delayed [Ca<sup>2+</sup>]<sub>cyt</sub> increase (type 3), and transient [Ca<sup>2+</sup>]<sub>cyt</sub> increase (Type 4). (b)-(c) Relative abundance (%) of SH-SY5Y cells that display type 2 (b) or type 3 (c) recordings without (Control) and with methadone treatment (Veh). Also shown are the effects of several treatments on the above mentioned relative abundance. Treatments include removal of extracellular calcium (0 Ca), L-type Ca<sup>2+</sup> channel blocker Nifedipine (2 μM); opioid receptor antagonist Naloxone (50 μM), mitochondrial uncoupler FCCP (1 μM); ATP synthase inhibitor oligomycin (10 μg/ml), mitochondria Na<sup>+</sup>/Ca<sup>2+</sup> exchanger CGP37157 (25 μM); superoxide dismutase mimetic TEMPOL (0.2 μM) or MPTP antagonist cyclosporine A (1 μM, CsA). All treatments were performed 5 min prior to addition of 0.5 mM methadone. Data represent results obtained in at least 3 independent experiments. \**P* < 0.05; Student's *t*-test versus basal conditions, (Veh).

1 and 4 represent, respectively, nonresponsive of cells and cells responding with transient rise in [Ca<sup>2+</sup>]<sub>cyt</sub> and amount 20.82 and 31.38% of the cells. However, types 2 and 3 displayed short or delayed continuous rise in [Ca<sup>2+</sup>]<sub>cyt</sub> that may be considered as early or delayed Ca<sup>2+</sup> deregulation according to the literature [26–29]. The abundance of cells showing deregulation (type 2 and type 3) amount to 32.83 and 14.92%, of the total, respectively.

We have analyzed the relative abundance of the four types of recordings described above in the absence (Control) and the presence (Methadone) of 0.5 mM methadone. The results obtained are shown in Figures 1(b) and 1(c). Clearly, methadone decreased type 4 (transient responsive cells) and increased types 2 and 3 (deregulated cells). These results

suggest that methadone may induce a short or delayed Ca<sup>2+</sup> deregulation in SH-SY5Y cells.

The mechanisms underlying the observed quantitative changes in the types of Ca<sup>2+</sup> responses mediated by methadone are unknown. Therefore, we performed a comprehensive pharmacological survey to study the possible contribution of different Ca<sup>2+</sup> related mechanism to the response to methadone. As illustrated in Figures 1(b) and 1(c) and discussed below, a 5 min pretreatment of SH-SY5Y cells with different conditions and drugs affected the methadone-induced [Ca<sup>2+</sup>]<sub>cyt</sub> response, monitored as changed frequencies of the different types of the responses. We found that in the absence of extracellular calcium (0 Ca) no change in responses to methadone was observed

( $n = 291$  cells). These results suggest that  $\text{Ca}^{2+}$  entry does not contribute to the reported changes in  $[\text{Ca}^{2+}]_{\text{cyt}}$ . To confirm that extracellular  $\text{Ca}^{2+}$  was not involved in these responses we tested the effect of methadone in the presence of nifedipine, a specific isopropyl L-type  $\text{Ca}^{2+}$  channel blocker. Nifedipine ( $2 \mu\text{M}$ ) did not modify the effects of methadone on the  $[\text{Ca}^{2+}]_{\text{cyt}}$  in SH-SY5Y cells ( $n = 226$  cells). So, we ruled out the involvement of this voltage-dependent channel family in methadone-induced  $[\text{Ca}^{2+}]_{\text{cyt}}$  variations. We asked then whether intracellular  $\text{Ca}^{2+}$  stores could contribute to the responses to methadone. Cells were treated with thapsigargin ( $1\text{--}100 \mu\text{M}$ ). To deplete intracellular  $\text{Ca}^{2+}$  stores before methadone treatment. As expected, thapsigargin induced a significant release in  $\text{Ca}^{2+}$  from the endoplasmic reticulum, causing a transient increase in the  $[\text{Ca}^{2+}]_i$  that failed to returned to the basal level within a 10-minute period. A detailed observation of the methadone induced rise in  $[\text{Ca}^{2+}]_i$  shows that cell responses were partially affected by this treatment, suggesting that the rise in calcium is partially due to release from thapsigargin-sensitive, intracellular  $\text{Ca}^{2+}$  stores ( $n = 146$  cells). We found that in untreated cells addition of thapsigargin induced a transient increase in  $[\text{Ca}^{2+}]_i$  that has been attributed to a leakage of  $\text{Ca}^{2+}$  from the endoplasmic reticulum. This effect of thapsigargin alone interferes the interpretation of the type 2 and 3 responses after methadone addition (data not shown).

Another possible mechanism is the activation of endogenous opioid receptors. To test this possibility, we treated the cells with  $50 \mu\text{M}$  Naloxone, the competitive antagonist of the  $\mu$ -opioid receptor. We found that this treatment did not modify the relative abundance of any of the four types of cell populations ( $n = 80$  cells, 4 exp). This result suggests that the changes in  $[\text{Ca}^{2+}]_{\text{cyt}}$  and DCD induced by methadone in SH-SY5Y cells are independent of opioid receptors. In agreement with the lack of  $\mu$ -opioid receptor participation as found here, it has been reported that methadone-toxic pathways are not mediated by  $\mu$  receptors [19, 30–32].

To test the possible contribution of mitochondria we used the mitochondrial uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP;  $1 \mu\text{M}$ )  $\text{Ca}^{2+}$  uptake by mitochondria depends strongly on mitochondrial potential ( $\Delta\Psi_m$ ). It is well established that FCCP collapses mitochondrial potential and abolishes the ability of mitochondria to take up  $\text{Ca}^{2+}$ . We found that in untreated cells, addition of FCCP induced a transient increase in  $[\text{Ca}^{2+}]_i$  that has been attributed to leakage of  $\text{Ca}^{2+}$  from depolarized mitochondria. We found that FCCP induced a 44% decrease in the appearance of type 2 cells whereas increased the relative abundance of cells showing a type 3 response by 2.7-fold in FCCP (Figures 1(b)–1(c)). FCCP abolished the presence of type 1 cells.

The above results suggest that mitochondria are likely involved in the response to methadone. Nevertheless, it must be taken into account that as the simple usage of protonophores does not allow a clear-cut study of the role of mitochondrial  $\text{Ca}^{2+}$  transport as they also may cause a lowering of the ATP/ADP ratio, thereby affecting ATP-dependent  $\text{Ca}^{2+}$  pumps [14]. An approach that has been previously exploited to investigate the role of mitochondria

in synaptosomal  $\text{Ca}^{2+}$  homeostasis involves inhibition of mitochondrial ATP synthesis by oligomycin and application of glycolysis as the source of ATP and independent manipulation of  $\Delta\Psi_m$  with specific respiratory chain inhibitors [33, 34]. Inhibition of ATP synthase by oligomycin prevents mitochondrial oxidative phosphorylation, but unlike protonophore addition, it does not cause hydrolysis of cytoplasmically generated ATP. It has been reported that DCD might result from a failure in  $\text{Ca}^{2+}$  extrusion caused by cytoplasmic ATP depletion [26]. Therefore, we tested the effects of a short, 5 min, incubation with  $10 \mu\text{g/ml}$  oligomycin on  $\text{Ca}^{2+}$  responses. Under these conditions, oligomycin alone did not modify the  $[\text{Ca}^{2+}]_{\text{cyt}}$  responses during the 30 min recording period. However, oligomycin did alter the  $[\text{Ca}^{2+}]_{\text{cyt}}$  responses to methadone. Specifically, the abundance of SH-SY5Y cells showing a type 2 response were increased whereas the pool of cells showing a DCD-related type 3 response were lost. Additionally, oligomycin induced a 3.3-fold increase in cells showing a type 4 response. To test further the contribution of mitochondria we investigated the possible role of the mitochondrial  $\text{Ca}^{2+}/2\text{Na}^{+}$  exchanger. Mitochondrial  $\text{Ca}^{2+}$  efflux is normally primarily regulated by a  $\text{Ca}^{2+}/2\text{Na}^{+}$  exchanger. To block mitochondrial  $\text{Ca}^{2+}$  exit we used CGP37157 ( $50 \mu\text{M}$ ), an inhibitor of the  $\text{Ca}^{2+}/2\text{Na}^{+}$ -exchanger. A five min exposure of cells to CGP37157 significantly increased the proportion of cells showing no rise in  $[\text{Ca}^{2+}]_{\text{cyt}}$  in response to methadone during the entire  $[\text{Ca}^{2+}]_{\text{cyt}}$  measurement period (type 1) (data not shown) because CGP37157 resulted in a drastic decrease in the population of cells showing either type 2 (74%) or type 3 (85%) responses. In addition, the relative abundance of cells showing a type 1 response returned to the value obtained in untreated cells. These results support data suggesting the relevance of mitochondria in methadone-induced DCD (Figures 1(b)–1(c)).

To test contribution of reactive oxygen species we used the cell-permeable, small molecule compound TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy) to mimic superoxide dismutase activity. In the presence of TEMPOL ( $0.2 \mu\text{M}$ ;  $n = 80$  cells), cells responded to methadone in a different way. Specifically, TEMPOL decreased the number of cells showing a type 2 response by  $\sim 70\%$ , whereas the abundance of cells showing a type 3 response was largely increased (3.5 fold). Types 1 and 4 were nearly not present. Consistent with our results, Nicholls et al. [28] suggested that enhanced ROS is a consequence rather than a cause of DCD. In their studies, they applied a novel technique to monitor the bioenergetic status of in situ mitochondria in cultured neurons in a model of glutamate excitotoxicity. In agreement with this, a general ineffectiveness of antioxidants to decrease DCD in the presence of glutamate has been observed [35]. Finally, we tested the contribution of the mitochondrial permeability transition (MPTP). Additional efflux of mitochondrial  $\text{Ca}^{2+}$  can occur by induction of MPTP formation, which is dependent on the mitochondrial matrix  $\text{Ca}^{2+}$  concentration and can be inhibited by cyclosporine A [16, 17]. To evaluate MPTP participation we administered CsA ( $1 \mu\text{M}$ ). CsA diminished the occurrence of response type 2 by 36% ( $n = 89$  cells) in cell cultures challenged with  $0.5 \text{ mM}$

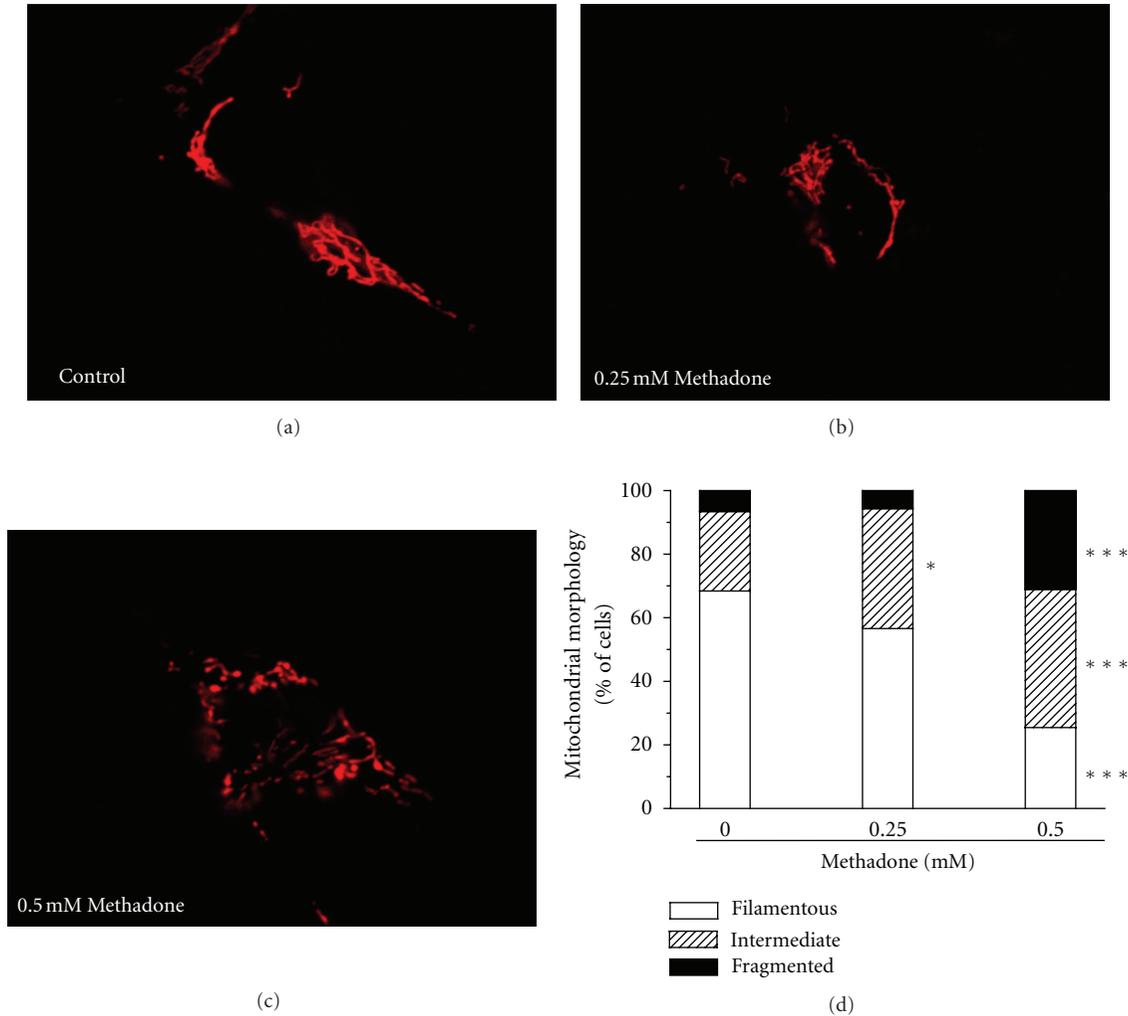


FIGURE 2: Methadone alters mitochondrial morphology. Morphology of methadone-treated cells was studied by confocal imaging of SH-SY5Y cells transfected with the pDsRed2-mito vector. Twenty-four hours after transfection, cell cultures were incubated for 3 h in the absence or presence of 0.25 or 0.5 mM methadone. Shown are images of the representative mitochondrial morphology in non-treated cells (control, (a)) or cells treated with methadone ((b), 0.25 mM; (c), 0.5 mM). Scale bar indicates 10  $\mu$ m. (d), The fractions of cells with filamentous, intermediate or punctuate mitochondrial patterns were determined in at least 6 independent cultures. (\* $P < 0.05$ ; \*\*\* $P < 0.001$ ; Student's  $t$ -test versus basal conditions as indicated.)

methadone. Moreover, consistent with the hypothesized role of the pore in DCD, CsA induced a 2-fold increase in cells showing type 4 response. However, interpretation of these results is difficult because CsA may also inhibit the mitochondrial  $Ca^{2+}$  uniporter in some instances.

Taken together, our data indicate that only drugs affecting mitochondrial handling of  $Ca^{2+}$ , such as oligomycin, FCCP, CGP 37157, and cyclosporine A, were able to modulate methadone-induced delayed calcium deregulation in SH-SY5Y cells. We therefore conclude that methadone-induced dyshomeostasis is caused by improper functioning of mechanisms that directly control mitochondrial activity rather than a participation of plasma membrane  $Ca^{2+}$  channels or opioid receptors.

Next, the effect of methadone on mitochondrial morphology was studied in SH-SY5Y cells transfected with

pDsRed2-mito. In untreated cultures, mitochondria presented a long and tubular morphology (Figure 2(a)), which became dramatically shorter and rounder upon three hours of methadone treatment (Figures 2(b)–2(d)). Cell counting of the different mitochondrial morphologies (filamentous, mixed and fragmented) indicated that methadone, in a dose-dependent manner, induced mitochondrial fragmentation (Figure 2(d)). This effect seems contradictory to our earlier observations that methadone failed to induce mitochondrial swelling in isolated rat liver mitochondria [19]. Possibly, the fragmentation effect of methadone on mitochondria is mediated by calcium. In fact, our data support this hypothesis, and indicate a role of DCD in methadone-induced toxicity. In agreement with this, it has been reported that the MPTP opens under pseudopathological conditions with relatively high  $Ca^{2+}$  and low ATP concentrations [15]

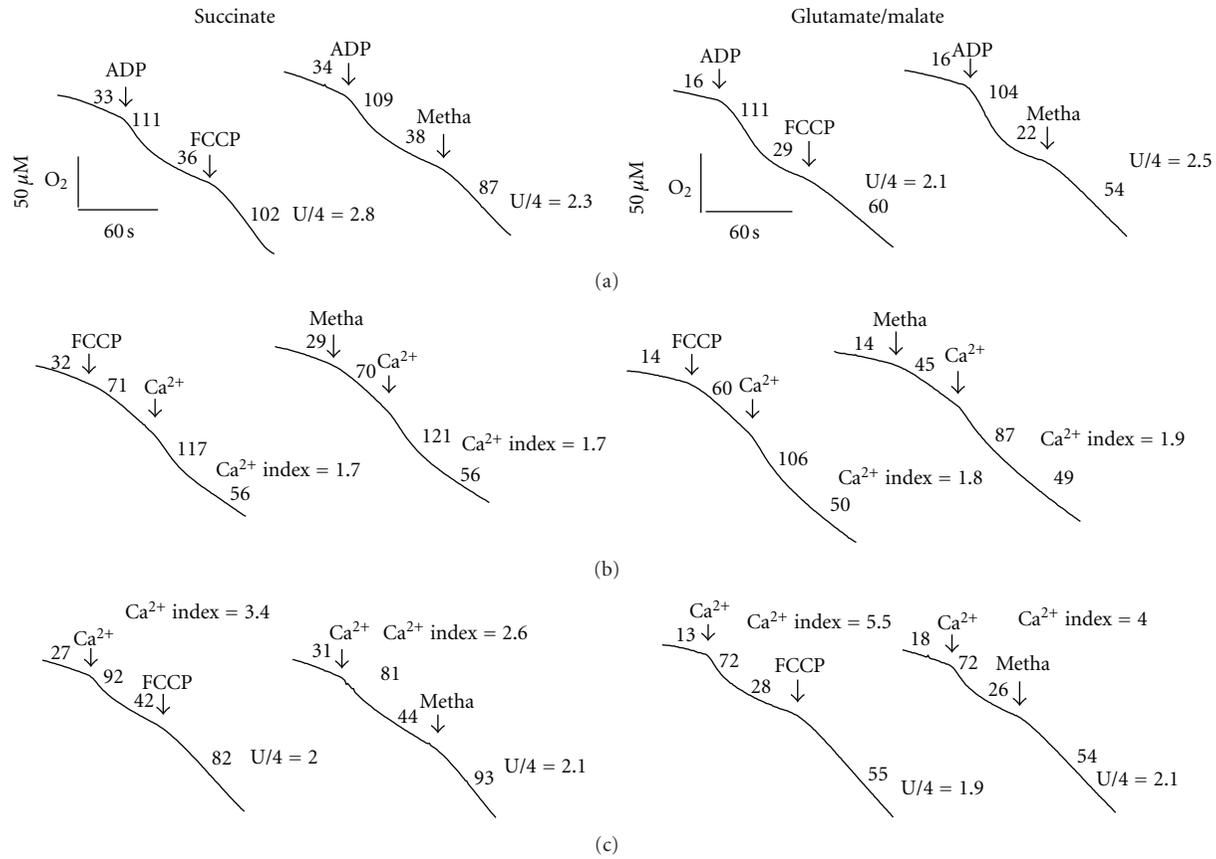


FIGURE 3: Methadone affects mitochondrial Ca<sup>2+</sup> uptake in a respiratory substrate-dependent way. Oxygen uptake by isolated liver mitochondria was determined using a Clark electrode in the presence of 5 mM succinate or 2.5 mM glutamate/2.5 mM malate as substrates. (a) Uncoupling capacity of FCCP and methadone. (b) The effect of FCCP and methadone on Ca<sup>2+</sup> uptake. (c) The effect of Ca<sup>2+</sup> uptake on the uncoupling capacity of FCCP and methadone. Additions: 250 μM ADP, 200 μM CaCl<sub>2</sub>, 0.4 μM FCCP, and 0.5 mM methadone. Traces shown are the means obtained from four independent mitochondria preparations. Numbers on the traces indicate respiration rates at 37°C in nmol oxygen · mg protein<sup>-1</sup> · min<sup>-1</sup>.

as was the case in our previous experiments with SH-SY5Y cells [19]. The rupture of the mitochondrial membrane caused by Ca<sup>2+</sup> overload reduces the number of “healthy” mitochondria and this will affect crucial neuronal functions including synaptic transmission and axonal transport.

Finally, the effect of methadone on mitochondrial Ca<sup>2+</sup> uptake was studied (Figure 3). We used a Clark electrode and applied different respiratory substrates, namely succinate and glutamate/malate. The respiratory chain is less dependent on the presence of  $\Delta\Psi_m$  for succinate than for glutamate/malate. Methadone is known to cause uncoupling [19]. Therefore, as a control, 0.4 μM FCCP was used because this concentration of FCCP resulted in an increase of oxygen-uptake rate comparable with the uptake rate calculated for 0.5 mM methadone (Figure 3(a)). The calculated values of the state U (uncoupled state) to state 4 (resting state) ratios (U/4 ratios) were as follows: for succinate 2.8 ± 0.5 (+FCCP) and 2.3 ± 0.4 (+methadone), and for glutamate/malate 2.1 ± 0.5 (+FCCP) and 2.5 ± 0.4 (+methadone). Then, we checked the effect of FCCP and methadone on Ca<sup>2+</sup> uptake by mitochondria (Figures 3(b) and 3(c)). For this purpose

a Ca<sup>2+</sup> index was calculated, which denotes the ratio of oxygen-uptake rate triggered by addition of 200 μM Ca<sup>2+</sup> to previous oxygen uptake rate. The values of the Ca<sup>2+</sup> index were as follows: for succinate 3.0 ± 0.7 (control), 1.7 ± 0.3 (+FCCP), and 1.7 ± 0.4 (+methadone), and for glutamate/malate 4.8 ± 0.3 (control), 1.8 ± 0.4 (+FCCP), and 1.9 ± 0.5 (+methadone). Thus, the effects of FCCP and methadone on Ca<sup>2+</sup> uptake are comparable, although in the presence of glutamate/malate the effect appears to be much more pronounced. This probably results from a stronger uncoupling effect of methadone on glutamate/malate access to the respiratory chain and a consecutive additional impairment of Ca<sup>2+</sup> uptake. Therefore, the effect of methadone on Ca<sup>2+</sup> uptake by mitochondria may be dependent on the respiratory substrates. On the other hand, when the values of the U/4 ratios calculated in the absence and presence of Ca<sup>2+</sup> uptake were compared (Figures 3(a) and 3(c)), a distinctive decrease was observed in traces recorded in the presence of Ca<sup>2+</sup> uptake (Figure 3(c)). The calculated values of U/4 ratio decreased as follows: for succinate from 2.8 ± 0.5 to 2.0 ± 0.2 (+FCCP) and 2.3 ± 0.4 to 2.1 ± 0.3 (+methadone) and for

glutamate/malate  $2.1 \pm 0.5$  to  $1.9 \pm 0.3$  (+FCCP) and  $2.5 \pm 0.4$  to  $2.1 \pm 0.2$  (+methadone). This could be caused by the reduction of a  $\Delta\Psi$  component of the protomotive force as a result of  $\text{Ca}^{2+}$  uptake, leading to a decrease of uncoupling capacity by FCCP and methadone.

The data presented indicate that methadone induces DCD in SH-SY5Y cells by altering the capacity of mitochondria to handle calcium, and correlates with distinct changes of mitochondrial morphology. These morphological changes, in turn, can be associated with mitochondrial damage and cell death. Interestingly, swollen mitochondria have been observed in the context of neurodegenerative diseases [36, 37]. An imbalance in mitochondrial  $\text{Ca}^{2+}$  homeostasis might be important for both early and late stages of the observed side effects and, perhaps account for some of the observed clinical symptoms, for example, memory impairment.

## Abbreviations

FCCP:	Carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone
$[\text{Ca}^{2+}]_{\text{cyt}}$ :	Cytosolic $\text{Ca}^{2+}$ concentrations
DCD:	Delayed calcium deregulation
$\Delta\Psi\text{m}$ :	Mitochondrial inner membrane potential
MPTP:	Mitochondrial permeability transition pore
ROS:	Reactive oxygen species.

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## Review Article

# Chelators in the Treatment of Iron Accumulation in Parkinson's Disease

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Iron is an essential element in the metabolism of all cells. Elevated levels of the metal have been found in the brains of patients of numerous neurodegenerative disorders, including Parkinson's disease (PD). The pathogenesis of PD is largely unknown, although it is thought through studies with experimental models that oxidative stress and dysfunction of brain iron homeostasis, usually a tightly regulated process, play significant roles in the death of dopaminergic neurons. Accumulation of iron is present at affected neurons and associated microglia in the substantia nigra of PD patients. This additional free-iron has the capacity to generate reactive oxygen species, promote the aggregation of  $\alpha$ -synuclein protein, and exacerbate or even cause neurodegeneration. There are various treatments aimed at reversing this pathologic increase in iron content, comprising both synthetic and natural iron chelators. These include established drugs, which have been used to treat other disorders related to iron accumulation. This paper will discuss how iron dysregulation occurs and the link between increased iron and oxidative stress in PD, including the mechanism by which these processes lead to cell death, before assessing the current pharmacotherapies aimed at restoring normal iron redox and new chelation strategies undergoing research.

## 1. Introduction

Parkinson's disease is a chronic, progressive disorder and the second most common neurodegenerative disease after Alzheimer's disease, with an overall prevalence in the general population of 0.3% [1]. The incidence of PD increases with age, making this the most important risk factor. The cardinal symptoms of tremor, rigidity and postural instability result from the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and its projections in the nigrostriatal tract [2]. Symptoms do not occur until dopamine levels have been reduced by 70-80%, meaning that neuroprotection is a crucial but difficult task. In most PD patients the disease is idiopathic: a combination of environmental factors and genetic susceptibility. Only 5% of cases are purely genetic, with a number of genes identified, serving a vital role in early onset forms of the disease. The majority of cases are multifactorial—a combination of

factors including accumulation of toxic protein aggregates, oxidative stress and inflammation.

A further pathological feature of PD is the abnormal accumulation of iron at affected neurons. Iron plays a vital role in various physiological functions including DNA synthesis, mitochondrial respiration, and oxygen transport. Neuronally, iron is involved in myelination and neurotransmission and is the most abundant metal in the brain. Crucially in relation to PD, the metal acts as a cofactor for tyrosine hydroxylase (TH), the enzyme at the rate-limiting step in the synthesis of dopamine. The importance of iron in this process is underlined by *in vitro* studies showing that TH activity is stimulated dose dependently by iron [3]. While iron is important physiologically in these actions, in excess the metal can be toxic through oxidative stress. The locations of iron accumulation in neurological disorders mirror the regions affected by the relevant condition. This situation is maintained in PD as increased levels of iron have been found

in the substantia nigra of PD patients [4–6] and subsequently implicated in numerous neurological disorders with parkinsonism symptoms [7]. It is not clear whether this is a cause of or a development secondary to neuronal degradation [8]. However, infusion of ferric iron into the SNpc can be used to create a model of dose-related, progressive parkinsonism including a reduction in dopaminergic activity [9]. This can be attenuated by treatment with the lazaroid U-74389G [10], showing that iron may play a prominent causative role in the death of neurons by oxidative stress and lipid peroxidation. It has also been found that chronic exposure (more than 20 years) to iron and other metals leads to an increased risk of developing the disease [11], again demonstrating that excess iron may, at least in part, induce PD pathogenesis.

Increased iron content is caused by a number of factors (reviewed in [7]), including a disturbed or “leaky” blood-brain barrier (BBB), occupational exposure [11] and disruption of the body’s iron storage and transport mechanisms. Iron distribution and storage is normally tightly regulated in the body due to the deleterious effects that iron deficiency and, as relevant in this discussion, overload have. This complex homeostasis is maintained by the differential expression of proteins that regulate its cellular uptake, utilisation, and storage.

The access of iron to cells is controlled primarily by transferrin receptors; its storage by the protein ferritin and pigment melanin (reviewed in [12]), a by-product of dopamine oxidation. Iron binds to transferrin after carefully controlled absorption from the duodenum and circulates in the blood. It is taken into cells via transferrin receptors and stored in the centre of metalloproteins. Excess iron is stored as ferritin and lost when cells are shed in the gut. Stored iron is mobilised from hepatocytes and tissue macrophages in response to an acute need, with increased intestinal absorption requested when demand (primarily by erythroid cells for heme synthesis) exceeds the supply of stored iron [13]. The levels of ferritin are crucial: iron is relatively nontoxic when bound to ferritin but alterations in unbound (free) iron can cause problems, therefore ferritin levels must be closely regulated. At the posttranscriptional level, appropriate cellular iron storage is maintained by the iron regulatory proteins 1 and 2 (IRP1 and IRP2, resp.). When iron levels are low, IRPs bind to iron responsive elements (IREs) on the 3′- and 5′-untranslated regions of their respective mRNAs, thus inhibiting translation of ferritin RNA and thereby decreasing the iron-storage capacity and stimulating translation of the transferrin receptor mRNA, a glycoprotein which controls levels of free-iron. The system then works in the opposite direction once sufficient iron has been taken up to downregulate the process [14, 15], helping maintain a storage capacity relative to the level of iron and the body’s current demands. The importance of ferritin has been demonstrated through overexpression of H (heavy chain) ferritin in dopaminergic neurons [16]. The second important storage protein is intimately related to nigral neurons. These neurons produce the dark pigment neuromelanin, which can bind heavy metals, particularly iron. Loss of melanised neurons is correlated with an

abundance of nonheme iron ( $\text{Fe}^{3+}$ ) and a significant increase in redox activity, which is most pronounced in patients with the greatest loss of neuromelanised cells [17].

This change in redox state can contribute to oxidative stress and induce further cell death. This paper will summarise briefly the factors contributing to a dysregulation of iron in parkinsonian patients and its role in the disease pathology before discussing the methods aimed at restoring iron homeostasis.

## 2. Molecular Basis of Iron Dysregulation in PD

The full role of iron in the pathogenesis of PD has been frequently reviewed [18–20] with various interpretations placed on its significance. Iron toxicity occurs when the levels of iron exceed the binding capacity of transferrin leading to an excess of reactive, unbound iron in the body. It is then sequestered in cells where creating an overload can induce deleterious effects.

Iron exists in two forms in living organisms: its reduced form,  $\text{Fe}^{2+}$ , and the oxidised  $\text{Fe}^{3+}$  state. The transfer between these states, from ferrous iron ( $\text{Fe}^{2+}$ ) to the ferric form ( $\text{Fe}^{3+}$ ) in a catalytic reaction with hydrogen peroxide (or molecular oxygen) known as the Fenton reaction, can yield the highly toxic hydroxyl radical ( $\cdot\text{OH}$ ) via the Haber Weiss reaction [21]. The Fenton reaction is a normal metabolic process, occurring at times including electron transfer in mitochondria and readily in the cytoplasm where a large proportion of iron is reduced. However, in PD patients the ratio of  $\text{Fe}^{2+}:\text{Fe}^{3+}$  is 1:3 rather than 1:1, as in control brains [22], although this figure can vary between sources [5]. The iron redox is, therefore, in favour of  $\text{Fe}^{3+}$ . The presence of additional iron together with a diminished supply of antioxidants leads to an increased generation of hydroxyl radicals through various reactions in the microglia, producing a cascade of destructive events including oxidative stress, lipid peroxidation, and eventually apoptosis [23]. The iron increase in the pathology of PD is not dependent on systemic iron imbalance, with local dysregulation of the redox contributing to the overload in the respective tissues [13].

Under physiological conditions, the free radical species produced in this reaction are sequestered and inactivated by the body’s army of antioxidants, including glutathione (GSH), superoxide dismutase, and catalase. However, a tip in the balance of the Fenton reaction due to excess ferrous iron means there is an overwhelming amount of free radicals produced, which disrupts the normal cellular redox state. The endogenous cellular defences (which are relatively low for the amount of oxygen the brain consumes) are compromised, and oxidative stress follows, triggering a cascade of deleterious effects throughout the cell. Depleted glutathione levels have been forwarded as an early factor in PD pathogenesis [24], as asymptomatic cases of the disease show a similar pattern of depletion as later in the disease [25]. But certainly, an increase in antioxidant production in combination with a compromised cellular defence system will lead to an increased vulnerability of these cells.

Maintaining iron redox through processes such as iron transport sequestration and release is tightly regulated [26]. Any deviation from the normal redox state can have serious consequences for cells and the organism as a whole due to the potential iron possesses to become toxic. Ferritin, capable of sequestering up to 4500  $\text{Fe}^{3+}$  atoms [27] (although it is rarely saturated [28]), represents an important endogenous capacity to limit the presence of redox-active iron. The protein is primarily cytosolic and exists in two subunits with different tasks. The ferroxidase activity of the H-ferritin subunit converts harmful labile  $\text{Fe}^{2+}$  to relatively nontoxic  $\text{Fe}^{3+}$ , thereby keeping it in this unreactive form [15, 29]. The L- (light chain) ferritin subunit stabilises this complex and promotes iron's long-term storage [27, 30, 31]. The direct impact of these components in the iron regulatory system can be evaluated when studying inherited disorders. Mutations in the gene coding for L-ferritin have been reported to cause a basal ganglia disorder similar to PD, highlighting the neurological consequences that excess iron can have [32].

Imbalance in regulation mechanisms [12] leads to an increased reactive free-iron pool. Immunohistochemistry studies show a 60% reduction in iron-transferrin receptor binding in PD [33], as well as a decrease in transferrin binding sites [34]. It has been found that in PD brains, excessive iron is not mirrored by an increase in ferritin levels [35, 36]—normally brain H-ferritin levels parallel the increased iron accumulation which occurs with age [37]. Furthermore, in dopamine neurons iron stored within the ferritin core can be reduced readily by products of dopamine oxidation such as superoxide and 6-OHDA, adding a further level of vulnerability to this neuronal population. In an aged individual, the ferritin can become heavily burdened with the excess iron, which accumulates with age. Within lysosomes, ferritin may become degraded releasing the bound iron and further increasing the level of reactive iron [38, 39]. Therefore, in PD the iron is unbound and free to initiate a range of cytotoxic and inflammatory effects, such as the activation of redox-sensitive transcriptional factor nuclear factor kappa-B (NF $\kappa$ B) and cytokine release from activated microglia [40].

Oxidative stress is instrumental in PD pathogenesis, as confirmed by significant neurochemical, histological, biochemical, and physical evidence and as shown in several models, including 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [41] and rotenone [42], which exert their neurotoxic effects by free-radical-mediated mitochondrial dysfunction and oxidative stress [43, 44]. MPTP has also been implicated in exacerbating iron-related biochemical abnormalities [45] through an increased expression of the divalent metal transporter 1 (DMT1) [46]. Oxidative stress is at a high basal level in the SNpc as the autoxidation of dopamine produces semiquinones, a toxic species themselves, which can also lead to the generation of reactive oxygen species [47] making dopaminergic neurons particularly vulnerable to iron excess and these subsequent effects. This level is augmented in PD, partly due to the presence of additional iron. An excess of iron may lead to a vast increase in the production of free radicals, which overwhelms the

natural defensive mechanisms and causes damage at several cellular levels.

Oxidative stress may also impair the ubiquitin-proteasome system, thereby inhibiting cells' ability to clear degraded proteins. Iron released from neuromelanin increases oxidative stress in mitochondria, disrupting mitochondrial function and reducing the ATP-dependent proteasomal activity of 26S (the proteasome involved in the ubiquitin system)—effects which could be reversed in one study by superoxide dismutase and the iron chelator deferoxamine [48]. Failure of the proteasomal system to clear these proteins, which include excess  $\alpha$ -synuclein, could lead to the formation of the proteinaceous inclusions Lewy bodies, the pathological hallmark of PD [2, 49], and dopamine-dependent neurotoxicity [50]. The metal has been implicated in the formation of these protein aggregates [51]. Along with other free radical generators (dopamine and hydrogen peroxide), iron promotes the aggregation of intracellular aggregates containing  $\alpha$ -synuclein and ubiquitin in a human neuroblastoma cell line overexpressing A53T and A30P proteins [52].

The process of lipid peroxidation, another event upregulated in PD patients [53], may be induced and can propagate several effects that threaten cell viability. It can result in the production of 4-hydroxy-2-nonenol (HNE) [54], a highly reactive lipophilic  $\alpha,\beta$ -alkenal capable of inducing apoptosis through a cascade of caspase activation [55], in addition to promoting DNA fragmentation and contributing to oxidative stress [56]. It has been found that lipid peroxidation can cause protein aggregation [18], producing Lewy bodies. Iron may constitute a link between the pathogenic events of oxidative damage and protein aggregation, with iron accumulating in Lewy bodies in PD [57] and promoting alpha-synuclein aggregation [58], an event reversed by the administration of an iron chelator [59].

It is, therefore, the proficiency of unbound iron to generate free radicals and induce oxidative stress, which is at the centre of their deleterious effects. This relationship is summarised in Figure 1. An imbalance in the normal redox state of iron, due to an age-related accumulation of iron in combination with dysregulation of the metal's storage, transport, and excretion systems, lead to increased levels of ferrous iron, which produce free radicals that overwhelm endogenous cell defences and generate a cascade of cytotoxic effects. The intimacy of iron storage sites in neuromelanin to dopaminergic neurons in the substantia nigra mean these cells are particularly at risk of iron overload-induced death.

### 3. Iron Chelators as a Treatment

The correlation between iron accumulation in the brain and PD has logically led to the theory that chelators of iron could help slow the development of the disease by mopping up the unbound, free radical-enhancing iron in the brain. This mode of treatment has precedent: copper chelation using D-penicillamine has been effective in the removal of neuronal copper in Wilson's disease [60]. The treatment of iron dysregulation in PD at least holds the

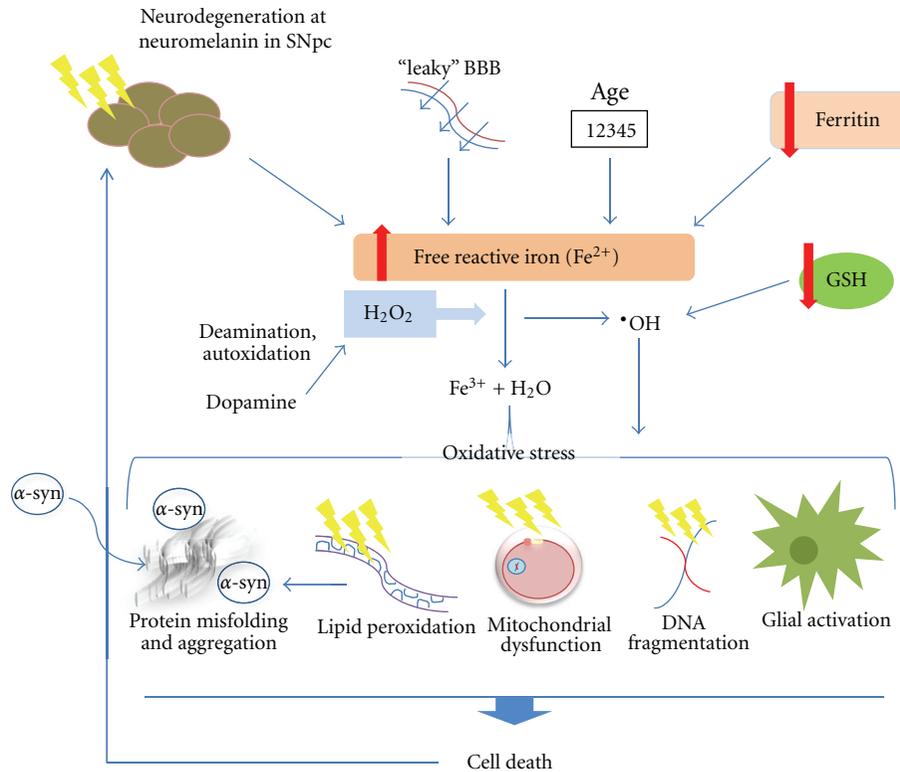


FIGURE 1: Iron-mediated cell death in PD. Reduced storage capacity in PD due to decreased ferritin expression and degeneration of nigral melatonin-containing neurons causes an increase in the reactive  $\text{Fe}^{2+}$  iron pool. Age-related increases in iron and a leaky BBB cause further iron accumulation. The transfer of the free iron to ferric iron,  $\text{Fe}^{3+}$ , in the hydrogen peroxide-mediated Fenton reaction produces the highly toxic hydroxyl radical. A compromised level of glutathione exacerbates the levels of free radicals, whilst the deamination and autoxidation of dopamine produces further  $\text{H}_2\text{O}_2$ . The subsequent oxidative stress can then elicit a range of cytotoxic reactions including protein misfolding, lipid peroxidation (which, in turn, can cause  $\alpha$ -synuclein aggregation), mitochondrial dysfunction, and activation of glial cells. These various insults can induce cell death by apoptosis, causing further degeneration.

advantage over Alzheimer's disease (AD) that only one metal need be targeted, whereas with AD copper and zinc have also been associated with the disease pathology. Moreover, chelation of copper using D-penicillamine is ineffective against MPTP-induced dopamine depletion in mice [61]. Clear guidelines exist on the design and structure of a suitable iron chelator [62]. Potential chelators must have the ability to selectively scavenge excess intracellular iron and turn into a nontoxic product, which can be safely excreted. Since chelation therapy would be maintained for life, it is important that the agent does not impinge upon levels of other metals taken in by the diet. Chelators should only access the intracellular reactive labile iron pool, that is, iron that is not bound in a ferritin-iron complex as this is essential for normal physiological functions. The need for chelators to readily cross the BBB means that the size of potential chelators is important—300 Da being the stated maximum [63]. There are further substances that have found neuroprotective benefits in conditions of iron accumulation, but this is often due primarily to their antioxidant properties and/or by increasing mitochondrial activity. This paper will discuss those with proven iron chelating abilities, which often work in combination with some other neuroprotective

abilities. Table 1 summarises the information outlined in the following sections, with the modes of action of the chelators shown in Figure 2.

**3.1. Chemical Chelators.** Chemical iron chelators are already available clinically for various conditions. The use of them in neurodegenerative disorders has been restricted primarily by their ability (or lack thereof) to cross the BBB in therapeutically efficacious concentrations. But steps have been taken to overcome these limitations, which has produced a range of effective drugs to address iron overload in PD by various means.

**3.2. Desferal.** Desferrioxamine (also known as deferoxamine, DFO, or desferal) is a cell-impermeable, highly selective chelator, which can be taken up by the cell through endocytosis. It is a hexadentate iron chelator (i.e., it binds all six of iron's electrochemical coordination sites) and has been the most widely used iron chelator over the last 30 years [78], having been developed for the treatment of secondary iron overload (such as in  $\beta$ -thalassemia [79]), with the target primarily being the excess iron present in the liver and spleen.

TABLE 1: Iron chelators in brief. Summary of key information regarding iron chelators currently undergoing research as possible PD therapies.

Chelator name	BBB-permeable	Stage of research	Relevant findings	References
Synthetic				
Desferal	No	Clinically used for systemic iron accumulation. Cellular and animal models of PD	Neuroprotective in rat 6-OHDA model	[64]
Deferiprone	Yes	Phase II trials	Efficacious. Can reduce iron levels but not always with symptomatic improvement	[65, 66]
Apomorphine	Yes	Animal models	Effective against iron-induced toxicity and MPTP-induced cell death	[67]
VK-28	Yes	Animal models	Protective in 6-OHDA rat model	[68]
M30	Yes	Animals models	MAO-A and -B inhibitor. Selective. Effective in MPTP mouse model	[69]
M10	Yes	Cell culture	Hydroxide scavenger. Inhibits lipid peroxidation	[70, 71]
CQ	Yes	Animal models	Neuroprotective in MPTP mouse model	[72, 73]
Natural				
EGCG	Yes	Animal models	Multiple protective actions. Can be used in combination with rasagiline. Effective in MPTP mouse model but not in 6-OHDA rat model	[74, 75]
Phytic acid	Unknown	Cell culture	Protects against MPP <sup>+</sup> and 6-OHDA toxicity in normal and excess iron	[76, 77]

Desferal has also been used successfully to chelate iron in cases of aceruloplasminaemia, as assessed by MRI imaging, which parallels with improved neurological function [80]. However, this chelator has been unsuccessful previously at removing small increases in iron, such as in the joints of rheumatoid arthritis patients, due to adverse effects, which have been attributed to the high doses used [81]. Some factors make it unclear whether desferal would be suitable to treat excessive iron related to neurodegenerative diseases namely, whether it is able to remove excess iron without interfering with normal iron metabolism and if these small, hydrophobic molecules can cross the BBB.

Desferal is reported to attenuate iron-induced oxidative stress and mitochondrial dysfunction and prevent  $\alpha$ -synuclein aggregation in the human neuroblastoma cell line SK-N-SH in culture [82]. Desferal was effective against MPP<sup>+</sup>-induced toxicity in a microdialysis study [83]. The drug also provided neuroprotection *in vivo* in a concentration-dependent manner in the 6-OHDA model of PD [22, 84]. The drug has been effective against dopaminergic neuron loss in rats [85] and in MPTP-treated mice [86] with

iron overload. Desferal has been shown to attenuate nigral cell death induced by lactacystin [87]. However, the high hydrophobic nature and subsequent poor BBB permeability of desferal means that in these *in vivo* studies it was delivered by intracerebroventricular (ICV) injection—clearly not a viable option for clinical use. Therefore, effective chelators that can be delivered peripherally and effect iron levels in the brain are required. More recently, systemic administration of desferal at 30 mg/kg has been shown to be neuroprotective in the 6-hydroxydopamine (6-OHDA) model of PD and reduced hydroxyl radical formation [64]. There has been much progress delineating the mechanism and safe doses of desferal. However, the difficulty of administering the drug clinically limits its use sharply.

**3.3. Deferiprone.** The chelator deferiprone, used in the treatment of thalassaemia major, has the great advantage of being orally active; meaning the complex and mostly impractical administration routes of desferal may be avoided. It has been reported to be successful after 6 months of therapy in

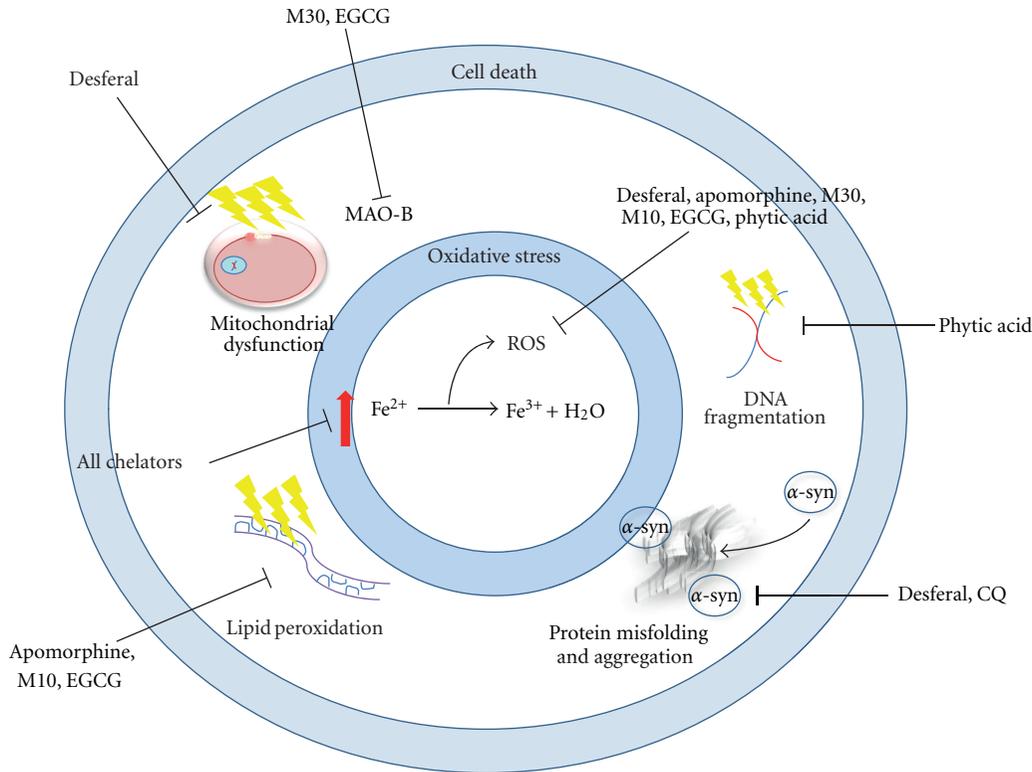


FIGURE 2: Action of iron chelators targeting PD. All iron chelators mop up excess free, reactive iron, thus reducing the reduction of  $Fe^{2+}$  to  $Fe^{3+}$ —a reaction that produces various ROS, such as the hydroxyl radical. Oxidative stress resulting from the generation of ROS produces a range of deleterious insults, which can be targeted with the multiple actions of inhibitors. This can attenuate the cell death that these events induce. Chelators with antioxidant properties inhibit the production of ROS, in an environment of diminished antioxidant activity. The dopamine-oxidising enzyme MAO-B, which resides in the outer membrane of mitochondria, can also be inhibited by some chelators.

one case of neurodegeneration associated with brain iron overload, with an improvement of gait and reduction in dyskinesias [65], demonstrating that the drug can cross the BBB in efficacious concentrations. This led to the development of a Phase II trial for deferiprone to assess its safety and efficacy as a chelator of excess iron in the treatment for PD, with low doses being favoured (15 mg/kg/day). Patients are to be assessed every 3 months by unified PD rating scale (UPDRS) and, when possible, by magnetic resonance imaging [88]. Further clinical trials are assessing deferiprone at different doses in aged individuals [89, 90]. A smaller Phase II pilot study has already been completed, assessing the safety and efficacy of deferiprone on targeting globus pallidus iron in patients affected by pantothenate kinase-associated neurodegeneration (PKAN), a genetic disorder where levels of cysteine, an efficient iron binder, increase in the basal ganglia. The study, which nine patients completed, shows that 6 months of treatment leads to a significant reduction in iron content in this brain region, as measured by MRI, although the clinical status of patients did not change [66]. However, the patients in the study were not aged (the range being 7–39 years old), so whether deferiprone would be equally well tolerated in older PD patients is not certain. But the clear efficacy in reducing iron content in this form of

neurodegeneration with brain iron accumulation (NBIA) is promising and may be well transferred to PD patients. Earlier intervention could be required to provide a symptomatic benefit.

**3.4. Apomorphine.** Pretreatment with R-apomorphine, a D1- and D2-receptor agonist, which can be used as a Levodopa replacement therapy in late-stage PD, shows antioxidant and iron-chelating abilities in the MPTP model due to its catechol structure [91]. The authors report that the dopamine agonist property is not behind the neuroprotective ability of the drug in this model, with a combination of iron chelating and, primarily, radical scavenging capacities accounting for the protection afforded [92]. This is because the nondopaminergic receptor agonist isomer of the drug, S-apomorphine, retains the same neuroprotective properties [93]. A broad spectrum of neuroprotective abilities is shown again by R-apomorphine *in vitro* [94]. These studies confirm that both inhibit the reduction in GSH, again confirming the benefit of a drug with multiple neuroprotective actions. The comprehensive assessment of R-apomorphine by [67] demonstrates the drug's neuroprotective actions against 6-OHDA- and iron-induced toxicity *in vitro* as well as in MPTP

mouse models. These include inhibition of iron-mediated lipid peroxidation, meaning this agent elicits an extensive range of actions to counter the damage caused by iron accumulation.

**3.5. Hydroxyquinolines.** Hydroxyquinoline is a bidentate chelator containing the 8-hydroxyquinoline (8-HQ) moiety. This structure forms stable 5-membered chelate rings with  $\text{Fe}^{3+}$  [95]. 8-HQ, a highly lipophilic complex, readily penetrates cell membranes and the BBB and, therefore, has been used as the base for orally effective chelators, including VK-28, M30, and clioquinol, all of which will be discussed.

VK-28 is an iron chelator with a potency comparable to desferal. However, in contrast to desferal, VK-28 is BBB-permeable. It is neuroprotective against 6-OHDA toxicity in rats when given either by ICV or intraperitoneal routes without altering peripheral iron metabolism. In the absence of the toxin, VK-28 has no effect on the basal levels of transmitters such as dopamine, showing that the iron-dependent rate-limiting enzymes TH and tryptophan hydroxylase are not affected [68].

Since VK-28 so far meets all the needs of an iron chelator, derivatives of VK-28 have been generated. M30, a potent brain-selective inhibitor of monoamine oxidase-A and -B (MAO-A and MAO-B, resp.) based around the pharmacore of VK-28, is effective in both cell culture [70] and the MPTP mouse model [69]. It also restores levels of the antiapoptotic protein Bcl-2 in mice treated with microinjections of lactacystin [71]. Both M30 and parent molecule VK28 showed behavioural improvements in this model [70]. M30 also prevents iron-dependent hydroxyl radical generation [96]. Part of this bifunctional protection is explained by the drug sharing some structural units with rasagiline, a selective irreversible MAO-B inhibitor [97]. M10 is another hydroxyquinoline derivative with radical scavenging and iron-chelating properties. It is a potent hydroxide scavenger and has been shown to be as effective as rasagiline in PC10 cell culture and inhibits lipid peroxidation with an  $\text{IC}_{50}$  value comparable to desferal [96].

Antibiotics have been shown to have iron-chelator properties. 5-chloro-7-iodo-hydroxyquinoline (or clioquinol/CQ) was at the centre of one of the worst drug disasters of the 20th century, when thousands of people worldwide developed subacute myelo-optic neuropathy (SMON) after using the drug to treat intestinal infections. This pathology was later linked to the drug's promotion of vitamin B12 excretion [72]. It has long been known that clioquinol chelates iron [98]. More recently it has been studied for its iron-chelating properties in neurodegenerative diseases. CQ has been reported to be effective against  $\beta$ -amyloid aggregation in AD transgenic mice [99] in a study targeting copper and zinc interactions, showing that CQ is not a selective iron chelator. However, despite this apparent drawback when considering the guidelines outlined [78], the converse was the case, as copper can also facilitate  $\text{Fe}^{2+}$  toxicity [99]. Toxic side effects have been a problem when using other iron chelators; the low lipid solubility of which hinders their ability to cross the BBB meaning higher doses are required.

The lipophilic nature of CQ allows for its effective use at lower concentrations [72, 73]. CQ provided neuroprotection in MPTP-treated mice [73] after 8 weeks of oral treatment in addition to a reduction in motor dysfunction. CQ led to decreased bioavailable iron levels in normal mice [72], with the drug well tolerated in both studies, an important consideration to make since any clinical treatment is likely to be for life.

**3.6. Natural Chelators.** This group includes components of plant polyphenols, such as epigallocatechin gallate (EGCG), a green tea extract (catechin). These compounds have been utilised over many years for their antioxidant properties and have been investigated for their potential use in several diverse areas including oncology, cardiology, and neurology. The favourable properties of green tea (GT) are attributed to their high content of antioxidant polyphenolic flavanoids, of which there is a vast array—over 4000 flavanoids have been identified [100]. GT catechins possess structures which infer metal chelating properties—the 3',4'-dihydroxyl group and the gallate groups are present. These may neutralise ferric iron to produce a redox-inactive form [101]. The compounds are nontoxic and readily cross the BBB, meaning the ease of administration is a major advantage, with epidemiological evidence showing that drinking two cups of green tea a day reduces the risk of PD [102].

**3.7. EGCG.** The most abundant and pharmacologically active green tea extract, EGCG, has been shown to attenuate striatal dopamine and TH loss as well as nigral dopaminergic cell death in MPTP-treated mice [103]. The authors propose various mechanisms for this protection including acting as an antioxidant, preventing lipid peroxidation and inhibiting MAO-B in addition to its role as an iron chelator. An agent which could work by various pathways would be highly beneficial due to the multifactorial nature of PD. Catechins have also been shown to regulate various signalling pathways involved in cellular survival and downregulate proapoptotic pathways (reviewed in [104]), as well as inhibit catechol-O-methyltransferase (COMT) activity [105]. Together with its iron-chelating activities, this data mean green tea extracts are powerful agents to use when cell death may be mediated by oxidative stress.

EGCG has been tested in combination with the established anti-PD drug rasagiline [74]. EGCG is relatively a far weaker MAO-B inhibitor than rasagiline (with an  $\text{IC}_{50}$  of  $662 \mu\text{M}$  compared to  $6 \text{ nM}$  [103]); therefore, if this pharmacological requirement could be supplemented by another drug, the neuroprotective effect may be augmented. It was found that the two act synergistically to provide neuroprotection in the MPTP mouse model [74].

However, EGCG has proven less successful in the 6-OHDA rat model as only subtle behavioural improvements in the absence of neuroprotective benefits were produced when given orally [75]. This may be due to the doses administered ( $1\text{--}2 \text{ mg/kg}$ : physiological dose), which, although effective against MPTP-induced neuronal degradation [103], were too low for this particular model due to the mechanism

of toxicity. Therefore, it is important to consider the actions of individual PD models when evaluating the effectiveness of these compounds.

**3.8. Phytic Acid.** Phytic acid (IP6, myoinositol hexakisphosphate) is an antinutrient and has previously been shown to be effective in cancer through an antioxidant effect [106]. The agent has been studied for its beneficial antioxidant properties on hydroxyl radical formation enhanced by  $\text{Fe}^{2+}$  induced by MPP<sup>+</sup> in rat striata. Here phytic acid chelated iron required for radical formation via the Fenton reaction [107]. It has since been demonstrated to be protective against MPP<sup>+</sup>-induced toxicity in immortalised rat mesencephalic/dopaminergic cells by attenuating caspase-3 activity and DNA fragmentation, and increasing cell viability in both conditions of normal and excess intracellular iron content [76]. This effect was replicated in a cell culture model utilising 6-OHDA [77]. Phytic acid has been forwarded as a safer alternative to synthetic iron chelators, such as desferal, although there remains some concern about the compound's ability to cross the BBB. Studies in PD models are also limited at this stage. Further work is required with this compound to fully assess its potential clinical benefit.

These groups of natural chelators meet all the requirements for an iron chelator to be effective in providing neuroprotection in PD. The range and vast number of compounds available mean there are many more similar structures which can be screened. Further tests in models of PD would allow them to be effectively evaluated.

## 4. Conclusions

There is strong evidence that iron accumulation causes cell death, particularly in the substantia nigra through oxidative stress mechanisms. Deposits of the metal are found in the substantia nigra of PD patients, whilst local administration of the metal can cause degeneration and reductions in dopamine levels. Iron is involved in various biochemical reactions, with the Fenton reaction at the centre of this redox balance. The transfer between the two ion states is a tightly regulated process, in which storage and transfer proteins work concurrently to maintain physiological levels of iron. An influx of the metal causes an increase in the levels of reactive ferric iron and results in more harmful reactive species. Deleterious consequences including lipid peroxidation, reduction in levels of endogenous antioxidants and stimulation of cellular apoptotic cascades escalate cell death in these areas. Iron chelators have the potential to address the balance in the available level of the reactive iron form (labile iron pool) to prevent the overproduction of oxidative species.

Established iron chelators, which have been used in conditions of iron excess for many years, such as desferrioxamine/desferal, have now found a new use in neurodegenerative diseases. But desferal's inability to cross the BBB means this drug will never be a feasible option in its initial form. However, the drug's use as a base for orally active agents,

which retain these iron chelating activities, is more significant. Of the putative treatments, deferiprone is certainly at the most advanced levels of research. Having found success in experimental models, the recent commissioning of Phase II clinical trials investigating the agent will provide further information regarding the safety and tolerability in aged patients.

A major advantage of these new pharmacotherapies is the mixture of neuroprotective actions they possess. In addition to iron chelation, recent therapies also have MAO-B inhibition capabilities and antioxidant properties. This allows for a more comprehensive mode of action, which is what is required in PD where a plethora of pathologies exist. Antioxidant actions of natural substances, such as the green tea extract EGCG, have been coupled with iron chelation activities to provide natural alternatives.

However, due to the ubiquitous nature of iron, it is important to consider whether iron chelators will provide the cellular specificity required to remove excess iron from the appropriate tissue, without affecting systemic iron homeostasis. The dose of chelator is an important factor to consider in treatment so that the correct tissues are penetrated and systemic iron levels are not altered. Moreover, subcellular locations of iron may need to be recognised. Li and colleagues [108] discuss the importance of specificity of intracellular targets for iron chelation in relation to Friedreich's ataxia (FA). Iron chelators, therefore, may be required to remove iron in a highly selective manner whilst stabilising iron levels elsewhere even within the same cell. Initial clinical trials [65] demonstrate that doses of deferiprone are well tolerated over the long term (6 months) with no stated propagation of a challenge to systemic iron homeostasis. The early successes of these patient studies demonstrate that this issue may not be as pertinent in PD as in FA. Furthermore, deferiprone itself has been shown to be highly selective when used at low concentrations in FA patients—relocating iron from areas of accumulation in the brain to ferritin, thereby preventing deprivation in other tissues [109].

The presence of increased levels of iron in the areas affected by degeneration in PD together with the effects this dysregulation have, mean reversing this redox imbalance is an important aim for any neuroprotective therapy. Iron chelators initially used for other medical applications have been built upon with their functions adapted to suit the pathology of PD. With some treatments now reaching clinical trials, it is hoped that an efficacious and tolerable iron chelation therapy, which is effective at attenuating neurodegeneration, is close to being achieved.

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## Review Article

# Oxidative DNA Damage in Neurons: Implication of Ku in Neuronal Homeostasis and Survival

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Oxidative DNA damage is produced by reactive oxygen species (ROS) which are generated by exogenous and endogenous sources and continuously challenge the cell. One of the most severe DNA lesions is the double-strand break (DSB), which is mainly repaired by nonhomologous end joining (NHEJ) pathway in mammals. NHEJ directly joins the broken ends, without using the homologous template. Ku70/86 heterodimer, also known as Ku, is the first component of NHEJ as it directly binds DNA and recruits other NHEJ factors to promote the repair of the broken ends. Neurons are particularly metabolically active, displaying high rates of transcription and translation, which are associated with high metabolic and mitochondrial activity as well as oxygen consumption. In such a way, excessive oxygen radicals can be generated and constantly attack DNA, thereby producing several lesions. This condition, together with defective DNA repair systems, can lead to a high accumulation of DNA damage resulting in neurodegenerative processes and defects in neurodevelopment. In light of recent findings, in this paper, we will discuss the possible implication of Ku in neurodevelopment and in mediating the DNA repair dysfunction observed in certain neurodegenerations.

## 1. Oxidative DNA Lesions and Repair Systems

Reactive oxygen species (ROS) are constantly produced inside the cell and mediate different oxidative reactions with various cellular molecules (phospholipids, proteins, RNA, and DNA) [1]. In particular, ROS are genotoxic and capable to be harmful to DNA by generating various oxidative DNA lesions with base or sugar damage [2]. ROS can be produced endogenously as side effect of normal cellular metabolism, in particular by the mitochondrial oxidative metabolism [3]; or exogenously, by chemical and physical stress (e.g., ionizing or ultraviolet radiations) [4, 5]. The modifications or alterations inflicted on DNA have potentially serious consequences for the cell. Five main classes of hydroxyl radical-mediated oxidative damage may be generated: oxidized bases, abasic sites, DNA-DNA intrastrand adducts, DNA strand breaks (single-strand break, SSB, and double-strand break, DSB), and DNA-protein cross-links [1, 6].

The low redox potential of guanine renders this base particularly vulnerable although the number of different lesions is not higher than with other bases. The most thoroughly examined guanine oxidation product is the 8-oxo-2'-deoxyguanosine (8-OHG), which is both mutagenic and carcinogenic in that it can pair with either cytosine or adenine causing GC to AT transversions [7, 8]. The oxidation of nucleotides in the DNA does not lead to direct breaks of the DNA. However, when hydroxyl radical attacks the sugar-phosphate backbone, it can generate SSB. If two reactions of that type occur in close vicinity (clusters), a DSB formation is possible [9]. Breaks of both DNA strands could also occur after conversion of labile lesion to SSB or after enzymatic processing of base damage [9]. Nevertheless, if left unrepaired, or repaired incorrectly, DNA lesions may result in massive loss of genetic information, genomic rearrangements, or cell death. Therefore, the cell has evolved a number of pathways to repair DNA damage.

The four major pathways for repairing damage to DNA are mismatch repair (MMR), nucleotide excision repair (NER), base excision repair (BER), and double-strand break repair (DSBR). DNA mismatch repair (MMR) is a highly conserved pathway that removes base-base mismatches and insertion-deletion loops that arise during DNA replication and recombination [10]. The nucleotide excision repair (NER) is a complex DNA repair system that recognizes bulky, helix-distorting lesions, such as pyrimidine dimers and 6–4 photoproducts, intrastrand crosslinks [11], DNA-protein cross-links [12], and some DNA adducts caused by oxidative damage [13, 14]. NER involves the excision of a single-stranded lesion-containing oligonucleotide fragment, thus creating a single-strand gap in the DNA. This gap is subsequently filled during repair synthesis by a DNA polymerase using the undamaged strand as a template [15]. There is, however, an alternative NER pathway that is coupled to active transcription and is termed transcription-coupled repair [16]. BER involves the removal of one nucleotide (short-patch BER, SP-BER) or 2–13 nucleotides (long-patch BER, LP-BER) by a glycosylase action (i.e., 8-oxoguanine DNA glycosylase, OGG1), in which the other strand is used as a template to repair the specific lesion [17]. In SP-BER, the DNA polymerase beta (POLB) plays a crucial role carrying out two distinct and essential enzymatic reactions: it uses its DNA polymerase activity to fill in the one-nucleotide gap, and it also uses its 5′-deoxyribosephosphatase activity to cleave the 5′ phosphate to allow for efficient ligation (lyase activity) [18, 19]. SSB can be induced during the repair of 8-oxoG by BER pathway, or it can occur in the absence of BER when hydroxyl radical attacks and breaks directly the sugar-phosphate DNA backbone, without creating a base damage. Thus, SSB is always integrated in the BER pathway, but it can be triggered in the absence of BER [15]. SSB utilizes, in fact, many of the same proteins and follows essentially the same procedure, as BER. SSB has also two subpathways, short-patch (SP) and long-patch (LP), similar to BER [20]. When both strands of DNA are damaged, the cell activates the double-strand break repair (DSBR), which involves one of two mechanisms: homologous recombination (HR) or nonhomologous end joining (NHEJ) [21]. The two pathways differ in their fidelity and their template requirements. HR uses an undamaged DNA template on the sister chromatid or homologous chromosome to repair the break, leading to the reconstitution of the original sequence. In fact, HR is restricted to the late S to G2/M phase of the cell cycle, when a sister chromatid is available in proliferating cells [22]. In contrast, NHEJ involves binding of Ku70/86 heterodimer to the two DNA termini and putting them directly back together, without the use of a homologous template. In fact, NHEJ is an error-prone process: hence, it is not able to restore the sequence information in the DNA, causing the accumulation of randomly located mutations in the genome of each somatic cell of an organism [23]. However, NHEJ preserves the phosphodiester backbone and the molecular integrity of the chromosome, avoiding the loss of several hundreds of genes on entire chromosomal arms or segments [24]. Nevertheless, NHEJ is evolutionarily conserved throughout the animal kingdom and is the predominant double-strand

break repair pathway in mammalian cells [25], because it functions either throughout the cell cycle or in postmitotic differentiated cells [15, 22], and because it does not require the sequence of a sister chromatid.

## 2. NHEJ in the Nervous System

Since the mature nervous system is largely postmitotic, NHEJ is the main DNA DSB repair pathway in the brain [15, 26]. In fact, several observations in the mouse knockout models of NHEJ factors, reviewed in [27], and different analyses of NHEJ activity in mature rat brain [26, 28, 29] point towards the importance of NHEJ in neuronal function and homeostasis. Neurons are particular in being terminally differentiated, postmitotic cells, while also being extremely metabolically active. They, in fact, display high rates of transcription and translation, which are associated with high metabolic rate and mitochondrial activity, and thus with a high rate of oxygen consumption. The nervous system is also very rich in polyunsaturated fatty acids (PUFAs) and has a high content of transition metals and ascorbate levels, which together act as potent oxygen radical-generating system. On the other hand, it possesses a relative paucity of antioxidant systems compared with other organs, so that the nervous system is highly vulnerable to oxidative stress. Excessive levels of ROS can be therefore generated and constantly attack DNA producing several lesions [30–32]. Among them, DSB, although being less frequent, is one of the most toxic and mutagenic lesions [9]. Furthermore, defective DNA repair systems in neurons can lead to a high accumulation of DNA damage, such as chromosomal breaks. In particular, during neural development, defects in NHEJ can result in neuropathology (i.e., neurodegeneration and microcephaly), suggesting that responding to DNA DSBs is essential for neural homeostasis [33–35].

Development of the nervous system occurs in a basic pattern of proliferation, differentiation, migration, and maturation. The nervous system generates from proliferative ventricular zones that form neural precursor cells. Two main classes of cells make up the nervous system, neurons, and glia, these also encompassing many specialized subtypes. As these cells exit the cell cycle, they migrate and differentiate, establishing the nervous system's shape. Moreover, the development of nervous system is characterized by a massive apoptosis from the early stages of proliferation until the later stages of functional maturation, constituting a part of the process that generates the system's sophisticated cytoarchitecture and connectivity. Inactivation of critical proteins involved in NHEJ pathway has been demonstrated to have detrimental effects in neurodevelopment [36–40]. The DNA ligase IV-deficient mouse shows massive cell death in the developing nervous system and embryonic lethality [36]. This dramatic apoptosis may be a reflection of the propensity of damaged neuronal cells to undergo apoptosis rather than to mature into differentiated neurons. Individuals with mutations in LIG4 exhibit immunodeficiency, developmental delay, growth retardation, and microcephaly, a disease that has been termed LIG4 syndrome [40]. The fact that knockout mice lacking functional Lig4 are not viable [36] indicates

that the mutations in the LIG4 syndrome patients might be hypomorphic alleles. However, considering the high level of apoptosis detected in Lig4-deficient mice, it is possible that LIG4 syndrome patients also experience elevated neuronal apoptosis during development, this possibly underlying the reported microcephaly and developmental delay [36, 40].

Furthermore, in mature brain, the inability to respond to DNA DSBs may lead to neurodegenerative disorders. In particular, it has been reported that genes involved in signaling pathway which coordinates cell cycle arrest after DNA DSB, such as the ataxia telangiectasia mutated gene (ATM), are associated, when defective, with neurodegenerative disorders (ataxia-telangiectasia and ataxia-telangiectasia-linked disorder, AT and ATLD) [15]. ATM is a protein kinase whose DNA damage-induced phosphorylation of various substrates is involved in cell cycle regulation or maintenance of genomic stability [41–43]. Moreover, the high oxidative DNA damage and the decreased DNA repair observed in Alzheimer's patients [44, 45] have been correlated to defects in the NHEJ repair process, although a precise *locus* or gene(s) affected in this pathway has not yet been identified [46].

**2.1. Multiple Functions of Ku.** Like most DNA repair processes, the NHEJ pathway of DSBs requires three enzymatic activities: (i) nucleases to remove damaged DNA, (ii) polymerases to aid in the repair, and (iii) a ligase to restore the phosphodiester backbone [25]. However, the first step is the recognition of the lesion. Ku is, indeed, deputed to this function. Ku is a heterodimer formed of two subunits: Ku70 and Ku86 (also termed Ku80) [47]. In particular, the complex binds directly the two broken DNA termini so protecting them from excessive degradation and ultimately preparing them for ligation [24]. As shown in Figure 1, the Ku heterodimer is capable of interacting with the nuclease (Artemis-DNA-PKcs) complex, the polymerases (pol  $\mu$  and pol  $\lambda$ ), and the ligase (XLF-XRCC4-DNA Lig IV) complex. Ku first recruits the catalytic subunit of the DNA-PK (DNA-PKcs) and Artemis, this preventing the premature processing of DNA ends, in the repair site [24]. It likely changes conformation once it slides onto the DNA end, since Ku complexes with DNA-PKcs are not detected except when Ku is bound to a DNA end [48]. The new-formed complex (DNA-PK) is able to phosphorylate itself, Artemis, and different substrates. The autophosphorylation causes a conformational change, and DNA-PKcs dissociate from DNA allowing the recruitment of several end-processing enzymes, including XRCC4, DNA Lig IV, and Cernunnos/XLF [23]. The consequence of these activities is the rejoining of DNA ends and the repair of DNA damage. DSBs can also induce the phosphorylation of histone H2AX in the vicinity of DSBs by members of the phosphatidylinositol-3 kinase (PI3K) family, such as DNA-PKcs, ATM, and ATR. This is considered a crucial signal for the cell in order to activate the DNA repair response, since it serves as a site for the accumulation and retention of the central components of the signaling cascade initiated by DNA damage [49].

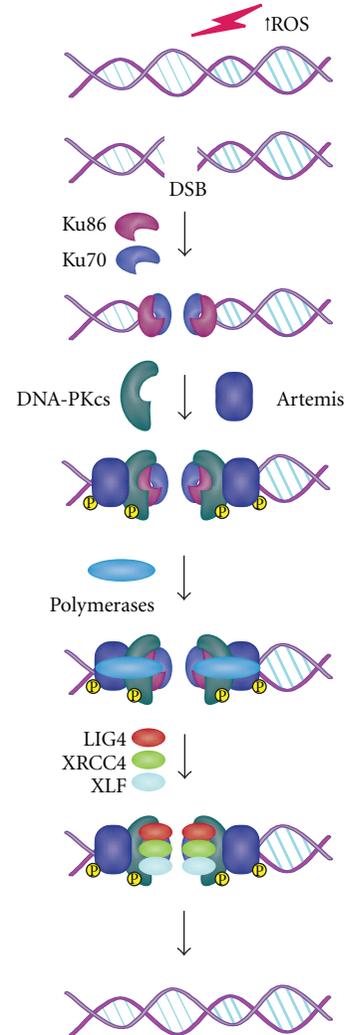


FIGURE 1: ROS can generate double-strand breaks with heterogeneous incompatible DNA ends. Following DSB formation, Ku70 and Ku86 form the heterodimer Ku, which can bind directly the two broken DNA termini. Ku, likely changing conformation once it slides onto the DNA end, recruits DNA-PKcs and Artemis to form the DNA-PK complex, which brings the two DNA ends close together and protects them from excessive degradation. DNA-PK phosphorylates itself and also mediates a regulatory phosphorylation of other NHEJ components, such as Artemis. Subsequently, the DNA polymerases (including the pol X polymerases, pol  $\mu$  and  $\lambda$ ) synthesize each DNA segment necessary for the repair. Finally, the DNA-PK complex recruits the LIG4-XRCC4-XLF complex in order to perform the ligation of the DNA termini, after which the DNA-repair factors dissociate.

The Ku complex was originally found as a major target of autoantibodies taken from Japanese patients with scleromyotonia overlap syndrome [50, 51]. Current knowledge on Ku deals with its fundamental role in DNA repair. That said, the Ku complex is also implicated in other cellular processes, including telomere maintenance, antigen receptor gene arrangements (VDJ recombination), regulation of specific gene transcription and apoptosis [47]. For instance,

Ku has been found to protect telomeres from inappropriate degradation and interchromosomal recombination; to contribute to the tethering of telomeres to the nuclear periphery; to regulate telomerase [52]. Wang and coworkers (2009) have also demonstrated that Ku plays an essential role in human cells, because it prevents dramatic telomere loss. In particular, they showed that the cell death that resulted from the conditional knockout of Ku86 in a human somatic cell line was associated with massive telomere loss [53].

Variable (V), diversity (D), and joining (J) gene-segment recombination is initiated by the generation of sequence-specific DSBs by an enzyme complex (which consists of the recombination-activating gene (RAG)1 and RAG2 proteins) at the ends of two coding segments that have to be joined. The subsequent processing and repair of the resulting structures is performed by NHEJ; therefore, Ku is involved in this process [54]. This kind of DSBs is not pathologic but functions so as to rearrange the genome to generate diversity in the immune system. Moreover, Ku70 can be involved in the regulation of mitochondrial apoptotic pathway by sequestering Bax from the mitochondria, in an acetylation-sensitive manner, and by mediating Bax deubiquitylation [55, 56]. In particular, it has been demonstrated that, under normal growth conditions, Ku70 is maintained in a nonacetylated state by the histone deacetylases (HDACs), such as the NAD<sup>+</sup>-dependent SIRT1, which enables its association with Bax. After apoptotic stimuli, the acetyltransferases CBP and PCAF acetylate specific lysines on Ku70, resulting in a conformational change of Ku70 and the Bax release, that can act, in such a way, to initiate apoptosis [57]. Sawada and coworkers (2003) characterised for the first time the interaction between Bax and Ku70, identifying the Bax-binding domain of Ku70 within amino acids 578–583 [58]. They also found out that the cell permeable pentapeptide designed from the Bax-inhibiting domain of Ku70 is able to inhibit Bax-induced cell death [59]. These extensive studies were published in 2003 but retracted in 2007. However, several works dealing with Bax-Ku70 interaction, as well as with the cytoprotective effect of Bax-inhibiting pentapeptide, collectively confirmed the reproducibility of the former findings [55–57, 60–62]. Indeed, Bax-inhibiting peptides were abundantly used in a number of works, which point out the relevance of Bax-Ku70 interaction in apoptosis induction, that is, in the susceptibility of cell death of human laminin- $\alpha$ 2-deficient myotubes and mouse models of congenital muscular dystrophy [60]; in the protection of cells from polyglutamine toxicity caused by Ku70 acetylation [61]; or in the reduction of neuronal death and behavioral deficits following global cerebral ischemia [62].

Regarding its transcriptional function, the Ku complex has been reported to act in both a sequence-nonspecific and -specific manner. This factor has been found to be associated with RNA polymerase II elongation sites, without a sequence-specific DNA binding [63]. Ku can interact with the sugar bonds of DNA through the central ring of the protein formed by the two subunits Ku70 and Ku86, in a sequence nonspecific manner, which is already known being the way to bind DSBs [47]. On the other hand, there are several reports indicating that the Ku complex is

a transcription modulator. In particular, it has been reported (i) to repress human  $\alpha$ -myosin heavy-chain promoter during heart failure [64]; (ii) to contribute to ERBB2 oncogene overexpression in breast cancer cells by the interaction with activator protein-2 [65]; (iii) to regulate S100A9 gene expression, which is a member of a multigenic family of nonubiquitous cytoplasmic Ca<sup>2+</sup>-binding proteins, alongside poly(ADP-ribose)polymerase-1 [66]; to act as corepressor in farnesoid X receptor-mediated gene expression [67]; (iv) to function as transcriptional recycling coactivator of the androgen receptor [68]. In addition, we have recently reported that Ku is involved in the repression of the proapoptotic gene *Apaf1* upon DNA damage [69]. Actually, we still do not know which is the Ku70 and/or Ku86 domain(s) implicated in the specific interaction with DNA and how it occurs. However, we can speculate that the C-terminus domain of Ku70, called SAP (SAF-A/B, Acinus, and PIAS) [70], which is found in proteins involved in chromatin remodelling [71, 72], could have a role in this process. We cannot exclude that Ku-related transcription function is the result of the interaction with other still characterised factors, which specifically bind promoter elements, at least in those cases where Ku is reported acting as a cofactor [64–68]. In this scenario, Ku could represent a chromatin-remodelling factor conferring to chromatin an open/close structure, even though being still capable to bind the DNA in a sequence-nonspecific manner.

### 3. Ku Involvement in Neuronal Homeostasis

Ku is involved in the signalling pathway which follows the DNA DSB repair. As above mentioned, it can regulate the mitochondrial pathway of apoptosis either by negatively modulating the expression of the proapoptotic gene *Apaf1* [69] or by interacting with Bax [55–59], with both these functions mostly observed in cells of neuronal origin. Accordingly, we have demonstrated the Ku mediates dynamic modulation of *Apaf1* in neural progenitors deriving from the telencephalon of embryonic stage 14 mice [69]. Moreover, a large body of evidence obtained in neuroblastoma cells [55, 73], primary cortical neurons [61], retinal ganglion cells [74], and in mouse brain [62] argues for Ku70 negatively affecting the proapoptotic activity of Bax. In such a way, Ku would exert a double prosurvival role within the cell, by participating in the DNA repair response and by preventing the mitochondrial apoptotic pathway.

The dysfunction of Ku has been demonstrated to have detrimental effects in the nervous system where the NHEJ pathway is the fundamental DSBs repair system used by neurons and where the apoptotic cell death can regulate neural development (Figure 2). For instance, the lack of either Ku70 and/or Ku86 in knockout mice results in dramatic apoptosis of many types of developing embryonic neurons of spinal cord, cerebral cortex, midbrain, and hindbrain [75]. Apoptosis has also been found in the embryonic retina in Ku-null animals [76]. Notably, the neuronal apoptosis observed in the Ku70 and Ku86 knockouts is not as severe as in knockouts of the other NHEJ components, and, in fact, the Ku70

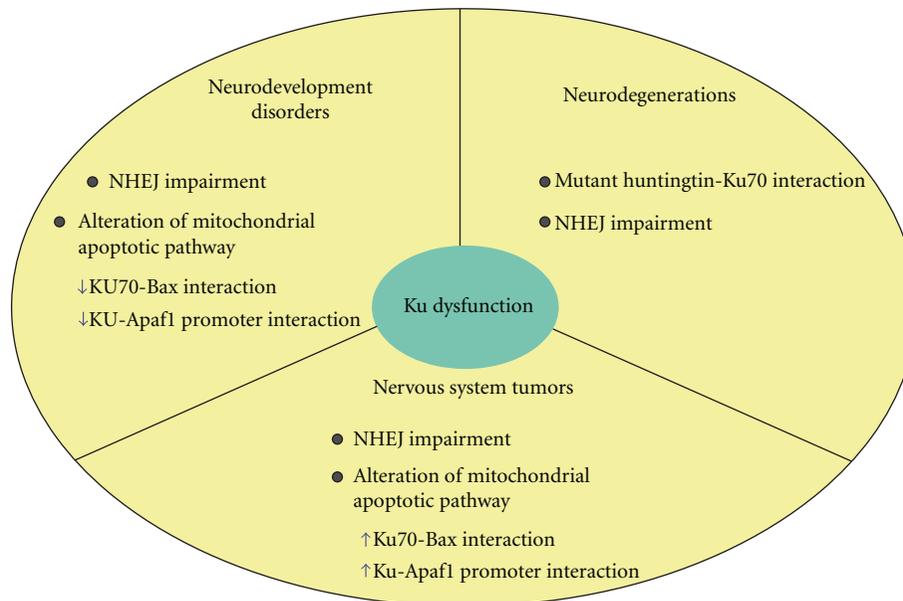


FIGURE 2: Ku dysfunction can lead to pathological states of the nervous system. Besides NHEJ impairment, which results in DNA damage accumulation, Ku-related alterations of the mitochondrial apoptotic pathway can play a crucial role in neurodevelopment disorders and neoplastic transformations. In particular, Bax-Ku70 and/or Apaf1 promoter-Ku interaction can be decreased and lead to massive apoptosis upon Ku loss of function, as observable in Ku-related disorders of neurodevelopment. Alternatively, Ku gain of function in tumors of the nervous system may lead to an increase of Ku binding to Bax and/or the Apaf1 promoter, thus leading to evade apoptosis and to increase chemoresistance. On the other hand, in a mature nervous system, Ku sequestration mediated by mutant huntingtin can contribute to the HD pathology, by leading to an altered DSBs repair by NHEJ.

and Ku80 knockouts survive into adulthood, even though no apoptosis was found in the nervous system of *DNA-PK<sub>CS</sub>*-null mice [75]. It has been suggested that neuronal cells might be particularly prone to apoptosis in response to DSBs during neurodevelopment, perhaps in order to eliminate damaged neurons and guarantee a sufficient cell replacement for maintaining neural development [77]. In *Ku70*<sup>-/-</sup> mice, migrating cortical neurons, which normally undergo oxidative DNA damage, fail to repair DNA lesion by NHEJ and undergo apoptosis [78]. In addition, it has been demonstrated that the DNA-PK complex, including Ku, can promote survival of young neurons in embryonic mouse retina, thus confirming the critical role of Ku in regulating neurogenesis [79]. In this regard, it has to be also considered that the Ku70/Bax interaction is probably abolished in a Ku-depleted background. This interaction is crucial in regulating the mitochondrial apoptotic pathway by preventing Bax localization into the mitochondria [55–62], and its absence could contribute to the massive apoptosis observed in nervous system of *Ku* knockout mice. Also, the specific Ku-mediated modulation of the apoptotic gene *Apaf1* observed in neural progenitors could account for the excessive cell death observed in Ku-depleted backgrounds [69]. All the above-mentioned processes argue for Ku playing a critical role in neuron survival, either by participating in DSB repair through NHEJ or by regulating apoptosis. According to these assumptions, Ku could also have a role in the chemoresistance of tumors originating from the nervous system (Figure 2). As part of their mechanism of action, most

chemotherapeutics affect DNA integrity through the generation of different lesions, such as DSBs. Indeed, it has been reported a correlation between resistance to chemo- and/or radiotherapy and a high Ku70–Ku86 expression/activity [80–82]. Moreover, some reports have shown the modulation of Ku70 acetylation status and Ku70-Bax interaction, by means of HDAC inhibitors, both effects inducing chemosensitivity in medulloblastoma and neuroblastoma cells [55, 73, 83]. All this confirms a pivotal role for Ku in neuronal homeostasis and survival.

Enokido and coworkers (2010) have also demonstrated a critical involvement of Ku70 in the pathogenesis of the Huntington's disease (HD) [84]. In particular, they found that mutant huntingtin specifically interacts with Ku70, resulting in Ku70 sequestration and its inability to function in the NHEJ pathway. This leads to the impairment of the DNA repair process by NHEJ and to the accumulation of the DNA damage in neurons (Figure 2). In fact, DNA damage repair has been recently shown to be a critical component of several polyglutamine (polyQ) disease pathologies, such as HD [85]. Also, it has been demonstrated that accumulated oxidative DNA damage triggers activation of the single-base excision repair enzyme oxoguanine glycosylase 1 (OGG1), thereby enhancing the CAG repeat instability during aging in somatic cells of polyQ patients [85]. The work of Enokido and colleagues (2010) definitely demonstrates that Ku70 is the mediator of the DNA repair dysfunction. In their experimental conditions, a specific Ku70 sequestration by the mutant huntingtin prevented an efficient DNA damage

repair by the NHEJ pathway. They also showed that Ku70 supplementation rescues phenotypes of HD mouse model [84]. More recently, it has been demonstrated that, in *Drosophila* models of HD, huntingtin and Ku70 coexpression recovers lifespan, locomotive activity, and eye degeneration, thus supporting the hypothesis that Ku70 is a critical and conserved mediator of the HD pathology [86].

#### 4. Conclusions

As discussed above, a critical process for maintaining homeostasis in the brain is an effective response to oxidative DNA damage. One of the most serious DNA lesions is DSB, and the NHEJ is the predominant pathway used by the cells to repair this kind of damage. Among NHEJ proteins, Ku is a key factor in regulating the cellular response to DSBs, since it directly promotes DNA repair by binding the broken ends, and participating in the modulation of critical apoptotic proteins, such as Bax and Apaf1. The genetic manipulation of Ku in mice has shown how it can be fundamental for brain development and homeostasis. However, the regulation of NHEJ and the mitochondrial apoptotic pathway could also represent two independent functions that Ku exerts in neurons. This could explain some important inconsistencies, such as the fact that the sole absence of LigIV or XRCC4 is sufficient to lead a massive apoptosis and a lethal phenotype in knock-out mice for these genes, still having a functionally active Ku. In fact, Ku could bind and repress the *Apaf1* gene far more in differentiated postmitotic neurons than in neural progenitors, as our recent findings in neuronal precursor cells bear out [69]. In fact, Apaf1 has to be expressed at high levels in these cells, in order to guarantee the potential of displaying apoptosis. On the other hand, mature neurons have to avoid cell death, surviving as long as the organism does. For this reason, Apaf1 silencing mediated by Ku could be a strategy which neurons adopt to escape apoptosis, thereby highlighting the pivotal function of Ku as a key regulator of neuron survival.

Finally, the discovery that exogenous Ku70 expression rescues abnormal behavior and pathological phenotypes in a mouse model of HD [84, 86], where DNA repair is impaired, provides the final evidence that Ku plays a crucial role in regulating brain homeostasis. Thus, Ku could be a promising therapeutic target in this pathology.

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## Review Article

# Nitric Oxide Inactivation Mechanisms in the Brain: Role in Bioenergetics and Neurodegeneration

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During the last decades nitric oxide ( $\cdot\text{NO}$ ) has emerged as a critical physiological signaling molecule in mammalian tissues, notably in the brain.  $\cdot\text{NO}$  may modify the activity of regulatory proteins via direct reaction with the heme moiety, or indirectly, via S-nitrosylation of thiol groups or nitration of tyrosine residues. However, a conceptual understanding of how  $\cdot\text{NO}$  bioactivity is carried out in biological systems is hampered by the lack of knowledge on its dynamics *in vivo*. Key questions still lacking concrete and definitive answers include those related with quantitative issues of its concentration dynamics and diffusion, summarized in the how much, how long, and how far trilogy. For instance, a major problem is the lack of knowledge of what constitutes a physiological  $\cdot\text{NO}$  concentration and what constitutes a pathological one and how is  $\cdot\text{NO}$  concentration regulated. The ambient  $\cdot\text{NO}$  concentration reflects the balance between the rate of synthesis and the rate of breakdown. Much has been learnt about the mechanism of  $\cdot\text{NO}$  synthesis, but the inactivation pathways of  $\cdot\text{NO}$  has been almost completely ignored. We have recently addressed these issues *in vivo* on basis of microelectrode technology that allows a fine-tuned spatial and temporal measurement  $\cdot\text{NO}$  concentration dynamics in the brain.

## 1. Introduction

Nitric oxide ( $\cdot\text{NO}$ ) is a small and diffusible free radical synthesized by a family of nitric oxide synthases (NOS) that participates in a wide range of signaling pathways in biological tissues, mediating physiologic processes such as vasodilation, memory and learning, neuronal development, regulation of immune response, among many others [1–5]. In the brain,  $\cdot\text{NO}$  is mainly synthesized in synaptic terminals by a neuronal NOS isoform, acting as a neuromodulator [3, 4]. The radical nature, small size, and hydrophobicity support the notion that  $\cdot\text{NO}$  lacks specific interactions with receptors, and yet these properties confer to this molecule a great versatility concerning interactions with biological targets. The outcome of these interactions is dictated by  $\cdot\text{NO}$  concentration dynamics, ranging from physiological to pathological effects leading to cell death. This dual role anticipates a tight regulation of  $\cdot\text{NO}$  concentration dynamics under physiologic conditions.

The major aspects that characterize  $\cdot\text{NO}$  neuroactivity and its regulation are discussed in this paper.

## 2. The Interactions of $\cdot\text{NO}$ with Biological Targets: Redox and Functional Impact

Nitric oxide is able to rapidly diffuse in tissue and interact with a variety of biological targets involved in relevant physiological processes. Two main mechanisms that stabilize the unpaired electron of  $\cdot\text{NO}$  are its reaction with other free radicals and interactions with d-orbitals of transition metals [6]. Among the latter, the interactions of  $\cdot\text{NO}$  with iron are the most relevant in biological systems due to the abundance of proteins containing iron, most notably heme proteins, involved in numerous physiological processes. Essentially,  $\cdot\text{NO}$  can interact with iron complexes by three ways: (a) binding to iron, (b) reaction with dioxygen iron complexes, and (c) reaction with high valent oxo-complexes [7].  $\cdot\text{NO}$

can bind to both ferrous and ferric heme proteins, but the binding to Fe(II) is generally faster and more reversible than to Fe(III) [8]. Actually, the majority of biologically relevant  $\bullet$ NO reactions with heme proteins involve the reversible binding of  $\bullet$ NO to ferrous iron in proteins, a process called nitrosylation [7]. For instance, the binding of  $\bullet$ NO to ferrous heme activates the enzyme soluble guanylyl cyclase (sGC) [3], which is the best characterized signaling target of  $\bullet$ NO and inhibits cytochrome c oxidase (CcO), a crucial enzyme for mitochondrial respiration [9]. The interaction between  $\bullet$ NO and ferrous hemoglobin is also biologically relevant by binding to the deoxy-hemoglobin heme or as a means to degrade  $\bullet$ NO via reaction with oxy-hemoglobin, resulting in the oxidation of the ferrous heme and formation of nitrate.

These interactions are relatively fast, exhibiting rate constants of  $2\text{--}4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$  [8]. Given the abundance of hemoglobin in the vasculature, they constitute the main pathway of  $\bullet$ NO removal in that compartment and significantly contribute to shape the dynamics of  $\bullet$ NO at the neighboring tissues [10].

A critical aspect of the interactions of  $\bullet$ NO with heme-proteins able to transduce a transient  $\bullet$ NO concentration change into a biological response is their different sensitivity for  $\bullet$ NO. The most sensitive  $\bullet$ NO physiologic signaling target is sGC with half-maximal activation at 10 nM [11], mediating most of the known  $\bullet$ NO biological effects [3]. For higher  $\bullet$ NO concentrations inhibition of CcO occurs, being half-maximal at  $\approx 120$  nM, under resting metabolic conditions and physiological  $\text{O}_2$  tension [9, 12].

The indirect effects of  $\bullet$ NO require it to react with molecular oxygen or superoxide anion radical ( $\text{O}_2^{\bullet-}$ ) to produce reactive oxygen and nitrogen species (RONS) such as nitrogen dioxide ( $\bullet\text{NO}_2$ ), nitrogen trioxide ( $\text{N}_2\text{O}_3$ ), and peroxyxynitrite ( $\text{ONOO}^-$ ).  $\text{ONOO}^-$  and  $\bullet\text{NO}_2$  are potent oxidants ( $>1.0$  V NHE) [13] and can both oxidize and nitrate protein residues and lipids. The product of  $\bullet$ NO autooxidation,  $\text{N}_2\text{O}_3$ , is a mild oxidant and will preferably nitrosate nucleophiles such as amines and thiols [14, 15].

S-Nitrosation is a covalent posttranslational modification associated to  $\bullet$ NO-dependent signaling, which refers to the incorporation of a nitroso group ( $-\text{NO}$ ) to a thiol group (in cysteine residues). There are several mechanisms by which S-nitrosation can occur, mainly through (a) involvement of  $\text{N}_2\text{O}_3$ , (b) direct interaction of  $\bullet$ NO with a thiyl radical (radical-radical interaction), and (c) transfer of a nitroso group from a nitrosylated metal or nitrosothiol (transnitrosation) [16]. S-Nitrosation mediated by  $\text{N}_2\text{O}_3$  somehow defines the microenvironment in which the modification predominantly occurs. Because the reaction of  $\bullet$ NO with  $\text{O}_2$  is slow, it requires high levels of both species, a condition favored by the proximity of sources of  $\bullet$ NO production and by hydrophobic environment (where both species accumulate). Also, the life time of  $\text{N}_2\text{O}_3$  is increased in hydrophobic compartments [7]. These factors limit S-nitrosation and confer selectivity to the reaction as only cysteine residues found within a particular microenvironment in a protein are prone to be nitrosated (reviewed by [17]).

Several proteins have been described as being regulated by S-Nitrosation and ensued physiologic and pathologic

consequences described [18, 19]. To mention some, S-nitrosation of N-methyl-D-aspartate receptors (NMDAR) has been shown to inactivate the receptor, thereby possibly protecting against excessive NMDAR activation and consequent excitotoxicity [20]. S-Nitrosation of 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid receptor (AMPA) regulatory proteins (stargazin and N-ethylmaleimide-sensitive factor) may increase surface expression of the receptor [21, 22]. Other examples include nitrosation of caspases-3 and -8 and poly(ADP-ribose) polymerase [23–25], protecting against apoptosis. In turn, S-nitrosation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) inhibits its dehydrogenase activity and induces an acyl phosphatase activity in the enzyme, resulting in the uncoupling of glycolytic flux from ATP synthesis [26]. Nitrosated GAPDH can also translocate to the nucleus, enabling it to degrade selected target proteins and affect apoptosis [27].

The nitration of proteins and lipids by  $\bullet$ NO-derived reactive species is a further posttranslational modification of proteins by which  $\bullet$ NO can accomplish functional diversity in cellular processes. It is currently accepted that protein nitration is an *in vivo* protein modification that translates into functional alterations in physiological and pathological settings [28]. Nitration results from the addition of a nitro  $-\text{NO}_2$  group to aromatic and aliphatic residues in proteins or to the aliphatic chain of fatty acids, mainly mediated by  $\text{ONOO}^-$  and  $\bullet\text{NO}_2$ . In proteins, tyrosine residues are the key target for nitration by  $\text{ONOO}^-$  (reviewed in [29]). 3-Nitrotyrosine (3-NT) has been used as a marker of pathological events associated to oxidative stress. Indeed, 3-NT immunoreactivity has been found in early stages of several neurodegenerative disorders in human autopsy samples as well as in animal models (reviewed in [28, 30]).

Protein nitration is a very selective modification. Not all tyrosine residues present in a given protein can suffer nitration. Protein folding, the surrounding local environment (namely, the presence of glutamate residues), and the nitration agent all contribute to direct nitration towards specific tyrosine residues [31]. Examples of nitration targets with relevance in the context of the nervous system and neurodegeneration may include the following: (1) neurofilament L in human ALS neurons, preventing assembly and possibly axonal transport, both pathological hallmarks of ALS [32–34]; (2) tyrosine hydroxylase, the rate-limiting enzyme in catecholamine synthesis, causing loss of enzyme function in the MPTP-induced Parkinson's disease model [35–37], affording a possible cause of dopamine deficiency prior to cell death; (3) nitration of Mn superoxide dismutase inactivates this mitochondrial antioxidant enzyme, with implications several disease states (reviewed in [38]); (4) Lewy bodies in Parkinson's disease have been found to contain nitrated  $\alpha$ -synuclein, which tends to form oligomers [39].

In sum, at low  $\bullet$ NO concentrations, direct  $\bullet$ NO interactions with transition metal centers via reversible binding are likely to predominate as a physiological signal transduction mechanism.

On the other hand, the actual concentration at which  $\bullet$ NO-dependent nitrosative and nitrative chemistry occurs,

either from a physiological or deleterious view point, is not known and likely depends on the particular tissue redox conditions. A rough estimate has been made in cellular preparations, indicating that nitrosative stress becomes significant when the •NO concentration reaches near-micromolar levels [5, 40].

From the lines of evidence shown above it becomes clear that the concentration dynamics of •NO (the profile of change in time and space) is an essential aspect of its biology. Nitric oxide signals, having different amplitude, time course, and spatial distribution may thus encode different signaling messages, possibly mediated by different biological targets. Paradoxically, in spite of being one of the most studied endogenous molecules during the last decades, the exceeding majority of studies are of qualitative and phenomenological nature, lacking the critical quantitative information on •NO dynamics. Under this scenario it is not a surprise that several dogmas have undermined the knowledge of •NO biological actions.

Therefore, the monitoring of •NO concentration profiles *in vivo*, allowing to unravel its endogenous concentration profiles and the major factors regulating its concentration, is a critical piece of knowledge to understand the mechanisms by which •NO affects cell and tissue function in the brain.

### 3. The Transient •NO Change from the Background and the Mechanisms That Regulate Its Profile

**3.1. •NO Production in the Brain.** The •NO signaling in CNS is intimately associated to the glutamate system. In glutamatergic synapses, •NO synthesis involves the stimulation of ionotropic glutamate receptors (iGluR), particularly NMDA subtype, and consequent influx of  $\text{Ca}^{2+}$  to the cytosol that, upon binding to calmodulin, activates neuronal isoform of NOS (nNOS). The  $\alpha$ -splice variant of nNOS is particular in that it possesses an N-terminal PDZ motif [41], which allows the enzyme to bind to other PDZ-containing proteins, such as the synaptic density scaffold protein PSD-95 [42]. The functional impact of this association is very relevant, as PSD-95 simultaneously binds to the NR2 subunit of the NMDAR [43, 44], thus forming a supramolecular complex that places the  $\text{Ca}^{2+}$ -dependent nNOS under the direct effect of  $\text{Ca}^{2+}$  influx through the activated NMDAR channel [45]. Upon  $\text{Ca}^{2+}$  influx, providing that substrates (L-arginine,  $\text{O}_2$ ) and several other cofactors (NADPH, FMN, FAD, tetrahydrobiopterin, heme) are available, nNOS catalyses the conversion of L-arginine to L-citrulline and •NO [46, 47]. However, the regulation of •NO synthesis by nNOS goes beyond  $\text{Ca}^{2+}$  dynamics, also involving, among other factors, specific adaptor proteins [48] and posttranslational modifications [49, 50]. In addition to the diverse regulatory mechanisms associated to •NO synthesis, the distribution of nNOS within a particular volume is suggested to influence the •NO volume signaling [51]. In essence, the abovementioned notions suggest an intricate regulatory process for •NO production that may translate into distinct concentration dynamics.

**3.1.1. Measurement of Nitric Oxide Concentration Dynamics In Vitro and In Vivo.** The measurement of •NO in real time by electrochemical methods with microelectrodes inserted into the •NO diffusion field is an important tool to characterize •NO concentration dynamics [52]. Glutamate-dependent •NO production in hippocampus is of particular relevance because of the involvement of •NO in the regulation of plasticity, such as learning and memory, and cell death associated with neurodegeneration [53]. Electrochemical detection presents several advantages in relation to other methods to measure •NO in biological systems, as it allows direct and real-time measurement. Moreover, the small size of the carbon fiber microelectrodes affords their use in nervous tissue, with minimal perturbation of the natural environment, and confers high spatial resolution to the measurements [54, 55].

In hippocampal slices, chemically modified carbon fiber microelectrodes with suitable analytical properties for •NO-selective measurement [56] have been used to perform real-time recording of endogenous •NO concentration dynamics evoked by activation of ionotropic GluR. By using this methodological approach we were able to show that NMDA-evoked •NO concentration dynamics is heterogeneous along the trisynaptic loop in the rat hippocampus [57]. We also provided evidence that the AMPAR in addition to the NMDAR could contribute to the fine tuning of glutamate-dependent •NO production [58] and that NMDA-evoked •NO production inhibits tissue  $\text{O}_2$  consumption for submicromolar concentrations [59].

The use of this approach has also allowed the characterization of endogenous •NO concentration dynamics produced in rat brain *in vivo* upon glutamatergic stimulation. In the hippocampus we found that both endogenous and synthetic agonists of ionotropic GluR (glutamate, NMDA, and AMPA) promoted transient increases of extracellular •NO concentration, although with different kinetics. Pharmacological modulation suggested that •NO overflow elicited by glutamate resulted from an integrated activation of both subtypes of ionotropic GluR [60]. Glutamate-evoked •NO concentration changes were further characterized along the hippocampal trisynaptic loop (CA1, CA3, and dentate gyrus), as well as in cerebral cortex and striatum, showing that while glutamate induced transitory increases in •NO levels in all regions, regional-specific concentration profiles were observed (unpublished data).

**3.2. •NO Diffusion and Half-Life.** Together with •NO production, the rates of diffusion and inactivation are key determinants of •NO concentration dynamics in tissues. However, unlike the mechanisms of •NO synthesis, the physiologic processes underlying the termination of •NO signals *in vivo* (inactivation) remain unclear, particularly in the brain. Inactivation of classical neurotransmitters (e.g., dopamine or glutamate) relies on their rapid removal from the extracellular space via intracellular uptake processes [61–63]. However, given the uncommon physicochemical properties of •NO, its consumption via chemical reactions is generally accepted as the most probable route of inactivation. Although several biochemical mechanisms have

been proposed to fulfill that role, the exact extent of their effect on  $\bullet\text{NO}$  concentration dynamics in brain tissue has remained uncertain. Another layer of complexity is added by the effect of diffusional processes on the signal. Although  $\bullet\text{NO}$  has been frequently considered as a molecule that can freely diffuse in tissues [64–66], with a diffusion coefficient estimated in aqueous solution of  $2 \times 10^{-5}$  to  $4.5 \times 10^{-5}$   $\text{cm}^2/\text{s}$  [67–69], it has been observed that  $\bullet\text{NO}$  diffusion is hindered across the aortic wall [70].

Recently, we have obtained experimental data that allowed the characterization of  $\bullet\text{NO}$  diffusion in the rat brain *in vivo*. The studies were conducted by using  $\bullet\text{NO}$ -selective carbon fiber microelectrodes to monitor  $\bullet\text{NO}$  increases following local application of small volumes (few nanoliters) of  $\bullet\text{NO}$  solution from a micropipette located 200–350  $\mu\text{m}$  away. These microelectrode/micropipette arrays were stereotaxically inserted in the brain of anesthetized rats [71].

The resulting electrochemical signals were fitted to an equation that describes the diffusion of a compound and takes into account a first-order kinetics of inactivation. This approach allowed the estimation of the  $\bullet\text{NO}$  diffusion coefficient and half-life in tissue. We found that  $\bullet\text{NO}$  is highly diffusible and short lived in the brain, having an effective diffusion coefficient ( $D_{\text{NO}}^*$ ) of  $2.2 \times 10^{-5}$   $\text{cm}^2/\text{s}$  and a half-life of 0.64 s in the rat cortex.

We have also investigated possible pathways of  $\bullet\text{NO}$  diffusion by testing the concepts of free, hindered, and enhanced diffusion. The  $D_{\text{NO}}$  obtained in agarose gel, a model used to evaluate  $\bullet\text{NO}$ -free diffusion, was  $2.6 \times 10^{-5}$   $\text{cm}^2/\text{s}$ , only 14% higher than the *in vivo*  $D_{\text{NO}}^*$ , suggesting that  $\bullet\text{NO}$  could freely diffuse in the brain. Accordingly, we found that  $\bullet\text{NO}$  diffusion in brain tissue is distinct from a molecule of similar size that remains in the extracellular space (nitrite), but importantly, our data indicated that  $\bullet\text{NO}$  diffusion is hindered by conditions that mimic intracellular macromolecular crowding [71].

These latter lines of evidence suggest that neither  $\bullet\text{NO}$  diffusion through the extracellular space nor a homogeneous diffusion in the tissue through brain cells provides a reasonable conceptual explanation for the similarity between the  $D_{\text{NO}}^*$  obtained *in vivo* and the  $D_{\text{NO}}$  found in agarose gel. Thus, as previously suggested for  $\text{O}_2$  [72, 73], it is likely that  $\bullet\text{NO}$  diffusion in nervous tissue is facilitated by certain physiological processes. A likely candidate is  $\bullet\text{NO}$  partition in hydrophobic media, such as cell membranes and myelin sheaths, which may constitute low-resistance pathways that facilitate  $\bullet\text{NO}$  diffusion in nervous tissue, resulting in an increased diffusion rate.

Together with diffusion, the quantification of  $\bullet\text{NO}$  half-life in the brain is also important to better understand the dynamics of  $\bullet\text{NO}$ , produced during brain function.  $\bullet\text{NO}$  half-life is a kinetic parameter essential for the definition of its basal concentrations and diffusion radius. Some studies have estimated the  $\bullet\text{NO}$  half-life in biological tissues in the range between 5 and 15 s [66]. Studies performed on isolated cell preparations of brain and liver extrapolated values for *in vivo* of around 100 ms [74, 75], in accordance with the half-life obtained in heart muscle [76]. In intact brain tissue,

a half-life of 10 ms was reported in acute cerebellar slices for  $[\bullet\text{NO}]$  below 10 nM [77] which was 60 fold slower in organotypic cerebellar slices [78]. Although variability exists in the data, these works support the notion that  $\bullet\text{NO}$  is a short-lived messenger, in agreement with the biological necessity to maintain  $\bullet\text{NO}$  levels within the physiological range. Our *in vivo* approach, based on the fitting of a diffusion/inactivation equation to the signals of exogenously applied  $\bullet\text{NO}$  *in vivo* in the rat brain cortex, allowed the estimation of a half-life of 0.64 s in that brain region, thus providing quantitative experimental evidence for a sub-second  $\bullet\text{NO}$  half-life *in vivo*.

**3.3. Mechanisms of  $\bullet\text{NO}$  Inactivation.** Several studies have found  $\text{O}_2$ -dependent mechanisms of  $\bullet\text{NO}$  consumption/inactivation in *in vitro* preparations but the direct reaction between  $\bullet\text{NO}$  and  $\text{O}_2$  is too slow to account for significant  $\bullet\text{NO}$  consumption if one considers the low concentration of the reactants *in vivo* (particularly  $\bullet\text{NO}$ ). Nevertheless, the favored partition of these molecules in the hydrophobic phase of cell membranes greatly accelerates the reaction, possibly accounting for  $\bullet\text{NO}$  consumption in tissues [79].

As mentioned above,  $\bullet\text{NO}$  direct biological activity may play out through the reaction with transition metals, in particular the iron contained in heme proteins. One such target for  $\bullet\text{NO}$  is Cytochrome c oxidase (CcO), the terminal complex of the mitochondrial respiratory chain. In the mid-1990s,  $\bullet\text{NO}$  was shown to bind to and inhibit CcO [80] and block mitochondrial respiration in preparations as diverse as isolated mitochondria [81] synaptosomes [82], and primary cell cultures [83, 84].

The initial assessment of  $\bullet\text{NO}$  interaction with CcO suggested an inhibition mechanism based on the high-affinity, reversible binding of  $\bullet\text{NO}$  to the enzyme's binuclear active site, in competition with  $\text{O}_2$ . [81, 82, 85]. In accordance with this model that prevails under high enzyme turnover conditions,  $\bullet\text{NO}$  binds to the fully reduced binuclear center (heme  $a_3/\text{Cu}_B$ ) and its removal from the active binuclear sites returns CcO to a fully active state.

A second low-affinity inhibitory site has been proposed for the fully oxidized enzyme—the  $\text{Cu}_B$  in the binuclear center-rendering CcO inactive [86, 87]. This is an uncompetitive mechanism of inhibition of CcO by  $\bullet\text{NO}$ . Contrary to the simple on/off mechanism observed in the competitive model, bound  $\bullet\text{NO}$  reduces the enzyme and is itself oxidized to  $\text{NO}_2^-$ . Enzyme inhibition is reverted by dissociation of  $\text{NO}_2^-$  upon further reduction [88, 89]. This uncompetitive inhibition mechanism is favored by high  $[\text{O}_2]$  and low turnover and its main biological role seems to be to consume  $\bullet\text{NO}$ , thus shaping  $\bullet\text{NO}$  concentration dynamics in the tissue [90, 91].

In mitochondria,  $\bullet\text{NO}$  can also be consumed by the nearly diffusion-limited reaction with  $\text{O}_2^{\bullet-}$  [92], generated as a byproduct of cellular respiration. However, the physiologic relevance of this reaction is questionable, since  $\text{O}_2^{\bullet-}$  dismutation by MnSOD greatly decreases  $\text{O}_2^{\bullet-}$  concentration (in spite of the favoured competition of  $\bullet\text{NO}$  over SOD to  $\text{O}_2^{\bullet-}$ ) to values in the pM range under physiologic conditions [7, 93].

Several heme-containing enzymes catalyze redox reactions that can consume •NO in dispersed preparations. Among others, a flavoheme protein in several mammalian cell lines [94], lipoxygenases, prostaglandin H synthase and cyclooxygenase-1 in platelets [95–97], peroxidases [98], and cytochrome P450 oxidoreductase [99] have been identified.

Proteins from the globin family are other likely candidates to participate in •NO inactivation in brain tissue due to their rapid reaction with •NO ( $k \approx 10^7 \text{ M}^{-1} \text{ s}^{-1}$ ). In several brain regions, neurons express neuroglobin (Ngb), cytoglobin, and also hemoglobin [100–102]. The exact physiologic functions of these proteins in the brain tissue are not clear. It was observed that the overexpression of Ngb protects the brain against ischemic damage [103] and cells overexpressing Ngb are more resistant to •NO cytotoxicity, suggesting a possible role of •NO scavenging in the neuroprotective mechanism of Ngb. However, given the low intraneuronal concentration of these proteins ( $<1 \mu\text{M}$ ), the function of Ngb as a robust •NO scavenger would require its association with a putative met-Ngb reductase to rapidly regenerate ferrous heme upon reaction with •NO. Yet, this functional association has not been reported [104]. Neuronal hemoglobin appears to have a role in intracellular oxygen storage or transport but insufficient data exists regarding its capacity to metabolize •NO in neurons [102]. Conversely, it is widely accepted that hemoglobin is the major •NO sink in the vasculature, due to its high concentration in erythrocytes (ca. 20 mM) and high reaction rate with •NO [105]. The effectiveness of this reaction on the regulation of •NO concentration in the extravascular compartment has been disputed since erythrocytic packing of hemoglobin and intravascular flow can decrease the effectiveness of hemoglobin scavenging of •NO by 3–4 orders of magnitude [105–107].

The uncertainty regarding how •NO is inactivated in the brain in physiologic conditions is related to difficulties in its direct measurement in intact tissue. A study on •NO inactivation in cerebellar slices of rat brain found that •NO was inactivated by an unknown mechanism that could not be explained by any known mechanism of •NO consumption [77].

**3.3.1. Pathways of •NO Inactivation In Vivo.** A strategy to the study of the mechanisms that govern •NO inactivation *in vivo* has included the recordings of •NO signals by •NO-selective microelectrodes, following local application of small volumes of exogenous •NO in the brain [10]. The decay of •NO signals obtained by means of this approach was very sensitive to experimental conditions impairing vascular function *in vivo*. First, global ischemia induced a 90% decrease in the •NO signals decay rate constant ( $k$ ), suggesting that •NO inactivation is nearly abolished during this condition. Second, the  $k$  values of •NO signals decay were 3–5-fold higher *in vivo* than in brain slices of cortex and hippocampus, which lack functional vasculature. Finally, impairing the microcirculation in the brain *in vivo* by inducing hemorrhagic shock induced an average 50% decrease in  $k$ . Comparatively, modulation of  $\text{O}_2$  tension in the brain *in vivo*, either by inducing hypoxia or hyperoxia, caused

only small changes in •NO decay (20%), thus demonstrating that scavenging by circulating red blood cells constitutes the major •NO inactivation pathway in the brain (Figure 1).

The •NO half-life in tissue is thereby expected to be dependent on the vascular density, which may be tuned to meet specific local signaling requirements. Accordingly, using microelectrode arrays that allow monitoring •NO in four brain sites simultaneously, we observed that the probability of •NO detection following its local application is apparently related with the local vascular density [10].

## 4. The Functional Impact of •NO in Brain Tissue

**4.1. •NO as a Neuromodulator.** sGC is the best characterized signaling target for •NO and is often considered a receptor-like molecule for •NO in cells for the role of •NO as a neuromodulator is mainly mediated by its binding to sGC, leading to transient increases in cGMP.

The most widespread mechanism associated with cGMP is the activation of the cGMP-dependent protein kinase (PKG). Several substrates for PKG have been identified and many of its actions are exerted at the level of phosphatases, thereby affecting the levels of phosphorylation of effector proteins [108]. Other mechanism able to mediate •NO signaling effects downstream sGC is the cGMP activation of cyclic nucleotide-gated (CNG) ion channels [109] and hyperpolarization-activated cyclic nucleotide-modulated (HCN) channels [110]. CNG and HCN are nonselective cation channels that allow the passage of several ions, including  $\text{Na}^+$ ,  $\text{K}^+$ , and  $\text{Ca}^{2+}$ . For instance, studies reported that, in neurons expressing these ionic channels, the •NO-cGMP pathway cause a pre- or postsynaptic membrane depolarization (depending on the location of the channels) thereby modulating neuronal excitability [3].

It is noteworthy that the actions of •NO through the •NO-cGMP pathway obey no general rules. Despite employing the same transduction mechanism (cGMP), the physiological outcomes are tissue/cell specific since different cell populations may arbor different cGMP targets. Thus, the great variability of cGMP targets among different populations of neurons seems to explain the wide range of reported •NO effects in the nervous system. To cite some examples, •NO has been implicated in the modulation of neuronal excitability, synaptic plasticity, modulation of neurotransmitter release, regulation of rhythmic activity, and neurovascular coupling [3, 4, 46, 111].

**4.2. Regulation of Mitochondrial Respiration.** The average  $[\text{O}_2]$  in capillaries is  $30 \mu\text{M}$  and typically cells experience an intracellular concentration of  $\text{O}_2$  of around  $3 \mu\text{M}$  [112]. The high affinity of CcO for its substrate guarantees sustained mitochondrial phosphorylation with a large safety margin regarding  $\text{O}_2$  [113]. •NO will compete with  $\text{O}_2$  for binding to the active site of CcO, inhibiting respiration, raising the enzyme's  $K_m$  and limiting  $\text{O}_2$  usage even under normoxic conditions.

The physiological and/or pathophysiological impact of •NO inhibition of CcO will depend on the fraction of enzyme that is effectively inhibited and if this decreases

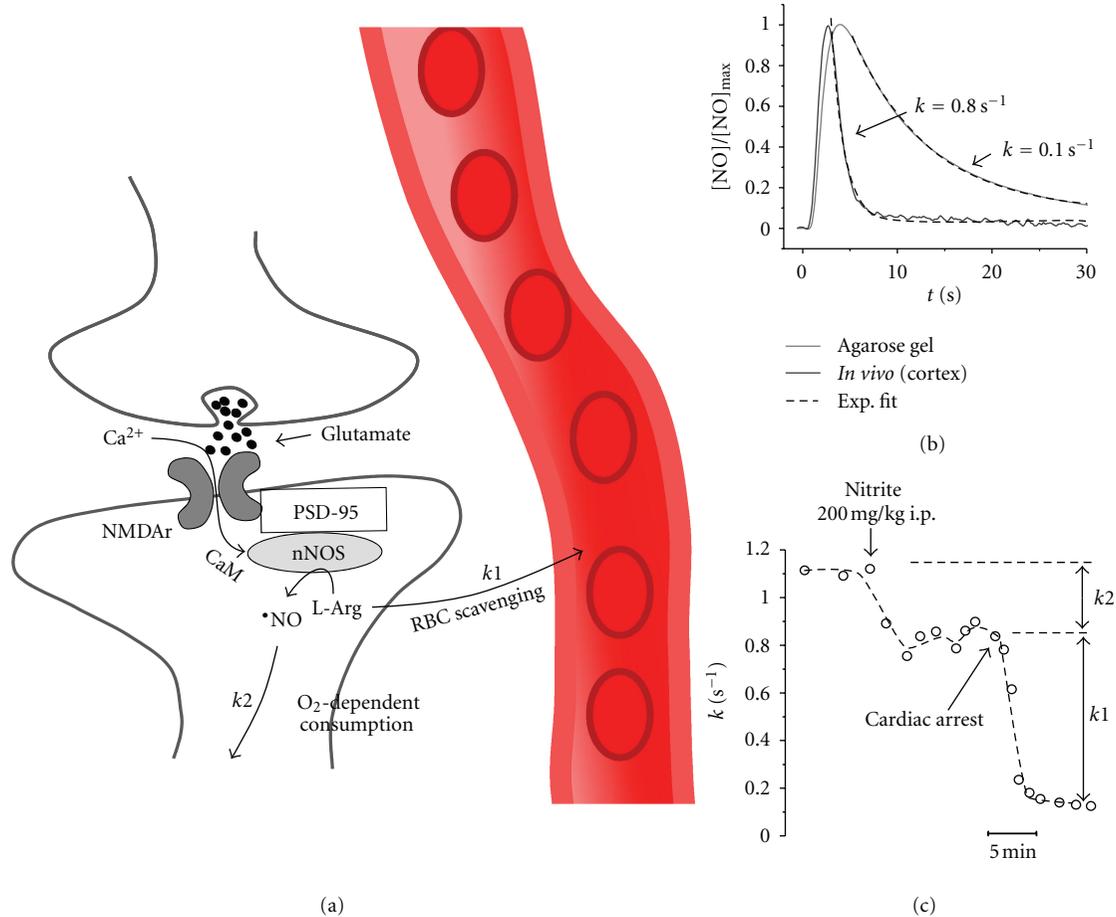


FIGURE 1: The major pathways of  $\bullet$ NO production and inactivation in the brain. (a)  $\bullet$ NO is synthesized following calcium entrance into the postsynaptic density (upon glutamate activation of NMDA receptors). Calcium activates nNOS by promoting Calmodulin (CaM) binding to the enzyme.  $\bullet$ NO rapidly diffuses to neighboring tissue, being inactivated both by O<sub>2</sub>-dependent mechanisms and by scavenging by circulating erythrocytes (RBCs). (b) Typical electrochemical signals obtained using microelectrodes in the rat brain *in vivo* and in agarose gel following local application of small volumes (few nL) of  $\bullet$ NO solution. First-order decay constant values ( $k$ ) were used to quantify the decay profiles. (c) Anoxia, induced by a nitrite lethal dose, induced a 20% decrease in  $k$  ( $k_2$ ), in contrast with a large decrease in  $k$  following cardiac arrest, suggesting that the major route of  $\bullet$ NO inactivation in the brain *in vivo* is by circulating RBCs scavenging ( $k_1$ ). Adapted from [10].

O<sub>2</sub> consumption and oxidative phosphorylation [114]. The overall electron flow in the respiratory chain is typically regulated by complex I in state 4 respiring mitochondria and under these conditions, a fractional inhibition of CcO by  $\bullet$ NO produces no net change on O<sub>2</sub> consumption, contrary to what occurs in state 3 respiring mitochondria [115, 116]. An immediate consequence of decreased O<sub>2</sub> consumption is the increase in O<sub>2</sub> availability and increased tissue oxygenation at sites farther away from blood vessels [74], an effect that may act in synergy with  $\bullet$ NO-induced vasodilation. Alternatively, increased [O<sub>2</sub>] may render it available to participate in signaling pathways such as the activation of hypoxia-inducible factor [117].

A key notion which has emerged in the literature is that mitochondrial cell signaling cascades are intimately linked to  $\bullet$ NO regulation of CcO function [118]. The interaction of  $\bullet$ NO with CcO may inhibit enzyme activity

without producing a net effect in cellular respiration: steady-state and kinetic modeling [119] have revealed that the  $\bullet$ NO-CcO interaction can lead to an accumulation of reduced cytochromes upstream of CcO without producing a net effect on cellular respiration, but with consequences in redox signaling pathways such as increased mitochondrial production RONS, notably O<sub>2</sub><sup>-•</sup> and H<sub>2</sub>O<sub>2</sub> [114, 120, 121], both of which can impact downstream signaling cascades.

The situation changes dramatically when inhibition of CcO is severe and persistent-excessive inhibition of mitochondrial respiration results in bioenergetics dysfunction and cellular damage, conditions associated with aging and neurodegeneration. Under such conditions, excess O<sub>2</sub><sup>-•</sup> may react with  $\bullet$ NO yielding ONOO<sup>-</sup>, which unlike  $\bullet$ NO can irreversibly block all complexes of the mitochondrial respiratory chain through oxidation and nitration chemistry [122–124]. In 2005, Shiva et al. proposed the term nitroxia to

describe this pathological situation resulting from a deregulation of the  $\bullet\text{NO}$ -CcO signaling pathway with increased production of RONS leading to mitochondrial and cellular oxidation and nitration chemistry [125].

**4.3. Neurovascular Coupling.**  $\bullet\text{NO}$  has been suggested as a mediator of the neurovascular coupling, the active mechanism enlarging vessel diameter in response to the rising of metabolic demands imposed by neuronal activity [126]. Indeed,  $\bullet\text{NO}$  appears well suited to mediate this process as it is a potent vasodilator, is released during enhanced neuronal activity, and, as previously discussed, is also highly diffusible. During the years, the role of  $\bullet\text{NO}$  in neurovascular coupling has been strengthened by the observation that the increase in cerebral blood flow dependent on neuronal activation is repressed by NOS inhibitors [127–130]. However, contradictory reports have also shown the lack of effect of NOS inhibitors, pointing towards a lack of  $\bullet\text{NO}$ -mediated effect associated to neurovascular coupling [131–133]. As a matter of fact, although it is plausible that neuronal-derived  $\bullet\text{NO}$  is involved in the regulation of cerebral blood flow, concrete evidence is still missing, as well as the elucidation of the underlying mechanism [134]. Recently, by using an experimental approach that allowed the simultaneous measurement of  $\bullet\text{NO}$  concentration dynamics and cerebral blood flow changes *in vivo* upon glutamatergic activation in hippocampus we were able to establish the temporal, amplitude, and spatial association of both events. Furthermore, the coupling between neuronal activation and local cerebral blood flow changes mediated by neuronal-derived  $\bullet\text{NO}$  occurs in the hippocampus regardless of the intermediacy of other cellular players such as astrocytes (unpublished data).

The mechanism of neuronal-derived  $\bullet\text{NO}$ , via volume signaling, mediating neurovascular coupling, may be a non-canonical fashion to underlie a process of vital importance for the brain to preserve its structural and functional integrity [135]. However, the dual interaction of  $\bullet\text{NO}$  concentration dynamics and vasculature assists the hypothesis of a highly and intrinsically controlled mechanism to match blood supply with the metabolic demands imposed by increased neuronal activity. While  $\bullet\text{NO}$  triggers the increase in cerebral blood flow, in turn, the increase in the cerebral blood flow, by way of hemoglobin-dependent inactivation of  $\bullet\text{NO}$ , helps to shape the  $\bullet\text{NO}$  signal.

**4.4. Neurodegeneration.** A key tenet of  $\bullet\text{NO}$  bioactivity is that besides participating in important physiological functions as those previously mentioned, it has also been implicated in pathological processes associated with several neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), and ischemic brain injury (reviewed by [111, 136]). The pathological role of  $\bullet\text{NO}$  involves pathways that are only partially different from those underlying its physiological actions but which are associated to other stress conditions. In AD, the three NOS isoforms are suggested to operate as central mediators of amyloid- $\beta$  ( $A\beta$ ) action, contributing to the maintenance, self-perpetuation, and

progression of the disease [137], although data regarding changes in NOS in AD are highly inconsistent [138]. In cell-free assays,  $A\beta$  peptides were shown to strongly inhibit constitutive NOS (eNOS and nNOS) [139]. Conversely, other reports have shown  $A\beta$ -dependent enhancement of nNOS activity [140], and memory impairment appears to be correlated with the increase in nNOS expression and  $\bullet\text{NO}$  levels [141].

A larger consensus exists regarding the inducible isoform of NOS, which seems to be overexpressed in AD [138]. Various studies have reported that  $A\beta$  stimulates microglial and astrocytic  $\bullet\text{NO}$  production [142–144]. Moreover, modifications of iNOS expression are suggested to importantly contribute to AD progression. The ablation of iNOS from a transgenic mouse model of AD protected the AD-like mice from cerebral plaque formation and increased  $A\beta$  levels, astrocytosis, and microgliosis [145]. Moreover, astrocytic-derived  $\bullet\text{NO}$  triggers tau hyperphosphorylation in hippocampal neurons [146] and  $A\beta$ -mediated inhibition of NMDAR-dependent LTP requires iNOS activity [147].

The majority of neurotoxic effects of  $\bullet\text{NO}$  supporting the development of AD pathological mechanisms are due to indirect reactions of  $\bullet\text{NO}$ , promoting posttranslational protein modifications, namely, nitration and S-nitrosation. Indeed, high levels of nitrotyrosine have been found in brains from AD patients [148–151]. Nitrated proteins were described to be associated with  $A\beta$  deposition [150], and recently  $A\beta$  itself was shown to be a target for  $\bullet\text{NO}$  bioactivity. Nitrated  $A\beta$  is characterized by an accelerated aggregation rate, being detected in the core of plaques of APP/PS1 mice and AD brains [152]. Also tau protein and synaptophysin are potential targets for nitration with relevant consequences in terms of AD progression [153].

Furthermore, lines of evidence suggest that a number of proteins are S-nitrosated in AD. An example with important impact in neurodegeneration is the S-nitrosation of endoplasmic reticulum (ER) chaperone protein-dissulfide isomerase (PDI). The modification of an active cysteine in the PDI promotes the inhibition of both isomerase and chaperone activities, resulting in abnormal accumulation of misfolded and polyubiquitinated proteins, ER stress, and ultimately in cell death [154, 155]. Also dynamin-related protein 1 (Drp1) is a target for S-nitrosation, being hyperactivated, a mechanism suggested to underlie  $A\beta$ -related mitochondrial fission and neuronal injury [156].

Finally, it should be remarked that  $\bullet\text{NO}$  may also have a protective role in the development of AD pathology. Under physiological conditions, endothelium-derived  $\bullet\text{NO}$  evidenced a protective action against  $A\beta$  accumulation through direct modulation of  $A\beta$ , APP, and BACE-1 levels [157].

## 5. Novel Perspectives on the Mechanisms Underlying Deregulation of $\bullet\text{NO}$ Dynamics in Disease

As mentioned in the sections above, the excessive activation of NOSs is commonly regarded as the main mechanism

leading to the buildup of cytotoxic •NO concentrations in pathological conditions ranging from AD, PD, multiple sclerosis (MS) to ischemic damage [158, 159]. Despite the view of the excessive •NO production in neurodegeneration (although the quantitation *in vivo* requires refinements), the notions presented in the previous sections of this paper also highlight the role of the pathways of •NO diffusion and inactivation in the regulation of •NO levels in brain tissue. Accordingly, a pathological change of the mechanisms controlling diffusion and inactivation might also cause a deregulation in •NO concentration dynamics with consequences for tissue homeostasis. Indeed, given the recently found importance of the vasculature on the regulation of •NO inactivation *in vivo* [10], there are several pathological situations in which a vascular impairment might greatly account for deregulation of •NO levels, including ischemia-reperfusion, AD, and MS.

It is known that, in the brain of AD patients, vascular dysfunction appears in the early stages of the disease, which manifests as a characteristic oligemia in some brain regions [160]. This condition might contribute to increase •NO concentration due to lowered inactivation.

Conversely, the blood brain barrier breakdown that occurs in MS [161] might contribute to increase •NO inactivation, thereby lowering •NO concentration. But, alterations in inactivation might also be beneficial in some circumstances. We observed a great decrease in •NO inactivation rate in the brain during global ischemia [10], which might potentiate •NO accumulation in the affected tissue, formed from either residual NOS activity or NOS-independent •NO synthesis mechanisms, such as ischemic reduction of nitrite [162]. Interestingly, preischemic administration of •NO donors or nitrite *in vivo* decreases brain ischemia/reperfusion infarct volume in models of focal ischemia [162]. The vasodilatory action of •NO may explain its neuroprotective role during ischemia by enhancing microcirculation in the regions adjacent to the affected area (penumbra). Thus, it is possible that the impairment of •NO inactivation in the brain region affected by ischemia is protective by increasing local •NO availability and consequently enhancing microcirculation in the adjacent tissue.

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## Research Article

# Kinetic Modeling of the Mitochondrial Energy Metabolism of Neuronal Cells: The Impact of Reduced $\alpha$ -Ketoglutarate Dehydrogenase Activities on ATP Production and Generation of Reactive Oxygen Species

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Reduced activity of brain  $\alpha$ -ketoglutarate dehydrogenase complex (KGDHC) occurs in a number of neurodegenerative diseases like Parkinson's disease and Alzheimer's disease. In order to quantify the relation between diminished KGDHC activity and the mitochondrial ATP generation, redox state, transmembrane potential, and generation of reactive oxygen species (ROS) by the respiratory chain (RC), we developed a detailed kinetic model. Model simulations revealed a threshold-like decline of the ATP production rate at about 60% inhibition of KGDHC accompanied by a significant increase of the mitochondrial membrane potential. By contrast, progressive inhibition of the enzyme aconitase had only little impact on these mitochondrial parameters. As KGDHC is susceptible to ROS-dependent inactivation, we also investigated the reduction state of those sites of the RC proposed to be involved in ROS production. The reduction state of all sites except one decreased with increasing degree of KGDHC inhibition suggesting an ROS-reducing effect of KGDHC inhibition. Our model underpins the important role of reduced KGDHC activity in the energetic breakdown of neuronal cells during development of neurodegenerative diseases.

## 1. Introduction

A decline in the activity of the thiamine-dependent enzyme complex  $\alpha$ -ketoglutarate dehydrogenase (KGDHC) in brain has been reported for numerous age-related neurodegenerative diseases [1, 2]. In Alzheimer's disease, reductions in brain KGDHC activity range from 25 to 75% [3–7] and are strongly correlated to the decline in cognition [8]. Variations in the amounts of KGDHC between different brain regions [9, 10] may account for the brain region-specific different vulnerabilities. Neurons containing high amount of KGDHC like cholinergic neurons in the nucleus basalis are particularly susceptible to Alzheimer's disease [3, 11].

The citric acid cycle is catalyzed by eight enzymes, among which KGDHC has the lowest activity [12]. Thus, KGDHC is considered one of the rate-limiting enzymes in the tricarboxylic acid cycle (TCAC). It has been proposed that reduced activity of this enzyme complex initiates

a cascade of adverse processes, including metabolic failure, mitochondrial membrane depolarization, calcium overload, and cytochrome c release, eventually leading to cell death [13]. The same cascade has been implicated in the massive death of dopaminergic neurons in the substantia nigra of patients with Parkinson's disease [14].

The molecular mechanisms underlying the age-dependent loss of brain KGDHC activity remain elusive. Inactivation of the enzyme complex by reactive oxygen species (ROS) is one possible explanation as KGDHC and the aconitase have been shown to be the main targets of ROS in the citric acid cycle [15].

To further clarify the implications of reduced activities of the TCAC enzymes KGDHC and aconitase for the mitochondrial energy metabolism and the formation of ROS by the respiratory chain, we developed and applied a detailed kinetic model encompassing the TCAC, the respiratory chain (RC), translocation of adenine nucleotides between

mitochondrial matrix and the cytosol, oxidative phosphorylation, and ion transport across the inner mitochondrial membrane. The submodel of the RC describes the electron transport as a multistep process whereby some of the intermediate redox sites allow electron transfer to molecular oxygen under formation of the superoxide anion (ROS).

## 2. Model

The reaction scheme of the kinetic model is shown in Figure 1. It comprises the reactions of the citric acid cycle, the respiratory chain, oxidative phosphorylation, mitochondrial ATP generation, the exchange of adenine nucleotides exchange between mitochondrial matrix and cytosol, and the transport of small ions across the inner mitochondrial membrane. Since more than 90 percent of the ATP produced in neuronal cells is derived from oxidative phosphorylation, we omitted the glycolytic pathway while putting the supply of pyruvate and its uptake into the mitochondrial matrix to a fixed value.

The kinetic model focuses on the mitochondrial-derived ATP production and is compartmentalized into cytosol and mitochondria. The model consists of 184 state variables describing the neuronal citric acid cycle, the respiratory chain, the oxidative phosphorylation, the mitochondrial ATP generation, the nucleotide exchange between the mitochondrial matrix and the cytosol, and the electrophysiological coupling between the pathways. Since more than 90 percent of the ATP produced in neuronal cells is derived from oxidative phosphorylation [16], we omitted the glycolytic pathway. It is assumed that the glycolytic pathway is not limiting in the provision of pyruvate, so cytosolic pyruvate was kept constant. Since cytosolic redox equivalents are not considered, the aspartate/malate shuttle is not part of the model. Mitochondrial calcium is a potent activator of PDH, IDH, and KGDHC. Mitochondrial calcium concentration does vary in a physiological range depending on mitochondrial membrane potential [17, 18]. For simplicity, we modeled the mitochondrial calcium concentration as linear function of the mitochondrial membrane potential.

In order to include the potential formation of ROS in our model, we developed a detailed submodel of the respiratory chain that takes into account the substructure of complexes I and III composed of several prosthetic groups and iron sulfur clusters (see Figure 2).

The functional parts of complex I are the flavine mononucleotide (FMN), eight iron-sulfur clusters (i.e., N3, N1a, N1b, N4, N5, N6a, N6b, and N2), and the docking site for ubiquinone. FMN accepts two electrons from NADH forming fully reduced flavine. The electrons are then successively transferred to the subsequent iron-sulfur clusters. When the first electron is moved, the flavin exists as a flavin radical. From the terminal iron-sulfur cluster N2, the electron is transferred to ubiquinone forming a bound semiubiquinone (SQ). A second electron transported from N2 to SQ generates ubiquinol that is released from complex I. This electron transfer takes place in the arm of complex I extending into the mitochondrial matrix, whereas the ubiquinone/ubiquinol conversion is located at the n-site

of the inner mitochondria membrane. The transfer of one electron from N6b to N2 and from N2 to ubiquinone or semiubiquinone is linked with the export of one proton from the matrix into the cytosol. In the model, the electron state of complex I is represented as an array of integer numbers where the reduction of the flavinmononucleotide, each iron-sulfur cluster, and the existence of the bound semiubiquinone are encoded. All reactions are modeled as reversible mass action kinetics. Model simulations show that lumping together the complexes N1b, N4, N5, N6a, and N6b gives similar results as the full model but reduces the number of state variables from 1536 to 96.

Each state of complex III is described as an array describing the reduction and binding states of its functional parts: cytochrome c1 ( $c_1$ ), the iron sulfur cluster (Fe-S), the binding site for ubisemiquinone at p-site ( $SQ_p$ ), the low b-type heme ( $b_L$ ), the high b-type heme ( $b_H$ ), and the binding site for ubisemiquinone at n-site ( $SQ_n$ ). The spatial arrangement of these redox carriers enables the transduction of two electrons from one molecule ubiquinol to two molecules of cytochrome c via the q-cycle mechanism. Ubiquinol diffuses from n- to p-site, reacts with oxidized Fe-S, thereby reducing it, and generates a bound semi-ubiquinon at p-site. The bound semi-ubiquinon reduces  $b_L$  at p-site and free ubiquinon diffuses back to n-site. Only after release of ubiquinone from p-site, reduced Fe-S is able to transfer its electron to oxidized cytochrome c1, which passes it on to cytochrome c. The reduced  $b_L$  transfers its electron to oxidized  $b_H$ . The reduced  $b_H$  reacts with n-site ubiquinone to bound semi-ubiquinon. In a second round, reduced  $b_H$  transfers its electron to bound semi-ubiquinon. In this way one electron is recycled in each turnaround of ubiquinol, and two protons are transferred from the mitochondrial matrix to the intermembrane space/cytosol per electron transferred to cytochrome c. Importantly, it is assumed that electrons are transferred from ubiquinol at p-side in a two-step process, first reducing the Fe-S and binding of  $SQ_p$  and afterward reducing  $b_L$ , and release of  $Q_p$ . As long as  $SQ_p$  is bound, Fe-S cannot transfer the electron to  $c_1$ . This gives 48 state variable and 88 reactions for complex III. All electron transitions in complexes I and III are modeled as reversible mass action kinetics. For further details, see supplemental information in Supplementary Material available online at doi:10.1155/2012/757594.

While kinetic models of the citric acid cycle are available for heart and liver mitochondria [19], we developed a model for brain tissue. Our submodel of the respiratory chain extends existing models for complex III [20] by inclusion of cytochrome c1 and  $SQ_n$  into the state space and detailed modeling of complex I.

The detailed kinetic equations for the reactions and transporters (see supplemental information) are specific for neuronal and brain tissue and based on extensive literature research.  $V_{max}$  values for the reactions were determined by fitting simulated metabolite concentrations to experimentally determined values.

The model was implemented in MATLAB (the MathWorks, version R2011b). The extended model of complex I of the respiratory chain was implemented in C++ (Microsoft

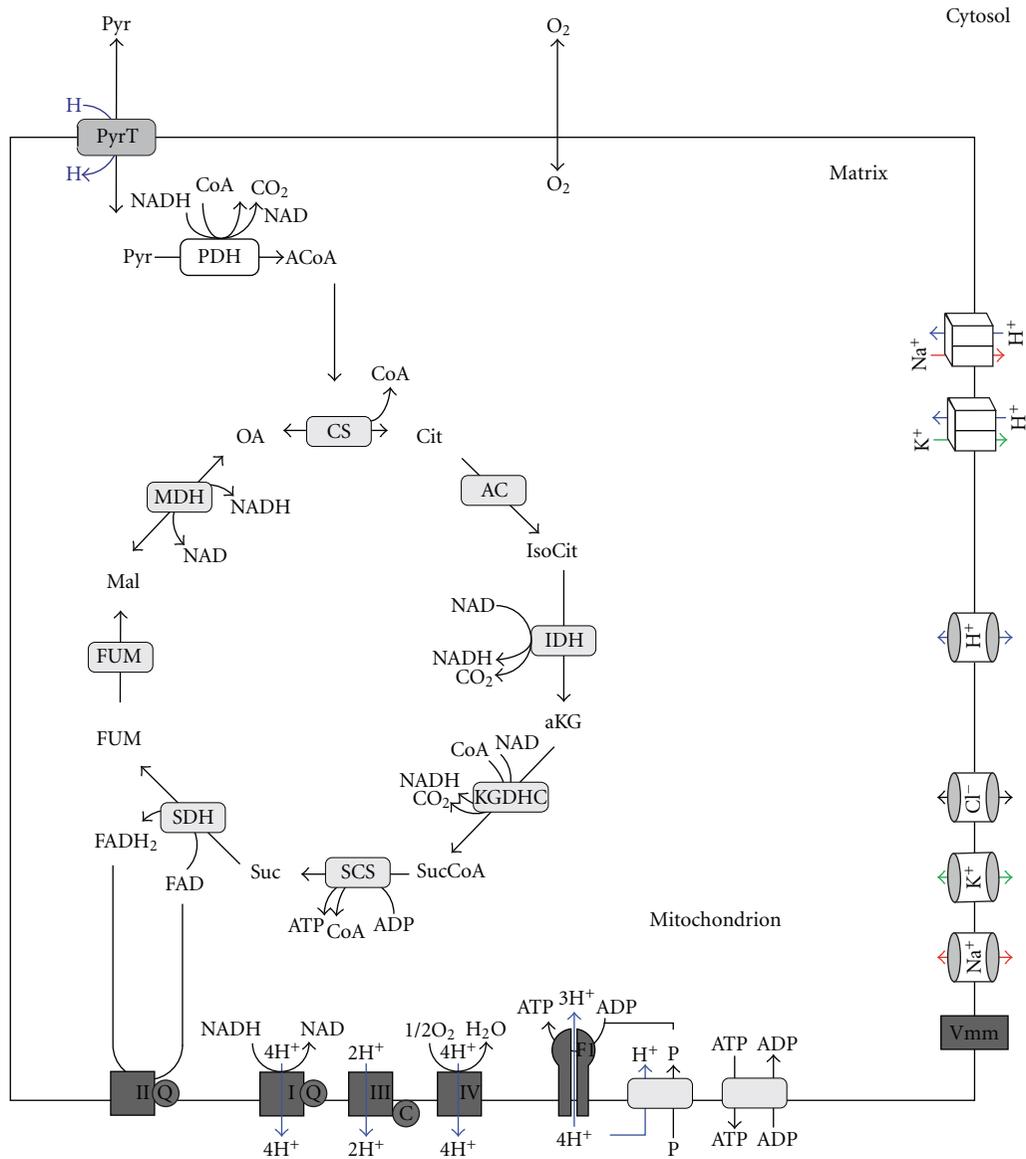


FIGURE 1: Schematic of the mathematical model. Pyruvate (Pyr) is the only substrate of the TCA cycle. Pyruvate is decarboxylated by pyruvate dehydrogenase (PDH) to acetyl-CoA (ACoA), which is then condensed with oxaloacetate (OA) to citrate (Cit) via the citrate synthase (CS). Citrate is converted to isocitrate (IsoCit) by the aconitase (AC), which is further converted to  $\alpha$ -ketoglutarate (aKG) via the isocitrate dehydrogenase (IDH) producing NADH from NAD in the process. The  $\alpha$ -ketoglutarate dehydrogenase complex (KGDHC) catalyses the reaction of  $\alpha$ -ketoglutarate with Coenzyme A to succinyl-CoA (SucCoA) under reduction of NAD to NADH. Succinyl-CoA is further metabolized by succinyl-CoA synthase (SCS) to succinate (Suc) by phosphorylating ADP to ATP (substrate-chain phosphorylation). Succinate is dehydrogenated to fumarate (Fum) by the succinate dehydrogenase (SDH, complex II) reducing ubiquinone to ubiquinol (see legend of Figure 2). Fumerase (FUM) converts fumarate to malate (Mal), which is oxidized by malate dehydrogenase (MDH) again producing one NADH and regenerating the initial oxalacetate so the cycle can start over again. In summary, PDH and the TCA cycle produce one ATP from ADP, one ubiquinol from ubiquinone, and four NADHs from NAD while oxidizing one pyruvate to three CO<sub>2</sub>. Oxidation of NADH and/or succinate in the respiratory chain, is coupled to transmembrane proton pumping which generates a proton gradient and a mitochondrial membrane potential. The proton gradient is used to fuel pyruvate uptake from the cytosol into the matrix via pyruvate transporter, pumping of sodium, potassium from the matrix into the intermembrane space/cytosol, phosphate transport from the cytosol into the matrix space, and ATP generation by the F<sub>0</sub>F<sub>1</sub>-ATPase. The mitochondrial membrane potential drives the ATP/ADP exchange between the matrix and the intermembrane space/cytosol. The model also comprises the passive exchange of protons, sodium, potassium and chloride between the matrix and the intermembrane space/cytosol driven by electrodiffusion as well as the mitochondrial membrane potential. Cytosolic ATP is hydrolyzed to ADP and phosphate to meet the energy demand of the cell.

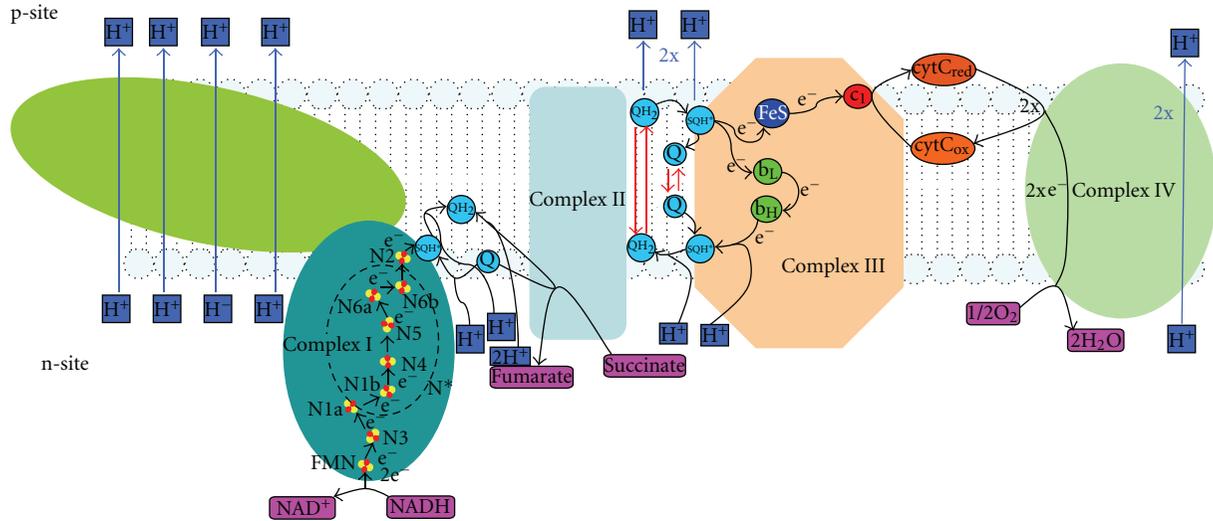


FIGURE 2: Schematic of the respiratory chain. The respiratory chain: in complex I, NADH is oxidized to NAD, while four protons are pumped from the mitochondrial matrix into the intermembrane space/cytosol. Concomitantly ubiquinone (Q), residing in the inner membranous space, is reduced to ubiquinol ( $\text{QH}_2$ ) along with the uptake of two matrix protons. In Complex II, succinate is oxidized to fumarate while ubiquinone is reduced to ubiquinol. In this reduction two protons are taken up from the matrix space, but no protons are pumped across the mitochondrial membrane. In complex III, innermembranous ubiquinol is oxidized to ubiquinone. Via the q-cycle mechanism, two protons are taken up from the matrix space, and four protons are released into the inter membrane space/cytosol. The two electrons are consecutively transferred via Fe-S cluster to cytochrome c1 and reduce two molecules of cytochrome c. In complex IV, two molecules of reduced cytochrome c are oxidized, and oxygen is reduced to water along with the transduction of two protons from the matrix space into the inter membrane space/cytosol. With either NADH or succinate as substrates, the respiratory chain pumps ten and six protons, respectively, from the matrix space to the inter membrane space/cytosol, and one molecule of water is formed.

Visual C++ 2008 Express Edition) and integrated with help of the ODE integration package CVODE (SUNDIALS). The developed source code can be provided on request.

### 3. Results

**3.1. Analysis of Normal Mitochondrial Energy Metabolism.** First, we defined a normal reference state where the cytosolic ATP consumption rate, which is equal to the mitochondrial ATP production rate under steady-state conditions, amounts to about 30% of the maximal consumption rate and where in concordance with experimental data [30–32] 24% of the proton gradient is utilized by the proton leak of the inner mitochondrial membrane, 16% by pumping of potassium ions and 60% by the FoF1-ATPase and phosphate uptake. Next, we calibrated our model (unknown  $V_{\max}$  values) such that measured intramitochondrial metabolite concentrations (Figure 3, green bars) were reproduced. We then varied the ATP consumption rate up to its maximal possible value and calculated steady-state metabolite concentrations (Figure 3, blue bars).

To check the reliability of our model, we compared load-dependent changes of further model parameters with experimental observation reported for various tissues (Figure 4). The membrane potential is remarkably stable between  $-150$  mV and  $-120$  mV over a wide range of ATP consumption rates [33]. However, beyond a 2.5-fold increase of the ATP consumption rate, a small further increase in the ATP consumption rate was accompanied by a large drop

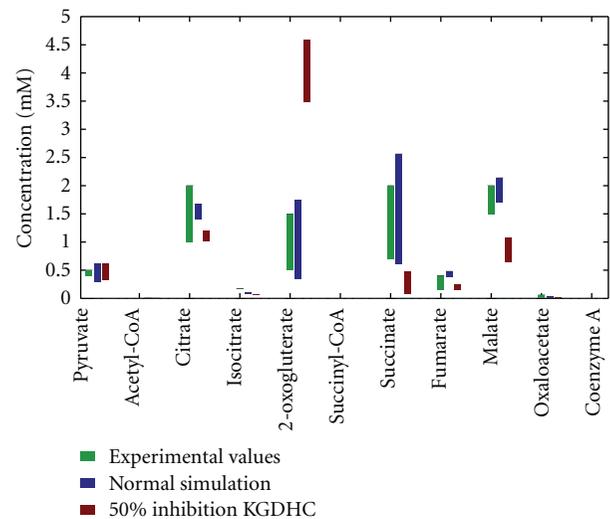


FIGURE 3: Comparison of simulated and experimentally determined concentrations of TCAC intermediates. Green bars indicate the concentration range of reported experimental values [21–29]. Blue bars (normal state) and red bars (50% inhibition of KGDHC) indicate variations of concentration when varying the energetic load between 33% and 100% of maximum.

in the membrane potential, hinting to metabolic failure. The mitochondrial redox potential (expressed through the NADH/NAD ratio) showed a quasilinear decline from 0.3 to 0.1 for ATP consumption rates up to the 2.5-fold of

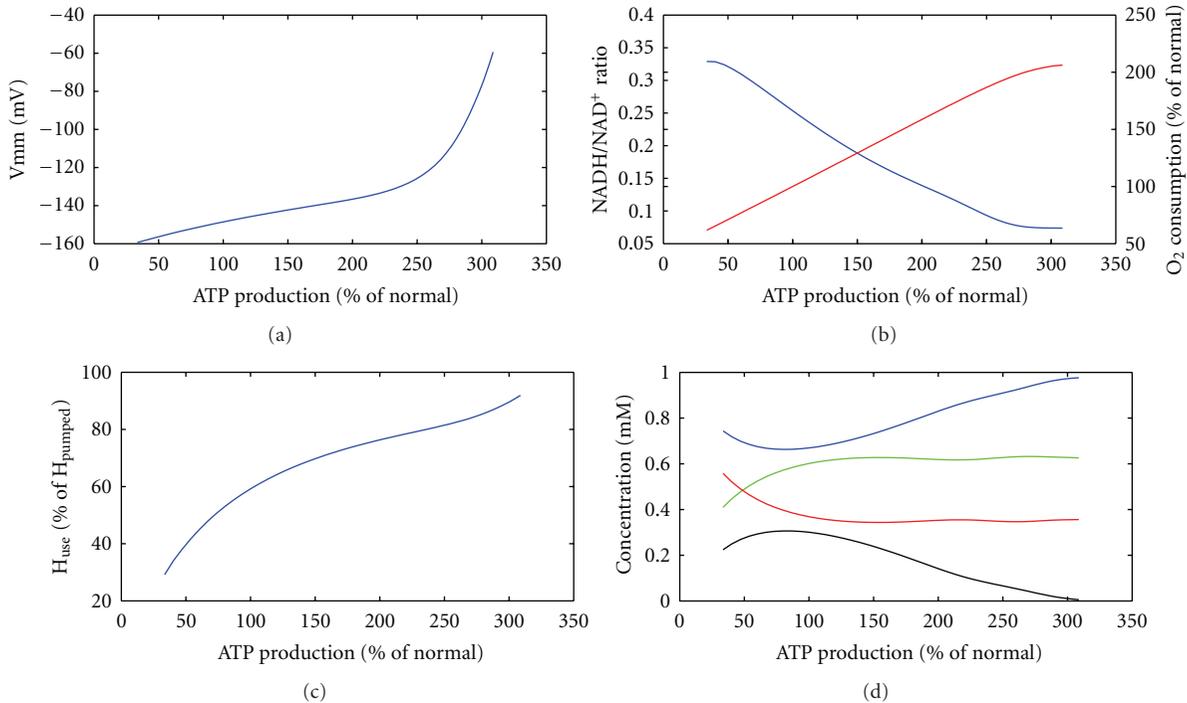


FIGURE 4: System characteristics under energetic challenge. Energetic demand was varied and behaviour of system variables determined. (a) mitochondrial membrane potential; (b) blue NADH to NAD ratio, red oxygen consumption rate; (c) share of created proton gradient used for ATP synthesis; (d) blue: ubiquinol at p-site, green: ubiquinol at n-site, red: ubiquinol at n-site, black: ubiquinol at p-site. ATP production and oxygen consumption are normalized to the reference state of the system.

the normal. Concurrently, the oxygen consumption rate doubled. The fact that oxygen consumption only doubled at threefold increased ATP consumption is accounted for by increased efficiency of ATP production, that is, the share of ATP generation in the utilization of the proton gradient increases from initially 60% to over 90% (Figure 4(c)). The total ubiquinol to ubiquinon ratio (at n-site + p-site) varied between 1.5 and about 0.5, in agreement with experimental data [29]. At the mitochondrial p-site, this ratio dropped from about 1 to zero at maximal indicating that ubiquinol diffusion becomes rate limiting at high ATP consumption rates [34].

Since multiple sites for mitochondrial ROS production in complex I and complex III have been suggested in the literature, we monitored the occupation states of the disputed ROS producing sites at varying ATP consumption rate (see Figure 5). From our model simulations, we concluded that the fully reduced flavin, the semi-ubiquinone bound at n-site in complex I, and the semiubiquinone bound at p-site in complex III are in agreement with expected dependencies on the membrane potential, while the flavin radical and the semiubiquinone at p-site can be ruled out as main ROS producers.

**3.2. Analysis of Mitochondrial Energy Metabolism at Reduced KGDHC Activities.** Next, we investigated the effects of KGDHC and aconitase inhibition on the energy metabolism. Simulations were performed with increasing degree of inhibition of the KGDHC from 0 to 70% (see Figure 6).

The maximal ATP production capacity decreased very slowly until about 50% inhibition of the enzyme. Higher inhibition resulted at first in an approximately linear decrease of the maximal ATP production capacity and finally also in a linear reduction of the ATP production rate in the reference state, that is, the energy demand of the normal load state can be satisfied until about 60% inhibition. At an inhibition of about 70%, the system is close to collapse as can be seen by the highly depolarized membrane potential. Successive depletion of NADH (green curve) caused depletion of reduced cytochrome c (black curve) in a nonlinear manner which is ultimately responsible for the metabolic failure seen at high inhibition states. Notably, as long as reduced cytochrome c is not fully exhausted, the membrane potential can be kept close to the reference value. Caution must be used at interpreting the curves in regimes with strongly depolarized mitochondrial membrane ( $> -80$  mV), since mechanisms not modeled (like initiation of apoptotic pathway/transition pore opening) are likely to dominate the cellular behavior.

There occurred also profound shifts in the metabolite concentrations of the citric acid cycle intermediates. Figure 3 shows the range of the citric acid cycle intermediates under varied energetic load conditions at 50% inhibition of the KGDHC (red bars). A-ketoglutarate was vastly increased and thus partially compensated for the loss in enzyme activity through higher substrate availability. Other metabolites of the TCAC had lower concentrations compared to the normal case.

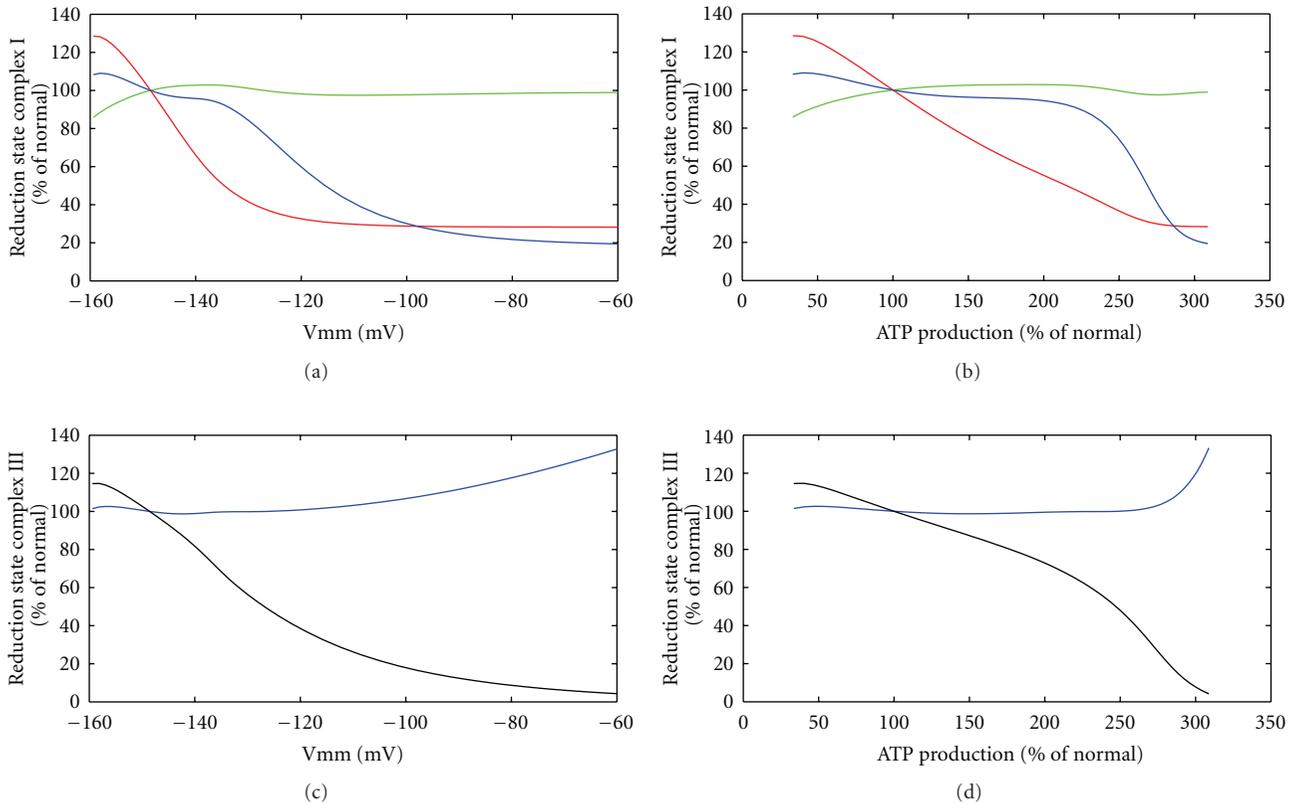


FIGURE 5: Potential ROS producing states in the RC. ROS producing states of complex I ((a) and (b)) and complex III ((c) and (d)) are depicted versus the mitochondrial membrane potential ((a) and (c)) or the ATP production rate. Red: fully reduced flavin, green: flavin radical, blue: semi-ubiquinon at n-site bound to the respective complex, black: semi-ubiquinon at p-site bound to complex III. ATP production and occupation of ROS producing states are normalized to the reference state of the system.

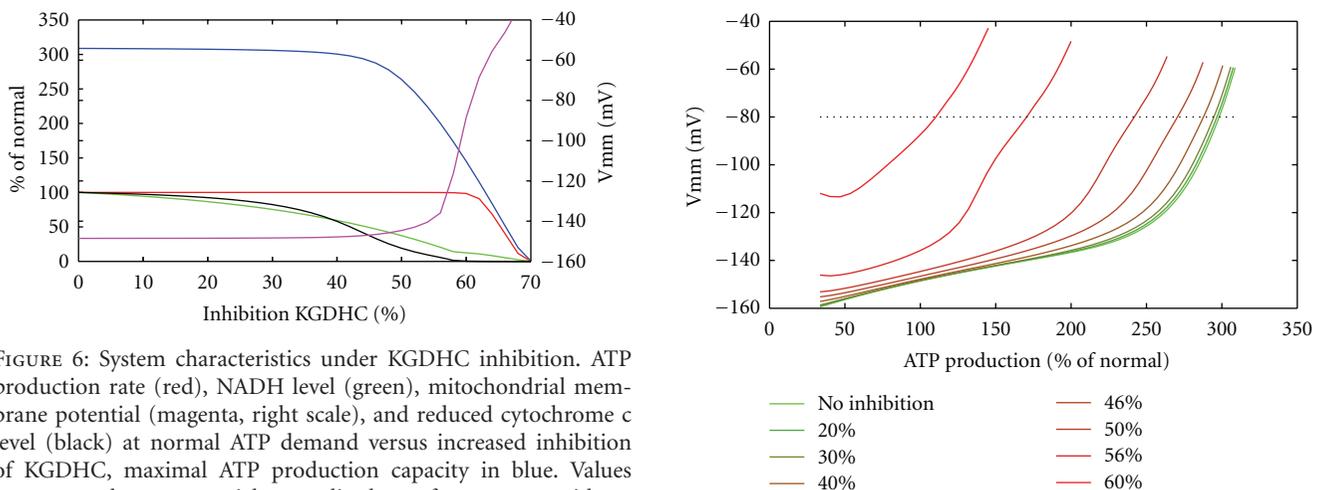


FIGURE 6: System characteristics under KGDHC inhibition. ATP production rate (red), NADH level (green), mitochondrial membrane potential (magenta, right scale), and reduced cytochrome c level (black) at normal ATP demand versus increased inhibition of KGDHC, maximal ATP production capacity in blue. Values except membrane potential normalized to reference state without inhibition.

The dependence of the mitochondrial membrane potential on the ATP consumption rate at different inhibitions of the KGDHC is shown in Figure 7. At moderate inhibition of the KGDHC < 30%, the increase of the membrane potential was only 20 mV up to 2.5-fold elevated energetic load, that is, a significant rise of the membrane potential occurred only

FIGURE 7: Mitochondrial membrane potential characteristics at inhibition of KGDHC. Mitochondrial membrane potential versus the ATP production rate at different inhibition levels of KGDHC. Dotted line: -80 mV membrane potential level.

at very high energetic load. At KGDHC inhibition of about 60%, the membrane potential was already elevated at normal ATP consumption rate, and the membrane depolarized at

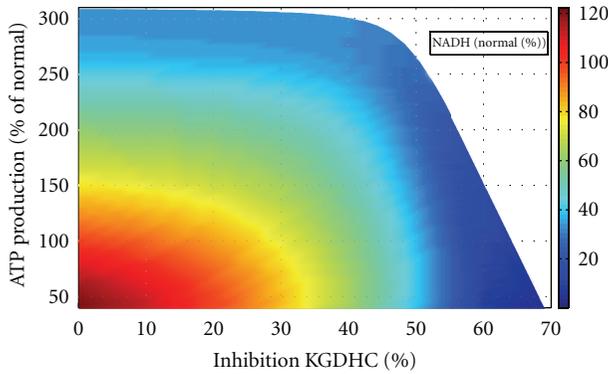


FIGURE 8: NADH level characteristics at varying ATP demand and KGDHC inhibition. NADH level is depicted as colour value (right scale). NADH level and ATP production are normalized to the reference state without KGDHC inhibition.

much smaller increase of the ATP demand. Given pathological states of mitochondria to occur at values of the membrane potential above  $-80$  mV (dotted line in Figure 7), increasing KGDHC inhibition of the membrane potential resulted in a dramatic reduction of the tolerable maximal energetic load and successive membrane depolarization at normal energy demand.

The redox state of the RC and thus the residual energetic capacity of the mitochondrion is common level. Figure 8 shows the combined impact of KGDHC inhibition and energetic load on the mitochondrial NADH level. Generally, progressive inhibition of the KGDHC is equivalent to progressive increase of energetic load. For example, relative inhibition of about 40% has the same effect as a 1.5-fold increase of the energetic load by the mitochondrial NADH. The effect of KGDHC and aconitase inhibition on mitochondrial NADH content has been determined experimentally [15]. Figure 9 demonstrates that our simulations are in good concordance with these experimental data. Inhibition of the KGDHC has a much stronger effect on the NADH content than inhibition of the aconitase. Whereas half-reduction of the NADH level is already achieved with about 40% inhibition of the KGDHC, the same effect requires about 95% inhibition of the aconitase.

**3.3. ROS Production in the Respiratory Chain at Inhibited KGDHC.** Next, we investigated the influence of KGDHC inhibition on ROS generation by the RC. To this end, we calculated the occupation state of ROS generating sites in the presence of KGDHC inhibition. Since our simulation of the normal case suggested that the flavin radical in complex I and the semiquinone at n-site in complex III can be discarded as major ROS generating sites, only the fully reduced flavin, the semiquinone bound at n-site of complex I, and the semiquinone bound at p-site of complex III are shown. With increasing degree of KGDHC inhibition, there was a remarkable reduction in the occupation state of the fully reduced flavin in complex I as well as  $SQ_p$  of complex III at all workloads (see Figure 10), while the changes of  $SQ_n$  of complex I were negligibly small. Above 2.5-fold

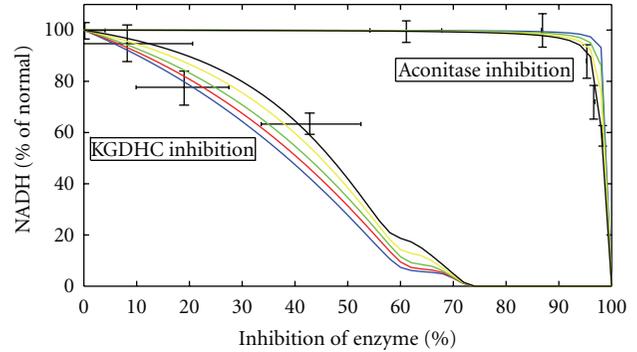


FIGURE 9: NADH level comparison experiment and simulation. Experimental determined NADH level as black points with error bars at different inhibition levels of KGDHC and aconitase (data from [15]). Different coloured curves vary in the basal ATP demand of the system from low (blue) to increased (black). Green curves are for the ATP demand of the reference state. NADH levels are normalized to the NADH level without enzyme inhibition respective to the ATP demand.

increase of the energetic load corresponding to a rise of the membrane potential above  $-100$  mV, the RC is almost completely oxidized so that additional KGDHC inhibition has only a marginal effect on the occupation state of the considered ROS generating sites.

## 4. Discussion

Decreased KGDHC activity in neuronal cells of the brain is associated with a number of neurodegenerative diseases. To understand the impact of reduced KGDHC on the mitochondrial energy metabolism of neurons, we developed a detailed mathematical model comprising the central components of mitochondrial ATP generation.

In agreement with experimental findings, our model simulations showed a steady decline of the mitochondrial NADH level with progressive KGDHC inhibition, whereas up to 95% inhibition of the aconitase had virtually no impact. This finding underpins the notion of KGDHC to be a rate-limiting enzyme of the TCAC. Decline of NADH fluorescence at inhibition of KGDHC has been considered to be indicative for a reduced ATP-generating capacity of mitochondria [15]. The advantage of our mathematical model is to enable predictions of the relationship between NADH decline and ATP production rate. These simulations suggest that a reduced NADH content does not translate linearly to reduced energy production. This is due to a compensatory change in the level of TCAC intermediates upon KGDHC inhibition, thus rendering the flux through the TCAC almost constant over a wide range of inhibition at normal or moderately enhanced energetic workload. Increased akg levels activate the remaining intact KGDHC to reestablish the original flux through the enzyme. This leads to an increased akg content (see Figure 3), a marker for KGDHC impairment, since akg levels are also elevated in the blood/urine. Since akg is in equilibrium with glutamate by a deaminase reaction, this might lead to glutamate poisoning,

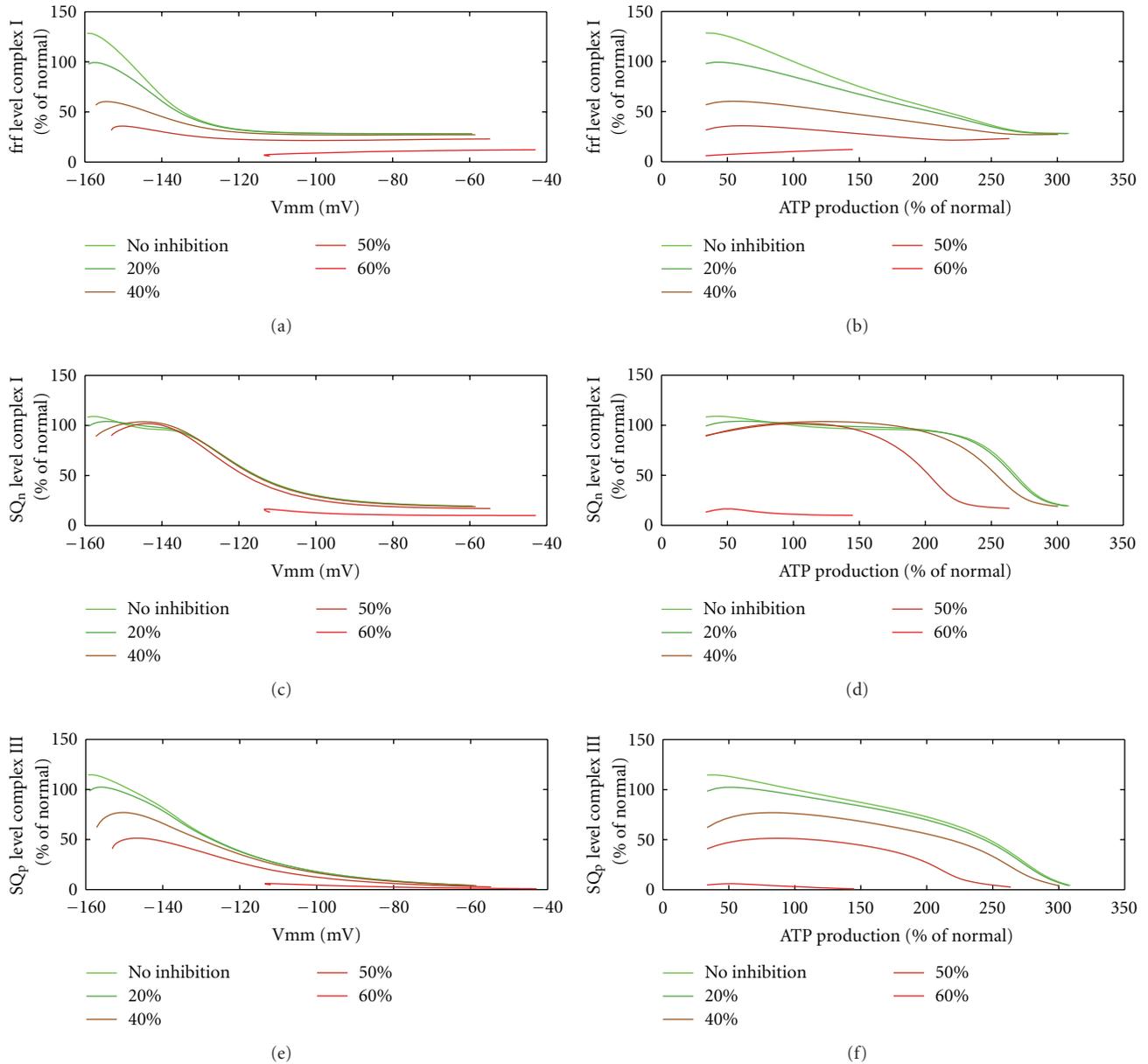


FIGURE 10: ROS production at inhibition of KGDHC. Levels of fully reduced flavin ((a) and (b)), semi-ubiquinone at n-site bound to complex I ((c) and (d)) and semi-ubiquinone at p-site bound to complex III ((e) and (f)) at various inhibitions (a) of KGDHC. The levels are depicted versus the mitochondrial membrane potential ((a), (c), and (e)) and the ATP production rate ((b), (d) and (f)). Values except membrane potential are normalized to the reference state.

another pathological feature associate with KGDHC inhibition [35].

Our simulation also revealed that the impairment of the energy metabolism depends on the functional state of the neuron. At high inhibition and/or high energetic workload, the energetic output is severely compromised, and functionality may not be maintained. This goes along with a marked membrane depolarization.

There is striking evidence for a correlation between reduced KGDHC activity and the loss of glutamatergic neurons seen for example, in Alzheimer's and Parkinson's disease [11]. Programmed cell death through the apoptotic

pathway requires the simultaneous occurrence of three different events: depolarization of the mitochondrial membrane potential, low intramitochondrial ATP concentrations, and a mitochondrial overload with calcium. The first two conditions are fulfilled when neurons with reduced KGDHC activity are challenged with high ATP demand that normally can be accomplished by the cell. When the cytosolic ATP levels are low, calcium is not sufficiently pumped out of the cell into the extracellular space, and the resulting increased cytosolic calcium concentration finally leads to mitochondrial calcium overload. Thus, strongly reduced KGDHC activity might directly lead to apoptosis.

One of the peculiarities of neurodegenerative diseases is the strong age dependence. It is believed that the accumulation of mitochondrial damage through ROS is one of the determining factors in brain aging and performance as well as a key factor in the development of neurodegenerative diseases [36]. One of the harmful effects of ROS is the inactivation of enzymes of the citric acid cycle. Citric acid cycle enzymes that are especially susceptible to oxidative stress are the aconitase and the KGDHC [15, 37]. Aconitase shows the highest susceptibility towards ROS, because of its sulfur-iron complex, but aconitase inhibition remains irrelevant if it does not exceed 90 percent (see Figure 9 and [15]). KGDHC is tightly bound to the inner mitochondrial membrane [38] and might be part of a citric acid cycle super-complex [39]. It binds to complex I of the mitochondrial respiratory chain [40], which might make it a prominent target of ROS due to the close spatial proximity to the ROS generating sites. Thus, ROS is one possible reason for KGDHC deficiency.

There is no general agreement on the relative importance of various proposed ROS producing sites of the RC. Dependence of ROS production from the membrane potential has been measured [41, 42]. Comparing these measured characteristics with the occupation status of the disputed ROS generating sites, modeling can help to identify or exclude sites as relevant producers of ROS (see Figure 5). Our calculations show that the occupation state as function of the membrane potential of the fully reduced flavin, the SQ<sub>n</sub> site in complex I, and the SQ<sub>p</sub> site in complex III, but not the flavin radical in complex I, and the SQ<sub>n</sub> site in complex III, is in agreement with measured dependencies. For complex I, our findings are in agreement with experimental results in [43] excluding the flavin radical but showing the fully reduced flavin to act as ROS producing site of complex I. For complex III, our findings are in agreement with other reports (see, e.g. [44]).

Since KGDHC is especially susceptible to ROS, we examined if the reduction of KGDHC activity has an effect on the occupation state of the confirmed relevant ROS producing sites (Figure 10). We found that there is a significant decrease in the occupation state of the fully reduced flavin in complex I and SQ<sub>p</sub> of complex III for all ATP demands, that is, KGDHC inhibition reduces ROS production in the respiratory chain by lowering the redox state of the mitochondrion. Since KGDHC itself is also an ROS producer and ROS production by this enzyme decreases with decreasing mitochondrial redox state [45], endogenous ROS production by KGDHC is also reduced in case of KGDHC deficiency.

It has to be noted that our model does not include the impairment of RC complexes by ROS as it has been observed in neurodegenerative diseases (see, e.g., [46]). A reduction in the activity of complex I results in increase in ROS production [47]. Hence, structural and functional impairment of the RC might override the inhibitory effect of reduced KGDHC on ROS production.

Here, we focused on the effects of KGDHC inhibition on the energy metabolism and showed that for a given degree of inhibition the impairment depends crucially on the functional state of the cell. For ROS production, KGDHC

inhibition alone does reduce the ROS generation in the respiratory chain. Nevertheless, the detailed description of the RC in our model provided further arguments for the relative importance of ROS producing sites proposed in the literature.

Taken together, our model helps to elucidate the causal chain of molecular events connecting reduced KGDHC activity to the energetic breakdown of neuronal cells during development of neurodegenerative diseases.

## Abbreviations

Fe-S:	Iron sulfur
FMN:	Flavin mononucleotide
KGDHC:	$\alpha$ -ketoglutarate dehydrogenase
RC:	Respiratory chain
ROS:	Reactive oxygen species
SQ:	Semi-ubiquinon
TCAC:	Tricarmonic acid cycle/citric acid cycle
Q:	Ubiquinon
QH <sub>2</sub> :	Ubiquinol.

## Acknowledgment

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## Review Article

# Mitochondrial- and Endoplasmic Reticulum-Associated Oxidative Stress in Alzheimer's Disease: From Pathogenesis to Biomarkers

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Alzheimer's disease (AD) is the most common cause of dementia in the elderly, affecting several million of people worldwide. Pathological changes in the AD brain include the presence of amyloid plaques, neurofibrillary tangles, loss of neurons and synapses, and oxidative damage. These changes strongly associate with mitochondrial dysfunction and stress of the endoplasmic reticulum (ER). Mitochondrial dysfunction is intimately linked to the production of reactive oxygen species (ROS) and mitochondrial-driven apoptosis, which appear to be aggravated in the brain of AD patients. Concomitantly, mitochondria are closely associated with ER, and the deleterious crosstalk between both organelles has been shown to be involved in neuronal degeneration in AD. Stimuli that enhance expression of normal and/or folding-defective proteins activate an adaptive unfolded protein response (UPR) that, if unresolved, can cause apoptotic cell death. ER stress also induces the generation of ROS that, together with mitochondrial ROS and decreased activity of several antioxidant defenses, promotes chronic oxidative stress. In this paper we discuss the critical role of mitochondrial and ER dysfunction in oxidative injury in AD cellular and animal models, as well as in biological fluids from AD patients. Progress in developing peripheral and cerebrospinal fluid biomarkers related to oxidative stress will also be summarized.

## 1. General Introduction

Alzheimer's disease (AD) is the most common form of dementia with a progressive course. AD pathology evidences neuronal damage in specific vulnerable brain regions and circuits involved in memory and language, namely, the hippocampus and cerebral cortex, which appears to be preceded by synaptic and neuronal dysfunction. From a pathology perspective, the presence of extracellular plaques, mainly composed of amyloid beta peptide ( $A\beta$ ), a 39- to 42-aminoacid residue peptide, derived from the processing of amyloid precursor protein (APP), and intraneuronal neurofibrillary tangles, consisting of tau protein aggregates, constitute important hallmarks of the disease and serve, as a dividing line between AD and other dementias [1–4]. Demented individuals who do not have plaques and tangles does not qualify for a diagnosis of AD, but the simple presence of plaques and tangles do not distinguish demented from

nondemented individuals since brains of aged nondemented individuals frequently contain plaques and tangles [3].

Although the etiology of AD is largely unknown, it has been hypothesized that multiple factors, including genetic components, oxidative stress, intracellular and/or extracellular accumulation of  $A\beta$ , excitotoxicity, inflammation, mitochondrial dysfunction, alteration of cytoskeleton and synapse components and neuronal loss, may play important roles in the onset of the disease [5]. One hypothesis that may account for the heterogeneous nature of AD and the fact that aging is the most obvious risk factor is the increased generation of reactive oxygen species (ROS); indeed, neurons are extremely sensitive to attack by destructive free radicals [6].

## 2. Evidence of Oxidative Stress in AD Brain

The "oxidative stress theory" of aging holds that a progressive and irreversible accumulation of oxidative damage caused by

ROS impacts on critical aspects of the senescence process, contributing to impaired physiological function, increasing incidence of disease, along with a reduction in life span [7]. Although low and intermediary levels of ROS are physiologically important, high ROS concentrations above the clearance capacity of the cell cause oxidative stress, mitochondrial dysfunction, cellular damage, and, in numerous cases, cell death [8], thus pointing oxidative stress as a potential unifying mechanism contributing to age-related pathologies [7] and, in particular, to AD [9, 10].

Lipid peroxidation is one of the major outcomes of free-radical-mediated injury leading to the generation of a variety of relatively stable end products. The ones that have been most extensively studied, both in brain and biological fluids, such as cerebrospinal fluid (CSF), plasma, urine of AD and mild cognitive impairment (MCI) patients, are malondialdehyde (MDA), trans-4-hydroxy-2-nonenal (HNE), and F<sub>2</sub>-isoprostanes (F<sub>2</sub>-IsoPs). Indeed, several studies have demonstrated significantly increased levels of MDA and thiobarbituric acid reactive substances (TBARS) in AD [11–13] and MCI brains [14], particularly in regions where neurofibrillary tangles and senile plaques typically accumulate. HNE, one of the most toxic products of lipid peroxidation, is, like MDA, diffusible and highly reactive with other biomolecules being able to covalently modify proteins, thus affecting their function. Increased levels of free HNE and HNE-protein adducts have been described in the brains of MCI and AD patients compared to controls [15–19]. In addition, increased levels of F<sub>2</sub>-IsoPs have been documented in different brain regions of AD in comparison to cognitively normal individuals [20–22]. This increase of F<sub>2</sub>-IsoPs was demonstrated to be specific of AD-type dementia and did not occur in cases of frontotemporal dementia [21]. F<sub>2</sub>-IsoPs have also been investigated in brain of MCI subjects. Increased levels of these lipid peroxidation products were documented in different brain regions of MCI subjects compared to controls [23]; however these data were not confirmed by other authors [24].

Within proteins, all amino acids can be attacked by ROS, but sulphur-containing and aromatic amino acids are the most susceptible. The oxidation of amino acids mainly leads to the formation of carbonyl groups, while peroxyxynitrite can nitrate tyrosine groups and form the stable compound 3-nitrotyrosine (3-NT). Increased levels of protein carbonyls have been detected in the superior and middle temporal gyri of patients with early-stage AD and MCI and also in the hippocampus and parietal lobe of AD patients compared to controls [14, 25, 26], but unchanged in the cerebellum, which is consistent with the regional pattern of histological changes in AD. On the other hand, increased 3-NT immunoreactivity has been also detected in regions of the cerebral cortex affected by neurodegeneration in AD patients [27], with a distribution similar to protein carbonyls. Moreover, high levels of protein nitration were found in inferior parietal lobes and hippocampi of MCI patients [28]. Protein oxidation in AD does not seem to be a random process but rather involves specifically more susceptible proteins that have been identified through redox proteomic studies [29]. Many of the proteins that have been

identified so far, as oxidatively modified in the brain of AD patients and MCI subjects, are either mitochondrial proteins or proteins that are known to interact with mitochondria; these include glyceraldehyde 3-phosphate dehydrogenase (GAPDH), voltage-dependent anion channel (VDAC), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), adenosine triphosphate (ATP) synthase-alpha chain, beta-actin and/or aconitase [18, 30–32].

ROS, and particularly the hydroxyl radical, can react with all components of the DNA molecule, causing different kinds of damage. DNA injury has been investigated in AD and MCI subjects mainly through the analysis of DNA strand breaks and the presence of specific oxidized DNA bases and adducts, of which 8-hydroxy-2'-deoxyguanosine (8-OHdG) is the most commonly investigated. Several postmortem studies have reported significant DNA fragmentation in the brain of AD subjects compared to nondemented controls, especially in areas that are more prone to neurodegeneration [33–36]. A buildup of 8-OHdG was detected in brain tissue from AD subjects, that was most prominent in mitochondrial DNA (mtDNA) of the parietal cortex [37]. These results were confirmed by another report showing that the presence of oxidized nucleosides was inversely related to the neurofibrillary tangle content [38], further suggesting that DNA oxidation could precede lesion formation. This hypothesis was further corroborated by a study by Wang and coauthors [39] who observed higher indices of oxidation in mtDNA from neocortical regions of MCI subjects compared to controls, but similar to the ones observed in AD patients, suggesting that DNA oxidation was indeed an early event in the pathogenesis of the disease. RNA is more vulnerable to oxidation than DNA and can be easily attacked by the hydroxyl radical. Several studies evaluated the levels of 8-hydroxyguanosine (8-OHG) as a marker of oxidative damage to RNA. Immunohistochemical analysis of neurons in particularly vulnerable brain areas of AD patients showed a marked accumulation of 8-OHG, that was negatively correlated with the duration of the disease and the extent of A $\beta$  deposition [40]. These findings have been further extended by Shan and collaborators that showed a large increase in the extent of messenger RNA (mRNA) oxidation in the frontal cortex, but not in the cerebellum of AD patients [41, 42]. It was also demonstrated that increased levels of 8-OHG in the parahippocampal gyrus were already present in MCI subjects, compared to controls, but similar to the levels found in AD patients [43], suggesting that RNA oxidative damage is an early event in AD pathology.

Very recently, multiple biochemical markers of oxidative stress and antioxidant defenses were analyzed in frontal cortex postmitochondrial supernatant, mitochondrial, and synaptic fractions from age-matched noncognitively impaired, mild cognitive impairment (MCI), mild AD, and AD subjects [44]. In this study, a strong correlation was observed between levels of synaptic lipid peroxidation, protein oxidation and nitration, and the subjects' global cognitive status. Changes in levels of the antioxidants glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) also strongly correlated with the minimal status examination (MMSE) score [45]. Previous studies found both

increased [11] and reduced activity of antioxidant enzymes in AD [12] and MCI brain [46].

In studies assessing oxidative damage in brain, the possibility of artifacts due to postmortem delay cannot be completely ruled out. However, in most of the referred studies postmortem interval was conveniently short (1–5 hours), matched between patients and control samples and therefore should not have a significant effect on the discussed parameters. In fact, a few studies [33, 47] have examined the influence of postmortem delay in oxidative damage measures, and similar levels have been found in rapid (<1 h) and conventional autopsy tissue (up to 8 hours). Overall, these findings support the idea that the unbalance between ROS generation and detoxification by antioxidants is an early event that plays an important role in the progression of the disease.

### 3. ROS Generation and Mitochondrial Dysfunction

In cells, multiple pathways and enzymes can generate ROS. These include, as an example, complexes I and III of the mitochondrial respiratory chain in the mitochondrion, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), xanthine oxidase, or nitric oxide synthase (NOS) [8]. Mitochondria produce ROS and reactive nitrogen species (RNS) during the normal aerobic activity. This accounts for the generation of superoxide ( $O_2^{\bullet-}$ ), mainly produced at complex I and complex III of the electron transport chain, and nitric oxide ( $\bullet NO$ ).  $\bullet NO$  controls mitochondrial respiration and both cytotoxic, as well as cytoprotective effects have been described to be due to this RNS. Depression of ATP synthesis through oxidative phosphorylation by  $\bullet NO$  has been mainly attributed to the inhibition of mitochondrial complex IV. In fact,  $\bullet NO$ -induced inhibition of complex IV is completely and quickly reverted upon its removal, suggesting that the inhibition of mitochondrial complex IV by  $\bullet NO$  can be better described as a functional control of cell respiration [48]. Importantly, if these two molecules ( $O_2^{\bullet-}$  and  $\bullet NO$ ) encounter each other, they undergo a fast spontaneous reaction leading to production of peroxynitrite ( $ONOO^-$ ). For this purpose, classical antioxidant pathways, such as superoxide dismutase (SOD2 in the matrix and also SOD1 at the intermembrane space) and the glutathione cycle, play a relevant role in detoxifying increased mitochondrial ROS levels. Although it is unclear whether the decline in antioxidants precedes the increase in oxidants during AD progression, their levels are certainly not capable of neutralizing enhanced ROS generation [44]. Thus, mitochondria require efficient expression of antioxidant enzymes. In this perspective, oxidative stress is also seen as an imbalance that has its origins in genes and in the way in which gene expression is regulated. At the center of this new focus is a transcription factor named nuclear factor (erythroid-derived 2)-like 2, or Nrf2 (described further in this paper), the “master regulator” of the antioxidant response, modulating the expression of hundreds of genes, including the familiar antioxidant enzymes [49].

Evidence from AD postmortem brain, as well as cellular and animal AD models, shows that  $A\beta$  triggers mitochondrial dysfunction by interaction with different mitochondrial targets, including the outer mitochondrial membrane OMM, intermembrane space, inner mitochondrial membrane IMM, and the matrix. The consequent impairment of oxidative phosphorylation, ROS production, mitochondrial dynamics, and the interaction with mitochondrial proteins [50] may be related to a toxic effect caused by intracellular  $A\beta$ . Indeed,  $A\beta$  has been described to accumulate intracellularly, a process linked to early stages in the neuropathological phenotype of AD [51]. Within the cells, aggregated  $A\beta_{1-42}$  may appear as dense packed granules [52]. Moreover, intracellular  $A\beta$  is present in mitochondria from brains of AD transgenic mice and AD patients.  $A\beta$  progressively accumulates in mitochondria and is associated with decreased activity of complexes III and IV and a reduction in the rate of oxygen consumption [53]. Importantly,  $A\beta$  can be transported into mitochondria via the translocase of the outer membrane (TOM) machinery in a process independent of the mitochondrial membrane potential [54].

Concordantly, many studies have shown mitochondrial abnormalities in AD, as expressed both by energy deficits and the potentially toxic production of free radicals [6]. Imaging and biochemical studies in brain and peripheral samples obtained from AD patients revealed alterations in both extramitochondrial and mitochondrial metabolic pathways. Accordingly, reduced cerebral glucose transport and pyruvate levels through glycolysis were observed in the temporal cortex of AD subjects. Moreover, deregulation of tricarboxylic acid cycle and oxidative phosphorylation system coupled to altered mitochondrial dynamics were also found [55, 56], along with the well-defined deficit in mitochondrial complex IV [57]. Thus, mitochondria are susceptible organelles in AD, largely contributing for disease-related ROS generation and AD pathogenesis.

Both mitochondrial ROS production and  $Ca^{2+}$  handling (which is necessary for the activity of mitochondrial dehydrogenases) are considered the centre of important biological processes, and their deregulation has been implicated in a number of human pathologies, including neurodegenerative diseases like AD. Due to localized high  $Ca^{2+}$  concentration in microdomains close to mitochondria,  $Ca^{2+}$  is rapidly accumulated within mitochondria (e.g., [58]) influencing energy function by activating mitochondrial matrix dehydrogenases to produce more NADH, donating more electrons through complex I, and thus driving the synthesis of ATP. Thus, the role of mitochondria as reservoirs of  $Ca^{2+}$  and apoptotic proteins and producers of ROS is pathologically linked to neurotoxicity in both AD and aging brain. However, most investigators agree that mitochondria from AD subjects differ from those of age-matched, nondemented subjects [3, 59–61]. The role of mitochondrial ROS as inducers of  $Ca^{2+}$  deregulation is well established, and a major cause of ROS production has been linked to  $Ca^{2+}$  deregulation, along with reduced mitochondrial ATP levels. Thus, oxidative stress and  $Ca^{2+}$  regulation are intricately linked and can cooperatively contribute to AD pathogenesis [60, 61].

Apart from producing ROS and RNS, mitochondria are susceptible targets for oxidant molecules. These can attack mitochondrial lipids, proteins, and DNA. In fact, the lack of histones in mtDNA renders them vulnerable organelles to oxidative stress [7, 8]. Mitochondrial-targeted ROS scavengers, without interfering with physiological ROS signaling, therefore represent a promising novel therapeutic approach to the treatment of neurodegenerative diseases like AD [8, 60]. In recent studies the mitochondrial antioxidant MitoQ (mitoquinone mesylate: [10-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadienyl) decyl triphenylphosphonium methanesulfonate]) prevented increased production of ROS and the loss of mitochondrial membrane potential in cortical neurons subjected to A $\beta$  and further prevented cognitive decline, synaptic loss, caspases activation, and oxidative stress in female of 3xTg-AD mice [62].

To better access mitochondrial dynamics and how A $\beta$  affects the function of this organelle, researchers mainly use *in vitro* strategies. In pyramidal neurons from the hippocampus of AD patients, the levels of intracellular A $\beta$ 1-40 and -42 were found to be 3 and 10  $\mu$ M, respectively, higher than those found in control individuals [63], which are in the range of the concentrations used in numerous *in vitro* studies. In fact, by using isolated rat brain mitochondria treated with A $\beta$ , both mitochondrial transmembrane potential and the mitochondrial capacity to accumulate Ca $^{2+}$  were shown to be decreased and to cause a complete uncoupling of respiration [64]. Moreover, mitochondrial accumulation of A $\beta$  reduced oxygen consumption and mitochondrial electron transport chain activity [65, 66]. The progressive accumulation of A $\beta$  within this organelle was shown to be linked to mitochondrial abnormalities, like mtDNA defects and altered mitochondrial gene expression, along with changes in mitochondrial dynamics [67], axonal transport, and also synaptic degeneration [50, 60].

Deregulated Ca $^{2+}$  levels are also detrimental to mitochondrial function, and therefore impaired Ca $^{2+}$  homeostasis may play a role in ROS generation, A $\beta$  aggregation, and damage to mitochondria in AD [68]. A $\beta$  can further promote intracellular Ca $^{2+}$  increase in a deleterious positive feedback loop [69], suggesting that A $\beta$  accumulation can deregulate Ca $^{2+}$  levels and vice versa. In fact, L-, P- and N-type Ca $^{2+}$  channels activity can be modulated by A $\beta$ , an effect apparently mediated primarily by A $\beta$ -induced ROS production [68]. A $\beta$  was also shown to promote excessive release of Ca $^{2+}$  from endoplasmic reticulum (ER), which may underlie mitochondrial Ca $^{2+}$  dyshomeostasis and ROS generation, thereby disturbing organelle functioning and, ultimately, damaging neurons [55], as described above.

The mild or gradual energy disturbance, described above, may influence ROS generation (namely, through disruption of the mitochondrial respiratory chain) and cause the oxidative damage of different molecules and the formation of the high conductance mitochondrial cyclophilin D-associated permeability transition pore (PTP) [70]. This is followed by the release of proapoptotic factors, particularly cytochrome c and apoptosis-inducing factor (AIF) and the activation of caspases in charge of the “execution” phase of the apoptotic cascade [71]. In this perspective, apoptosis

through the intrinsic pathway has been largely described to play an essential role in AD pathogenesis [72]. In response to apoptotic signals, loss of mitochondrial membrane potential associates with mitochondrial membrane permeabilization to evoke cytochrome c release and the activation of the initiator caspase-9. Nevertheless, evidence of apoptosis has been largely controversial in AD. Although many reports support the occurrence of mitochondrial-linked apoptosis, as observed following exposure to A $\beta$ , other researchers have not seen an increase in apoptosis. Previous reports described that the hippocampus of AD brains displayed DNA fragmentation, but only few cells showed morphological characteristics of apoptosis [73]. This has been opposed by studies in cell and animal models of AD overexpressing the antiapoptotic protein Bcl-2. In this regard, we previously showed that Bcl-2 is neuroprotective against apoptotic cell death caused by A $\beta$ (25–35) [74]. Additionally, overexpression of Bcl-2 in 3xTg-AD mice improved place recognition memory, reduced caspase activation, and attenuated APP processing, leading to decreased formation of extracellular plaques and neurofibrillary tangles [75].

*3.1. Oxidative Stress and Synaptic Loss: The Relevance of Synaptic Mitochondria.* Synapses are sites of high energy demand and extensive Ca $^{2+}$  fluctuations since synaptic transmission requires high levels of ATP and constant regulation of intracellular Ca $^{2+}$  concentration, rendering synaptic mitochondria vital for maintenance of synaptic function and transmission [59].

Recent studies in postmortem frontal cortex obtained from MCI individuals or mild/moderate and late-stage AD patients demonstrated a significant disease-dependent increase in oxidative markers mainly localized to the synapses. Interestingly, the levels of oxidative markers significantly correlate with MMSE suggesting an involvement of oxidative stress in AD-related synaptic loss [44]. A recent study also demonstrated mitochondrial morphologic alterations in neurons obtained from different brain areas of postmortem human AD brains concomitantly, with loss of dendritic branches and depletion of dendritic spines [76]. In AD, synaptic dysfunction and the loss of synapses are in fact early pathological features, probably due to defects in synaptic mitochondria, which lead to alterations in cognitive function [44], and, interestingly, this seems to be related to ROS production and altered Ca $^{2+}$  dynamics at the synapse [61]. In mouse hippocampal neurons, A $\beta$  was demonstrated to impair mitochondrial movements, reduce mitochondrial length, and cause synaptic degeneration [77]. Compared with nonsynaptic mitochondria, synaptic mitochondria showed a greater degree of age-dependent accumulation of A $\beta$  and mitochondrial alterations. The fact that synaptic mitochondria, especially A $\beta$ -rich synaptic mitochondria, are more susceptible to A $\beta$ -induced damage highlights the central importance of synaptic mitochondrial dysfunction to the development of synaptic degeneration in AD [59]. Indeed, synaptic mitochondria are more sensitive to ROS than nonsynaptic mitochondria [78].

In AD, synapses are the primary sites of  $\text{Ca}^{2+}$  deregulation due to overactivation of glutamate receptors. These receptors are concentrated on postsynaptic spines of neuronal dendrites where they are subjected to particularly high levels of  $\text{Ca}^{2+}$  influx, oxidative stress, and ATP demand. Therefore, they are likely sites at which neurodegenerative processes are initiated in aging and early AD, thus playing an important role in decreased synaptic function. In addition, the apoptotic process has been shown to be activated locally in synaptic compartments after exposure to  $\text{A}\beta$  in vulnerable AD neuronal populations [79].

With this in mind, in the next section we discuss the role of *N*-methyl-*D*-aspartate receptors (NMDARs), a subtype of glutamate receptors, in mitochondrial  $\text{Ca}^{2+}$  regulation and ROS formation in AD-associated neurodegeneration.

**3.2. Role of NMDA Receptors in AD.** Ionotropic glutamate receptors mediate most excitatory neuronal transmission in the brain and play essential roles in the regulation of synaptic activity. In fact,  $\text{Ca}^{2+}$  influx through NMDARs induced by synaptic activity is required for many types of synaptic plasticity and underlies some forms of learning and memory. Very recently, the selective roles for GluN2A and GluN2B subunits of the NMDARs in long-term potentiation (LTP) and long-term depression (LTD), respectively, were reported [80]. However, excessive  $\text{Ca}^{2+}$  influx due to overactivation of NMDARs may result in excitotoxic cell death in many neurological disorders, including AD [81] (Figure 1).

Depending on their specific response to different pharmacological agents, ionotropic glutamate receptors can be subdivided into NMDARs,  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors [81, 82].  $\text{A}\beta$  oligomers were shown to induce inward currents, intracellular  $\text{Ca}^{2+}$  increase, mitochondrial  $\text{Ca}^{2+}$  overload, oxidative stress, mitochondrial membrane depolarization, and apoptotic cell death through a mechanism requiring NMDAR and AMPAR activation in both rat cortical neurons and hippocampal organotypic slices [69].

Functional NMDARs are heterotetramers composed of two glycine-binding GluN1 subunits assembling with two glutamate-binding GluN2 (GluN2A–GluN2D) subunits or, alternatively, GluN3 (GluN3A and/or GluN3B) subunits which can replace GluN2 [83]. The most widely expressed NMDARs contain the obligatory subunit GluN1 plus either GluN2B or GluN2A or a mixture of the two. GluN2B and GluN2D subunits are expressed at high levels in early developmental stages (prenatally), whereas GluN2A and GluN2C expression is first detected near birth [84]. NMDARs exhibit high  $\text{Ca}^{2+}$  permeability and voltage-dependent channel block by extracellular  $\text{Mg}^{2+}$  [81], properties of both physiological and pathological importance. Channel blockade by  $\text{Mg}^{2+}$  reduces  $\text{Ca}^{2+}$  influx at membrane voltages near rest but is relieved during neuronal excitation [81].

Recent studies have reported activation of the ROS-producing NOX after NMDAR stimulation in response to intrastriatal administration of glutamate in mice. In contrast, mice lacking NOX2 were less vulnerable to excitotoxicity,

presented reduced levels of ROS production and protein nitrosylation, decreased microglial reactivity and calpain activation, suggesting that NOX is stimulated by  $\text{Ca}^{2+}$  entry through ionotropic glutamate receptors [85]. Recent results also demonstrate that not only glutamate excitotoxicity and/or oxidative stress alter mitochondrial fission/fusion, but that an imbalance in mitochondrial fission/fusion in turn leads to NMDAR upregulation and oxidative stress [86], suggesting a new vicious cycle involved in neurodegeneration that includes glutamate excitotoxicity, oxidative stress, and mitochondrial dynamics.

Although NMDARs activation is essential for memory formation, therapeutic actions of memantine, an uncompetitive open channel blocker of NMDARs, include slowing of neuronal loss due to NMDARs excitotoxicity, thus correcting for an excitation-inhibition imbalance. Indeed, memantine is widely prescribed as a memory-preserving drug for moderate- to late-stage AD patients [87], suggesting that the therapeutic effect of memantine derives predominantly from NMDARs inhibition. However, it appears paradoxical that inhibition of NMDARs slows memory loss associated with AD, considering that NMDARs activation is essential for memory formation.

$\text{A}\beta$  oligomers were previously reported to coimmunoprecipitate with extracellular domains of the GluN1 subunit, suggesting a direct interaction of  $\text{A}\beta$  with NMDARs [88]. Using transfected HEK293 cells, it has previously been shown that  $\text{A}\beta$  mediates necrotic cell death through changes in  $\text{Ca}^{2+}$  homeostasis in HEK293 cells selectively expressing GluN1/GluN2A subunits, but not GluN1/GluN2B subunits [84]. However, in rat primary cortical cultures it was recently demonstrated that  $\text{A}\beta_{1-42}$  preparation containing both oligomers (in higher percentage) and monomers directly interacts with cell function by disturbing intracellular  $\text{Ca}^{2+}$  homeostasis through activation of GluN2B-containing NMDARs [89]. Moreover, the same preparation of  $\text{A}\beta_{1-42}$  induced microtubule disassembly, reduced neurite length and DNA fragmentation in mature hippocampal cells, which were largely prevented by the selective NMDAR antagonists MK-801 (noncompetitive antagonist), memantine and ifenprodil (GluN2B subunit antagonist), suggesting a role for extrasynaptic GluN2B-containing NMDARs in  $\text{A}\beta$  toxicity, as recently shown by Mota and colleagues (in press).

Application of  $\text{A}\beta$  monomers and low-*n* oligomers (dimers and trimers) secreted from Chinese hamster ovary cells that stably overexpress human APP bearing the Val717Phe familial AD mutation was shown to mimic a state of partial NMDAR blockade, reducing NMDAR activity and NMDAR-dependent  $\text{Ca}^{2+}$  influx [90]. Accordingly, neurons from a genetic mouse model of AD were found to express reduced amounts of surface GluN1 subunit [91], and  $\text{A}\beta_{1-42}$  was also found to reduce surface expression of the GluN1 subunit, in both cortical and hippocampal neurons [91, 92]. On the other hand, GluN2A- and GluN2B-NMDARs appear to have opposite roles in regulating intracellular  $\text{Ca}^{2+}$  in the presence of  $\text{A}\beta_{1-42}$  in rat cortical cultures [89]. These findings support the concept that dysregulation

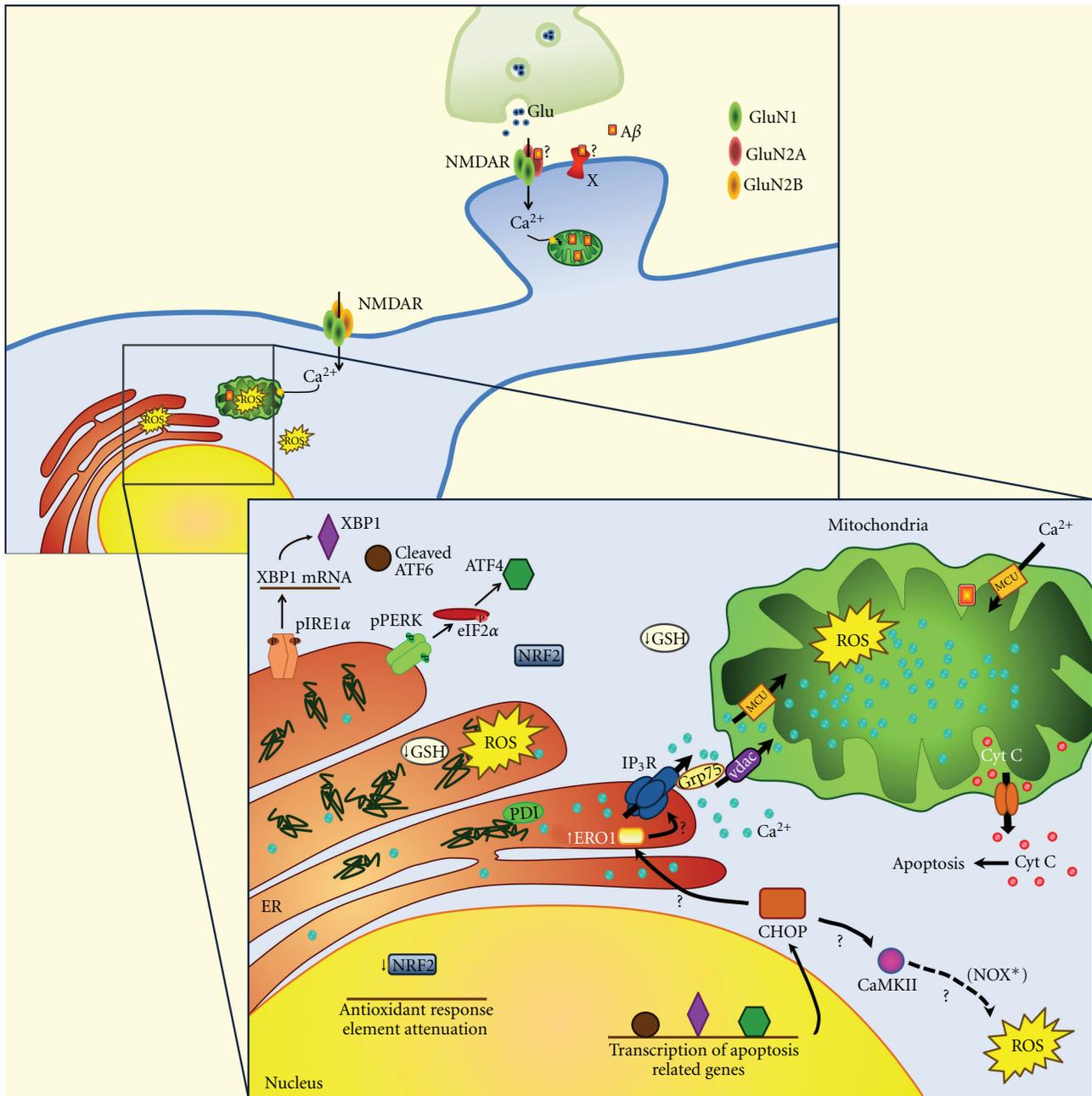


FIGURE 1: Sources of reactive oxygen species in Alzheimer's disease. Extracellular accumulation of A $\beta$  may direct or indirectly alter NMDARs-mediated glutamatergic neurotransmission with concomitant cytosolic Ca<sup>2+</sup> increase and impaired synaptic activity. Excitotoxic increase in glutamatergic neurotransmission may activate extrasynaptic NMDARs leading to a massive increase in the intracellular Ca<sup>2+</sup>, which is rapidly taken up by mitochondria and ER. Mitochondria Ca<sup>2+</sup> overload promotes the generation of ROS. Additionally, the ER may also promote ROS production. Decreased PDI activity may lead to polyubiquitinated proteins accumulation, which may thus induce the UPR, mediated by IRE1 $\alpha$ , PERK, and ATF6 pathways. In order to cope with the need to balance disulfide bond formation, the activity of ERO1 $\alpha$  is increased leading to the production of ROS that are able to directly attack and affect IP<sub>3</sub>R function. Since the ER and the mitochondria are in close proximity, the Ca<sup>2+</sup> released from the ER, through the IP<sub>3</sub>R, can then enter directly into mitochondria, through the VDAC or the MCU, leading to the increase in mitochondrial Ca<sup>2+</sup> content, inducing mitochondrial ROS production. As a result of prolonged ER stress, CHOP may induce ERO1 $\alpha$  upregulation or activate the enzyme CaMKII, which can further activate NOX, localized at the plasma membrane, enhancing cytosolic ROS production. As a consequence, protective antioxidant defenses such as GSH are depleted. In addition, Nrf2, which normally translocates to the nucleus where it activates the antioxidant response element, may be retained in the cytosol.

of intracellular  $\text{Ca}^{2+}$  homeostasis is induced by a possible interaction of  $\text{A}\beta$  with NMDARs, particularly of the GluN2B subtype. In addition, it was also demonstrated that in the AD brain and human cortical neurons, excitatory synapses containing the GluN2B subunit of the NMDAR appear to be the main sites of oligomer accumulation. In this study,  $\text{A}\beta$  oligomers colocalized with synaptic markers, and this effect was counteracted by ifenprodil and memantine, blocking the ion channel formed by the NMDAR [93].

There is a growing body of evidence that NMDAR activity has the potential to promote survival or death in neurons of the central nervous system [94], which may be related to differences in synaptic versus extrasynaptic NMDAR signaling. It was recently demonstrated that extrasynaptic, but not synaptic, NMDARs activity stimulates neuronal amyloidogenic  $\beta$ -secretase-mediated APP processing and increases  $\text{A}\beta$  production in primary cultures of cortical neurons [95]. Interestingly, in this study, memantine inhibited extrasynaptic NMDAR-induced APP protein expression as well as neuronal  $\text{A}\beta$  release in a dose-dependent manner. In fact, the differences between synaptic and extrasynaptic pools could be due to the way they are activated: brief saturating activation in the case of synaptic NMDARs, compared with chronic, low-level activation of extrasynaptic NMDARs by bath application of glutamate. Differences in the properties of intracellular  $\text{Ca}^{2+}$  transients evoked by these different stimuli may differentially affect signaling, even if the overall  $\text{Ca}^{2+}$  load is similar [96, 97].

$\text{Ca}^{2+}$  influx through NMDARs activation also seems to have opposite consequences on neuronal fate, according to their cellular localization [98, 99]. Stimulation of synaptic NMDARs induces prosurvival events through the activation of cAMP response element-binding protein (CREB) [100] and the extracellular signal-regulated kinase (ERK) cascade [101]. Conversely,  $\text{Ca}^{2+}$  influx through extrasynaptic NMDARs overrides these functions coupling to a dominant CREB shut-off pathway causing CREB dephosphorylation, which is less well tolerated, triggering decreased mitochondrial membrane potential and cell death [99]. Thus, a distinct NMDARs activation signaling pathway was postulated, depending on their localization. Synaptic stimuli evoke  $\text{Ca}^{2+}$  entry through both GluN2A- and GluN2B-containing NMDARs and, in contrast to excitotoxic activation of extrasynaptic NMDARs, produce only low-amplitude cytoplasmic  $\text{Ca}^{2+}$  spikes and modest nondamaging mitochondrial  $\text{Ca}^{2+}$  accumulation [102]. However, NMDAR signaling can also be due to differences in the composition of the NMDARs as opposed to the location of the receptors. Thus, it has been suggested that excitotoxicity is triggered by the selective activation of NMDARs containing the GluN2B subunit [103, 104] irrespective of its location (synaptic or extrasynaptic), as GluN2A-containing NMDARs promote survival [104]. Accordingly,  $\text{Ca}^{2+}$  entering through GluN2A or GluN2B subunits-containing NMDARs was shown to have antiapoptotic activity or mitochondrial dysfunction and cell death, respectively [100].

## 4. ER and Oxidative Stress in AD

### 4.1. ER Stress and ER-Mitochondria Crosstalk in AD

**4.1.1. ER Stress in AD.** The ER is a multifunctional organelle that plays a central role in many essential cellular activities, such as folding, assembly and quality control of secretory and membrane proteins, disulfide bond formation, glycosylation, lipid biosynthesis,  $\text{Ca}^{2+}$  storage and signaling. Under stress conditions, such as perturbed  $\text{Ca}^{2+}$  homeostasis or redox status, elevated secretory protein synthesis rates, altered glycosylation levels, and hypercholesterolemia, unfolded or misfolded proteins accumulate in the ER lumen leading to ER stress [105]. To relieve stress and reestablish homeostasis, the ER activates intracellular signal transduction pathways, collectively termed the unfolded protein response (UPR), which reduces the influx of newly synthesized proteins into the ER through induction of general translational arrest and induces the transcriptional upregulation of genes that enhance the ER protein-folding capacity and quality control. During UPR, the ER also employs proteasomal (ER-associated degradation, ERAD) and autophagic pathways to degrade mis- or unfolded proteins [106]. Three specialized ER stress-sensing proteins involved in the canonical mammalian UPR pathway have been identified: protein kinase R-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1  $\alpha$  (IRE1 $\alpha$ ) and activating transcription factor 6 (ATF6). Upon ER stress, the ER chaperone glucose-regulated protein 78 (Grp78) dissociates from these ER transmembrane sensors and promotes their activation, inducing phosphorylation and oligomerization of IRE1, and PERK, and translocation of ATF6 to the Golgi where it is cleaved by Site 1 and Site 2 proteases (S1P and S2P). Active IRE1 $\alpha$  processes the mRNA encoding X-box binding protein 1 (XBP1), a transcription factor that upregulates genes encoding mediators of protein folding, ERAD, organelle biogenesis, and protein quality control. PERK activation reduces protein load in the ER by decreasing general protein synthesis through phosphorylation of the initiation factor eukaryotic initiation factor 2 (eIF2 $\alpha$ ) which paradoxically increases selective translation of activating transcription factor 4 (ATF4) mRNA. The ATF4 protein is a member of the bZIP family of transcription factors that activates the expression of several UPR target genes involved in antioxidant responses, apoptosis, and autophagy. In ER stressed cells, ATF6 is cleaved at the Golgi apparatus, and the released cytosolic domain translocates to the nucleus where it increases the expression of ER chaperones, ERAD-related genes, and proteins involved in organelle biogenesis. However, when ER stress is prolonged or too severe, these adaptive mechanisms fail to restore protein-folding homeostasis, thus shifting adaptive programs toward the induction of apoptotic signaling to eliminate irreversibly damaged cells [107].

Unresolved and prolonged ER stress leads to perturbed  $\text{Ca}^{2+}$  homeostasis, increased protein accumulation, loss of ER function, and activation of apoptotic cascades [106]. Under these conditions, the level of the UPR-induced cell death mediator C/EBP-homologous protein (CHOP) increases [108] and activates the transcription of GADD34,

which interacts with protein phosphatase I to catalyze eIF2 $\alpha$  dephosphorylation [109, 110]. Dephosphorylated eIF2 $\alpha$  in turn increases protein synthesis and oxidation leading to ER protein overload [111]. CHOP also represses the transcription of the antiapoptotic Bcl-2 protein [112]. Accordingly, deletion of CHOP gene partially protects both cells and animals from ER stress-mediated cell death [113]. The UPR is known to initiate other proapoptotic events as well, including c-Jun N-terminal kinase (JNK) phosphorylation, cleavage of ER-specific caspases such as caspase-12, and disruption of cellular Ca<sup>2+</sup> homeostasis [114].

In the past few years, ER stress has been largely implicated in the pathogenesis of multiple human diseases, including neurodegenerative disorders [107, 115]. Several studies support that UPR activation upon ER stress is one of the main players in synaptic dysfunction and neuronal death occurring in AD [116–118]. In postmortem brain tissues from AD patients, a significant increase in the levels of ER stress markers, including phospho-PERK, phospho-eIF2 $\alpha$ , and phospho-IRE1 $\alpha$ , the transcription factor XBP1, the chaperone Grp78, and the downstream mediator of cell death CHOP has been reported, compared with age-matched controls, suggesting that the prolonged activation of the ER stress response is involved in the neurodegenerative process in AD [119–122]. Furthermore, recent studies revealed a connection between UPR activation and autophagic pathology in AD brain since the levels of microtubule-associated protein light chain 3 (LC3), an autophagosome marker, are increased in neurons displaying UPR activation [123]. Recent evidence obtained in an AD transgenic mice model, in which caspase-12, Grp78 and CHOP are strongly up-regulated, further implicates ER stress induction in the pathogenesis of AD [124]. Familial AD-linked presenilin-1 (PS-1) mutations downregulate the UPR and lead to ER stress vulnerability [125]. The mechanisms by which mutant PS-1 affects the ER stress response are attributed to the inhibited activation of ER stress transducers such as IRE1 $\alpha$ , PERK, and ATF6. On the other hand, in sporadic AD, it was found that the aberrant splicing isoform (PS2V), generated by exon 5 skipping of the presenilin-2 (PS-2) gene transcript, downregulates the signaling pathway of the UPR [126].

Familial and sporadic AD are both associated with increased A $\beta$  levels in brain parenchyma. Several evidences support that A $\beta$  deposition and ER stress are interrelated events in AD. A global molecular profile of hippocampal and cortical gene expression revealed that ER stress-related genes are differentially regulated during the initial and intermediate stages of A $\beta$  deposition [127]. ER stress was shown to enhance  $\gamma$ -secretase activity, as well as A $\beta$  secretion [128]. On the other hand, it was proposed that A $\beta$  is generated within the ER lumen as a result of deficits in axonal transport [129]. It was also found that in transgenic mice expressing APP(E693 $\Delta$ ) (APP(OSK)) intraneuronal A $\beta$  oligomers accumulate in the ER in hippocampal neurons and cause cell death by inducing ER stress [130]. Additionally, the involvement of caspase-12 activation in A $\beta$ -induced synaptic toxicity was recently demonstrated in cortical and hippocampal synaptosomes isolated from 3xTg-AD mice [131]. Several evidences demonstrate that A $\beta$  is also able to

trigger an ER stress response *in vitro* [132–134]. In primary cortical neurons, both fibrillar and oligomeric A $\beta$  have been shown to upregulate Grp78 concomitantly with activation of the ER stress-mediated apoptotic cell death pathway [135, 136]. How A $\beta$  causes ER stress is presently unclear. However, recent evidences obtained in cultured hippocampal neurons support that interaction of A $\beta$  oligomers with NMDAR, in particular with the GluN2B subunits, occurs upstream of deregulation of ER Ca<sup>2+</sup> homeostasis and upregulation of ER stress markers (Costa et al., unpublished data).

Perturbation of ER Ca<sup>2+</sup> homeostasis, a trigger for the accumulation of unfolded or misfolded proteins and activation of the ER stress response, seems to play an important role in the onset or progression of neuronal dysfunction in AD [117, 137]. Significantly, a markedly decrease of calreticulin immunoreactivity (ER Ca<sup>2+</sup> binding protein) was described in AD postmortem brain [138]. Recent studies in AD transgenic mice have shown that enhanced Ca<sup>2+</sup> response is associated with increased levels of ryanodine receptors and altering synaptic transmission and plasticity mechanisms before the onset of histopathology and cognitive deficits [139, 140]. Moreover, mutant PS-1 interacts with the inositol-1,4,5-trisphosphate (IP<sub>3</sub>) receptor (IP<sub>3</sub>R)-associated Ca<sup>2+</sup> release channel, resulting in Ca<sup>2+</sup> signalling abnormalities [141, 142] that have been suggested to be an early pathogenic event in AD involved in presynaptic dysfunction [143]. Recently, it was discovered that PS-1 and PS-2 can form low-conductance channels, leading to passive ER Ca<sup>2+</sup> leak [144]. These results provided potential explanation for abnormal Ca<sup>2+</sup> signaling observed in familial AD cells with mutations in PSs. Several findings also implicate A $\beta$  as a trigger of ER Ca<sup>2+</sup> dyshomeostasis. APP overexpression was shown to potentiate CHOP induction and cell death in response to ER Ca<sup>2+</sup> depletion [145]. Similarly, A $\beta$  depletes ER Ca<sup>2+</sup> through IP<sub>3</sub>R- and RyR-mediated Ca<sup>2+</sup> release, thus increasing intracellular Ca<sup>2+</sup> levels and compromising cell survival [136, 146]. In addition, A $\beta$ -induced perturbation of intracellular Ca<sup>2+</sup> homeostasis in neurons was shown to be correlated with an increase of the specific isoform of the ryanodine Ca<sup>2+</sup> channel RyR3 expression and activity [147].

Recent evidences suggest that strategies able to ameliorate ER stress can prevent A $\beta$  pathology. 4-Phenylbutyrate (PBA), acting through its chemical chaperone-like activity and via the transcriptional activation of a cluster of proteins required for the induction of synaptic plasticity and structural remodeling, was shown to mitigate ER stress. In the Tg2576 mouse model of AD, ER stress was accompanied by reversal of learning deficits, clearance of intraneuronal A $\beta$  accumulation, and restoration of dendritic spine densities of hippocampal CA1 pyramidal neurons [148]. Additionally, the same authors demonstrated that chronic administration of PBA, starting before the onset of disease symptoms, prevents age-related memory deficits in Tg2576 mice, associated to a decrease in A $\beta$  pathology and inflammation [148]. Wiley and colleagues [149] also demonstrated that PBA ameliorates the cognitive and pathological features of AD in the APP-swePS1delta9 AD transgenic mice. In APP-overexpressing cells, PBA blocked the repressive effects of the ER stressors tunicamycin and thapsigargin upon APP proteolysis, UPR

activation, and apoptosis [150]. Furthermore, silencing CHOP gene expression was shown to protect against AD-like pathology triggered by 27-hydroxycholesterol in rabbit hippocampus [151]. Recently, it was demonstrated that activation of the PERK-eIF2 $\alpha$  UPR pathway prevents A $\beta$ -induced neuronal ER stress [152]. Furthermore, the active form of the transcription factor XBP1 was shown to be neuroprotective in flies expressing A $\beta$  and mammalian cultured neurons treated with A $\beta$  oligomers, which was mediated by the downregulation of RyR3, preventing the accumulation of free Ca<sup>2+</sup> in the cytosol [153]. In addition, dantrolene and xestospongins C, pharmacological inhibitors of ER Ca<sup>2+</sup> release, were shown to prevent A $\beta$ -induced apoptotic cell death [154, 155].

*4.1.2. ER-Mitochondria Crosstalk in AD.* ER stress-induced apoptotic cell death involves a mitochondrial component [156, 157]. ER directly communicates with mitochondria through close contacts referred as mitochondria-associated membranes (MAMs) that promote Ca<sup>2+</sup> transfer from ER to mitochondria thus maintaining mitochondrial metabolism and cell survival [158–160]. The molecular bridges that regulate the contacts between ER and mitochondria include the IP<sub>3</sub>R on the ER and the VDAC, which are physically coupled through the cytosolic chaperone glucose-regulated protein 75 kDa (Grp75) [161]. In addition, the dynamin-related GTPase mitofusin 2 (Mfn2) on the ER forms homoheterodimers with Mfn1 or Mfn2 on mitochondria to keep the tight contacts between the two organelles. Moreover, PACS-2 (mainly localized at the ER) and dynamin-related GTPase protein 1 (Drp1) indirectly control the distance between the two organelles through regulation of mitochondrial morphology and distribution [162]. The chaperone Sigma-1 receptor (Sig-1R) is able to sense Ca<sup>2+</sup> concentrations in the ER and controls the amount of Ca<sup>2+</sup> released through the IP<sub>3</sub>R that can be transmitted to mitochondria [163].

Disruption of contact sites and impairment of Ca<sup>2+</sup> coupling between ER and mitochondria have profound consequences for cellular function and in extreme cases lead to apoptosis. In fact, decreasing the space between both organelles promotes mitochondrial Ca<sup>2+</sup> overload that can lead to the opening of the PTP, dissipation of the mitochondrial membrane potential and activation of apoptotic cell death [164], and, on the other hand, an increase in the distance between the two compartments inhibits Ca<sup>2+</sup> transmission, compromising Ca<sup>2+</sup>-dependent regulation of mitochondrial metabolism and consequently cell viability [165]. Accordingly, during the adaptive phase of ER stress, an early increase in cellular bioenergetics and mitochondrial metabolism occurs [166] but during the cell death response, ER stress exerts profound deleterious effects on mitochondrial function [167] and activates an apoptotic pathway which depends crucially upon Ca<sup>2+</sup> transfer from the ER to the mitochondria [135, 168]. The MAM is responsible for this transfer since its disruption, achieved by siRNA knockdown of PACS-2, results in the inhibition of ER Ca<sup>2+</sup> release and apoptosis onset [162]. Furthermore,

apoptotic stimuli known to act through Ca<sup>2+</sup> release from the ER induce a prolonged increase in the mitochondrial Ca<sup>2+</sup> concentration [154, 155, 169, 170].

Several members of the Bcl-2 family, such as Bcl-2 itself, Bax and Bak, naturally localize to both mitochondria and the ER and modulate Ca<sup>2+</sup> content in both organelles, controlling the amount of ER-releasable Ca<sup>2+</sup> that can reach mitochondria triggering apoptotic cell death [154, 171–176]. Transmission of a Ca<sup>2+</sup> signal from ER to mitochondria was demonstrated to be associated with IP<sub>3</sub>-induced opening of PTP and, in turn, cytochrome c release [177]. Similarly, phosphorylation of IP<sub>3</sub>R by Akt reduces cellular sensitivity to apoptotic stimuli through a mechanism that involves diminished Ca<sup>2+</sup> flux from the ER to the mitochondria [178]. Cytochrome c released from mitochondria can also bind to ER IP<sub>3</sub>R and promotes Ca<sup>2+</sup> release through this channel [179]. Released ER Ca<sup>2+</sup> triggers the extrusion of a large amount of cytochrome c from all the mitochondria in the cell, amplifying the death signal [180, 181]. It has been reported that mobilization of Drp1 to mitochondria, under ER Ca<sup>2+</sup> release conditions, can trigger mitochondrial cristae remodelling, facilitating cytochrome c release and subsequent apoptosis [182, 183]. However, recruitment of Drp1 to mitochondria upon sustained Ca<sup>2+</sup> release from the ER was described to protect from apoptosis by fragmenting the mitochondrial network and blocking Ca<sup>2+</sup> transmission [184].

Despite the evidence that demonstrates the involvement of mitochondrial and ER dysfunction in AD pathogenesis [55], the role of ER-mitochondria crosstalk in this neurodegenerative disorder has not been clarified so far. It was recently shown that PS-1 and PS-2 are highly enriched in a subcompartment of the ER that is related with MAM [185]. In SH-SY5Y cells and primary neuronal cultures, overexpression of PS-2, and more drastically its familial AD mutants, was demonstrated to increase the physical interaction between ER and mitochondria thus facilitating mitochondrial Ca<sup>2+</sup> uptake [186]. Moreover, the association of hyperphosphorylated tau with ER membranes was detected in AD brains and also in the brain of asymptomatic mice that overexpress mutant tau [187]. Interestingly, these mice exhibited more contacts between ER membranes and mitochondria, suggesting that accumulation of tau at the surface of ER membranes might contribute to tau-induced neurodegeneration through impairment of mitochondrial function [187]. Recent studies performed in mtDNA-depleted  $\rho$ 0 cells challenged with toxic A $\beta$  described the activation of an ER stress-induced apoptotic cell death pathway that requires the presence of a functional mitochondrial [188]. In A $\beta$ -treated cortical neurons, it was previously demonstrated that Ca<sup>2+</sup> released from ER, through IP<sub>3</sub>R and RyR channels [146], is implicated in the depolarization of the mitochondrial membrane, release of cytochrome c upon translocation of Bax to mitochondria and activation of caspase-9 [135, 136], thus implicating the ER/mitochondria crosstalk in neurodegeneration occurring upon A $\beta$  exposure. This communication was also corroborated by the evidence obtained with cybrids, which recapitulate the mitochondrial defect (inhibition of complex IV of the electron transport chain) observed in AD [9, 189]. In these cells, markers

of ER stress-induced apoptotic cell death were shown to be increased by  $A\beta$  treatment in comparison with controls suggesting that  $A\beta$ -induced ER stress is enhanced under mitochondrial dysfunction conditions [190].

**4.2. ER-Driven ROS Production.** Numerous evidences clearly implicate oxidative stress in AD pathogenesis. In this respect, the first thing that comes to our mind is mitochondrial-driven ROS generation. However, could ER be another important source of ROS in AD? Mainly during protein synthesis, 25% of cellular ROS are produced in the ER as a consequence of the activity of oxidoreductases, a family of proteins that catalyze protein folding reactions [191–193]. After the entry of nascent proteins in the ER, disulfide bond formation must occur to ensure their correct maturation and function. This reaction is catalyzed by the protein disulfide isomerase (PDI) that accepts electrons from thiol residues in the polypeptide chain substrate leading to its oxidation [194, 195]. To continue its activity, PDI must be reoxidized, a process that is guaranteed by oxidoreductin 1 (ERO1) [196]. In order to recycle itself, ERO1 transfers electrons to molecular oxygen, leading to the production of ROS. In AD patients, no substantial alterations were observed in PDI levels when compared to controls [197]; however this may not imply about its net activity. In fact, it was reported that the activity of PDI may be inhibited by  $\cdot\text{NO}$ , since increased levels of S-nitrosylated PDI were found in the brain of sporadic AD patients [198]. As a consequence, polyubiquitinated proteins accumulate, which may thus activate the UPR [198].

The ROS formation due to ERO1 activity is not exclusively linked to protein folding. ERO1 is retained in the ER through its interaction with PDI and the ERp44 [199, 200]. Beside this interaction, ERp44 also binds to the  $\text{IP}_3\text{R}$  leading to its inhibition, a process that is dependent on pH,  $\text{Ca}^{2+}$  concentration and redox state [201]. In this way, ERp44 works as a sensor of the environment in the ER lumen. When this ERp44- $\text{IP}_3\text{R}$  connection is disrupted, ER  $\text{Ca}^{2+}$  is released through this channel into the cytosol. This process may rely on the presence of ERO1, since prolonged ERO1 activation is expected to originate a hyperoxidizing environment in the ER lumen [202], which may lead to the formation of disulfide bonds in the  $\text{IP}_3\text{R}$  [201], disrupting the repressive interaction between ERp44 and  $\text{IP}_3\text{R}$  [203]. Interestingly, ERO1 $\alpha$ , one of the two ERO1 proteins expressed in human, was described to be localized on MAM [204], which is highly enriched in  $\text{IP}_3\text{R}$  [205], suggesting that human ERO1 $\alpha$  regulates  $\text{IP}_3\text{R}$ - $\text{Ca}^{2+}$  signaling on the MAM [204]. The  $\text{Ca}^{2+}$  released from ER can then enter directly into mitochondria, through the OMM VDAC or the IMM  $\text{Ca}^{2+}$  uniporter (MCU), leading to the increase in mitochondrial  $\text{Ca}^{2+}$  content [206], ROS production, and the opening of the PTP [207, 208]. This sequence of events is expected to occur in AD and can be hypothesized to underlie the increase in cellular ROS triggered upon ER  $\text{Ca}^{2+}$  release observed in  $A\beta$ -treated cortical neurons [135] (Figure 1).

Several ER functions, such as chaperone-mediated protein folding and refolding and the maintenance of  $\text{Ca}^{2+}$

gradients, are ATP-dependent processes. During the UPR, ER chaperones like Grp78 are upregulated, and consequently higher levels of ATP must be delivered to the ER, requiring an increase in ATP production by the mitochondrial respiratory chain, with the consequent enhancement of ROS production [209]. Similarly, the ER  $\text{Ca}^{2+}$  leak that occurs under prolonged stress conditions could obligate the sarco(endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) to increase the rate of entry of  $\text{Ca}^{2+}$  to the ER lumen, causing ATP depletion and subsequent increase of ROS production within the mitochondria.

Another consequence of UPR activation, in an attempt to recover from protein unfolding or misfolding, is the depletion of the antioxidant GSH. The function of GSH in the ER needs to be fully elucidated; however it has been suggested that GSH acts as a reductant [210], either by maintaining ER oxidoreductases in a reduced state or by directly reducing nonnative disulphide bonds in substrate folding proteins [211]. This may explain why the ER lumen contains a relatively high concentration of oxidized glutathione (GSSG), driving the GSH: GSSG ratio to approximately 3 : 1 [192, 212]. During UPR, the overload of unfolded proteins enhances ERO1 activity, leading to an increase in oxidized PDI levels, which requires higher levels of GSH. The subsequent conversion to GSSG leads to a depletion of the GSH pool. Another hypothesis for this decrease is that the stimulation of ERO1 activity increases the generation of ROS that reacts with GSH, decreasing its levels, which further increases ROS levels (Figure 1). In AD, contradictory results concerning GSH levels have emerged. Adams and colleagues [213] have suggested that GSH levels increase in the AD brain as a compensatory mechanism following damage in specific brain regions. In an opposite manner, Aksenov and coworkers [214] have reported that GSH metabolism is compromised in affected brain regions of AD patients. Moreover, GSH levels were described to be decreased in red blood cells from male AD patients and in experimental models of AD [215, 216]. It has been previously shown that GSH levels decrease in cortical neurons treated with  $A\beta$ , and this decrease was correlated with the release of  $\text{Ca}^{2+}$  from the ER [168]. This datum is further supported by previous results showing that depletion of GSH occurs in neurons treated with  $A\beta$  fibrils [217]. Therefore,  $A\beta$ -driven GSH depletion might contribute to the impairment of quality control mechanisms operating at the ER, leading to the accumulation of unfolded/misfolded proteins.

GSH is not the only antioxidant defense that may be reduced in AD as a consequence of ER stress and ROS formation. When the UPR is induced, the ER senses the increase in ROS and increases antioxidant defenses, namely, through the PERK signaling pathway that coordinates the convergence of ER and oxidative stress. One of these antioxidant responses involves the phosphorylation of Nrf2 by PERK, followed by its dissociation from the microtubule-associated protein Keap1 (Kelch-like ECH-associated protein 1), which allows the dislocation of Nrf2 from the cytosol to the nucleus [209, 218]. Once in the nucleus, Nrf2 binds to the antioxidant response element (ARE) to activate the transcription of several phase II detoxification enzymes and

antioxidant enzymes [219]. Nrf2 activation also contributes to the maintenance of GSH levels, which in turn buffers the accumulation of ROS during the UPR [220]. Several studies allow us to speculate that the increase in ROS observed in AD may be linked, at least in part, to a deregulation of Nrf2 activity (Figure 1). Indeed, not only Nrf2 was described to be predominantly cytoplasmic in hippocampal neurons from AD patients, resulting in decreased nuclear levels [221], but also Nrf2-ARE pathway was shown to be attenuated in APP/PS1 transgenic mouse brain at the time of A $\beta$  deposition [222]. The potential protective role of Nrf2 in AD is further supported by the demonstration of a significant reduction in spatial learning deficits of aged APP/PS1 mice, observed when Nrf2 is overexpressed in this AD model [222].

When ER stress is prolonged, UPR signaling pathways ultimately lead to apoptosis. CHOP is one of the mediators of ER stress-mediated apoptotic cell death. Li and colleagues [223] have demonstrated that CHOP induces ERO1 $\alpha$  upregulation, which causes the activation of the ER IP $_3$ R. The Ca $^{2+}$  released from ER can enter the mitochondria, promoting ROS generation as described above, but can also activate the enzyme calcium/calmodulin-dependent protein kinase II (CaMKII), which triggers mitochondrial-mediated apoptosis [224] (Figure 1). CaMKII can further induce NOX that activates a protein kinase R (PKR-) activating protein, leading to sustained PKR-mediated CHOP expression, amplifying the pathway induced by this ER stress-related transcription factor [225]. In AD patients, during the initial stages of the disease, the expression of all 3 isoforms of NOX was shown to be significantly increased [226], activating NOX-associated pathways and contributing to AD progression [45]. The connection between CHOP upregulation and NOX signaling in AD remains to be further clarified but it seems to be a good target for future therapeutic perspectives. Another positive feedback is played by ROS itself that can sensitize both Ca $^{2+}$ -release channels and SERCA at the ER membrane [227–229]. ROS or RNS can oxidize critical thiols in the RyR, causing Ca $^{2+}$  release [230]. On the other hand, oxidation of SERCA inhibits their ability to transport Ca $^{2+}$  to the ER lumen, increasing cytosolic Ca $^{2+}$  concentration [228].

From the data exposed above it is possible to conclude that the ER could be, by its nature, an important source of ROS in AD, which impacts on cell survival upon perturbation of normal ER function. Due to the close communication between ER and mitochondria, ER stress occurring in AD brain can be expanded to the mitochondria releasing its malicious oxidative power that can further trigger apoptotic cell death pathways. Therefore, targeting these cellular sources of ROS may bring strong therapeutical outcomes for this neurodegenerative disease.

## 5. Oxidative Stress Markers in Biological Fluids from AD Patients

With the move towards development of disease-modifying treatments, there is a need for more accurate diagnosis of AD in its early stages. Therefore, much attention has been paid to the identification and validation of biological markers

of the disease. Markers that specifically reflect the onset of pathology may have a profound impact both on early diagnosis and on detection of treatment effects in the near future. Established CSF biomarkers exist for early AD: total and hyperphosphorylated tau (tau and p-tau) that reflect AD-type axonal degeneration and the 42 amino acid isoform of amyloid  $\beta$  (A $\beta$ 1-42) that reflects senile plaque pathology [231]. These biomarkers have recently been incorporated in the new proposed revised criteria for AD [232, 233]. However, these classical markers do not capture all the pathological changes that take place in the brain of AD patients, and its clinical application is limited by the invasive nature of its collection. Impaired bioenergetics, increased production of ROS, and oxidative injury are, as seen above, important features of AD pathology that occur early in the course of the disease. These findings have spurred the development of assays for markers that reflect these processes both in tissue, CSF and peripheral fluids.

The methodology mostly used to assess oxidative damage is through the detection of products of free radical attack against biomolecules (lipids, proteins, and nucleic acids). Additionally, several compounds of the antioxidant defense system can be measured and used as complementary information regarding the oxidant/antioxidant balance of the organism.

Results on lipid peroxidation in plasma and peripheral blood cells have been inconsistent, with several authors demonstrating increased levels of free MDA or TBARS in serum/plasma [234–239] or in erythrocytes [240, 241] of AD patients and MCI subjects, whereas others did not confirm these findings [242–244]. Interestingly, a few studies have shown that the highest TBARS levels were found in APOE- $\epsilon$ 4 carriers [13, 241], suggesting that APOE genotype affects the extent of the oxidative stress-induced damage.

Free HNE has also been assessed in ventricular CSF from patients with AD, and significantly elevated levels were found in comparison to age-matched controls, while no differences were detected in the levels of HNE-protein adducts [245]. Similar to what has been reported for MDA and TBARS, the results of the determination of HNE in peripheral fluids of AD and MCI subjects have been somewhat inconclusive. Some authors have demonstrated elevated plasma levels of HNE in AD patients, compared to controls [243, 246], while others did not observe any differences [247]. An interesting study [248] reported increased levels of MDA and HNE in peripheral cells (skin fibroblasts and lymphoblasts) derived from familial AD patients, carrying APP and PS-1 mutations, while no differences in these lipid peroxidation markers were found between sporadic AD cases and controls.

Increased levels of F $_2$ -IsoPs were also found both in postmortem ventricular CSF from AD patients [20, 249] and in lumbar CSF collected *in vivo* [250, 251], correlating with clinical severity and other biomarkers of the disease, like CSF A $\beta$ 1-42 and tau [252, 253]. Several studies on MCI subjects also found increased levels of CSF F $_2$ -IsoPs [251, 254, 255], including longitudinal studies, that have shown that CSF F $_2$ -IsoPs levels rise after 12-month followup [254] and that the rate of increase is higher in MCI subjects that progress to AD, compared to healthy controls and stable MCI [256]. In

fact, longitudinal evaluation of CSF F<sub>2</sub>-IsoPs seems to be useful in predicting future cognitive deterioration both in cognitive normal and MCI subjects and in increasing the diagnostic accuracy of prodromal AD [255, 257]. One study in particular [258] suggests that the determination of CSF isoprostanes could be useful in monitoring the effectiveness of experimental antioxidant treatments. The quantification of isoprostanes in peripheral fluids of AD patients and MCI subjects has however yielded conflicting results. Some studies have found elevated levels of F<sub>2</sub>-IsoPs in the urine [252, 259] and plasma of AD patients [252] and MCI subjects [251], but further studies did not confirm these results [260, 261].

Overall, it seems that data regarding oxidative damage to lipids in the central nervous system is fairly consistent in showing increased markers of lipid peroxidation in early stages of AD. However, when moving to peripheral fluids, results are rather conflicting. Methodological differences could in part explain these contrary results. Furthermore, multiple physiological and pathological conditions can influence the levels of lipid oxidative damage in peripheral fluids, such as diet, physical activity, smoking habits, and comorbidities like diabetes, cardiovascular disease, and cancer that are known to increase oxidative damage. Therefore, when analysing the levels of lipid peroxidation markers in peripheral fluids in AD patients and MCI subjects, it is extremely important to control for potential confounders.

Protein carbonyls are usually detected with 2, 4-dinitrophenylhydrazine (DNPH) by a simple spectrophotometric assay. Carbonyl content has also been studied in plasma, with some studies failing to show an increase in this protein oxidation marker in AD patients [243, 262] and MCI subjects [241]. Recent studies, however, have demonstrated increased plasma concentrations of protein carbonyls in AD patients and MCI subjects, compared to controls [263], and also in peripheral lymphocytes isolated from AD patients [246].

Protein nitration, detected by nitrotyrosine immunoreactivity, has been studied not only in the brain but also in CSF. By employing sensitive HPLC methods, five- to eightfold increases in the levels of 3-NT have been found in the ventricular and lumbar CSF of AD patients when compared with cognitively normal controls [264, 265]. These results, however, were not confirmed by a different study using a gas chromatography coupled with mass spectroscopy approach [266], where the majority of AD patients had 3-NT CSF levels similar to the controls. The discrepancies between these studies are probably due to the different sample preparation and analysis methods and to the possible *in vitro* formation of 3-NT in the CSF samples. Similarly to what has been shown for protein carbonyls, increased levels of 3-NT have also been reported in plasma and lymphocytes of AD patients compared to controls [124].

DNA injury, assessed through increased levels of 8-OHdG, has also been shown in intact DNA extracted from ventricular CSF [267, 268] or in lumbar CSF of AD patients [269]. Studies using DNA extracted from peripheral tissue have also demonstrated increased levels of DNA oxidation, thus suggesting the systemic nature of oxidative damage in AD. Increased levels of 8-OHdG and oxidized

purines and pyrimidines in the peripheral lymphocytes and leukocytes of AD and MCI patients have been demonstrated [235, 270, 271] and also an increased urinary excretion of oxidized nucleosides in AD patients [272]. The potential of DNA oxidation levels as a biomarker for AD has been questioned, however, due to the overlap between AD and controls and to its lack of specificity, as increased DNA oxidation seems to be present in other neurodegenerative conditions, such as amyotrophic lateral sclerosis and Parkinson's disease [272]. Besides DNA, oxidation of RNA can also be used as a marker of oxidative stress, through the determination of 8-OHG levels. Interestingly, those were found to be fivefold increased in the CSF of AD patients compared to controls, being unaltered in the serum [273].

Contradictory results have been reported regarding the peripheral activity of cellular antioxidant enzymes in AD patients. While some studies have not found any differences in the activity of these enzymes in red blood cells of AD or MCI subjects as compared to controls [234, 241, 242], others have reported an increased activity of glutathione peroxidase, catalase, and superoxide dismutase [236, 239, 240], but decreased activity of the latter enzymes has also been found [238, 274, 275]. Regarding nonenzymatic antioxidants, including glutathione, uric acid, carotene, lycopene, vitamins A, C, and E, work from several groups has demonstrated decreased plasmatic levels in AD patients [234, 269, 276] and MCI subjects [263, 275, 276], with some authors suggesting that progression to AD might be related to depletion of antioxidant defenses [277]. One of the most investigated nonenzymatic antioxidants is probably vitamin E, the most powerful chain-breaking antioxidant [278], with reduced levels reported not only in plasma [234, 241, 275, 276] but also in CSF [279] and brain parenchyma of AD patients [280]. Antioxidant intervention in animal models of AD showed a significant reduction in oxidative stress, A $\beta$  deposition, and also behavioral improvements [281, 282]. However, in AD clinical trials, antioxidants have shown only a marginal positive effect on disease progression [283, 284], and subsequent MCI trials with antioxidants indicate that vitamin E ingestion has no benefit on the risk of progression to AD [285, 286]. The lack of success of these trials [287–289] likely arises from a combination of factors, including using the wrong dose in an unbalanced monotherapy, not monitoring the drug levels and surrogate markers for the *in vivo* therapeutic effect of the drug of interest and starting the therapy very late in the disease stage. The failure of simple antioxidants to reverse ROS damage has prompted the need of other mitochondrial-targeted therapies, such as acetyl-L-carnitine-carnitine (a compound that acts as an intracellular carrier of acetyl groups across the inner mitochondrial membrane), MitoVitE (a compound that results from the conjugation of vitamin E with the lipophilic triphenylphosphonium cation—TPP<sup>+</sup>—making the antioxidant selectively accumulate inside the mitochondria), Szeto-Schiller peptides (small cell permeable antioxidants that target mitochondria in a potential-independent manner), or Dimebon (the Russian antihistamine laterpirdine), as reviewed elsewhere [290, 291].

The failure of antioxidant therapy to attenuate disease progression [285, 286] might also be explained by the fact that oxidative stress could be a necessary but insufficient factor for the development of disease, that is dependent upon additional factor(s) for the onset of underlying pathogenesis. Nevertheless, early intervention to prevent chronic oxidative stress, and thereby ameliorate one of the factors for the development of the disease, should influence and reduce the risk of ever developing the disease. Indeed, the role of oxidative stress in the pathogenesis of AD has moved from an epiphenomenon to one of the earliest events in disease pathogenesis, occurring prior to the onset of symptoms and associated with the brain regions typically affected in the disease [14, 28, 38–40, 43, 251]. The hypothesis, based on *in vitro* cell culture experiments, that A $\beta$  causes oxidative stress [1] has been challenged by *in vivo* studies where oxidative stress chronologically precedes A $\beta$  deposition. In fact, A $\beta$  accumulation is associated with reduced levels of oxidative stress [38–40]. Therefore, the identification of valuable reliable peripheral markers of oxidative damage would be of utmost importance for researchers and clinicians. Currently there isn't no single biomarker of oxidative stress. The standardization of assessment methods and the consideration of potential confounders are critical to reduce the inconsistencies that have been reported between studies. Moreover, many of these studies have been done by comparing AD patients and/or MCI subjects with healthy controls and not with other neurodegenerative diseases, so specificity is still an issue. Oxidative stress has been found increasingly implicated in a number of neurodegenerative disorders including AD, Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) [292]. However, even if a process is not specific to AD pathogenesis, such as oxidative damage, its biomarkers may be useful in the context of clinical and imaging studies to monitor disease progression and optimize therapy. Increased sensitivity and specificity can probably be achieved by using a panel of different biochemical indices that target different pathological processes and can provide a more accurate picture of the oxidative balance of the organism.

## 6. Concluding Remarks

Alzheimer's disease (AD) is the most common age-related dementia. It is a slowly progressive and chronic neurodegenerative disorder, in which cognitive impairment is related to synapses degeneration and neuronal death occurring in the limbic system and specific regions of the cerebral cortex. The accumulation of A $\beta$  in senile plaques and the intraneuronal aggregates of hyperphosphorylated tau protein are recognized hallmarks of the disease whose cause still remains unknown.

Several lines of evidence show that mitochondria dysfunction, Ca<sup>2+</sup> deregulation, and oxidative stress are prominent factors in AD cellular pathology. Mitochondria, where free oxygen radicals are generated as by-products from the electron transport chain and from enzymes of the tricarboxylic acid cycle, are main sources and simultaneously main targets of ROS.

Toxic A $\beta$  oligomers may induce Ca<sup>2+</sup> influx into neurons, rendering neurons vulnerable to excitotoxicity, through the activation of glutamate NMDAR, and apoptosis. Glutamate excitotoxicity and/or oxidative stress have been shown to alter mitochondrial fission/fusion and an imbalance in mitochondria dynamics in turn leads to NMDAR upregulation and oxidative stress. In addition, A $\beta$  accumulates in mitochondria and thereby impairs the activity of mitochondria respiratory chain and reduces ATP synthesis and the mitochondria Ca<sup>2+</sup> buffering capacity, causing elevated cytoplasmic Ca<sup>2+</sup> levels and oxidative stress.

A $\beta$  was also shown to promote ER stress and excessive release of Ca<sup>2+</sup> from ER which may underlie mitochondrial Ca<sup>2+</sup> dyshomeostasis and ROS generation, thereby disturbing organelle functioning and, ultimately, damaging neurons.

Mitochondria and the ER are closely linked morphologically and functionally, and considerable crosstalk of cell death proteins, promoted by ROS and high Ca<sup>2+</sup> levels, occurs between these two organelles. The Ca<sup>2+</sup> transport systems of the ER are also sensitive to oxidative stress being directly exposed to ER/mitochondria-generated ROS. The resulting abnormal cellular Ca<sup>2+</sup> load can trigger cell death by activating proteases, reinforcing signals leading to caspase activation, such as cytochrome c release from mitochondria, or by triggering other catabolic processes mediated by lipases and nucleases.

A $\beta$ -associated Ca<sup>2+</sup> deregulation, impaired bioenergetics, increased production of ROS, and oxidative injury to lipids, proteins, and nucleic acids, associated to impairment of antioxidant defences, are important features of AD cellular pathology that occur early in the course of the disease. It can be hypothesized that the progression to AD may be related to the incapacity of the antioxidant system to counterbalance the oxidative injury, leading to disruption of cell redox signaling. In this context, development of reliable oxidative stress biomarkers and new antioxidant strategies should be proposed as primary prevention measures, even before significant plaque deposition or cognitive decline.

## Abbreviations

ATF6:	Activating transcription factor 6
CaMKII:	Calcium/calmodulin-dependent protein kinase II
CHOP:	C/EBP-homologous protein
ER:	Endoplasmic reticulum
GSH:	Glutathione
IP <sub>3</sub> R:	Inositol-1,4,5-trisphosphate receptor
IRE1 $\alpha$ :	Inositol-requiring enzyme 1 $\alpha$
MCU:	Mitochondrial Ca <sup>2+</sup> uniporter
NOX:	NADPH oxidase
NMDARs:	N-methyl-D-aspartate receptors
Nrf2:	Nuclear factor (erythroid-derived 2)-like 2
PDI:	Protein disulphide isomerase
PERK:	Protein kinase R-like endoplasmic reticulum kinase
ROS:	Reactive oxygen species

UPR: Unfolded protein response  
VDAC: Voltage-dependent anion channel.

## Authors' Contribution

E. Ferreiro, I. Baldeiras, and I. L. Ferreira contributed equally to the paper.

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## Review Article

# Reactive Oxygen Species-Mediated Control of Mitochondrial Biogenesis

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Mitochondrial biogenesis is a complex process. It necessitates the contribution of both the nuclear and the mitochondrial genomes and therefore crosstalk between the nucleus and mitochondria. It is now well established that cellular mitochondrial content can vary according to a number of stimuli and physiological states in eukaryotes. The knowledge of the actors and signals regulating the mitochondrial biogenesis is thus of high importance. The cellular redox state has been considered for a long time as a key element in the regulation of various processes. In this paper, we report the involvement of the oxidative stress in the regulation of some actors of mitochondrial biogenesis.

## 1. Introduction

Mitochondria are organelles that have critical functions in eukaryotic cells. Besides their well-known involvement in energy and intermediary metabolism (i.e., ATP synthesis, thermoregulation, heme biosynthesis), mitochondria also play a crucial role in both calcium homeostasis and apoptosis. Mitochondrial dysfunction has been associated with numerous pathologies including neurodegenerative diseases [1], diabetes [2], and aging [3, 4]. ATP synthesis by mitochondria is mostly generated through oxidative phosphorylation (OXPHOS) (Figures 1 and 2). Enzymatic complexes of the mitochondrial respiratory chain couple the oxidation of reducing agents such as NADH and FADH<sub>2</sub> to proton extrusion toward the intermembrane space. Due to the low proton permeability of the inner mitochondrial membrane, this proton extrusion results in the establishment of an electrochemical potential difference in protons across this membrane. This proton electrochemical potential difference is, in turn, used for ATP synthesis by the F<sub>0</sub>F<sub>1</sub>-ATP synthase complexes.

Mitochondria (and chloroplasts) are unique among eukaryotic extranuclear organelles in that they contain their own genome (mtDNA). In mammalian cells, mtDNA is a circular molecule, which encodes for 13 mRNAs, 22 tRNAs, and

2 rRNAs. All 13 mRNAs encode subunits of the OXPHOS. However, mitochondria are genetically semiautonomous in that they rely strongly on the nuclear genome for their biological function. Indeed, all the remaining mitochondrial proteins, including protein machineries involved in mtDNA replication, transcription, and translation, are encoded by the nuclear genome (Figures 1 and 2).

Consequently, mitochondrial biogenesis is a highly regulated process that involves the coordinated expression of two distinct genomes. This represents an important field of research notably because it has been well established in a wide range of cell types that mitochondrial content within the cell can vary massively depending on the physiological state [5, 6]. For example, a decrease in mitochondrial content has been described in numerous pathologies such as type 2 diabetes [7]. The signals and actors involved in the regulation of mitochondrial biogenesis are thus of high importance. Some of these pathologies are also associated with an oxidative stress, which raises the question of a possible regulation of mitochondrial biogenesis by the cellular redox state that would include ROS levels and glutathione redox state.

In this paper, we focus on some actors of mitochondrial biogenesis—the increase of the mitochondrial enzymatic content—at the transcriptional level in both mammalian

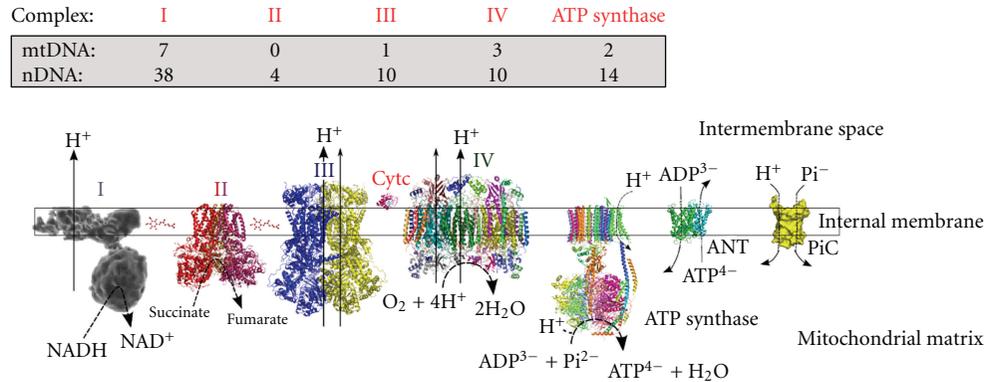


FIGURE 1: The mammalian oxidative phosphorylation (OXPHOS) system. Depicted are the four respiratory complexes (I–IV), electron carriers coenzyme Q and cytochrome c, the ATP synthase complex, the ADP/ATP carrier (ANC); and the phosphate carrier (PiC). Arrows at complexes I, III, and IV illustrate the proton pumping to the intermembrane space. Indicated are the number of complex subunits encoded by mitochondrial (mtDNA) and nuclear (nDNA) genomes.

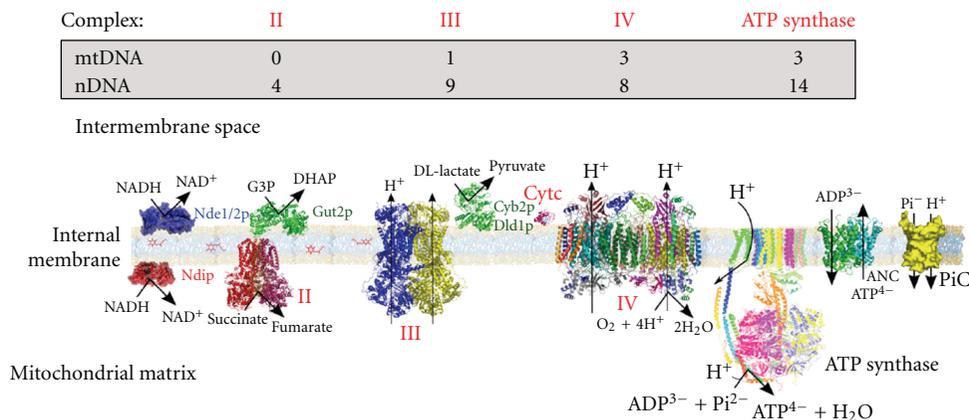


FIGURE 2: The *Saccharomyces cerevisiae* oxidative phosphorylation (OXPHOS) system. The main differences with the mammalian OXPHOS system are the absence of complex I that is substituted by external and internal NADH dehydrogenases, and the presence of D, L-lactate dehydrogenases, which transfer electrons directly to cytochrome c. Indicated are the number of protein subunits encoded by mitochondrial (mtDNA) and nuclear (nDNA) genomes.

cells and the yeast *Saccharomyces cerevisiae* and on their regulation by the cellular redox state.

## 2. Modulation of the Mitochondrial Content in Mammalian Cells

**2.1. Overview.** One of the best illustrations of the variation of the cellular mitochondrial content is the adaptation to energy demand [6]. In the skeletal muscle, in the 1960s, experiments have demonstrated an increase in the number and the size of mitochondria associated with an increase in the activity of mitochondrial marker enzymes in response to exercise [8, 9]. Twenty years later, chronic contractile activity produced by electrical stimulation was shown to increase mRNA levels encoding both nuclear and mitochondrial gene products [10]. These modifications are part of the phenomenon of exercise-induced muscle plasticity [11, 12] and are presumably an adaptation in order to adequately match ATP synthesis to ATP consumption by the contractile activity.

Proliferation of mitochondria also occurs during adaptive thermogenesis in brown fat during cold exposure. This proliferation coincides with an increase in the expression of UCP-1, an uncoupling protein that dissipates the proton gradient, leading to an increase in mitochondrial respiration and to heat production [13–15].

**2.2. Transcription Factors.** Nuclear respiratory factors (NRF-1 and NRF-2) were the first identified nuclear transcription factors governing respiratory gene expression in mammalian cells. NRF-1 was discovered when studying the regulation of the cytochrome *c* encoding gene [16, 17]. This protein binds to DNA as a homodimer and functions as a positive regulator of gene transcription. Inactivation of NRF-1 results in early embryonic lethality, pointing out its essential function [18]. NRF-2 was identified by the analysis of the regulation of cytochrome *c* oxidase (COX) subunits encoding genes [19]. It is a complex of five subunits that shares some target genes with NRF-1. Both NRF-1 and NRF-2 are well known to

regulate the transcription of many *respiratory* genes, that is, subunits of complex I, complex II, complex III, COX and ATP synthase, genes encoding proteins involved in mtDNA transcription and replication, as well as genes encoding proteins involved in mitochondrial protein import [17, 20–23]. Peroxisome proliferator-associated receptors (PPAR $\alpha$ , PPAR $\beta$ , PPAR $\gamma$ ) and ERR $\alpha$  are nuclear hormone receptors associated with mitochondrial metabolism. These proteins act as heterodimers and seem to coordinate the expression of genes involved in both fatty acid oxidation and the respiratory chain [24–26].

The PGC-1 family of coactivators (PGC-1 $\alpha$ , PGC-1 $\beta$ , and PRC) plays a central role in the transcriptional regulation of mitochondrial biogenesis. PGC-1 $\alpha$  was primarily identified as a key actor of adaptative thermogenesis [27]. PGC-1 $\beta$  and PRC were discovered through research of sequence similarity to PGC-1 $\alpha$  [28, 29]. These coactivators act as coordinators of the activity of numerous transcription factors involved in the mitochondrial biogenesis process. Indeed, the PGC-1 proteins, through binding to other transcription factors such as NRF-1, PPARs, and ERR $\alpha$ , regulate their activity and are involved in an increase in the expression of transcription factors like NRF-1 and NRF-2 [30]. Overexpression of PGC-1 $\alpha$  or PGC-1 $\beta$  in cultured cells and transgenic mice results in an increase of cellular mitochondrial content [30, 31]. Overexpression in the skeletal muscle induces the conversion to oxidative type muscle fibers [32, 33]. These results strengthen the importance of the PGC-1 family in mitochondrial biogenesis.

**2.3. Regulation of Mitochondrial Biogenesis by the Oxidative Stress.** Mitochondria play an important role in the cellular redox homeostasis due to their main function—that is, oxidation of reduced NADH and FADH<sub>2</sub>—but also through their involvement in ROS metabolism. Indeed, mitochondria are considered as one of the main sites of ROS production in the cell [34]. Considered for a long time as toxic byproducts of oxidative metabolism, ROS are now also considered as signaling molecules, which mediate redox regulation of multiple processes such as cell proliferation, differentiation and apoptosis [34, 35]. Moreover, a regulation of transcription factors involved in mitochondrial biogenesis by oxidative stress has been reported (see the following).

In the late 1990s, an increase in the expression level of mitochondrial proteins mRNAs by oxidative stress was reported. Indeed, the addition of antimycin A—a well-known inhibitor of respiratory chain complex III which increases mitochondrial ROS production—to human fibroblasts, at a concentration partially inhibiting the cellular respiratory, rate led to an increase in cytochrome c1 and cytochrome b mRNA levels [36]. In accordance with this study, another team showed that addition of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to lung fibroblasts led to an increase in the expression of the transcription factor NRF-1, which is involved in mitochondrial biogenesis [37]. Moreover, lipopolysaccharide-induced oxidative damage leads to the upregulation of NRF-1 and NRF-2 and the redox regulation of NRF-1 binding to target promoters is supposed to be mediated through Akt-dependent phosphorylation [38].

St-Pierre et al. [39] have shown that treatment of mouse embryonic cells with H<sub>2</sub>O<sub>2</sub>, increases PGC-1 $\alpha$  and PGC-1 $\beta$  mRNA levels. Previously, several studies showed that PGC-1 $\alpha$  and PGC-1 $\beta$  regulate the expression of genes encoding enzymes involved in the ROS defense system, that is, catalase and superoxide dismutases (SODs) [40–42]. Moreover, PGC-1 $\alpha$  expression is important for resistance to oxidative damage increase, neurodegeneration, and apoptotic cell death [39, 41]. Thus, those results depict a process where there is a tight link between the biogenesis of a main ROS source production, that is, mitochondria, and the anti-ROS system. Furthermore, PGC-1 $\alpha$  induction by oxidative stress is partly mediated by binding of the cAMP responsive element binding protein (CREB) in the PGC-1 $\alpha$  promoter [39].

In skeletal muscle cells, data provided by the Hood team described a ROS-mediated regulation of PGC-1 $\alpha$  transcription [43]. Indeed, treatment of C<sub>2</sub>C<sub>12</sub> cells with H<sub>2</sub>O<sub>2</sub> resulted in an increase in PGC-1 $\alpha$  mRNA that was prevented by pretreatment of these cells with an antioxidant: N-acetyl-L-cysteine (NAC). In this paper, the induction of PGC-1 $\alpha$  seemed to depend on the activation of the AMP-activated protein kinase (AMPK) by H<sub>2</sub>O<sub>2</sub>. That activation increased the DNA binding activity of the transcription factor USF1 to the PGC-1 $\alpha$  promoter, resulting in an increase in PGC-1 $\alpha$  expression. This work is one of the numerous *in vitro* and *in vivo* studies related to the highly debated question of the relationships between skeletal muscle, oxidative stress, and physical exercise. Since the 1980s, a stimulus role for skeletal muscle adaptation to exercise has been suggested [44]. In recent years, different groups reported that oral administration of antioxidants prevents the exercise-induced adaptation of muscle mitochondria probably through preventing the induction of transcription factors such as PGC-1 $\alpha$  [45, 46]. Thus, ROS produced during exercise could stimulate mitochondrial biogenesis. However, contradictory results have also been provided about the inhibitory effect of antioxidant addition [47, 48] and this research area is still a strong matter of debate [49, 50].

Diabetes consists in a group of metabolic diseases characterized by defects in the control of glucose and insulin homeostasis, which are a major public health issue. Type 2 diabetes, the most widespread type of diabetes, is known to be associated with alterations in mitochondrial density and mitochondrial dysfunctions in the skeletal muscles of patients [2, 51]. Transcriptomic profile analysis of type 2 diabetes patients revealed a reduced expression level of genes encoding proteins involved in the OXPHOS system [52, 53]. Hyperglycemia is known to increase intracellular ROS levels. This is supposed to occur via several mechanisms [54], one of them being an hyperpolarization of the mitochondrial inner membrane-favorable conditions for ROS production by the mitochondrial respiratory chain [55]—due to impairment in the regulatory capacity of the mitochondrial respiratory chain. Bonnard et al. showed that in hyperglycemic and hyperlipidemic mice, an increase in muscle ROS production is associated with mitochondrial alterations and a decrease in the expression of PGC1- $\alpha$  mRNA and some of its target genes. In a model of hyperglycemia-associated oxidative stress (streptozotocin treated mice), NAC treatment restores

mitochondrial density and structure [56]. In conclusion, this study suggests that hyperglycemia and hyperlipidemia-induced ROS production in skeletal muscle leads to mitochondrial dysfunction due to a decrease in the expression of PGC1- $\alpha$  and its target genes. Although these results seem contradictory if we compare them to the Hood team's, they might be reconciled through the physiological state of the cell. Indeed, downregulation of AMPK activity has been shown in animal models of insulin resistance, high fat feeding, and glucose infusion [57, 58]. Thus, energetic parameters should be considered to interpret the consequences of the oxidative stress on mitochondrial biogenesis.

### 3. Modulation of Mitochondrial Content in the Yeast *Saccharomyces cerevisiae*

**3.1. Overview.** The budding yeast *Saccharomyces cerevisiae* is the unicellular eukaryotic microorganism with the best annotated complete genome sequence. It has been widely used to study molecular mechanisms underlying diverse biological aspects such as mitochondrial functions. Indeed, when yeast cells are grown on nonfermentable carbon source, that is, lactate or ethanol, mitochondria are the unique source of ATP. As stated previously, cells adapt to their energy needs by adjusting their mitochondrial enzymatic content resulting in a capacity to modulate the ATP turnover [6]. In living cells, growth is the result of coupling between substrate catabolism and multiple metabolic processes taking place during net biomass formation and cell maintenance. A crucial parameter for growth description is its yield, that is, the efficiency of the transformation from substrate consumption to biomass formation. When yeast cells are grown on a purely respiratory substrate, biomass generation is entirely connected to substrate oxidation through oxidative phosphorylations and, hence, to oxygen consumption. We have previously shown that, in nonfermentable media, the growth yield is identical regardless of the strain, growth phase, and respiratory substrate used [59]. This homeostasis is the consequence of a strict linear relationship between growth and respiratory rate. Moreover, the oxygen consumption rate was strictly controlled by the cellular content in respiratory chains in such a way that, *in vivo*, the steady state of oxidative phosphorylation was kept constant. The cAMP signaling pathway is now well known to be involved in the regulation of mitochondrial biogenesis, both in mammalian cells and in yeast, even though the molecular mechanisms of this process are not well defined. It has been shown that treatment of human preadipocytes with forskolin, which leads to an overactivation of the Ras/cAMP pathway, increased mitochondrial DNA copy number [60]. In yeast, we showed that overactivation of the Ras/cAMP pathway leads to an increase in the cell mitochondrial content [61, 62]. Yeast has three A kinase catalytic subunits, which have greater than 75% identity and are encoded by the TPK (*TPK1*, *TPK2*, and *TPK3*) genes [63]. Although they are redundant for viability and functions such as glycogen storage regulation, the three A kinases are not redundant for other functions [64–66]. We have shown that in the absence of the yeast protein kinase Tpk3p only, there

is a significant decrease in cellular mitochondrial content, when cells are grown in nonfermentable medium [67]. This generates a drastic decrease in cell growth in the  $\Delta tpk3$  cells versus the wild type cells, since when yeast cells are grown on respiratory substrate, energy transformation processes involve oxidative phosphorylation [59].

**3.2. The HAP Complex and the Regulation of Mitochondrial Biogenesis by Redox Agents in the Yeast *Saccharomyces cerevisiae*.** Similarly to what was shown in mammalian cells, the first identification of transcriptional factors regulating mitochondrial biogenesis in *S. cerevisiae* resulted from the study of the regulation of the cytochrome c gene expression (*CYC1*). The master regulator of mitochondrial biogenesis in *S. cerevisiae* is the HAP complex. It is constituted of four subunits: Hap2p, Hap3p, Hap4p, and Hap5p (Figure 3). Subunits 2, 3, and 5 are DNA binding subunits whereas Hap4p is the activator of the complex [68–72]. As illustrated in Figure 3, the HAP complex regulates the expression of many genes encoding proteins involved in mitochondrial functions [73, 74]. In accordance with that key role, the absence of any subunits of the HAP complex leads to a growth defect on nonfermentable medium (i.e., lactate or ethanol). During growth of *Saccharomyces cerevisiae* on fermentable medium (containing high glucose concentration (i.e., 2% (*p/v*))), there is a repression of the expression of several genes encoding mitochondrial proteins [75–77]. Under these conditions, it has been shown that overexpression of Hap4p, the activator subunit of the HAP complex, was sufficient to derepress those genes [74, 78]. These results strengthen the main role played by the HAP complex in the regulation of mitochondrial biogenesis. For a long time, the only known signal regulating the HAP complex was the carbon source. Indeed, whereas Hap2p, Hap3p, and Hap5p are constitutively expressed, Hap4p expression is maintained at a very low level during growth on fermentable substrates. Growth on nonfermentable substrates strongly induces Hap4p expression and thus the activity of the HAP complex [71].

As stipulated before, in  $\Delta tpk3$  cells, there is a decrease in mitochondrial content. Moreover, in these cells we have shown that the activity of ROS detoxifying enzymes such as catalase and superoxide dismutases is strongly induced. Mitochondria isolated from  $\Delta tpk3$  cells have a high H<sub>2</sub>O<sub>2</sub> production rate and an elevated level of protein carbonylation [79]. These results led us to hypothesize that the  $\Delta tpk3$  cells were subjected to an oxidative stress. Treatment of  $\Delta tpk3$  cells with an antioxidant such as NAC, as well as Sod1p (superoxide dismutase isoform 1) overexpression, leads to a full restoration of growth, cellular respiratory rates, and mitochondrial content. This clearly indicates that the decrease in mitochondrial content of the  $\Delta tpk3$  cells is due to an increase in mitochondrial ROS production. In order to understand the link between ROS and mitochondrial biogenesis, we assessed HAP complex activity in these cells. We were able to show that HAP complex activity was reduced in  $\Delta tpk3$  cells and restored to the wild type level upon antioxidant treatment. Moreover, addition to wild type cells of hydrogen peroxide or antimycin A decreases Hap4p level, the activator

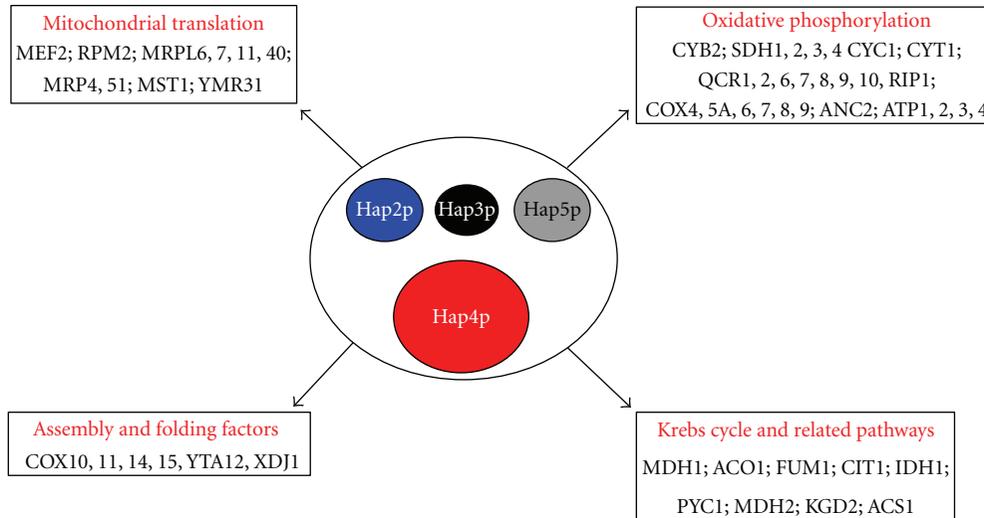


FIGURE 3: The HAP complex: a master regulator of the mitochondrial biogenesis in the yeast *Saccharomyces cerevisiae*. The four subunits constituting the complex are represented here. Size differences illustrate the difference in the predicted molecular weights of each subunit. The mitochondrial proteins encoding genes regulated by the complex are also indicated. See text for references.

subunit of the HAP complex [79]. Thus, ROS regulates mitochondrial biogenesis in the yeast *Saccharomyces cerevisiae* through the regulation of HAP complex activity by decreasing the Hap4p protein level. This work illustrates a mitochondrial control-quality process in which the main transcription factors involved in mitochondrial biogenesis are able to sense a mitochondrial dysfunction and then decrease the biogenesis of these dysfunctional mitochondria.

Those observations are reinforced by our recent study linking the glutathione redox state and the mitochondrial biogenesis. Glutathione is considered as the major redox buffer of the cell because of both its high concentration (1–10 mM) and its low redox potential ( $E_{hc} = 240$  mV) [80, 81]. Reduced glutathione (GSH) is a cosubstrate in many ROS detoxifying reactions and protein oxidation repairs (i.e., glutathione peroxidase, glutaredoxins) producing its oxidized form (GSSG). The glutathione reductase, encoded by the *GLR1* gene in the yeast *Saccharomyces cerevisiae*, is a very important enzyme in the glutathione system because it regenerates GSH. The deletion of *GLR1* leads to a decrease in the 2GSH/GSSG ratio. In  $\Delta glr1$  cells growing in non-fermentable medium, we observed a very low Hap4p level associated with a low mitochondrial content. The addition of reduced glutathione to  $\Delta glr1$  cells induced an increase in this ratio and so resulted in a more reduced cell redox state [82]. Under such conditions, we showed that both Hap4p level and mitochondrial content were restored [83].

In 2010, the transcription factor Hcm1p was identified as another possible regulator of mitochondrial metabolism in the yeast *Saccharomyces cerevisiae* [84]. Indeed, the over-expression of Hcm1p leads to an increase in the cellular respiratory rate and its absence impairs cell growth on ethanol. Interestingly, the nucleus localization of Hcm1p is increased by addition of hydrogen peroxide and Hcm1p increases the expression of antioxidant enzymes such as Sod2p. Thus, Hcm1p could link mitochondrial biogenesis and the ROS

detoxification systems. Although the mechanisms clearly explaining the link between Hcm1p—formerly known for its involvement in the regulation of the spindle pole—have not been shown, this result could represent a novel piece to the puzzle of the relationship between the oxidative stress and the regulation of the mitochondrial biogenesis.

#### 4. Conclusion

Due to its dependence on two physically distinct genomes, the biogenesis of mitochondria has to be a well-coordinated and regulated process. The identification of nuclear transcription factors regulating the expression of both mitochondrial and nuclear genes encoding for mitochondrial proteins was a great advance in that domain. In the landscape of the regulating signals of the mitochondrial biogenesis, the cellular redox state is becoming an important actor. Mitochondria being one of the main sites of ROS production, and many pathologies being associated with both decrease in mitochondrial content and oxidative stress, these studies naturally raise the question of the crosstalk between the mitochondria and the nucleus. Both decrease and increase of mitochondrial biogenesis in oxidative conditions have been reported. In mammalian cells, the tight link between the regulation of mitochondrial biogenesis and the antioxidant systems has been mechanistically well described. Thus, it seems logic that an increase of the mitochondrial biogenesis by ROS relies on a regulatory system, which is built to *prevent* the cell from the ROS production due to increase of the mitochondrial metabolism. However, in conditions of excessive oxidative stress as in the case of some pathologies and/or severe dysfunction of the mitochondrial respiratory chain, a decrease in mitochondrial biogenesis could be considered as a quality-control process through the decrease of dysfunctional mitochondria by ROS. Because of the key roles played by mitochondria in energy metabolism and many

other processes, alternative regulatory pathways relying, for example, on energetic parameters must be taken in consideration. More detailed analyses, notably in regard to the eventual signal specificity according to the nature of the oxidative stress, will certainly be necessary to improve our understanding of the relationships between the oxidative stress and the mitochondrial biogenesis process.

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## Review Article

# Energy and Redox Homeostasis in Tumor Cells

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Cancer cells display abnormal morphology, chromosomes, and metabolism. This review will focus on the metabolism of tumor cells integrating the available data by way of a functional approach. The first part contains a comprehensive introduction to bioenergetics, mitochondria, and the mechanisms of production and degradation of reactive oxygen species. This will be followed by a discussion on the oxidative metabolism of tumor cells including the morphology, biogenesis, and networking of mitochondria. Tumor cells overexpress proteins that favor fission, such as GTPase dynamin-related protein 1 (Drp1). The interplay between proapoptotic members of the Bcl-2 family that promotes Drp 1-dependent mitochondrial fragmentation and fusogenic antiapoptotic proteins such as Opa-1 will be presented. It will be argued that contrary to the widespread belief that in cancer cells, aerobic glycolysis completely replaces oxidative metabolism, a misrepresentation of Warburg's original results, mitochondria of tumor cells are fully viable and functional. Cancer cells also carry out oxidative metabolism and generally conform to the orthodox model of ATP production maintaining as well an intact electron transport system. Finally, data will be presented indicating that the key to tumor cell survival in an ROS rich environment depends on the overexpression of antioxidant enzymes and high levels of the nonenzymatic antioxidant scavengers.

## 1. A Brief Prelude

Every biochemical reaction within living cells involves the transduction of some degree of free energy that is ultimately derived from the oxidation of dietary nutrients. Most of this free energy is made biologically available as reversible phosphorylation reactions involving adenosine triphosphate (ATP) that is continuously being produced and utilized by cells to drive thermodynamically nonspontaneous reactions, such as ion transport, muscle contraction, protein synthesis, and DNA replication. Just as an example of the stupendous biological power, the amount of free energy transduced by our body during light walking is about  $3,18 \times 10^{-3}$  W/g, which is roughly 16.000 times more than the fusion reactions that take place in the Sun core [1]. It is known that phosphate esterification into ATP can occur by several processes but the best known are the phosphocreatine-ATP shuttle, glycolysis, and oxidative phosphorylation [2]. Oxidative phosphorylation is capable of producing significantly more ATP per mole of substrate than glycolysis in reactions completely dependent on the availability of oxygen. However,

the utilization of oxygen by cells, albeit the advantages of oxidative phosphorylation, is not without consequence, since partially reduced oxygen intermediates, the so-called reactive oxygen species (ROS) play key roles in cellular redox homeostasis [3] that may have a role in tumorigenesis.

## 2. Mitochondria and Bioenergetics

*2.1. Fermentation, Pasteur, Warburg, and Crabtree.* Fermentation was the first metabolic pathway to be fully known, thanks to the key findings of many researchers such as Louis Pasteur, who defined the biological nature of the process and Eduard Buchner, who showed that cell-free extracts could carry out fermentation. Later, Otto Meyerhoff experimentally demonstrated that a process similar to fermentation occurred in skeletal muscles, although generating a different final product, lactate [4]. He also showed that, in the absence of oxygen, glycogen was converted to lactate and when oxygen was present lactate was converted back to glycogen, establishing the cyclic nature of lactate metabolism in muscles (the *lactate shuttle*). In the context of cancer,

when cells that use glucose as the main substrate to drive ATP synthesis are subjected to hypoxia, as happens to the cells located in the center of the tumor mass, glucose uptake and metabolism increase significantly in order to maintain cellular ATP levels. Since under limited oxygen availability the oxidative phosphorylation machinery is not fully operational, other pathways are recruited in order to supply the energy demand. The reversible nature of increased glucose uptake and metabolism when cells experiment hypoxia is known as the *Pasteur Effect*. Also relevant is the reversible repressive effect of glucose over respiration, known as the *Crabtree effect* [5]. Thus, in spite of a functional oxidative phosphorylation machinery, most solid tumors exhibit a reversibly switch of their metabolism towards lactic fermentation, even under normoxia. Thus, contrasting with the *Pasteur Effect*, the limitation of respiration in the *Crabtree effect* is not due to oxygen availability, but rather to an acute repressive signaling cascade triggered by glucose over the mitochondrial function. For this reason, sometimes the *Crabtree effect* is also referred to as *Reverse* or *Inverted Pasteur Effect*. However, the molecular mechanisms that underlie the *Crabtree effect* remain elusive. Finally, the long-term metabolic reprogramming that takes place in many cancer cells and which bears on cancer is known as the *Warburg effect* [6]. Otto Warburg observed that cancer cells displayed decreased respiration and enhanced lactate production, suggesting that they depended mainly on fermentative metabolism for ATP generation [7]. It is commonly assumed that tumors manifesting the *Warburg effect* do so because the oxidative phosphorylation machinery is somehow impaired. In this context a growing body of evidence shows in fact that the oxidative phosphorylation is preserved in many cancer cells, as will be discussed in the following section. The point that should be stressed here regarding the main difference between *Crabtree* and *Warburg effects* is that in the former the oxidative phosphorylation is rapidly and reversibly downregulated by the repressive effect of glucose, whereas in the latter, there is a long-lasting irreversible effect favoring fermentation due to the increased expression of proteins involved in glucose transport and metabolism [5].

### 2.2. Mitochondria and the Processes of Energy Transduction.

Structurally, mitochondria are organelles enclosed by two very distinct membranes: an outer membrane, moderately selective, and an inner membrane which is protein rich and highly selective. These compartments are structurally and functionally different. The tricarboxylic acid (TCA) cycle enzymes are located within this compartment, whereas the proteins that comprise the electron transport system (ETS) occur in the inner mitochondrial membrane. The redox reactions mediated by different compounds from ubiquinone to iron/copper-sulphur clusters, cytochromes, and finally oxygen reduction to water (respiration) take place at the inner mitochondrial membrane. Recently, an effort to identify the whole set of mitochondrial proteins in different tissues of mice, rat, and human demonstrated that this organelle is composed of almost 1100 different proteins [8].

The ETS is essentially composed of proteins that contain an array of redox centers making up the complexes commonly listed from I to IV [2]. It is important to mention, however, that respiration can be promoted by multiple sites of electrons entry to the ETS in which electrons converge at the ubiquinone reduction (Q-junction) [9]. Importantly, the free energy released during the electrons transport by the ETS complexes is linked to the transport of protons across the inner mitochondrial membrane. Due to its proton impermeable nature, an electrochemical gradient is established [10]. This electrochemical proton gradient has two components; one chemical ( $\Delta\text{pH}$ ) and the other electrical ( $\Delta\psi$ ) in nature, which together represent the protonmotive force (*pmf*). The free energy accumulated in the form of *pmf* can be converted to chemical energy by means of the complex molecular motor activity of the  $\text{F}_1\text{F}_0$  ATP synthase, which allows the return of protons back to the mitochondrial matrix coupled to ATP production [11]. *pmf* is important not only for ATP synthesis but for many processes such as the control of substrate transport to mitochondrial matrix, respiratory rates [12], calcium homeostasis [13], ROS generation [14] and heat production [15].

2.3. *Redox Reactions in the ETS.* The first ETS redox centers were described in the nineteenth century by Charles MacMunn. In 1883, he found a peculiar pigment (*myohematin*) in the muscle of insects, whose light absorption pattern was quite similar to heme. MacMunn proposed the respiratory nature of these pigments and suggested that they were not derived from hemoglobin since they were found in organisms that knowingly did not have it [16]. Decades later the parasitologist David Keilin revisited the problem using an ingenious device, the microspectrophotometer. During his studies, Keilin found the very same four absorption bands identified by MacMunn not only in the fly, but in *Bacillus subtilis* and in baker's yeast. Keilin called the ubiquitous colored pigments *cytochromes*. Eventually, Keilin also determined that light absorption pattern of the four bands changed distinctly when metabolic poisons were administered, or when yeasts were deprived of oxygen. He concluded that the intensity of light absorption bands resulted from the cytochromes reduction. As a result, it became paramount to understand how cytochromes supported respiration.

Besides heme-containing cytochromes, it is known today that many distinct redox centers are involved in the electron transport along the ETS such as the iron/copper-sulphur clusters, the flavin-containing enzymes, and ubiquinone. These compounds differ not only in composition, but also in the number of electrons transported and their redox potentials. An interesting feature of the ETS is the presence of two mobile electron transfers: the nonproteic organic molecule ubiquinone (UQ) and cytochrome *c*. Although UQ is quite hydrophobic, it is highly mobile and promotes the bridging between complexes I, II, Glycerol-3 phosphate dehydrogenase, and electron transfer flavoprotein-ubiquinone oxidoreductase with complex III [2]. Unlike the cytochromes and iron/copper-sulphur clusters, UQ can be reduced by two electrons, generating the fully reduced form ubiquinol ( $\text{UQH}_2$ ). However, during its redox cycle, UQ can

be partially reduced, generating an unstable ubisemiquinone ( $UQ^{\bullet-}$ ) radical. Cytochrome *c* is a small heme protein which is loosely bound to the inner mitochondrial membrane and is responsible for the transport of a single electron. Cytochrome *c* also participates as a major inducer of apoptosis, when released by the mitochondria in response to proapoptotic stimuli, such as calcium and oxidative stress conditions [17]. Cytochrome *c* is bound to inner mitochondrial membrane by means of a direct interaction with cardiolipin which can be disrupted when cardiolipin is oxidatively modified in redox imbalance [18].

The ETS complex I, or NADH:ubiquinone oxidoreductase, is considered one of the largest known membrane proteins and can be visualized by electron microscopy, which reveals its characteristic “L” shape [19]. The structure of this huge protein complex was recently elucidated [20]. Complex I activity couples the transfer of two electrons from NADH to ubiquinone, in parallel with the translocation of four protons across the inner mitochondrial membrane. This activity provides about 40% of the proton-motive force generation coupled to mitochondrial ATP synthesis. In mammals, complex I contains 45 subunits resulting in an apparent molecular mass of about 1 MDa and it has been implicated in many human neurodegenerative diseases.

Complex II, also known as succinate dehydrogenase, converts succinate to fumarate, which is the only TCA cycle reaction taking place at the inner mitochondrial membrane. The electrons from succinate oxidation directly contribute to UQ reduction and oxidative phosphorylation as well. The elucidation of complex II structure revealed that the architecture of its redox centers is arranged in a way that prevents ROS production at the FAD site [21, 22]. Complex II contains four subunits, two of which are integral membrane, while the other two face the mitochondrial matrix, which contains covalently bound FAD and three iron-sulphur clusters.

Glycerol 3-phosphate dehydrogenase (G3PDH) has two isoforms, the cytosolic (*c*G3PDH) and the mitochondrial (*m*G3PDH). The *m*G3PDH is bound to the inner membrane facing the mitochondrial intermembrane space [23] and transfer electrons generated from dehydrogenation of G3P to UQ. The activity of this enzyme is closely associated to the oxidation of cytosolic NADH from the glycolytic pathway, regenerating the “pool” of  $NAD^+$  from glycolysis.

The other component of ETS contributing to electrons entry is the electron transfer flavoprotein-ubiquinone oxidoreductase (ETF-QO), which is an intrinsic membrane protein located in the inner mitochondrial membrane. ETF-QO contains an FAD molecule and a [4Fe4S] cluster. The protein represents the only input site of the ETS for electrons derived from nine flavoprotein acyl-CoA dehydrogenases and two N-methyl dehydrogenases, which are eventually transferred to UQ [24]. Incidentally, of the four distinct ETS electrons entry sites, only complex I contributes to the  $\Delta p$ , as it couples the energy of electrons transport to the proton translocation across inner mitochondrial membrane.

Complex III, also called ubiquinol:cytochrome *c* oxidoreductase, couples the transfer of two electrons from  $UQH_2$  to cytochrome *c* generating a proton gradient across the inner

mitochondrial membrane. The redox centers involved in complex III activity are the cytochrome *b*, which has two heme type b (BL and BH), cytochrome *c*<sub>1</sub>, and the *Rieske* iron sulphur protein [2Fe-2S] [25]. Complex III is a dimer. Each monomer consists of 11 different polypeptide subunits yielding a total of 240 kDa [25]. The presence of two active sites in complex III is an essential feature for the operation of Q cycle [26]. One of the sites is responsible for  $UQH_2$  oxidation and proton translocation to the intermembrane space and is located near the cytoplasmic side of the inner membrane (Qp). The other site is responsible for reducing the UQ, capturing electrons from the inner side of the membrane and is located near the matrix side (Qn). Since  $UQH_2$  donates two electrons and the cytochromes are reduced by only one, electron transfer from the  $UQH_2$  to complex III is bifurcated. The first step comprises the oxidation of  $UQH_2$  at the Qp site of complex III. One of its electrons is transferred to the *Rieske* iron sulphur protein while the other is transported to heme *b*<sub>L</sub>. There are two fundamental reasons for the passage of the two electrons from  $UQH_2$  to cytochrome *c*<sub>1</sub> and cytochrome *b*. The first, and more obvious, is the fact that UQ is reduced by two electrons and two protons, while cytochrome *c* by a single electron. The other is structural. Because the *Rieske* center is mobile in complex III and directs the electrons to reduce cytochrome *c*, eventually it returns back to the Qp site [27]. Thus, at the same time, the *Rieske* center is close to cytochrome *c*, and distant from the site receiving the Qp of another  $UQH_2$  electron [25]. During the Q cycle, for each pair of electrons, two protons are consumed from the matrix and four protons are released into the intermembrane space, promoting the net transport of two protons.

The electron present at the cytochrome *c* is then transferred to the terminal ETS complex IV, also known as cytochrome *c* oxidase. The mammalian complex IV is composed of 13 subunits and contains several redox centers such as two hemes, one cytochrome *a* and cytochrome *a*<sub>3</sub>, and two copper centers, the  $Cu_A$  and  $Cu_B$  centers. In fact, the site of oxygen reduction to water is composed of a binuclear center which contains cytochrome *a*<sub>3</sub> and  $Cu_B$ . Complex IV catalyzes the transfer of four electrons from four reduced cytochrome *c* to oxygen, completely reducing it to two water molecules [28]. Oxygen reduction involves a complex redox cycle in which  $Cu_A$  and  $Cu_B$  centers, as well as the heme *a*, heme *a*<sub>3</sub> and a tyrosine residue participate. Firstly, the oxygen molecule binds to the enzyme complex at the heme *a*<sub>3</sub>- $Cu_B$  binuclear center on its fully reduced state in the following redox configuration: heme  $a_3^{+2}$ - $Cu_B^{+1}$ . In fact, molecular oxygen binds to the binuclear center at the heme  $a_3^{+2}$  site and then the bonds between oxygen atoms are disrupted in such a way that one of the oxygen atoms remains bound to heme *a*<sub>3</sub> site and the other one, to the  $Cu_B$  center. During this step, two electrons are transferred from the heme  $a_3^{+2}$  to the oxygen atom bound, adopting the heme  $a_3^{+4}$  oxidation state (ferryl). The other oxygen atom is reduced by means of transfer of two electrons, one originating from  $Cu_B^{+1}$  center, which becomes  $Cu_B^{+2}$ , and the other one from the tyrosine 244 residue, which is cross-linked to histidine 240 where the  $Cu_B$  is bound. In the next step, the tyrosine is regenerated

with electrons from one reduced cytochrome *c* molecule which is transferred via heme *a*. An additional electron is transferred from one reduced cytochrome *c* molecule, through heme *a*, to the heme  $a_3^{+4}$ , which converts to its  $+3$  redox state. Complete enzyme regeneration is achieved by further delivery of two electrons, from two reduced cytochrome *c* molecules, through heme *a*, to the active site, restoring the heme  $a_3^{+2}$  and  $\text{Cu}_B^{+1}$  redox configuration and allowing each of the two oxygen atoms originally in oxygen to dissociate as water. Interestingly, the very same site where oxygen binds to the cytochrome *c* oxidase, the heme  $a_3^{+2}$  at the binuclear center, is also able to bind other ligands such as cyanide (CN<sup>-</sup>) carbon monoxide (CO) and nitric oxide (NO), all of which are respiration inhibitors [2].

During the sequential redox reactions of electrons transport along the ETS to their final acceptor, molecular oxygen, a significant part of the energy is conserved as protons are transported from the mitochondrial matrix to the intermembrane space, generating the  $\Delta p$ , specifically at the complexes I, III, and IV. As proton transport is thermodynamically unfavorable, coupling the energy released by the electrons transport at complexes I, III, and IV overcomes the energy barrier.

**2.4. The Chemiosmotic-Dependent ATP Synthesis.** The ETS-dependent proton transport across the inner mitochondrial membrane allows the  $\Delta p$  formation and represents the ultimate energy source by which mitochondrial ATP is synthesized [10]. One of the first proposals was based on the idea that electrons passage through the ETS would release a finite amount of energy that would be trapped in the form of a phosphorylated intermediate "X" which would then transfer its "high energy phosphate" to ADP by specific enzymes [29]. Initial efforts to identify intermediate "X," which for a while was thought to be phosphohistidine, failed.

Then, a revolutionary idea was proposed by Mitchell in 1961 [30]. Mitchell's hypothesis was based on the concept known as *vectorial metabolism*, which stated that substrates could be transported across a membrane by an enzyme with a particular orientation against a chemical and electrical gradient. This could only be achieved if it was coupled to another thermodynamically favorable reaction such as ATP hydrolysis. Mitchell's chemiosmotic hypothesis explained mitochondrial ATP synthesis, which in several ways resembles the fuel cell-type, and set the basis for a concept hinging on the coupling of electron transfer to ADP phosphorylation. The supramolecular organization of the enzymes at the inner mitochondrial membrane is an essential component to be considered in this proposal and Mitchell referred to it as anisotropy. In essence, the anisotropic enzymes would act as molecular charge splitters across the inner mitochondrial membrane, in which the redox reactions at the ETS complexes resulted in the separation of protons and hydroxyl anions to each side of membrane, creating the so-called protonmotive force ( $\Delta p$ ). As respiration proceeded, the resulting increase in the  $\Delta p$  would drive the separation of protons and hydroxyl anions at the active center of the ATPase, allowing ADP and inorganic phosphate dehydration and ATP formation.

In addition, this ingenious proposal offered explanations for many other observations such as (i) the effect of uncouplers, (ii) the regulation of redox state of the ETS components by the magnitude of the electrochemical potential, (iii) the photochemical phosphorylation in chloroplasts, and (iv) the swelling and shrinkage effects on mitochondria associated to the changes in the electrochemical potential. This hypothesis was extensively validated experimentally, being eventually consolidated in 1967 after Mitchell's answers to the criticisms raised by Slater [10]. Although the respiration coupled to ATP synthesis was mechanistically demonstrated, it did not explain how the ATP molecules were produced by the ATPase using the energy accumulated in the form of  $\Delta p$ .

The fundamental basis of the mechanism by which the  $F_1F_0$  ATP synthase complex produces ATP at the expenses of the  $\Delta p$  was made possible by the research conducted in the Laboratories of Efraim Racker, John E. Walker, Paul D. Boyer and many others. In fact, a mitochondrial ATPase activity was directly involved in the mechanism by which ADP phosphorylation is coupled to electron transport [31]. This complex was first observed by electron microscopy in the 1960s [32]. Two distinct subcomplexes were seen: one associated to the inner mitochondrial membrane ( $F_0$ ) and the other facing towards the matrix ( $F_1$ ). Purification of the whole  $F_1F_0$  ATP synthase was achieved as well as and the characterization of both  $F_1$  and  $F_0$  activities [33]. In 1973, Paul Boyer observed that the exchange of labeled oxygen between inorganic phosphate and water was not blocked by uncouplers of oxidative phosphorylation, but was inhibited by oligomycin (a compound that blocks  $F_1F_0$  ATP synthase activity). Interpretation of these results led to the suggestion that ATP might be formed at the catalytic site of this complex. Thus, a significant part of the energy transduced by oxidative phosphorylation is utilized to drive the release of preformed ATP from the enzyme [11]. Later, in 1977, Boyer advanced this concept by proposing an alternating site model for oxidative phosphorylation, in which ATP is formed at one site of the enzyme site, but is transiently tightly bound. This ATP was not released from enzyme unless ADP and Pi bound a second enzyme site and the ATPase complex became energized [34]. Therefore, net ATP formation by oxidative phosphorylation occurs by a cooperative mechanism involving alternate conformational changes in the  $\beta$  subunits of  $F_1$ , promoted by the passage of protons through the  $F_0$  site of this complex [35].

The passage of protons from the intermembrane space to the mitochondrial matrix mediated by the  $F_0$  site would drive a rotational movement of the whole  $F_0$  which, in turn, would transfer the rotation movement to the  $\gamma$  subunit and then to the  $\beta$  subunits at the  $F_1$  site. As the interactions between the  $\gamma$  subunit and each one of the three  $\beta$  subunits are unique,  $\gamma$  subunit rotation induces a specific conformation in each of the  $\beta$  subunits (open, loose, and tight). When the  $\beta$  subunit adopts an open conformation, ATP is released from the enzyme and the active site becomes empty, while the neighboring  $\beta$  subunit adopts a *loose* conformation, binding ADP, and inorganic phosphate. Finally, the third  $\beta$  subunit is in the closed conformation, expelling water from its active site, allowing the thermodynamically spontaneous ATP synthesis.

**2.5. Mitochondrial Redox Metabolism.** Since a long time, it was known that oxygen played essential biological functions ranging from biomolecule modification to cellular respiration. However, life arose long before oxygen could accumulate in the atmosphere in order to be utilized by cytochrome *c* oxidase. In fact, evidence indicates that organisms in the primitive Earth had simpler metabolic pathways that were not able to fully utilize the energy contained in nutrients. Also, the process of respiration seems to have emerged before the occurrence of significant amounts of oxygen in the atmosphere, as a result of photosynthetic activity. Evidence supporting this interpretation was derived from microorganisms that utilize electron acceptors other than oxygen. Examples are iron, sulfate, vanadium, and even uranium. Along Earth's evolutionary history, organisms that were able to use the sunlight as an energy source to allow water oxidation coupled to molecular oxygen production had a clear advantage. From the energy perspective, oxygen utilization allows a more efficient use of the energy stored in the nutrients through the process of oxidative phosphorylation. Thus, organisms lacking oxygen transport and storage systems, relying simply on its diffusion to the inner parts of its body, exhibited a growth rate that strongly limited by oxygen availability. This idea seems to offer an excellent explanation for the large number of giant fossil records aged approximately 300 million years that lived when atmospheric oxygen levels reached about 35%.

Oxygen is not an inert gas and its toxicity was firstly reported by Paul Bert way back in 1878. He showed that oxygen was toxic to a number of invertebrates as well as fungi, germinating seeds, birds, and even other higher animals. In the central nervous system of mammals, oxygen toxicity was referred to as the "Paul Bert effect." The mechanisms underlying cellular oxygen toxicity were further studied by Rebecca Gerschman in 1954 who proposed that oxygen potentiated cell death induced by X-ray irradiation [36]. The conclusion was that oxygen and ionizing radiation share mechanisms that possibly involved the formation of "oxidizing free radicals." A free radical is defined as any atom or molecule that has at least one unpaired electron in an orbital [37]. The term ROS is used to designate not only oxygen-derived free radicals, but also nonradical oxygen species that are capable to generate highly reactive oxygen radicals, such as hydroxyl radical [37]. Because free radicals have unpaired electrons, they tend to achieve stability by donating or removing electrons from adjacent biomolecules such as sugars, lipids, and proteins, resulting in their structural modification. The accumulation of altered or damaged biomolecules by free radicals is associated to a multitude of functional changes in cells, such as apoptosis, mutations, inhibition of enzyme activities, and oxidative stress [37].

Much of the biomedical interest regarding the ROS are due their potential role in the pathogenesis of many diseases and also in aging. In this regard, the seminal work of Harman in 1956, established the well-known "Free-radical theory of aging," which stated that aging is a result of chronic oxidative modification of biomolecules and structures within the cells that ultimately culminate

in death [38]. According to Harman, the cellular free radicals would probably arise by reactions involving molecular oxygen as a result of dehydrogenase activity. Later in 1969, McCord and Fridovich made a central contribution by establishing a link between biology and free-radical chemistry. A copper-containing enzyme, previously identified by Keilin in 1939 as hemocuprein, was found to have a key activity of dismutating superoxide radicals into oxygen and hydrogen peroxide in bovine erythrocytes [39].

Most of the oxygen consumed by the cell is completely reduced to H<sub>2</sub>O by cytochrome *c* oxidase. However, a small portion of this is partially reduced by mitochondria, generating ROS. Complexes I, II, and III of ETS are sources of ROS. In fact, the ETS is not only capable of generating free radicals such as the superoxide radical (O<sub>2</sub><sup>•-</sup>), but also ROS such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). The first demonstration of mitochondrial ROS formation was made in 1966 by Jensen, who showed that succinate and NADH were able to support hydrogen peroxide formation and this was strongly potentiated by the metabolic poison antimycin A [40]. In the cell, ROS production usually occurs during electron transport along ETS, however, some pathophysiological conditions can increase mitochondrial ROS production because they reduce the activity of ETS or decrease ADP content in mitochondria leading to an increase in the magnitude of the membrane potential, a condition associated with increased ROS "leakage" [41]. Further developments have shown that not only the oxygen pressure, but also the magnitude of the membrane potential strongly affected mitochondrial ROS generation [42]. However, mitochondria are not the only cellular source of ROS. NADPH oxidases, peroxisomes, and endoplasmic reticulum also represent important sites of ROS production.

The imbalance between ROS generation and removal might lead to the so-called oxidative stress. The antioxidant defenses found in biological systems to avoid oxidative stress may be divided into preventive (inhibition of ROS generation), scavengers (suppression of unpaired electrons), and repair (repair of molecules damaged by ROS). The UCPs and hexokinase bound to mitochondria act as preventive antioxidant systems, because both mechanisms aim to reduce membrane potential, promoting mitochondrial depolarization. Cancer cells as well are characterized among other features, by a high expression of hexokinase bound to mitochondria through the VDAC protein. Originally, it was thought that the VDAC bound hexokinase had an exclusive role in the maintenance of the high glycolytic flux typical of cancer cells, but similarly to the mouse brain cells, hexokinase of tumor cells may also maintain the redox balance through an ADP recycling mechanism [43, 44]. The remaining two groups of scavengers are enzymatic (enzymes like superoxide dismutase, catalase and glutathione peroxidase) and nonenzymatic ( $\alpha$ -tocopherol, thioredoxin). Examples of the repair antioxidant defenses are the poly (ADP ribose) polymerase (PARP) and aldehyde dehydrogenase.

### 3. ROS and Cancer

In the light of Otto Warburg's original observations concerning aerobic glycolysis, it became pertinent to ask whether the mitochondria of tumor cells were functional or not. This question is by no means easily answered and even after 85 years into the post-Warburg era, the issue remains conflicting, or at best riddled with misconceptions. One example of this situation is the oft repeated puzzlement about the cancer cell's selection of the less energy efficient anaerobic glycolysis over the more ATP-rich tricarboxylic acid cycle in conjunction with oxidative phosphorylation. Actually, what cancer cells frequently do is to combine the best of the two pathways in order to sustain the intense proliferation as well as the metastasis that accompanies the transformed state. The glycolytic pathway does not just generate ATP and lactate as the end product. Many of its intermediate metabolites are recognizably anaplerotic in nature as is the case of 3-phosphoglycerate which acts as a precursor to serine, glycine, and cysteine synthesis, all of which are essential for the anabolic condition seen in tumorigenesis. Indeed, it has been recently shown that in human breast cancer, one of the genes that consistently displays a gain in copy number is phosphoglycerate dehydrogenase a result that highlights the association between glucose uptake and aminoacid synthesis [45]. Besides, it must be remembered that glycolysis is a much faster process in terms of ATP synthesis, so that when not hindered by limited amounts of glucose, tumor cells are not badly off. When dealing with the term functionality with regards to mitochondria it must be taken into account that the organelles are a hub to many essential cellular processes. Whilst mitochondria of tumor cells may perform some functions as well as those from normal cells others may be deficient and thus there is a need to specify which of these bear on cancer causally. This question will be addressed below with emphasis on those pathways that are at the same time anomalous and, therefore, amenable to interference by specific inhibitors, especially those connected to the bioenergetics scenario associated to cell transformation.

Functionality of the mitochondria has been addressed in several ways. Many papers dealing with the properties of the organelle, particularly when comparing tumor and normal cells, approach the question in a rather loose and sectorized manner. For example, it is common to refer to the physiological status of the mitochondria in terms of its morphology, its respiratory function including ATP synthesis, its role as regulators of intracellular  $\text{Ca}^{2+}$  homeostasis, or as essential elements in the processes of cell proliferation and apoptosis. Frequently in the literature, the mitochondria are considered functional or not on the basis of analysis that may not be entirely informative of the status of the organelle. For example, in some cases, conclusions about the integrity of mitochondria, or the cell as a whole, are based solely on the ability of certain enzymes to reduce tetrazolium salts and on the assumption that if the enzymes are active, then the organelle and the cells are also viable. Clearly, then, it becomes important to consider what weight should one attribute to these individual parameters and

whether they could represent bona fide markers regarding the general health of the mitochondria and sufficient to classify them as dysfunctional. Perhaps a better description for the supposedly aberrant behavior of the organelles within the context of malignant transformation would be "deviant." The questions that naturally follow this initial discussion are deviant mitochondria are the cause or consequence of cancer? If mitochondria are in fact key elements in cell transformation, which alterations predispose cells to cancer?

*3.1. Morphology.* It is not simple to define what is the normal morphology of mitochondria, mainly because they exhibit considerable plasticity and change their shape radically even when functioning within normal cells. Mitochondria may display transient shape changes as a result of energy demand, that is, oxidative phosphorylation (OXPHOS). A condensed appearance has been associated to mitochondria actively undergoing OXPHOS, whereas the orthodox conformation reflected diminished oxygen consumption. Since in many types of cancer cells the mitochondria have been shown to respire normally, even though with less intensity than glycolysis, it may be difficult to observe morphologies that diverge significantly from the condensed appearance. In other words, the cancer-related morphology of mitochondria may be merely a reflection of the occurrence, or not of the Warburg effect (see below).

Morphology of the organelle refers also to the dynamic processes of fission and fusion with one another and with other organelles that mitochondria undergo in parallel with the cell cycle. Mitochondrial fusion and elongation produces a branched tubular network spreading throughout the cytosol that characterizes what is generally known as mitochondrial networking. Although the mechanism of mitochondrial fission, fusion and elongation, is not yet fully understood, some of the key players in this process have been identified and it was the analysis of these components that suggested to a certain degree the distinction between normal and tumor cells. In normal cells, mitochondrial fission occurs in synchrony with cell division. As the cells enter mitosis, mitochondria too begin to fragment, an event which is largely regulated by a GTPase dynamin-related protein 1 (Drp 1), a major component of the fission apparatus. The fission machinery also requires the presence of hFis 1 which is integrated in the outer membrane of the mitochondria. It is thought that interaction between hFis 1 and Drp 1 alters the conformation of the latter leading to the formation of a constricting ring around the mitochondria which ultimately produces fragmentation [46, 47]. In turn, Drp 1 activity itself depends on posttranslational modifications, namely, phosphorylation catalyzed by Cdk 1/cyclin. In addition, the half-life of Drp 1 can be modified. It is enhanced by sumoylation by SUMO1 which protects Drp 1 from degradation via proteasome and decreased by deSUMOylation mediated by the protease SenP5. Incidentally, these reactions illustrate quite well how mitochondrial fission responds to elements that normally control the cell cycle and thus becomes synchronous with cytokinesis. Exception should be made to tissues in which cells do not proliferate, such as in muscle. Although the dynamin-related GTPases are the core

components of the mitochondrial fission mechanism, recent data have implicated other proteins as upstream regulators of the Drp 1/hFis 1 complex and hence of mitochondrial networking [48, 49].

Upon completion of cytokinesis, mitochondria reconnect again through fusion, a complex event that involves the merging of the double lipid membrane of the mitochondria. This is mediated by profusion proteins located on the surfaces of the inner and outer membranes, the complex formed between Mgm1p and the optic atrophy protein OPA1 together with mitofusins (Mfn1 and Mfn2), respectively. The mitofusins also play a role in tethering the mitochondria to other organelles such as the endoplasmic reticulum [50]. This type of interaction does affect  $\text{Ca}^{2+}$  signaling and exemplifies how mitochondrial networking could have far reaching effects on metabolism as well. By the same token, loss of Mfn2 is known to affect the expression of the subunits that make up the respiratory complexes leading to reduced cellular oxygen consumption. Independently of direct actions of mitofusins on mitochondrial tethering, Mfn2 also regulates the ERK/MAPK signaling pathway a feature that can have a direct bearing on tumorigenesis as it will be discussed ahead [51].

So what is the consensus regarding mitochondria morphology when comparing normal and tumor cells? Within the context of mitochondrial networking, it has been proposed that mitochondria of normal cells spend most of their time in a fragmented mode (postfission state) before they fuse again [52, 53]. Along those lines it has been reported that tumor cells also display a higher frequency of fragmented mitochondria [54], which would indicate the prevalence of fission events in those organelles. A situation that could be considered analogous would be apoptosis. Reports have shown that when cells are subjected to overexpression of proapoptotic members of the Bcl-2 family, they exhibit a higher rate of Drp-1-dependent mitochondrial fragmentation and that over expression of fusogenic proteins such as Opa-1 (promoting mitochondrial fusion) protects from apoptosis [55, 56]. Our own results confirm this interpretation indirectly. The mitochondria of H460 cells treated with sodium butyrate, which inhibited cell proliferation, were shown to be more elongated than controls. This was accompanied by a higher expression of Mfn1 suggesting that mitochondrial fusion could be associated to lower rates of cell proliferation [57]. Taken together, the data indicate that except for those cases in which oxidative stress directly induces mitochondrial fission [58], the balance between mitochondrial fusion and fission might depend primarily on the proliferative (or apoptotic) status of the cells. Along those lines, it would be interesting to compare the variations of mitochondrial morphology in different types of synchronized cultures of tumor cells versus that of the normal cell counterparts. *In vivo*, however, there would be experimental complications since it is known that cell doubling time and mitotic indexes are highly heterogeneous even within tumors and also when different types of cancer are compared. In conclusion, so far the morphology of mitochondria cannot be unequivocally associated to cell transformation. The structural mitochondrial alterations

observed in many types of tumors cannot be ascribed to any specific neoplasm and this question remains largely unresolved.

*3.2. Are Mitochondria of Tumor Cells Dysfunctional?* Papers that address the intermediary metabolism of tumor cells typically begin the discussion by quoting the Warburg effect and usually mention that glycolysis replaces mitochondrial oxidative phosphorylation as the principal source of cellular ATP. Frequently, these opening remarks are followed by statements that indicate that the high glycolytic flux adaptation occurs because the mitochondria of tumor cells are dysfunctional or partially disabled. However, the aerobic glycolysis described by Warburg did not imply that the mitochondria were dysfunctional since he himself acknowledged that tumor cells continued to consume oxygen at levels comparable to those of normal cells. In other words, what Warburg really noted was that tumor cells did not exhibit the Pasteur effect, that is, glycolysis in tumor cells persisted even in the presence of oxygen.

The longstanding notion that mitochondria are somehow defective presumably derives from the fact that researchers approach the problem in a nonholistic fashion and frequently assume that if one set of results obtained from tumor cells significantly differs from the normal cell counterparts, other downstream events, even if not investigated, will vary as well. Some metabolic modifications have indeed been detected that when considered individually justified the belief that the mitochondria of cancer cells were somehow impaired. These include the preference for particular respiratory substrates, rates of electron transport, and the activities of enzymes involved in oxidative phosphorylation [59]. Albeit those early reports, data have accumulated to the effect that recently, there has been a shift in opinion. There is now a tendency to accept that mitochondria of cancer cells rarely present defects and seem to retain their capacity to carry out oxidative phosphorylation and consume oxygen with levels comparable to those of normal cells, much as Warburg himself had stated [60]. According to this view, the enhanced lactate production observed in tumor cells does not necessarily imply the cessation of mitochondrial activity, as confirmed by several experiments in which oxygen consumption of cancer cells was evaluated. Such results also indicate some degree of independence between cytoplasmic glycolysis and mitochondrial metabolism acquired by the tumor cells which may explain the absence of the Pasteur effect in cancer cells through the loss of a regulatory interface. In this respect, it was shown that the cytotoxic effect of 2-deoxyglucose (2-DG) on A549 lung cancer cells depended on inactivation of mitochondria in  $p53^{-/-}$  cells. The observation that the Warburg effect was only evidenced in cells in which mitochondria were impaired supported the idea of a functional link between glycolysis and the oxidative reactions of the organelle [61]. Furthermore, the often quoted idea that in cancer cells the mitochondria stop respiring in order to save carbon skeletons for the biosynthesis of other biomolecules required for rapid growth is not compatible with the observation that tumors actually excrete high amounts of lactate [62]. That is not to say that

the macromolecules and lipids found in the intramitochondrial milieu do not display alterations that while observable may not significantly compromise mitochondrial respiratory function. The enhanced production of reactive species of oxygen (ROS) and reactive nitrogen species (RNS) associated to cancer cells (discussed in Section 3.3) can definitely cause damage to proteins, DNA, RNA, and membranes. However, the mutations produced in mtDNA are not necessarily synonymous with tumorigenesis. It is known that germline mutations of mitochondrial DNA can cause diseases that affect children and adults ranging from mitochondrial myopathies to retinitis pigmentosa and possibly even to autism, but not cancer. In an analogous manner, sporadic mutations that may result from oxidative damage due to elevated ROS in the mitochondria are suggested by many to be the culprits of tumorigenesis. This has been difficult to demonstrate, however, mainly because there is no strong evidence showing that the mtDNA mutations are driver mutations. Also, the majority of somatic mutations found in mitochondrial DNA are not harmful to the cells and populational surveys showed that the so called specific cancer mutations (varying from 30–100%) are frequently found in mitochondrial DNA of individuals with no history of cancer. Hence, it is thought that ROS-induced mtDNA mutations may actually occur as a consequence of metabolic reconfiguration of cancer cells. Nevertheless, papers abound that correlate the mitochondrial mutations to several types of cancer [63].

How to resolve this quandary? There is a very tight connection between the nuclear and mitochondrial genomes coordinating the expression of exons encoding different subunits of proteins making up the electron transport chain. Thus, it would be desirable to separate the individual contributions of nuclear and mitochondrial DNA mutations to the cancer phenotype. After the advent of the technique of cytoplasmic hybrids (cybrids), or transmitochondrial cybrids, it became possible to repopulate cells from which mitochondria had been depleted, with exogenous mitochondria present in enucleated cells. In this manner, the information obtained from the fused cells, for example, cancer cells, should highlight which events could be assigned to the mitochondria alone [64]. Results obtained with this experimental approach have demonstrated that many of the respiratory alterations ascribed to mitochondria of cancer cells (and other pathologies) could be reproduced in the “transplanted” cells. For example, Imanishi and collaborators [65] were able to show that mitochondrial respiration defects observed in human breast cancer cells caused by mtDNA mutations were responsible for the expression of high metastatic potential in recipient cells. Interestingly, these experiments with cybrids demonstrated that the mutated mtDNA affected metastasis, but not cell transformation. Results that confirmed that the increase in metastatic potential was acquired were also obtained in a mouse model in which the transferred mtDNA had a mutation in the NADH dehydrogenase subunit 6, a deficiency that augmented the production of ROS. In these experiments, the metastatic potential of the cybrids was enhanced and use of a scavenger such as N-Acetyl cysteine was able to counteract it [66]. Correlations exist showing

that ROS and RNS are able to inhibit the process of anoikis, that is, the occurrence of apoptosis as a result of loss of cell adhesion. In normal cells, anoikis prevents detached cells from colonizing different tissues. According to this, the oxidative stress generated by ROS and RNS overproduction in tumor cells would favor metastasis by means of activation of prosurvival signaling pathways that in turn would inhibit anoikis and in this manner boost the progression of cancer [67]. It would be very interesting if it could be demonstrated that alterations in the mtDNA alone and by extension the higher production of ROS could be responsible for metastasis. Other parameters such as diminished cellular oxygen consumption and ATP synthesis observed in human breast cancer cells could also be successfully passed onto the cybrids and thus were firmly interpreted as derived from mitochondria [68]. Diminished respiratory rates, except when connected to damage or reversal of the electron transport chain, however, would generate less ROS a result which would go against the grain according to the canonic view of ROS-induced tumorigenesis.

Although with the aid of the cybrid technology, the role of mitochondria in tumorigenesis could be better appreciated, caution should still be exercised to avoid misinterpreting, or overstating certain selected parameters. Some of those have limited information. For instance, when studying mitochondrial respiratory control, values for the P/O ratio, a very popular analysis, do not really define whether mitochondria are functional or not since on its own they reflect proton leak as a result in fluctuations of states 3 and 4 of respiration. Others, as in the case of experiments using isolated mitochondria, may result from manifestations that occur independently of the regulatory grid that normally control the organelles. Thus, experiments generating data based on morphology (see Section 3.1) as well as evaluation of reactions to specific stressors have to be interpreted with reserve because they abrogate the innate responses connected to HIF-1 $\alpha$  and hypoxia. In addition, when using intact cells to investigate mitochondrial function, researchers have to make use of detergents such as digitonin in order to allow access of cell-impermeant substrates to the organelle. This experimental resource may produce artifacts due to damage to the mitochondria outer membrane resulting in the release of components such as cytochrome *c* that in turn may trigger cellular responses independently of the bioenergetics analysis being carried out. In a recent review, those questions have been clearly and carefully dissected pointing out the pros and cons of each approach [69].

The importance of mitochondria as fully functional organelles in cancer cells has been strengthened by considering the recently proposed hypothesis that metabolically they actually conform to the orthodox model of ATP production via the regular set of mitochondrial oxidative reactions, like the TCA cycle, oxidative phosphorylation, and the anaplerotic glutamine utilization pathway. According to this empirically based hypothesis, cancer cells are not considered as rogue cells that become immortalized and manage to live independently of other tissues. Reports have described a lactate shuttle that is formed between stromal cells and the cancer cells, in which the former predominantly

glycolytic, feed the latter, the oxidative tumor cells that utilize mitochondria to their full capacity. This hypothesis maintains that aerobic glycolysis, the hallmark of many tumors, is actually carried out by the cancer associated fibroblasts rather than the cancer cells themselves [70, 71]. With the metabolic symbiosis thus established, the stromal fibroblasts would undergo autophagy and mitophagy and as a result secrete and supply lactate to the cancer cells. In turn the fibroblasts would profit from the available mitochondria in the latter. This phenomenon was termed the “reverse Warburg effect” and the autophagy/mitophagy occurring in the stromal fibroblasts would be induced by oxidative stress triggered by the cancer cells. The proponents of this model went as far as mentioning that, in some cases, the Warburg effect might in fact represent an *in vitro* artifact. Although a similar metabolic symbiosis seems to occur in normal cells, it remains to be demonstrated whether the reverse Warburg effect could be extrapolated to other types of cancer [72]. At any rate lactate fueled respiration, a feature of metabolic symbiosis, has been demonstrated in tumor cells in mice, thus strengthening the idea of the interdependence that exists between normal and tumor cells [73].

In conclusion, it can be safely stated that the mitochondria of tumor cells are functional and that they may have a significant role in the maintenance of proliferation and metastasis.

**3.3. ROS and RNS Production in Tumor Cells.** This section tries to appraise the role of ROS and RNS in cancer formation. At the same time, it introduces the question whether the oxidative stress signature of cancer cells can indeed be a prime target for therapy. ROS and RNS can be regarded as decidedly toxic to the cells by considering many of their direct or indirect effects on biomolecule targets. However, toxicity, or for that matter the adjuvant role in carcinogenesis that ROS and RNS have appeared to depend ultimately on their final concentrations at a given instant and on the duration of the stimulus. In consequence, ROS and RNS levels depend on the regulation of the pathways that generate them as well as those that degrade them (scavengers). When ROS and RNS concentrations are within a certain range of the concentration gradient, “physiological” steady-state levels of cellular ROS and RNS perform the housekeeping coordination of metabolic and genetic processes. As such  $H_2O_2$ , for example, stimulates cell proliferation by acting as modulators of various transcription factors that in turn influence several important cellular processes. Among the transcription factors, NF- $\kappa$ B, Nrf2, p53, HIF-1 $\alpha$ , and STAT3 could be mentioned. The mechanism whereby ROS modulate transcription factors involves the reversible oxidation of cysteine residues of proteins belonging to signaling pathways such as protein tyrosine kinases and phosphatases, lipid phosphatases, proteases, and signaling effectors. Exposure of proteins to the oxidative environment generates dityrosine residues that can be considered as oxidation markers. Apart from its effect on proliferation,  $H_2O_2$  also mediates cell differentiation and migration. As opposed to the physiological role of ROS in normal cells, the oxidative stress is characterized by situations in which the levels of molecular

oxygen or its ROS derivatives increase above the threshold of normality and produce widespread irreversible oxidation of aminoacids, polydesaturation of fatty acids, and mutations on DNA and RNA. Mutations in nucleic acids occur by formation of C5-OH and C6-OH adducts of thymine and cytosine or similarly with purines, or by reaction with the sugar moiety of the polymer leading in some cases to single or double-strand breaks, intrastrand cross-links, and protein-DNA cross-links [74–76]. This destabilizing effect of ROS on DNA can promote genomic instability which as a consequence may predispose the cells to malignant transformation. In this context, cancer cells that are able to survive in a supposedly hostile microenvironment of high ROS and RNS could be regarded as a subpopulation that were selected in terms of their peculiar metabolic adaptations. This view, however, is not without controversy. Before discussing the possible mechanisms of adaptation, it is relevant to inquire whether there is a consensus concerning the ROS associated etiology of cancer.

What then, if there is a pattern, is the ROS/RNS phenotype of cancer cells? Are ROS and RNS able to act as stimulants of cell transformation cells by subverting the normal control network through a sustained oxidative environment, or do they act directly producing harmful modifications on lipids, proteins and RNA/DNA? Or both? The earlier work of Szatrowski and Nathan [77] showed that relatively large amounts of hydrogen peroxide, comparable only to polymorphonuclear leukocytes and monocytes, were produced by a number of tumors. Since then, the list has grown and many reports indicate that cancer cells produce massive amounts of ROS to levels that exceed the capacity of the antioxidant enzymes that are normally in charge of ROS detoxification. Adding to this, there is evidence showing that excessive ROS production causes the progressive inactivation of the antioxidant enzymatic systems, a condition that favors the maintenance of high concentration of ROS and the induction of a chronic oxidative state. Results published by several groups indicate that it is this situation that sets the scene for transformation. According to this hypothesis, the maintenance of parameters such as cell proliferation within the boundaries of normalcy would depend primarily on the redox balance established between ROS production and the activity of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, glutathione reductase, catalase, and thioredoxin as well as the nonenzymatic antioxidants scavengers glutathione and vitamins C and D.

In contrast, increased levels of ROS, that may result from a failure of the antioxidant system rather than from overproduction, have been associated to chronic degenerative conditions such as cancer, aging, diabetes, and cardiovascular diseases [78, 79]. The site of the oxidative stress has been narrowed down to mitochondria and was covered extensively in a recent review [80] that highlights the prooxidant role of the altered organelles as protagonists of tumorigenesis. Corroborating this view, the high levels of ROS associated to the pathophysiology of a wide variety of cancers have prompted clinicians to examine the amount of dityrosine residues in proteins exposed to an oxidative environment. Proteins that are highly susceptible to oxidative attack have in fact been

termed as advanced oxidation protein products (AOPP) and currently serve as markers for monitoring cancer patients [81]. Numerous other reports have generalized the notion that cancer cells are more challenged by oxidative stress than normal cells [82]. Among the types of cancer that have been linked to increased oxidative stress are bladder, brain, breast, cervical, gastric, liver, lung, melanoma, multiple myeloma, leukemia, lymphoma, ovarian, pancreatic, and prostate [83]. In a number of reports, it has been found that genes involved in oncogenic pathways and those linked to tumor suppression are frequently mutated in transformed cells all of them sharing the common feature of increased amounts of ROS [84–88]. Finally, the hypothesis that ROS and RNS are early effectors of tumorigenesis is in agreement with data revolving around the inflammatory reactions. A hegemonic view on the etiology of cancer states that when an inflammatory condition lasts long enough it behaves as a prodrome to cancer. The normal course of an inflammatory reaction produced by a number of different agents including bacteria, carcinogens, and radiation progresses to a stage in which mast cells and leukocytes are mobilized to the sites of lesion. These cells produce what could be described as respiratory bursts that in turn contribute towards the increase in local production of ROS. The respiratory bursts are actually amplified several fold by other inflammatory cells attracted to the site of inflammation by chemokines and cytokines released by the former cells, so that the oxidative stress actually propagates to neighboring cells. In this context, it is known that the extent of tumor associated macrophage infiltrates correlates well with the prognosis of certain types of cancer. If inflammation is not reversed, it could then create a vicious circle whose outcome is chronic ROS-induced oxidative stress and ultimately, cell transformation. The occurrence of inflammatory reactions with the participation of cell infiltrates in premalignant senescent hepatocytes has also been demonstrated in inflammation-based mouse models of hepatocellular carcinoma [89].

One question could be raised concerning the increase in ROS as an oncogenic factor. Why, in view of the known roles of high ROS in inducing senescence and apoptosis, cancer cells do not undergo apoptosis? Presumably in these transformed cells the pathways connected to senescence and apoptosis are somehow blocked. One proposed mechanism for this inactivation involves p38 MAPK pathway. In normal cells, it is known that elevated ROS induces apoptosis via the p38 $\alpha$  MAPK. In contrast, human cell lines in which p38 $\alpha$  is inactivated are refractory to ROS-induced apoptosis which suggests that deficiencies in this pathway, as well as those which involve p53 (often mutated in most cancers) allow cancer cells to remain viable in the presence of high ROS [90]. The question whether cancer cells survive in a hostile ROS environment as a result of enhanced activity of antioxidant enzymes and compounds must also be considered.

Many papers attest to the fact that tumor cells are usually well adapted to high levels of ROS and that their viability is only possible due to enhancement of antioxidant activity [60]. However, it should be borne in mind that tumor cells, which occur in a tumor in different stages of transformation,

should exhibit heterogeneous metabolic profiles. This would be due to oncogenic gain-of-function and/or loss of tumor suppressors. These alterations may generate a mosaic of metabolic patterns resulting from differentially expressed enzymes which in the same tumor would reveal increased ROS levels and downregulated antioxidant systems. Such a scenario is compatible with the model of waves of gene expression proposed by Smolková and collaborators [5].

As alluded some findings cannot be generalized to all types of tumors and to all situations. Some points that require further discussion are listed below.

(a) *Source of ROS*: scientists do not agree as to the source of ROS. Many reports state flatly that mitochondria are the main producers of ROS and highlight that organelle as the site where the tumorigenic process begins [91]. Others call attention to the fact that there are alternative and perhaps more important intra- and extracellular ROS generating reactions that include the endoplasmic reticulum, peroxisomes, the cytosol, plasma membrane, and the extracellular space [92]. Among the most important nonmitochondrial sources of ROS is the NADPH oxidase family of enzymes. These are bound to the plasma membranes and to the membranes of phagosomes. Of the seven oxidases that comprise the NADPH family, Nox1, Nox2, and Nox4 are expressed by several types of cancer cells, including colon, prostate, gliomas, melanomas, pancreatic adenocarcinomas, renal carcinomas, and ovarian. Other incidental nonmitochondrial sources of ROS include those that are generated by external factors such as carcinogens and radiation that are known effectors of inflammatory reactions that usually precede cell transformation (see below).

In the context of cancer, the NADPH oxidases should be highlighted not only because of their quantitative contributions to ROS formation, but also as promoters of tumor-induced angiogenesis, an important process adjuvant to tumor growth and metastasis [93]. Supporting the role of NADPH derived superoxides as promoters of tumor induced angiogenesis, experiments have shown that antioxidants such as vitamins C and E reduced the expression of vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor 2 (VEGFR-2) in mice [94]. Likewise, *in vivo* experiments demonstrated that the scavenger NAC was able to inhibit sarcoma-induced angiogenesis, thus strengthening the ROS-induced tumorigenesis model [95].

(b) *Are ROS tumorigenic?* There is a growing consensus as to the role of ROS in tumorigenesis. In many works, ROS are clearly singled out as the main tumorigenic factors [96, 97]. Some reports, however, indicate that ROS are harmful and tumor cells are only able to survive thanks to efficient or exacerbated antioxidant defense systems [98, 99]. Put another way, mitochondria of tumor cells, which as mentioned above are believed to be fully functional and which according to many are the main producers of ROS, play the simultaneous role of providing energy to the cells, and also of introduce the tenuously controlled oxidative stress, the sword of Damocles as it were [70]. Interestingly, ROS production seems to be much higher in mitochondria in which damage to the respiratory chain occurs, or mitochondria that are actively undergoing reverse

electron transport, than those that are normally producing ATP [100]. This situation illustrates then how the processes of autophagy and mitophagy are beneficial to the cells as removers of potential sources of cell transforming ROS [53]. Adding support to the toxic effect of ROS to cancer cells, it is worth mentioning that both, radiotherapy and chemotherapy, normally employed to eradicate cancer cells do so by producing ROS-mediated oxidative stress [97]. Although it is known that ROS have multiple downstream effects, what is emerging as an explanation to conciliate all the conflicting data is that the overall outcome of the ROS-triggered activations/inhibitions depends primarily on their final concentration. In order to even out the data in the literature that show a wide variation in ROS concentrations produced by different types of tumor cells, it would be important to show whether the observed oscillations are actually due to the activities of antioxidant enzymes or the presence of higher levels of free radical scavengers in these cells. This seems to be the case of cancer stem cells that appear to have lower level of ROS than normal cells [101]. Likewise, hematopoietic systems display low levels of ROS although the progenitor cells myeloid produce high levels of ROS. If the hypothesis of antioxidants as the regulators of ROS and hence as pace makers of tumorigenesis proves to be right, the management of cancer should afford many strategies of interference based not only on the use of antioxidants, but on inhibitors acting on ROS downstream signaling pathways. Distinction should be made among the ROS, however. Nitric oxide (NO), for example, has been shown to have antiproliferative effects in both, normal and tumor cells [102, 103], and it has been demonstrated that superexpression of nitric oxide synthase (iNOS) caused inhibition of proliferation of pancreatic tumor cells. When considering the effects of ROS on transformed cells, another issue has to be taken into account which involves the plasticity of tumor cells regarding exposure to ROS. Results of experiments in which tumor cells in culture were incubated in the presence of increasing amounts of ROS showed that they responded by displaying an enhanced tolerance to the oxidative stress. Results obtained by Onul et al. [104] evidenced further that A549 lung cancer cells adapted to long-term high levels of hydrogen peroxide grew better in culture than the parental cell line and were more resistant to the chemotherapeutic agent Doxorubicin. Interestingly, those adapted cells definitely favored a more anaerobic metabolic profile suggesting that the survival strategy adopted might be independent of mitochondria. The same standing applies for high concentrations of nitric oxide (NO). In breast tumor cell lines, high concentrations of NO were shown to induce a phenotypic change [105]. Thus, it may well be that it is the metabolic adaptation of tumour-initiating cells that could dictate the development of cancer rather than HIF activation or other signaling pathways that have been known to affect solid tumor growth. Whether the cells adapted to high ROS would be resistant to antioxidant therapy remains to be investigated. In conclusion, the individual contributions of different cell compartments to the final ROS concentration within the cells may predispose them to transformation, mainly in those cases when the antioxidant systems are not

effective in counteracting the oxidative stress. Excess of ROS is also harmful to cancer cells.

*3.4. Different Tumors, Different Biochemistries.* Attempts to build a grand unifying model of metabolic reprogramming in cancer cells that would make biochemical sense are perhaps premature. As it became clear from the preceding discussion, the available data do not always fit into coherent mechanisms and so far ideas do not converge to a single stratagem to combat cancer cells. Apart from the controversies, experimental difficulties, and conflicting interpretations, there are inherent differences that have to be considered before one draws a standard biochemical profile analysis of different tumors. Firstly, normal tissues have individual metabolic rates that would certainly influence the type of metabolism occurring in the cognate transformed cells. Slow-twitch and fast-twitch muscle tissue, for example, obtain ATP preferentially from different pathways. The same applies for several other tissues in which the metabolic diversity reflects the presence of specific isoforms of enzymes, as occurs in liver and brain tissue. It is plausible then to imagine situations in which as a function of distinct metabolic rates, variable levels of ROS would be produced that could cause different types of local lesions. Secondly, individuals are themselves different when considering their biochemical buildup. The emerging field of pharmacogenomics recognizes these differences and the trend is now, taking advantage on available high throughput technology, to carry out individual genome and transcriptome analysis for patients undergoing long term chemotherapy. However, biochemical diversity seems to transcend genes. Population studies have suggested that the majority of cancers is in fact of the sporadic type and hence would reflect the life style of individuals more than their genetic background. So pharmacogenomics must be complemented by “pharmacometabolomics” which could be adapted to the individual needs according to the redox profile of the tumors under treatment. ROS-based treatment would thus select combinations of prooxidant and antioxidants agents that would best counteract the anomalous oxidative stress generated, preferably at the transformative stage. In other words, the prooxidant, and antioxidant medication would be tissue tailored. That the antioxidant, system renders itself as a target for chemotherapy was eloquently shown by Raj and collaborators [99] using a murine model. In this work, cancer cells from different types of tumors, but not normal cells, were effectively killed by piperlongumine, a small molecule derived from the plant *Piper longum*. In addition, the authors were able to demonstrate that the cytotoxic effect of piperlongumine was achieved through interference with the antioxidant systems of the tumor cells. Such an approach has the added advantage that inhibitors targeting antioxidant systems could also be prescribed on a preventive setting.

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## Review Article

# Reticulon Protein-1C: A New Hope in the Treatment of Different Neuronal Diseases

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Reticulons (RTNs) are a group of membrane proteins localized on the ER and known to regulate ER structure and functions. Several studies have suggested that RTNs are involved in different important cellular functions such as changes in calcium homeostasis, ER-stress-mediated cell death, and autophagy. RTNs have been demonstrated to exert a cancer specific proapoptotic function via the interaction or the modulation of specific proteins. Reticulons have also been implicated in different signaling pathways which are at the basis of the pathogenesis of several neurodegenerative diseases. In this paper we discuss the accumulating evidence identifying RTN-1C protein as a promising target in the treatment of different pathologies such as cancer or neurodegenerative disorders.

## 1. Introduction

Neuronal death occurs by necrosis or apoptosis, which differ morphologically and biochemically. Necrosis is the result of extreme perturbation of the cellular environment, as occurs in ischemic insults or trauma. In contrast apoptosis is dependent on intracellular pathways which lead to cellular commitment to a defined series of steps leading to cell suicide. Apoptosis is an important mechanism in normal cell turnover and in growth and development, as well as in aging. Alteration of this machinery results in the evolution of cancer and autoimmune or degenerative diseases. The process of neuronal apoptosis involves two principal pathways that converge on activation of caspases: the cell surface death receptor pathway and the mitochondrial pathway [1]. However, a role in the initiation of neuronal apoptotic cell death by other organelles, including endoplasmic reticulum (ER), is now well established [2].

Disruption of ER homeostasis interferes with protein folding and leads to the accumulation of unfolded and misfolded proteins in the ER lumen. This condition, designated "ER stress," can be triggered by stimuli that perturb ER function, including depletion of Ca<sup>2+</sup> stores, reduction of disulphide bonds, overexpression of certain proteins, and

nutrient/glucose deprivation [3]. To maintain homeostasis, the ER mounts an unfolded protein response (UPR), as a self-protective mechanism, which results in transcriptional induction of UPR genes, translational attenuation of global protein synthesis, and ER-associated protein degradation (ERAD) [3]. However, if these adaptive responses are not sufficient to relieve the ER stress, the cell dies through apoptosis [3]. Interestingly, prolonged ER stress is at the basis of the pathogenesis of several neuronal disorders [4, 5]. In fact, recent studies have reported that many human disorders have their origin from dysfunctions in the endoplasmic reticulum and that regulation of some important UPR mediators may be potential targets for modulating ER stress response [6].

For example, ER-stress-dependent apoptosis is important in ischemia-reperfusion injury and in neurodegenerative diseases (such as Alzheimer's and Parkinson's), where abnormalities have been identified in protein folding or secretion in the Golgi-ER compartment [4].

In the last few years a new family of proteins, reticulons (RTN), primarily localized on the ER membrane, has attracted particular interest due to their implication in different cellular processes [7]. They play important role in bending and shaping the ER membrane, in trafficking of

material from the ER to the Golgi apparatus, and in apoptosis [8–10]. In particular, studies from different groups have indicated that RTN proteins show a proapoptotic function mediated by the induction of endoplasmic reticulum stress [11, 12] and also act as key regulators of ER morphology and nuclear envelope formation [13].

The mammalian reticulon family of proteins consists of four members (RTN1–RTN4); the specific functions for most of them are presently poorly understood although RTN4 (also called Nogo) has been widely demonstrated to be an inhibitor of axonal extension and neurite outgrowth [14]. RTN genes are expressed as multiple N-terminal isoforms that are generated by the use of different promoters or alternative splicing events [15]. Their C-terminal reticulon domain is evolutionarily conserved while the specificity of the N-terminal region contributes to their interaction with a vast array of proteins [16–19]. Although the reticulon genes are expressed in many tissues and are conserved in different phyla, suggesting a basic function in cell physiology and a universal role in the eukaryotic system, some of them (RTN-1A, RTN-1C, Nogo-A and RTN-2) are preferentially expressed in nervous tissues [20].

Reticulons were originally identified as markers for carcinomas with neuroendocrine characteristics [21]. In particular, the reticulon family gene 1 (RTN-1) was characterized by antibodies that stained a subset of neuroendocrine tissues and neoplasms [21] and was formerly called neuroendocrine-specific protein.

There is no doubt that cellular homeostasis of RTNs is important for normal cellular function. In particular, the pattern of RTNs localization in the ER, Golgi, and plasma membrane strongly suggests the existence of trafficking functions in the secretory compartment. In line with this finding it has been demonstrated that reticulons interact with several proteins regulating endo- and exocytosis processes [22].

They also have been identified as proteins showing a peculiar and almost exclusive localization to the tubular ER [23]. Moreover they contain a domain of ~200 amino acids (RHD), including two hydrophobic segments of unusual length (30–35 residues), which are thought to form a hairpin in the membrane of endoplasmic reticulum [23]. These structural properties, together with reticulons' ability to oligomerize, allow these proteins to generate and/or stabilize tubules *in vitro* and to play a key role in the mechanism by which the tubular network of the endoplasmic reticulum is generated and maintained [23]. It has been also demonstrated that reticulon proteins are deeply involved in nuclear envelope formation during mitosis in metazoans by a chromatin-mediated reorganization of the tubular ER [13]. In fact, it has been suggested that the levels of reticulons directly affect the balance between tubules and sheets in the ER and contribute to nuclear envelope formation [13].

On the other hand, more recent studies have expanded the biological functions of RTNs in several neuronal disorders [24–26], including cancer of cells of neuronal origin or neurodegeneration pathologies such as Alzheimer's disease and amyotrophic lateral sclerosis (ALS) [27]. It has been also demonstrated that reticulons participate in and regulate the pathogenesis of other diseases such as atherosclerosis [28].

## 2. RTN-1C and Brain Tumors

Cancer is characterized by an imbalance between cell division and cell death, caused by homeostatic changes occurring during the transformation process, the consequence of which results in dysregulation of apoptosis. Different therapeutic approaches have been used to counteract tumors growth and induce apoptosis, largely based on the induction of p53-dependent DNA damage. However, since more than 50% of tumors are defective in p53-family transcriptional activity, with mutations resulting in abrogated protein function and genetic instability, many tumors are insensitive to these treatments, leaving an acute need for novel therapies able to induce apoptosis independently of p53. This need is apparent even in tumors lacking in (or with rare occurrence of) p53 mutations, such as neuroectodermal tumors, a family of tumors notoriously resistant to apoptosis due to defective activation of apoptotic signalling pathways mediating either death receptor ligation or DNA damage [29–31]. Furthermore, these tumors remain resistant to p53-dependent damage despite p53 status being rarely mutated [32–34] as reported for many other types of cancer [35]. The poor response to chemotherapy thus results in poor survival rates, leaving us in acute need of novel therapeutic strategies. It has been suggested that inducing apoptosis via ER stress may represent a novel way to kill cancer cells that are resistant to apoptosis mediated by death-receptor ligation or DNA damage [36].

Reticulons are expressed in most neuroendocrine tumors; they are considered to be highly sensitive and specific markers of neuroendocrine differentiation for use in the diagnosis. In this context it is interesting that reticulons can exert a cancer-specific proapoptotic function. Such evidence is particularly pertinent for the RTN-1C family member. The first experimental results in this regard detected RTN-1C capability of interacting with glucosylceramide synthase (GCS) (Figure 2), a key enzyme in the biosynthesis of glycosphingolipids, and implicated in many biological phenomena, including multidrug resistance (MDR) [37]. Various studies have demonstrated a direct correlation between the development of MDR and increased levels of glucosylceramide [37, 38], with GCS being suggested as a candidate target for cancer therapy.

GCS inhibition, by both antisense and the specific inhibitor (D-threo)-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP), results in a drastic decrease of apoptosis induced by the p53-independent chemotherapeutic agent *N*-(4-hydroxyphenyl)retinamide (fenretinide) in neuroepithelioma cells [39]. Significantly it has been demonstrated that RTN-1C not only interacts with GCS at Golgi/ER interface but also modulates its catalytic activity *in situ* and affects the apoptotic response to fenretinide-induced apoptosis via a p53 independent mechanism [40].

Thus GCS role in fenretinide-induced apoptosis is influenced by RTN-1C, which provides a functional link between Golgi and ER in this response. These results confirmed the possibility of developing a targeted therapy for cancer using a combination of p53-dependent and p53-independent pathways. In line with this, we demonstrated that RTN-1C

regulates in a mutually exclusive way ER stress versus DNA-damage-induced cell death [11]. In fact, the increase of RTN-1C protein levels *per se* results in endoplasmic reticulum stress-induced cell death, mediated by an increase of cytosolic  $Ca^{2+}$ . This increase also significantly sensitizes cells to different endoplasmic reticulum stress inducers [11]. In line with these findings, the reduction of RTN-1C, by means of antisense DNA expression, reduced the response to ER stressors [11]. In the presence of high RTN-1C levels, genotoxic drugs become ineffective as a consequence of the cytoplasm translocation of p53 protein; conversely the silencing of endogenous RTN-1C boots the effectiveness of genotoxic drugs [11]. These data indicate that RTN-1C is able to modulate the cellular sensitivity to different apoptotic pathways and so it represents a promising molecular target for new drug development.

Other interesting data provided by experiments demonstrated that RTN-1C expression is directly correlated to calreticulin (CRT) exposure on the plasma membrane; this is through a mechanism mediated by the reduction of endoplasmic reticulum  $Ca^{2+}$  [41]. Such an event has been proved to be important for the activation of anticancer immune response and cell death. In fact, CRT exposure has been correlated to immunogenic cell death after anthracycline and  $\gamma$ -radiation in mouse models and has the potential for rendering conventional chemotherapies immunogenic [42, 43].

More recently it has been reported that the synthetic peptide corresponding to the C-terminal region (aa 179–208) of the human RTN-1C is able to bind DNA. The C-terminus of RTN-1C is characterized by the presence of an H4 histone consensus motif [PS000047 HISTONE\_H4] (GAKRH) [44] (Figure 1), the lysine present in this consensus sequence being one of the four residues that can be acetylated and modulate the H4 histone interaction with DNA [45].

Lysine acetylation is a reversible and highly regulated posttranslational modification, initially discovered on histones, and known to regulate diverse protein properties including DNA-protein interaction, subcellular localization, transcriptional activity and protein stability [46]. Acetylation modifies the lysine residues of both histone and nonhistone target proteins, such as p53, and is now recognized as an important regulatory step in gene transcription. Moreover lysine acetylation and its regulatory enzymes (acetyltransferases, HAT and deacetylases, HDAC) have been intimately associated with many different diseases, such as cancer and neurodegenerative disorders [47, 48] and with different cellular functions including stress response and apoptosis [49]. In this regard epigenetic regulation of gene transcription has recently attracted wide interest in the field of cancer especially because several genes implicated in oncogenesis are regulated by acetylation and deacetylation [50]. Modification of proteins by histone acetyltransferases or histone deacetylases plays an important role in the control of gene expression while their dysregulation has been linked to malignant transformation and other diseases [48]. Histone deacetylases are recognized as one of the most promising targets for cancer treatment, many HDACs inhibitors currently undergoing clinical trials [51, 52]. These enzymes are nowadays considered among the best

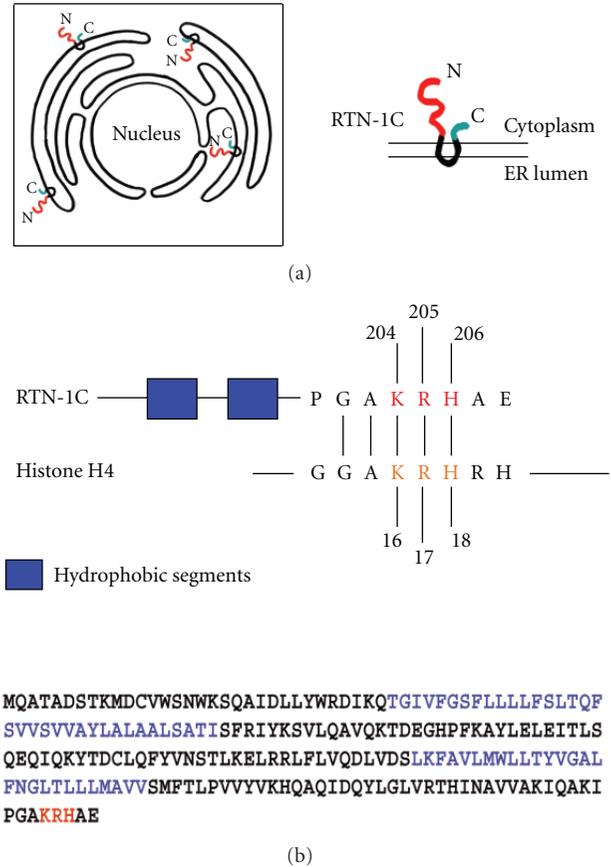


FIGURE 1: (a) Scheme showing the reticulons distribution on endoplasmic reticulum and the physical connection between nuclear envelope and ER membrane. (b) Schematic diagram of RTN-1C and histone H4 proteins showing the shared GAKRH motif. The blue aminoacids indicate the two hydrophobic segments of RTN-1C protein. The red aminoacids indicate the three positive charges in the H4 consensus motif. RTN-1C is acetylated on Lys 204.

potential targets for antineoplastic therapy [51]. HDACs are overexpressed in many cancer cells, and the death-inducing capability of different HDAC inhibitors correlates with their inhibitory potency. Interestingly these compounds have been demonstrated to induce apoptosis mediated by caspase activation as well as caspase-independent autophagic cell death [53]. Induction of two modes of programmed cell death by HDAC inhibitors indicates that these drugs might be particularly valuable when treating cancers with apoptotic defects.

In particular, inhibition of HDACs results in growth arrest and apoptosis of cancer cells while their deregulation has been linked to autophagy induction [54], suggesting that the epigenetic regulation of gene expression may be a fertile area for the development of anti-cancer strategies.

As previously reported, an H4 histone consensus motif has been identified in the C-terminal domain of RTN-1C. All the three positively charged amino acids (KRH) in the H4 consensus are essential for RTN-1C-mediated apoptosis. The function of this reticulon protein has been shown

to be modulated by posttranslational acetylation on lysine 204. Worth noting is a direct correlation between RTN-1C acetylation and HDACs activities; in fact the reticulon protein is able to negatively modulate their enzymatic activity (Figure 2). Moreover, RTN-1C protein is able to bind to DNA, this interaction being regulated by the acetylation process [45].

Considering the critical role of lysine acetylation in regulating diverse cellular functions, such as cancer development and human brain disorders [48, 51], HDAC substrates could represent candidate proteins relevant to human disease and therapeutic targets for drug design. In this context RTN-1C not only represents an important candidate for the development of new epigenetic therapeutic strategies, but also a novel protein with affinity for DNA, which could regulate the interaction between the ER/nuclear envelope membranes and chromatin.

### 3. RTN-1C and Autophagy

Another interesting connection between RTN-1C and cancer development may be the reticulon possible involvement in autophagy induction. Autophagic cell death is another important physiological cell death process, involved in development and stress responses. Furthermore, like apoptosis, autophagic cell death is involved in tumorigenesis, even if currently there is no cancer therapeutic approaches that specifically target the autophagic cell death machine.

It is now well accepted that there is a complex interplay between apoptosis and autophagy and that the molecular regulators are interconnected; numerous death stimuli are capable of activating either pathway and both pathways share several genes that are critical for their respective execution [55]. In particular, several reports have indicated a functional link between ER stress signaling pathways and autophagy. This is highlighted firstly by the possibility that endoplasmic reticulum stress response may lead to the induction of autophagy, and secondly by the fact that deregulation of this process causes several disorders characterized by the accumulation of toxic proteins in the ER [56]. The cross-talk between apoptosis and autophagy is critical and represents a key factor in the outcome of death-related pathologies such as cancer, its development and treatment. In particular, accumulating evidence suggests that ER stress is linked to autophagy [57, 58], where inhibition of the apoptosis pathway induces activation of the autophagy programme and vice versa [55, 59]. Moreover it has been shown that proper function of ER is required for autophagosome formation; when the ER senses the accumulation of unfolded misfolded protein, it can signal the induction of autophagy to overcome the resulting stress [60]. Finally, the possibility that autophagy could act as degradation system for unfolded protein accumulated in the ER in addition to ERAD has been demonstrated [61]. Interestingly a role for autophagy in the prion disease process has recently been suggested. In this regard it has been reported that cytoplasmic prion aggregates lead to endoplasmic reticulum stress activation of reticulon 3, impairment of ubiquitin-proteasome system, induction of

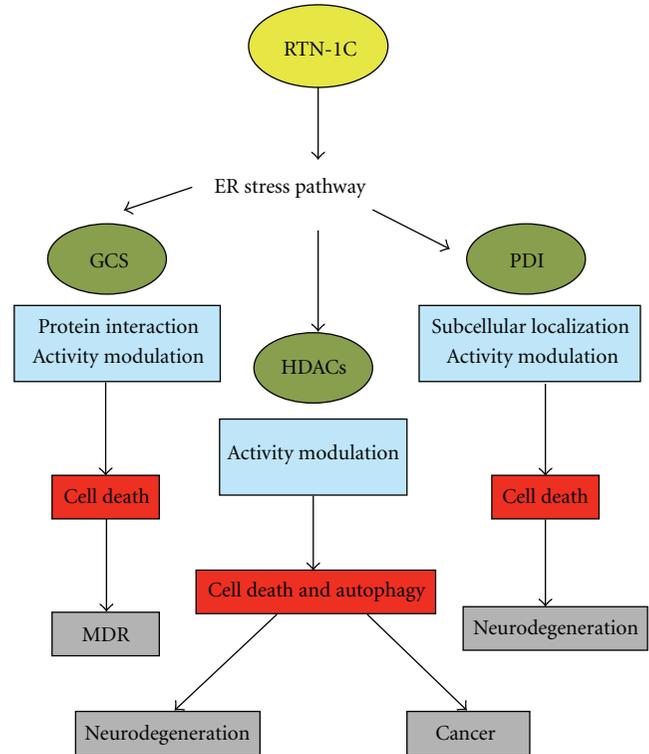


FIGURE 2: Schematic representation of the different RTN-1C-induced signaling pathways. Modulation of RTN-1C expression triggers the ER stress pathway and the regulation of different proteins (green ovals) at different levels (blue boxes). These events affect cellular processes (red boxes) which are at the basis of several human pathological settings (grey boxes).

autophagy, and apoptosis [62]. In this context it seems that RTN3 negatively regulates autophagy.

It has been also suggested that inducing apoptosis via ER-stress and/or autophagy may represent a novel way to kill chemoresistant cells and a promising approach for enhancing efficiency of cancer chemotherapy [37, 63]. Interesting pilot data (manuscript submitted) show that in neuroblastoma cells the modulation of RTN-1C expression disrupts  $Ca^{2+}$  signalling and induces  $Ca^{2+}$ -dependent autophagy. We can assume that autophagy may act as a prosurvival mechanism in response to ER stress and the accumulation of unfolded or misfolded proteins; thus autophagy machinery is perhaps activated to degrade the UPR proteins. Conversely, when the stress becomes severe and prolonged, these fine regulatory mechanisms are not sufficient and the cell undergoes apoptosis. According to this the upregulation of RTN1-C expression results in ER stress induction, finally leading to apoptotic cell death [11]. These data are a compelling reason to further explore the role of RTN-1C in autophagy induction and to understand to what extent and by which mechanism ER-stressed cells escape autophagy protection and commit apoptosis.

Future studies should define the molecular details dictating the alternative behaviour of the different RTN family members in autophagy regulation.

#### 4. RTN-1C and Neurodegeneration

Another exciting frontier of reticulon research is the field of neurodegenerative diseases. It has been widely demonstrated that the ER stress pathway is implicated in various neurodegenerative pathologies [6]. For example UPR activation has been shown to be an early event in the brain of Alzheimer's patients [64]. It has also been observed that motoneurons of amyotrophic lateral sclerosis (ALS) patients are characterized by ER alterations, so suggesting that the UPR response is activated and that ER stress may be involved in the neurodegeneration of these cells in early stages of ALS [65]. In this context, reticulons have been shown to play an important role in the nervous system both in normal and pathological settings, and recent studies have expanded the biological functions of RTNs in several CNS disorders, including Alzheimer's disease (AD) [66–68]. The latter, AD, is a progressive neurodegenerative disorder characterized by cognitive deficits and extensive neuronal loss. Several pathological changes have been described in postmortem brains of AD patients, including beta-amyloid (A $\beta$ ) plaques, intracellular neurofibrillary tangles formed by the hyperphosphorylated tau protein, inflammation, and extensive cell death [69]. One of the earliest molecular events in AD patients involves disturbances in calcium homeostasis [70]. Interestingly, it was found that all four human reticulon proteins can modulate BACE1 enzymatic activity [71] thought to contribute to early synaptic loss leading to the initial cognitive decline. In particular, reticulon proteins block access of BACE1 to APP and reduce the cleavage of this protein [72]. Thus, changes in the expression of reticulon proteins in AD brain are likely to affect cellular A $\beta$  and the formation of amyloid plaques in AD [71, 73].

Studies carried out on postmortem brain tissues have revealed changes in reticulon expression in the temporal and frontal cortex of patients with Alzheimer's disease [74]. In particular, it has been demonstrated that the expression of RTN-1C is significantly reduced in the frontal cortex of Alzheimer's disease patients compared to controls [74]. Finally altered RTN expression has been directly correlated to significant effects on cellular trafficking and abnormality in exocytosis or endocytosis; these may represent one of the mechanisms that could lead to neurodegenerative disorders [75, 76].

We have recently shown, by the use of microarray analysis of the whole human genome, that RTN-1C is able to specifically regulate gene expression, modulating transcript clusters implicated in the onset of neurodegenerative disorders [77].

*In vivo* studies have also established that enhanced expression of this reticulon family member in the cerebral cortex results in ER stress, leading to a neurodegenerative process characterized by an abnormal synaptic plasticity at corticostriatal synapses [77].

Another important mechanism involved in neurodegeneration process is the regulation of calcium homeostasis. Dynamic changes in calcium concentration within the ER and alteration of ER calcium homeostasis may be important in regulation of cell function and survival and may trigger various forms of neurodegeneration and/or neuropathy [78, 79].

In fact, disruption of the ER calcium homeostasis triggers ER stress response, which in turn may trigger a cascade of events leading to survival or death of neuronal cells.

Interestingly, RTN-1C modulates the expression of genes which has been demonstrated to be affected in schizophrenia with a common thread related to Ca<sup>2+</sup> signalling [80]. It has been observed that schizophrenic patients displayed elevated mobilization of Ca<sup>2+</sup> from intracellular stores in response to receptor stimulation; this suggests that increased cytosolic calcium may be the primary molecular abnormality in this pathology [81]. In keeping with this finding, it is known that the reticulon protein controls cytosolic Ca<sup>2+</sup> levels by depleting the ER Ca<sup>2+</sup> stores [11]. Matching these findings, reticulon overexpression has been observed in the cortex of patients affected by different neuronal pathologies including schizophrenia [82]. Furthermore, numerous studies have shown that the ER, whose function is strictly regulated by RTN-1C, plays a number of essential roles in synaptic transmission and plasticity at many central synapses [81]. Conversely, perturbation of ER Ca<sup>2+</sup> homeostasis is critically involved in aberrant forms of synaptic plasticity in mouse models of AD and schizophrenia [4, 83, 84]. In this context, it has been recently established that transgenic mice overexpressing reticulon 3 develop dystrophic neurites with impairment of spatial learning abilities and hippocampal long-term potentiation (LTP), thus resembling AD-like phenotype [85]. RTN-1C involvement in neurodegeneration processes was also investigated by the use of a transgenic RTN-1C mouse model. These mice exhibit maladaptive synaptic plasticity and show dysregulation of two plasticity-related genes DARPP32 and NOS2a whose induction/correct modulation is required for normal expression of bidirectional plasticity [86, 87]. Interestingly, Meyer-Lindenberg et al. [88] identified a specific haplotype of DARPP32 gene, associated with the risk for schizophrenia in a family-based association analysis. Altogether, these data highlight an as yet unknown role for RTN-1C for the regulation of higher brain functions associated with motor learning.

Reticulons have recently been implicated in other important neurodegenerative disorders; amyotrophic lateral sclerosis, which is a rapidly progressing fatal neurodegenerative disease, is characterized by the presence of protein inclusion in motor neurons. The induction of ER-stress-mediated apoptosis has proved to be an important event in the pathogenesis of ALS; moreover ER occurs early in the disease and involves the upregulation of protein disulphide isomerase (PDI), an important endoplasmic reticulum chaperone [89]. Endoplasmic reticulum is the primary site for synthesis and folding of secreted and membrane-bound proteins. The accumulation of unfolded and misfolded proteins in ER triggers a wide range of human neurodegenerative disorders. It is now evident that molecules that regulate the ER stress response represent potential candidates as drug targets to tackle these diseases. Protein disulphide isomerase is a chaperone involved in ER stress pathway, its activity being an important cellular defence against protein misfolding. The regulation of PDI activity may be a way of modulating ER stress responses and consequently the balance between apoptosis or survival in stressed cells. An increase of PDI activity

TABLE 1: Correlation between some RTNs cellular functions and their potential involvement in different human diseases.

Cellular functions	Human diseases
ER homeostasis	Cancer and neurodegenerative disorders (i.e., ALS, Alzheimer's disease)
Calcium homeostasis	Neuronal pathologies (i.e., schizophrenia)
Apoptotic response	Cancer, neurodegenerative disorders (i.e., ALS, Alzheimer's disease Parkinson's disease)
Membrane trafficking	Neurodegenerative disorders (i.e., Parkinson's disease)
Autophagy	Cancer, neurodegenerative disorders (i.e., prion disease, Alzheimer's disease, Huntington's disease)

could represent a means of counteracting protein inclusion formation typical in neurodegenerative diseases [90].

Several reports suggested that PDI function is particularly important for neuronal cell death because it is able to attenuate the neurotoxicity associated with the accumulation of aggregated proteins which is responsible for neurodegenerative processes [90]. It has been suggested that PDI activity is impaired in ALS. In addition anti-PDI-antibody immunopositive inclusions have been found in neurofibrillary tangles (NFTs) of the brain of Alzheimer's patients [91].

Interestingly, a very recent paper has shown that the reticulon family proteins, and in particular RTN-1C, represent novel regulators of PDI intracellular localization and that this phenomenon could be an important modulating factor in amyotrophic lateral sclerosis [92]. In this context, recent data (manuscript submitted) have demonstrated that the reticulon-1C family member is able to cause a dramatic intracellular PDI redistribution, from a diffuse to a punctate pattern which is not simply the result of ER stress induction. More importantly, RTN1-C significantly increases PDI enzymatic activity by modulating S-nitrosylation reactions (Figure 2). These results are in line with previously known experimental evidence that PDI functional activities (chaperone and isomerase) are regulated by S-nitrosylation processes [90]. Interestingly, it has been reported that inhibition of PDI activity by S-nitrosylation is strongly associated with mutant Cu/Zn superoxide dismutase toxicity in amyotrophic lateral sclerosis disease and that a small molecule mimicking the PDI active site protects against mutant superoxide dismutase 1 inclusion formation [89]. Moreover in models of Parkinson's disease, one of the S-nitrosylated targets found is the PDI [90]. Thus, based on these findings, RTN-1C is a good potential candidate for the modulation of PDI function in ameliorating aggregation and toxicity of mutated proteins.

Another interesting link between neurodegenerative disease and reticulons concerns the latter's previously mentioned involvement in autophagic signaling pathway. Autophagy dysfunction has been extensively described in neurodegenerative conditions linked to protein misfolding and aggregation [93]. Pharmacological induction of autophagy can enhance the clearance of intracytoplasmic aggregate-prone proteins, such as mutant forms of huntingtin, and ameliorate pathology in cell and animal models of neurodegenerative diseases [94].

Recent studies have reported that autophagy is the major degradational pathway following UPR activation in neuronal cells and constitutes a connection between UPR activation and autophagic pathology in AD brain [95]. In

line with this assumption, a recent work has reported that RTN3 negatively regulates autophagy blocking the clearance of cyPrP aggregates and thus providing a clue regarding the potential for inducing autophagy for the treatment of prion disease and other neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Huntington's disease.

We have recently found that (manuscript submitted) RTN-1C induces the activation of a  $Ca^{2+}$ -dependent autophagic pathway which is paralleled by changes in mitochondrial morphology and mitochondrial fission and fusion machinery. RTN-1C-mediated ER stress condition not only triggers alterations in  $Ca^{2+}$  homeostasis and mitochondrial dynamics, but is also capable of inducing an autophagic response. In the context of neurodegeneration, autophagy may constitute a prosurvival mechanism in response to ER stress and the accumulation of unfolded or misfolded proteins; reticulon-1C structural ER protein may modulate the neuronal stress at the basis of neurodegenerative pathologies.

Finally very interesting recent work has reported that reticulons are implicated in axonopathy and, in particular, in hereditary spastic paraplegias (HSPs), a group of genetically heterogeneous neurodegenerative conditions. Specifically RTN-2 has been found to be mutated by a complete deletion or a frameshift mutation, producing a truncated protein which is the cause of HSP [96]. These findings are most likely correlated to an abnormal reticulon function in the morphogenesis of the ER resulting in axonal degeneration.

## 5. Conclusion

Reticulons are a family of integral membrane proteins implicated in a variety of important biological functions such as ER morphology and organization, nuclear envelope formation, calcium homeostasis, and cell death. In the last few years many experimental data have expanded the reticulons' functions to a wide array essential for neuronal cell homeostasis Table 1. RTNs deregulations have been implicated in several human diseases such as cancer development and/or neurodegenerative disorders. However, for most of the reticulon proteins the specific role and the biochemical mechanisms at the basis of their biological function are still unknown.

Studies from our group have focused on the reticulon-1C isoform of RTN1 gene which we originally identified as a GCS interacting protein. Considering the role of this enzyme in the mechanism of cancer development and MDR, we have been interested in characterizing the role of RTN-1C in tumour cells of neuroectodermal origin. During the last few

years, we have obtained a series of very interesting results in the field of cancer development: these indicate that reticulon-1C is a promising molecular target for novel therapeutic approaches. We next expanded RTN-1C function to the modulation of neurodegeneration processes. We discovered that it participates in different signaling pathways, from ER-stress-induced cell death to autophagy, through the regulation of different enzymes such as HDACs or PDIs; future studies however would need to define the molecular details regulating the RTN-1C biological functions essential for clarifying its involvement in neural cell pathologies.

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## Review Article

# The Role of Mitochondrial NADPH-Dependent Isocitrate Dehydrogenase in Cancer Cells

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Isocitrate dehydrogenase 2 (IDH2) is located in the mitochondrial matrix. IDH2 acts in the forward Krebs cycle as an NADP<sup>+</sup>-consuming enzyme, providing NADPH for maintenance of the reduced glutathione and peroxiredoxin systems and for self-maintenance by reactivation of cysteine-inactivated IDH2 by glutaredoxin 2. In highly respiring cells, the resulting NAD<sup>+</sup> accumulation then induces sirtuin-3-mediated activating IDH2 deacetylation, thus increasing its protective function. Reductive carboxylation of 2-oxoglutarate by IDH2 (in the reverse Krebs cycle direction), which consumes NADPH, may follow glutaminolysis of glutamine to 2-oxoglutarate in cancer cells. When the reverse aconitase reaction and citrate efflux are added, this overall “anoxic” glutaminolysis mode may help highly malignant tumors survive aglycemia during hypoxia. Intermittent glycolysis would hypothetically be required to provide ATP. When oxidative phosphorylation is dormant, this mode causes substantial oxidative stress. Arg172 mutants of human IDH2—frequently found with similar mutants of cytosolic IDH1 in grade 2 and 3 gliomas, secondary glioblastomas, and acute myeloid leukemia—catalyze reductive carboxylation of 2-oxoglutarate and reduction to D-2-hydroxyglutarate, which strengthens the neoplastic phenotype by competitive inhibition of histone demethylation and 5-methylcytosine hydroxylation, leading to genome-wide histone and DNA methylation alternations. D-2-hydroxyglutarate also interferes with proline hydroxylation and thus may stabilize hypoxia-induced factor  $\alpha$ .

## 1. Oxidative Phosphorylation and Glutaminolysis in Cancer Cells

*1.1. Strategies for Survival of Malignant Tumors.* During malignant transformation, cells undergo stages of gene expression reprogramming and mutagenesis that alter their metabolic phenotype(s) [1–5]. Initial stimuli (not all known) dysregulate information signaling and activate oncogenes and/or cancer stem cells, resulting in a partial glycolytic “Warburg” phenotype [1–5] in which pyruvate is diverted, at least to a certain extent, from oxidative phosphorylation (OXPHOS). High proliferation and impaired angiogenesis subsequently cause hypoxia in certain regions within a growing tumor, and then hypoxia-mediated metabolic reprogramming (such as that promoted by hypoxia-induced factor, HIF [6–8]) further intensifies the glycolytic phenotype

and may nearly completely divert pyruvate from pyruvate dehydrogenase (PDH), that is, from OXPHOS. The sustained high rate of cell proliferation, however, results in aglycemia, initiating the revival of OXPHOS in conjunction with the promotion of glutaminolysis [1, 2, 9, 10]. The overall glutaminolysis provides cytosolic pyruvate/lactate and also yields NADPH via citrate export from mitochondria and subsequent ATP-citrate lyase and malic enzyme reactions. This compensates for the reduced net energy production by the glycolytic pathway and pentose phosphate pathway (PPP). Pyruvate imported into mitochondria is the precursor of not only acetyl-CoA but also citrate, which is required for fatty acid synthesis and hence for phospholipid synthesis, so it is essential for cell growth [1–5]. The final established phenotype is exemplified by human glioblastoma cells, which, despite their low respiration, maintain a constant

pyruvate flux through PDH and hence partial OXPHOS [9]. Oxaloacetate, however, may also be provided by the pyruvate carboxylase reaction [11].

**1.2. Glutaminolysis at Rejuvenated OXPHOS.** For the purpose of this paper, we shall use the term “glutaminolysis” in a more general way than just the transformation of glutamine to 2-oxoglutarate (2OG). We categorize glutaminolysis according to the fate of 2OG after its initial formation from glutamine [1]. If 2OG resulting from glutamine acts in the forward Krebs cycle (despite possible ongoing citrate extrusion and truncation of the cycle so that aconitase and “classic” NAD<sup>+</sup>-dependent isocitrate dehydrogenase (IDH3) reactions are not required), we define the system of metabolic reactions involved as “OXPHOS glutaminolysis.” This term points out to its essential dependence on succinate dehydrogenase (Complex II) and hence on respiration and OXPHOS. In contrast, when the reductive carboxylation of 2OG by isocitrate dehydrogenase 2 (IDH2) (in the counter Krebs cycle direction) consuming NADPH follows glutaminolysis of glutamine to 2OG and when the reverse aconitase reaction and citrate efflux are added, we define that system as “reductive carboxylation glutaminolysis” (RCG), also referred to as “anoxic glutaminolysis.” The latter term denotes the absolute independence of oxygen (respiration).

In general, glutaminolysis is an anaplerotic pathway of the Krebs cycle. Although it acts frequently in broad cancer types, glutaminolysis is not universal for all cancers [3–5]. In cancer cells employing OXPHOS glutaminolysis, glutamine can fully compensate for the lack of glucose in terms of energy generation and syntheses of precursors for anabolic pathways [3–5]. Thus, to survive under conditions of limited glucose, highly glycolytic cancer cells may adapt to glutaminolysis, which in its OXPHOS mode restores OXPHOS and may restore also at least partial PDH function [1, 3, 12–14]. In normal cells, mitochondrial glutaminase catabolizes glutamine to produce ammonia and glutamate, which is further transaminated by glutamate dehydrogenase into 2OG to feed the Krebs cycle [15]. In malignant tumors, negative allosteric effectors, such as GTP, inhibit glutamate dehydrogenase, resulting in a move toward glutaminolysis, where glutamate and pyruvate are reactants in a transamination reaction that produces, for example, alanine and 2OG by alanine aminotransferase (transaminase) [15]. In cancer cells, 2OG usually feeds the forward-running Krebs cycle truncated after citrate synthase during citrate extrusion, so that aconitase and “classic” NAD<sup>+</sup>-dependent IDH3 reactions are not required [1, 5]. This OXPHOS glutaminolytic mode is strictly dependent on oxaloacetate and acetyl-CoA, that is, on the citrate synthase reaction; hence, it proceeds only in cells in which oxaloacetate is provided by malate dehydrogenase fed by the Krebs cycle as well as by malate import from the cytosol, where malate originates from ATP-citrate lyase reaction. Likewise incomplete inhibition of PDH restores acetyl-CoA in mitochondria (the pyruvate pool is split between the PDH and transaminase reactions). An alternative oxaloacetate source is provided by pyruvate carboxylase reaction [11].

Also, the mitochondrial malic enzyme may contribute to this pool by producing pyruvate from malate [16]. Citrate is extruded from mitochondria and converted to oxaloacetate and acetyl-CoA by ATP citrate lyase [17]. Acetyl-CoA is then used to produce fatty acids by fatty acid synthase and cholesterol for general lipid synthesis, which is essential for cancer cell proliferation [18, 19]. In glioblastoma, if there is excess NADH in the cytoplasm (produced by aerobic glycolysis), the cytosolic oxaloacetate is converted first to malate by malate dehydrogenase and then to pyruvate by the cytosolic malic enzyme, thus may also contribute to lactate production [9, 20]. The cytosolic malic enzyme also produces NADPH as another factor required for lipid biosynthesis. Moreover, alanine that is released by transaminase is used for cytosolic amino acid transformations and protein synthesis [1, 4, 5].

**1.3. Glutaminolysis Independent of OXPHOS.** Hypothetically, malignant tumors may survive on intermittent OXPHOS-independent RCG (also termed “anoxic” glutaminolysis) in parallel with intermittent glycolytic periods [1, 2]. RCG utilizes reductive carboxylation of 2OG by the reverse reaction of mitochondrial IDH2 at the expense of NADPH, followed by the reverse aconitase reaction and citrate efflux from the matrix [1–3, 21–23]. NADPH is provided by the malic enzyme converting malate to pyruvate and might also be provided by the mitochondrial transhydrogenase [24]. The OXPHOS independence of this mode means that it may proceed at any level of hypoxia and even at anoxia, thus increasing malignancy [1, 3]. However, it does not produce ATP so parallel glycolytic periods are required [1]. The reductive carboxylation involves IDH2, which converts 2OG to isocitrate, from which the reverse aconitase reaction produces citrate, which is again exported from the mitochondrial matrix to the cytosol for fatty acid and lipid synthesis. Note that acetyl-CoA, and hence the PDH reaction, is not required in this mode.

## 2. Isocitrate Dehydrogenase Enzyme Isoforms

**2.1. Overview of IDH Isoforms.** All eukaryot genomes contain three *IDH* genes. *IDH3* encodes a mitochondrial matrix NAD<sup>+</sup>-dependent octameric IDH3 (4 $\alpha$ 2 $\beta$ 2 $\gamma$  subunits [25]) that acts in the Krebs cycle. IDH3 is allosterically positively regulated by Ca<sup>2+</sup>, ADP, and citrate and negatively regulated by ATP, NADH, and NADPH. The two other *IDH* genes, *IDH1* and *IDH2*, encode cytosolic and mitochondrial matrix NADP<sup>+</sup>-dependent (or NADPH-dependent) IDH1 and IDH2, respectively, which are structurally and genetically unrelated to IDH3 [26] (Table 1). IDH3 irreversibly decarboxylates isocitrate to yield 2OG while reducing NAD<sup>+</sup> to NADH, whereas IDH1 and IDH2 catalyze reversible reactions, either decarboxylating isocitrate to 2OG while reducing NADP<sup>+</sup> to NADPH or acting in the reductive carboxylation reaction to convert 2OG to isocitrate while oxidizing NADPH to NADP<sup>+</sup>.

Heterozygous mutations in *IDH2* at Arg172 and at the analogous residue Arg132 in *IDH1* are frequently found in grade 2 and 3 gliomas, secondary glioblastomas, and

TABLE 1: Kinetics of IDH isoforms as compared to homodimeric mutant enzymes with clinically relevant mutations. Unless specified “reductive”, forward reactions were measured. “n.d.”: not determined.

Enzyme	Organism/tissue	Mutation	conditions	$K_m$ isocitrate ( $\mu\text{M}$ )	$K_i$ , 2-oxo- glutarate ( $\mu\text{M}$ )	$K_m$ NADP <sup>+</sup> ( $\mu\text{M}$ )	$V_{\max}$ ( $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ )	Reference
IDH1	Human	wt		65	1900	49	44000 s <sup>-1</sup>	[96]
	Human	R132H		370	24	84	38 s <sup>-1</sup>	[96]
	Human	R132H	Reductive	2OG: 965	n.d.	NADPH: 0.44	1000 s <sup>-1</sup>	[96]
	Human	wt		6.4	n.d.	n.d.	14	[101]
	Human	R132H		1280	n.d.	n.d.	0.8	[101]
	Rat liver	wt		120	n.d.	150	70	[102]
IDH2	Porcine	wt		8.4	n.d.	5.6	43	[103]
	Porcine	wt		6	n.d.	5	40	[51]
	Porcine	R133Q		990	n.d.	11	21	[51]
	Rat liver	wt		70	n.d.	60	66	[102]
	Rat heart	wt		45	80	46	16	[104]
	Rat heart	wt	Ischemic	17	250	46	38	[104]
IDH3	Human	wt	No ADP	2000	n.d.	NAD <sup>+</sup> : 60	26	[105]
	Human	wt	+1 mM ADP	50	n.d.	n.d.	n.d.	[105]

acute myeloid leukemia (AML [27]), but they occur less frequently in primary glioblastomas and other cancers [28–37]. No homozygous deletions of *IDH1* and *IDH2* have been found, as has been observed for classic tumor suppressors. Nevertheless, mutated IDH1 and IDH2 exhibit a neomorphic enzyme activity, reducing 2OG to D-2-hydroxyglutarate while converting NADPH to NADP<sup>+</sup> [23, 29, 36, 38–40]. Interestingly, the D-2-hydroxyglutarate thus formed further promotes neoplasia by competitive inhibition of histone demethylation and 5-methyl-cytosine hydroxylation, leading to genome-wide alternations in the methylation of histones and DNA [40]. It has also been reported that glioblastoma SF188 cells produce D-2-hydroxyglutarate, in spite of lacking the above-described mutations [41].

Moreover, IDH2, like ~20% of other mitochondrial enzymes [42, 43], is acetylated at lysines, which inactivates the enzymatic activity. In turn, deacetylation of IDH2 by the mitochondrial matrix deacetylase sirtuin 3 (SIRT3) activates the enzyme to produce more NADPH [44]. In nonmalignant cells, the cytosolic IDH1 is involved in lipid metabolism and glucose sensing. IDH2 was traditionally considered to be involved in the regulation of OXPHOS and redox homeostasis [45] (see Section 4), and its involvement in reductive carboxylation has been recognized only recently (Sections 2.4 and 3).

**2.2. Specific Enzymatic Properties of IDH2.** NADP<sup>+</sup>-dependent oxidative decarboxylation of isocitrate to 2OG, as the major function of IDH2 in nonmalignant cells, contributes substantially to the control of mitochondrial redox balance and the prevention of oxidative damage [45, 46]. Because the IDH2 reaction is reversible, it may act in a “reverse” Krebs cycle mode in the reductive carboxylation reaction

(see Section 2.4). IDH2 contains an N-terminal mitochondrial addressing sequence and hence is imported to the mitochondrial matrix [45], although localization to nuclei has also been reported [47]. The *IDH2* locus is adjacent to the gene for the  $\alpha$  subunit of IDH3 [48]. IDH2 expression in heart, skeletal muscle, and lymphocytes is quite substantial; lower levels are found in liver, kidney, and lung [45, 47]. IDH2 has also been found in cultured rat neurons, astrocytes, oligodendrocytes, and microglia [49]. Unlike IDH1, the 94-kDa IDH2 (EC 1.1.1.42) is a homodimeric enzyme of two 413-amino acid subunits, each 47 kDa [50, 51] (Figure 1). IDH2 function requires a divalent metal ion, and bound Mn<sup>2+</sup> yields the maximum activity [52]. The structure of the Mn<sup>2+</sup>-isocitrate binding site was mapped from the solved crystal structure of porcine IDH2 [50]. Within the site, Thr78, Ser95, and Asn97 (of the porcine sequence) donate a hydrogen bond to the C3 carboxyl, whereas Asp252 and Asp275 coordinate Mn<sup>2+</sup>. An additional six Arg residues provide hydrogen bonds with isocitrate oxygens [50]. Hydrogen bonding of Lys212 with other residues between the two subunits was also noted. The NADP<sup>+</sup> binding site was originally predicted from the *E. coli* IDH structures, positioning the 2-hydroxyl-bound phosphate to interact with His315 and Lys374 of porcine IDH2 [50]. Porcine Arg83 enhances NADP<sup>+</sup> affinity by hydrogen bonding with the 3'-OH of the nicotinamide ribose, and Asn328 provides a hydrogen bond to the N1 of adenine [53]. For efficient coenzyme site function, a hydroxyl group must be present at position 373 (Thr373 of the porcine sequence), whereas Asp375 and Lys260 contribute to coenzyme affinity and catalysis [54, 55].

Within the numbering of the human IDH2 sequence, mutations in Arg172 (an analog of frequently mutated Arg132 of cytosolic IDH1 [56]) are detected in gliomas [23, 36, 37], and mutations in Arg172 and Arg140 (which



and Arg140 mutants of IDH2 [23, 36, 38, 39] and glioblastoma SF188 cells under hypoxia [41] convert 2OG to D-2-hydroxy-glutarate in this “reverse-reaction” mode. This reductive carboxylation would proceed better *in vivo* when followed by the reverse aconitase reaction and subsequent citrate export from the mitochondrial matrix. Reductive carboxylation was demonstrated for IDH2 in 2002 [65] and was indicated for cancer cells in transformed brown adipocytes [22], pediatric glioma SF188 cells [23, 41], and UOK262 cells (derived from a renal tumor in a patient with hereditary leiomyomatosis, these cells are defective in respiration and devoid of fumarate hydratase activity) [2]. Reductive carboxylation accompanied by citrate efflux has also been found in quiescent fibroblasts and is enhanced in contact-inhibited fibroblasts [66]. IDH2 silencing in SF188 cells results in diminished conversion of glutamine to citrate [23, 41]. Recently, reductive carboxylation was detected in human osteosarcoma 143B cells in which the mitochondrial DNA encoded a loss-of-function mutation in respiratory chain Complex III (CYTB 143B cells) [2]. Because only low-level reductive carboxylation was detected in wild-type 143B cells, the authors suggested that the impairment of OXPHOS, such as given by mutant mitochondrial DNA, induces RCG. Silencing of either IDH1 or IDH2 reduces the growth of both wild-type and CYTB 143B cells [2]. Moreover, unlike in wild-type 143B cells, *de novo* fatty acid synthesis from glutamine as a precursor is prevalent in CYTB 143B cells [2]. Reductive carboxylation in fumarate hydratase—devoid UOK262 cells, which are defective in respiration, has also been identified in parallel with OXPHOS glutaminolysis [2]. Interestingly, inhibition of respiration in mouse embryonic fibroblasts via administration of antimycin, rotenone, or metformin induces a switch towards RCG [2]. Thus, these data provide additional support for the authors’ hypothesis that RCG is a common cellular response to impaired mitochondrial metabolism [2].

### 3. Contribution of IDH2 to Glutaminolysis That Is Independent of OXPHOS

**3.1. Evidence for Reverse Reactions in the Krebs Cycle.** The reverse IDH2 reaction was also considered such that IDH2 acts together with the forward reaction of IDH3 in a dissipative isocitrate/2OG cycle [63] (see Section 4.2). The reductive carboxylation reaction and the overall RCG may indeed proceed together with the forward decarboxylation reaction [2, 22]. The best evidence was obtained by tracking the metabolites of <sup>13</sup>C-labeled glutamine, such as the appearance of <sup>13</sup>C-label in citrate [2, 22, 23, 41].

**3.2. RCG in Cancer Cells.** The first demonstrations of RCG in cancer cells [22, 23, 41] are consistent with the recent findings that mutant IDH2 in gliomas and AML also produce D-2-hydroxyglutarate from 2OG by “alternate” reduction. Because these mutants are heterozygous, both RCG and the production of D-2-hydroxyglutarate might occur. The former reaction involves the nonmutant NADPH-dependent IDH2 “reverse” reaction followed by isocitrate conversion

to citrate and by citrate export. The mutant IDH2 (but maybe also wild-type IDH2, see [41]) acting in a “reverse” mode also produces D-2-hydroxyglutarate, which cannot be transformed by aconitase; however, it further enhances the malignant phenotype [40]. The importance of this neomorphic IDH2 activity for the cancer phenotype is valid even without consideration of D-2-hydroxyglutarate interference with epigenetics and the HIF pathway, because IDH2 depletes 2OG from the Krebs cycle.

The consumption of NADPH in the matrix is a consequence of the altered homeostasis of reactive oxygen species (ROS) in cancer cells (see Section 4.2) and is also possible due to NADPH production by the mitochondrial malic enzyme [1, 5] and transhydrogenase [24, 63, 67]. It is not known whether SIRT3-based activation also affects this reverse (NADPH-dependent) IDH2 reaction. Although NADH, rather than NAD<sup>+</sup>, accumulates in the mitochondrial matrix of highly glycolytic cancer cells in which OXPHOS is dormant (Figure 2(a)), NAD<sup>+</sup> might be produced by the inner membrane H<sup>+</sup> transhydrogenase from NADH with the simultaneous formation of NADPH from NADP<sup>+</sup> in the matrix [67], thereby activating SIRT3-mediated IDH2 deacetylation. Moreover, the usual acetylation of proteins may be retarded in highly glycolytic cancer cells; hence, no SIRT3-mediated deacetylation would be required.

**3.3. Intermittent Nature of RCG.** Unlike glycolysis, RCG does not form ATP. Hence, either RCG coexistence with glycolysis or intermittent glycolysis is expected under hypoxic and deep hypoxic conditions [1]; that is, RCG may help cancer cells survive aglycemia and hypoxia in malignant cells [68]. Nevertheless, before all ATP stores become consumed, glycolysis must be reestablished. If this happens, it may help the tumor cell survive even in anoxia. As clearly demonstrated by the examples of gliomas and AML with the oncogenic metabolite D-2-hydroxyglutarate, the establishment of RCG, even concomitantly with OXPHOS glutaminolysis (note that this mode does not require IDH2 and aconitase reactions), helps to accelerate the malignant phenotype. Recently, it has been demonstrated that hypoxia elevates RCG in SF188 cells in a HIF-dependent manner [41]. SF188 cells were able to proliferate at 0.5% O<sub>2</sub> even if such hypoxic conditions substantially diminished glucose-dependent production of citrate, that is, OXPHOS and forward Krebs cycle participation [41].

### 4. Role of IDH2 in ROS Homeostasis

**4.1. Regulation of ROS Homeostasis in Nonmalignant Cells.** A major function of IDH2 in nonmalignant cells, when acting within the forward Krebs cycle, is likely maintaining an adequate pool of reduced glutathione and peroxiredoxin by providing NADPH. This function improves the mitochondrial redox balance and prevents oxidative damage [45, 46, 69], including heat-shock-induced oxidative damage [70] and numerous consequent events of oxidative stress, such as

ROS-induced apoptosis [71, 72], apoptosis induced by ionizing radiation [73] and cadmium [74], and staurosporine-induced cell death [75]. The lack of IDH2 or its activity elevates cytosolic ROS, lipid peroxidation, and oxidative DNA damage and shortens cell survival after oxidant exposure [69, 71–73]. Also, susceptibility to curcumin-induced apoptosis has been demonstrated upon IDH2 silencing in HCT116 cells [76]. Cardiac hypertrophy development is attributed to a decrease in IDH2 activity owing to the lipoperoxidation product 4-hydroxynonenal and oxidative stress [77]. IDH2 is also protective for paraquat-mediated oxidative inactivation of aconitase in heart mitochondria [78]. Inactivation of IDH2 activity by various ROS insults [79] is an important factor that has to be accounted for in any consideration of oxidative stress in cells. The forward Krebs cycle activity of IDH2 is inactivated by 4-hydroxynonenal [80], singlet oxygen [81], hypochlorous acid [82], aluminum [83], nitric oxide [84], and peroxyxynitrite [85]. Peroxyxynitrite forms S-nitrosothiol adducts on Cys305 and Cys387 of IDH2 under nitrosative stress, such as that established in the liver of ethanol-fed rats [85]. Glycation-mediated IDH2 damage has also been reported [86].

IDH2 activity first increases and then decreases with age in fibroblasts and liver, kidney, and testes tissues of rats fed *ad libitum* but not of those fed a calorie-restricted diet [87]. Recently, caloric restriction has been proven to act via IDH2 deacetylation through SIRT3 and thus promote an antioxidant role for IDH2-produced NADPH [44]. Again, the activity within the forward Krebs cycle was considered. It is not known whether SIRT3-mediated deacetylation also activates the NADPH-dependent “reverse” reaction, that is, reductive carboxylation.

**4.2. The Dissipative Isocitrate/2OG Cycle.** The dissipative isocitrate/2OG cycle has been suggested based on the reductive carboxylation reaction of IDH2 (counter Krebs cycle reaction direction, NADPH dependent) in conjunction with the forward IDH3 reaction in the canonical Krebs cycle [63]. The cycle may manifest itself in the absence of citrate export from mitochondria, as normally occurs in non-malignant cells, since cycling is impossible when reversed aconitase reaction depletes isocitrate. The cycle would also be possible at sufficient reactant pools and inner membrane energization. Isocitrate formed by the reductive carboxylation reaction of IDH2 is processed back to 2OG by IDH3. Although in non-malignant cells Complex I regenerates NADH to NAD<sup>+</sup> and NADP<sup>+</sup> could be regenerated to NADPH by, for example, mitochondrial malic enzyme, with increasing malignancy (more dormant state of mitochondria and hence decreasing respiration), the mitochondrial inner membrane H<sup>+</sup> transhydrogenase [24, 63, 67] may alternatively transfer electrons from NADH and NADP<sup>+</sup> to NAD<sup>+</sup> and NADPH at the expense of the proton-motive force [24]. However, it remains to be determined, whether this cycle is possible with D-2-hydroxyglutarate. If D-2-hydroxyglutarate was metabolized by IDH3 in the canonical Krebs cycle, then the cycle would be automatically induced by the appearance of D-2-hydroxyglutarate at simultaneously

active H<sup>+</sup> transhydrogenase. Nevertheless OXPHOS cannot be completely dormant, since proton-motive force would be required for this normal “forward” transhydrogenase reaction [24].

**4.3. Consequences of Reductive Carboxylation for ROS Homeostasis in Cancer Cells.** Consider the situation in highly malignant cells in which energy is derived primarily from glycolysis disconnected from OXPHOS (Warburg phenotype) and high reductive carboxylation glutaminolysis takes place (Figure 2(a)). Presumably, OXPHOS impairment [2] or deep hypoxia [41] may set up this metabolic pattern. In this case, higher glucose-6-phosphate dehydrogenase activity (the first PPP enzyme) produces more NADPH [88]. It may be erroneously considered as antioxidant action; however, because the constitutively expressed NADPH oxidase isoform-4, NOX4 [89, 90], can consume a major portion of the excess NADPH and produce more superoxide and consequently release more H<sub>2</sub>O<sub>2</sub> into the cytosol, the overall reaction scheme may be prooxidant (Figure 2(a)). This contributes to a much higher oxidative stress state in highly malignant cancer cells. Recently, NOX4 was also suggested to have mitochondrial localization [91]. Probably, NOX4 has  $K_m$  in the same order of magnitude as IDH enzymes. The NOX4 consumption of NADPH leaves fewer redox equivalents for the reduction of cellular glutathione and other redox systems [92–94]. The cytosolic oxidative stress is further intensified by the slow electron transport in low respiring (dormant) mitochondria of highly malignant cancer cells [1–5, 68], resulting in more superoxide release to the cytosol as well as the mitochondrial matrix from the respiratory chain. The ongoing maximum reductive carboxylation reaction further contributes to the oxidative stress by consuming NADPH, thus leaving less NADPH for maintenance of the reduced glutathione pool. Moreover, as mentioned above, the accumulated NADH at slow respiration may lead to NAD<sup>+</sup> formation in the reversed H<sup>+</sup> transhydrogenase reaction by concomitant NADPH formation from NADP<sup>+</sup> to further feed the NADPH pool and hence reductive carboxylation. Simultaneously, NAD<sup>+</sup> may lead to SIRT3-mediated activation of IDH2, at least of its “forward mode” [44], but it is not known whether reductive carboxylation is also activated by deacetylation of IDH2.

**4.4. Consequences of IDH2 Reaction within the Forward Krebs Cycle for ROS Homeostasis in Cancer Cells.** We next consider an intermediate Warburg phenotype, characterized by the mixed use of sole glycolysis, that is, aerobic glycolysis producing lactate, and OXPHOS (Figure 2(b)). The latter may be represented either by OXPHOS pyruvate metabolism and/or by OXPHOS glutaminolysis. Under these conditions, considerable cytosolic oxidative stress is expected because of the elevated NOX4 activity, as described above. However, a lower mitochondrial contribution to the cytosolic oxidative stress exists owing to an intermediate level of respiration and hence lower superoxide release from mitochondria to both the cytosolic and matrix compartments [92]. There is also lower oxidative stress expected in the matrix owing to the

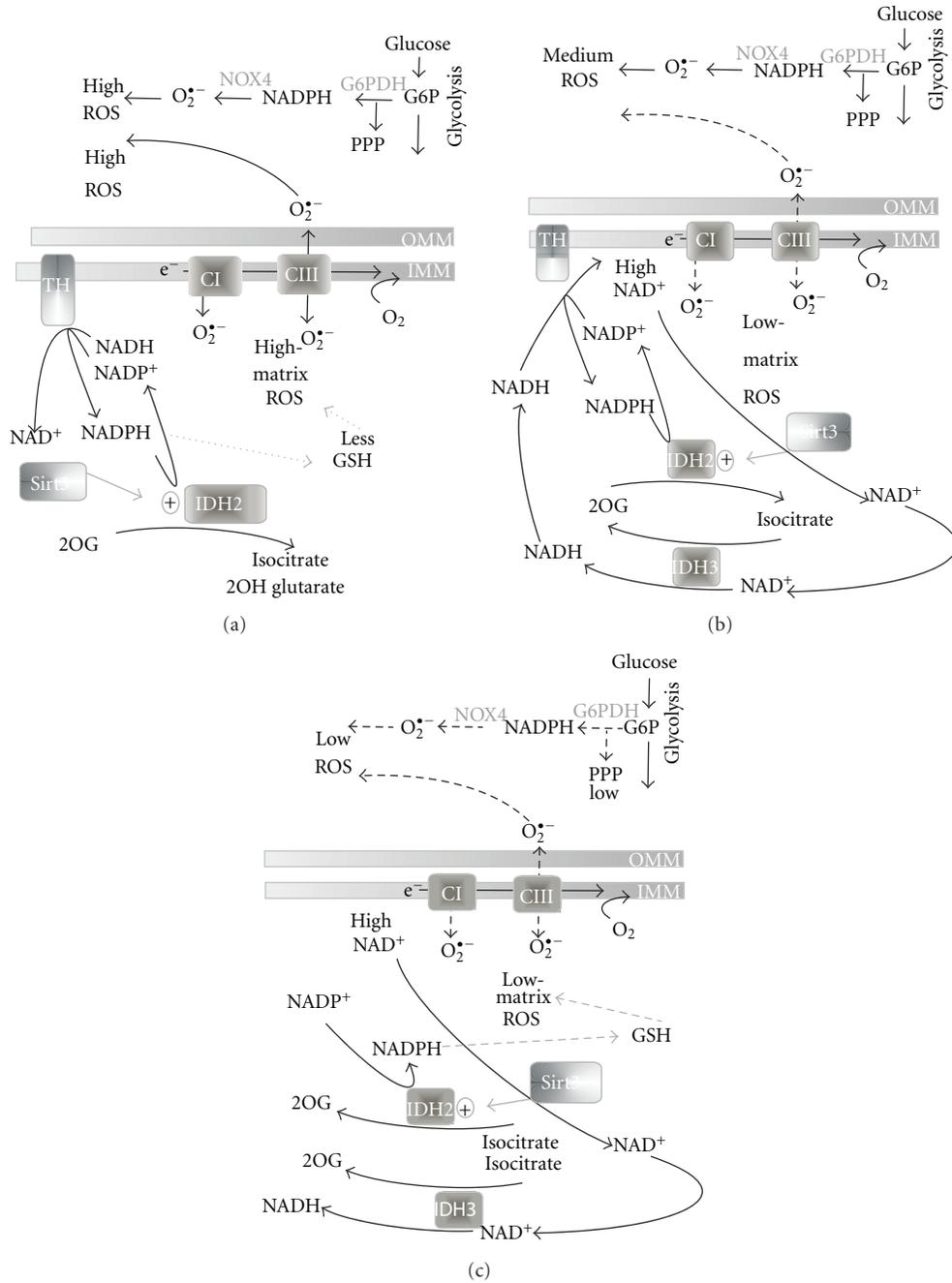


FIGURE 2: Consequences of IDH2 functions for redox homeostasis in cancer cells and nonmalignant cells.

possible ongoing dissipative isocitrate/2OG cycle, which by decreasing the proton-motive force [63] decreases mitochondrial superoxide formation [95]. This can be considered, however, only when citrate efflux from mitochondria is not dominant or when D-2-hydroxyglutarate would be cycling instead of isocitrate/2OG. If this is the case, NAD<sup>+</sup> rather than NADH would accumulate, further feeding the isocitrate/2OG cycle by simultaneous action of the forward IDH3 reaction and reverse (reductive carboxylation) reaction of IDH2. The accumulated NAD<sup>+</sup> would also promote SIRT3-mediated IDH2 deacetylation, consequently accelerating the

IDH2 branch of the reaction cycle (Figure 2(b)). Conditions similar to those described here may occur at hypoxia.

**4.5. Regulation of ROS Homeostasis in Nonmalignant Cells: IDH2 Contribution.** Finally, we consider the situation in non-malignant cells, in which OXPHOS predominates and the sole glycolysis and PPP activities are low (Figure 2(c)). In this case, low oxidative stress in the cytosol is a consequence of the negligible NOX4 activity and the low contribution of mitochondria to the cytosolic ROS pool. Under these normal

conditions, we assume that the forward IDH2 reaction generates NADPH, which further improves the reduced state of the mitochondrial matrix glutathione and peroxiredoxin systems. Indeed, ample evidence suggests that in cells with unattenuated OXPHOS, IDH2 plays an important antioxidant role that is further strengthened by NAD<sup>+</sup> accumulation in highly respiring cells. NAD<sup>+</sup> then induces SIRT3-mediated IDH2 deacetylation, thus increasing its protective function in NADPH formation for the maintenance of the reduced glutathione and peroxiredoxin systems and for self-maintenance by the reactivation of cystine-inactivated IDH2 by glutaredoxin-2 [59].

**4.6. IDH2-Dependent ROS Homeostasis at Glance.** (a) Situation in cancer cells with a prevalent Warburg phenotype and high reductive carboxylation. In the cytosol, higher glucose-6-phosphate dehydrogenase activity (G6PDH) produces higher NADPH within the pentose phosphate pathway (PPP). NADPH-oxidase isoform-4 (NOX4) thus produces more superoxide and consequently contributes to high levels of reactive oxygen species (ROS) in the cytosol. The cytosolic oxidative stress is further intensified by the slow electron transport in low-respiring (dormant) mitochondria, leading to higher superoxide release to the cytosol and matrix compartments. The ongoing maximum reductive carboxylation reaction further contributes to oxidative stress by consuming NADPH, thus leaving less for maintenance of the reduced glutathione pool. The accumulated NADH at slow respiration may lead to NAD<sup>+</sup> formation in the reverse H<sup>+</sup> transhydrogenase (TH) reaction (due to low proton/motive force) with concomitant NADPH formation from NADP<sup>+</sup> to further feed the NADPH pool and hence reductive carboxylation. Hypothetically, NAD<sup>+</sup> may lead to sirtuin 3 (SIRT3)-mediated deacetylation/activation (+) of IDH2, but it is not known whether reductive carboxylation is also activated by deacetylation of IDH2. (b) Situation in cancer cells with an intermediate Warburg phenotype and possible OXPHOS glutaminolysis. The major contribution to the cytosolic oxidative stress is the same as described above. However, a lower mitochondrial contribution to cytosolic ROS leads to intermediate oxidative stress under these conditions. Indeed, an intermediate or high respiration leads to much lower superoxide production and release from the mitochondria to both the cytosolic and matrix compartments (dashed arrows). Lower oxidative stress is also expected in the matrix owing to the ongoing dissipative isocitrate/2OG cycle, which decreases further mitochondrial superoxide formation by decreasing the proton-motive force. OXPHOS glutaminolysis may predominate under these conditions; hence, RCG might not be completed and the isocitrate/2OG cycle with forward H<sup>+</sup> transhydrogenase reaction may be initiated. NAD<sup>+</sup> would accumulate owing to high respiration, further feeding the isocitrate/2OG cycle. The accumulated NAD<sup>+</sup> would also promote SIRT3-mediated deacetylation/activation of IDH2. (c) Situation in nonmalignant cells. Aerobic glycolysis and PPP activity are low; consequently, low oxidative stress in the cytosol also results from the negligible NOX4 activity and the low

contribution of mitochondria to the cytosolic ROS. The forward IDH2 reaction thus forms NADPH, which further improves the reduced state in the mitochondrial matrix glutathione and peroxiredoxin systems. As in (b), the NAD<sup>+</sup> accumulation in highly respiring cells then induces SIRT3-mediated deacetylation/activation of IDH2.

## 5. Hypothetical IDH2 Involvement in Redox Signaling

As briefly described above, mutant IDH2 as well as IDH1 [96–100] produce D-2-hydroxy-glutarate, which can initiate an HIF-mediated “hypoxic type” of gene reprogramming even at normoxia [58]. Nevertheless, detail investigations of D-2-hydroxyglutarate effects on HIF signaling are required, since recently an opposite effect, diminishing HIF levels by inhibition of EGLN prolyl 4-hydroxylases, has been reported [99]. Also, non-mutated IDH2 acting in the 2OG/isocitrate cycle together with H<sup>+</sup> transhydrogenase [63] could contribute to the modulation of the ROS pool by initiating an impulse originating from Complex III to dissipate the proton-motive force, which reduces superoxide formation in the mitochondrial respiratory chain [95]. In contrast to mutant IDH2 activity, the ongoing dissipative 2OG/isocitrate cycle in the absence of citrate efflux from mitochondria would retard HIF signaling.

## 6. Future Perspectives

The discovery of mutant IDH2 and IDH1 in certain gliomas and AML and their production of the oncogenic metabolite D-2-hydroxyglutarate have unraveled a fascinating story of cancer self-acceleration via intermittent episodes of genome instability and metabolic remodeling and namely via epigenomic alterations [98, 100]. However, there are additional aspects to be clarified. First, the exact role of D-2-hydroxyglutarate must be further investigated to determine whether it promotes the reverse carboxylation mode of glutaminolysis and whether it acts in the dissipative 2OG/isocitrate cycle, which would then become the 2OG/D-2-hydroxyglutarate cycle, as we can now only speculate. Also, conditions under which D-2-hydroxyglutarate might be formed in non-mutant IDH2 should be defined. Second, the role of SIRT3 has to be established to determine whether it prevents or accelerates malignancy via IDH2. In particular, the question whether SIRT3 activates reductive carboxylation must be resolved. Finally, the role of IDH2 in other cancer types distinct from AML, gliomas, and renal tumors of hereditary leiomyomatosis should be investigated.

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## Review Article

# Altered Gene Expression, Mitochondrial Damage and Oxidative Stress: Converging Routes in Motor Neuron Degeneration

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Motor neuron diseases (MNDs) are a rather heterogeneous group of diseases, with either sporadic or genetic origin or both, all characterized by the progressive degeneration of motor neurons. At the cellular level, MNDs share features such as protein misfolding and aggregation, mitochondrial damage and energy deficit, and excitotoxicity and calcium mishandling. This is particularly well demonstrated in ALS, where both sporadic and familial forms share the same symptoms and pathological phenotype, with a prominent role for mitochondrial damage and resulting oxidative stress. Based on recent data, however, altered control of gene expression seems to be a most relevant, and previously overlooked, player in MNDs. Here we discuss which may be the links that make pathways apparently as altered gene expression, mitochondrial damage, and oxidative stress converge to generate a similar motoneuron-toxic phenotype.

## 1. Introduction

Motor neuron diseases (MNDs) are a rather heterogeneous group of diseases, with either sporadic or genetic origin or both, all characterized by the progressive degeneration of motor neurons. All MNDs are primarily axonopathies of the motor neurons in which neuromuscular synapses are early targets of damage and death of motor neurons probably occurs following loss of the neuromuscular junctions [1]. MNDs may manifest as weakness, atrophy of muscles, difficulty in breathing, speaking, and swallowing, with symptoms and severity varying as a consequence of the different involvement of upper or lower motor neurons or both.

The most common and studied form in adults is Amyotrophic Lateral Sclerosis (ALS), followed by Progressive Bulbar Palsy (PBP), the rarer forms being Progressive Muscular Atrophy (PMA) and Primary Lateral Sclerosis (PLS). These conditions seem to form a continuum of diseases since only part of patients have a “pure” phenotype, while others with PBP or PLS eventually develop the widespread symptoms common to ALS [2]. In all these MNDs, onset of symptoms occurs mainly in people aged 40–70. Life expectancy is

between 2 to about 5 years after onset in ALS and 6 months to 3 years in PBP, while pure PLS patients may have a normal or near-to-normal life duration. MNDs also include Spinal and Bulbar Muscular Atrophy (SBMA), in which age of onset and severity of manifestations vary from adolescence to old age, but longevity is usually not compromised. Infantile MNDs include Spinal Muscular Atrophy (SMA) with an infantile or juvenile onset and Lethal Congenital Contracture Syndrome (LCCS), causing prenatal death and thus being the most severe form of motor neuron disease.

## 2. Aetiology of MNDs

LCCS1 is an autosomal recessive condition found in communities of the northeastern part of Finland with a prevalence of 1 in 25,250 births [3]. LCCS manifests *in utero* with a marked atrophy of spinal cord motor neurons and fetal immobility due to lack of anterior horn motor neurons, severe atrophy of the ventral spinal cord, and hypoplastic skeletal muscles. It is characterized by total immobility of the fetus, detectable at the 13th week of pregnancy and invariably leading to prenatal

death before the 32nd gestational week. The defective gene for LCCS1 is a 16-exon gene coding for GLE1, an mRNA export mediator that is known to interact with the nuclear pore complex and is expressed in the neural tube of 11-day-old mice embryos, specifically in the ventral cell population from which the motor neurons differentiate, and later in other tissues including somites, from which skeletal muscle and bone tissue differentiate [3]. The most frequent mutation in LCCS1 (FinMajor) does not dramatically alter the stability or localization of the protein GLE1 but is predicted to introduce three aminoacid residues in a region that may be critical in the interaction between GLE1 and a motor neuron-specific protein [3].

SMA is the most frequent genetic cause of infant mortality and exists in various forms invariably caused by a genetic defect. Patients with the most common form (proximal SMA) are either deleted for the nine-exon gene *SMN1*, encoding the ubiquitously expressed protein SMN (Survival Motor Neuron) or carry small mutations in the same gene. However, SMA patients always carry at least one copy of the gene *SMN2*, which encodes the same protein as *SMN1* and is only partially functional because of a critical, translationally silent single nucleotide C/T transition inside exon 7 that profoundly affects correct splicing. The clinical severity of SMA ranges from respiratory distress at birth associated with limited life expectancy (SMA1) to onset at older than 10 years and a normal life expectancy (SMA4) and is inversely related to the level of *SMN2* compensating for *SMN1* deletion [4].

SBMA (also called Kennedy's disease) is an X-linked recessive motor neuron disease in which only lower spinal cord and brain stem motor neurons are affected [5]. SBMA is caused by a polyglutamine expansion in the androgen receptor (AR) [6]; CAG repeat numbers range from 38 to 62 in SBMA patients, whereas healthy individuals have 10 to 36 CAG repeats. Symptoms appear in childhood or early adolescence [7]; SBMA is a rare disease, with the exception of some population in the Vasa region of Western Finland where it was estimated that the prevalence is 13 in 85,000 male inhabitants [8].

While SMA, SBMA, and LCCS1 are invariably familial diseases, adult-onset MNDs are both sporadic and familial. PBP, PMA, and PLS are usually sporadic. ALS occurs sporadically in the majority of cases [9]. Proposed risk factors for ALS include ingestion of high concentrations of  $\beta$ -methylamino-L-alanine [10], use of cholesterol-lowering drugs [11], intensive physical exercise [12] including football playing [13, 14] and service in the USA Army [15], possibly linked to intermittent occupational hypoxia [16] or to head injury [17–19]. Environmental factors also include cigarette smoking [18, 20], exposure to heavy metals [21], and pesticides or herbicides [22–24]. Approximately 10% of ALS cases is inherited, with multiple autosomal dominant and recessive forms that have been ascribed to mutations in a number of different genes, each of them accounting for a different percentage of cases (Table 1). Interestingly, ALS-associated mutated proteins are implicated in a wide range of cellular processes, from antioxidant response to axonal and vesicular transport, angiogenesis, endoplasmic reticulum (ER) stress and unfolded protein response (UPR), and, most noticeably, to RNA metabolism.

### 3. Multifactoriality of MNDs: The Role of Altered Gene Expression

At the cellular level, MNDs share features such as protein misfolding and aggregation, mitochondrial damage and energy deficit, excitotoxicity, and calcium mishandling [1], a condition often indicated as multifactoriality. This is particularly well demonstrated in ALS, where both sporadic and familial forms share the same symptoms and pathological phenotype, that are recapitulated in available animal and cell models, with a prominent role for mitochondrial damage and resulting oxidative stress (for an extended Review, see [25]). Oxidative stress is reported also in SMA [26] and reactive oxygen species (ROS) inhibit assembly and activity of SMN complex in a dose-dependent manner [27]. Mitochondrial damage seems to be invariably present in neurodegenerative conditions [28] including SMA [29–32] and SBMA [33], in which mitochondrial dysfunction may be due to the interaction between AR and cytochrome c oxidase subunit Vb (COXVb) [34].

Based on recent data, however, altered control of gene expression seems to be a most relevant, and previously overlooked, player in MNDs.

Several studies addressing epigenetic modifications, transcriptomics, and proteomics of models and tissues from patients indicate that the overall pattern of gene expression is modified in MNDs. Because of the known non-cell autonomous mechanism of death of motor neurons, studies in ALS have been performed in tissues [35, 36] and in neuronal and in nonneuronal cultured cells (astrocytes, muscle) and revealed that most of the deregulated genes are involved in defense responses, cytoskeletal dynamics, protein degradation system, and mitochondrial dysfunction in neurons [37], while the insulin-like growth factor-1 receptor and the RNA-binding protein ROD1 are the most downregulated genes in glia [38]. The pattern is altered also in muscle, in which many of deregulated genes are the same found in surgically denervated muscles, while others appear to be ALS-specific and include proteins clearly involved in the redox response (e.g., metallothionein-2 and thioredoxin-1) [39, 40]. In a recent proteomic study on embryonic stem cell from a severe SMA mouse model differentiated into motor neurons in vitro, Wu et al. reported that 6 proteins are downregulated and 14 upregulated in this model. Most of these proteins belong to the same categories altered in ALS models, that is, are involved in energy metabolism, cell stress response, protein degradation, and cytoskeleton stability [41].

As in other neurodegenerative conditions, alterations of transcription in MNDs may follow altered epigenetic control due to an unbalance between histone acetyl transferases (HATs) and histone deacetylases (HDACs, including sirtuins, SIRTs) activities [42]. These enzymes catalyze forward and reverse reactions of lysine residue acetylation; thus, HATs modify core histone tails thereby enhancing DNA accessibility to transcription factors (TFs), while HDACs activity in general results in transcriptional repression and gene silencing. Interestingly, various TFs, like RelA, E2F, p53, and GATA1, which form part of the transcription initiation complex, are themselves substrates susceptible to the action of HATs and HDACs.

TABLE 1: Genes involved in MNDs.

Gene	Protein	MND	Main known function
SOD1	Cu, Zn superoxide dismutase	ALS1	Antioxidant enzyme
ALS2	Alsin	ALS2	guanine nucleotide exchange factor for GTPases
SETX	Senataxin	ALS4	DNA/RNA metabolism and repair
SPG11	Spataxin	ALS5	Neuron differentiation and axonal transport
FUS/TLS	Fused in sarcoma	ALS6	RNA binding protein
VAPB	VAMP-associated protein B	ALS8	Trafficking between the endoplasmic reticulum and Golgi apparatus
TDP-43	TAR-DNA-binding protein-43	ALS9	DNA- and RNA-binding protein
ANG	Angiogenin	ALS10	Angiogenesis in response to hypoxia; possibly RNA metabolism
FIG4	PI(3,5)P(2)5-phosphatase	ALS11	Metabolism of phosphatidyl inositol bisphosphate and vesicle dynamic
OPTN	Optineurin	ALS12	Vesicular trafficking
nAChR	Neuronal nicotinic acetylcholine receptor	ALS	Glutamatergic pathway
CHMP2B	Charged multivesicular protein 2B	ALS	Chromatin-modifying protein/charged multivesicular body protein family
VCP	Valosin-containing protein	ALS	Membrane trafficking, organelle biogenesis, maturation of ubiquitin-containing autophagosomes
DAO	D-aminoacid oxidase	ALS	Oxidative deamination of D-aminoacid
UBQLN2	Ubiquilin2	ALS	Ubiquitin-proteasome response
Sig-1R	Sigma-1 receptor	ALS	ER chaperone, modulates calcium signaling through the IP3 receptor
C9ORF72	Unknown	ALS	Unknown
AR	Androgen receptor	SBMA	Androgen receptor
SMN	Survival Motor Neuron	SMA	RNA processing
GLE1	Nucleoporin GLE1	LCCS1	Export of mRNAs containing poly(A)

Evidence for the involvement of this kind of regulation in MNDs is accumulating, although still far from definitive, and unspecific HDAC inhibitors such as sodium phenylbutyrate, trichostatin A, and valproic acid have been tested as neuroprotective drugs for the treatment of ALS with some positive result [43–47]. It is interesting to note that valproic acid is also endowed with antioxidative and antiapoptotic properties. However, most likely only selected HDACs participate to onset or propagation of motor neuron damage and thus must be targeted for an effective therapy. This concept is strengthened by the observation that complexes formed by ALS-linked proteins TDP-43 and FUS/TLS control the expression level of HDAC6 [48].

The SMN gene has a reproducible pattern of histone acetylation that is largely conserved among different tissues and species [49] and several HDAC pan-inhibitors such as suberoylanilide hydroxamic acid (SAHA) [50], trichostatin A [51], and the benzamide M344 [52] increase SMN2 transcript and protein levels. Valproic acid is currently tested in phase I and II clinical trials for the treatment of SMA (<http://clinicaltrials.gov/>). However, valproic acid has also serious adverse effects in cell and mice models for SMA [53, 54] pointing again to the need of inhibition of selected HDACs in MNDs, especially in the light of a recent report that the SMN2 gene is differentially regulated by individual HDAC

proteins and silencing of HDAC5 and 6 enhances inclusion of an alternatively spliced exon in SMN2 [55]. Finally, oral administration of the HDAC inhibitor sodium butyrate has been tested also in a transgenic mouse model of SBMA with some positive outcome but only within a narrow range of drug dosage [56].

Epigenetic control of transcription may also occur via methylation by DNA methyltransferases (DNMTs) or histone methyltransferases (HMTs), both using S-adenosylmethionine (SAM or AdoMet) as the methyl donor. DNA methylation in eukaryotes occurs by the covalent modification of cytosine residues (on the fifth carbon) in CpG dinucleotides, leading to gene silencing. Methylation of histones (as well as transcription factors) occurs on lysine or arginine. Methylated lysine residues can carry up to three methyl moieties on their amine group, whereas arginine can be mono- or dimethylated on the guanidyl group. Lysine methylation of histones is associated with activation or repression of transcription, depending on the degree of methylation and on the residue location [57].

Methylation may be extremely relevant in MNDs if one considers, for instance, that recognition of some Sm proteins by the SMN complex (that mediates the assembly of the Sm proteins onto snRNAs involved in pre-mRNA splicing and histone mRNA processing) is dependent on symmetrical

dimethyl arginine modifications of their RG-rich tails [58, 59]. This methylation is achieved by PRMT (protein arginine methyltransferase) 5 or by PRMT7, two enzymes that function nonredundantly [60] and utilize SAM as methyl donor. Furthermore, the SMN2 gene is subject to gene silencing by DNA methylation and some HDAC inhibitors including vorinostat and romidepsin are able to bypass SMN2 gene silencing by DNA methylation, while others such as valproic acid and phenylbutyrate are not [61].

Other observations support the concept that MNDs may be considered as “RNA dysmetabolisms” [62]. As reported in Table 1, several of the genetic factors involved in MNDs encode proteins with a role in RNA metabolism, and some overlap may exist among different diseases. For instance, copy number abnormalities of the SMN genes have been reported in sporadic ALS, although decrease of SMN protein in the anterior horn cells of ALS patients may be only a secondary phenomenon [63, 64]. RNA metabolism, however, consists of several intertwined steps, such as pre-mRNA splicing, mRNA transport, translational regulation, or mRNA decay, and the precise RNA pathway that is affected in a single MND remains unknown because virtually every one of the involved RNA-binding proteins has been implicated in more than one of these steps. Thus, it is not clear why motor neurons are so vulnerable to mutations in RNA-binding proteins.

Very recently, familial ALS has been associated with an expansion of a noncoding GGGGCC hexanucleotide repeat in the gene *C9ORF72* [65, 66] that codes for an unknown protein. The transcribed GGGGCC repeat forms intracellular accumulations of RNA fragments in cells in the frontal cortex and the spinal cord from patients carrying the expansion [65]. These RNA *foci* are composed of the expanded nucleotide repeats that may disturb transcription by sequestering RNA-binding proteins involved in transcription regulation as observed for other expanded RNA repeats diseases [67] such as myotonic dystrophy [68]. Interestingly, the GGGGCC sequence also represents a potential binding site of several RNA-binding proteins including hnRNP A2/B1, a TDP-43 interactor [69, 70].

#### 4. Altered Gene Expression, Mitochondrial Damage, and Oxidative Stress in MNDs: Which Are the Links?

Which are the links among altered gene expression, mitochondrial damage, and oxidative stress in MNDs is not clear yet. While oxidative stress and mitochondrial dysfunction are obviously connected into a vicious cycle in which excess in ROS production may influence the functionality of the organelles, that in turn would produce excess ROS, the connection with altered gene expression in MNDs is still somewhat foggy.

A few considerations may help to shed some light on possible, not mutually exclusive, mechanisms.

In analogy to what has been proposed in development [71] and in cancer [72], an interplay among oxidative stress,

thiol redox signaling, and epigenetic modulation by methylation may be critical in motor neurons. The antioxidant capacity of cells is influenced by the production of glutathione (GSH), and increased GSH production influences DNA and histone methylation by limiting the availability of SAM, the cofactor utilized during epigenetic control of gene expression by DNA and histone methyltransferases [71]. The above mentioned forms of methylation, which are relevant in MNDs, are not directly linked, since they involve different enzymes and different targets. However, they all require the same methyl donor, which could be limiting in MNDs.

HDACs themselves seem to be linked to and modulated by oxidative stress. Pan-HDAC inhibition promotes neuronal protection against oxidative stress in a model of glutathione depletion [73], thus suggesting that HDACs are downstream mediator in the mechanisms of toxicity by ROS, while carbonylation of reactive cysteines of some, but not all, class I HDACs causes reduction of histone deacetylase activity and change in histones acetylation and transcription of genes repressed by these HDACs [74]. Thus, oxidative stress may be a modulator of gene expression through the modulation of DNA accessibility.

In turn, the activity of HDACs modulates alternative splicing of human genes when the nascent RNA is still associated with chromatin (in particular the splicing of hundreds of genes is altered upon HDAC inhibition) [75] but also the activity of various TFs. Noticeably, oxidative stress is also a modulator of several TFs and thus ROS and HDACs may concur in the generation of a pathological phenotype through the same mechanism. For instance, as reviewed by Rahman et al. [76], oxidative stress inhibits HDAC activity and activates HAT activity; this leads to NF- $\kappa$ B activation, which, in turn activates proinflammatory mediators. The antioxidant and/or anti-inflammatory effects of thiol molecules (GSH, N-acetyl-L-cysteine and Nacystelyn) and dietary polyphenols (e.g., curcumin and resveratrol) have a role in either the control of NF- $\kappa$ B activation or the modulation of HDAC. Thus, oxidative stress may regulate both TFs and chromatin remodeling which in turn impacts on proinflammatory responses.

Furthermore, SIRT6 (class III HDACs) control the expression or the activity of a number of proteins involved in redox regulation (Table 2). Among these proteins, some are mitochondrial and many have been involved in one or more MNDs by transcriptomic/proteomic studies [77–79].

Last, but not the least, we have reported that mitochondrial damage itself is a cause of modification in the abundance of selected splicing variants [80] and that defective RNA metabolism seems to play a role also in SOD1-linked ALS and to descend directly from mitochondrial stress [81].

#### 5. A Unifying Mechanism for MNDs?

From what summarized above, it is tempting to speculate that indeed all MNDs are mainly forms of RNA dysmetabolisms. Motor neurons seem to be exceedingly susceptible to defects in RNA transcription or processing; one appealing explanation is that they require that RNA is not only correctly

TABLE 2: Effects of class II HDACs (Sirtuins) on redox-related proteins.

Sirtuin	Target	Effect	Reference
SIRT1 (nucleus and mitochondria)	FOXO3a	↑ Transcriptional activity	[82]
	PGC-1 $\alpha$	↑ Transcriptional coactivation	[83, 84]
	HIF1 $\alpha$	↓ Transcriptional activity	[85]
	HIF2 $\alpha$	↑ Transcriptional activity	[86]
	eNOS	↑ Enzyme activity	[87]
SIRT2 (cytoplasm)	p53	Mediates transcriptional activity, depending on SIRT1 expression level	[88, 89]
	FOXO3a	↑ Transcriptional activity	[90]
	HIF1 $\alpha$	↓ Transcriptional activity	[91]
SIRT3 (mitochondria)	SOD2	↑ Enzyme activity	[92, 93]
	OTC	↑ Enzyme activity	[94]
	NDUFA9	↑ Enzyme activity	[95]
	GDH	↑ Enzyme activity	[96]
	IDH2	↑ Enzyme activity	[97]
SIRT4 (mitochondria)	GDH	↓ Enzyme activity	[98]
SIRT5 (mitochondria)	CPS1	↑ Enzyme activity	[99]
SIRT6 (mitochondria)	HIF1 $\alpha$	↓ Transcriptional activity	[100]
SIRT7 (nucleoli)	p53	Mediates transcriptional activity, depending on SIRT7 expression level	[101]

CPS1: carbamoyl phosphate synthetase 1; eNOS: endothelial nitric oxide synthase; FOXO3a: Forkhead box O3 a; GDH: glutamate dehydrogenase; HIF1 $\alpha$ : hypoxia-inducible factor 1, alpha subunit; HIF2 $\alpha$ : hypoxia-inducible factor 2, alpha subunit; IDH2: isocitrate dehydrogenase 2; NDUFA9: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9; OTC: ornithine transcarbamylase; PGC-1 $\alpha$ : Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; SOD2: superoxide dismutase 2.

transcribed and spliced, but also correctly transported along axons to neuromuscular junctions (NMJ). While there is no clear demonstration of the presence of mRNAs at the NMJs yet, this process (at least in ALS) might result from the known alterations in axonal transport that precedes onset of symptoms [102].

However, one form or the other of alteration of RNA expression may have different weight in different MNDs and, most importantly, RNA dysmetabolisms may be a primary event (for instance in SMA or in TDP43- and FUS/TLN1-linked ALS) or dysregulation of components of the genetic machinery (the HATs/HDACs system, transcription factors, the splicing complex) may be secondary to oxidative stress or energy failure. In turn, which step is the primary site of damage may dictate the severity of disease (age of onset, progression), and which cell type beside motor neurons is primarily affected may dictate the form of MND. This field surely deserves further investigation aimed to the individuation of novel therapeutic approaches for MNDs.

## Abbreviations

ALS: Amyotrophic lateral sclerosis  
 ALS2: Alsin  
 ANG: Angiogenin  
 AR: Androgen receptor  
 CHMP2B: Charged multivesicular protein 2B

COXVb: Cytochrome c oxidase subunit Vb  
 DAO: D-amino acid oxidase  
 DNMT: DNA methyltransferase  
 ER: Endoplasmic reticulum  
 FIG4: PI(3,5)P(2)5-phosphatase  
 FUS/TLN1: Fused in sarcoma/translocated in liposarcoma  
 GLE1: Nucleoporin GLE1  
 GSH: Glutathione  
 HAT: Histone acetyl transferase  
 HDAC: Histone deacetylase  
 HMT: Histone methyltransferase  
 LCCS: Lethal congenital contracture syndrome  
 MND: Motor neuron disease  
 nAChR: Neuronal nicotinic acetylcholine receptor  
 OPTN: Optineurin;  
 PBP: Progressive bulbar palsy  
 PLS: Primary lateral sclerosis  
 PMA: Progressive muscular atrophy  
 PRMT5: Protein arginine methyltransferase 5  
 PRMT7: Protein arginine methyltransferase 7  
 ROS: Reactive oxygen species  
 SAHA: Suberoylanilide hydroxamic acid  
 SAM: S-adenosylmethionine  
 SBMA: Spinal and bulbar muscular atrophy  
 SETX: Senataxin  
 Sig-1R Sigma: 1 receptor

SIRT:	Sirtuin
SMA:	Spinal muscular atrophy
SMN:	Survival motor neuron
SOD1:	Cu, Zn superoxide dismutase
SPG11:	Spataxin
TDP-43:	TAR DNA-binding protein 43
TF:	Transcription factor
UBQLN2:	Ubiquilin 2
UPR:	Unfolded protein response
VAPB:	VAMP-associated protein
VCP:	Valosin-containing protein.

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## Review Article

# Mitochondrial Stress Signalling: HTRA2 and Parkinson's Disease

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Mitochondria are cellular energy generators whose activity requires a continuous supply of oxygen. Recent genetic analysis has suggested that defects in mitochondrial quality control may be key factors in the development of Parkinson's disease (PD). Mitochondria have a crucial role in supplying energy to the brain, and their deterioration can affect the function and viability of neurons, contributing to neurodegeneration. These organelles can sow the seeds of their own demise because they generate damaging oxygen-free radicals as a byproduct of their intrinsic physiological functions. Mitochondria have therefore evolved specific molecular quality control mechanisms to compensate for the action of damaging agents such as oxygen-free radicals. PTEN-induced putative kinase 1 (PINK1) and high-temperature-regulated A2 (HTRA2), a mitochondrial protease, have recently been proposed to be key modulators of mitochondrial molecular quality control. Here, we review some of the most recent advances in our understanding of mitochondria stress-control pathways, focusing on how signalling by the p38 stress kinase pathway may regulate mitochondrial stress by modulating the activity of HTRA2 via PINK1 and cyclin-dependent kinase 5 (CDK5). We also propose how defects in this pathway may contribute to PD.

## 1. Introduction

In evolutionary terms, the increase in energetic demands resulting from the evolution of small, prokaryotic organisms to larger, eukaryotic cells was achieved through the internalisation of energy-generating factories that ultimately led to the emergence of modern mitochondria [1]. Such an increase in energy output also resulted in detrimental consequences because these organelles are the main intracellular sources of damaging oxygen-free radicals. These reactive oxygen species (ROS) can be destructive, attacking various cellular components, including DNA, proteins, lipids, and carbohydrates, but can also act as regulators of intracellular signalling pathways [2]. Eukaryotic cells have evolved strategies to cope with the damage caused by excess levels of damaging agents such as ROS. Recent findings from genetic studies suggest that the defective sensing of mitochondrial damage may play an important role in the development of neurodegenerative diseases such as Parkinson's disease (PD) (reviewed in [3]). An effective response to such damage can be described in

three different steps: (1) damaged components need to be recognised by sensing mechanisms, (2) the sensing mechanisms must convey a signal to damage suppressors, and (3) the activity of damage suppressors must be increased to promote the disposal of damaged cellular components.

In this paper, we provide an overview of the research that focuses on how pathways affecting mitochondrial quality control may play a role in the aetiology of PD. We place particular emphasis on a recently identified mitochondrial damage response pathway regulated by p38 kinase and summarise some of the recent molecular determinants of mitochondrial quality control regulated by this kinase.

## 2. Parkinson's Disease and Mitochondrial Dysfunction

PD is a common neurodegenerative disease characterised by the progressive loss of dopaminergic neurons in the nigrostriatal region of the brain. Most PD cases occur sporadically

(i.e., they are of unknown cause). However, 10–15% of PD patients have a family history of the disease, indicating that there is a strong genetic basis for this disease in this subgroup. The molecular pathogenesis of sporadic PD and the basis for selective dopaminergic neuron loss remain unknown, and it is unclear whether gene mutations are involved in the development of this disease in sporadic PD patients. Epidemiological studies consistently link exposure to pesticides to a higher incidence of PD. In particular, pesticides that cause an increase in ROS, such as rotenone and paraquat, have been shown to cause PD-like conditions in rodent models [4]. In addition, the mitochondrial toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was shown to be responsible for the onset of severe PD-like symptoms in a group of young drug users in the 1980s. Drugs such as rotenone, paraquat, and MPTP disrupt normal electron transport chain (ETC) in the mitochondria, leading to an increase in free radical generation. Through the generation of ROS, exposure to mitochondria-damaging agents may be important in the aetiology of PD in sporadic patients. Although mitochondrial dysfunction has been inconclusively linked to PD in the past few decades, genetic evidence indicating mitochondrial involvement in this disease was recently obtained. A major advance occurred in a recent study in which researchers identified disease-causing *PINK1* mutations in familial PD [5]. Mutations in the mitochondrial serine protease *HTRA2* were also reported to be associated with PD in sporadic patients [6]; however, the role of *HTRA2* in PD remains controversial [7].

### 3. Mitochondria as the Major Source of Intracellular ROS

Mitochondria are the powerhouses of eukaryotic cells and are responsible for most of the ATP synthesis via oxidative phosphorylation (OXPHOS). During OXPHOS, NADH, and FADH<sub>2</sub> produced by glycolysis and the tricarboxylic acid (TCA) cycle are used as electron donors and transported through the ETC via a series of redox reactions that involve four molecular complexes (Complexes I, II, III, and IV). Oxygen is used as the final electron acceptor and is reduced to water through the acquisition of four electrons. The transport of electrons through the ETC is coupled to the discharge of protons from the mitochondrial matrix to the intermembrane space. The discharge of protons leads to the generation of a proton gradient that is necessary for the synthesis of ATP from ADP by ATP synthase. During transport through the ETC, a small portion of electrons (1–3%) escape prematurely, mainly from Complexes I and III, and directly reduce oxygen, generating the superoxide anion (O<sub>2</sub><sup>•-</sup>). These ROS can interact with other molecules to form other types of ROS, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and the hydroxyl radical (OH<sup>•</sup>). In addition to the ETC, other mitochondrial components are known to generate ROS, such as  $\alpha$ -ketoglutarate dehydrogenase ( $\alpha$ -KGDH) and pyruvate dehydrogenase, which generate both the superoxide anion (O<sub>2</sub><sup>•-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) [8, 9]. In addition to the physiological ROS produced by normal mitochondrial activity, nonphysiological increases in ROS levels can

occur in conditions of stress, such as nutrient deprivation or hypoxia, or as a consequence of the deterioration of mitochondrial enzymes. The increase in ROS concentration, a condition known as oxidative stress, can be detrimental for the mitochondria and the entire cell because of the capacity of ROS to damage several cellular components, including proteins, lipids, and nucleic acids. To cope with the detrimental effects of ROS, mitochondria are equipped with several antioxidant systems that comprise a first line of defence against these toxic agents. Of these systems, the most important is manganese SOD (Mn-SOD). Mn-SOD is a highly efficient enzyme ( $K_{\text{cat}} \sim 10^9 \text{ M}^{-1} \text{ sec}^{-1}$ ) localised to the mitochondrial matrix that can quickly dismutate superoxide anions (O<sub>2</sub><sup>•-</sup>) to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and molecular oxygen (O<sub>2</sub>). In addition to Mn-SOD, mitochondrial antioxidant defences include the thioredoxin-2 (Trx2) system, formed by Trx2 and thioredoxin reductase 2 (TrxR2), and peroxiredoxin-3 (Prx3); both are involved in the scavenging of hydrogen peroxide (reviewed in [10]).

### 4. Mitogen-Activated Protein Kinases Are Mediators of ROS-Linked Signal Transduction

Paradoxically, even though mitochondria-generated ROS are clearly viewed as damaging agents, they can also play an active role in intracellular signalling. Hydrogen peroxide, but not the superoxide anion, can easily cross the mitochondrial membranes and diffuse into the cytosol, where it can negatively affect the structure and function of proteins by the specific oxidation of reactive cysteine residues to sulphenic acid (–SOH) (reviewed in [11]). Sulphenic acid is a very reactive and unstable chemical and, together with ROS, can further react with a second molecule of H<sub>2</sub>O<sub>2</sub> to form a sulfinic acid derivative (–SO<sub>2</sub>H). Oxidation to sulfinic acid is, with some exceptions, an irreversible modification that can permanently alter the structure and function of a protein and can ultimately result in cellular damage and death. Under physiological conditions, sulphenic acid derivatives can be reduced back to the thiolate form or transformed into a number of thiol adducts by reactions such as S-glutathionylation to form protein-GSH mixed disulphide, a stable and reversible oxidation state that can be efficiently reduced back by reactions catalysed by Trx and peroxiredoxins. The reversible oxidation of cysteines by ROS, in particular H<sub>2</sub>O<sub>2</sub>, allows these molecules to be classified as second messengers, which lead to the activation of signalling pathways. Of these, pathways mediated by mitogen-activated protein kinases (MAPKs) are some of the best studied and characterised.

MAPKs are a family of evolutionarily conserved serine/threonine kinases involved in the regulation of several cellular processes, such as growth, differentiation, and apoptosis. A typical MAPK cascade includes a MAPK kinase kinase (MAP3K) that phosphorylates a MAPK kinase (MAP2K), which in turn phosphorylates a MAPK. Active MAPKs, through direct phosphorylation, regulate the activity of many cytoplasmic and nuclear targets. The switch from a

redox signal to a phosphorylation cascade can occur at different levels of the MAPK pathway, such as at the level of the MAP3Ks, some of which (e.g., ASK1 and MEKK1) are redox-sensitive proteins. ASK1, in particular, plays a key role in the cellular response to oxidative stress because it can activate both JNK and p38 pathways through the phosphorylation of their upstream kinases MKK4/7 and MKK3/6, respectively [12]. The activation of ASK1 is regulated by its redox-sensitive binding with thioredoxin-1 (Trx1), an antioxidant protein involved in the reduction of disulphide bonds. Under physiological conditions, one of the two cysteines in the active site of Trx1 binds and inactivates ASK1; when oxidative stress occurs, the Trx1 cysteines form an intramolecular disulphide bond, and the protein loses its interaction with ASK1, which becomes active and can activate downstream factors [13, 14]. The ultimate effect of MAPK activation can range from cell proliferation to cell death and is strongly influenced by the duration and magnitude of their direct phosphorylation. MAPK phosphorylation depends on an equilibrium state between the activity of upstream kinases and specific MAPK phosphatases (MKPs). MKPs are a group of protein phosphatases, including tyrosine, serine/threonine, and dual-specificity MAPK phosphatases, that dephosphorylate MAPKs and block the signalling pathways on which they depend. ROS, in addition to being able to promote MAPK phosphorylation, are known to inhibit the activity of MKPs through the reversible oxidation of their reactive cysteine residues, thus contributing to a prolonged activation of MAPKs [15]. There are three main MAPK cascades in mammals: those mediated by the extracellular signal-regulated kinases (ERK1/2), the c-Jun NH<sub>2</sub>-terminal kinases or stress-activated kinases (JNK/SAPK) and p38. The ERK cascade is mainly involved in the control of cell proliferation and differentiation, whereas the p38 and JNK pathways are implicated in the control of cell survival and cell death because they are activated by environmental stresses, which are often associated with the generation of ROS [16, 17].

## 5. Modulation of Quality Control in Mitochondria

Numerous findings have suggested that disruptions in mitochondrial function and dynamics contribute to ageing and neurodegenerative diseases (reviewed in [22]). Cells have therefore developed molecular mechanisms to cope with the diverse challenges imposed on mitochondrial integrity. Mitochondria are thought to have at least two levels of defence mechanisms that ensure their integrity and viability in individual cells (reviewed in [23]). The first line of defence comprises highly specific molecular quality control machinery, including molecular chaperones and proteases that monitor the folding and assembly of mitochondrial proteins. Interestingly, both PINK1 and HTRA2 seem to be important modulators of molecular quality control in mitochondria. The deletion of *HTRA2* from mice results in an increase in ROS levels and an accumulation of misfolded proteins in brain mitochondria [24], and an analysis of postmortem brain tissue obtained from PD patients with

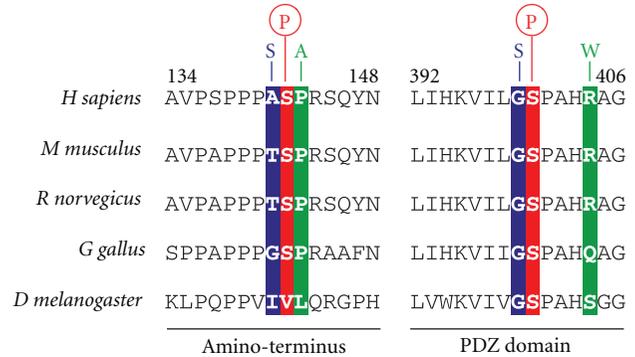


FIGURE 1: Mutations in *HTRA2* associated with Parkinson's disease lie in close proximity to p38 and CDK5 phosphorylation sites. Mutations in the amino-terminus (A141S [6] and P143A [18]) and the PDZ domain (G399S [6] and R404W [19]) are indicated. The relative positions of the phosphorylation sites in human *HTRA2* (see [20, 21]) are indicated by the circles above the serine residues. A sequence alignment with *HTRA2* orthologues from several species is shown.

mutations in *PINK1* revealed an increase in the levels of misfolded mitochondrial respiratory complexes in the brain [25].

Once mitochondrial molecular quality control is overwhelmed, a second line of defence, termed organellar quality control, is thought to take over. Organellar quality control relies on the dynamic nature of mitochondrial populations to ensure the disposal of defective mitochondrial components via mitochondrial fission and autophagy (reviewed in [3]). Mitochondrial dynamics are thought to be important for the control of mitochondrial turnover and bioenergetic efficiency. The combined functions of fusion, fission, and autophagy are now emerging as essential organellar quality control mechanisms that promote the sequestration, sorting, and elimination of functionally impaired mitochondria [26]. *PINK1* seems to play a key role in organellar quality control. *PINK1* is capable of recruiting parkin, a cytosolic ubiquitin ligase, to damaged mitochondria and targeting these organelles for autophagic clearance [27].

If both molecular and organellar quality control mechanisms fail, severe mitochondrial damage can lead to the uncontrolled release of mitochondrial proteins including cytochrome c. Once it reaches the cytosol, cytochrome c unleashes the apoptosis pathway of cell death (reviewed in [28]).

It is conceivable that a failure of either one of these quality control mechanisms in mitochondria ultimately results in the demise of dopaminergic neurons observed in PD and therefore plays a causative role in this disease.

## 6. Activation of Mitochondrial Damage Responses by the p38 Stress Kinase

As stated above, it is conceivable that upon mitochondrial damage, intracellular mechanisms act to convey a signal to damage suppressors to attempt to counteract such damage. In this context, recent evidence suggests that the p38 stress

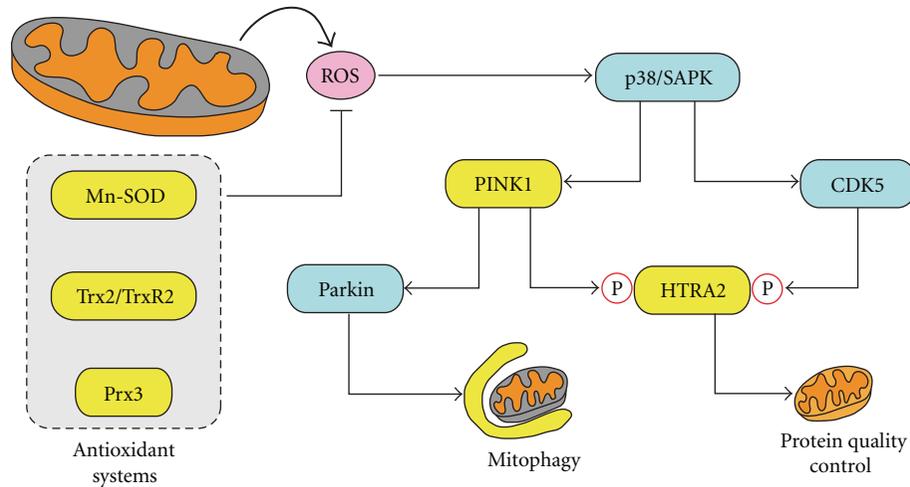


FIGURE 2: Modulation of mitochondrial quality control by the p38 stress kinase. When mitochondrial stress occurs, ROS production results in the activation of p38. Through its downstream effectors, PINK1 and CDK5, this pathway results in an increase in the proteolytic activity of mitochondrial HTRA2, which in turn contributes to the suppression of mitochondrial damage by enhancing protein quality control. By promoting the recruitment of cytosolic parkin to damaged mitochondria, PINK1 can also contribute to the clearance of these organelles through mitophagy. Antioxidant systems modulate the levels of ROS and can therefore affect signalling from p38 to either PINK1 or CDK5. Yellow: mitochondrial proteins; cyan: cytosolic proteins.

kinase may fit the role of such an intracellular sensor mechanism by conveying signals to mitochondrial proteins, such as the putative kinase PINK1 and the protease HTRA2, to activate mitochondrial quality control defence mechanisms. Disease-causing mutations in *PINK1* are linked to familial PD [5], whereas *HTRA2* mutations were reported to be present in sporadic PD patients [6, 19].

A role for HTRA2 as a proapoptotic factor was initially described by several groups [29–32]. However, more recently, *in vivo* studies in mice with a loss-of-function mutation in the *HTRA2* gene (S276C) and in *HTRA2* knockout mice showed that these animals are characterised by a lethal neurodegenerative disorder [33, 34] with an accumulation of unfolded proteins in the mitochondria [24]. This indicates that the activity of mitochondrial HTRA2 protease may be important for controlling the levels of misfolded proteins in mitochondria in a manner similar to its bacterial homologues DegP and DegS [35, 36]. As with many other proteases, the proteolytic activity of HTRA2 is tightly regulated to prevent unwanted proteolysis. Structural studies have shown that interactions between its protease domain and its regulatory PDZ domain keep the proteolytic activity of HTRA2 in check until the PDZ domain is engaged by binding to C-terminal PDZ-binding peptides or internal hydrophobic stretches in misfolded proteins [35, 36]. The activation of HTRA2 has also been shown to occur via the direct phosphorylation of S142 through an interaction with PINK1 [20]. This PINK1-mediated phosphorylation promoted by p38 results in an increase in HTRA2 proteolytic activity, which, in turn, increases its protective effects in mitochondria.

More recently, a novel phosphorylation site (S400) has been identified in HTRA2 [21]. This site lies in the PDZ domain of the protease, a region known to modulate its protease function [37], which may also be involved in

neuroprotection [20]. Phosphorylation of HTRA2 at S400 is promoted by CDK5, a serine/threonine kinase that is a member of the highly conserved family of cyclin-dependent kinases. This kinase is unique among its family members because it is neither activated by cyclins nor regulates the cell cycle (reviewed in Dhavan and Tsai [38]). Mice lacking *CDK5* die prematurely and demonstrate a disruption in neuronal layering [39]. Abnormal *CDK5* activity is associated with several neurodegenerative diseases. In particular, *CDK5* accumulates in neurons in Lewy bodies, the principal hallmark of PD. *CDK5*-dependent HTRA2 phosphorylation via the p38 pathway is involved in maintaining the mitochondrial membrane potential under stressful conditions and results in protection against cellular stress [21].

Curiously, the mutations in the *HTRA2* gene found in PD patients seem to lead to amino acid changes in residues that lie in close proximity to the identified phosphorylation sites in this protease. Both the A141S [6] and the P143A [18] mutations found in PD patients are near S142, whereas G399S [6] and R404W [19] are near the S400 phosphorylation site (Figure 1). These findings suggest that such mutations may affect the phosphorylation status of HTRA2 and therefore have a detrimental role in the activation of this enzyme downstream of p38 signalling.

## 7. Concluding Remarks

Taken together, the findings outlined in this paper are suggestive of a role for the p38 pathway in modulating molecular quality control in mitochondria. It is conceivable that following mitochondrial stress, mild ROS production results in the activation of a mitochondrial damage-sensing mechanism involving p38. This activation conveys a signal to downstream damage suppressors via PINK1 and CDK5, resulting in an increase in the proteolytic activity

of mitochondrial HTRA2, which then contributes to the suppression of mitochondrial damage by increasing the disposal of damaged mitochondrial components, such as misfolded proteins (Figure 2). Failure to activate such a pathway would result in an accumulation of mitochondrial damage and perhaps result in overwhelming levels of ROS. Such high levels of ROS would ultimately lead to the loss of integrity of the mitochondrial membranes, causing the release of proapoptotic proteins and eventual cell death.

Clearly, protein misfolding plays an important role in the development of PD. Although clear mechanisms for such protein misfolding pathologies are well established when accumulation occurs in the cytosol, cell nucleus, endoplasmic reticulum, and extracellular space, little is known about any causative role of protein aggregation in the mitochondria in PD.

Neurodegenerative diseases are a group of adult-onset pathologies of increasing clinical interest, considering the growing number of new cases each year and the increasing human lifespan, as age is one of the main risk factors. After many years of research, the crucial role of mitochondria in neurodegeneration has been established and is now widely accepted. Mutations of mitochondrial proteins or proteins involved in mitochondrial function have been found in familiar cases of such diseases, but the role of many of those proteins is still unclear. For example, HTRA2 is strongly implicated in neurodegeneration, in particular PD, but this has not been accompanied by a complete characterisation of its activity. A deeper comprehension of the role of the proteins involved in the control of mitochondrial homeostasis could provide better knowledge of the molecular mechanisms underlying the development of neurodegenerative disorders associated with mitochondrial dysfunction and could help the development of novel strategies able to effectively block the course of the disease.

## Abbreviations

ROS:	Reactive oxygen species
OXPPOS:	Oxidative phosphorylation
ETC:	Electron transport chain
ATP:	Adenosine triphosphate
NAD:	Nicotinamide adenine dinucleotide
FAD:	Flavin adenine dinucleotide
TCA:	Tricarboxylic acid
SOD:	Superoxide dismutase
MPTP:	1-Methyl-4-phenyl-1,2,3,6 tetrahydropyridine.

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## Research Article

# Early Decrease in Respiration and Uncoupling Event Independent of Cytochrome *c* Release in PC12 Cells Undergoing Apoptosis

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Cytochrome *c* is a key molecule in mitochondria-mediated apoptosis. It also plays a pivotal role in cell respiration. The switch between these two functions occurs at the moment of its release from mitochondria. This process is therefore extremely relevant for the fate of the cell. Since cytochrome *c* mediates respiration, we studied the changes in respiratory chain activity during the early stages of apoptosis in order to contribute to unravel the mechanisms of cytochrome *c* release. We found that, during staurosporine (STS)-induced apoptosis in PC12 cells, respiration is affected before the release of cytochrome *c*, as shown by a decrease in the endogenous uncoupled respiration and an uncoupling event, both occurring independently of cytochrome *c* release. The decline in the uncoupled respiration occurs also upon Bcl-2 overexpression (which inhibits cytochrome *c* release), while the uncoupling event is inhibited by Bcl-2. We also observed that the first stage of nuclear condensation during STS-induced apoptosis does not depend on the release of cytochrome *c* into the cytosol and is a reversible event. These findings may contribute to understand the mechanisms affecting mitochondria during the early stages of apoptosis and priming them for the release of apoptogenic factors.

## 1. Introduction

Mitochondria play a key role in apoptosis triggered by a wide variety of stimuli since they release important proapoptotic factors from their intermembrane space. The first mitochondrial apoptogenic molecule discovered was the hemoprotein cytochrome *c* [1]. Cytochrome *c* is involved in two critical cell processes. Normally, it acts as mobile electron carrier shuttling electrons between ubiquinol cytochrome *c* oxidoreductase (complex III) and cytochrome *c* oxidase (complex IV) of the respiratory chain allowing cell life. On the other hand, upon apoptotic induction, cytochrome *c* is released from mitochondria in the cytosol where it carries out a proapoptotic function by binding the adapter protein apoptosis protease-activating factor-1 (Apaf1). Consequently, it promotes, in presence of ATP/dATP, the assembly of the multiproteic complex apoptosome, which binds and activates the caspase-9, thereby initiating the activation of a caspase cascade which leads to apoptotic cell death [2, 3].

Mitochondrial outer membrane permeabilization (MOMP) and cytochrome *c* release from mitochondria during apoptosis are tightly regulated by the proteins of the Bcl-2 family. This family of proteins includes both anti-apoptotic members (e.g., Bcl-2 and Bcl-XL) repressing MOMP and release of apoptogenic factors from mitochondria and pro-apoptotic members promoting MOMP (e.g., Bax and Bak, Bid) [4, 5]. However, the detailed mechanisms of cytochrome *c* release are still unclear.

The phospholipid cardiolipin (CL) seems to have a key role in the process of the cytochrome *c* release [6]. Only 15% of cytochrome *c* is free in the intermembrane space, while most of it is attached to the outer leaflet of the mitochondrial inner membrane with the mitochondrion-specific anionic phospholipid CL. It has been recently found that posttranslational modifications or interaction with hydrophobic anions such as CL causes the activation of cytochrome *c* into a peroxidase with selective catalytic competence toward CL [7, 8]. Cytochrome *c* tightly bound to CL was proposed to possess

this peroxidase activity and to catalyze CL peroxidation, most likely by utilizing the high production of reactive oxygen species (ROS) generated in the mitochondria during the first stages of apoptosis. Cytochrome *c* has a lower affinity for peroxidized CL, and peroxidation of CL enables the dissociation of cytochrome *c* from mitochondrial inner membrane allowing the release of cytochrome *c* from mitochondria. CL oxidation is, indeed, mandatory for the release of cytochrome *c* [6]. However, the final release of cytochrome *c* requires additional steps such as the permeabilization of the outer membrane. CL is also involved in mitochondrial outer membrane permeabilization since it enables docking and activation of some pro-apoptotic Bcl-2 proteins [9–11].

These findings strongly suggest an active role of the membrane in modulating MOMP that has been underestimated so far. Cytochrome *c* is part of the respiratory chain whose complexes are located across the mitochondrial inner membrane, and membrane integrity greatly influences the respiratory activity of the cells. Based on this considerations, we analyzed the respiratory changes during apoptosis and their temporal relationship with the release of cytochrome *c* in order to get information useful to unravel the mechanisms of release of this hemoprotein from mitochondria. We therefore analyzed the respiratory activity of a rat cell line of neuronal derivation (pheochromocytoma-12, PC12 cells) by polarographic measurement of oxygen consumption in intact cells [12]. The PC12 line was originally cloned from a transplantable rat adrenal medullary pheochromocytoma. This cell line manifests many features of sympathicoblasts, the cells that give rise to postmitotic sympathetic neurons. Indeed, they respond to nerve growth factor (NGF) by shifting to a nonproliferating neurite-bearing phenotype and acquiring many of the properties characteristic of sympathetic neurons among which electrical excitability. For this reason the clonal PC12 cell line is widely used for various studies on neuronal cell differentiation and function. During staurosporine- (STS-) induced apoptosis in naïve PC12 cells, we observed an uncoupling event preceding the reduction of cytochrome *c* oxidase- (COX-) respiratory activity. Our investigation has also revealed different kinetics of decrease in 2,4-dinitrophenol- (DNP-) uncoupled and COX-dependent respiration with the former showing, at very early stage, a faster kinetics of decrease compared with the latter. This suggests an effect of STS on the respiratory activity, which is independent of cytochrome *c* release. This hypothesis is confirmed by our finding that overexpression of Bcl-2 protects from release of cytochrome *c* and massive decrease in respiration, while it has no effect on the early decrease in DNP-uncoupled respiration induced by STS.

## 2. Results

**2.1. Measurement of Respiration in Intact STS-Treated PC12 Cells Early Decrease in DNP-Uncoupled Respiration.** Since the cytochrome *c* is part of the respiratory chain as the electron donor for complex IV, it is expected that its release from mitochondria during apoptosis would cause changes in the respiratory activity of the cells. We analyzed the nature and

the extent of these changes by measuring the rate of oxygen consumption in naïve PC12 cells and in naïve PC12 cells undergoing apoptosis by treatment with the protein kinase inhibitor STS [13, 14].

We measured the oxygen consumption of the cells (endogenous respiration), the oxygen consumption in presence of the uncoupler DNP (DNP-uncoupled respiration, which represents the highest endogenous respiration rate that cells can reach being free of control by the proton gradient) and the rate of respiration measured in presence of the complex III inhibitor antimycin A, the artificial membrane-permeant electron donor TMPD (N,N,N',N'-tetramethyl-1,4-phenylendiamine), and the reducing agent ascorbate that maintains TMPD in a reduced state (TMPD-dependent respiration) [12]. Since antimycin A inhibits complex III and blocks the electron flux upstream of COX, the TMPD-dependent respiration provides a measure of the COX and cytochrome *c*-dependent oxygen consumption independently of the upstream segment of the respiratory chain [15]. The respiration of both untreated and STS-treated PC12 cells resulted being 100% antimycin A-sensitive (data not shown). Upon treatment with STS we observed a time-dependent decrease in the endogenous, DNP-uncoupled and TMPD-dependent respiration rates. After 5 hr of treatment with STS, the DNP-uncoupled respiration rate decreased to ~45% of the DNP-uncoupled respiration rate of untreated cell and to ~28,8% after 9 hr of treatment (Figures 1(a) and 2(b)). The oxygen consumption relative to prolonged period of STS treatment (9 h and 24 h) is overestimated since, being very light, it is difficult to collect all dying cells with the usual centrifugation speed; and it is not advisable to use higher speed since it would affect the respiration of alive cells.

The kinetics of decrease in TMPD-dependent COX respiration rate during STS-treatment was similar to the kinetics of decrease in endogenous and DNP-uncoupled respiration rates. However, very interestingly, by focusing on the first few hours of treatment, we observed that, the decrease in DNP-uncoupled respiration rate was significantly faster compared with the COX-dependent respiration rate decrease. In fact, after 1 hr of STS treatment, the TMPD-dependent respiration rate was decreased only by 4% relative to the rate of untreated cells, while the DNP-uncoupled respiration rate was decreased by 15% (Figure 1(b)). This difference remains constant with increasing time of STS-treatment. Since the COX-dependent respiration depends directly on cytochrome *c* and decreases less than the overall uncoupled respiration, this means that the uncoupled respiration is affected by some other modifications than the absence of cytochrome *c*. This finding suggests therefore that there is an impairment of the mitochondrial respiration soon after the apoptotic induction that is not due to the release of cytochrome *c*.

**2.2. Temporal Correlation between Respiration Changes, Cytochrome *c* Release and Nuclear Fragmentation during STS-Induced Apoptosis; Uncoupled Respiration Drop Precedes Cytochrome *c* Release.** To better understand the temporal correlation between decrease in respiration and cytochrome *c* release in STS-induced apoptosis we analyzed cytochrome

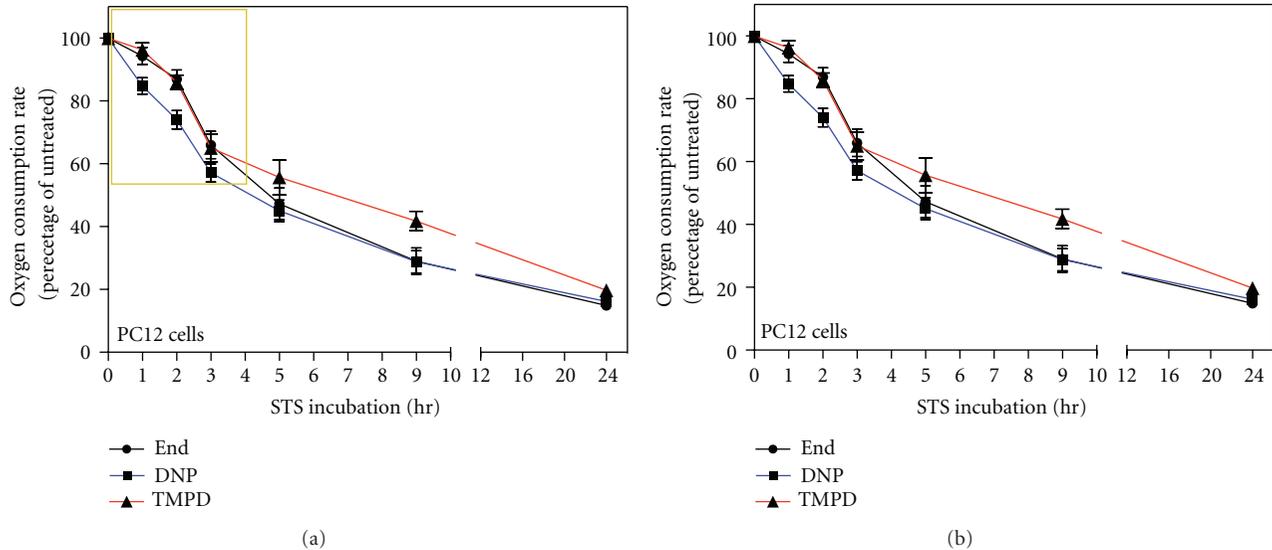


FIGURE 1: Decrease in endogenous, DNP-uncoupled, and TMPD-dependent respiration rates in intact PC12 cells treated with STS; early decrease in DNP-uncoupled respiration. (a) Oxygen consumption rates of  $2\ \mu\text{M}$  STS-treated PC12 cells measured at the indicated time points. Oxygen consumption rates were measured in TD buffer (endogenous respiration, End), in TD buffer containing DNP (DNP-uncoupled respiration, DNP) and in TD buffer containing DNP, antimycin A, ascorbate, and TMPD (TMPD-dependent respiration, TMPD). Data are expressed, at each time point, as percentages of the oxygen consumption of untreated cells (0 hr). To reduce the variability of the experiments we calculated the percentages with respect to the control of that particular experiment, and then we averaged the percentages. Data are expressed as means  $\pm$  SE (standard error) with  $n$  varying at each time point and being 31, 16, 9, 6, 7, 2, and 7, respectively, for the indicated time points (1 h, 2 h, 3 h, 5 h, 9 h, 24 h). (b) Magnification of (a) area surrounded by a yellow frame. Note that the ordinate scale starts at 50%.

*c* localization by confocal immunofluorescence microscopy.  $2\ \mu\text{M}$  STS-treatment of PC12 cells promoted nuclear fragmentation (Figure 2(a), STS 3 h; white arrow) and cytochrome *c* release from mitochondria as clearly resulted from the different immunolocalization of the mitochondrial marker Hsp60 and cytochrome *c* (Figure 2(a), STS 3 h; yellow arrows). Cell death was detected by DAPI staining and morphological analysis of nuclei. Figure 2(a) (white arrow) shows an example of cell considered apoptotic; it is shrunk and has a fragmented nucleus. By analyzing several fields, we calculated the percentage of cells showing cytochrome *c* release and the percentage of cells showing nuclear fragmentation (cell death) at each time point in order to compare these kinetics with the changes in respiration. Since cytochrome *c* release from mitochondria during apoptosis precedes nuclear fragmentation, we detected cells in which the cytochrome *c* had been released from mitochondria while the nucleus was not yet fragmented (Figure 2(a), STS 3 h; yellow arrowheads) but never the opposite. For this reason, we obtained a kinetics of cytochrome *c* release slightly more rapid than the kinetics of nuclear fragmentation (Figure 2(b)). Notably, once released from mitochondria, cytochrome *c* localizes throughout the cell and, as sometimes reported [16, 17], into the nucleus (Figure 2(a), STS 3 h; yellow arrows).

During the immunofluorescence staining, a high number of late stage apoptotic cells detached from the dish and were lost which means that we run the risk to underestimate their number. To solve this problem we calculated the real percentage of apoptotic cells by collecting all of them, also from

the supernatant, through high speed centrifugation and by staining all nuclei with DAPI. The difference between the percentage of cell death calculated by immunofluorescence on a dish and the one obtained by high speed centrifugation was used to correct the underestimated percentage of cytochrome *c* releasing cells evaluated by means of the immunofluorescence staining; dead cells detaching during the experiment are at the very final stages of apoptosis and have already released cytochrome *c* [18].

We compared these kinetics with the changes in respiration reported in Figure 1, and for this purpose we expressed data shown in Figure 2(b) as percentage of cells with mitochondrially localized cytochrome *c* and percentage of alive cells (Figure 2(c)). We observed that the progressively lower rate of respiration upon STS treatment highly correlates with the decreased number of alive cells due to apoptotic death. Interestingly, the decrease of cells with mitochondrial cytochrome *c* localization highly correlates with the kinetics of COX-respiration decrease, but such a decrease is slower than that observed in DNP-respiration (Figure 2(c)). Such an analysis strengthens the conclusion that the decrease in TMPD-dependent respiration is mainly due to cytochrome *c* release, while the decrease in DNP-uncoupled respiration precedes cytochrome *c* release and must be due to another mechanism of impairment of the respiratory chain system.

2.3. Respiration Rates, Cytochrome *c* Localization, and Nuclear Changes in Bcl-2 Transfected Cells DNP-Dependent Respiration Drop Occurs Also in Absence of Cytochrome *c* Release. To

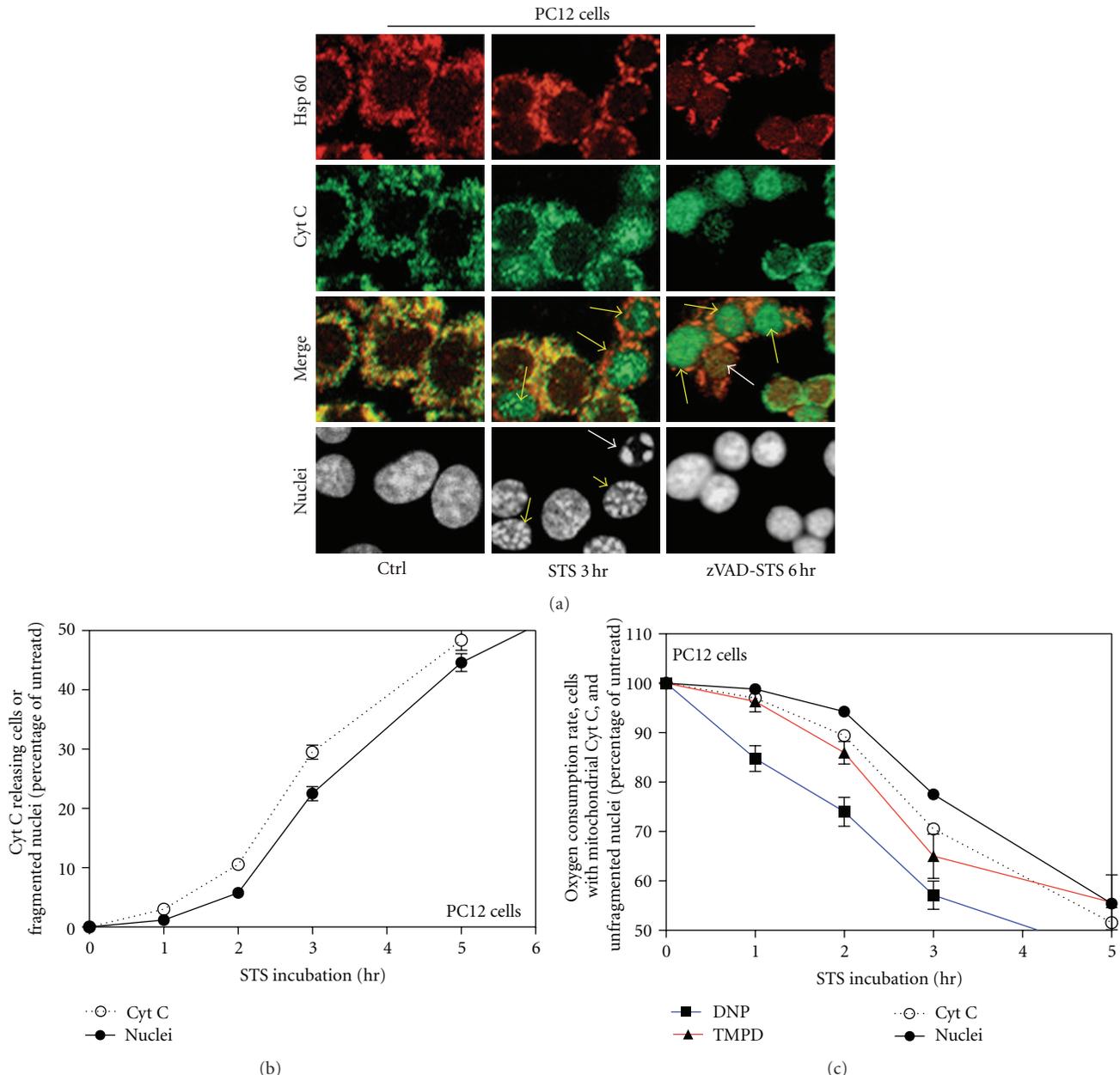
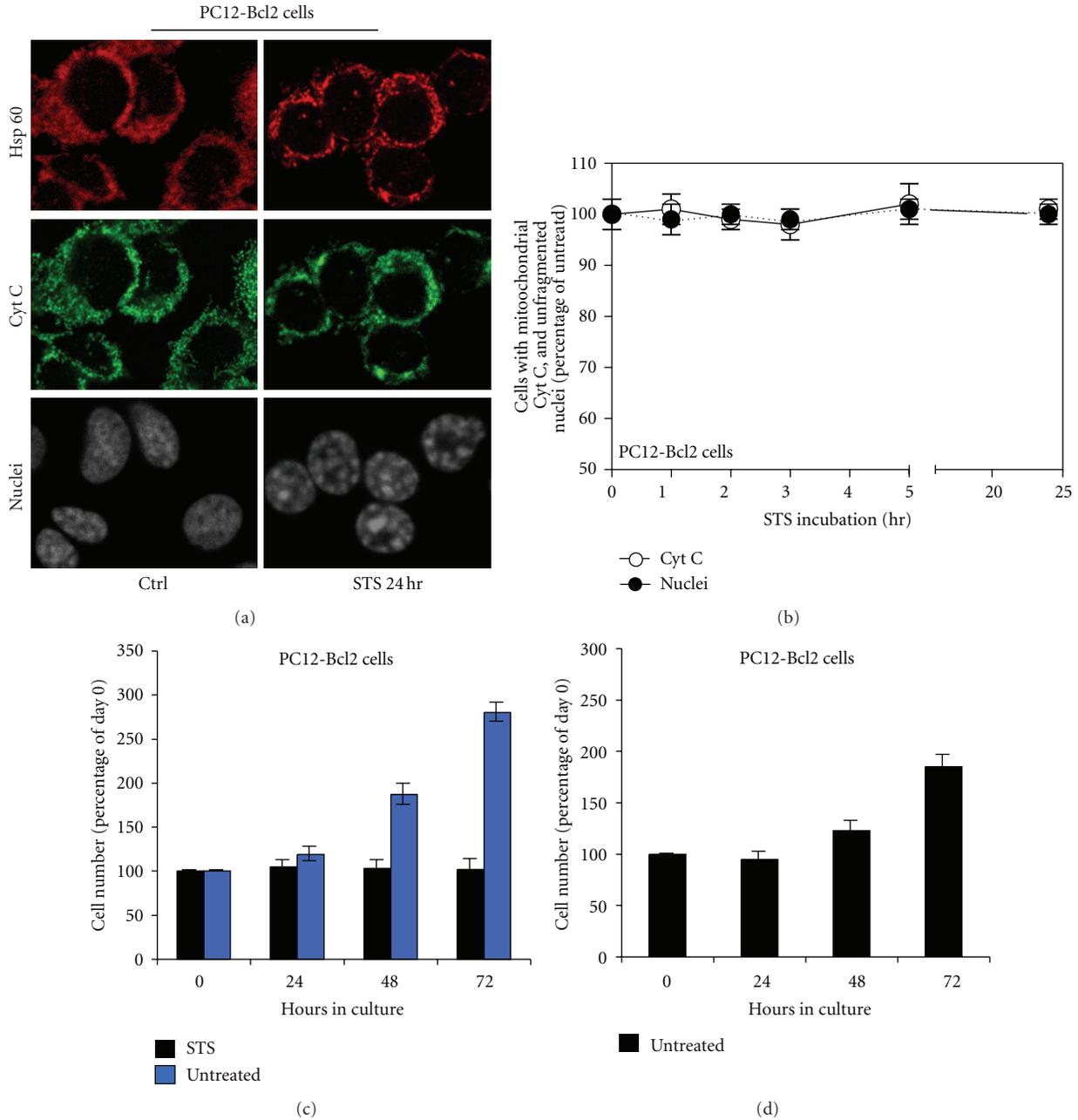


FIGURE 2: Cytochrome *c* release and nuclear apoptosis in STS-treated PC12 cells. (a) Triple labeling confocal immunofluorescence microscopy of cells untreated (Ctrl), 2  $\mu$ M STS-treated for 3 h (STS 3 hr), or 2  $\mu$ M STS-treated for 6 h with 30 min zVADfmk (100  $\mu$ M) pretreatment (zVAD-STS 6 hr). Pattern of Hsp60 (red), cytochrome *c* (green), merged patterns (yellow) and nuclear staining by SYTO-X (grey) of the same representative fields are shown. (b) Percentage of cytochrome *c* releasing and apoptotic PC12 cells treated with STS for the indicated time. Data calculated in 3 independent experiments for each time point. About 1000 cells were considered for each experiment at each time point. Some SE are small and are within the symbols. Note that the ordinate scale starts at 50%. (c) Comparison between data shown in Figure 2(b) but expressed as decrease of cells with mitochondrially localized cytochrome *c* and decrease of alive cells and data regarding decrease in respiration shown in Figure 1(b).

confirm that the early changes in respiration observed during apoptosis were really independent of cytochrome *c* release, we decided to study the respiration of PC12 cells stably overexpressing the apoptotic inhibitor Bcl-2 (PC12-Bcl-2 cells), which is known to block the release of cytochrome *c* from mitochondria [19, 20]. In fact, PC12-Bcl-2 cells treated with 2  $\mu$ M STS do not detach from the dish and do not undergo apoptosis as shown by DAPI staining (Figures 3(a)

and 3(b), nuclei). Moreover, the release of cytochrome *c* from mitochondria is inhibited. Indeed, also after prolonged exposure to STS, cytochrome *c* and Hsp60 maintain the same localization in PC12-Bcl-2 cells (Figures 3(a) and 3(b)).

The analysis of the respiration of these cells (Figure 4(a)) shows that Bcl-2 strongly protects against long-term respiratory decrease. In fact, after 24 h of treatment the uncoupled endogenous respiration decreases to  $\sim$ 44.5% of the rate



**FIGURE 3:** Cytochrome *c* localization and nuclear changes in STS-treated PC12-Bcl-2 cells. (a) Triple labeling confocal immunofluorescence microscopy of PC12-Bcl-2 cells untreated (Ctrl) and 2  $\mu$ M STS-treated for 24 h (STS 24 h). Pattern of Hsp60 (red), cytochrome *c* (green), and nuclear staining by SYTO-X (grey) of the same representative fields are shown. (b) Percentage of PC12-Bcl2 cells with mitochondrially localized cytochrome *c* and percentage of alive PC12-Bcl2 cells treated with STS for the indicated time. Data were calculated in 3 independent experiments for each time point. About 1000 cells were considered for each experiment at each time point. Note that the ordinate scale starts at 50%. (c) For quantification of cell proliferation, the same number of untreated or STS-treated PC12-Bcl2 cells was plated on several dishes and counted at the indicated times. Data were calculated in 3 independent experiments for each time point. (d) For quantification of cell proliferation, the same number of STS-treated PC12-Bcl2 cells were plated on several dishes and counted at the indicated times. At 24 h the medium was replaced in order to remove STS. Data were calculated in 3 independent experiments for each time point.

of untreated cells while in absence of Bcl-2, under STS treatment, respiration decreased to  $\sim$ 16% of the rate of untreated cells (or even less considered the overestimation cited) (Figure 1(a)). The difference observed for the COX-dependent respiration is even greater; indeed, after 24 h of treatment, PC12-Bcl-2 cell respiration decreases only to

$\sim$ 70.7% of the untreated cell rate, while the COX-dependent oxygen consumption of naive PC12 cells falls to  $\sim$ 19.8% of the control. The protection of the respiratory activity by Bcl-2 is mainly due to the fact that Bcl-2 blocks the release of the cytochrome *c* from mitochondria (Figures 3(a) and 3(b)). However, the early decrease in DNP-dependent

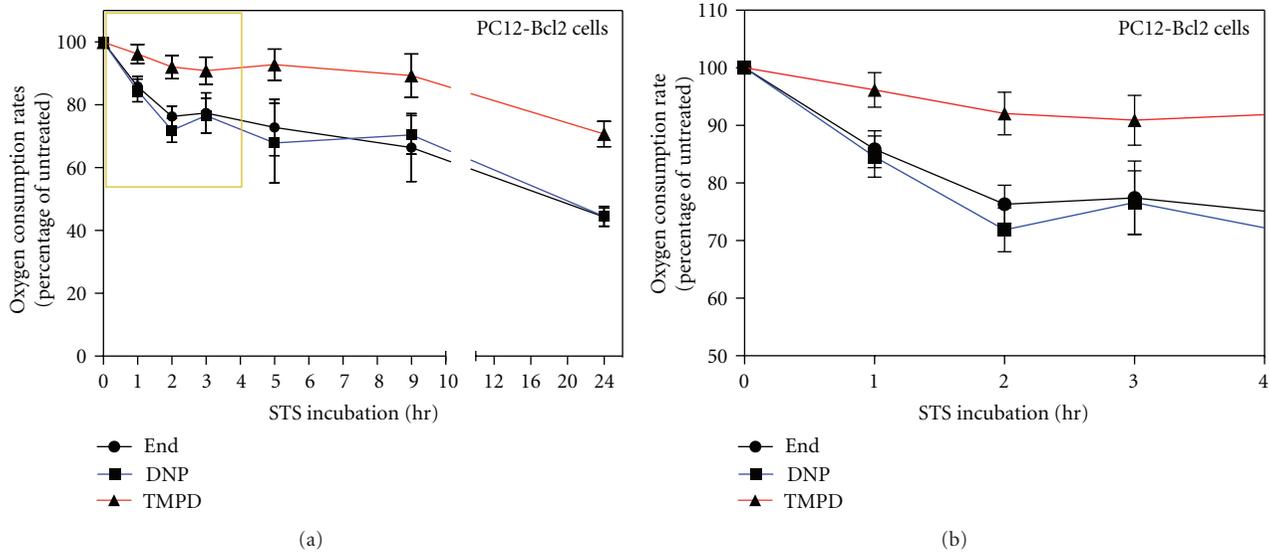


FIGURE 4: Decrease in endogenous, DNP-uncoupled, and TMPD-dependent respiration rates in intact PC12-Bcl-2 cells treated with STS; early decrease in DNP-uncoupled respiration. (a) Oxygen consumption rates of STS-treated PC12-Bcl-2 cells measured at the indicated time points. Oxygen consumption rates were measured in TD buffer (endogenous respiration, End), in TD buffer containing DNP (DNP-uncoupled respiration, DNP) and in TD buffer containing DNP, antimycin A, ascorbate and TMPD (TMPD-dependent respiration, TMPD). Data are expressed, at each time point, as percentages of the oxygen consumption of untreated cells (0 hr). The data represent the means  $\pm$  SE with  $n$  varying at each time point and being 17, 20, 12, 2, 3, 6, 8 respectively for the indicated time points (1 h, 2 h, 3 h, 5 h, 9 h, 24 h). (b) magnification of (a) area surrounded by a yellow frame. Note that the ordinate scale starts at 50%.

respiration that we have found during the first hours of apoptosis occurs also in Bcl-2-overexpressing cells, although cytochrome *c* is not released. Indeed, after 1 h of STS treatment, both in PC12-Bcl-2 and naive PC12 cells, DNP-uncoupled respiration decreases to  $\sim$ 84.6% of the respiration rate of untreated cells, and the COX-dependent respiration decreases only to  $\sim$ 96% of untreated cell respiration. After two hours of STS treatment, the uncoupled endogenous respiration falls to  $\sim$ 72% and  $\sim$ 74% of the control in PC12-Bcl-2 and naive PC12 cells, respectively, while the TMPD-dependent respiration reaches the 86% of the control in naive PC12 cells and remains almost unchanged in PC12-Bcl-2 cells (92%) (Figures 1 and 4).

Since in PC12-Bcl-2 cells cytochrome *c* is not released from mitochondria, this experiment confirms that, in the first phases of apoptosis, the maximum possible respiration rate achievable in the cell decreases and that the respiratory chain is impaired independently of the release of cytochrome *c*.

Moreover, the comparison between the respiration of naive PC12 and PC12 cells overexpressing Bcl-2 also shows that the ratio between the absolute values of DNP-uncoupled and endogenous respiration (DNP/End) decreases in PC12 cells immediately after STS treatment, while, in cells overexpressing Bcl-2 remains unchanged (Figure 5). This suggests an early uncoupling respiration event induced by STS that is prevented by Bcl-2.

The DNP-dependent respiration is uncoupled from the synthesis of ATP since it occurs when there is a dissipation of the proton gradient which cannot be used to provide the energy for the synthesis of ATP. In this condition, the consumption of oxygen is the highest possible since it is

free of control by the proton gradient and by the synthesis of ATP. DNP-dependent respiration rate is therefore higher than normal endogenous respiration since it is the maximum rate achievable by the respiratory chain. For this reason the ratio between DNP-dependent respiration and endogenous respiration (DNP/End) is higher than 1. If the ratio between DNP and End decreases, this means that endogenous respiration of the cell tends to become uncoupled. In the case of PC12-Bcl2 cells the ratio remains constant which means that there is no uncoupling, while in PC12 cells this ratio decreases and this indicates an early uncoupling event (Figure 5).

**2.4. Respiration Changes and Cytochrome *c* Release in STS-Treated PC12 Cells Are Not Dependent on Caspases and Do Rely Only on the Apoptotic Role of STS.** In order to define whether the decrease of respiration, under STS treatment, was caspase dependent we pretreated PC12 cells with the broad-spectrum caspase inhibitor *z*-Val-Ala-Asp (OMe)-CH<sub>2</sub>F (*z*-VADfmk) before STS incubation. While *z*-VADfmk blocks apoptosis also after 24 h of STS treatment (Figure 2(a), *z*VAD-STS 6h, nuclear morphology), it did not affect the extent of respiration rate reduction (Figures 6(a) and 6(b)). In fact, the decrease in endogenous, DNP-uncoupled and COX-dependent respiration upon STS treatment was perfectly comparable with the respiration rates measured in PC12 naive cells undergoing apoptosis (Figures 1(b)). Active caspases are indeed not required for the mitochondrial release of cytochrome *c* in STS-induced cell death [21]. In fact, the analysis of the localization of cytochrome *c* by confocal immunofluorescence over several hours after apoptotic induction shows a gradual release

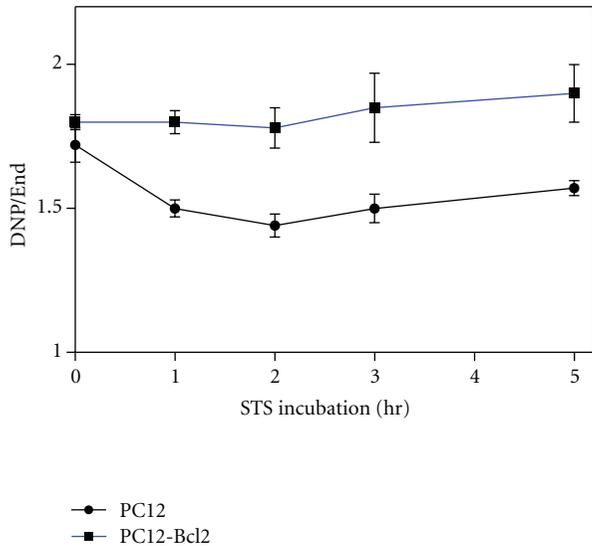


FIGURE 5: Uncoupling ratio behaviour in STS treated PC12 and PC12-Bcl-2 cells. Ratio between absolute DNP-uncoupled oxygen consumption and endogenous respiration expressed in nanomoles of oxygen consumed per minute and per mg of cellular proteins ( $\text{nmolO}_2/\text{min}/\text{mg}$ ) of PC12 and PC12-Bcl-2 cells under  $2\mu\text{M}$  STS-treatment for the indicated time. In detail, for the indicated time points (0h, 1h, 2h, 3h, 5h), endogenous respiration oxygen consumption values are 14,82; 13,99; 12,75; 10,12; 11,10  $\text{nmolO}_2/\text{min}/\text{mg}$  for PC12 cells and 15,82; 12,78; 10,94; 10,98; 9,81  $\text{nmolO}_2/\text{min}/\text{mg}$  for PC12-Bcl-2 cells. DNP-uncoupled oxygen consumption values are 25,50; 20,99; 18,36; 15,17; 17,43  $\text{nmolO}_2/\text{min}/\text{mg}$  and 28,48; 23,14; 19,48; 20,31; 18,64  $\text{nmolO}_2/\text{min}/\text{mg}$ .

of cytochrome *c* not affected by zVADfmk pretreatment (Figure 2(a), zVAD-STS 6 h, arrows).

To exclude the possibility that the early reduction of respiratory activity we detected during the first phase of apoptosis was due to the mere protein kinase inhibition activity of STS, without any relationship with its apoptotic induction, we tested bisindolylmaleimide-I (Bis-I), an STS-analog which is a protein kinase inhibitor without apoptotic functions [22, 23]. The treatment of PC12 cells with Bis-I shows that this drug does not induce apoptosis (data not shown) and does not inhibit the respiration of cells (Figure 6(c)). The lack of respiratory changes in cells treated with Bis-I confirms that the decrease in respiration and the uncoupling of respiration induced by STS correlate with its apoptotic role.

**2.5. Cytochrome *c* Independent and Reversible Nuclear Condensation Induced by STS.** As stated before, STS-treated PC12-Bcl-2 do not die and do not show nuclear fragmentation typical of apoptotic cells also after long treatment (Figures 3(a) and 3(b)). However, despite the absence of fragmentation, we can clearly observe a strong condensation of chromatin (Figure 3(a)), which is very similar to the first stage of condensation observed in STS-treated PC12 cells and preceding fragmentation (Figure 2(a), STS 3 h, nuclei yellow arrowheads). STS-treated PC12-Bcl-2 cells bearing

this strong nuclear condensation (Figure 3(a)) are alive since they respire (Figure 4) but do not replicate (Figure 3(c)); they do not die but stay in a quiescent state. In fact, when STS is removed by changing the medium, all nuclei regain a normal appearance and cells started replicating again (Figure 3(d)). We conclude that the first stage of nuclear modification in STS-induced apoptosis is not dependent on cytochrome *c* release and is not a point of no return of the apoptotic cascade.

### 3. Discussion

The overall decrease in respiration we detected during STS treatment of PC12 cells can obviously be related to the release of cytochrome *c*. However, we also found an impairment of the uncoupled respiration occurring in the first phase of STS-induced apoptosis and preceding the release of cytochrome *c*. This phenomenon was also reported in 143B cells [24], while in anti-Fas antibody-treated Jurkat cells, the loss of TMPD-dependent and of endogenous respiration occurred nearly simultaneously and followed cytochrome *c* release from mitochondria [15]. This difference in the sequence of events might rely on different pathways taking place in Fas- and STS-induced apoptosis.

Regarding the cause of this early respiration impairment independent of cytochrome *c* release, we hypothesize a modification of the mitochondrial inner membrane integrity likely to be present at the level of the phospholipid CL. Indeed, CL is intimately linked to the mitochondrial bioenergetic machinery and is also actively involved in the release of cytochrome *c*. It has also been demonstrated that the modifications of the binding between CL and cytochrome *c* and also the binding between CL and some Bcl-2 proteins precede and prime mitochondria for MOMP and for cytochrome *c* release [8]. CL is crucial for maintaining the integrity and the function of the mitochondrial inner membrane and is required for the optimal activity of most of the respiratory chain complexes. For these reasons, CL modifications (e.g., peroxidation by ROS and cytochrome *c* peroxidase activity or tBid binding or redistribution within and between membranes) immediately result in structural changes of the mitochondrial inner membrane which destabilize mitochondria and affect the activity of membrane embedded respiratory chain complexes [6]. This would impair respiration independently of cytochrome *c* release, as we found. Peroxidation of CL does occur following a wide variety of apoptotic stimuli also including STS. For all these reasons we hypothesize that the early changes in respirations observed might be due to perturbation of the mitochondrial membrane at the level of CL.

Our analysis also suggests that the first stage of apoptosis is characterized by an early uncoupling event. Indeed, in the first hours of STS treatment of PC12 cells, the ratio between the absolute DNP-uncoupled and the endogenous oxygen consumption decreases (Figure 5), indicating a difference between endogenous and DNP-uncoupled respiration decrease rate (Figure 1). This uncoupling event occurs after 1 h of STS treatment when TMPD respiration is only

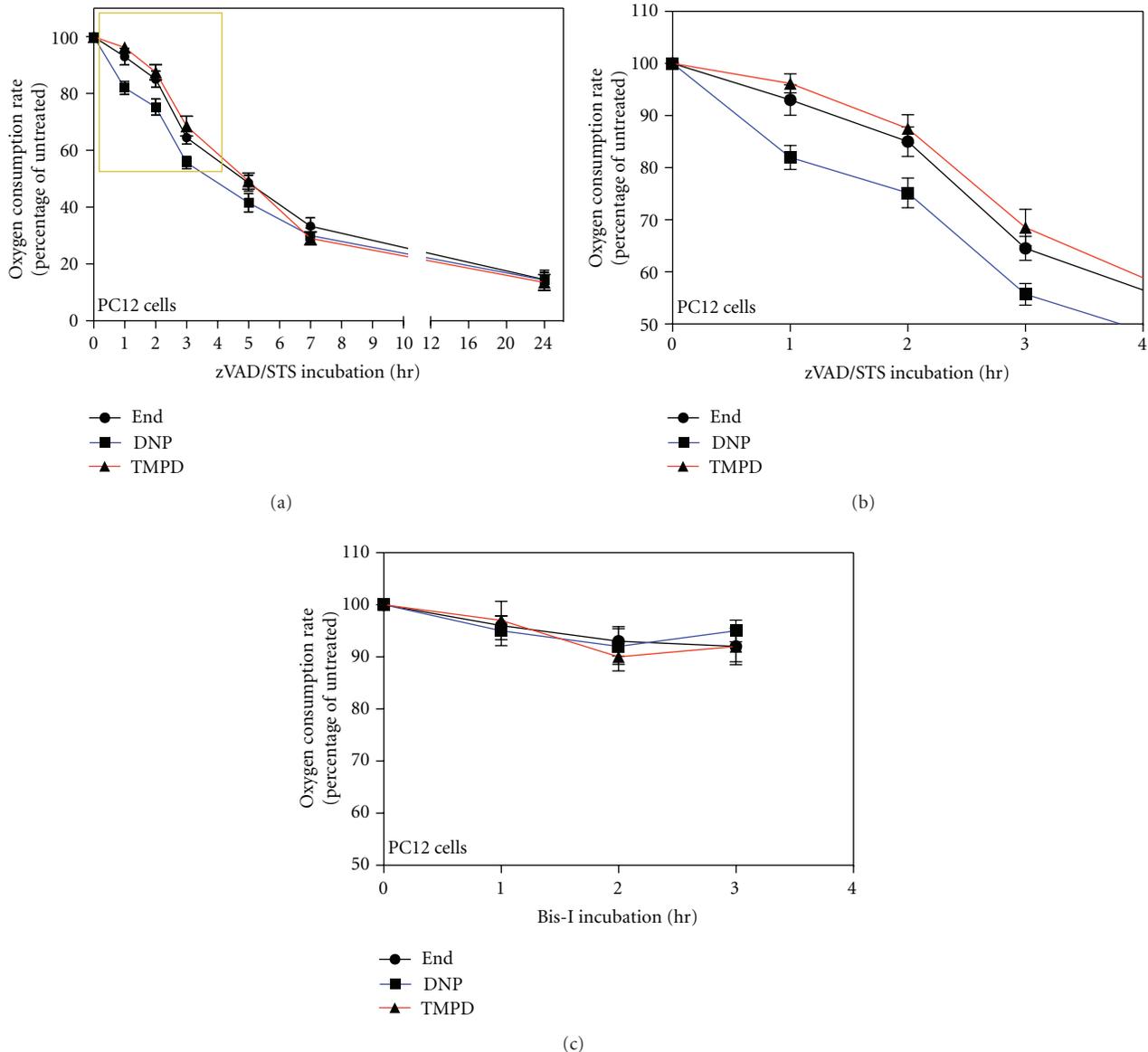


FIGURE 6: Decrease in endogenous, DNP-uncoupled, and TMPD-dependent respiration rates in STS-treated PC12 cells pretreated with zVADfmk and in Bis-I-treated cells. (a) Relative oxygen consumption rates of  $100 \mu\text{M}$  zVADfmk-pretreated,  $2 \mu\text{M}$  STS-treated PC12 cells for the indicated times. Oxygen consumption rates were measured in TD buffer (endogenous respiration, End), in TD buffer containing DNP (DNP-uncoupled respiration, DNP) and in TD buffer containing DNP, antimycin A, ascorbate and TMPD (TMPD-dependent respiration, TMPD). Data are expressed, at each time point, as percentages of the oxygen consumption of untreated cells (0 hr). Data are expressed as means  $\pm$  SE with  $n$  varying at each time point and being 8, 6, 6, 2, 3, 2, 3 for 1 h, 2 h, 3 h, 5 h, 9 h, and 24 h, respectively. (b) Magnification of the panel (a) area surrounded by a yellow frame. Note that the ordinate scale starts at 50%. (c) Relative oxygen consumption rates of Bis-I-treated PC12 cells for the indicated times. Data are expressed as means  $\pm$  SE with  $n$  varying at each time point and being 5, 6, and 5 for 1 h, 2 h, and 3 h respectively.

slightly affected, and it is therefore presumably independent of cytochrome *c* release. However, the uncoupling is blocked by the overexpression of Bcl-2 (Figures 4 and 5). Therefore, Bcl-2 does not prevent the early decrease in respiration induced by STS (Figure 4) but prevents the uncoupling of respiration, which might be the step following the decrease of the maximum rate of respiration and ultimately leading to cytochrome *c* release. We suggest that the uncoupling of respiration might correlate with the step of the cytochrome

*c* release process involving the Bcl-2 family of proteins. Interestingly, the uncoupling of respiration is known to be also caused by CL modifications, supporting our hypothesis of CL as early target for apoptotic changes in mitochondria.

Our experiments, in addition, show a strong degree of nuclear condensation (never reaching fragmentation) occurring in absence of cytochrome *c* release (Figure 3(a)). This partial chromatin condensation might be the first step of nuclear modifications leading ultimately to fragmentation,

and it has been reported to involve DNA double-strand breaks [25, 26]. We assessed that, in naïve PC12 cells, this early nuclear condensation occurring in Bcl2-overexpressing cells is reversible and that it is not a point of no return in the apoptotic pathway (Figures 3(c) and 3(d)). This confirms the finding that MOMP inhibitors allow cells with preapoptotic chromatin condensation to repair their DNA and to return to a normal nuclear morphology [26].

Finally, we observed that cytochrome *c* redistributed within the nuclei once lost its mitochondrial localization after induction of apoptosis, and, after long STS treatment, as previously reported [27], it became finally degraded (Figure 2(a), zVAD-STS 6 h, white arrow). No cells with cytochrome *c* localized only into the cytosol were detectable. This means that the nuclear localization of cytochrome *c* is quick, and indeed it occurs not only when the nuclei are fragmented but also when the chromatin is not yet fragmented or at a very early stage of condensation (Figure 2(a), yellow arrows). This nuclear cytochrome *c* accumulation was zVAD-independent (Figure 2(a), zVAD-STS 6 h).

In conclusion, we found that mitochondrial bioenergetics is perturbed previously and independently of the release of cytochrome *c* during apoptosis. This finding may shed new light on the mechanisms leading to the release of cytochrome *c*. Indeed, considering the interdependencies existing between bioenergetics and apoptosis [28], their coinvestigation in a holistic approach is strongly needed.

## 4. Material and Methods

**4.1. Cell Line and Culture Conditions.** PC12 cells were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere in F-12 K (GIBCO) medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 15% horse serum, 2.5% fetal bovine serum (FBS), and penicillin-streptomycin. For apoptotic induction, cells were seeded at 2 × 10<sup>5</sup>/mL into tissue culture dishes coated with 20 µg/mL poly-L-lysine (Sigma-Aldrich). After 2 days the medium was replaced, and after 2 hours 2 µM STS (Sigma-Aldrich) in DMSO was added. 100 µM zVADfmk (Kamiya Biochemicals) was added 30 min before STS. Bisindolylmaleimide GF 109203X was purchased from Calbiochem.

**4.2. Quantification of Apoptosis.** Apoptosis was assessed by nuclear morphology. At different time points upon STS treatment, both cell floating in the medium and cells adherent to the dish were collected by centrifugation, washed with PBS, fixed in 4% paraformaldehyde (PFA), and stained with 1 µg/mL of the fluorescent double-strand DNA-binding dye 4,6-diamidino-2-phenylindol (DAPI, Sigma-Aldrich) for 8 min. One drop of this preparation was analyzed on a slide by fluorescent microscopy. Cells with large nuclei containing uniformly stained chromatin were counted as alive cells, while cells containing fragmented nuclei and/or pyknotic nuclei were counted as dead cells. Values were obtained from 40–50 fields of 1–2 slides for a total of around

1000 cells/experiments. Each experiment was replicated 6–7 times with similar results.

**4.3. Transfection.** A human Bcl-2α cDNA [19] was subcloned from SFFV-Bcl-2 n1 into the neomycin resistance marker-containing plasmid pcDEF3 [29] under the control of the EF-1α promoter and then used for transfection into PC12 cells by electroporation. Exponentially growing PC12 cells (10<sup>7</sup>) were resuspended in 0.8 mL of PBS, mixed with 18 mg of Bcl-2-pcDEF3 and 5 mg of pGL1, and incubated on ice for 10 min. Electroporation was performed with a BioRad gene pulser by using a setting of 960 mF and 300 V. Cells then were incubated on ice for 10 min and plated in F12-K medium supplemented with 15% horse serum and 2.5% FBS on collagen coated dishes (BIOCOAT). After 48 h stable transfectant overexpressing Bcl-2 (PC12-Bcl-2 cells) was selected by using 0.3 mg/mL G418 (Geneticin; Invitrogen) for 2 weeks.

**4.4. Measurement of Respiration Rates in Intact Cells.** PC12 cells were suspended in TD buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na<sub>2</sub>HPO<sub>4</sub>, 25 mM Tris-HCl pH 7.4 at 25°C) at 3 × 10<sup>6</sup> cells/mL, and the respiration rate was measured in an oxygraph (Yellow Spring Instruments, model 5300). The same measurement was assessed after addition of 40 µM DNP or DNP and 20 nM antimycin A (Sigma-Aldrich) or DNP, Antimycin A, 400 µM TMPD (Fluka) and 10 mM ascorbate (Sigma-Aldrich) as described [12]. Oxygen consumption rate was expressed in nanomoles of oxygen consumed per minute and mg of cellular proteins (nmolO<sub>2</sub>/min/mg) as determined by the Bradford procedure (BioRad) and as percentage of the untreated PC12 cell respiration. The oxygen consumption rate measured in presence of cells, DNP, antimycin A, ascorbate, and TMPD was corrected by subtraction of the oxygen consumption rate due to the autooxidation of ascorbate/TMPD in absence of cells [15].

**4.5. Confocal Immunofluorescence Microscopy.** Cells were cultured on collagen-coated dishes at a concentration of 5 × 10<sup>5</sup> cells/mL. After 2 days cells were treated with STS, then washed with PBS, fixed in 4% PFA in PBS for 10 min at RT, washed, permeabilized with PBS containing 0.4% Triton X-100 for 5 min, and washed again with PBS. After blocking the cells with PBS containing 2% horse serum (HS-PBS) for 30 min, they were incubated with mouse anti-cytochrome *c* monoclonal antibody 6H2.B4 (BD Pharmingen) diluted 1 : 300 in HS-PBS and rabbit anti-Hsp60 antiserum (StressGene) diluted 1 : 200 for 1 h at 37°C in a humidified chamber. Following 3 washes in HS-PBS (5 min each), the cells were incubated with FITC conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) diluted 1 : 200 and lissamine-rhodamine conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) diluted 1 : 200 for 1 h at room temperature. The specimens were washed 3 min in HS-PBS and incubated with SYTOX Green (Molecular Probes) 25 nM for 15 min at room temperature. Cells were washed again 3 times (5 min each) in PBS, mounted in

Fluoroguard antifade reagent (BioRad), and analyzed on a Zeiss 510 laser scanning microscope.

**4.6. Immunoblot Analysis.** Same amount of cell lysates were analyzed by 15% SDS-PAGE. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (BioRad), and the membrane was treated for 1 h with blocking solution (0.02% Tween 20, 0.02% NaN<sub>3</sub>, 5% nonfat milk in PBS) at room temperature. The membrane was then incubated for 4 h at 4°C with mouse anti-Bcl-2 monoclonal antibody (sc-7382) (Santa Cruz Biotechnologies) diluted 1:1000 in blocking solution. It was then washed 3 times in PBS (10 min each) and 1 time with washing solution (150 mM NaCl, 50 mM Tris-HCl pH 7.5) and then incubated for 1 h at RT with sheep anti-mouse IgG peroxidase-linked (Amersham) diluted 1:2000 in washing solution containing 5% nonfat dried milk. Finally the membrane was washed 5 times in washing solution (10 min each), and specific protein complexes were identified using Super Signal West PICO chemiluminescence reagent (Pierce Biotechnology) by autoradiography.

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## Review Article

# Oxidative Stress, Tumor Microenvironment, and Metabolic Reprogramming: A Diabolic Liaison

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Conversely to normal cells, where deregulated oxidative stress drives the activation of death pathways, malignant cells exploit oxidative milieu for its advantage. Cancer cells are located in a very complex microenvironment together with stromal components that participate to enhance oxidative stress to promote tumor progression. Indeed, convincing experimental and clinical evidence underline the key role of oxidative stress in several tumor aspects thus affecting several characteristics of cancer cells. Oxidants influence the DNA mutational potential, intracellular signaling pathways controlling cell proliferation and survival and cell motility and invasiveness as well as control the reactivity of stromal components that is fundamental for cancer development and dissemination, inflammation, tissue repair, and *de novo* angiogenesis. This paper is focused on the role of oxidant species in the acquisition of two mandatory features for aggressive neoplastic cells, recently defined by Hanahan and Weinberg as new “hallmarks of cancer”: tumor microenvironment and metabolic reprogramming of cancer cells.

## 1. Introduction

With over 3 million novel cases each year in Europe, cancer is a main public health hitch with a vital need for new therapies. Hanahan and Weinberg defined in the 2000s the so-called hallmarks of cancers, mandatory characteristics of virtually all neoplastic cells, enabling them to grow in a foreign and hostile environment and allowing escaping endogenous protective systems [1]. These hallmarks are listed in our reinterpretation of the Hanahan and Weinberg picture (Figure 1). Firstly, we mention self-sufficiency in growth signals, that is, the ability of several cancer cells to produce in autocrine manner growth factors and cytokines, as well as the development of compensatory mechanisms enhancing growth factor receptor activation [2, 3]. The insensitivity to natural growth arrest signals, as the abolishment of cell contact inhibition, and the ability to evade apoptosis are two other intimately correlated cues of neoplastic cells [2–4]. Cancer cells evade apoptotic death due to lack of cell adhesion, a process called *anoikis*, as well as death induced by several chemotherapeutic drugs, thereby leading

to chemoresistance, at present the main obstacle to fight cancer dissemination [3–8]. Beside evasion from apoptotic death, cancer cells also escape senescence and the limiting in lifespan, overcoming immortalization. Last, they achieve two further features, which strongly facilitate dissemination of metastatic colonies and repopulation tumors elsewhere. Indeed the ability to recruit *de novo* formed vessels, the so-called neoangiogenesis or vasculogenesis, is mandatory first to grant nutrient supply once the tumor is grown and need new vasculature, and then to create a new way to reach the circulation and disseminate metastases to other organs [9]. Last, aggressive tumors increase their ability to invade surrounding tissues by enhancing their motility and ability to proteolytically degrade basal membrane and extracellular matrices [10].

Our paper is built upon mounting evidence that oxidative stress underlies many of the hallmarks of cancer as defined by Hanahan and Weinberg [29]. Studies in several cancers, including breast, prostate, and colon carcinoma, as well as melanoma, have clearly established that oxidative stress players are expressed aberrantly in cancers and

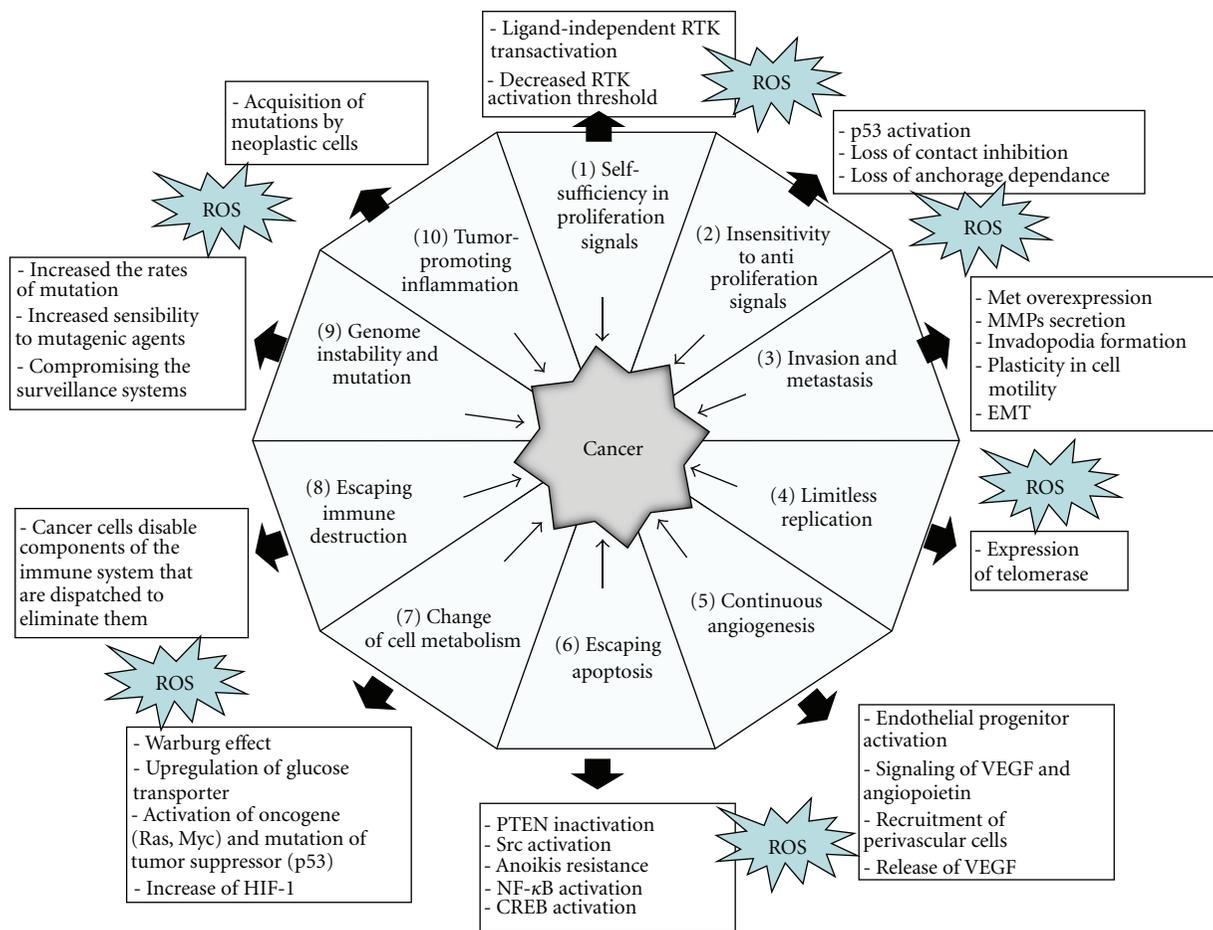


FIGURE 1: ROS play multiple roles in the hallmarks of cancers. Contribution of oxidants is indicated for each point (see text for details). (1) Self-sufficiency in proliferation signals: most normal cells wait for an external message before dividing. Conversely, cancer cells often counterfeit their individual proliferative messages. ROS play a role in ligand-independent RTK transactivation, decreased RTK activation threshold [11, 12]. (2) Insensitivity to antiproliferation signals: as the tumor enlarges, it squeezes adjacent tissues and therefore receives messages that would normally stop cell division. Malignant cells ignore these commands. ROS are involved in p53 activation, loss of contact inhibition, and loss of anchorage dependence [12–14]. (3) Invasion and metastasis: cancers usually lead to death only after they overcome their confines to the particular organ in which they arose. Cancer cells need to escape the primary tumour, invade matrix of different organs, find a suitable metastatic niche, and then grow in this secondary site. ROS play a role in Met overexpression, matrix metalloproteinase secretion, invadopodia formation, and plasticity in cell motility, EMT [12, 15–18]. (4) Limitless replication: healthy cells can divide no more than 70 times, but malignant cells need more than 70 cycles to make tumours. Hence tumours need to enforce the reproductive limit of cells. ROS are involved in expression of telomerase [12, 19]. (5) Continuous angiogenesis: tumour is characterized by a chronically activated angiogenesis due to an unbalanced mix of pro-angiogenic signals thus sustaining cancer “feeding.” ROS play a role in: endothelial progenitor activation, signalling of VEGF and angiopoietin, recruitment of perivascular cells, release of VEGF [20–22]. (6) Escaping apoptosis: in healthy cells, several conditions (including genetic damage or lack of ECM adhesion) activate a suicide program, but tumour cells bypass these mechanisms, thereby surviving to death messages. ROS are involved in PTEN inactivation, Src activation, Anoikis resistance, NF- $\kappa$ B activation, and CREB activation [4, 12, 23–25]. (7) Change of cell metabolism: tumours have the capability to modify or reprogram cellular metabolism to successfully carry on the neoplastic progression. ROS are involved in Warburg effect, upregulation of glucose transporter, activation of oncogene (Ras, Myc) and mutation of tumour suppressor (p53), and increase of HIF-1 [12, 26–28]. (8) Escaping immune destruction: tumours acquire the capability to evade natural immunological destruction by T and B lymphocytes, macrophages, and natural killer cells [29]. Furthermore, there are two additional characteristics facilitating the acquisition of aggressive features called “enabling characteristics.” (9) Genome instability and mutation: genomic alteration due to epigenetic mechanisms, the increase rate of mutation, or enhanced sensitivity to mutagenic agents can drive tumour progression. ROS are involved in increasing the rates of mutation, increasing sensitivity to mutagenic agents, and compromising the surveillance systems [12, 29, 30]. (10) Tumor promoting inflammation: innate immune cells, which are designed to fight infections and heal wounds, inadequately support the acquisition of hallmark capabilities with this leading to tumor expansion. ROS are involved in acquisition of mutations by neoplastic cells, thus accelerating their evolution towards heightened malignancy [29, 31].

positively affects mandatory steps of cancer initiation and progression, by acting on cell proliferation and anchorage independent cell growth, causing insensitivity to apoptosis, sustaining *de novo* angiogenesis, and by altering the migration/invasion programme through metabolic and epigenetic mechanisms (Figure 1). In this scenario, Reactive Oxygen Species (ROS) exert a key role affecting several hallmarks of cancer. Indeed, ROS are involved in proliferation by a ligand-independent transactivation of receptor tyrosine kinase and ERK activation as well as in promoting tissue invasion and metastatic dissemination due to metalloproteinase secretion/activation and epithelial mesenchymal transition. In addition, ROS are involved in tumor angiogenesis, through the release of vascular endothelial growth factor and angiopoietin and for evading apoptosis/*anoikis* [3, 4, 13, 20, 32–37].

In cancer cells, high levels of ROS can result from increased basal metabolic activity, mitochondrial dysfunction due to hypoxia or mitophagy, peroxisome activity, uncontrolled growth factor of cytokines signaling, and oncogene activity, as well as from enhanced activity of known ROS sources as NADPH oxidase (NOXes), cyclooxygenases (COXes), or lipoxygenases (LOXes) [11, 38, 39]. Compelling experimental and clinical evidence indicates that ROS can promote many aspects of tumour onset and progression towards a malignant phenotype. In general, the activity of oxidants on tumors can depend on (i) their mutagenic potential, a mandatory factor for tumor initiation [40], (ii) their effects on intracellular signaling pathways controlling cell proliferation and survival [32, 33]; (iii) their impact on cell motility and invasiveness [12, 40], and (iv) their recognized role in stromal reactivity, mandatory for cancer development and dissemination, like inflammation, tissue repair, and *de novo* angiogenesis [40, 41].

Consequences of the production of oxygen radicals on cancer biology are pleiotropic and complex. Currently, our incomplete knowledge of the entire network of reactions and effects profoundly hinders the implementation of novel and effective redox-based anticancer strategies. In fact, besides being directly involved in mutagenesis and genomic instability, ROS also contribute epigenetically to cancer development and progression, by acting as signalling intermediates downstream of mitogen receptors and adhesion molecules and as inducers of genetic programs leading to cell invasion and malignancy. Furthermore, oxidation of cell constituents is a general cause of cell stress and promotes spontaneous and therapy-induced tumor cell death by making cells more vulnerable. Resistance to oxidative stress appears to be a major mechanism of tumor chemo- and radioresistance. Such diverse biological effects likely reflect distinct biochemical mechanisms operating in different compartments within cells.

Recently, both tumor microenvironment and metabolic reprogramming have been included in the Hallmarks of Cancer model, in a revised perspective of the old hallmarks [29]. Both features, enabling cancer cells to achieve a more aggressive phenotype, have been correlated to oxidative stress and will be described below.

## 2. Tumor Microenvironment and Oxidative Stress

Beside cell-autonomous process involving genetically transformed cancer cells exposed to intrinsic oxidative stress, the importance of stromal cell types populating the tumoral microenvironment is now well established. Indeed, tumor microenvironment may affect evolution of cancers towards aggressiveness and metastatic dissemination through both structure- and function-based (matrix composition, hypoxia, acidity) or cell-based (cancer associated fibroblasts (CAFs) or macrophages (CAMs), endothelial precursors, etc.) mechanisms. Several factors, such as hypoxia or presence of CAFs or CAMs, have already been proven to elicit a prooxidant environment deeply affecting tumor progression and metastasis spread in several cancer models [2, 40–42] (Figure 2).

CAFs, originated either by resident fibroblasts or by recruitment of circulating mesenchymal stem cells [54, 55], become activated, in response to tumor-delivered factors, through a mesenchymal-mesenchymal transition (MMT) converting them into “activated fibroblasts” similarly to myofibroblasts [54, 56]. Fibroblasts activation is profoundly affected by oxidative stress in both neoplastic and fibrotic diseases [41, 47, 57, 58]. Oxidative stress in tumours can be either intrinsic or extrinsic. Indeed, in skin carcinogenesis model, TGF $\beta$ 1 increases the intracellular ROS level in stromal fibroblasts, which initiated the MMT and concomitant changes of gene expression, leading to the secretion of Hepatocyte Growth Factor, Interleukine-6, and Vascular Endothelial Growth Factor that result in proinvasive signals for migration of tumour cells [47]. In addition, Toullec et al. reported a link between myofibroblasts accumulation and the oxidative stress in different pathophysiological conditions (JunD-deficient animals, HER-2 amplified breast adenocarcinoma) [41], highlighting again the importance of oxidative stress in CAFs reactivity. Furthermore, in the diseased prostate stroma, MMT depends by Tumor Necrosis Factor  $\beta$ 1-generated oxidative stress through NOX4 activation that leads to downregulation of ROS-scavenging enzymes such as glutathione peroxidase 3, thioredoxin reductase 1 and the selenium transporter selenoprotein P plasma 1 [51]. Finally, senescence is another factor greatly affecting stromal oxidative stress. Indeed, DNA damage accumulation associated with ageing is involved in deregulation of ROS generation and decrease of antioxidant defences [59]. Indeed, senescent fibroblasts generate an inflammatory environment through the secretion of proinflammatory cytokines and proteases called senescence-activated secretory pathways, SASPs [60]. SASPs comprise soluble signalling factors, chemokines, insulin-like growth factor-1, secreted proteases, tissue-type plasminogen activators, the uPA receptor, and the plasminogen activator inhibitors, which concur to transform senescent fibroblasts into proinflammatory cells that promote tumor progression [49, 50, 60].

CAMs, that concur with CAFs to promote a prooxidant environment, have been recruited into several kinds of tumours, where they exert their effects by different mechanisms [42]. Firstly, the continuous generation of ROS, due to

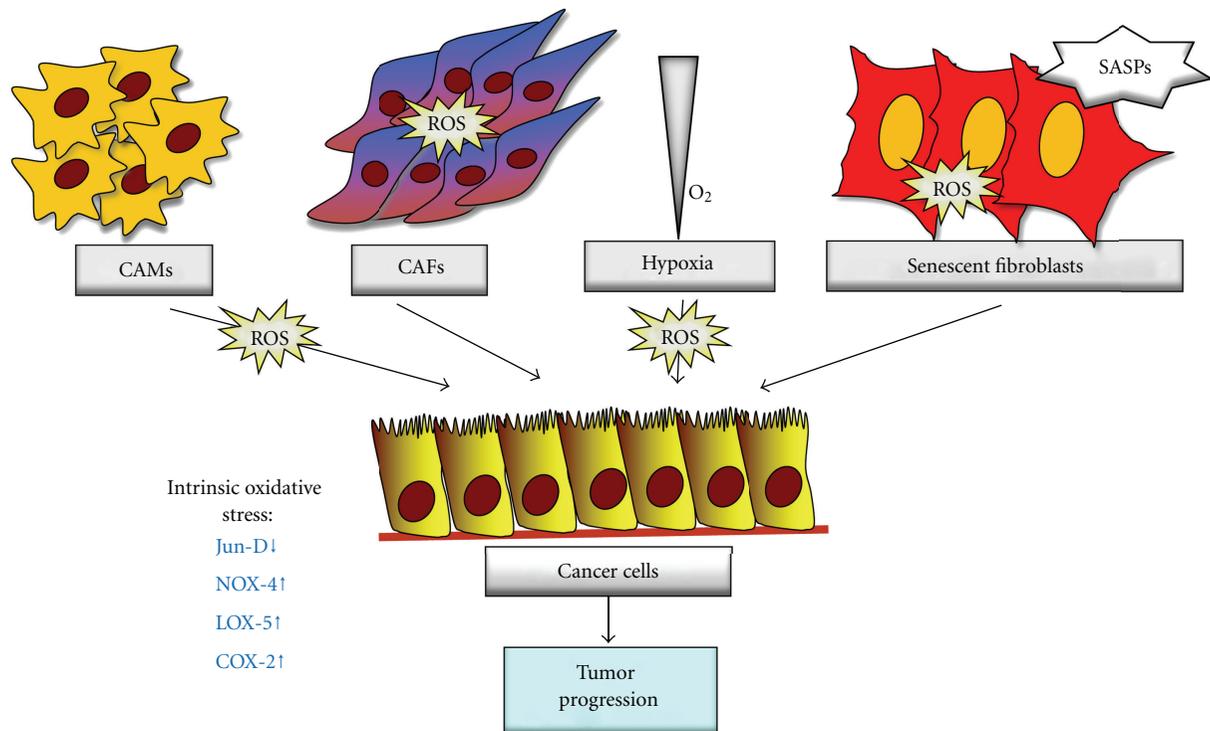


FIGURE 2: Oxidative stress in tumor microenvironment. Within microenvironment, oxidative stress can have intrinsic or extrinsic origin. Some stromal components can directly produce ROS. CAMs generate ROS through NOX2 activation and RNS through iNOS, while hypoxia produces oxidant species by deregulation of the complex III of mitochondrial electron transport or by NADPH oxidase activity [16, 42–46]. In response to extrinsic or intrinsic oxidative stress, CAFs became activated thus producing cytokines and proteases that affect tumour progression [41, 47, 48]. In addition, microenvironment or ageing-induced oxidative stress leads to secretion of “Senescent Activated Secretory Pathway” (SASP) by senescent fibroblasts affecting both stroma and cancer cells to promote cancer progression [49, 50]. Finally, cancer cells exacerbate oxidant environment by intrinsic production of oxidative stress through down-regulation of Jun D or enhanced of NOX-4, LOX-5 and COX-2 activity [41, 48, 51–53].

activation of macrophage NOX-2 and inducible Nitric Oxide Synthase, could directly promote invasion and metastasis, through CAFs recruitment or MMPs activation. Besides, CAMs secrete proinflammatory cytokines, which coordinate the inflammatory response in neighbouring stromal and cancer cells, leading to cancer cells dissemination [31, 61].

A decreased oxygen pressure (hypoxia) has been reported to be linked to an increase of intracellular/mitochondrial ROS that synergizes with other effects due to hypoxia to promote tumour progression [21, 43]. Mammalian cells respond to hypoxia by activating stress signal response, which triggers hypoxia-inducible factor (HIF-) 1 and -2 transcription helpful for adaptation and survival in the hostile milieu [62]. When cells become hypoxic, hydroxylation of the  $\alpha$  subunit of HIF is prevented, resulting in stabilization of the protein and activation of its transcriptional activity. HIF $\alpha$  stabilization occurs through ROS production due to electron transport chain failure or NADPH oxidase [21, 63]. Indeed, pharmacologic and genetic data point to ubiquinone cycle of complex III as the source of ROS generation during hypoxia to stabilize HIF1 $\alpha$  protein [44, 62, 64, 65]. Intratumoral hypoxia can produce several different effects on cancer cells, ranging from metabolic reprogramming towards a glycolytic phenotype, overexpression of ABC transporters, selection

of mutated cells whose apoptotic process is deficient, or protection from apoptotic inducers. Indeed, hypoxic cancer cells are more invasive, resistant to apoptosis and ultimately to chemotherapy and radiation therapy [66, 67]. Moreover, mounting evidence indicates that hypoxic cancer cells undergo exposure to oxidative stress, thereby developing adaptive strategies to survive in the hostile milieu [12, 22]. Of note, hypoxic cells can enhance their antioxidant capacity and hypoxia can behave as a promoting factor for this behaviour, with a possible correlation with resistance to therapy [11, 68].

It is important to underline that the adaptive strategies are indeed the antioxidant responses and that an anti-oxidant phenotype may result in increased aggressiveness.

We also recently reported that aggressive melanoma cells respond to hypoxia engaging a motogen escaping strategy, based on redox stabilization of HIF-1 and activation of the Met protooncogene, allowing a proteolytic motility enhancing metastatic dissemination to lungs [69]. In keeping with the key role exerted by ROS in sensing the effects of hypoxia, Gao et al. reported that the antitumorigenic effect of antioxidants as N-acetyl cysteine and vitamin C in murine models of Myc-mediated tumorigenesis is indeed HIF-1-dependent [70].

Thus, the adaptations to surrounding stromal cells, together with the intrinsic metabolic reprogramming of cancer cells (see below) lead to profoundly altered ROS production and sustained oxidative stress in tumor tissue [32, 33]. As a consequence, oxidant-sensitive transcription factors like Hypoxia Inducible Factor-1 (HIF-1) or Nuclear Factor  $\kappa$ -B (NF- $\kappa$ B) become active and play a mandatory role in eliciting a promigratory and proinflammatory response in cancer cells [71–73]. In addition, human prostate CAFs exert their propelling role for EMT in strict dependence on cyclooxygenase-2 (COX-2), NF- $\kappa$ B, and HIF-1, due to COX-2-mediated release of reactive oxygen species, which is mandatory for EMT, stemness, and dissemination of metastatic cells [48, 59]. These responses, similarly elicited by several components of tumor microenvironment, like cancer-associated fibroblasts, hypoxia, or acidity, embrace enhanced motility, survival to stressful environment, and reconfiguration of metabolism. The motile response is commonly recognised as Epithelial Mesenchymal Transition (EMT), an epigenetic transcriptional program leading cells to lose epithelial features and achieve mesenchymal-like motility [74–77]. EMT has been correlated with achievement of stem-cell like cues, as increase in the ratio of expression of CD44 and CD24, increase in CD133 expression, and enhancement of anchorage-independent growth and spheroid formation, as well as selection of tumor initiating cells able to disseminate metastases [78, 79]. Again, both EMT and stemness have been reported as redox-sensitive and to exploit prooxidant environment to drive metastatic dissemination and resistance to chemotherapies in several cancer models [48, 80].

Beside the role played by stromal cells, EMT can be also elicited by intratumoral hypoxia, acting in a biphasic manner. Hypoxia-induced migration include an early mitochondrial delivery of ROS, leading to activation of cell polarization and oriented migration; then, there is a second delayed phase, in which ROS act on HIF-1 $\alpha$  stabilization and VEGF expression, which sustains active motility [81]. In addition, stromal elements of tumor microenvironment regulate EMT and stemness through strengthening of hypoxic stimuli. By this way, CAFs are able to mimic the hypoxic stimuli, experiencing HIF-1 expression due to their oxidative stress, but without the real need for oxygen deprivation [41]. Indeed, exposure to reactive stromal fibroblasts engages an HIF-1 and NF- $\kappa$ B-mediated transcriptional response driving EMT, but that does not need hypoxia [48]. Of course it is likely that the appearance of intratumoral hypoxia should exacerbate this EMT programme, enhancing the motile response (Comito, unpublished results).

Oxidative stress during ovarian tumorigenesis has been recently correlated with a stress signature involving two miR-200 family members, miR141 and miR200a, already implicated in the control of EMT and stemness [82, 83]. In particular, the paper of Mateescu et al. demonstrates that high-grade human ovarian adenocarcinomas that accumulate miR-200a contain high level of ROS, which correlate with improved survival of patients in response to treatment and conclude that although oxidative stress promotes tumor growth, it also sensitizes tumor to treatment, which could

account for the limited success of antioxidants in clinical trials [84].

### 3. Metabolic Reconfiguration of Tumors Undergoing Oxidative Stress

Besides and in synergy with their altered perception of the tumor microenvironment, cancer cells undergo profound changes in their own intrinsic metabolism. The tendency of cancer cells to undergo Warburg metabolic reprogramming, characterized by increased activity in aerobic glycolysis and by lipid metabolism deregulation, is widely acknowledged. Recently both hypoxia and CAFs have been recognised to synergize in metabolic reprogramming of cancer cells, both establishing a sort of “Cori cycle” between glycolytic and respiring cells [85]. Indeed, hypoxia and/or contact with CAFs leads cancer cells to upload lactate, produced by neighbouring hypoxic cells or CAFs, which feeds aerobic cancer cells through respiration and anabolic functions [86, 87].

The reconfiguration of metabolism through oxidative stress occurring during cancer formation affects the metabolic flux and network topology of pathways in central carbon metabolism. It has been recently demonstrated that oxidative stress leads to mitophagy and limitation of oxidative phosphorylation [88] and to cysteine oxidation and inactivation of the M2 isoform of pyruvate kinase, with the consequence to enhance the level of glycolytic intermediates that are reconverted to the pentose phosphate cycle. This diverted pathway gives a key advantage to cells experiencing oxidative stress, which can use NADPH produced by pentose phosphate cycle and scavenge ROS, rescuing survival conditions. This adaptation was attributable to accumulation of phosphoenolpyruvate, due to redox inhibition of pyruvate kinase. Phosphoenolpyruvate acts as feedback inhibitor of the glycolytic enzyme triosephosphate isomerase, which activates the pentose phosphate pathway, increasing antioxidative metabolism and preventing ROS accumulation. NADPH also compensates for the oxidative stress caused in cancer cells undergoing nucleotide/fatty acid synthesis [89, 90]. These metabolic changes have effect on the transcriptome, allowing adaptation to cope with high ROS level, upregulating anti-oxidant defence systems, and helping cancer cells to reconfigure metabolic activity towards ROS detoxification, finally enhancing the ability to survive in a prooxidant environment.

Moreover, a crucial role in the regulation of Warburg effect in cancer cells has been proposed for mitochondrial SIRT3, which belongs to NAD-dependent deacetylase family, already involved in tumour metabolism. Indeed, the genetic loss of SIRT3 leads cancer cells to metabolic reprogramming towards glycolysis. This shift is mediated by an increase in cellular ROS generation that amplifies HIF-1 $\alpha$  stabilization and HIF-1-dependent gene expression, thereby driving the tumor phenotype [91]. In addition, SIRT3 has been proposed as tumour suppressor via its ability to suppress ROS and regulate HIF-1 $\alpha$  thus inhibiting tumour growth [92].

#### 4. Conclusions

The possibility to target cancer cell malignancy by intervention on both its metabolic reprogramming and its interplay with environmental factors is now attracting several scientists. Effects of intratumoral hypoxia and/or infiltrating CAFs should in principle be targeted by disrupting the Warburg metabolism in both cancer and stromal cells, as well as their reconfiguration towards the pentose phosphate pathway antioxidant strategy. Promising pharmacological approaches include drugs targeting the lactate shuttle, as well as inhibitors of glycolysis combined with inhibitors of autophagy, a compensatory mechanism for nutrient starved cancer cells. Before developing such strategies, it will be essential to deeply investigate all biochemical reactions producing ROS within cancer cells, as well as their exact targets and downstream effects.

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## Review Article

# Oxidative Stress-Induced Diseases via the ASK1 Signaling Pathway

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Apoptosis signal-regulating kinase 1 (ASK1) is a mitogen-activated protein kinase (MAPK) kinase kinase that activates the downstream MAPKs, c-Jun N-terminal kinase (JNK) and p38. ASK1 is activated by various types of stress, such as oxidative stress, endoplasmic reticulum stress, and infection, and regulates various cellular functions. Recently, it has been reported that ASK1 is associated with various diseases induced by oxidative stress. In this review, we introduce recent findings of the regulatory mechanisms of ASK1 and the oxidative stress-induced diseases mediated by the ASK1 signaling pathway.

## 1. Introduction

Cells are exposed to various types of external and internal stresses and need to respond to these stresses to maintain homeostasis. The mitogen-activated protein kinase (MAPK) pathway is one of the intracellular signaling systems that regulate various cellular functions, such as proliferation, differentiation, and apoptosis. Each MAPK pathway consists of three classes of protein kinases: MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K), and MAPK. MAP3K phosphorylates and thereby activates MAP2K, and activated MAP2K, in turn, phosphorylates and activates MAPK. Among MAPKs, c-Jun N-terminal kinase (JNK) and p38 MAPK respond to various types of stress, including reactive oxygen species (ROS), osmotic pressure, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and endoplasmic reticulum (ER) stress, and regulate apoptosis, inflammation, and morphogenesis through the phosphorylation of various target molecules [1]. MAP3Ks control the activation status of MAPKs, and thus, MAP3Ks are important for the regulation of various cellular responses.

Apoptosis signal-regulating kinase 1 (ASK1) is a member of the MAP3K family, which activates the MAPK kinase 4 (MKK4)/MKK7-JNK and MKK3/6-p38 pathways [1, 2]. ASK1 is preferentially activated in response to various types

of stress, such as ROS, TNF- $\alpha$ , lipopolysaccharide (LPS), and ER stress, and has pivotal roles in a wide variety of cellular responses, including apoptosis, differentiation, and inflammation [2–7]. Therefore, the excessive activation and dysregulation of ASK1 are closely linked to various diseases. Here, we focus on the molecular mechanisms of ASK1 activation and the involvement of ASK1 in oxidative stress-induced diseases.

## 2. Mechanisms of ROS-Induced ASK1 Activation

ASK1 forms a high molecular mass complex termed the ASK1 signalosome [8]. Within the signalosome, ASK1 is homooligomerized through its C-terminal coiled-coil (CCC) domain, a process that is critical for ASK1 activation. Thioredoxin (Trx), a redox-responsive protein, is included in the ASK1 signalosome, and the reduced form of Trx binds to the N-terminal region of ASK1 and inhibits its kinase activity. However, the oxidized form of Trx dissociates from ASK1 in response to ROS, and ASK1 is then activated by the autophosphorylation of Thr845 in its kinase domain [3, 9]. Upon ROS-dependent dissociation of Trx from ASK1, ASK1 appears to be tightly oligomerized through its N-terminal

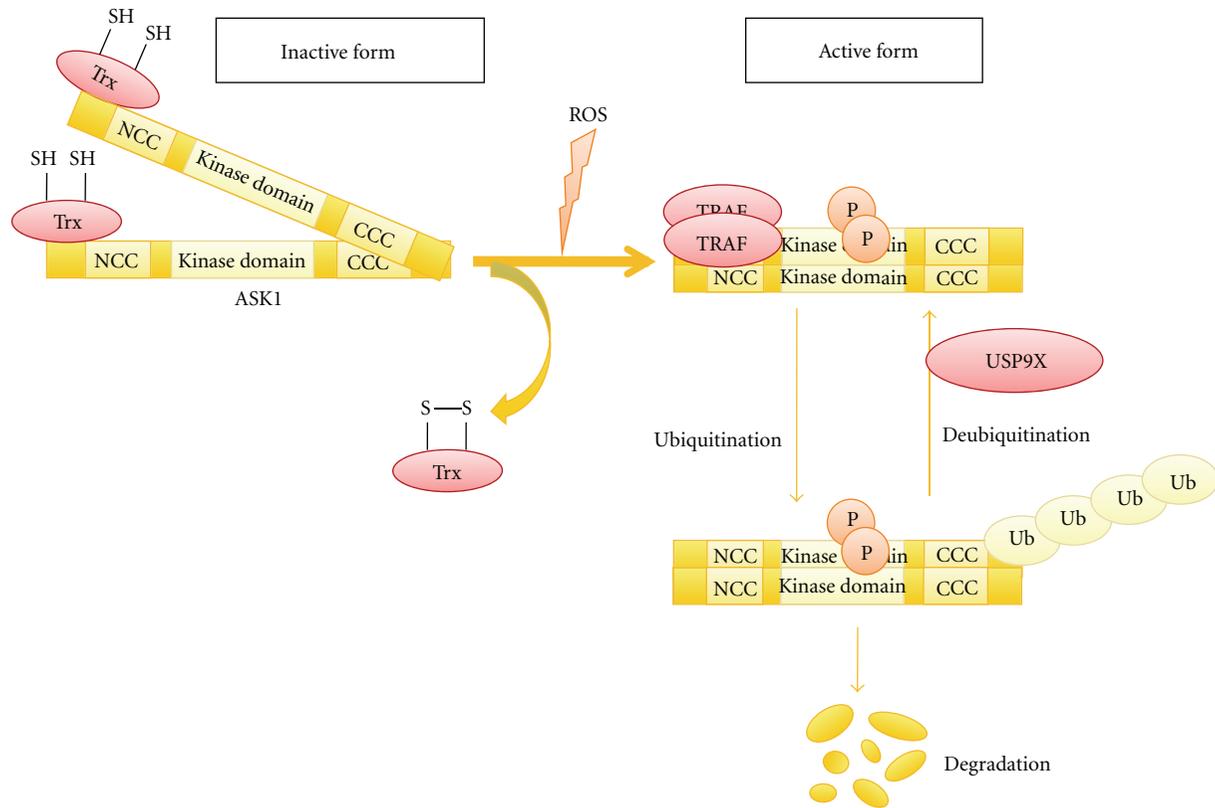


FIGURE 1: Mechanisms of ROS-induced ASK1 activation. Thioredoxin (Trx), a negative regulator of ASK1, is dissociated from the N-terminal region of ASK1 in response to ROS. Subsequently, TRAF2 and TRAF6 are recruited, thereby fully activating ASK1. The ROS-induced activation of ASK1 results in its ubiquitination and proteasome-dependent degradation. USP9X negatively regulates ASK1 degradation by deubiquitination, leading to the sustained activation of ASK1. CCC: C-terminal coiled-coil domain; NCC: N-terminal coiled-coil domain.

coiled-coil (NCC) domain, leading to the full activation of ASK1.

The ROS-stimulated ASK1 signalosome forms a much higher molecular mass complex than when not stimulated and contains various regulatory factors of ASK1, including TNF- $\alpha$  receptor-associated factor 2 (TRAF2), TRAF6, protein phosphatase 5 (PP5), and USP9X [8, 10, 11]. In response to ROS, the adaptor proteins TRAF2 and TRAF6 are recruited to the ASK1 signalosome and positively regulate ASK1 activity by promoting the homophilic interaction of its NCC domain [12]. TRAF2 and TRAF6 promote ASK1-dependent cell death and inflammatory cytokine production downstream of the TNF- $\alpha$  receptor and Toll-like receptor 4 (TLR4; a receptor for LPS), respectively. PP5 dephosphorylates the activating phosphorylation site of ASK1 in a ROS-dependent manner and negatively regulates ASK1 activity [10].

The USP9X deubiquitination enzyme also binds to ASK1 in response to oxidative stress. The oxidative stress-induced activation of ASK1 results in the ubiquitination and proteasome-dependent degradation of ASK1. Thus, USP9X positively regulates ASK1 activity and ASK1-dependent cell death through the deubiquitination and stabilization of ASK1 [11]. Therefore, ASK1 activity is regulated by both

phosphorylation and ubiquitination in response to oxidative stress (Figure 1).

### 3. Cancer and ASK1

Recently, it has been reported that ASK1 has an important role in skin tumorigenesis [22]. The ROS-induced activation of the ASK1-p38 pathway leads to the production of inflammatory cytokines, such as TNF- $\alpha$ , IL-6, and IL-1 $\beta$ , in dendritic cells and macrophages [6], and the ASK1-dependent production of inflammatory cytokines was found to be critical for chemically induced skin tumorigenesis during the promotion stage. In this case, ASK1 acts as a promoter of skin tumorigenesis. When ASK1 acts with ASK2, a functional binding partner of ASK1, during the initiation stage of skin tumorigenesis, ASK1 contributes to the induction of ROS-dependent apoptosis in epidermal keratinocytes. Because the ASK1-ASK2 complex functions as a tumor suppressor, the expression level of ASK2 appears to regulate the roles of ASK1 as a tumor promoter and suppressor. It has been observed that the expression of ASK2 was strongly reduced in various human gastrointestinal cancer cells and tissues compared with their normal counterparts [22]. Actually, ASK2 deficiency promotes chemically induced mouse skin

tumorigenesis, through the reduction of apoptosis in DNA-damaged epidermal keratinocytes during the initiation stage. ASK2 has been shown to activate ASK1 by direct phosphorylation.

ASK1 also acts as a tumor suppressor in hepatocarcinogenesis [17]. ASK1 is involved in death receptor-mediated apoptosis through the JNK-mediated phosphorylation of BimEL, a proapoptotic Bcl-2 family member, and the DNA damage-induced upregulation of p21 through the p38 pathway. ASK1 also contributes to the development of gastric cancer [21]; the expression level of ASK1 increased in human gastric cancer, and ASK1-deficient mice had both fewer and smaller tumors than wild-type mice. ASK1 upregulates the expression level of cyclin D1 through AP-1 activation, leading to cell proliferation. Moreover, cyclin D1 elevates ASK1 expression via the Rb-E2F pathway such that this positive feedback loop facilitates the development of gastric cancer. Thus, ASK1 functions as a tumor promoter and also as a tumor suppressor, depending on the cell type and cellular context, through the induction of various cellular responses, such as apoptosis, inflammation, and cell proliferation. Most recently, it has also been reported that frequent somatic mutations in ASK1 in metastatic melanoma were identified by exome sequencing [23]. Several mutations affect the kinase activity of ASK1. The Ile780Phe substitution in the kinase domain of ASK1 almost completely abolishes kinase activity, whereas the Glu663Lys substitution adjacent to the kinase domain reduces weakly, yet significantly, the kinase activity.

#### 4. Neurodegenerative Diseases and ASK1

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by two pathological findings: amyloid- $\beta$  ( $A\beta$ ) accumulation and neurofibrillary tangles.  $A\beta$  is the major component of senile plaques and induces neuronal cell death, and it has been reported that  $A\beta$  impairs mitochondrial redox activity and increases the generation of ROS, leading to apoptotic neuronal death.  $A\beta$  also activates ASK1 through the generation of ROS and induces JNK-mediated neuronal cell death [13]. It has been observed that  $A\beta$ -induced neuronal cell death decreases in ASK1-deficient mice, indicating that ROS-induced ASK1 activation by  $A\beta$  is an important step in the pathogenesis of AD.

Recently, it has also been reported that ASK1-mediated dopaminergic (DA) neuronal cell death is important for Parkinson's disease [14]. The peroxiredoxin 2 (Prx2) antioxidant enzyme inhibits DA toxin 6-hydroxydopamine-(6-OHDA-) induced ASK1 activation by modulating the redox status of Trx and inhibiting the dissociation of Trx from ASK1. Prx2 confers remarkable protection against 6-OHDA-induced DA neuronal loss via the suppression of the ASK1-dependent activation of JNK and p38. In addition, the oxidative stress-mediated activation of the ASK1-JNK pathway is associated with brain ischemia in the hippocampus [15]. Thus, ROS-induced ASK1 activation contributes to the pathogenesis of neurodegenerative diseases.

#### 5. Inflammation and ASK1

ASK1 also responds to biological stresses, such as bacterial and viral infection, and induces inflammation. Bacterial components, such as LPS, are recognized by TLRs and activate the downstream MAPK pathways. ASK1-deficient mice have been shown to be resistant to LPS-induced sepsis shock [6]. The LPS-induced p38 activation and production of inflammatory cytokines were reduced in splenocytes and dendritic cells derived from ASK1-deficient mice. Because LPS-induced p38 activation and cytokine production were suppressed by antioxidants, this implies that LPS-induced ASK1 activation is mediated by ROS generation. These results indicate that ASK1 is important for mammalian innate immunity. In addition, recent research has shown that the activation of the ASK1-p38 pathway through TLRs in glial cells is important for chemokine production in astrocytes and facilitates inflammation and neurotoxicity in multiple sclerosis (MS) [16]. An ASK1 deficiency or ASK1 inhibitor attenuated the sensitivity of experimental autoimmune encephalomyelitis, an animal model of MS, suggesting that ASK1 is a potential therapeutic target for the treatment of MS.

#### 6. Cardiac Diseases and ASK1

ASK1 is closely linked to cardiac diseases, such as cardiac hypertrophy, remodeling, and cardiac injury. It has been reported that in the left ventricle, ASK1 is activated by angiotensin-II-(Ang II-) induced ROS generation through the Ang II type 1 (AT1) receptor, resulting in cardiac hypertrophy and remodeling [18, 19]. Ang II-induced cardiac hypertrophy and remodeling, including cardiac hypertrophy-related mRNA upregulation, cardiomyocyte apoptosis, and interstitial fibrosis, were significantly attenuated in ASK1-deficient mice. ASK1 is also involved in Ang II-induced cardiac injury, such as capillary endothelial apoptosis, and a decrease in myocardial capillary density [20]. Cardiac injury was prevented by an AT1 receptor blocker through the inhibition of ROS generation and ASK1 activation in a mouse model of hypertensive decompensated cardiac hypertrophy and heart failure. ASK1 contributes to Ang II-induced cardiac diseases mediated by ROS generation.

#### 7. Diabetes and ASK1

Several studies have revealed that ASK1 is associated with the pathogenesis of diabetes. ASK1 has been shown to negatively regulate insulin receptor substrate-1 (IRS-1), a key mediator in insulin signaling, through JNK-mediated IRS-1 phosphorylation [24]. ASK1 is activated by TNF- $\alpha$ -stimulated ROS generation and is an important factor that causes insulin resistance. Hyperglycemia increases oxidative stress in various tissues, and it has been reported that the high glucose-induced activation of ASK1 contributes to endothelial cell senescence, leading to diabetes-related vascular aging mediated by oxidative stress [25]. These results suggest that ROS-mediated ASK1 activation is involved in

TABLE 1: ASK1-related diseases and pathologies.

Organ/tissue	Related diseases	Related pathologies	References
Nervous system	Alzheimer's disease	Neuronal death	[13]
	Parkinson's disease	Neuronal death	[14]
	Brain ischemia	Neuronal death	[15]
	Multiple sclerosis	Inflammation	[16]
Liver	Hepatocarcinogenesis	Apoptosis	[17]
Heart	Hypertrophy, remodeling	Cardiomyocyte apoptosis, interstitial fibrosis	[18, 19]
	Cardiac injury	Capillary endothelial apoptosis	[20]
Stomach	Gastric cancer	Cell proliferation	[21]
Skin	Skin tumorigenesis	Apoptosis, inflammation	[22]
Immune system	Infection	Septic shock	[6]

the pathogenesis of diabetes through the modulation of insulin signaling or cellular senescence.

## 8. Conclusions

Redox balance is important for the control of cellular responses, and the mode of cell reaction depends on the level of oxidative stress. As described above, the ASK1 signaling pathway is closely linked to various human diseases caused by oxidative stress and redox imbalance through the regulation of various cellular responses, such as apoptosis, inflammation, proliferation, and senescence. Recent studies have shown that ASK1 is a therapeutic candidate for these diseases (summarized in Table 1). ASK1 functions as an initial sensor of ROS generation and plays a pivotal role in signal transduction for the maintenance of homeostasis against redox imbalance. The excessive activation and dysregulation of ASK1 result in a wide range of diseases. Further studies will reveal the pathophysiological roles of ASK1 in oxidative stress-induced diseases, leading to the development of therapeutic strategies.

## Abbreviations

ASK1: Apoptosis signal-regulating kinase 1  
 MAPK: Mitogen-activated protein kinase  
 JNK: c-Jun N-terminal kinase  
 TNF- $\alpha$ : Tumor necrosis factor- $\alpha$   
 ROS: Reactive oxygen species  
 LPS: Lipopolysaccharide  
 Trx: Thioredoxin  
 TRAF: TNF- $\alpha$  receptor-associated factor  
 TLR: Toll-like receptor  
 Prx: Peroxiredoxin  
 Ang II: Angiotensin II.

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## Research Article

# S-Glutathionylation of Protein Disulfide Isomerase Regulates Estrogen Receptor $\alpha$ Stability and Function

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S-Glutathionylation of cysteine residues within target proteins is a posttranslational modification that alters structure and function. We have shown that S-glutathionylation of protein disulfide isomerase (PDI) disrupts protein folding and leads to the activation of the unfolded protein response (UPR). PDI is a molecular chaperone for estrogen receptor alpha (ER $\alpha$ ). Our present data show in breast cancer cells that S-glutathionylation of PDI interferes with its chaperone activity and abolishes its capacity to form a complex with ER $\alpha$ . Such drug treatment also reverses estradiol-induced upregulation of c-Myc, cyclinD1, and P21<sup>Cip</sup>, gene products involved in cell proliferation. Expression of an S-glutathionylation refractory PDI mutant diminishes the toxic effects of PABA/NO. Thus, redox regulation of PDI causes its S-glutathionylation, thereby mediating cell death through activation of the UPR and abrogation of ER $\alpha$  stability and signaling.

## 1. Introduction

Glutathione S-transferase pi (GSTP) is a biomarker protein in drug-resistant solid epithelial tumors, including ovarian, breast, liver, pancreatic, lung, and lymphoma [1]. In some cases, GSTP can be the most abundant protein in the tumor and, consequently, has the potential to serve as an important drug target [2–4]. One therapeutic approach has been to develop prodrugs that are substrates for GSTP and become cytotoxic when liberated in cancer cells, yet exhibit diminished activation/toxicity in normal tissue. PABA/NO (O<sup>2</sup>-[2,4-dinitro-5-[4-(N-methylamino) benzoyloxy]phenyl] 1-(N,N-dimethylamino) diazen-1-ium-1,2-diolate) [5] is a GSTP-activated prodrug that releases high levels of nitric oxide (NO) at physiological pH. This reaction results in the formation of a Meisenheimer-complex intermediate and subsequently the leaving group of the reaction generates two molecules of NO. Elevated NO levels lead to cytotoxic effects by forming RNS/ROS intermediates that can alter protein function directly through posttranslational modifications on redox sensitive cysteine residues (S-nitrosylation, P-SNO or S-glutathionylation, P-SSG) [3, 6].

Prior studies have shown that protein disulfide isomerase (PDI) is a molecular target of PABA/NO treatment in cancer cells [2, 5, 7–9]. PDI is the most abundant chaperone/isomerase in the endoplasmic reticulum and plays a pivotal role in protein folding through isomerase and chaperone activity. The active site cysteine residues are S-glutathionylated (PDI-SSG) following PABA/NO treatment. The functional consequences are reduced isomerase activity, accumulation of unfolded/misfolded proteins, and activation of the unfolded protein response (UPR). At least one mechanism of action for PABA/NO can be attributed to UPR-induced cell death.

PDI shares homology with the estrogen-binding domain of the estrogen receptor alpha (ER $\alpha$ ) [10]. Despite the KDEL sequence present in the C-terminus that retains PDI in the endoplasmic reticulum (ER), PDI is also found in non-ER locations [11]. With immune-affinity, steroid-affinity and site-specific DNA-affinity chromatography, Landel et al. have found that in extracts from MCF-7 cells and ER $\alpha$ -expressing CHO (CHO-ER $\alpha$ ) cells ER $\alpha$  forms complexes with PDI, Hsp70, and two unidentified proteins [12]. Moreover, PDI enhances the binding of purified ER $\alpha$  to

the estrogen response element (ERE) in a dose-dependent manner, suggesting that this accessory protein may be required for optimal interactions of ER $\alpha$  with EREs [12, 13]. Interestingly, PDI does not interact with estrogen receptor  $\beta$  (ER $\beta$ ) [10]. The DNA-binding domain and Hinge domain of ER $\alpha$  are the minimal structures sufficient for PDI-ER $\alpha$  interactions [13]. However, information regarding the corresponding domain(s) of PDI involved in this interaction is not presently available. The presence of estrogen neither is required for PDI-ER $\alpha$  interaction [13] nor has any effect on the stoichiometry of PDI association with ER $\alpha$  [12]. The PDI-ER $\alpha$  interaction may protect ER $\alpha$  from oxidation and alter ER $\alpha$  conformation so that the susceptibility of ER $\alpha$  to different proteases is altered [13].

The present study evaluates the efficacy of PABA/NO in ER $\alpha$ -positive breast cancer cells and interrogates the impact of S-glutathionylation of PDI on its chaperone activity, specifically with ER $\alpha$ . We hypothesize that S-glutathionylation of PDI not only blunts protein folding but also prevents protein interaction with ER $\alpha$ , destabilizing the receptor and subsequent ER $\alpha$  signaling (see scheme in Figure 1).

## 2. Material and Methods

**2.1. Reagents.** Reduced glutathione (GSH), oxidized glutathione (GSSG), and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent, and  $\beta$ -estradiol were purchased from Sigma (St. Louis, MO). Antibodies were purchased from the following sources: anti-PDI (Affinity BioReagents); anti-glutathionylation (Virogen), anti-actin (Abcam), anti-FLAG (Sigma), anti-ER $\alpha$ /anti-ER $\beta$  (Millipore), and anti-c-Myc/anti-Cyclin D1/anti-p21 (Cell Signaling). PABA/NO (O<sup>2</sup>-[2,4-dinitro-5-[4-(N-methylamino) benzoyloxy] phenyl]1-(N,N-dimethylamino) diazen-1-ium-1,2-diolate) was provided by Dr. Larry Keefer, Chemistry Section, Laboratory of Comparative Carcinogenesis, NCI at Frederick (MD).

**2.2. Expression and Purification of His-Tagged Recombinant PDIs.** His-tagged recombinant human PDI in the bacterial expression vector pET-28b was provided by Dr. Lana Lee (University of Windsor, Ontario) and expressed as previously described [8, 9]. The His-tagged PDI mutant FLFL was synthesized using the QuikChange Site-Directed Mutagenesis kit (Stratagene), in which histidine 55 and 399 were changed to phenylalanines, and lysine 57 and 401 were changed to leucines, simultaneously. All mutations were verified by DNA sequencing.

Purified plasmids were transformed into *Escherichia coli* BL21(DE3)pLysS strain, which were then induced with 1 mM IPTG at 37°C for 3 h and pelleted at 6000 rpm for 15 min at 4°C. Cells were lysed by sonication in lysis buffer (20 mM NaH<sub>2</sub>PO<sub>4</sub>, 500 mM NaCl). The lysates were centrifuged at 13,000 rpm for 30 min at 4°C and the pellets were discarded. The supernatants were incubated with Ni-NTA agarose beads (QIAGEN) for 1 h at 4°C. The beads were washed three times with wash buffer (20 mM

NaH<sub>2</sub>PO<sub>4</sub>, 500 mM NaCl, and 10 mM imidazole). His-PDIs were eluted with elution buffer (20 mM NaH<sub>2</sub>PO<sub>4</sub>, 500 mM NaCl, and 250 mM imidazole). The eluates were dialyzed against dialysis buffer (25 mM HEPES, pH7.3, 100 mM NaCl, 1 mM EDTA, and 1 mM DTT). Protein purity was assessed on Coomassie-stained gels. Protein concentrations were determined using the Bradford reagent with BSA as a standard.

**2.3. Expression of FLAG-Tagged PDIs in Mammalian Cells.** Full-length human PDI was amplified from a cDNA prepared from HL60 cells using the following primers: 5'-GACGGATCCATGCTGCGCCGCGCTCTGCTG-3' and 5'-GTCGAATTCTTACAGTTCATCTTTCACAGC-3'. The resulting PCR fragment was cloned into the BamHI/EcoRI sites of pCDNA3 vector for mammalian expression. A FLAG tag sequence DYKDDDDK was inserted between amino acids E497 and D498, upstream of the KDEL sequence [15]. FLAG-tagged PDI mutant FLFL was also generated using site-directed mutagenesis. All mutations were verified by DNA sequencing.

The human breast cancer line MCF7 was purchased from the American Type Culture Collection and grown as recommended by the supplier. Cells were maintained in  $\alpha$ MEM containing 10% FCS, 100  $\mu$ g/mL streptomycin, 100 units/mL penicillin, and 2 mmol/L l-glutamine at 37°C in a 5% CO<sub>2</sub> incubator. Expression plasmids containing FLAG-tagged PDI and FLFL mutant were transfected into MCF7 cells using GenJet reagent according the manufacturer's instruction. Cells (2  $\times$  10<sup>6</sup> per treatment group) were plated 24 h before drug treatment.

**2.4. Fluorescent Imaging of Native and PDI Expression Vectors.** To validate that overexpression of PDI (PDI-OE) does not lead to altered localization, transfected MCF7 cells (vector or PDI) were seeded onto LabTek II Chamber Slides and allowed to adhere overnight. Cells were rinsed 2X with PBS and fixed with 4% paraformaldehyde for 30 min. followed by permeabilization in 0.2% Triton X-100 in PBS. Nuclear staining was detected using 4',6-diamidino-2-phenylindole (DAPI). Nonspecific protein binding was blocked using 1% bovine serum albumin (BSA) for 30 min. Cells were then incubated for 1 h in primary anti-PDI (vector) or anti-FLAG (PDI-OE) antibody in PBS. Unbound primary antibody was removed by rinsing the cells 3X with PBS containing 0.05% Tween-20. The cells were incubated with Oregon Green secondary antibody. Following removal of the secondary antibody, the cells were imaged by fluorescent microscopy (Nikon eclipse E800, Nikon Instr., In. Lewisville, TX using Nikon DS-U1 software v. 5.03, Photometrics, Tucson, AZ).

**2.5. Protein Preparation.** Cells were harvested and washed with phosphate-buffered saline (PBS). Cell pellets were suspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 15 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, and 1 mM  $\beta$ -glycerophosphate with freshly added protease and phosphatase inhibitors, 5 mM NaF and 1 mM Na<sub>3</sub>VO<sub>4</sub>) and incubated for 30 min on

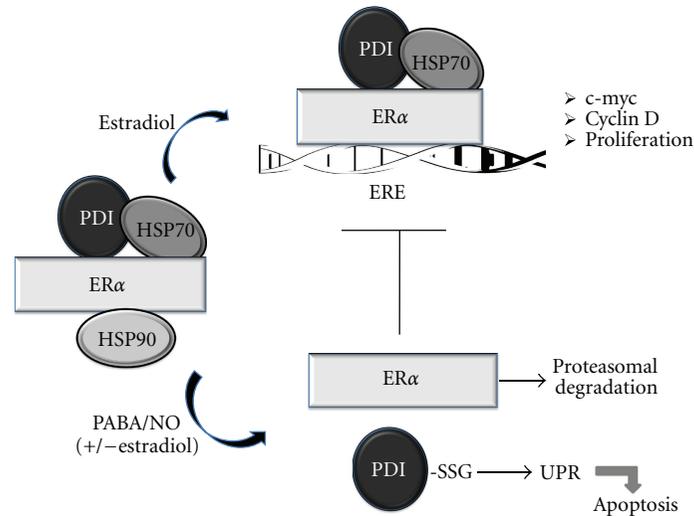


FIGURE 1: Model of the chaperone activity of PDI with ER $\alpha$ . S-Glutathionylation of PDI can interfere with protein folding, prevent protein interaction with ER $\alpha$ , and plausibly destabilize the receptor and subsequent ER $\alpha$  signaling.

ice. Lysates were sonicated for 10 sec and centrifuged for 30 min at 10,000 g at 4°C. Protein concentrations in the supernatant were assayed with the Bradford reagent (Bio-Rad Laboratories, Hercules, CA) using IgG as a standard.

**2.6. Immunoblot Analysis.** Equivalent amounts of protein were electrophoretically resolved under nonreducing conditions on 10% SDS-polyacrylamide gels (SDS-PAGE); unmodified proteins were separated under reducing conditions. Proteins were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Nonspecific binding was reduced by incubating the membrane in blocking buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 1  $\mu$ M protease inhibitors, 5 mM NaF, and 1 mM Na<sub>3</sub>VO<sub>4</sub>) containing 10% nonfat dried milk for 1 h. Membranes were incubated with the indicated antibody (blocking buffer containing 5% nonfat dried milk) at stated dilutions overnight at 4°C, washed 3x with PBS for 15 min, and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase for 1 h. The membranes were washed 3 times and developed with enhanced chemiluminescence detection reagents (Bio-Rad). The blots were scanned with a BioRad ChemiDoc system and visualized with a transilluminator. The images stored in a TIFF format. The relative intensity of bands was evaluated using Quantity One software (ver. 4.5.2; Bio-Rad) and plotted as arbitrary units (a.u.) in relation to actin.

**2.7. Spectroscopic Analysis of PDI In Vitro.** The structure of the S-glutathionylation refractory mutant (PDI-FLFL) was evaluated and compared to the wild type (PDI-WT) by protein tryptophan fluorescence scanning [8, 16]. The scan was recorded on an F 2500 spectrofluorometer (Hitachi) using 10  $\times$  10  $\times$  40 mm quartz cuvette, excitation and emission slits were 2.5 and 5.0 nm, respectively. The excitation wavelength was 295 nm to minimize an effect of protein tyrosines and

phenylalanines. Background spectra were subtracted from final emission of the protein. Quenching experiments were performed as follows: 1  $\mu$ M of PDI-WT or PDI-FLFL in 20 mM PB, pH = 7.4 was titrated with 0–5000 nM CsCl at room temperature under constant stirring. The Trp emission spectra (Ex. = 295 nm) were recorded and integrated using Felix32 standard software (PTI, NJ). All data were normalized to the emission proteins prior to addition of CsCl. Data represent the mean  $\pm$  SD for 3 independent experiments.

**2.8. PDI Activity Assay.** The enzyme activities of PDI-WT and PDI-FLFL were monitored using the turbidimetric assay of insulin disulphide reduction [17]. Briefly, 0.25  $\mu$ M purified human PDI was added to a solution of insulin (0–250  $\mu$ M) and GSH (500  $\mu$ M) in a 0.1 M K<sub>2</sub>HPO<sub>4</sub> buffer containing 2 mM EDTA (pH 7.0). DTT has been shown to completely reduce the disulfide bonds and is used as a positive control. The increase in turbidity was monitored ( $\lambda$  = 630) at 30 s intervals for 30 min. Mean values and S.E. were computed for each group,  $N$  = 3.

**2.9. Cytotoxicity Assays.** 10,000 cells were seeded in 96-well plates in 50  $\mu$ L medium. Increasing drug concentrations of PABA/NO were added to a final volume of 100  $\mu$ L and maintained in drug for 72 h. Following drug exposure, cell viability was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) conversion assay [14]. Each drug concentration was represented in quadruplicate and three independent experiments were conducted. Mean values and S.E. were computed for each group.

**2.10. Statistical Analysis.** Experimental data presented represent the mean value  $\pm$  SD for at least three independent experiments. Data were analyzed for statistically significant

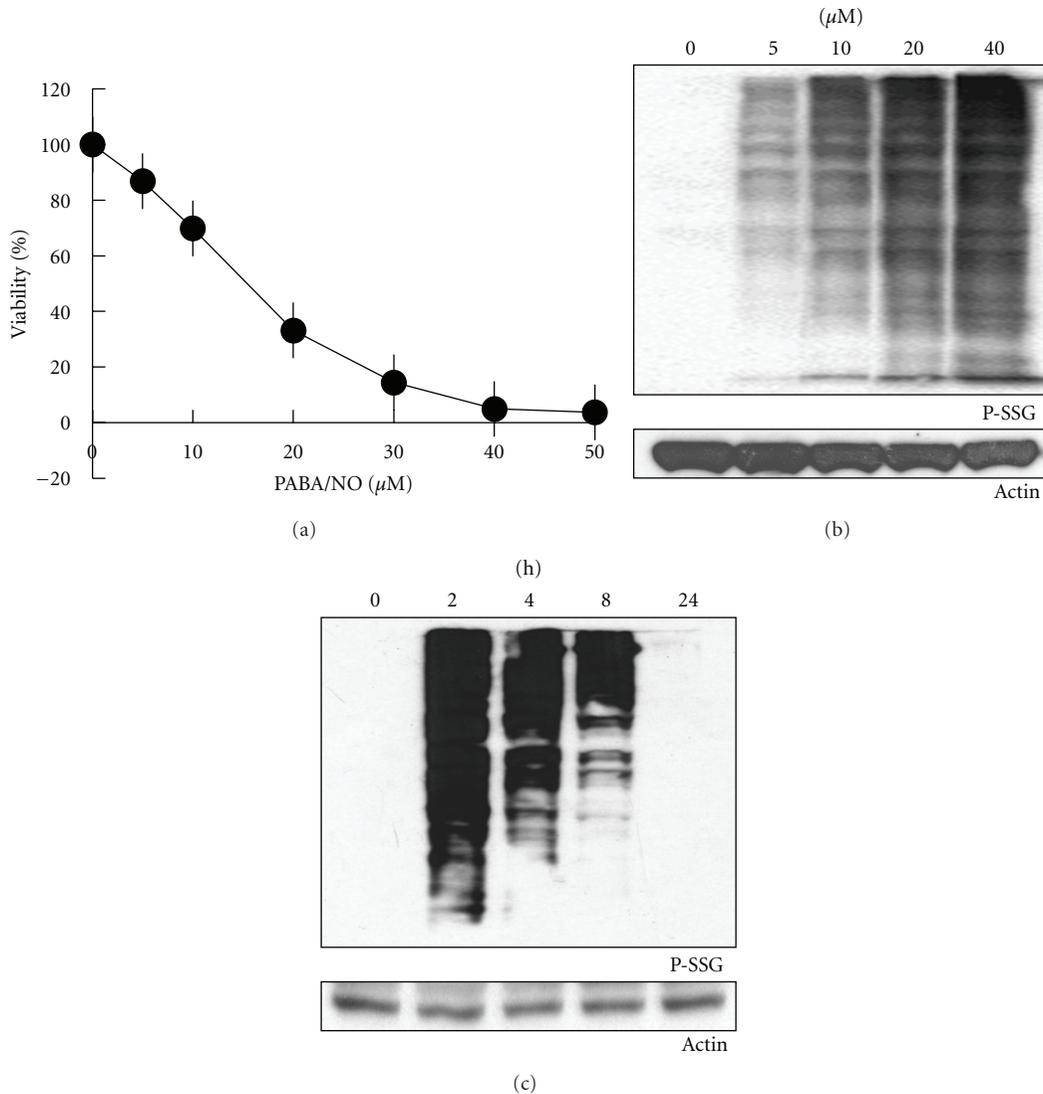


FIGURE 2: PABA/NO-induced S-glutathionylation of PDI *in vitro*. (a) MCF7 cells were seeded in a 96-well plate and treated with 0–50  $\mu\text{M}$  PABA/NO. Cell viability was measured at 72 h with the MTT assay [14]. Data represent the mean for 3 independent experiments  $\pm$  S.D. (b) MCF7 cells were treated with DMSO or PABA/NO at the indicated concentrations for 2 h. (c) MCF7 cells were treated with DMSO or 20  $\mu\text{M}$  PABA/NO for the indicated times. Cell lysates (40  $\mu\text{g}$ ) were separated and under nonreducing conditions and subjected to Western blot with anti-S-glutathionylation (P-S-SG) and antiactin antibodies.

differences between groups with Student's *t*-test using SigmaStat 3.5 (Systat Software Inc., San Jose, CA). Differences were considered statistically significant if the *P* value was  $<0.05$ .

### 3. Results

**3.1. S-Glutathionylation of PDI Disrupts the ER $\alpha$ -PDI Complex and Stability.** Prior studies have shown that PABA/NO induces dose-dependent cell death that is concurrent with S-glutathionylation and activation of the UPR in ovarian and leukemia cancer cells [8]. Using MCF7 cells, we confirmed the presence of a dose- (Figure 2(a)) and time-

(Figure 2). Under nonstressed conditions, PDI coimmunoprecipitates with ER $\alpha$ , Figure 3(a). However, PABA/NO-induced nitrosative stress-induced S-glutathionylation of PDI abrogates the interaction within 30 min following drug treatment (Figures 3(b) and 3(c)).

**3.2. PDI Levels Affect Drug-Induced ER $\alpha$  Proteasomal Degradation.** Chaperone functions of PDI are considered to be protective of ER $\alpha$  when exposed to either oxidative or nitrosative damage [13]. In fact, protein levels of ER $\alpha$  were diminished  $81 \pm 2\%$  following treatment with PABA/NO (Figure 4(a)). To determine if ER $\alpha$  levels were decreased as a consequence of proteasomal degradation, MCF7 cells were pretreated with proteasomal inhibitors (MG132 and

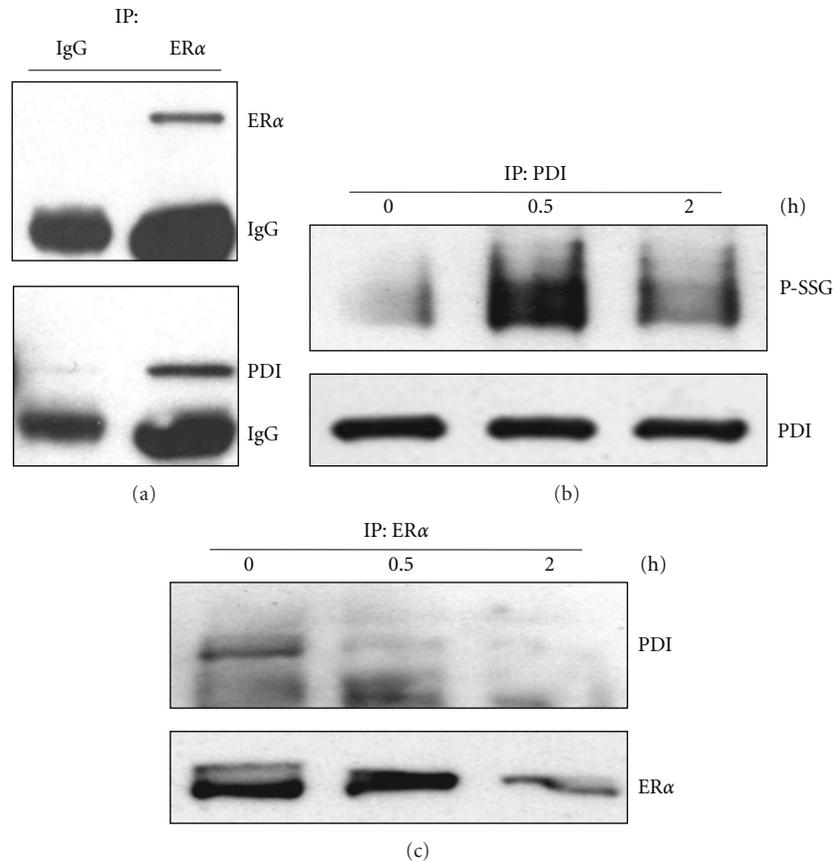


FIGURE 3: PABA/NO abrogates PDI-ER $\alpha$  interaction. (a) MCF7 cell lysates (1 mg) were incubated with anti-ER $\alpha$  antibody or control IgG at 4°C overnight. (b) MCF7 cells were treated with DMSO or 20  $\mu$ M PABA/NO for the indicated times. Cell lysates (300  $\mu$ g) were incubated with anti-PDI antibody at room temperature for 4 h. The precipitated endogenous PDI was subjected to Western blot with anti-S-glutathionylation (P-S-SG) and anti-PDI antibodies. (c) MCF7 cells were treated with DMSO or 20  $\mu$ M PABA/NO for the indicated times. Endogenous ER $\alpha$  was precipitated with the anti-ER $\alpha$  antibody at 4°C overnight. The precipitates were subjected to Western blot with anti-ER $\alpha$  and anti-PDI antibodies.

PS341) prior to subsequent PABA/NO exposures. Proteosomal inhibition blunted PABA/NO-induced ER $\alpha$  degradation (Figure 4(c)). To validate that PDI levels are critical to ER $\alpha$  stability, MCF7 cells were transfected with empty vector or with recombinant PDI to generate overexpressing cells. Figure 5(a) shows fluorescent images confirming that the sub-cellular localization of transfected, overexpressed PDI (right panel) is consistent with native PDI (left panel). Comparative analysis of vector or PDI-transfected cells treated with PABA/NO (Figure 5(b)) suggested that overexpression of PDI stabilized intracellular ER $\alpha$  levels.

**3.3. PDI-SSG Alters ER $\alpha$ -Mediated Gene Expression.** Previous studies have shown that estrogen stimulation can lead to activation of a range of proteins involved in cell proliferation [10]. We evaluated a cadre of such response proteins following  $\beta$ -estradiol  $\pm$  PABA/NO treatments (Figure 6). PABA/NO treatment appeared to counteract the expected estrogenic effects, leading to downregulation of c-Myc,

Cyclin D1, and p21. Such data are consistent with the antiproliferative and growth arrest effects of PABA/NO.

**3.4. S-Glutathionylation Refractory Mutant PDI Decreased PABA/NO-Induced Toxicity.** S-glutathionylation invariably occurs at cysteine residues found in a motif that exists in a low pKa environment [3]. Using site-directed mutagenesis we generated an S-glutathionylation refractory mutant, transfected it into host cells (PDI-FLFL), and evaluated its secondary and tertiary structure compared to PDI and PDI-SSG [8, 9]. S-Glutathionylation altered both the CD spectra and tryptophanyl fluorescence of native PDI. The tryptophanyl fluorescence scans of PDI-WT and PDI-FLFL have overlapping spectra ( $P < 0.05$ ), indicating the proteins are folded similarly (Figure 7(a)). Further validation that protein folding of PDI-FLFL was equivalent to PDI-WT was based upon the data showing quenching of the protein Trp fluorescence with CsCl (Figure 7(b)). There was no statistical difference between the PDI-WT protein

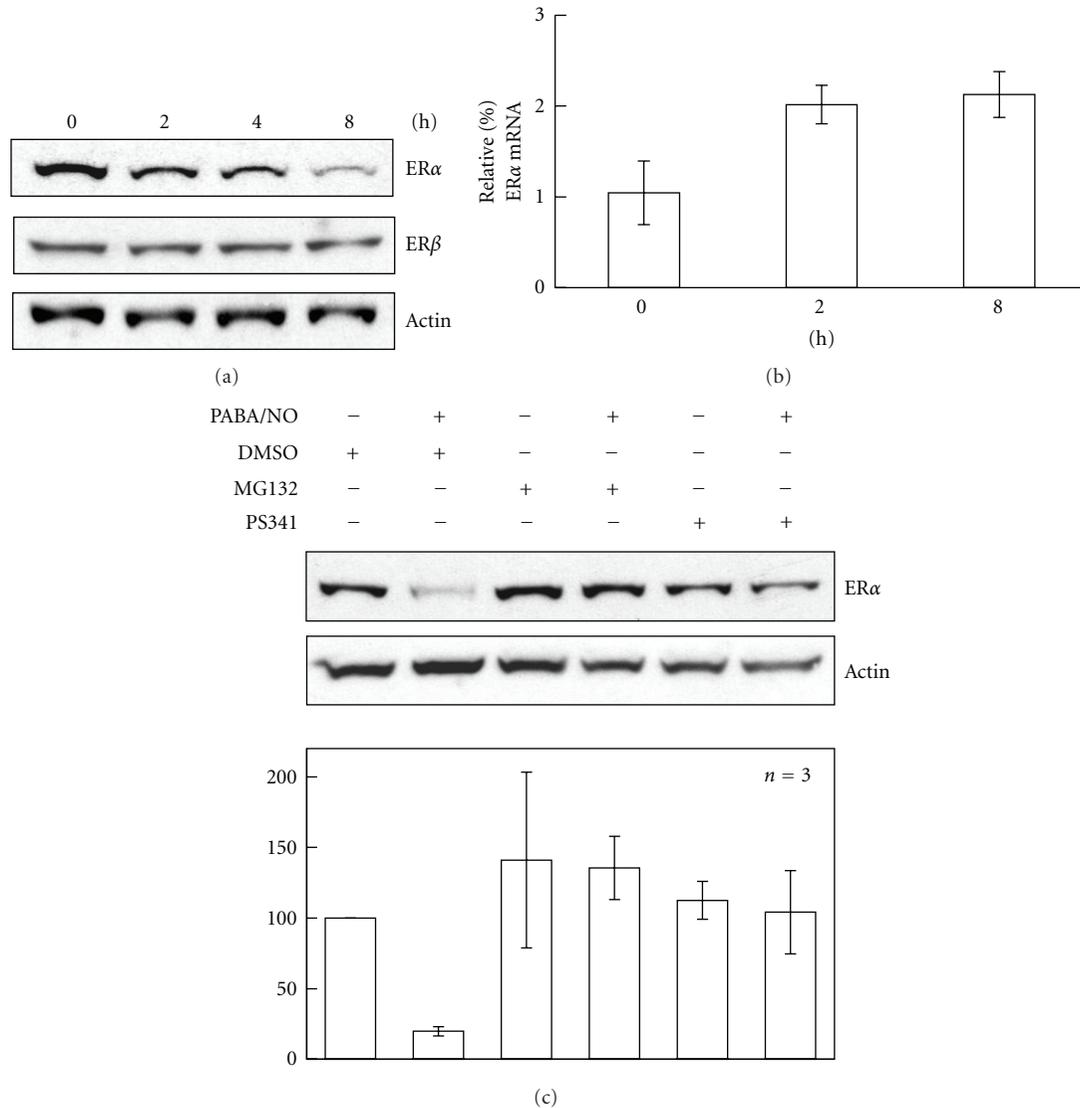


FIGURE 4: PABA/NO leads to ER $\alpha$  proteasomal degradation. (a) MCF7 cells were treated with DMSO or 20  $\mu$ M PABA/NO for the indicated times. Cell lysates (40  $\mu$ g) were subjected to Western blot with the indicated antibodies. (b) MCF7 cells were treated with DMSO or 20  $\mu$ M PABA/NO for the indicated times. (c) MCF7 cells were pretreated with proteasome inhibitors MG132 (1  $\mu$ M) or PS341 (10  $\mu$ M) for 2 h and then treated with 20  $\mu$ M PABA/NO for 6 h. Cell lysates (40  $\mu$ g) were subjected to Western blot with the indicated antibodies. Bar diagram showed the means and standard deviations determined from three independent experiments.

and the S-glutathionylation refractory mutant ( $P < 0.05$ ). Measurement of isomerase activity showed PDI and PDI-FLFL were essentially equivalent (Figure 7(c)). Recombinant proteins were incubated with PABA/NO and GSH for 30 min. PDI was S-glutathionylated in a dose-dependent manner, however PDI-FLFL was refractory (Figure 7(d)). PDI-WT and PDI-FLFL were transfected into MCF7 cells and treated with PABA/NO to induce S-glutathionylation and activate UPR-induced cell death. Following immunoprecipitation, PDI-WT was S-glutathionylated as detected by Western blot analysis. However, PDI-FLFL was not modified (Figure 7(e)). S-Glutathionylation of PDI is the trigger for ER $\alpha$ -PDI complex disruption and activation of the UPR. Consequently,

the toxic effects of PABA/NO were diminished in MCF7 cells transfected with the S-glutathionylation-resistant mutant, PDI-FLFL (Figure 7(f)).

#### 4. Discussion

ER $\alpha$  signaling is a contributing factor in the regulation of breast cancer cell proliferation. Targeting ER $\alpha$  signaling is an important and successful treatment strategy for most breast cancer patients that are ER $\alpha$  positive. In the preclinical evaluation of PABA/NO, PDI was identified as a molecular target of the drug [2, 5, 8, 18–20]. Specifically, PDI is S-glutathionylated on cysteine residues within the catalytic

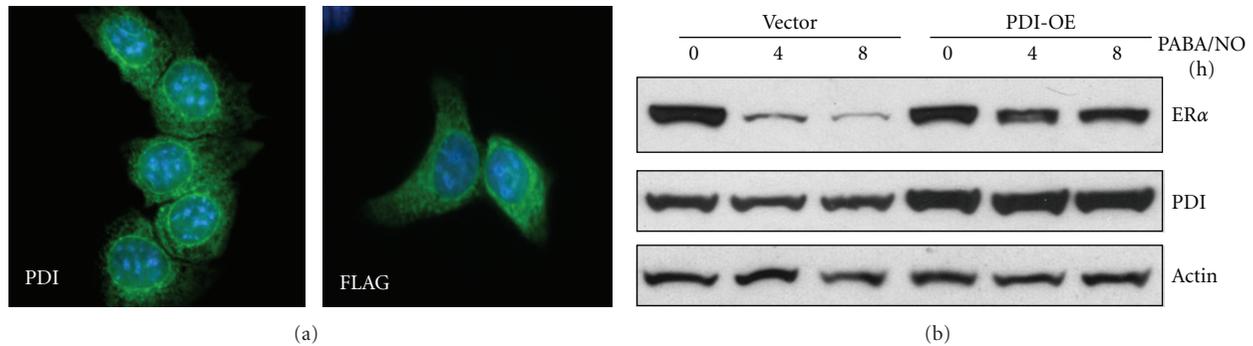


FIGURE 5: PDI expression levels affect PABA/NO-stimulated ER $\alpha$  degradation. (a) shows fluorescent images detailing similar subcellular distribution of PDI in normal and transfected cells. (b) shows Western blots of vector or PDI-transfected cells treated with PABA/NO for various times. In the transfected cells, over-expression of PDI stabilized intracellular ER $\alpha$  levels, preventing PABA/NO-induced S-glutathionylation from causing ER $\alpha$  degradation.

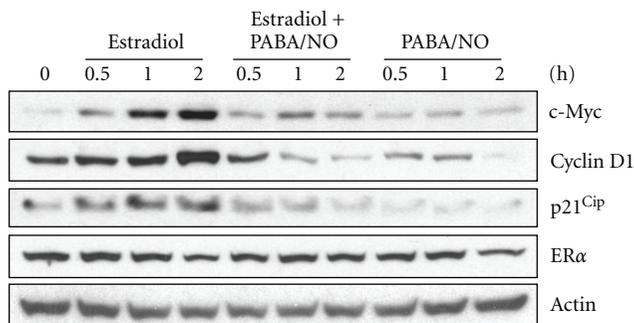


FIGURE 6: PABA/NO compromises ER $\alpha$ -mediated gene expression. MCF7 cells were treated with DMSO, 20 nM  $\beta$ -estradiol (E2), 20  $\mu$ M PABA/NO or both for the indicated times. Cell lysates (40  $\mu$ g) were subjected to Western blot with the indicated antibodies.

domains following nitrosative stress, thereby blunting isomerase activity [8, 9]. PDI has homology with the estrogen-binding domain of ER $\alpha$  and acts as a molecular chaperone that is required for ER $\alpha$ -mediated gene regulation [21]. In the present study we evaluated whether S-glutathionylation of PDI also impacts chaperone function. Our data suggest that S-glutathionylation of PDI leads to disruption of the PDI:ER $\alpha$  complex, proteosomal degradation of transcription factor, and diminished ER $\alpha$  mediated gene regulation.

PDI is organized into five domains (a, b, b', a', and c). There are two active sites in the catalytic domains a and a', each having two conserved cysteine residues that cycle between oxidized (disulfide) and reduced (dithiol) states to facilitate the folding and correct disulfide bond formation of its protein substrates [22]. The crystal structure of yeast PDI suggests that the a, b, b', and a' domains form a twisted U shape with the catalytic domains facing each other and an internal hydrophobic surface that interacts with substrate proteins [23]. Residues 101–144 of mature PDI have significant similarity (29.5% identity) with residues 350–392 of ER $\alpha$ . Moreover, residues 163–211 of mature PDI

are homologous with residues 304–349 of ER $\alpha$ . It is noteworthy that PDI segment 101–144 comprises the C-terminal part of the a domain, a short interdomain linker, and N-terminal part of the b domain, whereas segment 163–211 is exclusively located in the b domain. It would seem plausible that estrogen binding may cause a conformational change that interferes with the functional coordination between the catalytic domain and substrate-binding region, with the consequence that PDI enzymatic activity is suppressed. Accordingly, the apparent  $K_d$  value of PDI for estrogens such as  $\beta$ -estradiol has been determined to be 1.5–2  $\mu$ M [10]. The catalytic activity of PDI (measured by insulin degradation or reactivation of randomly oxidized RNase) is inhibited by estradiol *in vitro*, with an IC<sub>50</sub> of approximately 100 nM [21]. As an estrogen-binding protein, PDI can serve to modulate the intracellular estrogen reservoir and slow down estrogen metabolic disposition. Moreover, under certain conditions, PDI-bound estrogen can be released and augment the transcriptional activity of ER $\alpha$  [10].

S-Glutathionylation of proteins generally occurs on cysteine residues with a low pKa [3]. Using site-directed mutagenesis we generated a mutant PDI that is S-glutathionylation refractory with isomerase activity equivalent to native PDI. Transfection of PDI-FLFL into breast cancer cells resulted in diminished PABA/NO-induced toxicity as a result of S-glutathionylation refractory PDI. Hence the triggers for abolishing the ER $\alpha$ -PDI complex and activation of the UPR were blunted.

Our present data indicate that PDI is an ER $\alpha$ -interacting partner. Previously, studies have been designed to determine whether PDI regulates ER $\alpha$  protein levels. RNAi technology has been used to knock down PDI in MCF-7 cells but interpretation of results has not always been consistent. For example, Fu et al. demonstrated that PDI knockdown is associated with downregulation of ER $\alpha$  [10], whereas Schultz-Norton et al. reported the exact opposite finding that decreased expression of PDI leads to an increase in ER $\alpha$  protein levels [13]. Both groups have used at least two different RNAi sequences, thus a precise understanding of the relationship between PDI and ER $\alpha$  stability remains

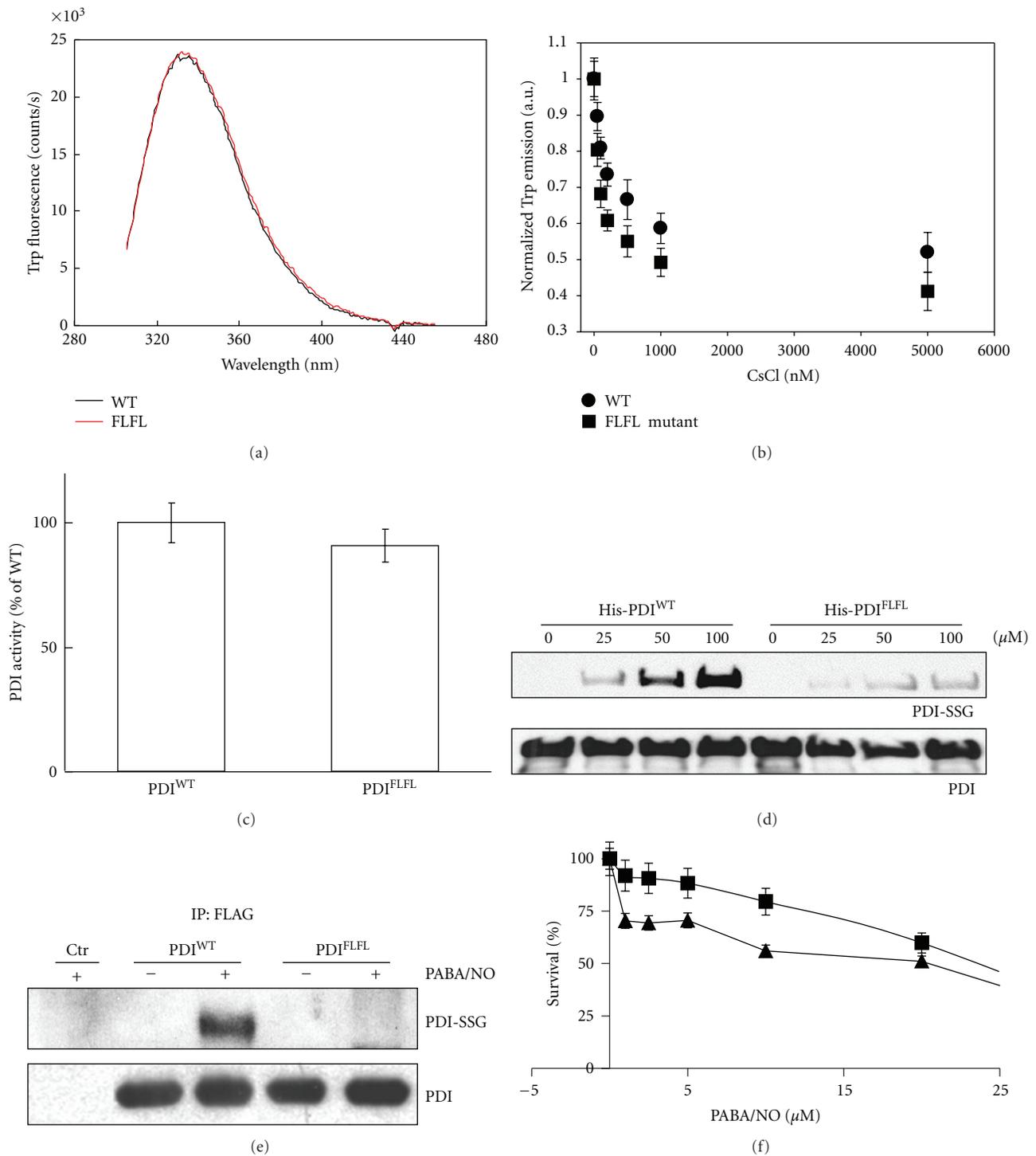


FIGURE 7: Mutations impair PABA/NO-induced S-glutathionylation of PDI. (a) Spectroscopic analysis of PDI-WT (black) and PDI-FLFL (red) was performed using tryptophanyl fluorescence of the purified proteins. (b) Quenching experiments were performed to further assess protein folding for PDI-WT (●) and PDI-FLFL (■). (c) The enzymatic activity of PDI-WT and PDI-FLFL was evaluated using the insulin turbidity assay. No statistical differences were observed in the folding (a-b) or isomerase activity (c),  $P > 0.05$ . (d) S-glutathionylation of PDI-WT and PDI-FLFL was evaluated following drug treatment. (e) MCF7 cells overexpressing PDI-WT or PDI-FLFL were treated with DMSO or 20  $\mu\text{M}$  PABA/NO. Following immunoprecipitation, the samples were evaluated by Western blot with anti-S-glutathionylation and anti-PDI antibodies. (f) MCF7 cells transfected with control (▲) or PDI-FLFL (■) were seeded in a 96-well plate and treated with 0–25  $\mu\text{M}$  PABA/NO. Cell viability was measured at 72 h with the MTT assay [14]. Data represent the mean for 3 independent experiments  $\pm$  S.D.

undefined. The results from the present study demonstrate that PDI levels do have a direct impact on ER $\alpha$  stability. Moreover, changes in redox homeostasis induced by nitrosative/oxidative stress cause S-glutathionylation of PDI and mediate cell death through activation of the UPR and abrogation of ER $\alpha$  stability and signaling.

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## Review Article

# The Yin and Yang of Nrf2-Regulated Selenoproteins in Carcinogenesis

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The NF-E2-related factor-2 (Nrf2) is a transcription factor which regulates the major cellular defense systems and thereby contributes to the prevention of many diseases including cancer. Selenium deficiency is associated with a higher cancer risk making also this essential trace element a promising candidate for cancer prevention. Two selenoproteins, thioredoxin reductase-1 (TrxR1) and glutathione peroxidase-2 (GPx2), are targets for Nrf2. Selenium deficiency activates Nrf2 as does a TrxR1 knockout making a synergism between both systems plausible. Although this might hold true for healthy cells, the interplay may turn into the opposite in cancer cells. The induction of the detoxifying and antioxidant enzymes by Nrf2 will make cancer cells chemoresistant and will protect them against oxidative damage. The essential role of TrxR1 in maintaining proliferation makes its upregulation in cancer cells detrimental. The anti-inflammatory potential of GPx2 will help to inhibit cancer initiation and inflammation-triggered promotion, but its growth supporting potential will also support tumor growth. This paper considers beneficial and adverse consequences of the activation of Nrf2 and the selenoproteins which appear to depend on the cancer stage.

## 1. Introduction

An adequate-to-high selenium supply and activation of Nrf2 by dietary compounds are considered to substantially help to prevent cancer development. Selenium exerts its effects mainly as part of selenoproteins with redox functions, and Nrf2 upregulates enzymes of the adaptive response. Thus, both systems are involved in the equipment of cells with a network of enzymes which are supposed to counteract the transformation of healthy into cancer cells by oxidative damage. However, not all attempts to prevent cancer by respective dietary supplementation/intervention ended up with a beneficial outcome; even harmful effects were observed.

The so-called Linxian trial was among the first large randomized, double-blind, primary prevention studies investigating a putative prevention of cancer by vitamins and trace elements. A mixture of selenium, vitamin E, and  $\beta$ -carotene, called factor D, significantly reduced total mortality, total cancer mortality, and most significantly mortality from

gastric cancer [1]. Although selenium was not given as a single component, according to subsequent studies it appeared to have the most efficient effects [2–4]. 10 years after completion of the Linxian trial, reduction in mortality remained 5% for total and 11% for gastric cancer [5]. Considering age, the effect of factor D was much stronger in individuals younger than 55 but almost absent in subjects older than 55 years. The effect on esophageal cancer was even reversed by age [5]. The findings may indicate that selenium supplementation is only helpful to rescue a marginal deficiency and that a benefit of the supplementation depends on the stage of carcinogenesis. Whereas selenium appears to prevent initiation of cancer in healthy cells at young age, in the elderly it may be harmful and rather support tumor growth of already initiated cells [5].

Nrf2 as regulator of the endogenous response system has generally been considered as beneficial, too. Since two selenoproteins, thioredoxin reductase-1 (TrxR1) and glutathione peroxidase-2 (GPx2), are induced by Nrf2, a synergism of both systems has been proposed [6]. Whereas this might hold

true for healthy cells, solely beneficial functions of Nrf2 have been questioned especially in cancer. Recent data revealed a “dark” side of Nrf2. Its upregulation in cancer cells provides an advantage for these cells to grow and, in addition, makes them resistant against chemotherapy (reviewed in [7, 8]). Thus, also a benefit of Nrf2 activation might depend on the cancer stage. Evidence to support this idea is summarized in view of the mutual regulation of selenium/selenoproteins and Nrf2 (see Figure 1).

## 2. Nrf2

Nrf2 is a transcription factor which is kept in the cytosol by Keap1. Keap1 acts as substrate adaptor for the Cul3-Rbx1 E3 ligase which ubiquitylates Nrf2 for proteasomal degradation. Dissociation of this complex is achieved by thiol modification of Keap1 preventing degradation and allowing newly synthesized Nrf2 to translocate into the nucleus. There it binds to the antioxidant/electrophile responsive element (ARE/EpRE) in the promoter region of its target genes. The mechanism of activation is complex but has become relatively clear in the very recent years and is described in multiple reviews [9–18].

Nrf2 regulates the expression of proteins that collectively favour cell survival. These comprise enzymes that directly or indirectly have antioxidant functions, are molecular chaperones and proteins that enhance glutathione synthesis and regeneration, belong to enzymes of the phase 2 detoxification drug metabolism systems, and recognize, repair, and remove damaged proteins and DNA. Also proteins that regulate the expression of other transcription factors, growth factors, and receptors and inhibit cytokine-mediated inflammation and autophagy are targets of Nrf2 (reviewed in [9, 19]). The realm of Nrf2 activators comprises endogenous signaling molecules produced during normal oxygen metabolism and under inflammation or other stress situations. Among activators are  $H_2O_2$ , ROOH,  $ONOO^-$ , oxoaldehydes, and ketones, or cyclopentenones, like 15-deoxy- $\Delta^{12,14}$ -prostaglandin  $J_2$ . Exogenous activators comprise dietary isothiocyanates, thiocarbamates, trivalent arsenicals, quinones, dithiolethiones, vicinal dimercaptanes, certain statins, and heavy metals [9, 12, 19]. Activation by the later group allows the conclusion that these compounds may act via initiation of a moderate oxidative/electrophilic stress.

The protective role of Nrf2 is also demonstrated by genetic ablation. Nrf2-deficient mice are more susceptible to carcinogen-induced cancers [20] and develop more severe intestinal inflammation and a higher number of aberrant crypts than controls upon dextran sulfate sodium treatment [21–23]. These findings indicate a role of Nrf2 in the prevention of carcinogenesis, especially if inflammation triggered. In contrast, without challenge, Nrf2-deficient mice did not show an obvious phenotype [24]. The Nrf2 system, thus, appears to be an emergency device that comes into play if a stress is severe enough that it can no longer be handled by constitutively active systems. An enhanced Nrf2 activity by moderate stress makes cells resistant to a subsequent more severe oxidative and electrophilic stress and, thus, works like

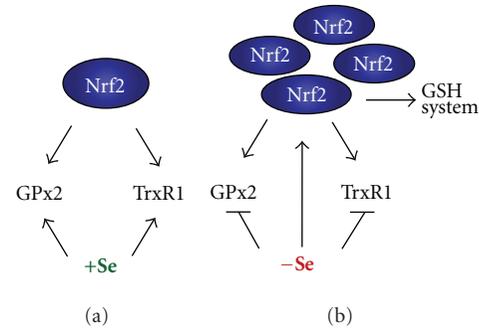


FIGURE 1: Interplay between Nrf2 and the selenoproteins thioredoxin reductase-1 (TrxR1) and glutathione peroxidase-2 (GPx2). (a) In the presence of selenium, the activation of Nrf2 leads to increased mRNA of both enzymes which can be translated into respective proteins. (b) Under selenium deficiency, Nrf2 is activated which in principle can lead to an induction of TrxR1 and GPx2 mRNA. Due to lack of selenium, the proteins cannot be synthesized. Decrease in TrxR1 further activates Nrf2, which subsequently upregulates enzymes of the glutathione (GSH) system. These, in part at least, can compensate the reduced TrxR1 activity. For details see text.

a vaccination. This way, by upregulation of defense systems, Nrf2 can prevent cancer initiation by elimination of reactive oxygen species and detoxification of carcinogens.

However, also this coin has two sides, since Nrf2 activation may not be beneficial under all circumstances. Not only normal but also tumor cells may benefit from the protective function of Nrf2 as evidenced by an increase of Nrf2 and its targets in many cancer cell lines (reviewed in [7, 17]). The physiological result of the upregulation, that is, inhibition of apoptosis and autophagy, and increase of proteasomal degradation of damaged proteins, provides a superior survival chance also for tumors. Accordingly, Nrf2 was expressed in a significantly higher proportion of endometrial serous carcinoma, the most aggressive subtype of endometrial cancer [25]. Nrf2 knockdown inhibited tumor growth from human cervical carcinoma cells in xenograft studies [26] and increased efficiency of chemotherapy in mice bearing subcutaneous tumors of these cells. In an urethane-induced lung cancer mouse model, Nrf2 deletion decreased tumorigenesis and facilitated death of early initiated cells by apoptosis [27]. These findings support cell survival properties of Nrf2 also in cancer cells.

A constitutive activity of Nrf2 is often reached by mutations in Keap1 and Nrf2 genes themselves [28]. Somatic mutations and loss of heterozygosity of Keap1 were first found in small cell lung carcinoma cell lines [29] and in non-small-cell lung cancers [30] associated with an upregulation of Nrf2 and respective target genes. RNAi-mediated downregulation of Nrf2 in these cells suppressed tumor growth in xenograft experiments and increased sensitivity to chemotherapy [31]. Somatic Keap1 mutations have also been found in cancers of gallbladder and hepatic bile duct [32] and prostate [33]. Nrf2 missense mutations were identified in lung cancers [34] and in squamous cell carcinomas of oesophagus and skin [35]. The mutations lie in the Nrf2-Keap1 interaction area, which

might similarly disturb the Nrf2/Keap1 complex formation as do mutations in the same areas of Keap1 [28].

A Keap1-independent increase in the basal Nrf2 level by oncogenic alleles of *Kras*, *Braf*, and *cMyc* has been described recently [36]. Whereas an ectopic transduction of mouse embryonic fibroblasts (MEFs) and NIH3T3 fibroblasts with K-Ras<sup>G12D</sup> led to an increase in the production of reactive oxygen species (ROS), inducible expression of endogenous K-Ras<sup>G12D</sup> in MEFs decreased ROS production and increased Nrf2 as well as enzymes of the Nrf2 antioxidant program. The K-Ras<sup>G12D</sup> mutation is commonly found in human pancreatic cancer [36]. Accordingly, higher NQO1 protein and lower levels of ROS biomarkers were detected in murine and human pancreatic intraepithelial neoplasia (PanIN) compared to normal tissue. The relevance of oncogene-mediated Nrf2 activation was proven in PanIN from Nrf2-deficient mice in which K-Ras<sup>G12D</sup>-induced proliferation and tumorigenesis was much less than in PanIN from wild-type (WT) mice.

A relatively novel system regulated by Nrf2 is the proteasome, a protease complex responsible for the degradation of proteins tagged with polyubiquitin chains [37]. The 26S proteasome consists of the catalytic 20S core subunit and the 19S regulatory particle, both consisting of different subunits. The 20S proteasome degrades oxidatively modified proteins and is activated upon mild oxidative stress [38]. The S5a subunit of the 19S proteasome and the  $\alpha$ -5 subunit of the 20S proteasome were enhanced in colon tumors compared to the surrounding normal tissue [39]. The higher levels correlated with an elevated nuclear localization of Nrf2. Activation of Nrf2 by electrophilic stress in human colon cancer cell lines further elevated these subunits and increased TRAIL-mediated NF $\kappa$ B activation leading to a protection against apoptosis [39].

Also upregulation of enzymes metabolizing xenobiotics will not always improve detoxification but increase the toxicity of xenobiotics as reviewed by Hayes et al. [40]. In fact, activated Nrf2 and upregulation of GSTP1 in hepatocarcinogenesis were the first hint to a supportive role of Nrf2 in cancer cells [41]. Thus, enhancement of Nrf2 and the resulting upregulation of multidrug resistance-associated proteins can help cancer cells to escape from chemotherapy [42, 43]. Chemoresistance has indeed been observed after treatment of breast cancer cells with tamoxifen [44] and of ovarian cancer cells with cisplatin [45] or other drugs [46]. Accordingly, knockdown of Nrf2 prevented resistance to tamoxifen in breast cancer cells [44] and resistance to doxorubicin in MEFs from Nrf2 knock-out (KO) mice [47].

However, Nrf2 is not activated in all types of cancer cells. It is even decreased in a high number of breast cancer cells compared to normal mammary epithelial cell lines. This coincides with variable but detectable levels of Keap1 and consistently increased mRNA and protein levels of Cul3, the ubiquitin ligase tagging Nrf2 for proteasomal degradation. Accordingly, downregulation of Cul3 in MCF-7 cells rescued Nrf2 and its targets [48]. A decrease of Nrf2 in the human breast cancer cells lines MDA-MB-231 and Hs578T is caused by silencing of the Keap1 RNA-stabilizing miR200a (miRNA) leading to a higher degradation of Nrf2 [49].

Taken together, cancer cells use all facets of the adaptive response to escape elimination. In consequence, under certain circumstances, the protective functions of Nrf2 can switch to procarcinogenic ones [7, 17, 28]. Nrf2 appears to be more active in some cancer cells and less in others, depending on the cell context, the nature of stress, and the cancer stage. Also Nrf2 targets may have dual roles in cancer which will here be discussed for the selenoproteins thioredoxin reductase-1 (TrxR1) [50, 51] and glutathione peroxidase-2 (GPx2) [52, 53].

### 3. TrxR1

Thioredoxin reductases (TrxRs) are a family of NADPH-dependent selenoflavoproteins (TrxR1, TrxR2, and TGR) present in almost all living cells (for reviews see [54, 55]). Together with thioredoxin (Trx) and NADPH, they build up the thioredoxin system. The system maintains a reducing environment in the cytosol, among others required for the redox regulation of gene expression via transcription factor activity. It, thus, is involved in DNA repair, angiogenesis, and inhibition of apoptosis. Moreover, during DNA synthesis Trx directly transduces electrons to ribonucleotide reductase which requires the continuous reduction of thereby oxidized Trx by TrxR. These functions underscore the pivotal role of TrxR in cell proliferation and survival [55, 56]. Due to its antioxidant function and its upregulation in cancer cell lines [57] and human gastrointestinal cancer tissue [58], TrxR was first expected to counteract malignant transformation. This hypothesis was supported by the fact that TrxR1 regulates the correct maturation of the tumor suppressor p53 [59, 60]. Furthermore, TrxR1 was the first selenoprotein identified as target of Nrf2 [50, 61] which at first glance was interpreted as support for a protective role [50, 62]. This might indeed hold true for the prevention of initiation of carcinogenesis in healthy cells.

However, it soon turned out that the beneficial effects of the Trx/TrxR system change to its opposite during the growth and progression phase of tumors. In fact, upregulation in cancer cells might also reflect the need of the enzyme for essential functions in cancer cells, the TrxR-dependent synthesis of deoxyribonucleotides [63]. Downregulation of TrxR1 by antisense RNA did not increase but inhibited growth of human hepatocarcinoma cells [64]. Also a knock-down of TrxR1 in lung carcinoma cells reversed their tumorigenicity and invasive potential in a xenograft model [65]. As underlying mechanism, the decreased expression of DNA polymerase  $\alpha$  was supposed [66]. Alternatively, the antiapoptotic function of TrxR1 may come into play. Reduced Trx is required to inhibit apoptosis signal-regulating kinase (ASK) [67]. Lack of TrxR1 will prevent inhibition of ASK making the elimination of malignant cells by apoptosis possible. Not surprisingly, TrxRs have been suggested as potential targets for anticancer drugs [68, 69]. The inhibitory mechanism of such drugs often is the same as used for the dissociation of Nrf2 from Keap1, namely, thiol modification. Modification of the selenol in the active centre of TrxR1 indeed leads to an inhibition of the enzyme activity. On the

other hand, the same drugs activate Nrf2 as evident from the upregulation of Nrf2 targets such as glutathione reductase, a glutathione peroxidase, and GST [69]. The thereby also upregulated TrxR1 might interfere with the inhibition of enzyme activity and facilitate cancer cell growth. This should be considered when selecting drugs to inhibit TrxR activity.

However, TrxR1 is not the only enzyme required for proliferation. Cells from mice with a differentiated hepatocyte-specific KO of TrxR1 were able to proliferate [70]. Proliferation of hepatocytes lacking TrxR1 was observed by *in vivo* staining [71] and MEFs from conditioned TrxR1 KO mouse embryos did not show impaired proliferation [72]. Thus, TrxR1 deletion appears to be compensated by another system. This most probably is the glutathione (GSH) system as demonstrated by a severely reduced replicative index if also GSH is depleted [71], and by an upregulation of GSH metabolizing enzymes in MEFs from conditioned TrxR1 KO mice [72]. The latter obviously is achieved in TrxR1-depleted cells by an activation of Nrf2 [73]. Deletion of TrxR1 in all parenchymal hepatocytes of mice resulted in a compensatory upregulation of Nrf2 targets including GSTs, GPx2, and sulfiredoxin [73]. It was, thus, concluded that ablation of *txnr1* encoding TrxR1 would mimic oxidative challenge and switch on a constitutively active Nrf2 pathway [73]. What is activating Nrf2 when TrxR1 is absent is unclear, but it fits with the observations made with TrxR1 inhibitors (see above) and the upregulation of Nrf2 targets in moderate selenium deficiency in mice [74, 75]. A challenging hypothesis would be that the thioredoxin system maintains critical thiol groups in Keap1 in the reduced state and, thereby, prevents Nrf2 release. That Trx is able to reduce Cys151 in Keap1 has recently indeed been shown [76]. This way TrxR1 would serve as turn-off signal for the Nrf2 system.

In sum, a critical balance between Nrf2 and TrxR1 activities might exist which appears worth to be further investigated.

#### 4. GPx2

The gastrointestinal glutathione peroxidase (GPx2) was first detected in the gastrointestinal system [77]. There it obviously plays a role in proliferating cells since its concentration is highest at crypt bases where proliferation takes place [78]. During human colon carcinogenesis, GPx2 is transiently increased with the highest expression in early adenoma and decreasing amounts in late stages of malignancy [78, 79]. It is also upregulated during the neoplastic transformation of squamous epithelial cells [80] and in lung adenocarcinomas of smokers [81] indicating that its expression is not restricted to the gastrointestinal system, but rather characteristic for rapidly dividing epithelial cells in general [82]. Evidences for a protective role are provided by genetically modified animals. A GPx2 KO rendered mice more susceptible to skin cancer development upon  $\gamma$ -irradiation [83]. Mice in which both GPx1 and GPx2 had been knocked out developed ileocolitis [84] and later intestinal cancer [85]. The lack of GPx2 was more detrimental, since one intact allele of GPx2 (but not of GPx1) was sufficient to prevent intestinal

inflammation [82]. Mechanistic studies with HT29 cells with a stable downregulation of GPx2 by siRNA revealed that GPx2 suppresses COX2 expression and PGE<sub>2</sub> production [86]. Furthermore, the siGPx2 cells exhibited an enhanced invasive potential and migrated faster in a wound healing assay [87]. Both effects obviously required the upregulated COX2 activity since celecoxib, a specific COX2 inhibitor, rescued the effects to the level observed in control cells. A protective role of GPx2 can also be inferred from its induction by Nrf2 [52]. Thus, the majority of the findings described so far characterize GPx2 as an anti-inflammatory enzyme.

The function of GPx2 as an anticarcinogenic enzyme is less clear, and evidence for an additional procarcinogenic role is increasing. Apoptosis at colonic crypt bases is drastically increased in GPx2 KO mice [88]. Inhibition of apoptosis may reflect the physiological function of GPx2 in crypt bases where it appears to support cell proliferation in the self-renewal of the intestinal mucosa. Cancer cells, however, will profit from not being eliminated by apoptosis which might well be the reason why the siGPx2 cells were not able to grow anchorage-independently and developed into much smaller tumors than WT cells when injected into nude mice [87]. Indeed, GPx2 expression is higher in proliferating cancer stem cells compared to their differentiated progeny [89]. Transcriptional regulation further points into a procarcinogenic direction. GPx2 is induced by  $\Delta$ Np63 [90], a transcription factor necessary for cell proliferation, and its overexpression inhibited oxidant-mediated apoptosis [90]. Activation of the GPx2 promoter by  $\beta$ -catenin [91], which is the key mediator in the Wnt pathway and constitutively active in most of intestinal cancers, can again be interpreted controversially, either as an attempt to counteract carcinogenesis or to sustain cancer cell growth.

Some tentative answers may be derived from a study using an inflammation triggered model of colon carcinogenesis, the azoxymethane (AOM)/dextran sulfate sodium (DSS) mouse model. In this study AOM/DSS treatment was combined with feeding WT and GPx2 KO mice a moderately Se-deficient, Se-adequate, and Se-supranutritional diet [92]. All AOM/DSS-treated mice developed colitis which was generally more severe in GPx2 KO mice than in WT mice under all Se states and especially high in moderate Se-deficiency. Inflammation and accordingly tumor formation were decreased under the Se supranutritional diet. Tumor numbers per animal tended to be higher in GPx2 KO mice at all selenium diets and were decreased by supranutritional selenium. In contrast, tumor size was smaller in GPx2 KO mice at the moderate selenium deficiency and in the supranutritional status, which correlates with the smaller tumors in nude mice developing from HT29 cells in which GPx2 was knocked down (see above). In the same experiment the effect of sulforaphane (SFN), a well-known Nrf2 activator, was tested. Surprisingly, it enhanced colitis in Se-poor WT and GPx2 KO mice but decreased it in Se-adequate mice to an identical score in both genotypes. The same dependency on selenium was observed for the reduction in the number of tumors and apoptotic cells by SFN in both GPx2 KO and WT mice. This indicates

that SFN needs a selenoprotein or a selenium-dependent process to act beneficially. However, this protein cannot possibly be GPx2 since SFN effects were the same in WT and GPx2 KO mice. The responsible selenoprotein might rather be GPx1 which is upregulated in the intestine of GPx2 KO mice [88], but can only be synthesized when selenium is available. Taken together, there is an interplay between selenium and Nrf2 activators, but this appears to be much more complex than a synergistic upregulation by Nrf2-mediated transcriptional activation and subsequent translation of a specific selenoprotein.

In short, GPx2 appears to be a protective enzyme with pronounced anti-inflammatory potential and antiapoptotic capacity. Consequences of an upregulated GPx2 might, however, differ between healthy and malignant tissue. In healthy tissue, GPx2 is required to maintain the normal self-renewing of the gastrointestinal epithelium and, as part of the adaptive response, to depress inflammatory processes. This way GPx2 can inhibit initiation of carcinogenesis. But once a cell has been programmed to proliferate in an uncontrolled way, GPx2, *inter alia* by inhibiting apoptosis, supports further growth, which does not appear particularly beneficial. This view is in line with the clinical study described in the beginning which revealed a decrease of esophageal cancer incidence by selenium only in younger but not in older participants [5]. Similarly, in recent N-nitrosomethylbenzylamine-induced esophageal squamous cell carcinoma (ESCC) study, the numbers of dysplasia and ESCC were significantly lower in rats on supplementation with selenium and vitamin E only during the early stage of tumor development or during the entire experimental period but not during the late stage [93].

## 5. Conclusions

The benefit of upregulation/activation of the Nrf2 pathway of the selenoproteins TrxR1 and GPx2 differs in healthy and in cancer cells. Via its physiological role in a program maintaining the cellular redox state and *inter alia* the endogenous defense systems and by preventing apoptosis and damage by a dysregulated redox homeostasis, Nrf2 might contribute to the prevention of cancer initiation in healthy cells.

The vital function of TrxR1 is explained by its role in the replication and proliferation of developing healthy cells. The physiological function of GPx2 appears to support proliferation of crypt base epithelial cells in the self-renewal of the gastrointestinal epithelium. Its antiapoptotic and anti-inflammatory properties might help to inhibit the initiation and promotion of carcinogenesis by proinflammatory mediators.

However, if a cell has been transformed into a malignant cell and the carcinogenic process has started, the cancer cells will equally profit from the protective roles of TrxR1, GPx2, and other Nrf2 programs and, accordingly, will grow unhampered.

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