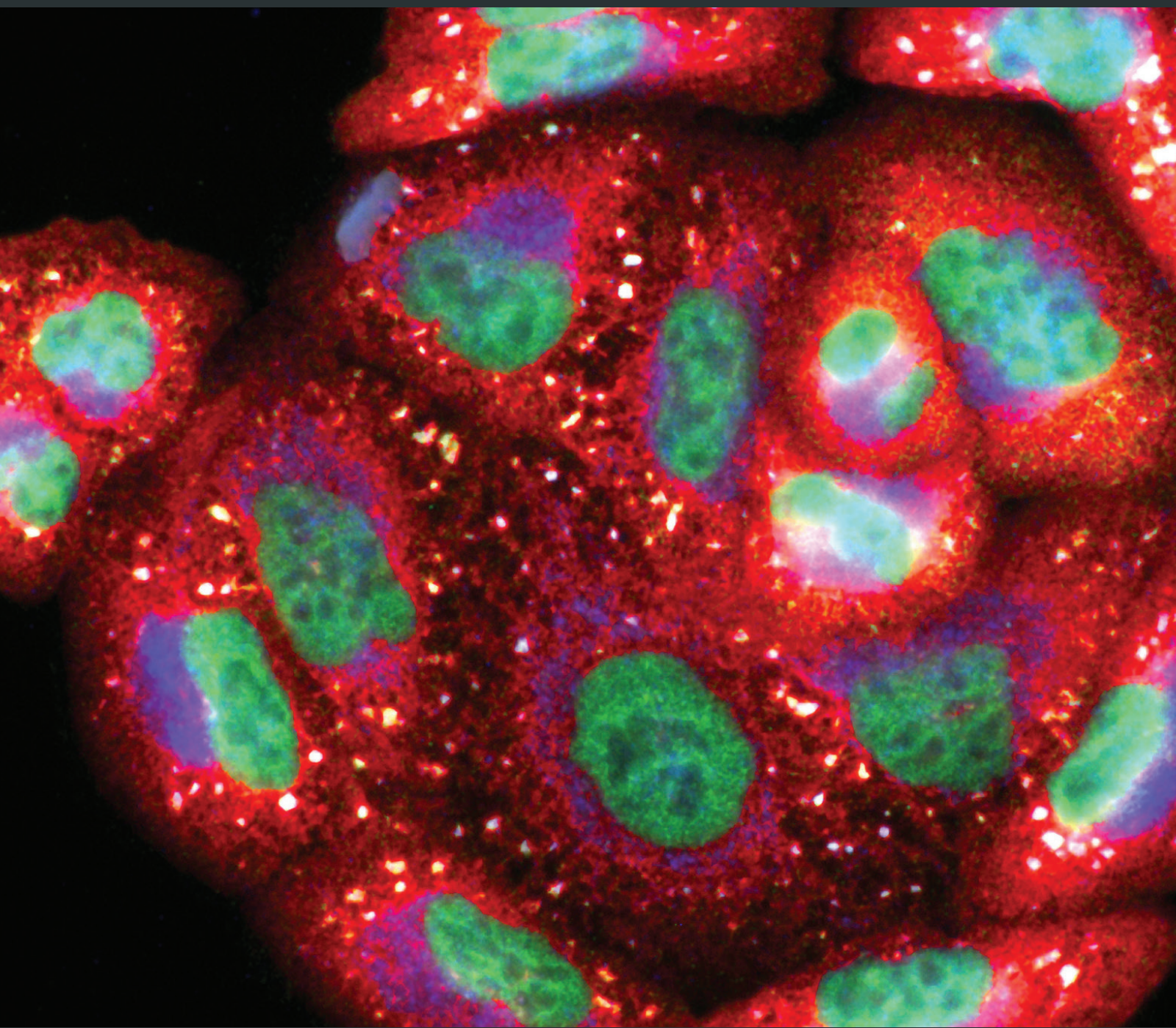
Oxidative Medicine and Cellular Longevity

Redox Status and Proteostasis in Ageing and Disease

Guest Editors: Federica Rizzi, Ioannis P. Trougakos, Gianfranco Pintus, and Gerasimos P. Sykiotis



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Editorial Board

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Editorial

Redox Status and Proteostasis in Ageing and Disease

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An extensive network of components, generally referred to as the proteostasis network (PN), safeguards the functionality and integrity of the proteome, thus ensuring an optimal and efficient cell function. Failure of the PN represents a common trait of several chronic and age-related pathological conditions. This special issue features a collection of reviews and original articles covering distinct aspects of the effect of redox imbalance on the integrated complex of adaptive molecular signaling required to actively maintain proteome stability and functionality. The broad range of pathologies covered reflects appropriately the central relevance of the PN across different disease areas.

In their review entitled "It Is All About U(biquitin): Role of Altered Ubiquitin-Proteasome System and UCHL1 in Alzheimer Disease" A. Tramutola et al. discuss the impairment of the proteasome system as a consequence of oxidative stress and how this contributes to Alzheimer disease (AD) onset and progression. In the review entitled "Killing Me Softly: Connotations to Unfolded Protein Response and Oxidative Stress in Alzheimer's Disease," B. Pająk et al. focused their attention on the possible causes of mitochondrial dysfunction in AD. Recent advances in the knowledge of mitochondria functions highlight that these organelles are extremely dynamic structures which not only represent the major bioenergetic hub of eukaryotic cells but also participate in the cellular signaling which control redox status and, likely, protein degradation. In "Cross Talk of Proteostasis and Mitostasis in Cellular Homeodynamics, Ageing, and Disease," S. Gumeni and I. P. Trougakos review the functional cross talk of proteostasis (homeostasis of the proteome) and mitostasis (mitochondrial homeostasis) in the maintenance of cellular homeodynamics; they also refer to the impairment of mitochondrial quality control and how this impacts on proteome stability during ageing and/or age-related diseases. The biological significance of compounds that modulate the cellular redox state (i.e., oxidants and antioxidants), their roles in brain health, and the impact of redox modulation as well as potential uses and limitations of natural antioxidant compounds in selected neuropsychiatric disorders are discussed by E. A. Fraunberger et al. in their review entitled "Redox Modulations, Antioxidants, and Neuropsychiatric Disorders." Oxidative stress has a major impact on the quality of membrane proteins highly expressed in the erythrocytes, which are required to preserve the structure and function of these cells. A. Pantaleo et al., in their original article entitled "Band 3 Erythrocyte Membrane Protein Acts as Redox Stress Sensor Leading to Its Phosphorylation by p^{72} Syk," present experimental data to support the hypothesis that band 3 acts as redox sensor regulating its own phosphorylation and that substances leading to protracted phosphorylation of band 3 may trigger a cascade of events culminating in hemolysis. Advances in understanding myocardial redox signaling pathways and promising antioxidant therapeutic approaches that may beneficially impact on myocardial physiology are presented by A. Arcaro et al. in "Novel Perspectives in Redox Biology and Pathophysiology of Failing Myocytes: Modulation of the Intramyocardial Redox Milieu for Therapeutic Interventions—A Review Article from the Working Group of Cardiac Cell Biology, Italian Society of Cardiology." The association between oxidative stress

biomarkers and cardiovascular risk factors as well as left ventricular hypertrophy in children with chronic renal failure is demonstrated by D. Drożdż et al. in the original research article entitled "Oxidative Stress Biomarkers and Left Ventricular Hypertrophy in Children with Chronic Kidney Disease." Finally, the paper from E. Al Jaaly et al. entitled "Pulmonary Protection Strategies in Cardiac Surgery: Are We Making Any Progress?" discusses the multifactorial mechanisms that relate to the activation of inflammatory and oxidative stress pathways and are involved in the development of pulmonary dysfunction.

The contributions selected for this special issue will concur to improve the understanding of the mechanisms that link failures of the cellular redox balance maintenance with the impairment of the protein quality control system, helping scientists to identify appropriate molecular targets for the development of new therapeutic strategies in the prevention and treatment of age-related diseases.

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Review Article

Cross Talk of Proteostasis and Mitostasis in Cellular Homeodynamics, Ageing, and Disease

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Mitochondria are highly dynamic organelles that provide essential metabolic functions and represent the major bioenergetic hub of eukaryotic cell. Therefore, maintenance of mitochondria activity is necessary for the proper cellular function and survival. To this end, several mechanisms that act at different levels and time points have been developed to ensure mitochondria quality control. An interconnected highly integrated system of mitochondrial and cytosolic chaperones and proteases along with the fission/fusion machinery represents the surveillance scaffold of mitostasis. Moreover, nonreversible mitochondrial damage targets the organelle to a specific autophagic removal, namely, mitophagy. Beyond the organelle dynamics, the constant interaction with the ubiquitin-proteasome-system (UPS) has become an emerging aspect of healthy mitochondria. Dysfunction of mitochondria and UPS increases with age and correlates with many age-related diseases including cancer and neurodegeneration. In this review, we discuss the functional cross talk of proteostasis and mitostasis in cellular homeodynamics and the impairment of mitochondrial quality control during ageing, cancer, and neurodegeneration.

1. Introduction

Cells express a pool of thousands of different proteins that need to be tightly controlled for proper cellular structure, organization, and function. The proteostasis network (PN) is an assembly of distinct dynamic molecular pathways that control the functionality of the proteome (proteome homeodynamics) during protein synthesis, folding, trafficking, and degradation. Failure of the PN is associated with broad range of diseases including cancer, neurodegeneration, and immunological and metabolic disorders [1]. Ageing leads to a gradual dysfunction of the proteostasis network and thus to proteome instability due to accumulation of damaged and/or misfolded proteins [2].

Mitochondria are the energy producing organelles in eukaryotic cell providing ATP through oxidative phosphorylation (OXPHOS). Moreover, mitochondria control cell death through apoptosis and supply Ca^{2+} and metabolites required for cellular homeodynamics [3]. We propose the term homeodynamics (instead of the term homeostasis) since cellular functionality obviously reflects a delicate highly dynamic balance of different (usually opposing in their final output) molecular pathways that aim towards a preset ideal equilibrium status rather than a static condition which is the true meaning of the word "-stasis" (from Greek *στάσις* "standing still"). In other words, the term homeostasis fails to illustrate the dynamic, adapting, and thus constantly remodelling nature of biological systems which determines survival (see also Rattan, 2014 and Demirovic and Rattan, 2013) [4, 5].

In line with this notion, mitochondria are highly dynamic organelles that undergo fission and fusion and move into the cell along the microtubules to generate the mitochondrial network [6]. Proper mitochondrial function also determines the functionality of most (if not all) of the other cellular organelles because of the specialized interacting functional networks that are generated; part of these networks is also established by contacts of the mitochondria with organelles (e.g., with endoplasmic reticulum, plasma membrane, and peroxisomes) [7–9]. For instance, the association of mitochondria with the endoplasmic reticulum (ER), in a juxtaposition known as Mitochondria-Associated Membrane (MAM), has an important role in controlling mitochondria

biogenesis, Ca^{2+} release, and lipid synthesis and apoptosis [10, 11]. In addition, the subcellular distribution of mitochondria can affect the cellular transcriptome and transcription rates. A recent study showed that mitochondria clustering around the perinuclear region can act as signaling for increased oxidative stress affecting hypoxia inducible promoters [12].

Mitochondrial dysfunction has also been associated with ageing and most of the so-called age-related diseases [13–17]. The maintenance of "healthy" and fully functional mitochondria is thus essential for cellular homeodynamics. A first check point and active surveillance is provided by the organelle itself. The mitochondria have their own chaperones and proteolytic enzymes that remove damaged or unfolded proteins [18–20]. Furthermore, impaired mitochondrial function and instability of the mitochondrial proteome activate a specific ubiquitin-proteasome response known as mitochondrial UPR (UPR^{mt}); UPR^{mt} thus provides a link between mitochondrial survival pathways and the multitasking UPS.

The plasticity of the mitochondria allows continuous changes of their shape and number, while their morphology is maintained by the equilibrium of fusion and fission events. Mitochondria undergo fusion and fission in order to avoid damage accumulation or respond to certain bioenergetics demands [21]. Fusion rearranges the matrix content of a damaged mitochondrion with a healthy one, diluting thus mutated DNA copies and unfolded proteome [22]. On the other hand, fission is important for mitochondria division and elimination of damaged mitochondria by autophagy [23]. If an extensive mitochondria damage persist the cells fate the apoptosis pathway releasing proapoptotic factors [24].

Herein, we will focus on cross talk of proteostasis and mitostasis in cellular homeodynamics, ageing, and disease.

2. Mitochondrial Chaperones and Proteases: Repair/Refold and Recycle

2.1. Chaperones. The mitochondrial proteome is composed of ~1500 peptides, of which only 13 are encoded by the mitochondrial genome. Therefore, the vast majority of mitochondrial proteins are synthesized in the cytosol and must be imported into the organelle [25, 26]. Most of the matrix proteins are transported in the mitochondria as precursor proteins, which are subsequently cleaved and assembled in multiprotein complexes (which can also be viewed as complex protein machines). Precursor proteins are transported across the narrow pores formed by the Translocase of the Outer Membrane (TOM) and the Translocase of the Inner Membrane (TIM) complexes, mostly in an unfolded state [27]. The whole process is under the surveillance of molecular chaperones in order to avoid the formation of protein aggregates or misfolded proteins (Figure 1). The nascent precursor peptide is bound by the cytosolic Hsp70 and Hsp90 chaperones that protect the hydrophobic segments of the peptide and keep them in unfolded conformation [28]. After the translocation in the mitochondria, the precursor peptide is bound to the matrix chaperones.

The two most dynamic networks of mitochondria chaperones are the mtHsp70 (an Hsp70 family member) and the multimeric Hsp60-Hsp10 machineries [29]. The mtHsp70 is part of the presequence translocase-associated import-motor (PAM) complex, which directly folds the incoming proteins. The mtHsp70 (via an ATP-dependent process) guides the translocation of the polypeptide chain through the translocase complexes of the outer and inner mitochondrial membranes and its complete unfolding [30].

Hsp60 forms large tetradecameric protein complexes consisting of two stacked rings that allow the accommodation of the unfolded polypeptide. The cavity of each ring gets closed by the Hsp10 cofactor. Conformational changes, after hydrolysis of ATP, lead to a more hydrophilic cavity which allows the folding of the polypeptide. The newly folded protein is then released after opening of the ring cavity by the dissociation of Hsp10 [31]. Hsp60 is required for the folding of new precursor peptides and plays an essential role in mitochondrial protein biogenesis [32].

An additional chaperone is Hsp78 (a member of the ClpB/Hsp104 family) which has a disaggregation function under stress conditions; Hsp78 is essential for the respiratory chain reaction and mitochondrial genome integrity under severe stress [33]. Mitochondrial chaperones deletion in yeast has lethal effects, indicating that heat shock proteins have an essential role in mitochondria quality control and protection of the organelle from unfolded protein aggregates and proteome instability [34].

2.1.1. The Hsp90-Type Chaperone TRAP1. TRAP1, also known as Hsp75, was initially identified as an Hsp90-like chaperone that interacts with the tumor necrosis factor (TNF) receptor and the retinoblastoma protein (Rb) [35]. However, later studies revealed that TRAP1 localizes in the mitochondrial matrix of mammalian cells [36, 37]. TRAP1 exhibits a significant sequence and structure similarity to the members of the Hsp90 family; these chaperones have a mitochondrial targeting sequence at their N-terminus (which is cleaved after mitochondrial translocation) and an ATP binding domain. The ATP binding site is the most conserved region between Hsp90 and TRAP1 [35, 38]. TRAP1 shows different functional characteristics from other chaperones and its expression in the cytosol could not rescue the Hsp90 loss of function phenotypes [35]. TRAP1 is thought to also play an important role in preventing cell death due to ROS accumulation. Specifically, downregulation of TRAP1 leads to ROS accumulation, while its overexpression suppresses ROS production [39, 40]. Moreover, TRAP1 regulates metabolic switch between oxidative phosphorylation and aerobic glycolysis [41]. Loss of TRAP1 in immortalized mouse fibroblasts and in human tumor cells resulted in increased mitochondrial respiration, as well as in increased oxygen consumption and ATP levels; these phenotypes were associated with suppression of aerobic glycolysis [41]. Further studies have shown that TRAP1 interacts with cyclophilin D and regulates the mitochondrial permeability transition pore to suppress cell death [42]. In addition, TRAP1 seems to promote neoplastic growth by inhibiting succinate dehydrogenase and downregulating cell respiration in colon carcinoma cells. It was reported that OXPHOS deregulation stabilizes the transcription factor HIF1α promoting tumor growth [43]. Also, it was found that

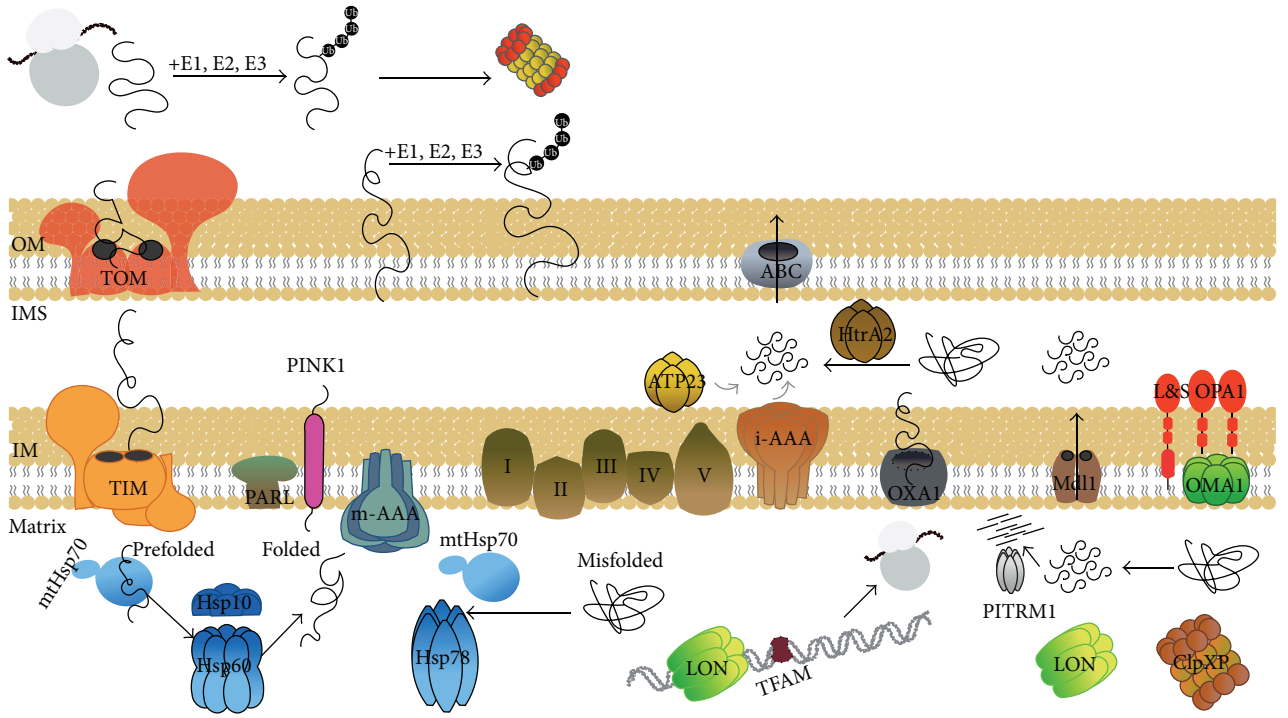

Figure 1: Mitochondrial quality control by molecular chaperones and proteases. Mitochondrial precursors synthesized in cytosol are imported in the mitochondrial matrix via the TOM and TIM translocases. Misfolded precursors are degraded by the 26S proteasome in the cytosol before they enter mitochondria; the 26S proteasome also degrades (following ubiquitination) proteins of the outer mitochondria membrane (OM). Precursors imported in the mitochondrial matrix are bound to chaperones (e.g., mtHsp70 and Hsp60/Hsp10) which then drive their proper folding; mtHsp70 along with Hsp78 also promote protein disaggregation during stress conditions. The polypeptides of the respiratory complex protein machines which are encoded by either mtDNA or genomic DNA are transported into the inner membrane (IM) by the Oxa1 peptide transporter. Damaged and/or unfolded matrix proteins are degraded by the LON, ClpXP, and m-AAA proteases, while the generated peptides can be further degraded by PITRM1; LON protease also degrades the TFAM transcription factor. Peptides generated by the ClpXP protein are transported across the inner mitochondrial membrane by the matrix ATP-dependent peptide transporter HAF-1 (Mdl1 in yeast). The PINK1 protein is encoded at the genomic DNA and after being transported at the IM it is processed by PARL. In the case of mitochondrial dysfunction or damage PINK1 translocates at the OM and facilitates the activation of autophagy/mitophagy machinery (see text). Similarly to PINK1, OPA1 is imported from the cytosol and is processed in long (L) and short (S) isoforms which are located at the IM and the intermembrane space (IMS), respectively. During mitochondrial dysfunction OPA1 isoforms are processed by OMA1 (and, likely, PARL), while unfolded, misfolded, and/or damaged proteins of the IMS are processed by the HtrA2 and i-AAA proteases; generated peptides are then released in the cytosol by the ATP binding cassette transporter (ABC transporter). Mitochondrial inner membrane protease ATP23 is thought to participate in the maintenance of the respiratory chain; however, its role still remains to be elucidated. Mentioned molecules along with their relative topologies and processing (arrows) are indicated in the figure.

TRAP1 is phosphorylated by PINK1 protein (see below) to promote cell survival [44]. Because of its cell protective role and since both the mRNA and proteins levels of TRAP1 are highly expressed in certain cancer cell lines and tumors, TRAP1 has been proposed as an anticancer therapeutic target [45, 46]. To this end, Gamitrinibs are the first mitochondria-targeted molecules which inhibit Hsp90 and TRAP1 and induce mitochondrial membrane permeabilization [45, 47]. Nevertheless, expression of TRAP1 in cancer cells is variable and in some cancers TRAP1 is even downregulated as compared to normal tissue counterparts [48, 49]. Therefore, further studies are needed to unequivocally demonstrate the role of TRAP1 in tumorigenesis.

2.2. Proteases. The mitochondrial respiratory chain is one of the main sources of endogenous reactive oxygen species (ROS). Generated ROS can oxidize (among others) the mitochondrial proteins and lead to accumulation of damaged and/or misfolded proteins [50, 51]. Therefore, loss of function proteins due to exposure to oxidative stress must be either *fold, hold, or degrade*; these options are mostly guided by the action of chaperones, since unfolded proteins that overcome the capacity of chaperones for refolding need to be removed by alternative pathways. The turnover of unfolded or damaged proteins is driven by a complex network of mitochondrial proteases that collaborate for this task with mitochondrial chaperones [52]. There are (a) the ATP-dependent proteases, namely, the LON protease and the Clp Protease Proteolytic subunit (CLPP) and the mitochondrial AAA (ATPases Associated with diverse cellular Activities) proteases of the inner mitochondrial membrane and matrix; (b) the two ATP independent proteases, the ATP23 and HtrA2; and (c) two

oligopeptidases, namely, the presequence protease (PITRM1, also known as PreP) and the mitochondrial oligopeptidase M (MEP, also known as neurolysin) [53] (Figure 1).

2.2.1. LON Protease. The LON protease, firstly identified in bacteria as La protein [54], is conserved among prokaryotes and eukaryotes. LON protein is encoded by the nuclear genome and belongs to the AAA+ protein family. This protease contains three domains of different functions: the N-terminal domain that interacts with protein substrates together with the second AAA+ domain (being involved in ATP binding and hydrolysis) and a third domain bearing the catalytic and proteolytic activity, respectively [55]. LON has a typical serine-lysine dyad at the active center and acts as homooligomeric complex of seven monomers in eukaryotes [56]. LON degrades oxidized and damaged proteins in association with chaperones which maintain the protein in unfolded state until the initiation of the proteolytic reaction [57]. Although the recognition mechanism of the target protein by LON still remains to be elucidated, it is thought that important features must be the overall structure of the protein and the exposed loops at the surface of substrate [58].

Notably, LON activity is not limited to misfolded and/or damaged proteins since several other proteins have been identified as LON substrates under normal conditions, including succinate dehydrogenase subunit 5, glutaminase C, cystathionine β-synthase, and cytochrome *c* oxidase subunit 4 isoform 1 [59–62].

Finally, LON protease has been associated with mitochondrial DNA regulation. LON binds to mitochondrial DNA and regulates mitochondrial DNA copy number and transcription by targeting the mitochondrial transcription factor A (TFAM) for degradation [63, 64]. Loss of the LON yeast homolog, PIM1, resulted in a respiratory deficient phenotype, whereas loss of LON function in human lung fibroblasts enhanced apoptosis and altered mitochondria morphology [65, 66]. Moreover, deficiency of LON in a mouse model showed alteration of OXPHOS and of mitochondrial respiratory chain activity [67].

Several experiments have shown the functional involvement of the LON protein in ageing, as well as in tumorigenic transformation [68, 69]. More specifically, LON overexpression increased lifespan and healthspan in *Podospora anserina*, while aged mice presented decreased protein levels of LON; these effects associate with oxidatively damaged mitochondrial proteins and mitochondrial dysfunction [66, 69]. Nevertheless, further studies are required to better clarify the functional involvement of LON in cancer and ageing, as well as in programmed cell death.

2.2.2. ClpP Protease. The ClpP protease is a large oligomeric protein complex being conserved from bacteria to higher eukaryotes [70, 71]. The proteolytic core of ClpP protease is formed of two stacked rings with 7 subunits each. ClpP is activated after the formation of a complex with ClpX, an AAA chaperone protein in the mitochondrial matrix; the chaperone partner component is involved in the initial recognition of the substrate polypeptide, its unfolding in an ATP-dependent way, and its translocation into the proteolytic chamber of the ClpP complex [72]. ClpP protease lacks homolog in yeast, but intriguingly it has identified a homolog for the ClpX chaperone, namely, the Mcx1 protein. However, deletion of Mcx1 in yeast did not show any prominent phenotype [73], while ClpP null mice demonstrate loss of fertility, failure of hearing and accumulation of ClpX and mtDNA [74]. Studies in human mitochondria have shown that there is a correlation of increased ClpP protein levels with the amount of mutated and unfolded proteins in mitochondria, suggesting a decisive role of this protease in the mitochondrial UPR response [75].

2.2.3. The Fts-H Type, AAA Proteases. The LON and ClpP proteases are soluble enzymes and therefore have no access to the membrane proteins or the proteins located in the intermembrane space. Thus, for the membrane protein quality control, mitochondria have separate proteolytic enzymes dedicated to proteolysis of membrane-integrated substrate proteins. These enzymes are mitochondrial AAA (ATPases Associated with diverse cellular Activities) proteins that belong to the filament-forming temperature-sensitive (Fts-H) protease family, named after the bacterial founding member. Members of this family have a zinc metalloprotease domain, a regulatory domain belonging to the AAA family, and a transmembrane domain [76, 77]. Two type members with different membrane topologies have been described, namely, the i-AAA members which expose their catalytic site in the intermembrane space and the m-AAA which expose their catalytic sites in the mitochondrial matrix.

The i-AAA proteases are involved in the degradation of nonassembled proteins in the intermembrane space [78]. Misfolded and/or mutated proteins are degraded to peptides and are further exported from the organelle or degraded to amino acids by various oligopeptidases.

A mitochondrial m-AAA protease was first described in yeast as a heterooligomeric complex composed of highly homologous subunits (Yta10p and Yta12p). The human m-AAA counterpart protease is composed of paraplegin and AFG3L2 [79, 80] which in human cells exist in two isoforms; one which forms an oligomer with paraplegin and another which forms homooligomers [81].

The AAA proteases have an important role in the proper assembly of the respiratory chain enzyme complexes [82]. Specifically, the biogenesis and assembly of the mitochondrial respiratory complexes is a complicated operation of proteins encoded by both nuclear and mitochondrial DNA, and thus the chances for accumulation of nonassembled subunits in the membrane increase. It is worth mentioning that the substrates of m-AAA protease in yeast are not only the respiratory complex components but this protease has a functional role in mediating proteolytic maturation of additional proteins [78], such as the mitochondrial ribosomal component MrpL32; this ribosomal component is also processed by the m-AAA human isoforms [83]. The m-AAA protease paraplegin AFG3L2 is also involved in OPA1 processing together with Presenilin-Associated Rhomboid-Like (PARL) protease in generating OPA1 isoforms [84]. Interestingly, these are not the only proteases being involved in OPA1 cleavage which is also cleaved by Yme1L, an i-AAA protease

anchored in the inner membrane which affects generation of OPA1 isoforms [85].

2.2.4. The HtrA2 Protease. The HtrA2 protease is conserved in animals and plants but not in yeast. HtrA2 consists of a serine protease domain and a PDZ domain involved in substrate binding and regulation of the enzymatic structure. Notably, this protease has the interesting ability to switch between protease and chaperone activity based on the temperature. Under normal conditions, HtrA2 acts mostly as a chaperone, but in stress conditions (e.g., due to temperature increase) HtrA2 exerts proteolytic activity and degrades the nonfunctional proteins [86]. Similarly, in bacteria, the HtrA2 homolog (HtrA/DegP) has a protein quality control role in the periplasmic space at elevated temperatures [87].

The human HtrA2/OMI localizes in the mitochondrial intermembrane space and its expression levels are increased during stress conditions [88]. Loss of HtrA2 increases the number of damaged mitochondria and of the unfolded respiratory chain subunits. Moreover, it was found that HtrA2 associates with programmed cell death, as well as with necrosis [89]; specifically, HtrA2 is released in the cytosol during apoptosis and cleaves antiapoptotic proteins [90, 91]. On the other hand, it was reported that HtrA2 is linked to the mitochondrial inner membrane and is being activated by PARL cleavage to prevent accumulation of proapoptotic proteins in the outer membrane [92]. HtrA2 has also been associated with alterations of mtDNA as loss of HtrA2 in mouse cells leads to accumulation of mtDNA mutations [93].

HtrA2 functionally interacts with the mitochondrial protein kinase PINK1 and mouse models lacking HtrA2 develop neurological defects reminiscent of Parkinson's Disease [94, 95]. HtrA2 knockout mice have decreased mitochondrial membrane potential and display mitochondrial uncoupling [96]. In addition, loss of HTRA2 results in ATP depletion and reduced mitochondrial mass [96]. Finally, studies in mice have revealed an implication of HtrA2/OMI in ageing [97]. Therefore, loss of HtrA2/OMI relates to both premature ageing and neurodegeneration.

2.2.5. The PITRM1 Proteases. PITRM1 is a highly conserved zinc metalloprotease known also as Presequence Peptidase (PreP). PITRM1 was identified in *Arabidopsis thaliana* as a protease that degrades targeting peptides in both mitochondria and chloroplasts [98]. PITRM1 localizes in the mitochondrial matrix and is involved in the cleavage of mitochondrial targeting peptides as well as unstructured peptides [98]. Human PITRM1 is a metalloendoprotease of the pitrilysin family [99], which is thought to have a role in mitochondria quality control with a broad range of predicted substrates. In humans, PITRM1 has been implicated in Alzheimer's Disease having a principal role in the degradation of the amyloid β-peptides [99] which inhibit peptide turnover and promote the accumulation of nonprocessed preproteins within mitochondria [100]. Incomplete processing of mitochondrial preproteins leads to their destabilization and accelerated turnover [101].

3. A Close Network with UPS

3.1. The UPS System. The proteasome is a large complicated protein machine of about 2.5 MDa. The 26S proteasome consists of the 20S core particle (CP) and the 19S regulatory particle (RP) [102, 103]. The 20S CP in eukaryotes consists of 28 α-type and β-type subunits organized in four rings [104]; it carries the catalytic center with the three peptidase activities, namely, the caspase-like, trypsin-like, and chymotrypsin-like peptidase activities [105, 106]. The 19S RP consists of 20 conserved subunits that form the two subcomplexes, known as the base and the lid [102, 107–109]. The lid is composed of nine non-ATPase subunits (Rpn3, Rpns5–9, Rpn11, Rpn12, and Rpn15), while the base is composed of six AAA-type ATPases (Rpt1–6) and three non-ATPases, namely, the Rpn1, Rpn2, and Rpn13 subunits [108, 110–113]. Proteasomes are mainly found in the nucleus and the cytosol [114].

UPS is responsible for the ATP-dependent degradation of either normal short-lived ubiquitinated proteins or misfolded, unfolded, and/or damaged proteins [115]. Ubiquitin (Ub), is a small 76 amino acid polypeptide that is attached to proteins as either a monomer or as a polyubiquitin chain by an enzymatic reaction; Ub is conserved among the eukaryotes but not in prokaryotes [115]. Notably, a small protein, known as prokaryotic ubiquitin-like protein (Pup), has been described in *Mycobacterium tuberculosis*; Pup modifies proteins posttranslationally for proteasome degradation. Pup contains an ubiquitin-like Gly-Gly motif, binds covalently the lysines residues, and targets proteins for proteolysis [116].

The conjugation of Ub to the polypeptide is orchestrated by a series of enzymes (ligases) known as Ub-activating enzymes (E1, E2, and E3). The E1 and E2 enzymes activate the ubiquitin in an ATP-dependent process, while the E3 ligase performs the final step ligating the carboxyl group of the C-terminal of Ub to the target protein [117]. Degradation of the targeted protein by (mainly) the proteasome requires polyubiquitination at lysine 48. However, ubiquitylation is also used for other cellular processes such as immune responses, protein endocytosis, DNA repair, or the assembly of signaling complexes [118, 119]. Proteasome localizes principally in nucleus and cytosol, while proteasome genes are also regulated in a tissue-specific manner during ageing and dietary restriction in liver and brain [113]. In support, studies of our group, and others, have shown the differential *in vivo* regulation of proteasome genes expression and proteasome peptidase activities in somatic tissues and gonads [120, 121].

3.2. UPS and Mitochondria. Mitochondrial outer membrane proteins have an important role in the regulation of metabolism, mitochondrial morphology, apoptosis, protein import into mitochondria, and other signaling pathways. Therefore, the maintenance of the outer membrane protein quality control is essential for the organelle function. A number of ubiquitin ligases have been localized on the mitochondrial outer membrane including MULAN, MARCH-V/MITOL, and Mdm30. These ligases affect mitochondrial dynamics by ubiquitinating the proteins being involved in mitochondria fusion and fission processes [122–125]. Notably,

no specific mitochondrial proteases have been identified at this compartment.

Several lines of evidence indicate the involvement of cytosolic UPS in mitochondrial outer membrane protein regulation and recycling during proteotoxic stress [126–128]. Mitochondrial Unfolded Protein Response (UPR^{mt}) induces outer mitochondrial membrane-associated degradation (OMMAD) and/or mitophagy or even apoptosis if the disruption of mitostasis and/or mitochondrial proteome stability is irreversible [20].

In addition, a role of proteasome in the biogenesis of precursor proteins and in controlling mitochondrial proteome fate has been proposed. Treatment of cells with MG132, a specific proteasome inhibitor, stabilized the precursor forms of OPA1 [129], while intramembrane space proteins that utilize the mitochondrial oxidative folding pathway (MIA pathway) can be ubiquitinated and degraded by the proteasome before they arrive at the mitochondria [130].

In yeast, Fzo1 (Mitofusin ortholog) degradation is mediated by the 26S proteasome [125]. Likewise, Mitofusin 1 (Mfn1) and Mitofusin 2 (Mfn2) (both involved in mitochondrial fusion; see below) are substrates of the UPS [131]. More specifically, after Parkin-mediated ubiquitination, both Mfn1 and Mfn2 can be degraded in a proteasome- and Vms1-p97/CDC48-dependent manner [132, 133]; Vms1-p97/CDC48 is an ubiquitin-selective chaperone that unfolds proteins and disassembles protein complexes and it is thought to play an important role in mitochondria quality control [134]. Vms1 localizes primarily to the cytosol but under stress conditions translocates to the mitochondria through its mitochondrial targeting domain and provides the main driving force for outer mitochondria protein extraction [135]. Furthermore, the association of four deubiquitinating enzymes (DUBs) (that drive an opposite to E3 ligases function) with mitochondria has been described. The Usp9x, Usp30, Usp36, and ataxin-3 may preserve mitochondrial protein degradation by editing or removing the degradative ubiquitin signal [113, 136–138]. However, further studies are needed to clarify which are the sensors of the OMMAD response and the detailed role of the UPS in mitochondria quality control. Given the multiplicity of enzymes involved and their differential subcellular localization it is essential to understand how these enzymes work together and regulate these processes.

Interestingly, additional evidence suggests a role of UPS not only in controlling the outer membrane protein quality but also in the regulation of the proteome of other mitochondrial compartments, such as the matrix [oligomycin sensitivity-conferring protein (OSCP), component of the mitochondrial membrane ATP synthase], the intramembrane space (Endonuclease G), and the inner membrane [Uncoupling Protein-2 and Uncoupling Protein-3 (UCP2 and UCP3)] [139–141]. Nevertheless, the exact mechanism of how UPS mediates the degradation of the inner mitochondria compartments proteins is still elusive and thus further studies are needed to define if and how these proteins are transported at the mitochondria outer membrane or if the UPS can directly access these compartments.

3.3. UPR^{mt}: A Mitochondria Specific Unfold Protein Response. The UPR^{mt} was firstly described in mammalian cells as a mitochondrial stress response. Depletion of mtDNA or overexpression of a nuclear-encoded aggregation-prone protein in mitochondrial matrix induced increased gene expression of the mitochondrial molecular chaperone Hsp60 and of the protease ClpP [142, 143]. Although UPR^{mt} has been studied in different model organisms, *C. elegans* has been a useful model for the comprehension of this pathway. The first described component of the UPR^{mt} is the C/EBP homology protein (CHOP). CHOP heterodimerizes with C/EBPβ and by binding to the promoter region of Hsp60 increases its transcription levels [60]. Further analysis of CHOP and C/EBPβ revealed that these proteins contain at their promoter region two additional conserved sequences, known as conserved Mitochondrial Unfolded Response Elements (MUREs) [144]. Activation of CHOP is not specific for mitochondrial stress but can also relate to ER stress conditions or even exposure to arsenate [145, 146].

Using a genome-wide RNAi screening various mediators of the UPR^{mt} have been identified. Accumulated unfolded proteins are processed by the ClpXP protein and transported across the inner mitochondrial membrane by the matrix ATP-dependent peptide transporter HAF-1 (Mdl1 in yeast) [147–149]. Deletion of ClpXP disrupts the proteolysis of unfolded mitochondrial proteins, whereas deletion of HAF-1 attenuates its activation during stress [148]. Both proteins are essential for the survival and normal lifespan during mitochondrial stress condition, underlying the important role of ClpXP and HAF-1 in mitochondria quality control. Another downstream component of HAF-1 is the bZip transcription factor ATFS-1 (Activating Transcription Factor associated with Stress). Under normal conditions, ATFS-1 is imported in mitochondria and degraded by the LON protease [150]. During mitochondrial stress ATFS-1 accumulates in the nucleus and activates transcription of UPR^{mt} genes [151]. Deletion of ClpP and HAF-1 prevented nuclear accumulation of ATFS-1 underlying its downstream activation in a HAF-1 dependent manner [147, 151]. DVE-1/UBL-5 is a protein complex that is necessary for the activation of UPR^{mt} response and acts downstream of ClpXP/HAF-1. DVE-1 is a conserved transcription factor that binds to Hsp60 promoter, while UBL-5 is an ubiquitin-like protein that is upregulated and binds to DVE-1 in response to mitochondrial stress [148, 152].

A growing number of studies underlie the involvement of UPR^{mt} in longevity. Specifically, reduction of the *C. elegans* NAD^+ levels decreased lifespan, while rescue experiments involving the protein deacetylase sir-2.1 (NAD-dependent enzyme) and activation of UPR^{mt} prevented the associated metabolic decline and extended lifespan [153]; in these experiments overexpression of deacetylase sir-2.1 induced lifespan extension in an UPR^{mt}-dependent manner. Furthermore, silencing of CCO-1, a subunit of Cytochrome *c* oxidase, in *C. elegans*, increased lifespan and induced UPR^{mt} [154]. Ribosomal protein S5 (Mrps5) was described as a candidate gene that regulates mouse lifespan. Knockdown of Mrps5 in worm increased lifespan and prompted activation of UPR^{mt} [155]; notably, however, UPR^{mt} activation does not always induce lifespan extension [156].

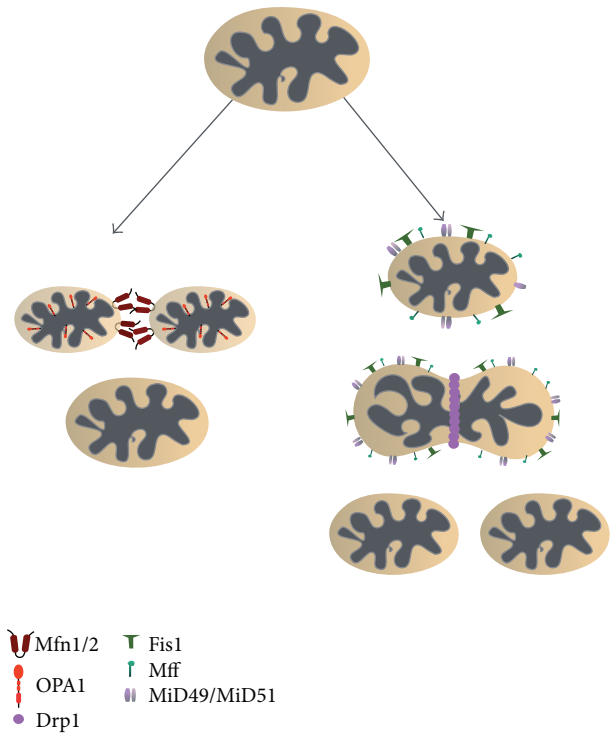

Figure 2: Mitochondrial dynamics. Mitochondrial morphology and cellular network are regulated by continuous balance and dynamic regulation of fusion and fission events. Fusion is mediated by the Mitofusin 1 (Mfn1) and Mitofusin 2 (Mfn2) GTPases of the OM, as well as from OPA1 of the IM (see Figure 1 for abbreviations). Mfn1 and Mfn2 promote fusion (via the interaction of their coiled-coil domains) of the OMs of two juxtaposed mitochondria and this event is followed by OPA1-mediated fusion of the IMs (left arrow). On the other hand, fission generates two daughter organelles from a mitochondrion. Drp1 is recruited to the mitochondria OM where it directly interacts with Fis1, Mff, and MiD49/MiD51. Then Drp1 generates a ring structure that constricts the mitochondrial membranes leading to the formation of two daughter mitochondria (right arrow).

Finally, as the components of the UPR^{mt} response are important for cell survival, many tumors and cancer cell lines display an accumulation of unfolded proteins and activated UPR^{mt} response [157, 158]. Nevertheless, the exact mechanism of the UPR^{mt} response in longevity and disease and what factors determine its activation in each case still remain to be elucidated.

4. Mitochondria Dynamics: Mix and Segregation

When the molecular pathways of chaperones and proteases are overwhelmed additional quality control mechanisms concerning the entire organelle homeodynamics are activated. Specifically, mitochondria undergo continuous cycles of fusion and fission in order to dilute damage. Both processes are regulated by a number of GTPases (guanosine triphosphatases) conserved from yeast to mammal (Figure 2). The importance of the fusion and fission events is highlighted by a number of disorders caused by mutations of the proteins involved in such processes (see below). Since mitochondria are double membrane organelles, fusion and fission processes involve proteins localized on both compartments.

4.1. Fission. Fission is an important process for the generation of new daughter mitochondria; this event is mainly driven by the dynamin-related protein 1 (Drp1). Drp1 is a cytosolic protein that translocates at the mitochondrial outer membrane to initiate the fission process. Once localized in the outer membrane, Drp1 is oligomerized into a spiral-like structure and constricts the outer and inner mitochondria membrane by inducing high curvature in a GTP hydrolysis-dependent way [159]. Fission is tightly regulated by several posttranslational modifications of Drp1. The first described is the phosphorylation by Cdk1/cyclin B which enhances mitochondrial fragmentation during mitosis [160]. Fission may also be inhibited by kinase A-mediated phosphorylation of Drp1 at Serine 637, a highly conserved Drp1 amino acid at metazoans. Phosphorylation at Ser^{637} inhibits GTPase activation of Drp1 and, likely, the recruitment of Drp1 to the outer membrane [161]. Other posttranslational events of Drp1, like nitrosylation and sumoylation, promote mitochondrial fission [162, 163]. Drp1 is also target of the ubiquitin ligase MARCH5/MITOL; in this case ubiquitination of Drp1 by this ligase does not target Drp1 for degradation but rather regulates the formation of membrane complexes and protein activity [164].

Recruitment of Drp1 to the mitochondrial membrane is mediated by receptor proteins. Specifically, the yeast homolog

of Drp1 (Dnm1p) is recruited by the receptor protein Fis1p [165]. In line with this finding, overexpression of Fis1 in mammalian cells promotes fission; however, its downregulation does not affect this process [166]. In eukaryotes, other interaction factors, like Mff, MiD49, and MiD51/Mief1, have been proposed to be functionally involved in Drp1-mediated fission [167–169]. The large number of factors which contribute to tight regulation of Drp1 function clearly highlights the importance of the fission event for mitochondria homeodynamics.

4.2. Fusion. During fusion, mitochondria mix their genetic content in order to complement deficit of damaged mitochondria. In contrast to fission, mitochondrial fusion is operated by three dynamically related GTPases proteins, namely, Mfn1, Mfn2, and Optic Atrophy 1 (OPA1). Mfn1 and Mfn2 are implicated in the fusion of the mitochondrial outer membrane, whereas OPA1 is involved in the fusion of the inner membrane [170, 171]. Mfns were firstly described in *Drosophila melanogaster* [fuzzy onions, (Fzo)]; Mfn homologs were later on also described in yeast (Fzo1) and in mammals (Mfn1 and Mfn2) [172, 173]. Mechanistically, the Mfn1 and Mfn2 proteins tether the outer membrane of the mitochondria by forming homo- and heterooligomers [174]. Downregulation of Mfn1 or Mfn2 in cells leads to mitochondrial fragmentation; additionally, lack of either Mfn1 or Mfn2 implies the total loss of fusion, evidencing that both proteins are essential for this mitochondrial process [170].

OPA1 is a conserved large GTPase of the dynamin family, imported at the mitochondrial membrane by its N-terminal sequence. Opal is involved in cristea remodelling and inner membrane fusion [175], while mutations of OPA1 lead to neuropathy of optic nerve known as dominant optic atrophy [176]. This GTPase has different splicing isoforms. Specifically, there are two types of forms, the long (L) form that is membrane anchored and the short (S) form that is found soluble in the intramembrane space [177]. The balance between these two pools of isoforms can regulate the fusion process since reduction of the membrane anchored forms by activation of the metalloprotease OMA1 during either stress conditions or decrease of the mitochondrial membrane potential suppresses the fusion events [178]. On the other hand, oxidative phosphorylation can enhance the mitochondrial inner membrane fusion [179]. Interestingly, loss of OPA1 results in loss of inner membrane fusion but does not affect the fusion of the outer membrane, suggesting that fusion-involved proteins can act in different phases and by distinct modes during this process [70].

4.3. Mitochondria Motility. Another important aspect of mitochondria dynamics is their motility and cellular distribution. The role and significance of this process are especially highlighted in neurons which need mitochondria energy at sites distant from the cell body [180]. The transport of the mitochondria is a cytoskeleton based movement [181]. In mammalian axons of neuronal cells, mitochondrial movement from the cell body to the synaptic junctions (known as anterograde movement) is driven by the kinesin-1 motor (KHC, Kif5b) and movement from the synaptic junctions to the cell body (the retrograde movement) is driven by dynein, whereas in yeast the transport is based on actin [182, 183]. The binding of the mitochondria to the kinesin-1 motor is mediated by the adapter proteins Milton and Mitochondrial Rho GTPase (Miro). Milton interacts with kinesin and directly binds to Miro located on the mitochondria outer membrane [184, 185]. Loss of Miro in *Drosophila* resulted in reduction of mitochondria from dendrites and axons [185].

Studies on a knockout mouse model have demonstrated that attachment of the mitochondria to the microtubule can also be regulated by the protein syntaphilin (SNPH). Neuronal depletion of SNPH increased axonal mitochondrial motility, whereas overexpression of SNPH augmented the number of immobile mitochondria [186].

The fusion and fission processes are closely related to the mitochondria motility. Mitochondria fragmentation induced by loss of Mfn1 reduces the mitochondrial motility, while loss of Drp1 in *Drosophila* leads to a decrease of synaptic mitochondria [170, 187]. On the other hand, deletion of Miro in yeast dramatically induces changes in the mitochondrial morphology but seemingly does not affect the fusion or fission processes [188].

5. Mitophagy: Remove the Damaged

When a mitochondrial damage or unrepairable dysfunction occurs, selective removal of mitochondria by autophagy takes place; this process is known as mitophagy, a term proposed by Lemasters in 2005 [189].

Autophagy is an evolutionarily conserved process that is responsible for the lysosome-mediated degradation of cytoplasmic components during a process where an isolated membrane named phagophore is generated upon autophagy signals [190]. The first upstream formed complex of this process in mammalian cells is the ULK1 complex which is composed of the ULK1 (Unc-51-Like Kinase 1 protein), ATG13, mTOR kinase, and the RB1CC1 (RB1-inducibile Coiled-Coil 1). Autophagy induction inhibits mTOR which under physiological conditions is phosphorylated and inhibits the ULK1 and ATG13 proteins of the complex [191, 192]. Phagophore nucleation requires the formation of a complex consisting of the vacuolar protein sorting (VPS) 34, VPS15, Beclin1, and the activating autophagy/beclin-1 regulator 1 (AMBRA1) [193]; in this process, B-cell lymphoma 2 (BCL-2) inhibits autophagy by binding Beclin1, while BCL-2-homology 3 (BH3-only) activates the VPS34 complex by displacement of the BCL-2 protein [194]. Furthermore, the phagophore expands after conjugation of ATG12 to ATG5 which interacts with ATG16 forming the ATG16L complex which then conjugates phosphatidylethanolamine (PE) to the procures of microtubule-associated protein 1 light chain 3 (LC3) until generation of the LC3 II receptor. Expansion of the phagophore continues until its edges surround the cargo, fuse, and form the autophagosome. Finally, the autophagosome fuses with lysosomes and its content is being degraded (Figure 3).

One of the most described pathways of mitophagy is the PINK1/Parkin-mediated autophagy [195]; notably, mutations in the Parkin and PINK1 genes are the most common

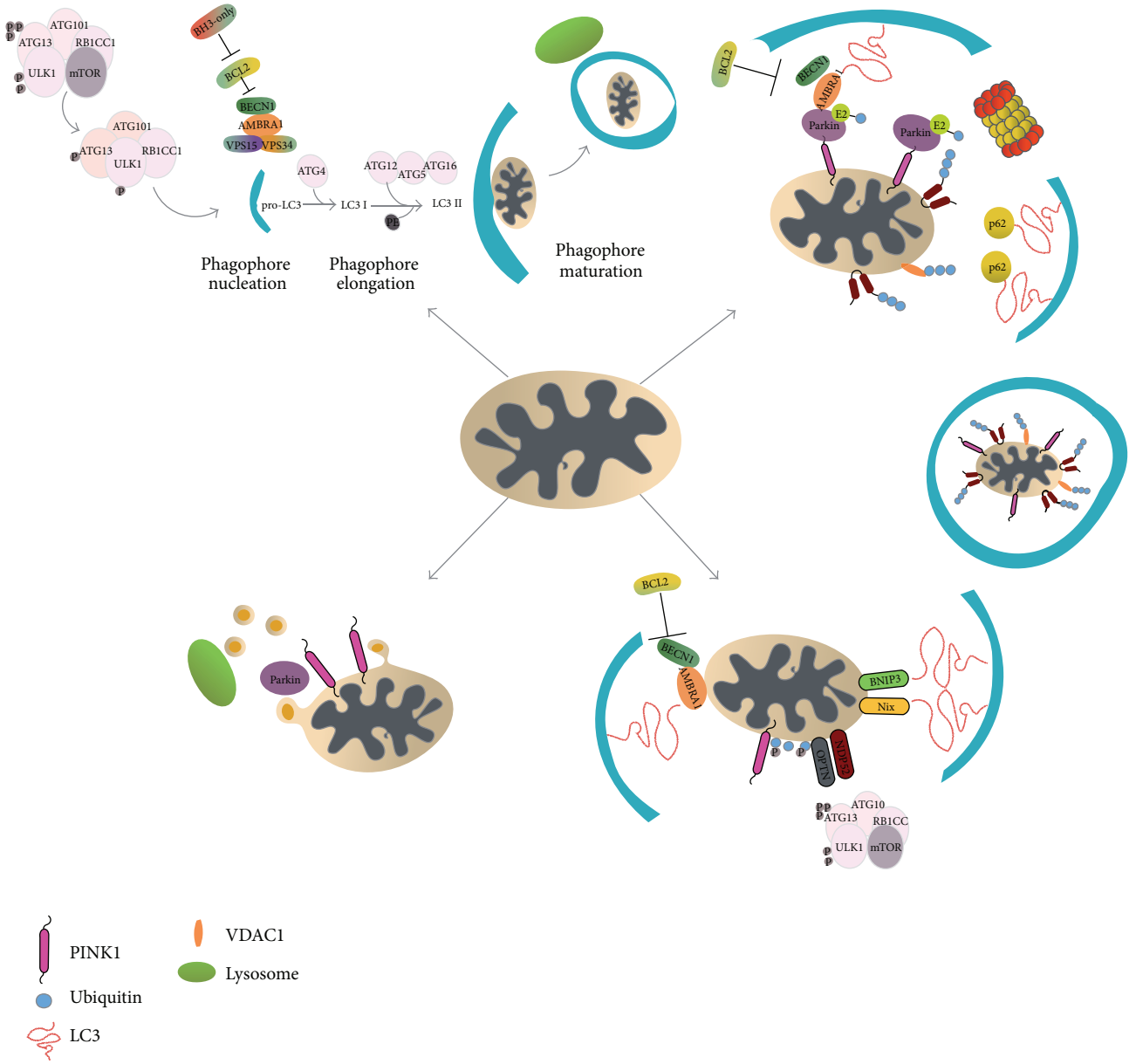

FIGURE 3: Pathways for the removal of damaged mitochondria. Unrepaired mitochondrial damage or reduced membrane potential ($\Delta\Psi_m$) prompts the removal of mitochondria by autophagy. Autophagy starts (upper left) with the upstream complex ULK1 which is composed from Unc-51-Like Kinase 1 protein (ULK1), ATG13, mTOR kinase, and RB1-inducibile Coiled-Coil 1 (RB1CC1). Inhibition of the mTOR kinase leads to the generation of the Beclin1-Vacuolar Protein Sorting (VPS) 34-VPS15 complex. B-cell lymphoma 2 (BCL-2) blocks the induction of autophagy by binding to Beclin1 and to the Activating Molecule in Beclin1-Regulated Autophagy (AMBRA1). Displacement by BH3-only proteins activates Beclin1-VPS 34-VPS15 and induces the phagophore generation. The phagophore is elongated by the autophagy proteins ATG12-ATG5 creating the ATG16L complex, which then conjugates phosphatidylethanolamine (PE) to the procures of microtubule-associated protein 1 Light Chain 3 (LC3) to generate the LC3 II receptor. Finally, the membrane engulfs the cargo, closes its ends, and fuses with lysosomes in order to degrade its content. Mitophagy can also occur in a PINK1/Parkin dependent pathway (lower left; upper right): PINK1 is exposed at the outer membrane, where it recruits the E3 ubiquitin ligase Parkin to mitochondria. Parkin ubiquitinates outer membrane proteins, such as Mfns and Voltage-Dependent Anion Channel (VDAC), which are then degraded by the 26S proteasome. Similarly, p62/SQSTM1 (Sequestosome 1) interacts with ubiquitinated mitochondrial proteins and recruits the autophagosome through its interaction with the LC3 receptor. An alternative PINK1/Park dependent pathway is the formation of cargo-selective vesicles (lower left) which are released from mitochondria (Mitochondria-Derived Vesicles, MDV) and fuse with lysosomes. The formation of MDV is induced by increased ROS levels and does not require mitochondrial depolarization and/or LC3 or ATG5 proteins. Mitophagy in a Parkin-independent way (lower right) may also occur since (a) the autophagy receptors NIX and BNIP3 can directly interact with the autophagosome through the LC3 receptor; (b) AMBRA1 if overexpressed in the mitochondria outer membrane interacts with the LC3 receptor and can induce autophagy by both Parkin dependent and Parkin-independent pathways; and (c) PINK1 phosphorylates the ubiquitin chains in mitochondria promoting the recruitment of NDP52 [also known as Calcium binding and Coil-Coil domain protein 2, (CALCOCO2)] and optineurin autophagy receptors; subsequently, ND52 and optineurin recruit the upstream machinery of autophagy and trigger mitophagy.

causes of recessive forms of Parkinson's Disease characterized by early onset [196, 197]. Specifically, the PINK1 gene encodes a serine/threonine kinase, which localizes in the outer membrane of depolarized mitochondria. Other forms of PINK1 that are processed by the rhomboid protease PARL can be found in the inner mitochondrial membrane or in the cytosol [198, 199]. Following PARL cleavage, PINK1 is degraded by mitochondrial proteases, and thus in most cells the levels of PINK1 that associate with mitochondria are undetectable or very low [198]. Parkin encodes a cytosolic E3 ubiquitin ligase that mediates polyubiquitination of its substrates (e.g., Mfn1 and Mfn2) on the outer mitochondrial membrane and initiates the mitophagic process [200, 201]. The ubiquitinated mitochondrial proteins can be degraded by either the autophagy machinery or the ubiquitin-proteasome system [202, 203]. *Drosophila* studies have shown that PINK1 and Parkin act in the same pathway since expression of Parkin in a background of mutated PINK1 in flies partially rescued the phenotype [204–206].

Mitochondrial depolarization stabilizes PINK1 on the outer mitochondrial membrane; this event directly phosphorylates Parkin and induces its recruitment in the mitochondria. Parkin then ubiquitinates the fusion proteins Mfn1 and Mfn2 and the proteins involved in mitochondrial trafficking, Miro1 and Miro2 [200, 203]. Moreover, the increased levels of Parkin induce ubiquitination of other outer mitochondrial membrane proteins, such as the voltage-dependent anion channel (VDAC) and the components of the TOM mitochondrial translocase complex [200, 203, 206–208]. Interestingly, Mfn1, Mfn2, and VDAC knockout mice still undergo mitophagy suggesting that the role of these proteins in mitophagy induction needs to be further investigated [209, 210]. After Parkin-mediated ubiquitination of the outer mitochondrial membrane proteins, the selective autophagy adapter protein p62/SQSTM1 (Sequestosome 1) is recruited to mitochondria where it is thought to promote autophagy due to its capacity to directly interact with the LC3 receptor (Figure 3) [132, 211, 212]. Mitochondrial depolarization with carbonyl cyanide m-chlorophenylhydrazone (CCCP) treatment induces the accumulation of histone deacetylase 6 (HDAC6) in the mitochondrial outer membrane. p62/SQSTM1 and HDAC6 interact with Ambra1 and Beclin1, prompting the accumulation of the autophagosome to mitochondria [213, 214]; interestingly, studies in p62 knockout mice showed that p62 also mediates mitochondrial perinuclear clustering [212]. Recently, optineurin was found to be recruited to mitochondria in order to induce autophagosome formation around the damaged mitochondria via LC3 receptor [215].

Several studies link the PINK1/Parkin pathway to mitochondrial dynamics, namely, fission/fusion and motility. Specifically, PINK1 phosphorylates the fusion protein Mfn2 and this event, likely, induces recruitment of Parkin to mitochondria [216]. Mitofusins not only are substrates for PINK1 and Parkin but also can regulate their proteasomal turnovers through ubiquitination [217]. Furthermore, studies in mammalian cells have shown that overexpression of PINK1 induced mitochondrial elongation, while its knockdown promoted fragmentation [218, 219]. Increasing fusion events prevent the degradation of mitochondria by starvation-induced autophagy [220]. Recently, several evidences link mitochondrial fission events and mitophagy. The yeast homolog of Drp1, Dnm1, is required in certain mitophagy types. Thus mitochondria fragmentation induced by fission probably facilitates autophagosome engulfment [221].

Like mitofusins, Miro is also phosphorylated by PINK1 and ubiquitinated by Parkin. Parkin-dependent ubiquitination of Miro leads to proteasomal degradation and arrest of mitochondrial motility [222]. Moreover, it was shown that Mfn2 interacts with Miro in the mitochondria axonal transport [223]; indeed, PINK1 and Parkin can affect Miro directly or indirectly by targeting Mfn2 to degradation. Also, genome screening studies have identified additional PINK1/Parkin regulators like SMURF1 (SMAD specific E3 ubiquitin protein ligase 1), ATPIF1/IF1 (ATPase inhibitory factor 1), and TOMM7 which, likely, promote autophagy [224–226].

Additional mechanisms that affect the PINK1/Parkin-mediated mitophagy include the activity of PI3K/AKT pathway in starvation conditions; this event attenuates mitophagy. On the other hand, mitophagy is enhanced by accumulation of unfolded proteins in the mitochondrial matrix or downregulation of the LONP1 peptidase (Human LON protease homolog) [227, 228]. Interestingly, lack of the PINK1 and Parkin yeast homologs does not seem to affect the removal of damaged mitochondria by autophagy. In another stressful condition, namely, nitrogen starvation, the Atg32/Atg11 complex recruits the fission machinery to interact with the Dnm1 protein and to induce mitochondria degradation by autophagy [221].

Despite the growing knowledge about the PINK1/Parkin pathway involvement in mitophagy, most of the studies are performed in models with altered expression of Parkin. The majority of the cell systems are treated with CCCP, which totally depolarize the mitochondrial membrane resulting in Parkin overexpression [229]; it is therefore still unclear to what extent endogenous Parkin mediates autophagy [230]. In fact, Parkin knockout mice presented failure of heart functionality and mitochondria aggregation, while no recruitment of Parkin on mitochondria was observed when it was overexpressed [231].

Although in the most studies mitophagy was induced artificially, in a recent work it was shown that constitutive mitophagy, which requires PINK1 and Parkin, occurs in mouse primary hippocampal neurons without mitochondrial membrane depolarization or drug treatment [232].

In addition, loss of Drp1 leads to mitochondria ubiquitination, accumulation of damaged mitochondria, and p62 mitochondrial targeting, independently from Parkin [233, 234]. Indeed, it seems that there must be additional proteins that regulate mitophagy in a Parkin-independent way. In line with this assumption, studies in *Drosophila* showed that the mitochondrial ubiquitin ligase 1 (MUL1) totally rescued the phenotype of PINK1/Parkin loss of function [235]. Other autophagy receptor proteins which have been shown to induce mitophagy in a Parkin-independent pathway include BNIP3 (BCL-2/Adenovirus E1B 19 kDa Interacting Protein 3) and NIX (also called BNIP3L) that interact

with the LC3 receptor and induce mitophagy in hypoxic conditions. Deletion of either BINP3 or NIX alone does not affect mitophagy, suggesting that both proteins are needed to promote mitophagy [236]. NIX null mice showed retention of mitochondria in erythrocytes and, likely, NIX is not required for mitophagy induction but rather acts as a receptor for targeting autophagosomes to mitochondria (e.g., like the Atg32 in yeast) [237]. Another protein that can induce Parkin-independent mitophagy is Cardiolipin, a phospholipid dimer of the mitochondrial inner membrane. Induced mitochondrial damage leads to translocation of Cardiolipin in the outer membrane followed by increased LC3 colocalization with damaged mitochondria [238].

Recently, two new Parkin-independent pathways have been described. Targeted overexpression of AMBRA1 at the mitochondrial outer membrane induces autophagy in both Parkin-dependent and Parkin-independent ways [239]. Similarly, a new Parkin-independent role of PINK1 in mitophagy was proposed [240]. Specifically, it was shown that PINK1 phosphorylation of ubiquitin molecules on mitochondrial membrane acts as an autophagic signal. PINK1, in the absence of Parkin, recruits NDP52 (also known as CALCOCO2, Calcium binding and Coil-Coil domain protein 2) and optineurin, but not p62, to mitochondria to activate (Parkin-independent) mitophagy. According to this new model phosphorylation of ubiquitins by PINK1 is needed to recruit Parkin and autophagy receptors to mitochondria. In the absence of Parkin, PINK1 induces blind levels of mitophagy using the relatively low basal ubiquitin levels on mitochondria. In the presence of Parkin the signal is amplified, since Parkin generates more ubiquitin chains on mitochondria which are subsequently phosphorylated by PINK1 enhancing the rate and levels of clearance [240] (Figure 3).

An additional mechanism for the removal of damaged mitochondria is the formation of mitochondria-derived vesicles (MDV) [241]. MDV are cargo-selective vesicles released from mitochondria which fuse with lysosomes and undergo hydrolytic degradation. The MDV formation is induced by increased ROS species and does not require mitochondrial depolarization [241]. Although MDV-mediated degradation is independent of the canonical autophagic proteins LC3 and ATG5, it still requires a PINK/Parkin functional pathway [241].

Overall, mitophagy is an important mitochondrial quality control mechanism that effectively removes damaged mitochondria in order to prevent oxidative stress and cellular death. Considering the growing number of proteins involved in this process, the variation in mitophagic events, and its functional implication in ageing and age-related diseases, further detailed studies are needed to clarify and better understand this highly dynamic process.

6. Mitochondria and Ageing

Ageing is a physiological process that occurs despite the presence of complex pathways of maintenance, defense, and repair, and it has been correlated with a number of diseases including cancer, neurodegenerative diseases, diabetes, and heart failure; notably, there are no evolutionary selected "gerontogenes" which function to cause ageing, while (among others) ageing correlates with increased proteome instability which leads to irreversible cellular damage and dysfunction [4, 5, 242–246].

In relation to mitochondria, generation of a transgenic mouse model with mutated mtDNA provided the first genetic evidence that mutated mtDNA leads to premature ageing [247]. Moreover, mitochondria are the primary source of ROS which seem to accumulate during ageing [248, 249], due to an (among others) age-related increase of mtDNA mutations which then increase ROS levels by affecting the respiratory chain [250–252]. Ageing decline of mitochondrial functionality is also associated with mitochondrial morphological alterations and decrease of mitochondria numbers [253, 254], as well as with a decrease of autophagic activity and reduced mitochondrial biogenesis [255, 256]; therefore, mitochondria dynamics seems to have an important role in the progression of ageing. Reduced expression of Mfn2 and Drp1 genes in the skeletal muscle of aged individuals suggested an impairment of fusion/fission event in skeletal muscle fibers; this could lead to loss of muscle strength and mass with age [257]. In support, reduced fission in mouse model is associated with muscle atrophy [258].

Several studies have shown that caloric or dietary reduction increases lifespan [259–261]. Insulin/IGF-1 signaling (IIS) and target of rapamycin (TOR) signaling pathways are the two main nutrient-sensing pathways that have been linked to lifespan regulation [262–264]. Studies in mice have shown that caloric restriction increases mitochondrial respiration and mitochondria biogenesis through sirtuin 1 activation [13, 265, 266]; in support, a diet that is rich in compounds that are known to impair mitochondrial functionality and accumulate during ageing, namely, advanced glycation end products (AGEs) or lipofuscin [267–269], reduced lifespan and affected proteasome activities in *Drosophila* [270]. Thus, endogenous or exogenous factors which affect the mitochondria bioenergetics and/or biogenesis have a direct impact on ageing and, likely, on age-related diseases (see below).

7. Mitochondria Quality Control and Cancer

The "Warburg Effect" was proposed by Warburg and suggested that cancer cells have a metabolic shift toward aerobic glycolysis (rather than oxidative phosphorylation), reduced mitochondrial respiration, and functionally altered mitochondria in order to provide sufficient energy for their growth [16]; nevertheless, in many types of cancer, tumor cells still depend on energy production by mitochondria and thus do not suppress mitochondrial bioenergetics.

Accumulation of mtDNA mutations along with increased levels of ROS (that enhance mutation on the mitochondrial genome) have been described as promoting factors of tumorigenesis [271–274]; in addition, many mtDNA mutations that associate with tumorigenesis were shown to inhibit OXPHOS [275, 276]. In support, exchange of mtDNA with pathogenic or normal mtDNA in cancer cells resulted in alterations of cancer cell phenotypes [277, 278], further underlying the important role of mtDNA in tumorigenesis. In addition, mutation of the mitochondrial transcription factor

A (TFAM) in some colorectal cancers was associated with mtDNA depletion, while its overexpression promoted cell proliferation [279, 280].

Increased ROS levels, which largely originate from dysfunctional mitochondria, promote the activation of a number of transcription factors, including nuclear respiratory factor 2 (NRF2), the nuclear factor-kappa beta (NF-κB), and the Hypoxia Inducible Factor 1α (HIF1α). The transcription factors NRF1 and NRF2 prompt the expression of the nuclear genes encoding subunits of the mitochondrial respiratory chain complexes and NRF2 activation increases synthesis of anabolic enzymes, NADPH production, and purine biosynthesis which all correlate with increased tumor growth [281]. Moreover, according to recent findings, NRF1 and NRF2 seem to be important in mitochondrial biogenesis and respiratory chain reactions [282, 283]; likewise, the role of NF-κB in tumorigenesis and mitochondria functionality has been adequately demonstrated in several studies [284]. NRF2 has been implicated in promotion of tumorigenesis by suppressing ROS levels and NRF2 knockout mice showed high levels of ROS and decreased tumorigenesis [285, 286]. In addition, Nrf2 was recently identified as a candidate transcriptional regulator of proteasome genes. Proteasome dysfunction in *Drosophila* induces high levels of reactive oxygen species that originated from malfunctioning mitochondria, triggering an Nrf2-dependent upregulation of the proteasome subunits [287].

The high proliferative rate of tumor cells leads (among others) to insufficient blood supply with nutrient and oxygen. Therefore hypoxic conditions are a feature of tumor cells *in vivo*. Hypoxia increases ROS levels that further stabilize HIFα transcription factor subunits so the cell can adapt to reduced oxygen levels [288, 289]. HIF1α binds to genomic hypoxia-responsive elements promoting the expression of a large number of genes including glycolytic enzymes and pyruvate dehydrogenase kinase-1 (PDK1 inhibits conversion of pyruvate to acetyl CoA) and it also inhibits LON protease that (among others) degrades COX4-1 subunit [62, 290, 291]. In addition, LON is thought to play an important role in metabolic reprogramming and cellular senescence and it also increases the oncogenic potential of tumor cells [67, 292, 293].

Since increased ROS levels are a common feature of cancer cells therapeutic approaches that aim to decrease intracellular ROS levels have been considered as a possible method to inhibit cancer growth [294–296]. However, these treatments can also affect normal cells where ROS play an import functional role (e.g., macrophages) [249]. Another reason why the use of these approaches has not been so successful is the fact that mitochondrial ROS are important signaling molecules and potent mitogens. Moreover, recently, it was shown that increased oxidative stress suppressed metastasis on melanoma cells [297], suggesting that increased levels of ROS may have an antioncogenic role; in line with this notion, antioxidants are frequently upregulated in cancer cells in order to suppress oxidative stress-mediated apoptotic effects and reduced proliferation [298].

Another factor being activated during tumorigenesis is peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α; a member of the PGC-1 family of coactivators) which is considered a key regulator of mitochondrial biogenesis and respiration. The PGC-1 family members potentiate the activity of other transcription factors and PGC-1α interacts with NRF1 and PPARα [299, 300]. PGC-1α can also reduce the generation of mitochondrial ROS and it also regulates the mitochondrial fusion machinery by activating Mfn2 [301]. High expression levels of PGC-1α were found to be induced by the melanocyte-specific transcription factor (MITF) in melanoma cells, while growth and progression of these melanoma cells were strongly dependent on PGC-1α expression levels [302]. Moreover, it was recently reported that Parkin regulates the expression of PGC-1α. Activation of Parkin promotes degradation of PARIS (a KRAB and zinc finger protein) which normally inhibits expression of PGC-1α by binding to insulin response sequences in the PGC-1α promoter [303].

Mitochondrial biogenesis is also controlled by the c-Myc protooncogene. c-Myc induces the activation of the PGC-1β factor; on the other hand, mitochondrial biogenesis is inhibited when HIF1 factors promote degradation of c-Myc [304].

Several tumor types have altered levels of mitophagy-related proteins. Parkin levels are downregulated in a number of different tumors, including ovarian, lung, and breast cancer, sporadic colorectal cancer, hepatocellular carcinoma, and pancreatic tumors, while PINK1 is overexpressed in adrenocortical (ACT) tumors [305–308]. Reportedly, the BNIP3 and NIX mitophagy genes are upregulated in different premalignant stages of some tumor types, while their expression is suppressed in invasive and malignant cancers [309–311]. Loss of BNIP3 probably leads to genome instability in pancreatic cancer, likely, due to increased ROS levels [312].

Finally, alterations of the mitochondrial fusion/fission rate and machinery have been also observed in tumors. More specifically, several reports indicate that fission (linked to upregulation of Drp1 or downregulation of Mfn2) is increased in a variety of tumors, including lung cancer and invasive breast carcinoma [313, 314]. Also, hypoxic conditions enhance the rate of fission events by modulating Drp1 activity, while enhancement of fission in U251 human glioblastoma cells promoted tumor cell migration [315, 316].

8. Mitochondria Quality Control and Neurodegeneration

Neuronal cells function and survival strongly depend on proper mitochondria functionality and activity, since axonal transport, neurotransmitter releasing, and ionic gradient can be severely impaired by dysfunctional mitochondria [317, 318]. In line with these facts a number of neurological disorders, including Alzheimer's Disease (AD), Parkinson's Disease (PD), and Huntington's Disease (HD), as well as amyotrophic lateral sclerosis (ALS), have been associated with the quality control of this organelle and the proteins involved. In support, mutations at PINK1 and Parkin genes are the most prevalent in patients with autosomal recessive PD early onset [319]. *Drosophila* PINK1 or Parkin loss of function exhibits muscle and neuron degenerations which are highly reminiscent of Parkinson's Disease [204]. Moreover,

the MitoPark mouse model (an animal model of Parkinson's Disease) is characterized by mitochondria fragmentation and respiratory deficiency in dopaminergic neurons [231]. Nevertheless, and despite the plethora of information which is available about these proteins, it still remains relatively unclear how PINK1/Parkin mitochondrial dysfunction leads to neurodegeneration. That is, likely, due to the fact that a great number of the studies about PINK1/Parkin are performed in cellular systems after artificially induced mitochondrial damage and depolarization leading probably to mitochondria and cell conditions which are significantly different, or at least with reduced similarity, with those found in neurological diseases. A main feature of PD is the Lewy body formation. The nonmitochondrial protein of alpha-synuclein is the major component of Lewy bodies [319]. Alpha-synuclein is degraded by proteasome and alpha-synuclein aggregates impaired normal proteasomal function [320, 321]; moreover, patients with sporadic or familial forms of PD display altered proteasome function [321].

AD is characterized by the formation of characteristic amyloid-β (Aβ) plaques and neurofibrillary tangles (as result of the association of mainly fibrillar forms of Aβ and tau protein with microtubules), impaired mitochondrial trafficking, and increased ROS levels [322]. Amyloid-β-peptide can accumulate at mitochondria and probably interacts with Drp1, while AD cellular models present decreased levels of Drp1 protein and increased expression of the Fis1 counterpart [323, 324]. UPS dysfunction seems to be also involved in AD disease, since the amyloid-β plaques formation impairs normal proteasomal function; this effect further fuels the formation of neurofibrillary tangles [325, 326].

Several other neurodegeneration diseases are associated with mitochondrial proteins dysfunction. Impaired fusion of the inner membrane due to Opal mutations leads to dominant optic atrophy, whether mutation of the outer membrane fusion protein Mfn2 is linked to peripheral neuropathy 30 Charcot-Marie-Tooth type 2A [176, 327]. Furthermore, mouse knockouts of Mfn1/2 and Opa1 genes result in embryonic lethality [170, 328], while mutations of the m-AAA subunit paraplegin lead to an autosomal recessive form of hereditary spastic paraplegia [79–81]. Mutations of the m-AAA subunit AFGL32 are linked to spinocerebellar ataxia [329], while mutations of Hsp60 in humans have been implicated in the pathogenesis of hereditary spastic paraplegia [330]. Finally, a mouse model lacking HtrA2 displayed neurodegeneration and PD-like phenotypes and missense mutations of HtrA2 have been reported in sporadic cases of PD [94, 331, 332].

The association of mitochondria function and dynamics with these neurological disorders highlights the central role of this organelle in proper functionality of neuronal cells. Besides mitochondria studies, even more data describe the UPS dysfunction in neurodegeneration disorders [333–335]. Nevertheless, more detailed research is required in relation to the functional involvement of UPS in neuronal cells function and how this system interacts with mitochondria in neuronal tissue.

9. Concluding Remarks

The vital role of mitochondria in cellular homeodynamics is clearly reflected in the severe effects of mitochondrial dysfunction on cellular functionality and human health, ageing, and age-related diseases (e.g., cancer or neurodegeneration).

Despite the growing knowledge about the molecular mechanisms that impose on mitochondrial function and structural preservation several controversial questions remain to be answered. For example, although mitochondria dysfunction (or altered function) seems to be a common feature in both neurodegeneration and cancer, the disease-specific alterations that determine the fate of the disorder need further detailed investigation efforts. In this line of research, the identification of the mitochondrial maintenance and/or signaling pathways that are specifically implicated in malignancy or neurodegeneration will, likely, reveal new disease-specific therapeutic approaches; similar efforts should aim at identifying how loss of mitostasis impacts on the progression of ageing.

An additional topic of exciting future research should of course relate to the identification of the molecular pathways that regulate the intense cross talk between the proteostatic and mitostatic modules in the young and aged somatic and reproductive tissues and how deterioration of one pathway affects the functionality of the other; these efforts will be particularly relevant given the UPS involvement in mitochondrial quality control and functionality and *vice versa*.

Moreover, the triggering event(s) that modulate the activation of the UPS systemic responses or mitophagy following mitochondrial damage clearly need further investigations. Most likely, the disrupted balance of ATP production (that initiates significant metabolic alterations) along with membrane depolarization and ROS accumulation influences the equilibrium between the selective removal of mitochondria by mitophagy or UPS-mediated degradation of damaged mitochondrial proteins.

Finally, another aspect of significant importance relates to the question whether UPS is also involved in the degradation of proteins of the internal mitochondrial compartments.

A better understanding of the mechanisms that regulate mitochondria quality control and their interconnection with the proteostasis modules (e.g., UPS) is relevant for human health since, besides the basic knowledge, mitochondria and proteasomes apart from impacting organismal healthspan are, likely, key therapeutic targets in main age-related diseases including cancer and neurodegeneration.

Abbreviations

ALS: Autophagy Lysosome System
OXPHOS: Oxidative phosphorylation
PDR: Proteome Damage Responses
PN: Proteostasis network
ROS: Reactive oxygen species
UPS: Ubiquitin-proteasome-system
UPR^{mt}: Mitochondrial Unfolded Protein Response
ULK1: Unc-51-Like Kinase 1 protein
RB1CC1: RB1-inducibile Coiled-Coil 1

Keap1:	Kelch-like ECH-associated protein 1
Nrf2:	NF-E2-related factor 2
Drp1:	Dynamin-related protein 1
OPA1:	Optic Atrophy 1
PARL:	Presenilin-Associated Rhomboid-Like
Miro:	Mitochondrial Rho GTPase
AMBRA1:	Activating autophagy/beclin-1 regulator 1
p62/SQSTM1:	Sequestosome 1
AD:	Alzheimer's Disease
PD:	Parkinson's Disease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Oxidative Stress Biomarkers and Left Ventricular Hypertrophy in Children with Chronic Kidney Disease

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Cardiovascular diseases remain the most frequent cause of morbidity and mortality in patients with chronic kidney disease (CKD). The aim of the study was to assess the association between oxidative stress biomarkers and cardiovascular risk factors and left ventricular hypertrophy in children with CKD. *Material and Methods*. The studied group consisted of 65 patients aged 1.4–18.6 (mean 11.2) years with stages 1 to 5 CKD. Serum oxidized low-density lipoprotein (oxLDL), protein carbonyl group, creatinine, cystatin C, albumin, lipids, high-sensitivity C-reactive protein, intercellular adhesion molecule-1, insulin, plasma renin activity, and aldosterone levels were measured. Patients were divided into groups depending on CKD stage. Anthropometric measurements, ambulatory blood pressure (BP) measurements, and echocardiography with left ventricular mass (LVM) calculation were performed. *Results*. Serum oxLDL strongly correlated with creatinine ($R = 0.246$; $p = 0.048$), cystatin C ($R = 0.346$; $p = 0.006$), total cholesterol ($R = 0.500$; $p < 0.001$), triglycerides ($R = 0.524$; $p < 0.001$), low-density lipoprotein concentrations ($R = 0.456$; $p < 0.001$), and 24 hour BP values of systolic ($R = 0.492$; $p = 0.002$), diastolic ($R = 0.515$; $p < 0.001$), and mean arterial pressure ($R = 0.537$; $p < 0.001$). A significant correlation between oxLDL levels and LVM z-scores ($R = 0.299$; $p = 0.016$) was found. *Conclusions*. Hypertension and dyslipidemia correlated with lipid oxidation in children with CKD. oxLDLs seem to be valuable markers of oxidative stress in CKD patients, correlating with left ventricular hypertrophy.

1. Introduction

An imbalance between the processes of formation of free radicals and their removal with a predominance of the production of reactive oxygen species (ROS) is referred to as oxidative stress. The uncontrolled increase in the concentration of free radicals is postulated to be one of the pathophysiological mechanisms of many diseases such as diabetes, atherosclerosis, vascular dementia, or neoplasms. Under physiological conditions, ROS and reactive nitrogen species (RNS) are constantly produced to defend the body against germs and are also of importance in the processes of cell signaling, tissue healing, and remodeling [1]. ROS include superoxide radical, hydrogen peroxide, and hydroxyl radical. The role of antioxidants in the body is fulfilled by enzymes: superoxide dismutase, catalase, oxidase, and glutathione peroxidase, and other substances such as glutathione, vitamins E and C, magnesium ions, zinc, albumin, ferritin, transferrin, and uric acid.

In patients with chronic kidney disease (CKD) treated conservatively and on dialysis, both the increased production of ROS and RNS and reduced antioxidant status have been

shown [2, 3]. Activation of the renin-angiotensin-aldosterone and sympathetic systems, as well as chronic inflammation, results in the production of oxidative stress markers. Low-density lipoprotein particle modified in the process of oxidation (oxLDL) develops atherogenic properties and becomes cytotoxic to vascular endothelial cells, stimulates the growth of smooth muscle, and attracts macrophages. oxLDL also inhibits macrophage mobility favoring their accumulation and formation of fatty streaks—the initial stage of the atherosclerotic process [4–6].

Oxidative stress is considered to be one of the cardiovascular risk factors in patients with CKD. In this group of patients higher prevalence of traditional risk factors (hypertension, dyslipidemia) and uremia-related ones (chronic inflammation, oxidative stress, endothelial dysfunction, anemia, fluid overload, and uremia toxins) is found. Many studies have shown increased morbidity and mortality from cardiovascular causes in adults with CKD. In children, because hard endpoints, such as stroke or cardiovascular death, are rarely evaluated, surrogate endpoints, such as left ventricular hypertrophy (LVH), are more frequently observed [7].

The aim of the study was to assess the association between oxidative stress biomarkers and cardiovascular risk factors and left ventricular hypertrophy in children with chronic kidney disease.

2. Material and Methods

Medical examinations were carried out from June 2008 to February 2011. The study was performed in accordance with the Declaration of Helsinki of 1975 for Human Research and approved by the Bioethical Committee of the Jagiellonian University (KBET/17/B/2006). The parents and patients were informed about the objective and method of performing the study and gave their informed consent.

2.1. Subjects. The inclusion criterion was the age of 0–21 years and diagnosed chronic kidney disease. The exclusion criteria were lack of consent of the patient or parents, congenital heart defects or other primary heart diseases, acute infections, or failure of other organs.

2.2. Blood Sampling and Biochemical Analysis. On admission blood samples were taken from all patients (fasting for 12 hours). Three samples were collected and centrifuged and plasma and serum were frozen at −80°C. Biochemical analyses necessary to determine kidney function were performed and urea, creatinine, cystatin C, electrolytes, albumin, aldosterone, and lipids concentrations were measured; plasma renin activity (PRA) was assessed. On the basis of serum creatinine and cystatin C, an estimated glomerular filtration rate (eGFR) with the Schwartz [8] and Filler [9] formulas was calculated. Patients were divided into groups depending on the stage of CKD [group 1: CKD stages 1 + 2 (GFR > 60), group 2: CKD stage 3 (GFR = 30–59), group 3: CKD stage 4 (GFR = 15–29 mL/min/1.73 m^2), group 4: dialyzed children].

To assess oxidative stress the concentration of oxidized LDL particles, as an effect of lipid oxidation, and the concentration of carbonyl groups resulting from oxidation of proteins were used. Concentrations of serum high sensitive C-reactive protein (hsCRP) (R&D Systems, USA); oxLDL (Mercodia Inc., Sweden); protein carbonyl groups (Cayman Chemical Company, USA); and intercellular adhesion molecule-1 (ICAM-1) (R&D Systems, USA) were determined with enzyme-linked immunosorbent assay (ELISA). Insulin levels (BioSource, Belgium) were measured using the IRMA method.

2.3. Anthropometric and Blood Pressure Measurements. During each visit, anthropometric parameters of patients, weight, height, and waist circumference, were measured. BMI was calculated by dividing weight in kilograms by height in meters squared. 24 h blood pressure monitoring (ambulatory blood pressure measurement, ABPM) using SpaceLabs 90207 device and cuff of appropriate size was performed. Blood pressure measurements were taken in an interval of 20 minutes during the day and every 30 minutes during the night. With the help of a licensed ABPM program mean values of systolic (SBP), diastolic (DBP), and mean blood pressure (mean arterial pressure, MAP) for the whole day were calculated. Hypertension was defined as BP values equal to or exceeding the 95th percentile for gender, age, and height [10]. The absolute values of height, weight, and BMI measurements were converted to z-scores based on data published by Palczewska and Niedźwiecka [11].

2.4. Left Ventricular Hypertrophy Assessment. Echocardiographic examinations were performed by an experienced cardiologist using HP 5500 unit with S4 and S8 variable frequency probes. In children on chronic hemodialysis echocardiography was performed on the day between two hemodialysis procedures, while in children on peritoneal dialysis, it was performed during the daily exchange, with a low volume of dialysate in the peritoneal cavity.

Left ventricular end-diastolic dimension (LVEDd), interventricular septal thickness at end diastole (IVSd), and left ventricular posterior wall thickness at end diastole (LVPWd) were measured by 2-dimensional guided M-mode echocardiography using the parasternal short-axis view at the level of the papillary muscles. Diameters and thickness were corrected for body surface area (BSA) and normal ranges were assessed according to values published by Kampmann et al. [12].

LV mass (LVM) was calculated by the formula described by Devereux and Reichek [13]. LVM z-score for height was calculated according to the method described by Foster et al. [14]. LVM index (LVMI) was obtained by dividing LVM by height$^{2.7}$ to normalize and linearize the relationship between LVM and height [15]. LV hypertrophy (LVH) was diagnosed when LVMI was over the 95th percentile for healthy children [16]. We used age-specific cut-off values provided by Khoury et al. [17].

2.5. Statistical Analysis. Qualitative values were compared by the chi-square test. Because data of the majority of variables did not show normal distribution, they are presented as median [25th–75th percentile]. Differences between the groups were compared using the Kruskal-Wallis test.

Spearman's rank correlation was used to relate levels of kidney function and oxidative stress markers. Statistical calculations were performed using a commercially available statistical package (Statistica PL). A value of $p < 0.05$ was considered significant in all statistical analyses.

3. Results

The studied group consisted of 65 patients (41 boys and 24 girls) aged 1.4 to 18.6 (mean 11.2) years with stage 1 to stage 5 CKD, who were under constant medical control in the University Children's Hospital in Krakow. Among diseases leading to the development of CKD in the examined children, the highest prevalence was noted in congenital abnormalities of the kidney and urinary tract, 31 (47.7%), followed by glomerulonephritis, 8 (12.3%), cystic disease, 7 (10.8%), and others, 19 (29.2%).

Clinical data and basic kidney function parameters depending on the stage of chronic kidney disease are presented in Table 1.

Several parameters of possible mechanisms (PRA, aldosterone, endothelial dysfunction-ICAM-1, inflammation-hsCRP, and hyperinsulinism) associated with oxidative stress were analyzed according to CKD stage in the studied group. There was a significant difference in median oxLDL concentration between CKD stages 3 and 5 (75.81 versus 98.89 U/L; $p = 0.019$). There were no significant differences in the concentration of most evaluated parameters (Table 2).

In the studied group 41 out of 65 patients were treated with angiotensin-converting enzyme inhibitors (ACEI) or angiotensin receptor blocker (ARB), 63.6% in stages 1 + 2, 72.2% in stage 3, 64.3% in stage 4, and 54.5% in stage 5, respectively. There were no differences between children on ACEI or ARB and without this treatment (data on request).

The average concentration of oxidized LDL was 86.94 U/L and of carbonyl groups 1.69 nmol/mg. Elevated concentrations of protein carbonyl groups (>4 nmol/mg) were observed in 6 of 54 patients. The median oxLDL concentration was significantly higher in girls than in boys (99.86 versus 81.06 U/L; $p = 0.024$).

In the studied population there was no correlation between oxLDL and age, CKD duration, weight, body mass index (BMI) in z-score, urea, high-density lipoprotein (HDL), albumin, insulin, hsCRP and ICAM-1, and aldosterone concentrations. Correlations of investigated parameters (aldosterone, PRA, carbonyl group, oxLDL, ICAM-1, hsCRP, and insulin) with kidney function markers were performed. The most pronounced correlations were found for oxLDL: its concentration significantly correlated with creatinine ($R = 0.246$; $p = 0.048$), cystatin ($R = 0.346$; $p = 0.006$), and eGFR calculated on their basis ($R = -0.266$; $p = 0.032$ and $R = -0.296$; $p = 0.027$, resp.). Furthermore oxLDL strongly correlated with total cholesterol ($R = 0.500$; $p < 0.001$), TGL ($R = 0.524$; $p < 0.001$), LDL ($R = 0.446$; $p < 0.001$), and 24 hour blood pressure values of SBP ($R = 0.492$; $p = 0.002$), DBP ($R = 0.515$; $p < 0.001$), and MAP ($R = 0.537$; $p < 0.001$) and negatively with PRA ($R = -0.264$; $p = 0.038$). hsCRP correlated significantly with creatinine ($R = 0.266$; $p = 0.033$), while insulin correlated with creatinine ($R = 0.333$; $p = 0.009$) and cystatin C ($R = 0.337$; $p = 0.010$) concentrations and eGFR calculated with the Filler formula ($R = -0.422$; $p = 0.002$).

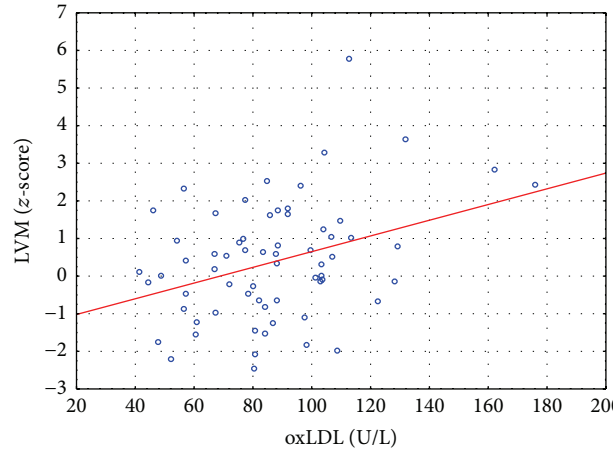

Figure 1: Scatterplot presenting the correlation between oxLDL concentrations and left ventricular mass (LVM) (z-score).

Patients were divided into 4 groups depending on the quartiles of oxLDL concentration and clinical, biochemical, and echocardiographic parameters between groups were compared. Children with high oxLDL concentrations were characterized by significantly higher blood pressure, triglycerides, and total and LDL cholesterol levels. In this group also higher left ventricular mass was found (Table 3).

Echocardiographic examinations revealed LVH in 34 children. There were no significant differences in median carbonyl groups concentrations between children with and without LVH (1.17 (0.62; 1.89) versus 1.29 (0.85; 1.96) nmol/mg; $p = 0.567$). A trend toward higher median oxLDL values in children with LVH was present (88.60 (76.24; 107.07) versus 81.06 (61.13; 98.35) U/L; $p = 0.084$), although it did not reach statistical significance.

There was a significant correlation between oxLDL concentration and LVPWT (z-score) ($R = 0.258$; $p = 0.038$). In the univariate analysis, LVM in z-scores correlated significantly with eGFR ($R = -0.427$; $p = 0.001$), MAP (z-score) ($R = 0.487$; $p = 0.002$), albumin ($R = -0.363$; $p = 0.004$), and oxLDL concentrations ($R = 0.299$; $p = 0.016$) (Figure 1). In the multivariate analysis, the single independent parameter was MAP ($B = 0.383$; $p = 0.001$). After exclusion of MAP from the equation 3 parameters correlated independently with LVM z-score (eGFR: $B = -0.01$; $p = 0.034$; albumin: $B = -0.025$; $p = 0.047$; and oxLDL: $B = 0.023$; $p = 0.001$).

4. Discussion

This is, to our knowledge, the first study comparing oxidative stress markers and left ventricular hypertrophy in children with chronic kidney disease.

Despite the enormous technological progress and the introduction of new medications into the treatment of

Table 1: Basic clinical data and kidney function parameters depending on CKD stage in the investigated group of 65 patients.

Parameter	CKD stage				p value
	1 + 2 (n = 11)	3 (n = 18)	4 (n = 14)	5 (n = 22)	
Age (years)	10.51 (5.04; 16.08)	11.33 (5.15; 16.33)	12.01 (8.70; 15.99)	11.61 (8.51; 15.20)	0.820
Height (z-score)*	0.078 (−0.300; 0.569)	−0.716 (−2.303; 0.060)	−0.868 (−1.484; −0.210)	−1.495 (−3.444; −0.130)	**0.013**
Body mass (z-score)**	−0.015 (−0.409; 0.419)	−0.980 (−1.635; 0.009)	−0.464 (−1.054; 0.228)	−1.587 (−3.714; −0.828)	**0.002**
BMI (kg/m^2)	16.5 (15.8; 22.1)	16.6 (14.7; 19.5)	18.1 (16.0; 20.4)	15.9 (14.5; 17.2)	0.224
BMI (z-score)***	0.316 (−0.318; 0.712)	−0.779 (−1.636; 0.433)	0.079 (−0.638; 0.712)	−1.252 (−1.599; −0.621)	**0.020**
Creatinine (μmol/L)****	69.3 (34.0; 93.8)	113.8 (95.0; 146.4)	274.9 (203.8; 311.9)	501.8 (414.3; 869.0)	<**0.001**
Cystatin C (mg/L)****	0.86 (0.68; 1.23)	1.45 (1.17; 1.86)	2.6 (2.23; 3.01)	4.81 (4.14; 6.53)	<**0.001**
eGFR Filler**** (mL/min/1.73 m^2)	109.46 (72.62; 141.28)	60.13 (45.64; 76.81)	29.49 (26.19; 36.49)	14.77 (9.78; 18.43)	<**0.001**

Values presented as median (25th–75th percentile).
BMI—body mass index; eGFR—estimated glomerular filtration rate.
*Statistically significant differences between stages 1 + 2 and 4, 1 + 2 and 5.
**Statistically significant differences between stages 1 + 2 and 3, 1 + 2 and 5, 4 and 5.
***Statistically significant differences between stages 4 and 5.
****Statistically significant differences between all stages.

Table 2: Selected parameters depending on CKD stage in the investigated group of 65 patients.

Parameter	CKD stage				p value
	1 + 2 (n = 11)	3 (n = 18)	4 (n = 14)	5 (n = 22)	
hsCRP (ng/mL)	171.6 (128.7; 464.3)	252.4 (106.5; 2574.9)	338.9 (143.0; 771.6)	365.6 (187.2; 878.1)	0.397
Carbonyl groups (nmol/mg)	1.15 (0.54; 1.32)	1.24 (0.87; 1.69)	1.64 (0.73; 2.41)	1.23 (0.66; 2.05)	0.454
oxLDL (U/L)*	80.65 (60.56; 109.68)	75.81 (56.66; 97.87)	82.31 (75.48; 91.92)	98.89 (82.16; 108.73)	**0.030**
Aldosterone (pg/mL)	186.1 (103.5; 298.7)	272.8 (142.3; 662.1)	471.00 (169.0; 1073.1)	192.3 (86.4; 711.4)	0.250
PRA (ng/mL/h)	4.99 (1.25; 8.38)	6.88 (3.41; 12.51)	4.69 (1.64; 9.98)	3.88 (2.01; 7.94)	0.521
ICAM-1 (ng/mL)	305.7 (289.4; 354.3)	319.1 (291.8; 446.3)	322.1 (290.3; 337.7)	329.2 (267.3; 408.9)	0.922
Insulin (μIU/mL)	9.0 (7.0; 10.6)	9.5 (8.1; 15.7)	12.8 (10.1; 16.8)	13.1 (6.9; 16.3)	0.162
Albumin (g/L)	45.0 (41.0; 47.0)	45.50 (41.0; 47.5)	46.8 (45.0; 49.1)	44.1 (37.7; 47.1)	0.127

Values presented as median (25th–75th percentile)
hsCRP—high sensitive C-reactive protein; oxLDL—oxidized low-density lipoprotein; PRA—plasma renin activity; ICAM-1—intercellular adhesion molecule-1.
*Statistically significant differences between stages 3 and 5.

patients with impaired renal function, this group is characterized by an increased cardiovascular risk. Mortality associated with cardiovascular causes is higher in children and adults with CKD compared to the general population [18]. According to data from the USA, the estimated survival time of children on dialysis is 40–60 years shorter than healthy peers [19].

CKD patients are exposed to high prevalence of traditional cardiovascular risk factors as well as nontraditional ones, such as inflammation, oxidative stress, and endothelial

Table 3: Investigated clinical, biochemical, and echocardiographic parameters in the groups with oxLDL quartiles.

Parameter	oxLDL (U/L)				p value
	41.4–67.4 (n = 16)	67.5–84.8 (n = 17)	86.0–103.5 (n = 16)	103.6–176.1 (n = 16)	
SBP 24 h (z-score)	0.11 (−2.64; 0.53)	−0.62 (−0.92; 0.56)	−0.33 (−1.41; 1.30)	1.86 (1.03; 2.73)	**0.009**
DBP 24 h (z-score)	−0.06 (−1.80; 0.27)	−0.77 (−2.14; 1.37)	−0.46 (−1.40; 1.78)	2.42 (1.16; 4.64)	**0.004**
MAP 24 h (z-score)	0.42 (−1.82; 0.79)	−0.32 (−1.09; 1.04)	0.00 (−0.86; 1.76)	2.32 (2.01; 4.45)	**0.003**
Total chol. (mmol/L)	4.19 (3.63; 4.81)	4.48 (4.13; 5.01)	4.67 (4.08; 5.62)	6.19 (5.39; 7.50)	**0.001**
TGL (mmol/L)	1.21 (0.90; 1.62)	1.30 (1.08; 1.47)	1.69 (1.28;2.35)	2.62 (1.63; 3.70)	**0.001**
HDL (mmol/L)	1.35 (1.08; 1.76)	1.35 (1.17; 1.76)	1.19 (1.03;1.43)	1.26 (0.97; 1.62)	0.417
LDL (mmol/L)	2.07 (1.70; 2.59)	2.43 (1.99; 2.64)	2.49 (2.08; 3.39)	3.26 (2.57; 4.01)	**0.005**
Cystatin (mg/L)	1.36 (1.09; 2.97)	2.36 (1.17; 3.46)	2.55 (1.61; 4.07)	4.24 (1.48; 6.12)	0.053
Albumin (g/L)	46.0 (44.1; 48.0)	46.0 (44.0; 47.7)	45.0 (38.3; 47.0)	43.0 (34.0; 47.6)	0.320
hsCRP (ng/mL)	267.5 (159.5; 688.2)	321.9 (162.5; 553.4)	459.2 (104.3; 1270.2)	273.2 (121.5; 486.4)	0.939
ICAM-1 (ng/mL)	295.5 (267.3; 339.4)	303.0 (288.5; 346.4)	339.9 (316.0; 405.8)	326.4 (278.4; 396.7)	0.373
Carbonyl groups (nmol/mg)	1.39 (0.87; 1.69)	1.73 (0.50; 2.25)	1.23 (0.96; 1.87)	0.92 (−0.54; 1.75)	0.736
LVM (z-score)	0.05 (−1.06; 0.76)	−0.27 (−0.99; 0.64)	0.73 (−0.41; 1.70)	1.01 (−0.05; 2.61)	**0.038**
LVEDd z-score	0.17 (−0.61; 0.85)	0.19 (−0.19; 0.61)	−0.03 (−0.60; 0.57)	0.22 (−0.31; 0.83)	0.765
IVSd z-score	0.99 (.07; 1.42)	0.31 (−0.06; 0.93)	1.06 (0.18; 1.72)	1.08 (0.56; 2.07)	0.082
LVPWT z-score	0.75 (0.18; 1.40)	1.32 (0.26; 1.58)	1.40 (0.50; 2.19)	1.45 (0.65; 2.37)	0.281

Values presented as median (25th–75th percentile).
SBP 24 h—systolic blood pressure 24 h; DBP 24 h—diastolic blood pressure 24 h; MAP 24 h—mean arterial pressure 24 h; TGL—triglycerides; HDL—high-density lipoprotein; LDL—low-density lipoprotein; hsCRP—high sensitive C-reactive protein; oxLDL—oxidized low-density lipoprotein; ICAM-1—intercellular adhesion molecule-1; LVM—left ventricular mass; LVEDd—left ventricular end-diastolic dimension; IVSd—interventricular septum at end diastole; LVPWd— left ventricular posterior wall thickness at end diastole.

dysfunction. These factors are responsible for accelerated atherosclerosis and heart damage. Fruchart et al. proposed a division of atherosclerosis risk factors into the old, the old/new, and the new [20]. The authors included into new agents, among others, triglycerides, oxidized LDL and anti-oxidized LDL antibodies, lipoprotein (a), homocysteine, and hsCRP, which indicates the role of oxidative stress and inflammation in atherosclerosis. Numerous studies [21–24] have shown increased concentrations of oxidative stress markers, such as advanced products of protein oxidation, malondialdehyde, and isoprostanes in patients with chronic kidney disease. Children on dialysis have demonstrated reduced antioxidant enzymes activity and decreased levels of trace elements [25]. According to different authors, oxidative stress plays a central role in the development and accelerated progression of atherosclerosis in patients with impaired renal function [23].

Our data suggest that uremia per se is a significant contributor to oxidative stress. In the studied group of children with chronic kidney disease a significant correlation between the concentrations of oxidized LDL and serum creatinine and cystatin C was demonstrated. The highest median concentration of oxLDLs was found in the group of children undergoing dialysis. Furthermore, a significant influence of traditional cardiovascular risk factors, hypertension and lipid disturbances, on the severity of oxidative stress in children with CKD was shown. oxLDL concentration correlated strongly with 24 hour systolic, diastolic, and mean arterial pressure values and with total cholesterol, triglycerides, and LDL cholesterol levels. The lack of correlation

between kidney function impairment and aldosterone level as well as plasma renin activity is probably connected with wide use of nephroprotection, starting at the early stage of chronic kidney disease. From the studied population 63% of children were treated with ACEI or ARB.

Statins are becoming more widely used in children, especially in those with familial hypercholesterolemia [26]. Given the high cardiovascular risk in patients with CKD and lack of efficacy of dietary restrictions in lipid normalization, it seems reasonable to execute clinical trials and determine the indications for statin therapy in these children and adolescents. A few recent studies have demonstrated that the use of statins may not only inhibit cholesterol synthesis but also have important pleiotropic effects, such as antioxidant and cytoprotective abilities. In the study of Chang et al. a significant reduction in CRP levels after 8 weeks of simvastatin therapy was observed in hemodialysis patients, which reflects the anti-inflammatory effect of statins [27]. In the study by Kumar et al., in a retrospective analysis of 257 dialyzed patients, the relation between statin therapy and lower CRP levels was found [28]. Furthermore, in another study a significant relationship between statin use and reduction of IL-6 levels was identified [29]. The result of anti-inflammatory effect of statins treatment may exceed beyond their lipid lowering effect in patients with CKD, but there is insufficient data for the pediatric population.

Over the last decades, several research studies investigating the role of oxidative stress in chronic kidney disease in adults were undertaken [30]. Müller et al. examining DNA damage showed a significantly higher degree of oxidative stress in hemodialyzed patients, compared to healthy volunteers [31]. Kaneda et al. found elevated AOPP concentrations in patients with ischemic heart disease and in those treated with hemodialysis. It should be pointed out that the severity of coronary heart disease correlated with AOPP quartiles [32]. In hemodialyzed patients the concentration of malondialdehyde (MDA)—a measure of lipid peroxidation—was significantly higher than that in patients with CKD treated conservatively and healthy subjects [33]. On the other hand, the concentration of an antioxidant—superoxide dismutase—was reduced. The combination of increased oxidative stress and lipid disorders leads to the progression of the process of atherosclerosis in patients with chronic kidney disease. Sakata and colleagues found an increased accumulation of advanced glycation end products in atherosclerotic lesions—from intimal thickening to atherosclerotic plaque—in the aorta of people with end-stage renal disease [34]. In the postmortem examination of aorta sections an increased content of pentosidine and MDA in the fraction of elastin in patients on hemodialysis was detected. The modification of elastin in the processes of glycoxidation and lipid peroxidation could lead to vascular lesions exaggeration in patients with end-stage renal disease [35].

The main factor influencing the increase in left ventricular mass in our study was elevated blood pressure. Several other parameters such as high oxLDL, low albumin concentrations, and low eGFR also correlated with LVM. The studied group of children with high oxLDL levels had significantly higher LVM and left ventricular posterior wall thickness. Future studies are needed to evaluate oxLDL as a biomarker of oxidative stress target organ damage in children. Holvoet et al. proposed evaluating the concentration of circulating oxidized LDLs as means of a more accurate cardiovascular risk assessment. Adults with coronary artery disease confirmed in angiography had significantly higher concentrations of oxLDLs. A significant correlation between oxLDLs and most of Framingham risk factors was also demonstrated [36]. In hemodialyzed patients, particularly vulnerable to oxidative stress, a beneficial effect of antioxidant usage in the form of large vitamin E doses on secondary prevention was shown in the SPACE study [37]. Treatment with vitamin E for a period of about two years reduced the risk of myocardial infarction, ischemic stroke, and peripheral vascular disease. The supply of a different antioxidant (N-acetyl-cysteine) led to a reduction in intracellular oxidative stress and the incidence of apoptosis in T cells in children on chronic hemodialysis [38].

In the studied population a significant correlation between hsCRP and serum creatinine concentrations was found. It should be stressed out that in children recorded hsCRP concentrations did not exceed 3 mg/L, a value that indicates an increased cardiovascular risk associated with the severity of chronic inflammation. In adults on hemodialysis significantly higher concentration of carbonyl groups in comparison to healthy subjects was observed. Danielski and coauthors suggested that increased oxidative stress associated with inflammation and phagocytic cell activation might preferentially increase aldehyde formation and oxidize thiol groups in proteins rather than promote lipid peroxidation [39]. In adults with diabetes mellitus protein carbonyl (PCO) content was higher than that in healthy controls and the hemodialysis procedure caused additional elevation of PCO levels [40]. The lack of a significant increase in protein carbonyl content in children with advanced CKD compared to adults can result from various diseases leading to impaired renal function. In adults, the most common causes of CKD are diabetes mellitus and long-lasting hypertension and in children, congenital abnormalities of kidney and urinary tract. In our study there was no correlation between insulin and oxLDL concentrations.

Chronic kidney disease is frequently accompanied by decreased albumin levels, both in adults and in children [41, 42]. Serum albumin concentration was found to be an independent predictor of mortality risk in a broad range of clinical and research settings in adults [43], especially in those with end-stage renal disease [44]. This increase in mortality was independent of malnutrition, a condition that until recently was thought to be the reason for reduced albumin levels [44]. Albumin is postulated to be major and predominant circulating antioxidant [45]. Several researchers have evaluated the relation between hypoalbuminemia and oxidative stress in adults with CKD; however no data is available for the pediatric population. Levels of inflammatory and oxidative stress biomarkers were increased in hypoalbuminemic compared with normoalbuminemic end-stage renal disease adults undergoing hemodialysis in a study by Danielski et al. [39]. Kaneko and coauthors found significantly lower serum levels of biological antioxidant potentials in adults with

idiopathic nephrotic syndrome [46]. In our study we found no correlation between albumin and oxLDL concentrations. Low albumin is related to overhydration and, as a result, high blood pressure [42] and left ventricular hypertrophy [47]. We demonstrated a significant negative correlation between albumin concentration and left ventricular mass expressed in z-score.

In the early stages of the atherosclerotic process there is an activation of adhesion molecules, which promotes adhesion of monocytes to the vascular wall and their migration to the intima. As a result of chronic inflammation adhesion molecules belonging to the immunoglobulin family: ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular adhesion molecule-1) are being expressed on endothelial cells. In the work of Ridker et al. the growing concentration of soluble ICAM-1 adhesion molecule was one of the major cardiovascular risk factors in postmenopausal women and the risk increased 2.6-fold between the lowest and highest quartiles of ICAM-1 levels [48]. In the studied population of children with CKD, no influence of the degree of renal function impairment on plasma ICAM-1 was found. Furthermore, no correlation of ICAM-1 with oxidative stress biomarkers was shown.

5. Conclusions

In children with chronic kidney disease an increase in the concentration of oxidized LDLs with the progression of the disease was found. This biomarker of oxidative stress was strongly correlated with 24 hour blood pressure values, triglycerides, and total and LDL cholesterol levels. Oxidized LDLs seem to be valuable markers of oxidative stress in CKD patients, correlating with left ventricular hypertrophy. In contrast to adults, the protein carbonyl content did not increase in advanced stages of CKD.

Conflict of Interests

All authors disclose no conflict of interests in relation to this work.

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Review Article

Killing Me Softly: Connotations to Unfolded Protein Response and Oxidative Stress in Alzheimer's Disease

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This review is focused on the possible causes of mitochondrial dysfunction in AD, underlying molecular mechanisms of this malfunction, possible causes and known consequences of APP, Aβ, and hyperphosphorylated tau presence in mitochondria, and the contribution of altered lipid metabolism (nonsterol isoprenoids) to pathological processes leading to increased formation and accumulation of the aforementioned hallmarks of AD. Abnormal protein folding and unfolded protein response seem to be the outcomes of impaired glycosylation due to metabolic disturbances in geranylgeraniol intermediary metabolism. The origin and consecutive fate of APP, Aβ, and tau are emphasized on intracellular trafficking apparently influenced by inaccurate posttranslational modifications. We hypothesize that incorrect intracellular processing of APP determines protein translocation to mitochondria in AD. Similarly, without obvious reasons, the passage of Aβ and tau to mitochondria is observed. APP targeted to mitochondria blocks the activity of protein translocase complex resulting in poor import of proteins central to oxidative phosphorylation. Besides, APP, Aβ, and neurofibrillary tangles of tau directly or indirectly impair mitochondrial biochemistry and bioenergetics, with concomitant generation of oxidative/nitrosative stress. Limited protective mechanisms are inadequate to prevent the free radical-mediated lesions. Finally, neuronal loss is observed in AD-affected brains typically by pathologic apoptosis.

1. Introduction

Alzheimer's disease (AD) is known for almost 120 years as a progressive fatal human neurodegenerative disease featured by decline in both memory and cognitive functions [1]. Early-onset familial AD (FAD) which accounts for less than 5% of cases is linked to mutations in *APP* gene on chromosome 21 or genes encoding components of γ-secretase (presenilin 1, presenilin 2) resulting in increased A$\beta_{42/40}$ ratio, where Aβ_{42} is highly fibrillogenic [2–4]. Sporadic, or late-onset, AD (SAD) which is a major form (over 95% of cases) has unknown etiology. While one out of nine people aged 65 or older has Alzheimer's, nearly one out of three people aged 85 or older has the disease [5]. The underlying molecular mechanisms that cause the formation of the hallmarks of FAD and SAD, namely, amyloid-β- (Aβ-) containing plaques and microtubule-associated protein tau-containing neurofibrillary tangles (NFTs), are not yet fully clarified. The Aβ peptide is a cleavage product of amyloid precursor protein (APP) by sequential action of β- and γ-secretases which release 39–43-amino-acid peptide from the C-terminal (cytoplasmic) end of transmembrane protein [6]. The outcome of the end-stage intracellular lesions in FAD and SAD is loss of neurons (brain atrophy) with most affected regions as frontal cortex, hippocampus, and amygdala [7]. Severe injuries are very selective and restricted to neurons as shown by the morphometric analyses of brain tissue slices obtained at autopsy from cases with diagnosis of AD faced up with cases with no clinical or pathological history of neurological disease [8]. Bulk of changes is characterized by significant reduction in mitochondria density, accumulation of mtDNA and proteins in cytoplasm and in the vacuoles associated

with lipofuscin once involved in mitophagy [9]. These observations show increased mitochondrial degradation products either by autophagy or by messed up proteolytic systems. Mitochondrial abnormalities to milder extent were also found in other cell types (endothelium, fibroblasts) obtained from patients with AD [10, 11]. We also observed extensive autophagy in cellular model of FAD and SAD [12].

For decades, the hypothesis of AD ("amyloid cascade hypothesis") of extracellular amyloid-β plaques and intracellular NFTs accumulations as clues in AD pathogenesis have been extensively examined with conflicting results. In the last decade, however, a new attractive hypothesis emerged from studies concerning mitochondria as key organelles for maintaining neuron functions and survival. A growing body of evidence supports the idea that dysfunctional mitochondria cause development of synaptic abnormalities, neuronal degeneration, and ultimately cell death as a consequence of unbearable oxidative stress in AD [13–16]. Numerous *in vitro* and *in vivo* experiments substantiated the so-called "vicious cycle hypothesis," pointing to the importance of mitochondria in the pathogenesis of AD [17–28]. Due to their limited glycolytic capacity (lack of salvage pathway), neurons are highly dependent on mitochondrial function for energy release and severely affected by the limited oxygen and glucose supply, making them especially susceptible to energy dyshomeostasis [29]. Moreover, mitochondria, which produce almost entire energy in neurons, have recently been found to be targeted by APP and Aβ [15, 17, 30–35]. The presence of APP and Aβ in mitochondria has detrimental consequences as both constituents cause perturbations in cellular energy homeostasis.

2. Neurons: Cells Extremely Susceptible to Energy Dyshomeostasis

Excitability is a basic attribute of neurons (as well as other excitable cells), as it encompasses the primary task to receive, analyze, and dispatch electronic signals within the neuronal network or to their cognate effectors. This function is achieved by the generation of electric currents, some of which are of high frequencies. These electric currents are evoked by the ion fluxes (Na^+, K^+, Ca^{2+}, and Cl^-) through channels located in the plasma membrane. Any change in the concentration of K^+ or Na^+ at the extra- or intracellular site of plasma membrane, respectively, activates Na^+/K^+-ATPase which restores the concentration gradient essential for excitability and also controls the cell volume. The active transport against the concentration gradient is entirely dependent on ATP delivered to Na^+/K^+-ATPase and other pumps (Ca^{2+}-ATPase, H^+-ATPase). ATP is hydrolyzed leading to phosphorylation of the pump at a highly conserved aspartate residue and subsequent release of ADP. Energy generation and energy consumption are tightly coupled to neuronal activity at the cellular level. Na^+/K^+-ATPase, a major energy-consuming enzyme, is well expressed in neurons rich in cytochrome *c* oxidase, an important enzyme of the energy-generating machinery and glutamatergic receptors that are mediators of neuronal activity [36]. Na^+/K^+-ATPase enzyme consumes the bulk of energy in the brain [37–39]. Nervous cells are highly enriched in mitochondria, the main energy supplying organelle, which provide ATP once they are sufficiently supplied with oxygen. Mitochondrial ATP is exchanged with cytosolic ADP through inner membrane adenine nucleotide translocase, so the intracellular location of mitochondria is crucial for availability of ATP and accelerated by cytosolic ADP. To meet energy requirements, mitochondria move regularly along the microtubular meshwork to the sites of higher ATP demand (high concentration of ADP), where they undergo the fusion process. Mitochondrial ATP is also indispensable energy donor for dynamins (kinesin, dynein), the proteins responsible for microtubule-associated axonal transport of the secretory vesicles. Obviously, any substantial interruption of the mitochondrial function, distribution, and fusion would affect the ATP delivery with resultant defects in neuronal activity.

3. APP Processing and Aβ Formation

First, widely expressed APP is a transmembrane glycoprotein, synthesized on three different templates (APP695, APP751, and APP770) which resulted from alternative splicing of the transcript [40, 41]. Second, after APP is synthesized on polysomes, this protein undergoes N-glycosylation in the ER. Once it is N-glycosylated, the APP is then transported to the Golgi apparatus. Golgi apparatus is a second chief site of APP posttranslational modifications including O- and N-glycosylations, phosphorylations, and sulphonations [42, 43]. Great deal of mature APP protein is stored in Golgi and trans-Golgi network (TGN), while approximately 10% of APP is unidirectionally (anterograde) transported by kinesins in TGN vesicles or in elongated tubular structures along microtubules in soma, dendrites, and axons [44, 45]. Third, APP glycoprotein embedded to plasma membrane is preferentially cleaved in the nonamyloidogenic pathway; alternatively it could be internalized via endocytosis [46]. Endosomic APP protein as well as its processed fragments can return to plasma membrane, can be proteolytically degraded in the lysosome, or can be transported from early endosome to TGN. Retention of APP in the endoplasmic reticulum/intermediate compartment (ER/IC) eliminated production of intracellular $A\beta_{40}$ but did not alter synthesis of fibrillogenic form ($A\beta_{42}$) [47]. Interestingly, the production of intracellular Aβ from wild-type APP695 appears to be a unique characteristic of postmitotic neurons, since intracellular Aβ was not detected in several nonneuronal cell lines [48]. Whether APP retromer (transported from early endosome to TGN) is also cleaved via amyloidogenic pathway is not clear due to conflicting observations [49, 50]. In neurons, APP695 is the major isoform and could be subject to sequential proteolytic cleavage by β- and γ-secretase to free Aβ. The β-secretase (BACE1, transmembrane aspartyl protease) initiates endoproteolytic cleavage giving rise to N-terminus of Aβ (β-secretase cleaved APP to βCTF as the intermediate) followed by γ-secretase (membrane-embedded aspartyl protease complex consisting of presenilin, PS), presenilin enhancer-2 (Pen-2), anterior pharynx defective-1 (Aph-1), and nicastrin, which reveals the C-terminus of Aβ [51]. Given that two PS (PS1 and PS2) and Aph-1 (Aph-1A and Aph-1B) variants exist, the processing

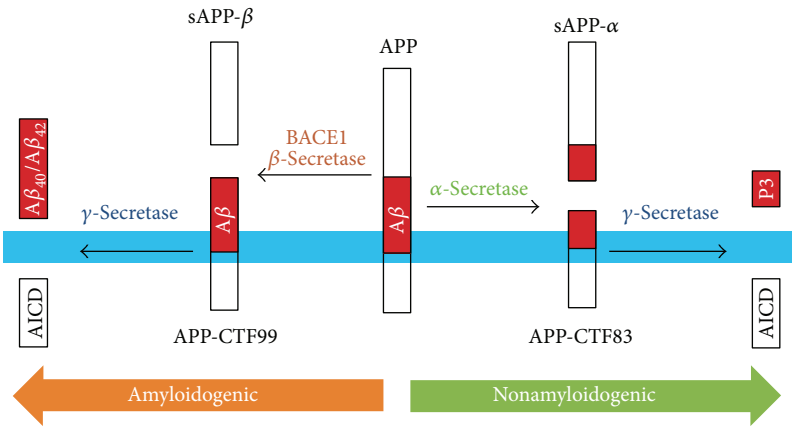

Figure 1: A diagram of amyloid precursor protein (APP) processing pathway. The transmembrane protein APP (membrane indicated in blue) can be processed by two pathways: the nonamyloidogenic α-secretase pathway and the amyloidogenic β-secretase pathway. In the nonamyloidogenic pathway, α-secretase cleaves in the middle of the β-amyloid (Aβ) region (red) to release the soluble APP-fragment sAPP-α. The APP C-terminal fragment 83 (APP-CTF83, αCTF) is then cleaved by γ-secretase to release the APP intracellular domain (AICD) and P3 fragment. In the amyloidogenic pathway, β-secretase cleaves APP to produce the soluble fragment sAPP-β. APP-CTF99 (βCTF) is then cleaved by γ-secretase to produce Aβ_{40}, Aβ_{42}, and AICD. Adopted from [179].

of APP by four different human γ-secretase complexes each acting at more than one βCTF site (ε-, ζ-, and γ-) leads to formation of several Aβ (Aβ_{37-43}), with Aβ_{40} and Aβ_{42} being predominant species. Finally, three end products are formed (sAPP-β, Aβ, and amyloid precursor protein intracellular domain, AICD). The key neuron α-secretase (ADAM10) cleaves APP inside Aβ polypeptide chain to αCTF as the intermediate, so after subsequent γ-secretase action on αCTF, three nonpathogenic fragments are formed (sAPP-α, P3 fragment, and AICD) (Figure 1).

As aforementioned, FAD is caused by mutations in *APP* and *PSEN* genes located on chromosomes 21 and 14, respectively, but the incidence of AD is also higher in dominantly inherited duplications of the APP locus in elderly individuals with Down's syndrome (trisomy of the 21st chromosome), pointing to important role played by APP and Aβ in AD. Mutations in APP located near the β-secretase cleavage site increase production of Aβ, whereas those near the γ-secretase cleavage site result in an increased ratio of Aβ_{42} to Aβ_{40} [52]. The ε4 allele of apolipoprotein E is the major risk factor for SAD. Thus, this particular *APOE* gene polymorphism increases disease risk in a dose-dependent manner and lowers the age of onset, as shown by Corder et al. [53]. One copy of APOE4 increases the risk of AD about fourfold (compared with the more common APOEε3/APOE3ε3 genotype), whereas two copies of APOE4 increase the risk of AD about 12-fold. The mechanism by which the amino acid difference between APOE3 and APOE4 increases the risk of AD remains to be established.

Widespread occurrence of APP and Aβ in nervous system brought about the assumption that both components might play physiological roles. More than few possible concepts have emerged, some validated by experimental data. The APP protein overexpression led to enhanced survival and growth of some cell types [54, 55]. Furthermore, secreted forms of APP (APP^{s}s: sAPPα and sAPPβ) were antiapoptotic [56] and have a potent neuroprotective action in cultured rat hippocampal and septal neurons and in human cortical neurons [57]. APP$^s{}_{695}$ and APP$^s{}_{751}$ protected neurons against hypoglycemic damage, and the neuroprotection was abolished by antibodies to a specific region common to both APP$^s{}_{695}$ and APP$^s{}_{751}$. Thus, APP^{s}s may normally play excitoprotective and neuromodulatory roles. Accordingly, APP was shown to stimulate axon branching and the maintenance and formation of synapses, neuronal survival, and neuritic outgrowth [58–60]. APP protein is highly expressed in axons and interacts with extracellular matrix components [61–64]. Similar to APP, Aβ was demonstrated to play a physiological role in synaptic plasticity as minute quantities of the peptide stimulated neurons and enhanced the release of neurotransmitter [65, 66]. Everything can change when the things go awry.

4. Perturbations in ER

In healthy cells including neurons, ER is a fundamental organelle for protein quality control in the secretory pathway, which prevents protein aberrant folding and aggregation [67]. A bulk of evidence shows the importance of ER in APP maturation and processing. With regard to APP intracellular processing, both secretases (α- and β-) have been identified in the ER together with γ-secretase which is present in mitochondria-associated membrane (MAM) subcompartment [48, 68]. This distinctive intracellular lipid-raft-like structure is involved in cholesterol and phospholipid metabolism, Ca^{2+} metabolism, and mitochondrial dynamics and becomes markedly augmented in AD [69]. MAM is responsible for the communication between the ER and the mitochondria with efficient transfer of Ca^{2+} from the ER to mitochondria supporting metabolic functions and cell

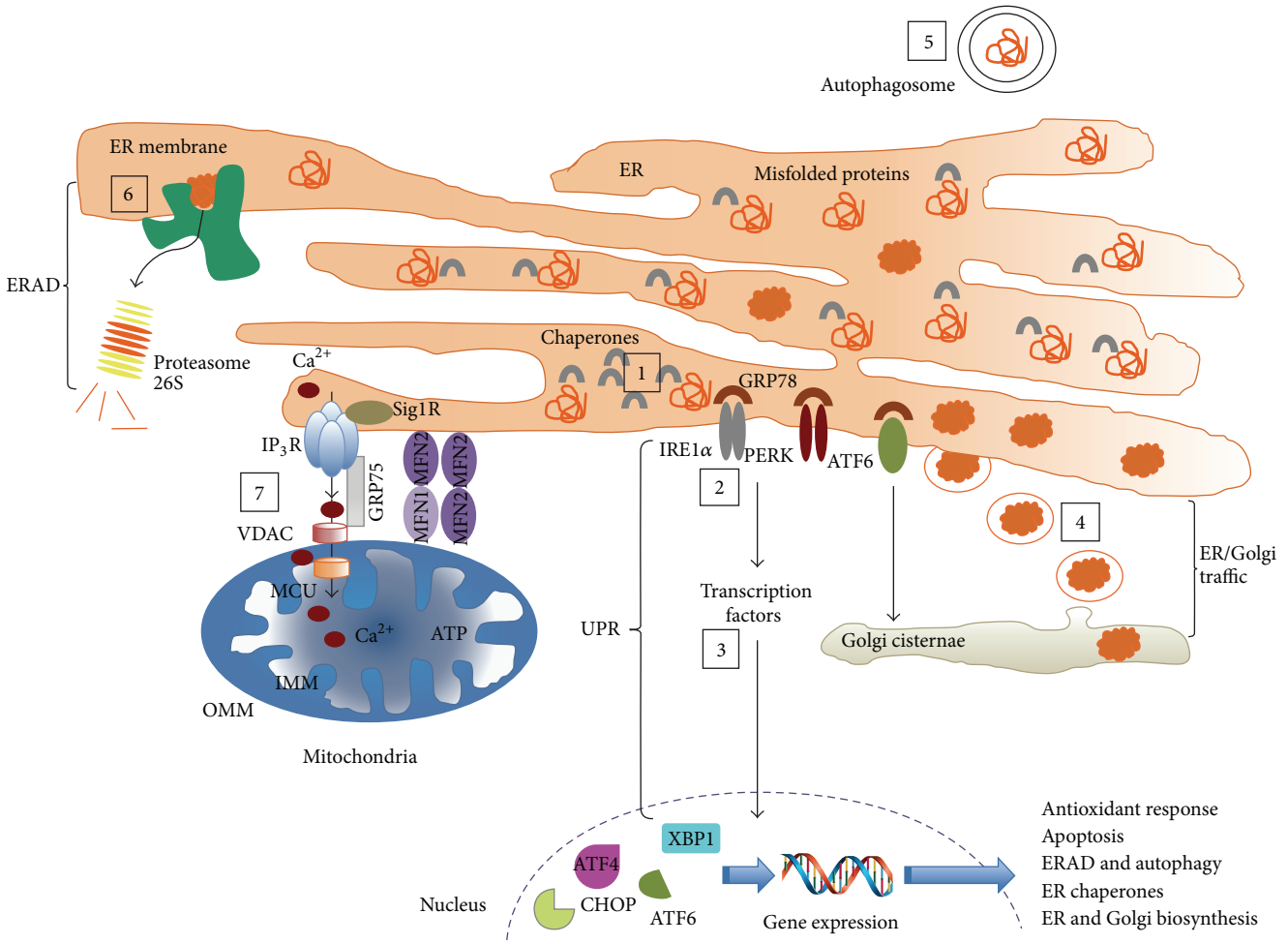

FIGURE 2: ER stress triggered by misfolded proteins in several neurodegenerative diseases. Abnormal conformations of the proteins APP, Aβ, and tau are implicated in the pathogenesis of AD. Alterations in the function of ER chaperones and UPR-related components, ERAD, ER/Golgi trafficking, and ER-to-mitochondria Ca^{2+} transfer have been suggested as underlying mechanisms of ER stress triggered by these disease-associated proteins. These proteins can accumulate and aggregate at the ER and their stable interaction with ER chaperones such as GRP78/BiP and PDI may trap ER chaperones, altering protein folding with concomitant ER stress. In addition, these proteins can lead to the oxidative modification of the active site of PDIs by nitrosylation leading to their enzymatic inactivation. Furthermore, some of these proteins alter the activity of the UPR stress sensors (IRE1α, PERK, and ATF6) as well as the activity/levels of downstream signaling mediators and transcription factors, including cleaved ATF6, ATF4, and spliced XBP1. As a result, genes implicated in autophagy and ERAD, antioxidant response, ER chaperones, and organelle's biosynthesis are upregulated. Moreover, these proteins block the exit of vesicles from the ER and alter the trafficking between ER and Golgi of properly folded proteins. The cellular responses controlled by UPR transcription factors, including the modulation of autophagy mediated degradation of protein aggregates, become compromised. Disease-related proteins can also interact with ERAD components, precluding the translocation of ERAD substrates from the ER to the cytosol, leading to the accumulation of abnormally folded proteins at the ER. Finally, Ca^{2+} released from the ER, mainly through the IP_3R, and its transfer to mitochondria can be impaired in the presence of disease-related proteins leading to mitochondrial Ca^{2+} overload and activation of apoptotic cell death pathways. AD: Alzheimer's disease; ATF6: activating transcription factor 6; ATF4: activating transcription factor 4; ER: endoplasmic reticulum; ERAD: endoplasmic-reticulum-associated protein degradation; IP_3R: inositol triphosphate receptor; IRE1α: inositol-requiring enzyme 1 alpha; PERK: protein kinase R- (PKR-) like ER kinase; UPR: unfolded protein response; XBP1: Xbox binding protein 1. Adopted from [67].

viability [70]. The molecular bridges between ER inositol 1,4,5-triphosphate receptor (IP_3R) and the voltage-dependent anion channels in the outer mitochondrial membrane are brought together through the cytosolic chaperone glucose-regulated protein 75 (GRP75) (Figure 2). Additionally, the dynamin-related GTPase mitofusin 2 (Mfn2) proteins located on the ER intermingle with Mfn1 or Mfn2 on mitochondria to tighten the connection. The distance between ER and mitochondria controlled by the phosphofurin acidic cluster sorting protein 2 (PACS-2) of ER and the dynamin-related GTPase protein 1 (Drp1) is crucial for cell survival, as either too long (lack of Ca^{2+} flux) or too short distance (Ca^{2+} overload) might lead to apoptosis [71]. Alternatively, impaired mitochondrial bioenergetics with reduced cellular ATP

levels stimulate autophagy. The molecular mechanism of ER-mediated autophagy is accurately regulated by Beclin 1 as well as ER membrane bound protein Bax inhibitor 1 (BI-1). Both proteins are capable of promoting autophagy through IP_3R-dependent mechanism [72].

5. APP Processing and ER Stress Response

The nature of APP processing is determined by the composition of membrane, with cholesterol rich lipid rafts as the site of amyloidogenic cleavage [73]. Consequently, one might expect that fate of APP is at least partly established by the representation of lipid rafts and possible access to β- versus α-secretase. Irrespective of the type of cleavage, there is one underestimated biochemical step in ER that might bring about damaging upshot. It is apparently the effectiveness of ER situated N-glycosylation which makes the APP molecule suitably folded. If misfolded/malfolded protein(s) accumulate in the ER, complex cascade of reactions known as the unfolded protein response (UPR) is triggered with the so-called endoplasmic reticulum stress response (ERS). Today, it is widely accepted that, during UPR, the ER sensors, protein kinase R- (PKR-) like ER kinase (PERK), activating protein kinase 6 (ATF6), and inositol-requiring enzyme 1 alpha (IRE1α), are freed from GRP78/BiP protein repression, which hereafter become activated. Without going into details of individual sensor action, a number of reactions occur at both the genomic and cytoplasmic level with selective degradation of mRNAs encoding protein(s) with abnormal folding and inhibition of protein translation, except for genes important for UPR, redox homeostasis, energy metabolism, and protein folding [67]. On the one hand, PERK phosphorylates eukaryotic initiation factor 2 alpha (eIF2α) to stop entrance of methionyl-tRNA to the ribosome; on the other hand, it allows translation of activating transcription factor 4 (ATF4) gene. IRE1α sets off alternative splicing of Xbox binding protein 1 (XBP1) transcript leading to activation of the transcription factor liable to stimulate ER/Golgi biogenesis and formation of proteins involved in endoplasmic-reticulum-associated protein degradation known as ERAD (Erdj4, $p58^{IPK}$, EDEM, RAMP-4, PDI-P5, and HEDJ; for details, see [67]). Finally, ATF6 is activated in the Golgi complex through proteolytic cleavage and translocates to nucleus where it cooperates with XBP1 in upregulation of chaperones and ERAD-related genes [43]. In principle, UPR is activated to restore ER homeostasis and stop the accumulation of aberrantly formed protein(s) but if the strength of ER stress is unbearable (meaning that it cannot be compensated by UPR) there is a path to activate apoptosis. Among other routes the most central role is played by the major proapoptotic transcription factor C/EBP homologous protein CHOP/growth arrest and DNA damage induced gene 153 GADD153. It downregulates the antiapoptotic protein Bcl-2 and upregulates proapoptotic Bax and Bak [74]. CHOP/GADD153 leads to excessive production of reactive oxygen species (ROS) within ER, subsequent depletion of reduced glutathione (GSH) and Ca^{2+} flux from the ER to cytoplasm through the IP_3R [75]. One hitherto unresolved issue is the physiologic importance of APP and GRP78/BiP interaction disclosed in coprecipitation study carried out by Yamamoto et al. [76]. Bulk of APP associated with GRP78/BiP was immature protein. Given that GRP78/BiP expression levels declined in samples of brain tissue obtained from FAD patients as demonstrated by Katayama et al. [77], few distinct scenarios are possible. First, binding to GRP78/BiP suggests ER accumulation of immature APP. Second, APP interaction with GRP78/BiP is a noticeable sign of UPR which is further validated by downregulation of GRP78/BiP in FAD patients. Finally, retention of APP in ER is most likely a result of amyloidogenic APP processing and as such it probably occurs in MAM, the cholesterol enriched domains. ER-mitochondria crossing point is therefore of particular interest in deciphering the links between the ER placed APP processing, UPR, and resulting cellular responses such as autophagy, apoptosis, and inflammatory reaction observed in AD (Figure 2).

6. Pathology of Tau Protein

Microtubule-associated protein tau controls assembly and prevents microtubules from severing. Microtubular network is fundamental component of cytoskeleton essential for intracellular transport of secretory vesicles and organelles (i.e., mitochondria). Glycogen synthase kinase 3 beta- (GSK-3β-) targeted hyperphosphorylation of tau causes this protein to dissociate from microtubules. Consequently, microtubules become fragmented and microtubule-dependent transport system fails. Furthermore, hyperphosphorylated tau (P-tau) is prone to form oligomers and toxic filaments, known as NFTs or tauopathy [78]. A variety of tau conformers were reported to exist, pointing to different tauopathies capable of self-propagation [79, 80]. Interestingly, intracellular tau inclusions define AD as clinical symptoms of disease observed when tauopathy is abundant together with intracellular Aβ deposits in neocortex [51, 81, 82]. People with abundant Aβ plaques, but no or only a few neurofibrillary lesions, do not have AD. Clinicopathological correlation studies have been crucial to generate hypotheses about the pathophysiology of the disease, by establishing the fact that there is a continuum between "normal" aging and AD dementia and that the amyloid plaque buildup occurs primarily before the onset of cognitive deficits, while neurofibrillary tangles, neuron loss, and particularly synaptic loss parallel the progression of cognitive decline [83]. Thus, misfolded proteins and descendant toxic filaments with a number of intermediates are critical for manifestation of AD, as fibrillogenic APP processing is not enough for onset of disease. Although the molecular mechanism of tauopathy is not deciphered in full, recent reports suggest ER stress as the starting point [84, 85]. This idea is substantiated by the elevated levels of ERS and UPR markers together with P-tau and GSK-3β in brains affected by AD [86]. Therefore, on the one hand, the incidence of UPR is strongly correlated with the presence of NFTs; on the other hand, aggregation of P-tau induces ERS with resultant UPR. Some lines of evidence confirmed UPR activation near the beginning of NFTs formation and point to the functional link between malformed tau protein and UPR. The *in vitro* experiments with phosphatase 2A inhibitor or phosphorylation activator demonstrated enhanced P-tau

formation in neurons in concert with the increased levels of PERK, eIF2α, and XBP1 transcript, apparent markers of UPR [87]. Furthermore, GRP78/BiP was found to encourage tau phosphorylation through facilitated substrate capture by GSK-3β [88]. GSK-3β seems to play dual role; first this kinase protects neurons from apoptosis as P-tau accumulation is strong molecular signal to trigger UPR with subsequent autophagy. Second, UPR raises GSK-3β activity through lysosomal degradation of inactive GSK-3β (P-Ser9-GSK-3β). To sum up, ERS and UPR are important molecular machines used to prevent cell viability turned on by tauopathy.

7. Oxidative Stress in ER

Sacs and tubes of ER delineate the compartment where the newly synthesized proteins undergo maturation to native state. Native state indicates properly folded, fully functional protein. Important reactions essential for protein folding of unbranched polypeptide chains include amino acids oxidation and glycosylation. As a result, redox homeostasis in ER is shifted to oxidative state so as to promote disulfide bond formation between adjacent cysteines. Oxidation of sulfhydryl groups required to make disulfide bonds is controlled by ER oxidase 1α (ERO1α). Next, disulfide bonds could be subject to posttranslational modification, disulfide exchange by protein disulfide isomerase (PDI). PDI is able to correct mispaired thiol residues by catalyzing the breakage and formation of correct disulfide bonds. These enzymes are fundamental for protein folding. Oxidation allows twisting of proteins, which is followed by N-glycosylation and/or C- and O-mannosylation. Protein N-glycosylation in eukaryotes is a complex process divided into several steps. First, there is a "call for" carrier lipids (polyisoprenyl phosphates such as dolichyl phosphates), the membrane lipids known to function as glycosyl transporters. In mammalian cells, the limiting substrate for dolichol biosynthesis is geranylgeraniol (GGOH) of mevalonate pathway. Dolichols, the longest aliphatic molecules synthesized in animal cells, have 18–21 α-isoprene saturated units (C90–105), critical for their recognition by the enzymes (glycosyltransferases) that glucosylate dolichyl phosphates [89]. Once dolichyl monophosphates (Dol-P) are formed in the ER membrane, the precursor oligosaccharide donor ($GLc_3Man_9GLCNAc_2$-P-P-dolichol) for protein N-glycosylation can be synthesized on the lumenal leaflet of ER. First, three sugar intermediates are produced (Man-P-Dol, Glc-P-Dol, GlcNAc-P-P-Dol, and $Man_5GlcNAc$-P-P-Dol) on the cytoplasmic leaflet of the ER. Next, enzyme flippases mediate transbilayer movement of the aforementioned intermediates to lumenal side of ER where conversion to $Glc_3Man_9GlcNAc_2$-P-P-Dol could be completed. $Glc_3Man_9GlcNAc_2$-P-P-Dol is also used for biosynthesis of glycosylphosphatidylinositol (GPI) anchors.

Taken together, lipid-mediated glycosylation plays a vital role in the appropriate protein folding and intracellular translocation of N-linked glycoproteins [90]. Likewise, it is important for protein O- and C-mannosylation, and GPI anchorage. Moreover, Dol-P availability in the ER is the rate-limiting factor in the production of glycolipid intermediates and N-glycosylation.

8. ER Stress and Apoptosis

It was shown that during ERS the ER resident proapoptotic cysteine protease known as caspase (caspase-12 in rat, caspase-4 in humans) is activated through cleavage. As a result caspase cascade is started via caspase-9 that in turn stimulates effector caspase-3 [91, 92]. The central role played by ER in programmed cell death is achieved by PERK branch where ATF4 induces the expression of CHOP/GADD153, which represses antiapoptotic Bcl-2 family proteins and simultaneously shuffles ER membrane Bax and Bak proteins into outer mitochondrial membrane. Consequently, pores are formed to leak the components of apoptosome from mitochondrial intermembrane space [93]. Another important mechanism of ERS-induced apoptosis is led by Ca^{2+}-dependent ERO1α-IP_3R pathway where ERO1α collaborates with IP_3R in Ca^{2+} efflux from ER to mitochondria via MAM [75, 94]. Accordingly, Ca^{2+} influx facilitates cytochrome *c* release from mitochondria; besides, cytochrome *c* can bind to ER IP_3R and the complex amplifies the apoptotic signal in a feedforward manner [95]. Last but not least, ERS-associated apoptotic programme is set off by c-Jun N-terminal kinase (JNK) as the effect of IRE1α complexed with TNF-receptor-associated factor 2 (TRAF2) activation of apoptosis-signal-regulating kinase 1 (ASK1) [96, 97].

What does ERS drive to induce apoptotic death in neurons? Actually, many reports indicate that APP and Aβ as well as hyperphosphorylated tau have been shown to block mitochondrial transport, which results in impaired energy storage and oxidative stress [98–101]. Indeed, accumulation of APP, Aβ, and NFTs in mitochondria led to reduced activity of some enzymes involved in substrate oxidation (tricarboxylic acid cycle), electron transport chain (ETC), and ATP synthase, as well as severely diminishing import of nuclear-encoded proteins [26, 27, 102–104]. One may ask if there is any additional link between ER and mitochondria other than MAM which could account for apoptotic signal. Though not directly, ER significantly contributes to oxidative stress in mitochondria of AD-affected subjects.

9. Abnormal APP Processing and Trafficking Culminate in ER Pathology of AD

From the morphological point of view, as neurons are highly specialized cells, soma, dendrites, and neurites are considerably distinct structures. Proteins needed by these compartments are delivered via microtubules once proteins have suitable sorting signals (i.e., APP trafficking from ER to plasma membrane is associated with several posttranslational modifications with oxidation and N-glycosylation). Additionally, the Golgi apparatus follows ER in subsequent APP adjustment (O- and N-glycosylation, phosphorylation, and sulphonation) [42]. Any inaccurate alteration of the APP molecule is potentially hazardous, as protein final destiny is missed causing its retention in ER or trans-Golgi network (TGN). In addition, other unusual settings for APP are possible as this large protein has few signal sequences hidden when APP is correctly folded. In the cells transfected with APP, this protein enters coat protein complex I (COPI)

vesicles and undergoes retrograde transport from cis end of the Golgi complex back to the ER [76]. Such response causes accumulation of APP in the tubulocisternal ER system together with aberrant intracellular translocation of the protein. Interestingly, the issue whether APP is subject to retrograde transport with successive fibrillogenic processing because of UPR and its interaction with GRP78/BiP is not clear, as the observations are inconsistent [77]. Nonetheless, accumulation of misfolded/malfolded proteins in the ER suggests disorganized process of posttranslational change. As anticipated, the accretion of proteins of anomalous pattern signals ERS and UPR followed by increased vulnerability to apoptotic cell death. Prior to decay, however, APP protein levels in the ER lessen by dint of cleavage with β- and γ-secretase [105, 106]. Products of this cleavage (sAPPβ, Aβ, and AICD fragment) all appreciably influence neuronal survival most likely through nonnative form of the APP substrate.

10. APP, Aβ, and NFTs Mark Mitochondria as Targeted in AD

Mitochondrial import of Aβ_{40} and Aβ_{42} peptides through the translocase TOM complex was blocked by preincubation of isolated mitochondria with antibodies raised against TOM proteins (TOM20, TOM40, and TOM70) [107]. Neither VDAC inhibition with antagonist antibodies nor inhibition of mitochondrial permeability transition pores (MPTP) or fall of mitochondrial membrane potential (MMP) affected uptake of Aβ [17]. With regard to APP, elegant study performed by Anandatheerthavarada and his colleagues [30] revealed that C-terminal truncated APP (lacking Aβ) targets mitochondria in cholinergic, GABAergic, dopaminergic, and glutamatergic neurons of AD brain by clogging up mitochondrial protein translocase complex TOM40/TIM22. Authors propose that occlusion of translocase is followed by blunted import of nuclear-encoded proteins vitally important for energy homeostasis. Mitochondrial APP protein transmembrane orientation indicates NH_2-terminal inside in contact with translocase, whereas COOH-terminal is facing cytoplasmic side. NH_2-terminus has mitochondrial signal sequence. Astonishingly, mitochondrial APP molecules were nonglycosylated giving rise to speculation that protein molecules that arrived at mitochondria have not achieved molecular maturity [31]. The accumulation of nonglycosylated APP species in mitochondrial import channels of AD brain was directly related to decreased mitochondrial functions as validated by the decline in cytochrome *c* oxidase activity (complex IV) and elevated levels of H_2O_2. Furthermore, in AD brain, the mitochondrial accumulation of nonglycosylated APP went along with a corresponding reduction in plasma membrane-associated APP. It suggests that AD brain has APP processing and trafficking severely affected by incomplete N-glycosylation, ensuing ER protein accumulation and exposure of the cryptic mitochondrial targeting signal for assisting chaperone proteins. Several lines of evidence indicate that not fully formed proteins are phosphorylated and bind to the cytosolic proteins required for movement from ER to mitochondria [108–110]. Moreover, ERS and UPR in AD seem to be incompetent and inefficient in elimination of malfolded proteins. In addition to APP also Aβ was frequently reported to occupy mitochondria although its origin and mechanism of mitochondria targeting mostly remain unexplored (except for involvement of protein translocase TOM complex). The possibility of mitochondrial Aβ generation has to be ruled out, as membrane orientation of arrested APP does not allow access to γ-secretase (γ-secretase activity in mitochondria was detected by independent study) [111]. Probably, Aβ species is derived from APP prior to its translocation (ER?), so Aβ may be transported to mitochondria independently of APP. Collectively, observations showing mitochondrial presence of APP, Aβ, and tau in aberrant configuration point toward the anomaly of protein folding at the level of ER and Golgi apparatus. Nonglycosylated molecule of APP suggests defective transfer of sugar core from dolichyl phosphate(s) and further modifications such as O- and N-glycosylations. One may admit that the lack of glycosyl residue brings about pathologic processing and trafficking of APP and its fragments. In point of fact, dolichol derivatives, mixture of polyprenols (acyclic isoprenoid alcohols) known as Ropren (Solagran Limited, Melbourne, Australia) commercially used to treat liver diseases, were tested in the treatment of AD in two separate trials conducted in 2005 and 2006 with promising results [http://www.asx.com.au/asxpdf/20071119/pdf/315x5nh4hm8wv7.pdf, http://www.asx.com.au/asxpdf/20070221/pdf/3111ztwcbqzkk9.pdf]. Further studies are urgently needed to test how important the glycosylation process is in the pathogenesis of AD. Dolichols are obtained from geranylgeraniol (GGOH) and the latter is an intermediate of mevalonate pathway. GGOH is a common substrate for dolichol and ubiquinone synthesis, but it is also necessary for protein prenylation. As both GGOH and farnesol (FOH) are engaged in protein prenylation more concern should be laid on the importance of these compounds in AD pathogenesis. Observations demonstrating mitochondrial relocalization of other proteins without posttranslational modification seemingly point to increased mitochondrial targeting of immature molecules resulting in mitochondrial dysfunction and acceleration of disease progression [34, 112–114].

11. Oxidative Stress in Mitochondria of AD Brains

Normal physiological functions of APP are thought to be involved in the stabilizing contact points between synapses and maintaining mitochondrial functions [60, 115]. Mitochondrial dysfunction was often observed regardless of the experimental model used to study AD [8, 29, 116–119]. It includes defects in oxidative phosphorylation, decreased ATP, decreased membrane potential, increased production of ROS/RNS, and perturbation in mitochondrial fusion and fission [15, 30, 31, 115, 120–122]. Hyperphosphorylated tau was also reported to impair mitochondrial functions [123]. Using proteomic approach, the strongest defects of the respiratory capacity were observed mainly at complexes I, IV, and ATP

synthase (complex V) at both protein and activity level [124]. While APP, Aβ, and hyperphosphorylated tau are potent inhibitors of mitochondrial import of nuclear-encoded proteins, apparently the pathology of each leads to metabolic harm in different way. In freshly isolated mitochondria from AD brains, the APP inhibited mitochondrial import of cytochrome *c* oxidase (COX) subunits IV and Vb [31]. Dysfunction of COX increases ROS production (incomplete reduction of oxygen molecules), reduces energy stores, and disturbs energy metabolism. Accordingly, in AD patients, deficiency of COX was found in brains and platelets [119, 125, 126]. Similar to APP, Aβ was found in mitochondria of transgenic mice and cellular and human AD models [17, 19, 33, 103, 127–130]. At present it is not clear whether the observed mitochondrial toxicity is due to APP or Aβ, or NFTs accumulation. Anyway, some regularity is observed with respect to most affected components of respiratory chain. NADH-ubiquinone oxidoreductase (complex I) activity is reduced to the utmost by hyperphosphorylated tau, whereas decreased activity of cytochrome *c* oxidase (complex IV) that resulted in mitochondrial dysfunction was observed during Aβ and APP accumulation [14, 15, 31, 32, 34]. Concomitantly, rise in the activity of antioxidant enzymes manganese superoxide dismutase (Mn-SOD) and catalase (CAT) was demonstrated in response to elevated levels of free radicals including superoxide anion radical ($O_2^{\bullet -}$), hydroperoxyl radical ($HO_2^{\bullet}$), hydroxyl radical ($OH^{\bullet}$), and nitric oxide radical ($NO^{\bullet}$) [15]. Consistent with observations of chronic respiratory chain dysfunction and mitochondrial oxidative stress, there are reports showing their contribution to tau pathology in AD [131]. In any case, free radicals that override antioxidant defense react with a wide variety of organic components causing lipid peroxidation to advanced lipid oxidation end products (ALE), cross-linking of proteins, nitrosylation of proteins, and mutations in DNA. Mitochondrial circular DNA (mtDNA) of ~16 kbp is devoid of repair systems meaning the buildup of lesions. There are 37 genes located in mtDNA with those encoding protein subunits of complex I (7), complex II (1), and complex IV (3) but not complex III. Interestingly, hallmarks of AD (APP, Aβ, and NFTs) mostly affect members of electron transfer chain (complexes I and IV) which rely exclusively on mitochondrially predetermined subunits. Maybe it is not simple coincidence, but the effect of inhibited import to mitochondria of nuclear-encoded subunits of complexes I and IV gives explanation for toxicity of APP or Aβ, or NFTs in mitochondria. There are additional findings in mitochondria affected by AD such as lower activity of pyruvate dehydrogenase (PDH) and oxoglutarate dehydrogenase (OGDH) [104, 132]. Inhibition of OGDH, the enzyme of tricarboxylic acid cycle, minimizes the NADH pool and electron number needed for ETC and mitochondrial membrane potential ($\Delta\psi_m$) to create and maintain proton gradient obligatory for ATP synthesis. There are also lines of evidence for direct inhibitory action of soluble oligomeric Aβ species on ABAD (Aβ-binding alcohol dehydrogenase) and internal membrane cyclophilin D (CypD) resulting in increased mitochondrial membrane permeability (MPTP), potentiated ROS production, synaptic loss, diminished activity of mitochondrial respiration, and finally cell death [129, 132–134]. CypD knockout prevents mitochondrial and neuronal perturbations and improves mitochondrial function in Alzheimer's disease mouse model [128, 135]. It has to be emphasized that oxidative/nitrosative stress affects the fusion and fission process of mitochondria. Fusion, that is, speedup by small GTPases mitofusins (Mfn1/Mfn2), improves efficiency of mitochondrial respiration and ATP production. Mitochondrial dynamics are severely imbalanced in AD cases in favour of fission, through elevated expression of the fission protein DLP1 (dynamin-like protein 1) associated with nitrosative stress stirred up by Aβ [136, 137]. Collectively, these observations indicate that toxic intracellular A$\beta_{42\text{-}43}$ oligomers differ in action from extracellular aggregates found in amyloid plaques of AD brains [138] (Figure 3).

12. Proteostasis in Mitochondria

Mitochondrial protein turnover grants the well-organized replacement of nonfunctional proteins into operational one. The first task is attained through proteolytic degradation of inner membrane and matrix proteins with local mitochondrial proteases [139] or outer membrane proteins through ubiquitin-proteasome system [140]. The second task is met by intramitochondrial protein synthesis but as almost 1500 different nuclear-encoded proteins have to be imported to complete mitochondrial proteome, a matter of capable import is fundamental for the function of the organelle. In extreme cases of cellular injuries observed in neurodegenerative diseases, damaged mitochondria with extended loss of the electrochemical potential are selectively removed by autophagy known as mitophagy [112]. APP and Aβ accumulation has also something to do with altered mitochondrial dynamics as fission takes advantage of fusion with resultant dysfunction of mitochondria and neurons [137]. AD neurons demonstrated selective mechanisms of proteolytic clearance of oxidatively and nitrosatively modified proteins in mitochondria. Insulin degrading enzyme (IDE) prevents formation of toxic insoluble fibrils from Aβ, as it cleaves Aβ prior to aggregation [141–143]. A novel zinc-metallopeptidase, Presequence Protease (PreP), a member of pitrilysin oligopeptidase family, degrades either intramitochondrially stored Aβ_{40} or Aβ_{42} protein. This protease seems to be highly sensitive to oxidative stress, as disulfide bridge formed between two proximal cysteine residues blocks its catalytic activity [144–149]. Another mitochondrial serine protease HtrA2/Omi occupies intermembrane space where it can cleave APP locked up in protein translocase complex [121]. Even though it is well established that HtrA2/Omi is released to the cytosol to amplify apoptosis through the degradation of antiapoptotic proteins and caspase activation [150–152], it is also implicated in proteolytic deletion of malfolded APP in ER [151]. From knockout studies on mice, it is obvious that HtrA2/Omi plays a significant shielding role as mice deficient in this protease exhibit neurodegenerative phenotype with weight loss and premature death [153]. Taken together, understanding the mechanisms of clearance of the unwanted proteins including APP, Aβ, and tau is vital in an attempt to get rid of them from mitochondria.

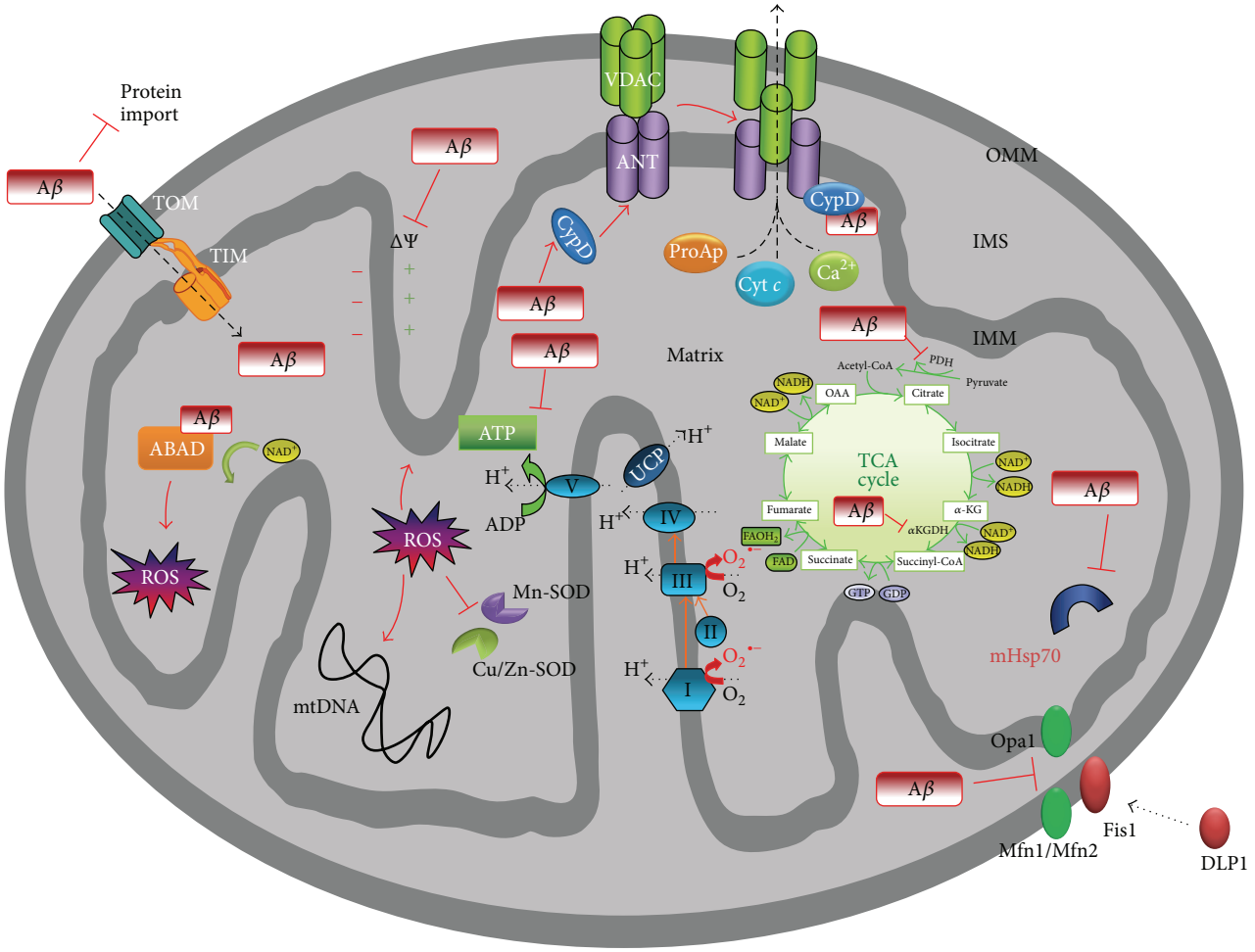

Figure 3: Amyloid-β-related mitochondrial impairment. Mitochondria were found to be the target for amyloid-β (Aβ), which interacts with several proteins, leading to mitochondrial dysfunction. Indeed, Aβ was found in the outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) as well as in the matrix. The interaction of Aβ with the OMM affects the transport of nuclear-encoded mitochondrial proteins, such as subunits of the electron transport chain complex IV, into the organelle via the translocase of the outer membrane (TOM) import machinery. Moreover, Aβ disturbs the activity of several enzymes, such as pyruvate dehydrogenase (PDH) and oxoglutarate dehydrogenase (OGDH), decreasing NADH reduction, and the electron transport chain enzyme complex IV, reducing the amount of hydrogen that is translocated from the matrix to the intermembrane space (IMS), thus impairing the mitochondrial membrane potential (MMP). Taken together, these events cause abnormal mitochondrial electron activities, leading to decreased complex V activity and so to a drop in ATP levels, in addition to increasing reactive oxygen species (ROS) generation. Moreover, ROS induce peroxidation of several mitochondrial macromolecules, such as mitochondrial DNA (mtDNA) and mitochondrial lipids, contributing to mitochondrial impairment in the mitochondrial matrix. The complex of Aβ bound to binding alcohol dehydrogenase (ABAD) impairs the binding of NAD^+ to ABAD, changes mitochondrial membrane permeability, and reduces activities of respiratory enzymes, inducing further ROS production and leading to mitochondrial failure. Aβ binding also activates Fis1 (fission protein) and promotes increased mitochondrial fragmentation; this increased mitochondrial fragmentation produces defective mitochondria that ultimately damage neurons. Furthermore, Aβ binding to cyclophilin D (CypD) enhances the protein translocation to the inner membrane, favouring the opening of the mitochondrial permeability transition pore, formed by the adenine nucleotide translocator (ANT) and voltage-dependent anion channels (VDACs). Cyt *c*: cytochrome *c*; DLP1: dynamin-like protein 1; PDH: pyruvate dehydrogenase; ProAp: proapoptotic factors; SOD: superoxide dismutase; TCA: tricarboxylic acid; TIM: translocase of the inner membrane. Adopted from [14].

Some hopes are associated with the application of at present indefinite modulators of proteolytic activity. HtrA2/Omi is, for example, activated by PTEN-induced putative kinase 1 (PINK1) upon phosphorylation at Ser142 residue [154]. Alternatively, accent has to be put on mitophagy of dysfunctional mitochondria and mitochondriogenesis.

13. Aging versus AD

The most common neurodegenerative disorder is represented by Alzheimer's disease, characterized by declining memory, reduced cognitive capacity, and progressive dementia, which are often fatal to elderly individuals above 65 years of age.

It is ranked as the fourth leading cause of death in modern societies where average life span increased greatly in the last two decades. As 95% of AD cases are diagnosed in older people, one might think that a causal relationship exists between aging and the onset of disease. Certainly, a number of similarities between getting old and being affected with AD could be listed. Historically, the free radical theory of aging by Harman [155] suggested aging as "side effect" of reactive oxygen species formed in mitochondrial respiratory chain. Apparently, free radicals, commonly generated by incomplete reduction of oxygen molecule at complexes I and III of mitochondrial ETC, are capable of damaging DNA, RNA, and proteins. They impair energy storage and lead to operational failure of mitochondria with progressive decline of cell viability. Almost identical conditions accompany AD and are an explicit step in pathogenesis of disease. As mtDNA is deprived of repair mechanisms, ROS-induced DNA strand breaks tend to accumulate with age or AD. Thus, mtDNA is a vulnerable target for ROS, but the reverse, the ROS generation due to the mutated mtDNA, is not convincingly confirmed [156]. Moreover, the evidence that mutated mtDNA accelerates the progress of aging is also questioned based on the results from study carried out on transgenic mice model [157]. Although some authors show inconsistency between the free radical theory and observations, cumulative evaluation of the scientific reports points to antioxidant defense systems as important factors in protection from premature aging [158]. Other mitochondrial components important for their function are hampered with age: adenine nucleotide translocase (ANT), nitric oxide synthase (NOS), and carnitine acyltransferase (CT) [159–161]. Actually, NOS activity is elevated in AD as reported from study performed on cellular model of disease [15]. We could not find any information about CT activity in AD, whereas ANT activity is noticeably inhibited by Aβ or hyperphosphorylated tau and this effect is reversed by mersalyl, a reversible alkylating agent of thiol groups [162]. Mitochondrial dysfunction, observed in transgenic mice models of AD and aging, demonstrates higher activity of genes controlling energy metabolism and apoptosis. Taken together, physiological aging and AD are associated with broad-spectrum dysfunction of mitochondria, but the foundations of mitochondrial decline are dissimilar. More discrepancies between physiological aging and AD were found with respect to ERS and UPR which play a significant role in cellular proteostasis. Dolichol was selected as an aging marker because the progressive increase in dolichol level was observed in aging brain [163]. In contrast, ubiquinone concentration which is also synthesized from geranylgeraniol diminishes with aging whereas cholesterol and dolichyl phosphate concentrations remain unaltered. In AD, decreased levels of dolichol were observed and increased levels of ubiquinone and dolichyl phosphate without any changes in brain cholesterol. AD cannot be regarded as a result of premature aging. The drop in dolichol and augmented dolichyl phosphate concentration points toward disturbed glycosylation in ER of diseased brain, while the increase in ubiquinone suggests efforts to protect the brain from oxidative stress induced by lipid peroxidation [164, 165].

14. Targeting ER Stress in AD Therapy

As ERS is a recognized factor in AD, drugs that interfere with ERS would theoretically have great therapeutic potential. There are several compounds grouped in classes that interact directly with components of the ERS (salubrinal, BiP inducer X (BIX), salicylamide analogs, flavonoids, guanabenz, and STF083010), chemical chaperons (4-phenylbutyric acid (PBA), tauroursodeoxycholic acid (TUDCA), and trimethylamine oxide (TMAO)), chemicals that inhibit protein degradation (Eeyarestatin, MG132, and Bortezomib), compounds with antioxidant activity (Edaravone, dibenzoylmethane derivatives, and N-acetyl cysteine (NAC)), and drugs controlling calcium signaling (dantrolene and carbazole derivatives) [166]. They may act by inducing transient translation arrest, upregulation of chaperone proteins, and augmented degradation of ER-associated misfolded proteins. Fundamental approach in the development of new therapy is the selection of appropriate molecular targets. In ER stress signaling, the aim is to alter the expression of ER stress-associated molecules that can rescue cells from the toxic effect of ERS. Recent efforts in establishing new promising drugs against AD are pointing to chemical chaperones such as PBA, TUDCA, or trimethylamine oxide (TMAO). These substances improve protein folding and alleviate native protein conformation [166]. It was shown on mouse models of AD that PBA, TUDCA, and TMAO stop Aβ accumulation and avoid the loss of dendritic spines [167]. Some observations even demonstrated improved memory and cognitive functions [168] associated with improved cell survival [169]. Salubrinal ((2E)-3-phenyl-*N*-[2,2,2-trichloro-1-[[(8-quinolinylamino)thioxomethyl]amino]ethyl]-2-propenamide, Sal) selectively inhibits growth arrest and DNA damage induced gene 34- (GADD34-) phosphatase complex (GADD34 associates with protein phosphatase 1 (PP1)) and promotes *in vitro* dephosphorylation of the alpha subunit of eIF-2α and IRE1α/ASK1/JNK signaling pathway being protective against ERS even induced by tunicamycin Tm [170]. In a great deal of experiments testing Sal in cultured cells and animal models of AD, this substance increased the viability of neuronal cells and Aβ toxicity [171, 172]. BIX (2-(3,4-dihydroxyphenyl)-2-oxoethyl ester thiocyanic acid) preferentially induced BiP mRNA in an ATF6-dependent manner leading to reduced Tm-induced death of neuronal cells [173]. Also DBM derivative 14–26 (2,2′-dimethoxydibenzoylmethane) was found to be neuroprotective for SH-SY5Y and PC-12 cells by decreasing expression of BiP and CHOP [174]. Dantrolene, a ryanodine receptor antagonist that inhibits abnormal calcium release from the ER, inhibited expression of both phosphorylated PERK and eIF2α. It also reduced CHOP expression and attenuated thapsigargin-induced apoptosis in PC-12 cells [175]. Neuroprotective effects similar to dantrolene were observed for ([9-(3-cyanobenzyl)-1,4-dimethylcarbazole]). This substance suppressed increases in intracellular Ca^{2+} in PC-12 cells treated with thapsigargin and reduced levels of BiP and CHOP [176].

Aforementioned compounds were chosen from others as the most potent ER stress inhibitors and persuasively protective to neuronal cells. 18 of 42 different compounds

were exploited in *in vivo* and *in vitro* models of central nervous system disorders with, in fact, improved cell or tissue viability [166]. Thus, the brain is the most frequently investigated organ in the context of ERS. From these experiments, it becomes clear that CHOP functions as proapoptotic factor. The roles of other specific ER stress molecules as molecular targets for pharmacological intervention are less clear and vary depending on cell type and context.

There are few underestimated modulations in APP processing that shed more light on current dogma of AD pathogenesis. The modulation of mevalonate pathway and cholesterol synthesis were reported to stimulate nonamyloidogenic pathway of APP processing [177]. Additionally, cholesterol derivative 27-hydroxycholesterol (27-OHC) was shown to induce ER stress which attenuated leptin-dependent viability by activating CHOP in SH-SY5Y neuroblastoma cells [178]. Irrespective of a number of compounds examined in AD, the call for new drugs modulating ER stress with healing effect is still waiting to be revealed.

Abbreviations

APOE: Apolipoprotein E
APP^s: Secreted form of APP
CHOP/GADD153: C/EBP homologous protein/growth arrest and DNA damage induced gene 153
Drp1: Dynamin-1-like protein
ERAD: Endoplasmic-reticulum-associated protein degradation
ERO1α: ER oxidase 1α
ERS: Endoplasmic reticulum stress
FOH: Farnesol
GGOH: Geranylgeraniol
GRP75: Glucose-regulated protein 75
GRP78/BiP: 78 kDa glucose-regulated protein
GSK-3β: Synthase kinase 3 beta
IP_3R: Inositol triphosphate receptor
IRE1α: Inositol-requiring enzyme 1 alpha
MAM: Mitochondria-associated membrane
MAP LC3: Microtubule-associated protein light chain 3
Mfn2: GTPase mitofusin 2
MMP: Mitochondrial membrane potential
MPTP: Mitochondrial permeability transition pore
NFT: Neurofibrillary tangles
PACS-2: Phosphofurin acidic cluster sorting protein 2
PDI: Protein disulfide isomerase
PERK: Protein kinase R- (PKR-) like ER kinase
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
RyR: Ryanodine receptor
TOM: Transporter outer membrane
TIM: Transporter inner membrane
UPR: Unfolded protein response
VDAC: Voltage-dependent anion channel
XBP-1: Xbox binding protein 1.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

It Is All about (U)biquitin: Role of Altered Ubiquitin-Proteasome System and UCHL1 in Alzheimer Disease

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Free radical-mediated damage to macromolecules and the resulting oxidative modification of different cellular components are a common feature of aging, and this process becomes much more pronounced in age-associated pathologies, including Alzheimer disease (AD). In particular, proteins are particularly sensitive to oxidative stress-induced damage and these irreversible modifications lead to the alteration of protein structure and function. In order to maintain cell homeostasis, these oxidized/damaged proteins have to be removed in order to prevent their toxic accumulation. It is generally accepted that the age-related accumulation of "aberrant" proteins results from both the increased occurrence of damage and the decreased efficiency of degradative systems. One of the most important cellular proteolytic systems responsible for the removal of oxidized proteins in the cytosol and in the nucleus is the proteasomal system. Several studies have demonstrated the impairment of the proteasome in AD thus suggesting a direct link between accumulation of oxidized/misfolded proteins and reduction of this clearance system. In this review we discuss the impairment of the proteasome system as a consequence of oxidative stress and how this contributes to AD neuropathology. Further, we focus the attention on the oxidative modifications of a key component of the ubiquitin-proteasome pathway, UCHL1, which lead to the impairment of its activity.

1. Introduction

The physiological aging process and age-related diseases share many common features among which are accumulations of oxidative damage, impaired mitochondrial activity, and reduced efficiency of clearance systems among others. In particular, the reduced activity of the "quality control system" (PQC), including the ubiquitin-proteasome system, autophagy, and other intracellular proteolytic enzymes, leads to the accumulation of oxidized/unfolded proteins that may contribute to neuronal loss. Deposits of aggregated, misfolded, and oxidized proteins accumulate normally over the lifespan in cells and tissues and enormously increase in neurodegenerative diseases [1]. Insoluble aggregates can be formed as a result of covalent cross-links among peptide chains, as in the case of amyloid-β-peptide (Aβ) in Alzheimer disease (AD), α-synuclein in Parkinson disease (PD), huntingtin in Huntington disease (HD), and SOD1 in amyotrophic lateral sclerosis (ALS).

Oxidative modification of a protein represents one of the major causes of its increased susceptibility to aggregate. Indeed, proteins are sensitive to oxidative stress-induced chemical modifications, undergoing several structural changes that are not always correctly recognized by the proteasome, thus generating impaired protein function. The balance between functional proteins, present in young/healthy cells, and damaged or altered proteins, present at higher concentration in aged/diseased cells, depends

mainly on their modification and turnover [2]. It is generally accepted that the age-related accumulation of "aberrant" proteins results from both the increased occurrence of damage and the decreased efficiency of degradative systems. Clearance of oxidatively modified proteins most often occurs through the proteasome system. The proteasome is the principal pathway to remove senescent and damaged proteins, and intact proteasome function is essential to preserve cellular homeostasis during oxidative stress conditions [3]. The age-related impairment of proteasome function in different cell types and organs has been widely demonstrated [4]. Such a decline of proteasome activity would therefore be expected to promote the accumulation of oxidized proteins with age. The reduced activity of the proteasome and other intracellular proteolytic machineries also has significant implications in the development of neurodegenerative diseases [4, 5].

What is the picture if oxidative stress targets members of the PQC? A number of studies suggest that oxidative stress can target the proteasome and impair its ability to correctly degrade oxidized proteins (reviewed in [6]). Further, ubiquitin-immunopositive inclusion bodies are commonly detected in the brain of patients suffering neurodegenerative diseases possibly as a result of impaired proteasome activity [4]. If from one side low levels of ROS are able to activate the expression of inducible proteasome subunits, at increasing ROS concentrations the proteasome subunits are susceptible to undergoing oxidative modifications [7], which ultimately result in impaired proteasome function. In addition, nondegradable protein aggregates and cross-linked proteins are able to bind to the proteasome, which makes the degradation of other misfolded and damaged proteins less efficient.

Intriguingly, recent studies proposed the impairment of proteasome activates autophagy, which might be a compensatory mechanism allowing eliminating ubiquitin-proteasome system (UPS) substrates [8, 9]. Indeed, treatment of both cells and mice with rapamycin, to induce autophagy, was able to protect against cell death caused by proteasome inhibition [10] and to protect against genetic loss of proteasome activity in *Drosophila* [8]. However, the exact mechanisms of the cross-talk between proteasome and autophagy are still not well understood. Among proposed mechanisms the activation of endoplasmic reticulum (ER) stress, due to the accumulation of misfolded proteins that leads to the induction of the unfolded protein response (UPR), is an interesting candidate. These different mechanisms may not be mutually exclusive and may also be of different importance in different cell types or at different time-points after the proteasome is inhibited [11].

In this review, we focus attention on the impairment of the proteasome system as a consequence of oxidative stress and how this impairment contributes to neurodegeneration. We suggest that reduced protein turnover may be caused by the selective oxidative damage of members of the proteasomal system that once targeted by oxidative stress are not able to fulfil their protective roles and contribute to the dysregulation of intracellular protein homeostasis. The complex interactions of these events in cellular protein and redox homeostasis in the brain are essential to design novel therapeutic intervention that may possibly retard the development of AD and other neurodegenerative diseases. AD is a disorder that leads to cognitive, behavioral, and memory deficits. The hallmarks of AD are the accumulation of Aβ into senile plaques and hyperphosphorylated tau into neurofibrillary tangles which consequent neuronal loss in select brain areas involved in learning and memory. Aβ is cleaved from amyloid-β protein precursor (APP) and comprises a set of 39–43 residue polypeptides that exert a range of neurotoxic effects that are considered to be important to the evolution of the pathology.

Ubiquitin-Proteasome System. In the case of protein misfolding and aggregation in cells, the PQC system uses three main parallel strategies to maintain protein homeostasis. The misfolded protein may be refolded to recover the protein's normal conformation. Different molecular chaperones, such as heat shock proteins (HSPs), play essential roles in protein refolding. Alternatively, if the protein cannot be refolded, it is targeted to the UPS or autophagy programs for degradation (Figure 1) [12].

The UPS is located in the cytosol and the nucleus, and it is responsible for the degradation of more than 70–80% of intracellular proteins. The UPS degrades misfolded/damaged proteins and removes proteins involved in many cellular processes, such as signal transduction, cell cycle regulation, and cell death, and, ultimately, regulates gene transcription [13, 14].

Most of the proteins targeted for proteasome degradation are covalently modified by ubiquitin, often in a polyubiquitin chain. Ubiquitin is an 8.5 kDa protein composed of 76 amino acids. The ubiquitin protein is transcribed from *UBB*, *ubiquitin C (UBC)*, *RPS27A*, *and UBCEP2* genes. However, only the first two genes encode for a polyubiquitin precursor that is involved in the UPS signaling cascade. The other genes encode ubiquitin that fuses to ribosomal proteins [15]. Ubiquitin can form polyubiquitin chains at seven lysine residues on the target protein: Lys-6, Lys-11, Lys-27, Lys-29, Lys-33, Lys-48, and Lys-63. These chains are formed by the successive attachment of monomers by an isopeptide bond, most frequently formed between the side chain of Lys-48 in one ubiquitin and the carboxyl group of the C-terminal Gly-76 of a neighbouring ubiquitin. Attachment of Lys-48 polyubiquitin chains to lysine residues on a protein results in at least a 10-fold increase in its degradation rate [16]. Polyubiquitin chains with linkages involving lysine residues on ubiquitin other than Lys-48 were found to play distinct roles. Ubiquitin is conjugated through the formation of an isopeptide bond between the ε-amino group of a lysine residue of the substrate and the C-terminal carboxylate [17]. First, ubiquitin needs to be activated by an E1 enzyme in an ATP-dependent reaction, which results in a high-energy thioester bond between the E1's active site cysteine and the carboxyl group of the ubiquitin protein. Then, E2 (ubiquitin-conjugating enzyme) receives ubiquitin from E1 and forms a similar thioester intermediate with ubiquitin. E3 (ubiquitin ligase) binds both E2 and the substrate and transfers the ubiquitin to the substrate [18]. The E3 ligase is a pivotal enzyme in the UPS cascade and is crucial for substrate specificity. E3 plays a critical role in selecting the substrates,

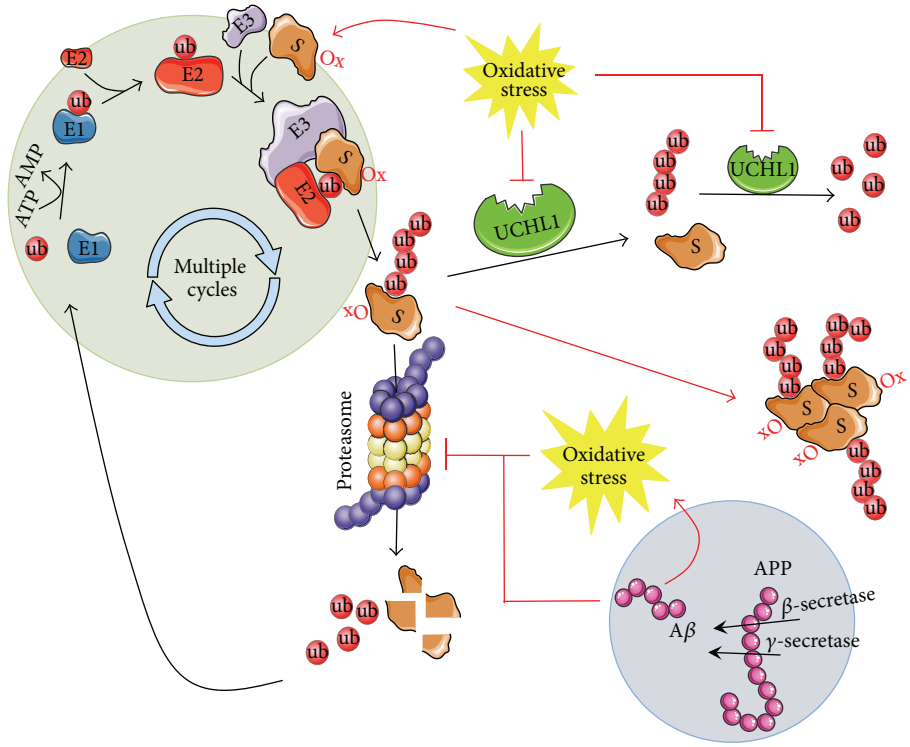

Figure 1: Oxidative stress impairs the functionality of the UPS in AD. Under physiological conditions the ubiquitin-proteasome system (UPS) mediates the clearance of misfolded proteins in order to prevent their toxic accumulation. Indeed, a target protein undergoes multiple cycles during which it is conjugated with one or more ubiquitin groups leading to mono- or polyubiquitinylated substrates (S). In particular, in the first step of this cycle, ubiquitin activating enzyme (E1) forms a thioester bond with ubiquitin and this reaction requires ATP as driving force. Subsequently, the ubiquitin group is transferred to ubiquitin-conjugating enzyme (E2), which works as a scaffold protein favoring the interaction between ubiquitin ligase (E3) and the target substrate, aimed at allowing the ligase to transfer the ubiquitin group from E2 to the substrate. After a number of cycles promoting the polyubiquitinylation of the substrate, this latter is driven to the proteasome for its degradation. Polyubiquitinylated substrates also can be targets of the activity of the ubiquitin carboxyterminal hydrolase L1 (UCLH1), which is highly expressed in neurons and hydrolyses small adducts of ubiquitin to generate the ubiquitin monomer. During the progression of Alzheimer disease (AD), increased amyloid-beta (Aβ) production and accumulation favor the augmentation of oxidative stress levels, which leads to protein oxidative modifications (Ox). Because oxidized proteins are neurotoxic, they would be eliminated through the UPS, but this does not seem to be the case in AD. Indeed, both Aβ and oxidative stress would promote the impairment of the UPS and the consequent accumulation of polyubiquitinylated proteins, which are visible as aggregates in AD brain. Arrows, promotion; lines, inhibition. Black, physiological conditions; red, pathological conditions.

and the regulation of either its catalytic activity or substrate interaction properties is important for the UPS signaling pathway. E3 ligase enzymes can be grouped into two classes: those that are homologous to the E6-AP carboxyl terminus (HECT) and the gene RING ligases. The two classes differ not only in their structure but also in the way they catalyze the last step of ubiquitinylation. The HECT ligases accept the activated ubiquitin from an E2 enzyme on a cysteine residue in the active domain and then transfer it to the substrate, whereas the RING ligases act as scaffold proteins by bringing together an E2 conjugating enzyme and the substrate [19]. The F-box and leucine rich repeat protein 2 (FBL2) is another component of the SCF (Skp1–Cullin1–F-box protein) E3 ubiquitin ligase complex that has been found to be decreased in the brains of AD patients [20]. Interestingly, the Watanabe group demonstrated that FBL2 impacts APP metabolism by interacting with APP to modulate APP ubiquitinylation. In detail, FBL2-mediated ubiquitinylation of APP inhibits its endocytosis [21].

In some circumstances, a fourth ubiquitinylation enzyme, known as the ubiquitin chain elongation factor E4, is necessary, together with the E1, E2, and E3 enzymes, to extend a polyubiquitin chain [22]. This cascade results in ubiquitinylation of the target protein. A polyubiquitin chain of at least four ubiquitin moieties is necessary for efficient translocation of the substrate to the proteasome [23]. Monoubiquitinylation is not associated with protein degradation, but with endocytosis, protein sorting, DNA damage response, and epigenetics [24–26].

Ubiquitinylation is a reversible posttranslational modification, and a family of proteases, the deubiquitinylating enzymes (DUBs), can remove ubiquitin from substrates, thereby regulating the ubiquitinylation process and recycling ubiquitin. The recycling of ubiquitin is critical for the

brain, which has a fixed amount of ubiquitin. DUBs are highly specific and have been grouped into five subfamilies: ubiquitin carboxyl-terminal hydrolases (UCH), ubiquitin-specific proteases (USP), ovarian tumor- (OTU-) like proteases, JAB1/MPN/Mov34 (JAMM/MPN) metalloproteases, and the Machado-Jakob disease proteases [27]. The state of substrate ubiquitinylation depends on the balance between ubiquitinylating and deubiquitinylating enzymes acting on a protein. Thus, cells developed a highly dynamic strategy based on a switch-on/switch-off type of mechanism that responds promptly to cellular requirements for proteolysis by the UPS. Ubiquitinylated substrates with a Lys-48-linked ubiquitin chain of sufficient length are targeted to the 26S proteasome for degradation.

The 26S proteasome is composed of a 20S catalytic core and two 19S regulatory caps on both ends of the 20S core. The 20S proteasome contains four stacked rings that form a barrel-shaped moiety with a central cavity [28]. These stacked rings include two noncatalytic outer rings called α-rings and two catalytic inner rings called β-rings. Three proteolytic activities are confined to the β-rings including chymotrypsin-like, caspase-like, and trypsin-like protease activities [19]. The 19S caps contain at least 18 subunits, with a base composed of six ATPases that exert a chaperone-like activity and a lid composed of eight subunits that recognize the polyubiquitin signals. The 19S proteasome binds and unfolds ubiquitinylated proteins and opens the entry gate of the 20S proteasome to allow protein in the central cavity [19].

Oxidation of a protein induces several reversible or irreversible alterations, including amino acid modification, fragmentation, or aggregation and causes increased susceptibility of the modified proteins towards proteolysis [29, 30]. It has been suggested that the oxidation of proteins causes the exposure of hydrophobic moieties to the surface via partial unfolding which are targeted by proteasome [31–33]. While the 26S proteasome degrades polyubiquitinylated proteins, the 20S proteasome by itself seems to be sufficient to degrade nonubiquitinylated oxidatively modified proteins in an ATP-independent manner; however, the exact mechanism is still unclear [13, 18, 19].

Deubiquitinylating Enzymes: UCHL1. DUBs function in the processing of ubiquitin precursors and ubiquitin adducts [34]. DUBs belong to a protease superfamily, and about 100 members are expressed in humans [35, 36]. The UCH class of DUBs consists of four proteins (Bap1, UCHL1, UCHL3, and UCHL5), which all have a conserved catalytic domain (UCH-domain) consisting of about 230 amino acids [35, 36]. Ubiquitin carboxyterminal hydrolase L1 (UCHL1) is a 223-amino-acid protein encoded by 9 exons [37]. The catalytic area of UCHL1 has a loop positioned over the active site, which limits the size of ubiquitin adducts that can be processed by it to small peptides [38]. UCHL1 is proposed to function largely by maintaining a stable pool of monoubiquitin for use in ubiquitinylation reactions, as showed in Figure 1 [27, 35]. Newly translated ubiquitin contains amino acids following the terminal glycine residue that is used for isopeptide bond formation. UCHL1 can cleave off these additional amino acids in order to expose the final glycine of ubiquitin for conjugation. UCHL1 can also help maintain the monoubiquitin pool by reversing accidental modifications that can form during ubiquitin activation [39]. Due to its specificity, UCHL1 does not remove ubiquitin from all proteins. It is this selectivity that offers unique advantages for drug discovery efforts. UCHL1 is also involved in the cotranslational processing of proubiquitin and ribosomal proteins translated as ubiquitin fusions [37]. In addition, UCHL1 can form dimers, whose form seems to act as another enzymatic activity in UCHL1, the ubiquitin ligase activity [40]. In the dimeric form, UCHL1 ligase activity produces Lys-63-linked ubiquitin chains to its substrates. In contrast to the well-recognized ubiquitinylation pathway with E1, E2, and E3 ligases, UCHL1 does not require ATP as a notable characteristic of this ligase. Interestingly, when they are polyubiquitinylated via Lys-63 of ubiquitin, the substrates escape from UPS-dependent protein degradation leading to their stabilization. The dual function of both addition and removal of monoubiquitin sets UCHL1 apart from other DUBs and makes it a special target for proper UPS function [41].

UCHL1 is among the most abundant proteins in the brain reaching 1-2% of total brain lysate and regulating the timing and the pattern of ubiquitinylation of brain proteins [37]. It is also one of the main enzymes that play a role in maintaining free ubiquitin levels in neurons [20, 21]. UCHL1 is also present in the peripheral nervous system, such as the dorsal root ganglion and trigeminal ganglion neurons [36]. UCHL1 is involved in synaptic activities and a reduction in UCHL1 function has been linked to neurodegenerative diseases [36, 42]. UCHL1 is also studied due to its association with various malignancies, including colorectal, breast, prostate, and lung cancers [43]. The *UCHL1* gene is known as *PARK5*, and its mutations are associated with Parkinson disease [42]; indeed the I93M mutation shows severely diminished hydrolase activity and lower E3 activity, while the S18Y mutant has greater hydrolase activity but lower E3 activity than WT [37, 44]. Further, deletion of one of the active site residues of the *UCHL1* gene is associated with gracile axonal dystrophy and leads to elevated oxidative damage in the brain [45].

UCHL1 is susceptible to oxidative damage and when this occurs it has aberrant functions analogous to mutated UCHL1 [46, 47]. Moreover, aberrant UCHL1 is able to interact with Lamp2a, Hsc70, and Hsp90 thus inhibiting chaperone mediated autophagy- (CMA-) dependent degradation and causing the accumulation of CMA substrates (e.g., α-synuclein) [48, 49].

2. Impairment of Ubiquitin-Proteasome System in Alzheimer Disease

The first observations about increased ubiquitin accumulation in specific structures characterizing human AD brain were showed about 30 years earlier. Indeed, ubiquitin was found to be covalently associated with the insoluble material of NFT and SP [50–52], thus suggesting that something in the degradative systems did not work properly. However, the presence of ubiquitin in abnormal aggregates was not associated with the subsequent proteolytic step [51]. Specifically,

Aβ inhibited the chymotrypsin-like activity but had no effect on the proteolytic activity of the protease chymotrypsin, suggesting that Aβ did not interact with the active site of the proteasome subunit [53]. Reduced chymotrypsin-like and peptidyl-glutamyl peptide-hydrolysing activity was found in AD brain [54].

The role of Aβ peptides in those processes was further investigated by Oh et al., who demonstrated that increased Aβ levels paralleled decreased chymotrypsin-like activity of the 26S proteasome in cortex and hippocampus of Tg2576 mice, a well characterized AD animal model that ubiquitously expressed Swedish mutant amyloid precursor protein (APP-swe) [55]. These results were also confirmed in B103 cells, a rat neuroblastoma cell line, in which Aβ treatment led to the inhibition of the proteasome activity [55], even though the mechanism involved in the inhibition of proteasome induced by Aβ is still unclear.

Because the effects mediated by Aβ can be dependent on its aggregation state, Cecarini and colleagues analyzed the impact of nonfibrillar, oligomeric, and fibrillar forms of Aβ on the proteasome activities in both the isolated 20S proteasome and SH-SY5Y cells [56]. They found a significant reduction only in the chymotrypsin-like activity in isolated 20S proteasome preparations treated with Aβ, independent of the aggregation state of this peptide [56]. Rather, these investigators showed a general decrease of the proteasome functionality especially upon treatment with the oligomeric and fibrillar forms in SH-SY5Y cells [56]. In fact, comparing these assays with that obtained using purified proteasomes, the tested activities were all significantly reduced. The marked decrease in proteasome functionality was also confirmed by the enhancement in the levels of ubiquitin protein conjugates. These results agree with the proposed toxic role of Aβ, possibly independent of its aggregation state.

While, on one hand, Aβ could be directly responsible for the proteasome impairment as cited above, on the other hand, the impairment of other members belonging to the UPS could also favor Aβ accumulation. In light of these findings Aβ is a part of a vicious cycle whereby its accumulation promotes the proteasome impairment responsible for further accumulation of Aβ-proteins.

Interestingly, Rosen et al. reported that the overexpression of the ubiquitin E3 ligase Parkin, which was found reduced in human AD brain, greatly decreased the levels of intracellular Aβ-42 in neurons [57]. This effect was abrogated by proteasome inhibition [57]. In addition, these researchers reported that intracellular Aβ-42 accumulation decreased cell viability and proteasome activity, while Parkin reversed both effects [57]. The importance of Parkin has been further highlighted in a subsequent study, where the overexpression of Parkin in APP/PS1 transgenic mice restored activity-dependent synaptic plasticity and rescued behavioral abnormalities [58].

Similarly, the ubiquitin ligase HRD1, which normally promotes APP ubiquitinylation and degradation resulting in decreased generation of Aβ, was found impaired in AD brain [59]. Indeed, suppression of HRD1 induced APP accumulation and increased production of Aβ *in vitro*, resulting in apoptosis [59]. In addition, Zhang et al. found that inhibition UCHL1 significantly increased β-secretase 1 (BACE1) protein level *in vitro* [60]. BACE1 half-life was reduced in cells overexpressing UCHL1 and decreased APP C-terminal fragment C99 and Aβ levels were observed [60].

Taken together, the impairment of members of the UPS different from the 26S proteasome itself, such as Parkin, HRD1, and UCHL1, may affect APP processing and Aβ production.

2.1. Oxidative Damage to UPS. Among the factors contributing to the impairment of UPS in AD, augmentation of the oxidative/nitrosative stress levels was proposed as conceivable causative effect [61]. Indeed, levels of oxidized proteins in AD are associated with loss of the activity of the 20S proteasome, which, as noted above, represents a major enzyme for the degradation of oxidized proteins [54, 62–64]. Interestingly, studies from the Davies and Grune groups showed that moderately oxidized proteins are preferentially recognized and degraded by the proteasome; however, severely oxidized proteins cannot be easily degraded and, instead, inhibit the proteasome [3, 65]. Further, studies have shown that prolonged oxidized proteins are more resistant to degradation by 20S proteasomes [66, 67]. Therefore, overloading the UPS by undegradable substrates, mutations, or oxidative damage may lead to the accumulation of abnormal proteins and to the selective degeneration of neurons.

In that context both the Butterfield group [46, 68] and others [69] demonstrated that UCLH1 is oxidatively modified in AD, establishing a link between the effect of oxidative stress on protein and the proteasomal dysfunction. Similarly, Saito et al. showed that HRD1 protein was insolubilized by oxidative stress but not by other AD-related molecules and stressors, such as amyloid-β, tau, and ER stress. Furthermore, these authors raised the possibility that modifications of HRD1 by 4-hydroxy-2-nonenal, decreased HRD1 protein solubility leading to the accumulation of HRD1 into the aggresome [70]. In addition, the identification of oxidative stress-induced modification of the heat shock cognate 71 seems to underlie the essential link between the folding and degradation machineries that once impaired by oxidative damage become critical for cell viability [71].

Nitric oxide- (NO-) induced S-nitrosylation of the protein disulphide isomerase (PDI) was proposed to have a role relating protein misfolding to neurodegeneration [72]. Indeed, S-nitrosylation inhibited PDI enzymatic activity and led to the accumulation of polyubiquitinylated proteins [72, 73]. S-nitrosylation also abrogated PDI-mediated attenuation of neuronal cell death triggered by ER stress, misfolded proteins, or proteasome inhibition [72]. Thus, PDI prevents neurotoxicity associated with ER stress and protein misfolding, but NO blocks this protective effect in neurodegenerative disorders through the S-nitrosylation of PDI [72].

Cecarini et al. also demonstrated that despite lack of differences in the amount of proteasome complex isolated from control, MCI, and AD brains, a large impairment in proteasome-mediated degradation of an oxidized protein was observed in MCI and AD subjects [74]. The impairment was associated with the elevation of proteasome oxidative

modifications such as protein carbonyls, 4-hydroxynonenal-conjugation, and neuroprostane-conjugation [74]. Intriguingly, the incubation of proteasome complexes with a reducing agent fully restored proteasome-mediated protein degradation in both MCI and AD samples, thus supporting a role for oxidative stress in promoting proteasome inactivation [74].

2.2. Mutant Ubiquitin UBB^{+1}. Together with Aβ and oxidative/nitrosative stress, a mutant form of ubiquitin, deriving from a molecular misreading of the *ubiquitin* gene and termed UBB^{+1}, was found to be selectively expressed in the brains of AD patients [75] and was reported to impair the proteasome activity *in vitro* [76].

Indeed, Lam and colleagues showed for the first time that (i) UBB^{+1} is polyubiquitinylated (UBB^{+1}-polyubiquitin); (ii) UBB^{+1}-polyubiquitin was strongly resistant to disassembly and accumulates in cells; and (iii) UBB^{+1}-polyubiquitin inhibited proteasomal activity, thus providing a likely mechanism of toxicity [76]. In accordance with these observations, overexpression of UBB^{+1} in neuroblastoma cells significantly induced nuclear fragmentation and cell death [77].

The complex nature of UBB^{+1} interactions was illustrated by the finding that on one hand the induction of UBB^{+1} expression in SH-SY5Y cells caused proteasome inhibition, while on the other hand UBB^{+1} also induced the expression of heat shock proteins, which conferred a subsequent resistance to tertbutyl hydroperoxide-mediated oxidative stress. Indeed, these authors concluded that although UBB^{+1}-expressing cells have a compromised ubiquitin-proteasome system, these cells are protected against oxidative stress conditions. However, which one of these two effects is prevalent does not emerge from the study and requires further investigations [78].

From the point of view of the mechanisms underlying UBB^{+1}-induced neurotoxicity, an ubiquitin-conjugating enzyme, E2-25K/Hip-2, which was found to be upregulated in the neurons exposed to Aβ42 and in the brain of AD patients, was proposed to have a role [76, 79]. E2-25K/Hip-2 seems to function both as an E2 ubiquitin-conjugating enzyme like other E2 proteins and as an unusual ubiquitin ligase to produce diubiquitin and unanchored polyubiquitin chains without any E3 ligase [80]. E2-25K/Hip-2 was shown to reduce proteasome activity [79]. Rather, E2-25K/Hip-2 was found to play a major role in Aβ neurotoxicity by promoting the polyubiquitinylation of UBB^{+1}, which leads to proteasome inactivation [79].

However, a protective role for UBB^{+1} was also reported. By using a triple transgenic mouse (APP/PS1/UBB^{+1}) obtained by crossing UBB^{+1} and APP/PS1 transgenic mice, van Tijn and colleagues showed a transient and significant decrease in Aβ deposition and soluble Aβ 1–42 levels in APP/PS1/UBB^{+1} transgenic mice compared to APP/PS1 mice at 6 months of age [81].

2.3. Dysfunction of UPS and Tau Aggregation. While Aβ was mainly found to both directly and indirectly trigger the inhibition of proteasome activity, probably the most investigated target in terms of proteins aggregation following proteasome inhibition in AD is tau. Studies in AD brain demonstrated that phosphorylated tau accumulated on both sides of the synapse, thus showing synaptic enrichment of this protein when compared with the cytoplasm [82]. The accumulation of p-tau at the synapse mirrors the accumulation of ubiquitinylated proteins in the same fraction, as well as the accumulation of proteasomes and related chaperones, consistent with the notion that tau aggregates are associated with impaired proteolysis mediated by the UPS [82].

Zhang et al. in 2005 reported for the first time that tau, both phosphorylated and nonphosphorylated, is degraded by the 26S proteasome in an ubiquitin- and ATP-dependent manner, suggesting that defect in the UPS would promote tau accumulation [83].

In agreement with the above, Cripps et al. reported that soluble paired helical filaments (PHF) of tau protein are ubiquitinylated at their microtubule-binding domain (at residues Lys-254, Lys-311, and Lys-353), suggesting that ubiquitinylation of PHF-tau may be an earlier pathological event and that ubiquitinylation could play a regulatory role in modulating the integrity of microtubules during the course of AD [84]. Through the use of tandem mass spectrometry, the same group highlighted that PHF-tau is modified by three polyubiquitin linkages at Lys-6, Lys-11, and Lys-48 [84]. Among these, Lys-48-linked polyubiquitinylation is the primary form of polyubiquitinylation with a minor portion of ubiquitin linked at Lys-6 and Lys-11 [84]. Because modification by Lys-48-linked polyubiquitin chains is known to serve as the essential means of targeting proteins for degradation by the ubiquitin-proteasome system, a failure of the UPS could play a role in tau accumulation in AD [84].

The role of PHF-tau was further highlighted by Gillardon et al., who proposed that the reduced peptidase activity observed in AD brain extracts is not an intrinsic property of the 20S proteasome but may be resulting from the presence of endogenous inhibitory proteins or substrates, for example, PHF-tau [85]. Indeed, these investigators found that proteasome activity was increased upon purification from AD brain [85], while the presence of cytosolic proteins, which had been removed during the purification process, led to proteasome inhibition [85].

Quite recently, HRD1 ubiquitin ligase, previously reported to favor APP degradation [59], was also identified as a negative regulator of tau phosphorylation in AD [86]. In fact, Shen et al. reported that HRD1 interacts with tau and promotes the degradation of both dephosphorylated and phosphorylated tau through the 26S proteasome [86].

An intriguing aspect about proteasome-mediated degradation of tau protein was the discovery that hyperphosphorylation of tau diminishes its recognition by the proteasome [87], thus questioning which form of proteasome is responsible for tau degradation: the classical ATP/ubiquitin-dependent 26S proteasome pathway and/or a 20S proteasome pathway not requiring ubiquitin or ATP? Indeed, the Poppek group reported that the ATP/ubiquitin-independent 20S proteasome could degrade tau *in vitro* [87], while evidence also exists that, under certain conditions, the tau protein is polyubiquitinylated and directed to the 26S proteasome

[83, 84, 88, 89]. Starting from such apparently conflicting reports, Grune and colleagues demonstrated *in vitro* that the normal turnover of the tau protein is catalyzed by the proteasome in an ATP/ubiquitin-independent manner and that the 20S proteasome is more important for normal tau turnover than is the 26S proteasome [90]. This interpretation seems reasonable, since the tau protein is largely unfolded and, therefore, should not require ATP for unfolding prior to degradation [90]. Conversely, under other conditions, including certain stress situations, the ATP/ubiquitin pathway and the 26S proteasome may be more important [90].

2.4. Role of ATP and Aggregated Proteins. Because ATP represents the essential driving force for UPS activity links between mitochondrial impairment and proteasome activity also have been evaluated. Huang et al. in 2013 demonstrated that cortical neurons treated with inhibitors of different elements of the electron transport chain showed a reduction in ubiquitinylated proteins and E1 activity as well as a calpain-mediated disassembly of the 26S proteasome [91]. Calpain activation promoted the cleavage of the microtubule-associated protein tau, leading to its accumulation [91]. Furthermore, all these changes paralleled increased 20S proteasome levels and activity [91]. The concomitant rise in the 20S proteasomes, which seem to degrade proteins in an unregulated and energy-independent manner, in the short-term may carry out the turnover of randomly unfolded oxidized proteins [91]. However, if chronic, this process could lead to neurodegeneration, as regulated protein degradation by the ubiquitin/proteasome pathway is essential for neuronal survival [91].

Although polyubiquitin aggregates are evident in AD brain, the identification of the proteins present in that structure is still elusive and studies on this are ongoing in our laboratories. Indeed, only a few examples emerge from the literature aimed at strengthening the role of the impaired UPS response in the progression of AD pathology.

β-Catenin, a member of the Wnt-signaling pathway, is a multifunctional protein that participates in cadherin-mediated cell adhesion and in transcriptional activation of Wnt target genes involved in development [92]. In the absence of a Wnt ligand, β-catenin is phosphorylated and is targeted for multiubiquitinylation by β-Trcp E3 ligase followed by rapid degradation by the 26S proteasome machinery [92]. Interestingly Ghanevati and Miller demonstrated that inhibition of the proteasome machinery in neuronal cultures leads to the progressive accumulation of phospho-β-catenin protein and formation of scattered, punctate cytoplasmic inclusions, which ultimately coalesced into a large cytoplasmic aggresome [92].

Striatal-enriched protein tyrosine phosphatase 61 (STEP61), the only isoform of this brain-specific family of phosphatases expressed in the cortex, localizes to postsynaptic terminals and the endoplasmic reticulum [93]. STEP61 associates with the NMDA receptor (NMDAR) complex, reduces NMDAR activity, and opposes the induction of LTP [93] with deleterious effects on cognitive functions. Kurup and colleagues demonstrated that STEP61 levels are elevated in aged transgenic AD model mice (Tg2576) and in AD brains and that Aβ is sufficient to increase STEP61 levels [93]. Increased STEP61 both in mice brain and in Aβ-treated cells had been found, thus suggesting an inhibition of the UPS [93]. The evidence outlined above highlights how a defective UPS activity is also associated with the accumulation of proteins, whose activation persists over the time, thus contributing to cognitive dysfunction in AD.

Studies have demonstrated that proteasome inhibition may occur also during normal aging [94, 95], which definitively represents one of the main risk factors for AD development. Thus, proteasome dysfunction together with other unknown mechanisms could contribute to protein aggregation throughout the lifetime. In that picture, the analysis of four molecular chaperones (Grp78, Grp94, PDI, and calnexin) revealed a marked decrease in aged rat hippocampus compared to young controls [96]. In addition, the levels of ubiquitinylated proteins were increased [96]. Thus, an age-related decrease in chaperone expression, together with an age-related decrease in proteasome activity [94, 97], conceivably could account for the increased content of ubiquitinylated proteins. Indeed, aged rats could be more predisposed to the formation of protein aggregates that in turn disrupt cellular functions and provide nucleation sites for the aggregation of other proteins. This scenario, together with environmental, genetic, and other unknown factors, could predispose development of age-related neurodegenerative disorders, such as AD.

2.5. Oxidative Modifications of UCHL1 in Alzheimer Disease. Protein ubiquitinylation can be regulated by modulating the enzyme levels or activity of deubiquitinylating enzymes such as UCHL1. This is an important mechanism for regulating a variety of cellular processes, including synaptic function, protein degradation, and neuronal apoptosis [98]. Thus, dysfunction of UCHL1 has been directly implicated in neurodegenerative diseases, such as AD. Of special interest is the finding that UCHL1 is involved, by maintenance of ubiquitinylation/deubiquitinylation machinery, in memory formation. In particular, the first study on UCHL1 was conducted in *Aplysia*, a model system used to investigate neuronal events associated with learning. Further studies confirmed the relevance of UCHL1 in synaptic function [99, 100].

Knockout mice for UCHL1 showed decreasing acetylcholine release from the synaptic terminal, which could be due to perturbed ubiquitin-dependent pathways as a result of decreased ubiquitin recycling. This reduction in content release is accompanied by hindered synaptic plasticity, nerve terminal retraction, and axonal degeneration [100]. In line with these findings, Zhang et al. showed that the gracile axonal dystrophy (gad) mutant mouse, which presents a deletion within the gene encoding for UCHL1, displays brain axonal degeneration [101]. These data suggest that the lower expression of UCHL1 may be partially responsible for cognitive impairment and Alzheimer pathophysiology. To consolidate these results and the involvement of UCHL1 in AD, Zhang and coworkers administered UCHL1 by intracranial injection of UCHL1-expressing rAAV into the hippocampus of the transgenic mice. Increased expression of

the enzyme reduced Aβ production, inhibited neuritic plaque formation, and improved memory deficits [101]. A second study on a double transgenic mouse model of AD further supports this evidence. These mice showed cognitive defects such as inhibition of LTP, and the protein level of UCHL1 was significantly decreased in hippocampus [102]. Transduction to hippocampal slices of UCHL1 fused to the domain of HIV-transactivator protein (TAT) significantly restored Aβ-induced inhibition of LTP and also reestablishes normal UCH activity, basal neurotransmission, and synaptic plasticity and improves associative memory in APP/PS1 mice [102]. Interestingly, exogenous UCHL1 ameliorated β-amyloid-induced synaptic and memory dysfunction in an AD mouse model. UCHL1 is important at synapses and suggests that increased UCHL1 activity could counteract certain symptoms in AD.

Furthermore, data from the Butterfield group demonstrated that UCHL1 reduced activity was due to specific oxidative modifications, which can block its normal functioning. In fact an increased amount of oxidatively modified UCHL1 was found in the brains of AD patients, compared to normal brains [46, 103]. Proteomics analyses showed that UCHL1 is a major target of oxidative damage in frontal cortex of AD subjects [46], which is extensively modified by carbonyl formation, methionine oxidation, and cysteine oxidation [69]. Moreover, in the neurofibrillary tangles of AD patients a deposition of proteins modified by HNE, a product of lipid peroxidation, was reported [104]. To support this view, *in vitro* data showed that addition of HNE induced the HNE modification of recombinant UCHL1 [105]. UCHL1 immunostaining displayed a prominent association between the enzyme and neurofibrillary tangles and the level of soluble UCHL1 protein is inversely proportional to the number of tangles in AD brains [69]. Potentially due to its sequestration in neurofibrillary tangles, soluble UCHL1 levels are decreased in postmortem AD brains [37]. In inherited AD, UCHL1 was oxidatively damaged [106].

It is well known that oxidative stress causes protein modification, which can result in altered protein function. A reduction in the levels of functional UCHL1 was speculated to contribute to the pathogenesis of AD. In light of this evidence, the hydrolase activity of HNE-modified UCHL1 was reduced to about 40–80% of nonmodified UCHL1 and was inversely correlated with the degree of modification [46, 69]. A recent study from our laboratory observed that UCHL1 is a target of oxidative damage also in Down Syndrome (DS) brains [47]. DS presents many common features of AD, such as early deposition of Aβ plaques and, above 40 years of age, development of AD-like dementia [107]. Similar to what is found in AD, UCHL1 enzyme activity was decreased about 30% in DS brain compared to controls [47]. These two events, oxidation and decreased activity of UCHL1, can be correlated in DS subjects similar to AD. Moreover, in DS subjects UCHL1 impairment is an early event occurring before clinical manifestation of dementia, thus contributing to neurodegenerative phenomena.

Overall these results discussed in this review show that in different animal models and human specimens aberrant UCHL1 activity is caused by oxidative modifications that in turn might lead to dysfunction of the neuronal ubiquitinylation/deubiquitinylation machinery, causing synaptic deterioration and neuronal degeneration in AD (Figure 1). Moreover, overexpression of UCHL1 delays AD progression in mouse models, and *UCHL1* gene therapy, to overexpress UCHL1, in the brain potentially could be a promising disease-modifying strategy for AD therapeutics.

3. Conclusions

A close connection between protein clearance network dysfunction and mechanisms of neurodegeneration is well documented. Potentially toxic oxidized and aggregated proteins harm neuronal cells once these are deprived of the cytoprotective functions of the PQC. In this scenario, a crucial role is played by oxidative stress that contributes to the buildup of oxidized/misfolded proteins. Concomitantly, oxidative stress targets members of the PQC, such as proteasome subunits and UCHL1, thus leading to its reduced ability to remove damaged/dysfunctional proteins. Taken together, these findings highlight that induction/protection of protein degradative system may represent an efficient therapeutic strategy for AD, as well as other neurodegenerative diseases.

Abbreviations

AD: Alzheimer disease
APP: Amyloid-β protein precursor
Aβ: Amyloid-β- (Aβ-) peptide
ATP: Adenosine triphosphate
BACE1: β-secretase 1
DS: Down Syndrome
DUBs: Deubiquitinylating enzymes
E2: Ubiquitin-conjugating enzyme
E3: Ubiquitin ligase
ER: Endoplasmic reticulum
FBL2: F-box and leucine rich repeat protein 2
HECT: E6-AP carboxyl terminus
HNE: 4-Hydroxynonenal
HRD1: ERAD-associated E3 ubiquitin protein ligase
LTP: Long Term potentiation
Lys: Lysine
MCI: Mild cognitive impairment
NFT: Neurofibrillary tangles
NO: Nitric oxide
OTU: Ovarian tumor-like proteases
PDI: Protein disulphide isomerase
PGJ2: Prostaglandin J2
PHF: Paired helical filaments
PQC: Protein quality control system
PS1: Presenilin 1
ROS: Reactive oxygen species
SCF: Skp1–Cullin1–F-box protein
SP: Senile plaques
STEP61: Striatal-enriched protein tyrosine phosphatase 61
SUMO-1: Small ubiquitin-like modifier protein 1
UBB^{+1}: Ubiquitin-B^{+1}
UCH: Ubiquitin carboxyl-terminal hydrolases
UCHL1: Ubiquitin carboxyterminal hydrolase L1
UPS: Ubiquitin-proteasome system

UPR: Unfolded protein response
USP: Ubiquitin-specific proteases.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Health.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Novel Perspectives in Redox Biology and Pathophysiology of Failing Myocytes: Modulation of the Intramyocardial Redox Milieu for Therapeutic Interventions—A Review Article from the Working Group of Cardiac Cell Biology, Italian Society of Cardiology

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The prevalence of heart failure (HF) is still increasing worldwide, with enormous human, social, and economic costs, in spite of huge efforts in understanding pathogenetic mechanisms and in developing effective therapies that have transformed this syndrome into a chronic disease. Myocardial redox imbalance is a hallmark of this syndrome, since excessive reactive oxygen and nitrogen species can behave as signaling molecules in the pathogenesis of hypertrophy and heart failure, leading to dysregulation of cellular calcium handling, of the contractile machinery, of myocardial energetics and metabolism, and of extracellular matrix deposition. Recently, following new interesting advances in understanding myocardial ROS and RNS signaling pathways, new promising therapeutical approaches with antioxidant properties are being developed, keeping in mind that scavenging ROS and RNS *tout court* is detrimental as well, since these molecules also play a role in physiological myocardial homeostasis.

1. Introduction

The prevalence of heart failure (HF) is still increasing worldwide, with enormous human, social, and economic costs [1–6], despite huge efforts in understanding pathogenetic mechanisms and in developing effective therapies that have transformed this syndrome into a chronic disease. Recently, following new interesting advances in understanding intracellular signaling pathways that control the main altered processes in the failing heart (such as cellular calcium handling and the contractile machinery, cardiac hypertrophy and dilatation, and myocardial energetics and metabolism), new promising therapeutical approaches are being developed. It is well established that cardiomyocytes of a failing heart are redox imbalanced, and, in this paper, we review and discuss the pathophysiology of HF, keeping in mind that ROS and RNS play an important role as signaling molecules in physiological myocardial homeostasis.

2. Heart Failure as a Systemic Disease

The etiology of heart dysfunction is heterogeneous, although individuals with HF have rather common symptoms as fatigue, shortness of breath, and fluid retention. Half of HF patients present with contractile failure and a dilated heart (systolic HF), while other patients have normal systolic function with a preserved ejection fraction (EF) and a nondilated, but often hypertrophied, heart. This latter is also named HF with preserved ejection fraction (HFpEF) [7].

Central to the pathogenesis of systolic HF is decreased left ventricular (LV) contractile function, due to an initial ischaemic insult (e.g., myocardial infarction, MI) or to nonischaemic insult (including genetic and inflammatory cardiomyopathies, hypertension, metabolic diseases, or toxic injury). These insults induce an inexorable series of compensatory responses in the body, including the retention of salt and water by the kidneys, the release of neurohormones, and the activation of intracellular signaling cascades in the heart and vasculature that modify cellular and organ morphology and function. Such responses initially offset reduced cardiac performance but then become part of the disease process, increasing organ failure and worsening clinical prognosis [1–7].

3. Neurohumoral Adaptations

When cardiac contractile dysfunction is established, the body responds by increasing release of sympathetic neurotransmitters, adrenaline and noradrenaline, and neurohormones, including angiotensin II (ATII), endothelin, and natriuretic peptides. These events contribute to maintaining cardiac output, increasing rate and intensity of heart contraction, and fluid retention. Such chronic stimulation becomes adverse and worsens prognosis of heart failure [8]. Indeed, current HF therapies mainly rely on antagonizing such neurohumoral activated pathways with β-adrenergic and angiotensin receptor blockade and angiotensin converting enzyme (ACE) inhibition and on hemodynamic control with nitrates and diuretics, with the net effect of producing vasodilation and lowering blood pressure, therefore unloading the heart [1–7]. Therapies based on blockade of β-adrenergic receptors (β-ARs), inhibition of angiotensin converting enzyme, blockade of the angiotensin II receptor AT1, and blockade of aldosterone receptor improved survival and symptoms in heart failure patients [1, 7, 9–11].

4. Energetic Breakdown in Heart Failure

The heart is an organ with limited capacity for storing energy. Thereby, to supply its high and constant workload, it needs substrates produced quickly and efficiently, mainly from circulating fatty acids (FA) rather than from glucose. A failing heart enters a state of inefficiency and of energy starvation, mainly due to a compromised regulation of energy metabolism, a reduced ATP availability, or an altered substrates utilization [7, 12].

A shift in energy metabolism from normal using of fatty acids (due to a decline in the expression of genes involved in fatty acid metabolism [13]) towards using glucose, which probably results in differences in substrates oxidation and thus mitochondrial function, has been observed in both ischaemic and nonischaemic heart failure [13–16]. Therefore, with this shift of metabolic profile, the myocardium relies on glycolysis for ATP generation [17, 18]. This situation has important fallouts in specific conditions such as heart failure associated with diabetes [19]. In this case, reduced FA oxidation is not accompanied by an increase in glucose or lactate oxidation to provide ATP, thus causing an energetic deficit in the failing heart that correlates with overall disease severity [13, 18]. Importantly, in diabetes, hyperglycemia per se, independently of FA utilization, is able to lead to cellular derangements and to adaptive and maladaptive processes involving, among many, the renin-angiotensin-aldosterone system, glucose transporters, and AGEs [20, 21]. In this setting, mitochondrial failure to generate enough ATP, coupled to increased ROS generation, with consequent ROS-induced posttranslational modifications of important proteins of the EC coupling machinery is directly involved in diabetic cardiomyopathy [21, 22].

In a failing heart not only ATP synthesis but also ATP storage is altered. Phosphocreatine is ATP storage molecule, which, in presence of ADP, is converted to creatine and ATP by creatine kinase, thereby generating rapidly energy when it is needed. The ratio of concentrations of phosphocreatine to ATP is used as a measure of energy balance. This ratio is found abnormal in heart failure together with the ATP flux [23].

5. Oxidative Stress and Heart Failure

Oxidative stress is commonly referred to as unbalanced ratio between production and scavenging of oxygen radicals with a detrimental oxidation of different substrates (proteins, lipids, nucleic acids, and others). The onset of a prooxidative condition can be due to a hyperactivation of different reactive species sources (see below) or to a depletion of antioxidant defenses or both. It is not clear whether oxidative stress is to be considered a cause, an index, or a mediator of heart failure. In the next paragraphs the sources and effects of prooxidant will be described in order to elucidate the role of oxygen radicals on the etiopathology of a failing heart (Figure 1).

6. Sources of Reactive Oxygen and Nitrogen Species in Heart Failure

Both excessive neurohormonal stimulation and energetic deficits with mitochondrial dysfunction lead to increased oxidative stress [21, 24] with production of excessive Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), widely recognized as promotors of both cardiac dysfunction and pathological remodeling of HF, which is characterized by altered excitation-contraction (EC) coupling with abnormally lower cardiac contractility and muscle relaxation velocities. Among other events linked to HF onset and progression are maladaptive hypertrophic myocardial response, extracellular matrix remodeling, altered tissue energetics,

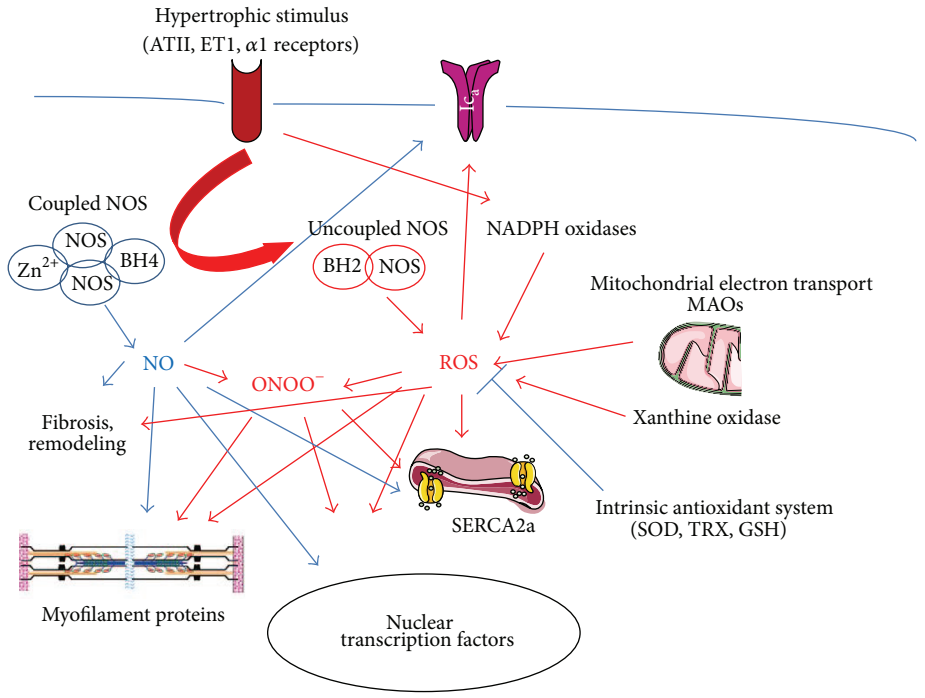

Figure 1: ROS promote heart failure by many mechanisms. Some of the deleterious effects of ROS are evidenced by red arrows. NO counteracts such effects (blue arrows). Modified from Tocchetti et al. [25].

loss of viable cardiomyocytes, vascular and capillary abnormalities, and inflammation [4, 7, 25–30]. Myocardial redox imbalance may be responsible, at least in part, for such abnormalities [22, 31]. ROS and RNS can be produced endogenously by cardiomyocytes by several cellular sources, including "direct" production such as NADPH oxidase system, lipoxygenases, cyclooxygenases, peroxidases, mitochondrial oxidative phosphorylation, nitric-oxide synthase 3 (NOS3 or eNOS) [25–27, 32–34] and "indirect" (free radicals production inducers) such as cytokines, growth factors, angiotensin II, catecholamines, pressure overload, xanthine oxidases, monoamine oxidases, enzymes of catecholamine and serotonin catabolism. Also, the myocardium is provided with endogenous nonenzymatic (i.e., glutathione, vitamins E and C, and β-carotene [33], lipoic acid, ubiquinone, and urate) and enzymatic systems that catabolize ROS physiologically generated [25].

NADPH oxidases are important cellular sources of ROS, crucial in many pathophysiological conditions that lead to cardiac diseases [25, 35–37]. The NADPH oxidase enzyme complex is composed of seven catalytic subunits, Nox1–Nox5 and Duox1 and Duox2. Nox2 and Nox4 are expressed in the heart and produce ROS by electron transfer from NADPH to molecular oxygen. In physiological states, Nox2 is quiescent and is stimulated by the translocation of regulatory proteins to activate the oxidase complex on the membrane [25, 38]. G-protein-coupled receptor agonists, cytokines, and growth factors can stimulate Nox2 to generate ROS. On the other hand, Nox4 is constitutively active and is modulated by its expression levels [25, 37]. Both Nox2 and Nox4 are key players in the pathogenesis of LV dysfunction. Indeed, after myocardial infarction, myocytes hypertrophy and apoptosis were significantly reduced in aortic rings of Nox2-deficient mice, with less LV dilation and better function compared to wild type mice [25, 37, 39]. Also, Nox2-containing NADPH oxidases play a role in ATII-induced hypertrophy independently of pressure overload [37]. The underlying mechanism at the base of the prohypertrophic Nox2 effect relies, at least in part, on the oxidation of mitochondrial proteins induced by increased production O_2^- that leads to mitochondrial dysfunction [39]. At the same time, Nox4-containing NADPH oxidases are important in the pathophysiology of cardiac hypertrophy from pressure overload: pressure overloaded hearts from c-Nox4$^{-/-}$ mice showed less hypertrophy and less interstitial fibrosis and apoptosis and had improved LV function compared to wild type [25, 37, 40]. Human failing hearts exhibit increased NADPH oxidase activation [41] together with the parallel activation of downstream signaling components ERK1/2, JNK, and p38 [42]. Also, Nox4 levels increase gradually in aging cardiomyocytes; interestingly, apoptosis is also increased upon enhanced Nox4 expression of caused apoptosis [25, 37]. Nox4 appears to produce mostly H_2O_2, while Nox2 generates mostly O_2^- [35, 36].

ROS can be also produced by xanthine oxidase (XO), an enzyme that belongs to the molybdoenzyme family (which comprises enzymes such as aldehyde oxidase and sulfite oxidase) [43]. Both O^{2-} and H_2O_2 XO can be generated by oxidative hydroxylation of purine substrates from XO. Inhibition of xanthine oxidoreductase improves cardiac structure and function in spontaneously hypertensive/HF rats [25, 44]. Also, XO can be activated by NAD(P)H oxidase [25, 45]. Compared to wild type animals, myocardial XO activity

did not increase after MI in p47phox−/− mice (genetically deprived of p47phox, the cytosolic NADPH oxidase component). Disappointingly, XO inhibitors, such as the purine analog allopurinol and the nonpurine analog febuxostat, when employed clinically, did not exert beneficial effects on human ischaemia/reperfusion and cardiac dysfunction [25], in spite of some success in animal studies [46–48]. Indeed, xanthine oxidase importance and role in the human heart have been questioned [49].

Because of their high-energy needs, cardiac myocytes possess a large number of mitochondria that not only can produce ATP but also can generate ROS as a by-product of mitochondrial respiration. Mitochondrial ROS are produced because the transfer of electrons via the electron transport chain is not totally efficient [50], with O_2^- being generated in the mitochondria at a measurable rate during physiological oxidative phosphorylation. Most of mitochondrial O_2^- possesses a relatively short half-life [43]. In the mitochondria, manganese superoxide dismutase (SOD) is located in matrix while copper/zinc SOD is in the intermembrane space: both of these enzymes can transform O_2^- into H_2O_2, that is not so reactive as O_2^- and can easily diffuse and behave as signaling molecule [51]. An alternative enzymatic reaction is operated by other antioxidant enzymes, such as glutathione peroxidase-1 and catalase, which can convert H_2O_2 to O_2 and H_2O [50]. Nevertheless, an imbalance between mitochondrial prooxidant and antioxidant systems can bring to mitochondrial oxidative stress. Differently from H_2O_2, OH (hydroxyl radical) cannot be catabolized by enzymatic reactions but can be quenched only by endogenous or food antioxidants. OH possesses a very short half-life and is very reactive *in vivo*; therefore it is believed to be a very dangerous molecule [52]. OH is a player in reperfusion injury, in HF, stroke, and MI, and in Ca^{2+} cycling and myofilament Ca^{2+} sensitivity in experimental myocardial preparations [33, 53].

Mitochondria produce more ROS during stress conditions, among many ischaemia/reperfusion and cardiac dysfunction [43, 54–56]. ROS can be generated not only on the inner mitochondrial membrane, but also on the outer mitochondrial membrane thanks to monoamine oxidases (MAOs) A and B during oxidative deamination of catecholamines and serotonin [57] (Figure 1).

7. Antioxidant Defenses

Antioxidants can be mainly divided into 2 groups: exogenous and endogenous. Antioxidants from exogenous sources are normally introduced with the diet and include (but are not limited to) vitamins (A and C), carotenoids, and flavonoids [58, 59]. On the other hand, endogenous compounds with antioxidant properties can be either of enzymatic origin (superoxide dismutase, GPx, and catalase) or nonenzymatic antioxidants (vitamin E, GSH, and bilirubin) [33]. The therapeutic approach to HF aimed at reducing oxidative stress would benefit from reducing radicals production and by enhancing antioxidant defenses reducing the ratio between the two.

8. The Double-Edged Role of Nitric-Oxide Synthases in Cardiac Dysfunction

Nitric-oxide synthases (NOSs) are extremely interesting molecules that produce NO by oxidizing the terminal guanidine nitrogen of L-arginine to L-citrulline. NOSs are present in 3 isoforms [43]: endothelial NOS3 (eNOS) and neuronal NOS1 (nNOS) are constitutively expressed in cardiomyocytes, while inducible NOS2 (iNOS) is absent in the normal myocardium, but its expression can be induced by proinflammatory mediators [25, 60–63]. NO is able to have diverse biological effects by posttranslational nitrosation/nitrosylation of specific cysteine thiol residues [43], mostly due to the cellular location in which NO is generated [28, 64]. NOS3 is mainly located into sarcolemmal caveolae and t tubules, where it interacts with caveolin-3 that modulates its activity and is connected with many cell surface receptors and β-adrenergic and bradykinin receptors [43, 65, 66]. NOS3-generated NO has a key role in depressing contractility and regulating β-adrenergic stimulation. On the opposite, NOS1 is usually described in the sarcoplasmic reticulum and coimmunoprecipitates with ryanodine receptors (RyRs), thus increasing contractility without altering ICa [25, 28, 61]. Hence, in contrast to NOS3, it appears that NOS1 has mainly a positive inotropic effect on the myocardium [43, 61].

Importantly, NO also plays an essential role in the maintenance of the O_2^-/NO homeostasis and can inhibit XO, thus behaving as an antioxidant [25, 67, 68]. In cardiac pathophysiology, maintenance of the nitroso/redox balance between RNS and ROS is critical [25, 27], since excessive oxidative and nitrosative stress are pivotal in many deleterious effects on the myocardium. Indeed, oxidative/nitrosative stress mediate cellular damage to organelles, DNA, proteins, lipids, and other macromolecules and can ultimately bring cardiomyocyte death [29]. Oxidative stress occurs when intrinsic antioxidant defenses are not able to protect from excessive ROS production.

Interestingly, in some pathological conditions, including HF, NOS can be uncoupled, and hence the flow of electrons from the reductase domain to the heme can be diverted to molecular oxygen instead of L-arginine, with following O_2^- production [69, 70]. Among the mechanisms that may be responsible for NOS3 uncoupling, tetrahydrobiopterin (BH4, a fundamental cofactor of NOS) deficiency has been often described [71–73]. Additionally, excessive ROS can further exacerbate NOS uncoupling [25]. NO generated by NOSs is able to react and interact with ROS. Indeed, in HF, ROS and RNS generated by different sources can decrease NO bioavailability. Such interactions can have a significant effect on myocardial contractility [33]. In failing hearts, beyond lower antioxidant defenses, diminished NO levels can bring a further increase in ROS because of NOS uncoupling [64] (Figure 1). Of notice, ROS such as superoxide can directly quench bioavailable NO even without affecting the expression and activity of NOS [74]. Superoxide anion (O_2^-) can react with NO, forming reactive species such as peroxynitrite, producing abnormalities in the nitroso-redox balance and further myocardial derangements [26, 30]. Importantly, in cardiomyocytes NO mediates S-nitrosylation of specific

cysteines [33, 75], with effects on Ca^{2+} fluxes and EC coupling [33, 76], but high levels of O_2^- can inhibit physiologic S-nitrosylation. High O_2^- concentrations interact with NO to form peroxynitrite that can produce numerous cytotoxic effects that may alter excitation-contraction coupling [26, 77, 78]. Additionally, in failing myocytes, NOS1 moves from its sarcoplasmic reticulum (SR) subcellular location to the sarcolemmal membrane [43, 79], disrupting the tight time- and substrate-dependent NOS regulation. Also, the high levels of NOS2 in failing myocytes appear to be, at least in part, a cause of the blunted myocardial inotropy after β-adrenergic stimulation [80, 81].

9. ROS-Mediated Alterations in Cardiac Dysfunction

In the heart, ROS stimulate transcription factors to promote hypertrophic signaling, therefore producing cardiac growth, remodeling, and dysfunction. ROS affect cardiac contractility and survival [4, 7, 25–30]. Cardiomyocytes apoptosis that is present in hypertrophy and HF contributes to development and progression of cardiac dysfunction [33, 82]. High levels of ROS have a key role in myocytes apoptosis. Indeed, at relatively low levels, ROS stimulates protein synthesis, while, at higher levels, there is activation of JNK and p38 MAPKs and Akt and induction of apoptosis [33]. Interestingly, in rat cardiomyocytes, H_2O_2 at low micromolar concentrations blunts contractile function significantly and activates ERK1/2 kinase with no effect on survival, while at higher micromolar concentrations H_2O_2 can stimulate apoptosis via JNK and p38 kinase [52].

ROS mediate the prohypertrophic signaling of alpha 1 adrenergic and angiotensin II pathways [83–87], by means of Ras thiol regulation [88]. In the heart, different signaling pathways involved in the modulation of cardiac hypertrophy, including protein kinase C (PKC), the MAPKs p38, JNK, apoptosis-signaling kinase 1 (ASK-1), and ERK1/2 [33], NF-κB, calcineurin, many tyrosine kinases, Akt, and Phosphatidyl-Inositol-3-Kinase (PI3K) [25–27, 89, 90], can be stimulated by ROS. Interestingly, H_2O_2 stimulates hypertrophy by activating PI3K in a time- and dose-dependent manner [91].

ROS can also stimulate myocardial fibrosis, thus contributing to myocardial remodeling [92, 93]: ROS can activate cardiac fibroblasts [94], regulate collagen synthesis [95], and activate posttranslationally matrix metalloproteases (MMPs) that are secreted in an inactive form [96].

Finally, ROS are able to regulate proteins of the excitation-contraction (EC) coupling machinery directly [97] (Figure 1). ROS oxidation of critical thiols on the RyR increases its open probability thus enhancing Ca^{2+} release, exacerbating Ca^{2+} overload and myocyte dysfunction [7, 98, 99]. ROS can also target sarcolemmal L-type Ca^{2+} channel, thus suppressing the Ca^{2+} current [100]. Additionally, they can blunt the activity of the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2), which plays an essential role in controlling Ca^{2+} cycling, with consequent myocytes dysfunction [33, 101]. Interestingly, low expression of SERCA2a can be already found in myocytes hypertrophied after ROS treatment [33]. Activation of Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) by ROS [102] is critically linked to remodeling of ionic homeostasis in various experimental hypertrophy models [103, 104] including familial hypertrophic cardiomyopathy [105]. In the myocardium, ROS can regulate the function of other important channels, including sodium channels, potassium channels, and ion exchangers, such as the Na^+/Ca^{2+} exchanger (NCX) and Na^+/H^+ exchanger type 1 [33, 106–110]. Also, in HF ROS can contribute to cardiac dysfunction by lowering myofilaments Ca^{2+} sensitivity [111, 112].

10. Antioxidant Therapeutics in Heart Failure

10.1. Standard Heart Failure Therapy That Possess Antioxidant Properties. During the last decades, treatment of HF has changed more than one time, along with the progressing pathophysiological knowledge of the disease. It initially focused on hemodynamic control and unloading of the heart with vasodilators and diuretics. Then, the concept that the compensatory neurohormonal response was no longer considered beneficial but rather worsening heart failure introduced inhibitors of renin-angiotensin-aldosterone system, as well as β-blockers, now used as current therapeutics. Then treatments focused on muscle stimulation in the weakened heart, but these therapeutics were set aside due to their detrimental effects when used in the long term [113] as demonstrated by several clinical trials [1, 4, 7, 114–118]. In the last years, implantable devices have had a remarkable impact on management of heart failure, since electrical devices controlled by microprocessors can deliver therapy, monitor disease, and prevent sudden cardiac death [2, 3, 7]. Interestingly, it has to be acknowledged that standard HF therapy is based on many drugs that possess redox properties (Table 1) [43]. For instance, current treatments with ACE inhibitors and ATII receptor blockers (ARBs) can limit ROS deleterious signaling [43, 119]. Indeed, ATII can induce hypertrophy via a Gαq mediated pathway that involves ROS generation and ROS-associated activation of various downstream signals [85, 120]. Consequently, in clinical practice, blockade of either ATII production or ATII binding to the AT1 receptor can prolong survival in patients. Notably, antioxidants that counteract ROS effects can also blunt ATII-induced hypertrophy [43, 86].

Spironolactone inhibits aldosterone actions, blunting the myocardial oxidant and profibrotic conditions that are a hallmark of HF. Indeed, aldosterone is able to activate NADPH oxidases, thus increasing ROS production [43, 121]. Similarly, correction of redox imbalance has been implicated in the therapeutic effects of eplerenone in HF [43, 122, 123].

Interestingly, recent studies on cardiac resynchronization therapy (CRT) demonstrate that the beneficial effects of this important device therapy also involve, among many, a redox-mechanism. In particular, in dyssynchronous HF, Cys294 of the mitochondrial F1-ATPase can form a disulfide bond with another cysteine residue, while introduction of CRT prevents disulfide formation with S-nitrosylation of Cys294 [124, 125].

Table 1: Properties of the main antioxidant therapeutics.

Components of standard heart failure therapy that possess antioxidant properties
ACEi, ARBs, ARNi, antialdosterone drugs: interference with RAAS signaling
Carvedilol: β1- and β2-adrenergic receptor blocker that also increase NO production or decrease inactivation
β3AR agonists: enhancement of myocardial β3-adrenergic coupling with NO-cGMP signaling
ARNi: enhancement of NPs/cGMP/PKG pathway
Drugs with redox effect that are not mainstream therapeutic approach in heart failure
PDE5 inhibition and BH4 supplementation: potentiating NO/cGMP/PKG signaling
Statins: NADPH oxidase inhibitors
Allopurinol: xanthine oxidases inhibitor
Ranolazine: inhibitor of elevated late I_{Na}
MAO inhibitors: blunting ROS production from MAOs
Novel therapeutic compounds that target ROS/RNS signaling pathways
SS-31 (MTP-131, Bendavia): direct action on mitochondrial function
Resveratrol: preservation of the LKB1-AMPK-eNOS signaling axis
HNO donors: improving Ca^{2+} cycling and myofilament Ca^{2+} sensitivity

ARNi: angiotensin receptor-neprilysin inhibitor.
AMPK: AMP-activated protein kinase.
NPs: natriuretic peptides.

Carvedilol is β1- and β2-adrenergic receptor antagonist with additional vasodilatory α1-blocking properties [126]. Its structure contains a carbazole moiety by which carvedilol can be considered also a potent antioxidant [126–128], as a result of increased NO production or decreased inactivation [126, 129].

The third-generation β-blocker nebivolol, by simultaneous stimulation of β3-adrenergic receptor (AR), can enhance NO signaling which is often lost in HF because of the lower NO bioavailability. The eNOS-dependency of nebivolol beneficial effects beyond conventional beta blockers was demonstrated in experimental models of post-MI and hypertrophy [130, 131]. A recent study [132] also showed that microdomain-targeted enhancement of myocardial β3AR/NO-cGMP signaling may be responsible, at least in part, for β1-adrenergic antagonist-mediated preservation of cardiac function in a volume-overloaded canine model. Additionally, the BEAT-HF trial (NCT01876433) is recently evaluating efficacy of oral treatment with a β3AR agonist in chronic HF, exploring also potential effects on diastolic function, symptoms, repolarization duration, and safety (Table 1).

10.2. Drugs with Redox Effect That Are Not Mainstream Therapeutic Approach to Heart Failure. Potentiating NO/cGMP signaling has provided beneficial effects on animal models of HF by means of phosphodiesterases 5 (PDE5) inhibition [133] and by BH4 supplementation [73]. cGMP/PKG (cGMP-dependent protein kinase) pathway negatively controls stress-response signaling. cGMP is generated upon natriuretic peptide binding to its receptors coupled to particulate guanylyl cyclase or upon NO activation of soluble guanylyl cyclase. Importantly, cGMP controls the activities of phosphodiesterases (which in turn control cAMP and cGMP hydrolysis) and can then activate PKG. This important kinase phosphorylates Ca^{2+} channels, myosin phosphatase, RGS2 (which negatively regulates G-protein-coupled receptors), and IRAG (which modulates inositol-1,4,5-trisphosphate-dependent Ca^{2+} signaling), troponin I, and phospholamban [134]. Enhancing cGMP/PKG signaling by inhibiting PDE5 seems to be able to attenuate and reverse cardiac hypertrophy induced by pressure overload [133] and blunt acute and chronic β-adrenergic stimulation and also protect against ischaemia-reperfusion injury and myocardial apoptosis induced by antitumoral agents [135–137]. Even though the first clinical trials with sildenafil in HF have been somehow disappointing, the concept that the cGMP pathway is a promising target to exploit has been corroborated by the recent results on the beneficial effects of neprilysin inhibition combined to ARBs [138].

Simvastatin (NADPH oxidase inhibitor) and allopurinol (xanthine oxidases inhibitor) both counteract oxidative stress and interfere with ROS-mediated hypertrophic signaling [139], blunting cardiac remodeling. In particular, statins can inhibit the isoprenylation and activation of Rac1 and other proteins of the Rho family, hence lowering NADPH oxidase activity [43, 140]. Additionally, it seems that statins have direct antioxidant effects on lipids, and it has been shown that the oxidation of LDL, VLDL, and HDL can be inhibited by hydroxyl metabolites of atorvastatin [43, 141]. Also, both short- and long-term therapies with statins can benefit endothelial dysfunction [43]. Recent work from Andres and colleagues [142] showed that acute cardioprotective effects elicited by simvastatin involve the protein Parkin that stimulates mitophagy and prevents mevalonate accumulation. The xanthine oxidase inhibitor allopurinol is currently studied to improve remodeling after MI in diabetic patients (clinicaltrials.gov: NCT01052272) [139].

Enhanced myocytes $[Na^+]_i$ has been recently shown to lower mitochondrial Ca^{2+} uptake, increasing ROS production [110]. The same group was able to prevent such enhanced ROS generation with an inhibitor of the mitochondrial Na^+/Ca^{2+} exchanger (mNCE), which decreased Na^+-induced Ca^{2+} exportation [109]. In turn, ROS could then activate Ca^{2+}/calmoduline kinase II [104, 143] that would increase late I_{Na} by interacting with the Na^+ channel [144, 145], thus generating a vicious cycle of high $[Na^+]_i$ and oxidative stress [110]. High $[Na^+]_i$ would then stimulate NCX and intracellular Na^+ would be exchanged with extracellular Ca^{2+} with consequent Ca^{2+} overload and electrical and mechanical dysfunction, in a scenario in which SERCA2a is inhibited and the RyR2 is activated by ROS [146, 147]. Hence, high $[Na^+]_i$ can be identified as an interesting therapeutic target for HF treatment [102]. Indeed, inhibiting the late I_{Na} with ranolazine has been proven beneficial in experimental HF [102, 148–151].

Other promising therapeutic targets are monoaminoxidases: MAO A and MAO B have been recently proposed to play a role in experimental hypertrophy and failure via increased generation of H_2O_2. Pharmacological or genetic manipulation of such enzymes could then prove beneficial in cardiac dysfunction [25, 152, 153] (Table 1).

10.3. Novel Therapeutic Compounds That Target the ROS/RNS Signaling Pathways. Other interesting compounds that may ameliorate cardiac function by acting on the redox milieu have been identified. SS-31 (MTP-131, Bendavia) [154] is a mitochondria-specific antioxidant that appears to decrease LV hypertrophy in a mouse model of ATII-induced hypertrophy [155] and improve postinfarction cardiac function preventing adverse left ventricular remodeling and restoring mitochondria-related gene expression in rats [156]. Four phase I trials with Bendavia have been completed, with an ongoing phase II trial in ischaemic cardiomyopathy [139, 157].

Resveratrol is a widely used antioxidant dietary supplement with promising experimental results on pressure overload cardiac hypertrophy, but beneficial effects on clinical hypertrophy have not yet been reported [139, 158].

Currently, in HF treatment the room for inotropic therapies such as dopamine, dobutamine, and milrinone is very limited by the mortality associated with long-term treatment with these drugs [115–117]. Nitroxyl (HNO) represents an alternative approach. HNO is a 1-electron-reduced and protonated sibling of NO and, like NO, is a gaseous signaling molecule and a potent vasodilator. Nevertheless, HNO appears to have distinct chemical and physiological properties and unique signaling pathways from those of NO [159, 160]. HNO was initially discovered to induce both venous and arterial dilation and positive inotropy in intact failing hearts. Following mechanistic studies have revealed multiple pathways that combine the strategies of these other approaches. Clinical interest in HNO is increasing in virtue of its positive inotropic effects. In vitro experiments suggested positive inotropic and lusitropic properties of HNO, while subsequent studies in healthy and heart failure dog models with the HNO donor Angeli's salt ($Na_2N_2O_3$) demonstrated significant improvements in load-independent LV contractility, associated with reductions in preload volume and diastolic pressure [161, 162]. These beneficial effects seem to be independent of cAMP/protein kinase A (PKA) and cGMP/PKG signaling [163] with no modification of L-type calcium channel activity [164], but rather related to modifications of specific cysteine residues on phospholamban [165, 166] and SERCA2a [167] and on myofilament proteins, correlating with increased Ca^{2+} sensitivity and force generation [168]. Recently, a new HNO donor, CXL-1020, has been developed, and both animal and clinical studies seem to confirm positive inotropic and lusitropic effects [118, 169–171] (Table 1).

11. Conclusions

ROS and RNS at physiological concentrations are beneficial molecules and play a role in the regulation of cellular signaling pathways [28]. ROS/RNS generation is finely regulated for proper myocardial homeostasis. Although oxidative and nitrosative stress can be deleterious and may therefore constitute a therapeutic target in HF, indiscriminate elimination of ROS and RNS by antioxidant treatments may not provide any improvement and may even impair physiological cellular functions, causing a complete loss of ROS/RNS signaling [172–175]. Indeed, antioxidants were shown to be able to counteract cardiac remodeling and improve contractility in many animal models of HF. However, when translated to the clinical arena, such therapeutic strategies [64] did not show the expected benefits or even worsened mortality [176], when the antioxidant effect was not paralleled by other pharmaceutical and biological properties, as for carvedilol [126]. Importantly, ROS biological effects on cardiomyocytes depend on the site of generation. Therefore, more specific, targeted, and "compartmentalized" antioxidant approaches that blunt local ROS/RNS production might be more successful in countering irreversible oxidative modifications. Furthermore, since in heart disease deranged mitochondria are the major generators of ROS, dictating the overall myocardial redox conditions, therapeutic strategies aimed at removing diseased mitochondria, thus promoting mitophagy, may help diminishing oxidative stress and ameliorating cardiac function [176].

Conflict of Interests

Carlo G. Tocchetti is a coinventor of the Canadian Patent no. 2,613,477: "Thiol Sensitive Positive Inotropes," issued on December 3, 2013.

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Research Article

Band 3 Erythrocyte Membrane Protein Acts as Redox Stress Sensor Leading to Its Phosphorylation by p^{72} Syk

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In erythrocytes, the regulation of the redox sensitive Tyr phosphorylation of band 3 and its functions are still partially defined. A role of band 3 oxidation in regulating its own phosphorylation has been previously suggested. The current study provides evidences to support this hypothesis: (i) in intact erythrocytes, at 2 mM concentration of GSH, band 3 oxidation, and phosphorylation, Syk translocation to the membrane and Syk phosphorylation responded to the same micromolar concentrations of oxidants showing identical temporal variations; (ii) the Cys residues located in the band 3 cytoplasmic domain are 20-fold more reactive than GSH; (iii) disulfide linked band 3 cytoplasmic domain docks Syk kinase; (iv) protein Tyr phosphatases are poorly inhibited at oxidant concentrations leading to massive band 3 oxidation and phosphorylation. We also observed that hemichromes binding to band 3 determined its irreversible oxidation and phosphorylation, progressive hemolysis, and serine hyperphosphorylation of different cytoskeleton proteins. Syk inhibitor suppressed the phosphorylation of band 3 also preventing serine phosphorylation changes and hemolysis. Our data suggest that band 3 acts as redox sensor regulating its own phosphorylation and that hemichromes leading to the protracted phosphorylation of band 3 may trigger a cascade of events finally leading to hemolysis.

1. Introduction

Due to their function in carrying oxygen and their high iron content, red blood cells (RBCs) are constantly exposed to oxidative stress [1]. In addition, RBCs may transiently experience oxidative stress when they are exposed to ROS crossing inflammatory tissues or interacting with oxidant contained in drugs or foods [2–4]. Moreover, a number of hemolytic disorders are also known to damage the RBC membrane increasing the production of free radicals originating from denatured hemoglobin species (hemichromes), invariably present in thalassemia, sickle cell disease [5–7] or with decreased ability of RBCs to deal with extracellular oxidants as in G6PD deficiency [8].

It is noteworthy that approximately 7% of world population is affected by those mutations which have been selected by malaria.

It is well known that RBCs respond to oxidative stress with a metabolic response finalized to maximize the production of NADPH and to regenerate the stores of GSH and thioredoxin. In parallel, RBCs also respond by activating tyrosine kinases determining the tyrosine (Tyr) phosphorylation of band 3, the most abundant RBC membrane protein and the major linkage between the cytoskeleton and the lipid bilayer [9–12]. In RBCs, hyperphosphorylation of band 3 has been constantly reported in all the prooxidant hemolytic disorders [13–15] and in malaria [16, 17], but the mechanisms

leading to its phosphorylation and its pathophysiological significance have been partially defined. We recently described that band 3 phosphorylation appears to be increased in intermediate thalassemia [18] and that this phenomenon is closely related to the formation of hemichromes. Band 3 phosphorylation and hemichromes formation have been also described in malaria infected RBC [19]. In both pathological situations, band 3 phosphorylation appears to play a permissive role in the release of membrane microparticles. Current knowledge appears to be still insufficient to explain the molecular details of the underlying mechanism, although some recent findings clearly indicate a role of band 3 phosphorylation in the regulation of metabolism mediated by the binding of deoxygenated hemoglobin (Hb) [20–22] and in the modification of the affinity between band 3 and ankyrin following oxidative stress [23].

The redox regulation of band 3 Tyr phosphorylation apparently involves different components. In a previous report, it has been demonstrated that oxidized band 3 is selectively phosphorylated [9]. Lyn is responsible for the phosphorylation of Tyr 359 and Syk is responsible for the phosphorylation of Tyr 8 and Tyr 21 [24–26]. Interestingly, all of those residues are located in the cytoplasmic domain of band 3.

Phosphatases (PTPs) have also been implicated in the phosphorylation of band 3 that follows oxidative stress [27–29] and inhibition of PTPs is due to the inhibitory Cys residue present in the catalytic site of some PTPs but the reactivity to H_2O_2 of the inhibitory Cys is 0.005-fold lower than GSH, indicating that, at its normal concentrations, GSH should very effectively protect PTPs from oxidative inhibition [30, 31]. Additional regulatory components could be also involved in the band 3 phosphorylation: Lyn kinase has been described to act as redox sensor [32]; Lyn activates Syk in different cell types and the role of Syk autophosphorylation remains to be elucidated [25, 26]. Moreover, all of those regulations have been mainly studied in immune cells and very little information is available on RBCs.

In the present report we performed a series of experiments to gain more information on the mechanisms that are involved in the Tyr phosphorylation of band 3 following a reversible membrane protein oxidation triggered by diamide and H_2O_2 and by hemichromes which cause irreversible oxidation.

2. Materials and Methods

2.1. Treatment of Red Blood Cells. Venous blood was drawn from healthy volunteers following informed consent and pelleted at 1000 g for 10 minutes at room temperature. After removal of the buffy coat, RBCs were again pelleted and washed 3 times with phosphate buffered saline (127 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 20 mM HEPES, 1 mM $MgCl_2$, and pH 7.4) in 5 mM glucose (PBS glucose) to obtain packed cells. RBCs were suspended at 20% hematocrit in PBS glucose and incubated at 37°C in 0.5 mM diamide at different incubation times (0, 30, 60, 120, 240, and 360 minutes) and then in the presence of different diamide concentrations. Separate experiments were also performed in 5 mM H_2O_2 or 1 mM phenylhydrazine (PHZ). pH was measured after 180 minutes and adjusted to 7.4 with NaOH. When necessary to avoid tyrosine phosphorylation, RBCs were pretreated with 10 μM of Syk inhibitor II (Calbiochem, USA), for 1 hour at 37°C in the dark, before oxidant treatment. For all protocols described, untreated controls were processed identically except that the inhibitor was omitted from the incubation. To prevent further phosphorylation of band 3, after incubation we washed the cells with cold buffer and membranes were immediately prepared.

2.2. RBC Membrane Preparation. Membrane proteins were prepared at 4°C on ice as previously described [9]. Briefly, 150 μL of packed RBCs was diluted into 1.5 mL of cold hemolysis buffer (HB) (5 mM sodium phosphate, 1 mM EDTA, pH 8) containing a protease and a phosphatase inhibitor cocktail and then washed up to 4 more times in the same buffer (until membranes became white) in a refrigerated Eppendorf microfuge at 25000 g. The preparations were stored frozen at −80°C until use. Membrane protein content was quantified using the CD Protein Assay (Bio-Rad).

2.3. SDS-PAGE. To perform one-dimensional electrophoresis, membrane proteins were solubilized in Laemmli Buffer [33] in a volume ratio of 1 : 1. 10 μg of proteins for analytical gels, 1 μg of proteins for anti-band 3, and 30 μg of proteins for anti-phosphotyrosine, anti-phosphoserine, and anti-Syk antibodies were loaded for western blot analysis and separated on 8% of polyacrylamide gel under reducing and nonreducing conditions. SDS-PAGE analysis was conducted by heating the sample for 5 minutes at 95°C and was run on the Bio-Rad mini-protean 3 setup.

2.4. Western Blot Analysis. Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes as previously described [16] and then probed with 5 different antibodies: monoclonal anti-band 3 antibody (B9277, Sigma-Aldrich, Saint Louis, MO) produced in mouse (directed to cdbd3) diluted to 1 : 50000; anti-phosphotyrosine (sc7020, Santa Cruz, CA); anti-phosphoserine (ab9332, Abcam, Cambridge, UK); polyclonal anti-band 3 (sc20657); and anti-Syk (sc28337). The final two produced in rabbit from Santa Cruz, CA, all are diluted to 1 : 2000. Secondary antibodies conjugated to infrared fluorescent dyes excitable at 680 nm or 800 nm (IRDye: anti-mouse 800 CW 926-32210, anti-mouse 680 CW 926-32220, and anti-rabbit 800 CW 926-32211, Li-COR, USA) were then used to visualize the desired antigens with a laser scanner (Odyssey, Licor, USA). Quantitative densitometry study of tyrosine phosphorylation was carried out, and Syk translocation and band 3 oxidation levels were measured analyzing western blot images by the Odyssey V3.0 software, and values were expressed as arbitrary units.

2.5. Measurement of Band 3 SH-Groups Reactivity in the Presence of Increasing GSH Concentrations. RBC membranes were diluted in HB to obtain a 5 μM band 3 concentration. Band 3 concentration was estimated measuring total

membrane proteins in packed membranes (approximately 4 mg/mL) considering that band 3 represents approximately 25% of total membrane proteins and a band 3 M.W. of 95.000 Da. Resuspended membranes were incubated for 10 minutes on ice, with 0.1 mM diamide in the presence of increasing concentrations of GSH. The reaction was stopped by washing the solution 3 times with HB. The percentage of oxidized band 3 was evaluated by western blot following nonreducing 8% SDS-PAGE and expressed as percentage of the maximal oxidation measured in the absence of GSH. In the absence of GSH an average of 95.2 ± 4.5% of the band 3 was found present in reducible aggregates with a M.W. >200.000 KDa.

2.6. PTP Activity Measurement. Erythrocyte PTP activity was measured using phosphorylated band 3 as substrate. Phosphorylated band 3 was obtained treating RBCs with 1 mM diamide. Membranes were prepared and incubated for 10 min at 37°C with the cytoplasmic fraction of RBCs treated with different concentrations of diamide. 10 μM Syk inhibitor II (Calbiochem, USA) was added to prevent further phosphorylation of band 3. The rate of band 3 dephosphorylation was expressed as PTP activity and as a percentage of maximal activity in untreated RBCs.

2.7. Hemoglobin Release Quantification. We used a simplified method to measure the relative changes of Hb in RBC cultures supernatant; after discarding RBC membranes by centrifugation, lysis was quantified by measuring hemoglobin absorbance at 405 nm in RBC supernatant and expressed in nmoles/mL [34].

2.8. Hemichrome Measurement. RBCs were solubilized in HB containing 1% Triton X-100, centrifuged at 15.000 g at 4°C. High molecular weight hemichrome aggregates were separated from the supernatant on a Sepharose CL-6B microcolumn. The hemichrome fraction was then diluted and quantified measuring heme absorbance at 560, 577, and 630 nm [35] and expressed as nmoles/mL of solubilized membranes.

2.9. Immunoprecipitation Studies. RBC membrane proteins were treated in the presence or the absence of 2 mM diamide, solubilized for 10 minutes on ice with 3 volumes of 1% Triton X-100 in HB. After centrifugation in a refrigerated Eppendorf microfuge at 15000 g, supernatants were collected and incubated with anti-mouse anti-band 3 cross-linked to Protein A-Sepharose (1 : 10) via bifunctional coupling reagent dimethyl pimelimidate for 2 hours at 4°C under gentle mixing. Beads were washed three times with 1% Triton X-100 in HB [9]. Laemmli buffer, containing 2% DTT (final concentrations), was added to packed beads (2 vol) and immunoprecipitated proteins were analyzed by immunoblotting using anti-band 3 and anti-Syk antibodies.

2.10. Cytoplasmic Domain of Band 3 Fragment Phosphorylation in Reconstituted Systems. To obtain the oxidized and nonoxidized cdbd3 fragment, RBCs were incubated with or without diamide (2 mM). Membranes were prepared as described above and cytoskeletal proteins were eliminated incubating the membranes with 0.1 M NaOH at 4°C (stripped membranes). Cdbd3 was then purified from RBC membranes as previously described [36]. The purity of cdbd3 was higher than 90%. After diamide treatment more than 60% of cdbd3 was present as disulfide cross-linked dimers. To measure band 3 phosphorylation in the presence of soluble oxidized and nonoxidized cdbd3, RBC membranes were incubated at 37°C for 10 minutes with RBC cytoplasm (diluted 1 : 10) as previously described [9]. The reaction was stopped by washing the membranes with HB. Band 3 tyrosine phosphorylation was then measured by western blotting as described above. The association between Syk and cdbd3 was tested incubating oxidized and nonoxidized cdbd3 with RBC cytoplasm at 37°C for 10 minutes and anti-band 3 immunoprecipitation was followed by western blot using anti-Syk antibody diluted 1 : 100.

2.11. Peptide Preparation for MS Analysis. Bands were excised from electrophoresis gels and were destained by doing several washes in 5 mM NH_4HCO_3/ACN (acetonitrile) (50/50 v/v) and successively dried with pure ACN. The gel slices were rehydrated for 45 minutes at 4°C in 20 μL of 5 mM NH_4HCO_3 digestion buffer containing 10 ng/μL of trypsin. Excess protease solution was removed and the volume was adjusted with 5 mM NH_4HCO_3 to cover the gel slices. Digestion was allowed to proceed overnight at 37°C.

2.12. Peptide Mass Fingerprinting by MALDI-TOF MS. Samples were loaded onto MALDI target using 1 μL of the tryptic digests mixed 1 : 1 with a solution of CHCA (alpha-cyano-4-hydroxycinnamic acid) (10 mg/mL in ACN/TFA 0.1%, 40/60). MS analysis of peptides from 1-DE gel bands was performed with a MALDI-TOF micro MX (Micromass, Manchester, UK) according to the tuning procedures suggested by the manufacturer. Peak lists were generated with Proteinlynx Data Preparation using the following parameters: external calibration with lock mass using mass 2465.1989 Da of ACTH fragment 18-39 background was subtracted using the adaptive mode, performing deisotoping with a threshold of 3%. The MS spectra were converted into pkl files using Mass Lynx 4.0. Peak lists containing the 20 most intense peaks of the spectrum were sent to MASCOT PMF search (http://www.matrixscience.com/) using a Swiss-Prot database (release 50.0, 30 May 2006). Search settings allowed one missed cleavage with the trypsin enzyme to be selected, carboxymethylated cysteine as fixed modification and oxidation of methionine as potential variable modification and a peptide tolerance of 50 ppm. Only protein identifications with significant Mascot scores ($p < 0.05$) were taken in consideration.

3. Results

3.1. Tyrosine and Serine Phosphorylative Response to Different Oxidant Species. Time dependent phosphorylative changes

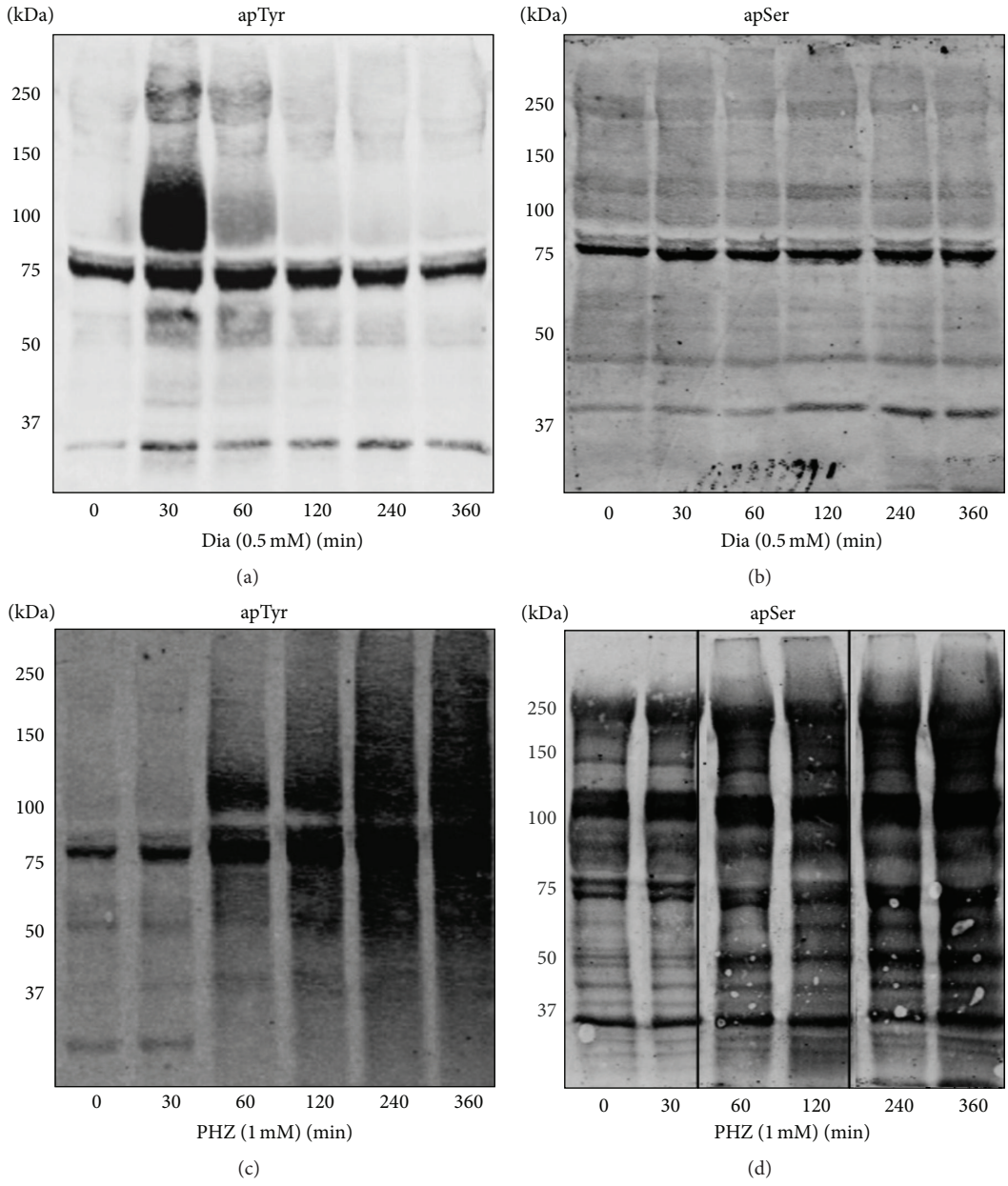

Figure 1: Time course of erythrocyte membrane proteins treated with oxidants. Erythrocytes were treated with 0.5 mM diamide (Dia) (panels (a) and (b)) and with 1 mM phenylhydrazine (PHZ) (panels (c) and (d)) at different incubation times. Membrane proteins were separated by 8% SDS-PAGE in the presence of reducing agent, blotted on nitrocellulose and stained with anti-phosphotyrosine (apTyr) and anti-phosphoserine (apSer) antibodies. Images were acquired using a laser IR fluorescence detector (Odyssey, Licor, USA). Results are representative of 4 separated experiments.

of the RBC membrane proteins have been measured comparing the effects of (i) diamide, a single electron oxidant that induces disulfide formation [9, 26], (ii) hydrogen peroxide (H_2O_2) that is physiologically generated from superoxide anion during methaemoglobin formation and by denatured hemoglobin products [37], and (iii) phenylhydrazine (PHZ) that reacts specifically with hemoglobin determining the formation of hemichromes which are capable of triggering ROS production [38, 39].

Diamide caused an intense and transient Tyr phosphorylation of band 3 and of proteins 4.1 and 4.2 though to a lesser extent and Ser phosphorylation changes in additional membrane protein (Figures 1(a), 1(b), and 2 and Table 1). H_2O_2 induced a phosphorylation response identical to diamide but at 10-fold higher concentration (data not shown). This is plausibly due to the potent scavenging activity of catalase and glutathione peroxidase in RBCs on H_2O_2.

Conversely PHZ caused a slow phosphorylation response measurable only after 60 minutes of incubation (Figures 1(c) and 1(d)). Tyr phosphorylation of band 3 was one of the earliest events but additional proteins were also phosphorylated at Tyr and Ser residues after 120–360 minutes of

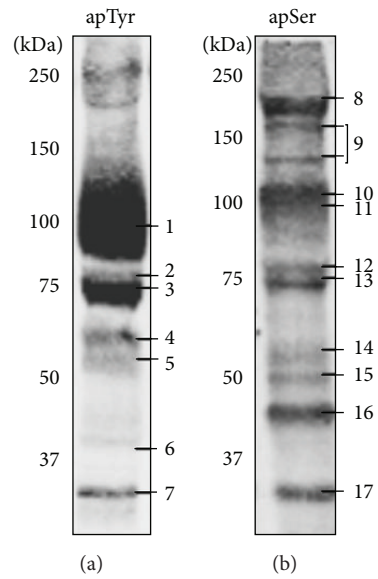

Figure 2: Protein phosphorylation analysis by mass spectrometry. Tyrosine phosphorylated proteins after 30-minute incubation of RBCs with 0.5 mM diamide (a) and serine phosphorylated proteins after 2-hour incubation of RBCs with 1 mM PHZ (b). Phosphorylated proteins were analyzed by mass spectrometry (MALDI-TOF) (Table 1). Band numbering in panels (a) and (b) identifies the proteins listed in Table 1.

Table 1

Band number	Protein name	Accession number	Phosphorylated peptide
1	Band 3	P02730	MEELQDD**Y**ED (Tyr 8) **Y**EDPDIPESQ (Tyr 21) PAKPDSSF**Y**K (Tyr 359)
2	Protein 4.1	P11171	V**Y**ECVVEKHA (Tyr 222)
3	Protein 4.2	P16452	Not identified
4	Catalase	P04040	KVWPHKD**Y**PL (Tyr 308)
5	P55	Q00013	AIRSQYAH**Y**F (Tyr 429)
6	Actin	P60709	GRDLTD**Y**LMK (Tyr 188)
7	G3PDH	P04406	PFIDLN**Y**MVY (Tyr 42)
8	Beta spectrin	P11277	ERT**S**PVSLW (Ser 2114)
9	Ankyrin	P16157	DQVVE**S**PAIP (Ser 856)
10	Alpha Adducin	P35611	REKSKKY**S**DV (Ser 408)
11	Beta Adducin	P35612	TP**S**FLKKSKK (Ser 713)
12	Protein 4.1	P11171	QEQYE**S**TIGF (Ser 461) RH**S**NLMLEDL (Ser 664)
13	Protein 4.2	P16452	LLNKRRG**S**VP (Ser 248)
14	Catalase	P04040	TFVQSG**S**HLA (Ser 517)
15	P55	Q00013	SC**S**PFGKKKK (Ser 243)
16	Actin	P60709	ANTVL**S**GGTT (Ser 300)
17	G3PDH	P04406	I**S**WYDNEFGY (Ser 312)

incubation. These data were also supported by the absence of reactivity when the proteins were treated with λ-phosphatase, which was used to remove phosphate groups from blotted proteins (data not shown). Control experiments to exclude a direct oxidant effect of PHZ (2 mM) on isolated membranes revealed no effect on band 3 sulfhydryl groups (data not shown). The lack of short term effects of PHZ on membrane protein phosphorylation is coherent with its specific action on hemoglobin and the slow formation of hemichromes [38, 39]. A list of phosphorylated proteins, identified by mass spectrometry, is shown in Figure 2 and Table 1. At least one phosphorylation site has been identified on each phosphoprotein with the exception of protein 4.2. It should be noticed that similar protein phosphorylation patterns have been previously observed in pathological situations characterized by high content of hemicromes such as malaria infected RBCs, G6PD deficiency, and thalassemia [16, 18].

3.2. Functional Relationships between Band 3 Oxidation and Its Tyr Phosphorylation. To obtain quantitative data on the relationships intercurring between band 3 oxidation and its Tyr phosphorylation, we performed parallel measurements of the detection limits of band 3 phosphorylation (Figure 3(a)) and its oxidative crosslinking (Figure 3(b)) starting from very low concentrations of diamide. This experiment revealed that both events become measurable at the same diamide concentration (10–25 μM) that, due to the buffering effect of cellular GSH, are not expected to exert an effect on protein thiols. We observed a dose-dependent increase of the phosphorylation signal (Figure 3(c)) and of the binding of Syk to the membrane (Figure 3(d)). Interestingly both phenomena became detectable in the same concentration range of diamide. Those data comprehensively indicate that, as previously suggested [9], Syk acts preferentially on oxidized band 3. To further investigate this hypothesis, we tested if purified oxidized cdbd3 fragment may exert a competitive inhibitory action on the phosphorylation of

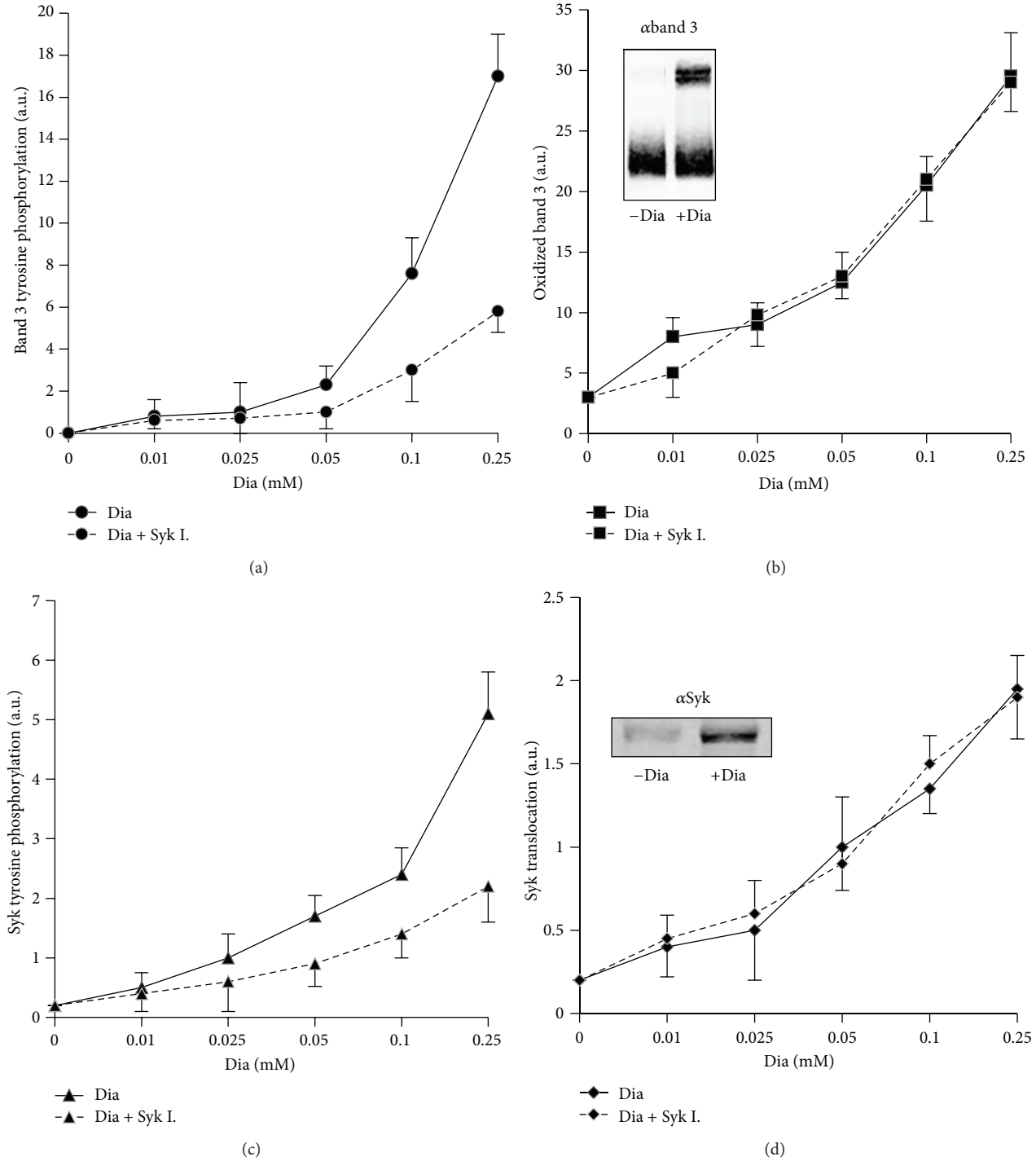

Figure 3: Band 3 modifications and Syk activation following diamide treatment. RBCs were treated with increasing concentration of diamide for 30 min in the presence or in the absence of 10 μM Syk inhibitor II (Syk I.). Band 3 tyrosine phosphorylation (panel (a)). Band 3 oxidative crosslinking (oxidized band 3, Dia) expressed as the amount of oligomeric band 3 (apparent M.W. higher than 200 KDa) under nonreducing conditions (panel (b)). Syk tyrosine phosphorylation measured in whole cellular extracts (panel (c)). Syk bound to the membranes (Syk translocation) (panel (d)). Western blotting was quantified using an IR fluorescence detection scanner (Odyssey, Licor, USA). Images were analyzed by Odyssey V3.0 software. Values are representative of 4 separated experiments and are expressed as arbitrary units (au); the bars represent the standard deviations.

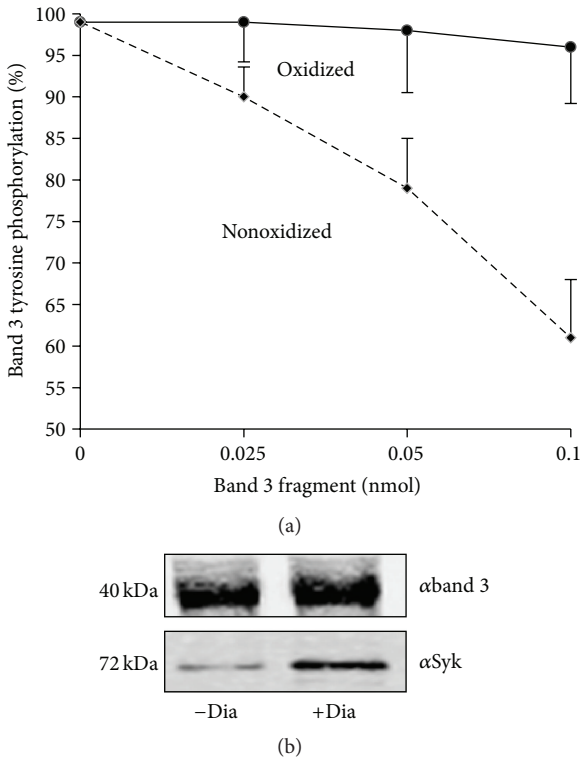

Figure 4: Competitive effect of oxidized and nonoxidized cdbd3 on band 3 phosphorylation and its association with Syk. Band 3 Tyr phosphorylation was measured in membranes obtained from diamide treated RBCs in the presence of RBC cytoplasm at increasing concentration of oxidized or nonoxidized cdbd3. The level of band 3 phosphorylation is displayed as percentage of its maximal phosphorylation absence of cdbd3 (panel (a)). Nonoxidized (−Dia) cdbd3 and oxidized (+Dia) cdbd3 were incubated with RBC cytoplasm and immunoprecipitated by anti-cdbd3 antibody. Immunoprecipitated proteins were western blotted with anti-band 3 (panel (a)) and anti-Syk antibody (panel (b)). Western blots are representative of 4 separated experiments.

band 3. Figure 4(a) shows that oxidized cdbd3 exerts a dose-dependent inhibitory effect on band 3 phosphorylation, while nonoxidized cdbd3 fragment did not exert any measurable effect. RBC membranes containing oxidized band 3 were used as PTPs substrate; on the contrary, experiment performed with purified band 3 provided unreproducible results possibly indicating that the requirement of a specific quaternary structure of oxidized band 3 is essential to allow the docking of Syk. Immunoprecipitation studies confirmed that Syk binds prevalently to the oxidized form of cdbd3 (5.8-fold higher, $p < 0.01$) while no significant difference has been observed between the amount of cdbd3 immunoprecipitated from oxidized and nonoxidized samples (Figure 4(b)).

3.3. Measurement of the Accessibility of the Band 3 Cys Residues. The characteristic accessibility of the two cysteins 201 and 317 located in the cytoplasmic domain of band 3 has been already demonstrated [36]. To obtain quantitative data on the relative accessibility of those Cys residues, we measured the effect of diamide (100 μM) in forming band 3 (5 μM) intermolecular disulfide bonds on increasing concentrations of GSH. This experiment showed that at 0.1 mM GSH concentration (20-fold higher than band 3) approximately 40% of band 3 was still oxidized by diamide; at 1 mM GSH concentration (200-fold higher than band 3) approximately 20% of band 3 was still oxidized (Figure 6(a)), indicating much higher accessibility of the Cys residues located in the cdbd3 than GSH. Those results are therefore in agreement with the observed oxidation of band 3 with low concentrations of diamide as the blood concentration of GSH is approximately 1 mM. Moreover, we have noticed that at low concentrations of diamide (50–100 μM) no oxidation of GSH was detectable (data not shown).

3.4. Comparative Analysis of Syk Kinase and Protein Tyr Phosphatase Activities following Sulfhydryl Group Oxidation. Erythrocyte PTPs have been implicated in promoting the Tyr phosphorylation of band 3 due to an inhibitory Cys residue located in their catalytic domain [10, 40].

To rule out the possibility that diamide treatment, at concentrations that induce band 3 phosphorylation, may also determine a substantial inhibition of PTPs, we compared the levels of band 3 phosphorylation and PTP inhibition at different diamide concentrations. Figure 6(b) shows that, after treating RBCs with 1 mM diamide that causes a nearly maximal band 3 phosphorylation, PTPs still display approximately 70% of their maximal activity. This finding is in agreement with the low reactivity of the regulatory Cys residue of PTPs that has been previously reported [30].

3.5. Effect of Syk Inhibitors on the Membrane Destabilization Induced by Phenylhydrazine . Differently from the effect of diamide that caused reversible changes, after phenylhydrazine treatment, band 3 oxidation, and its phosphorylation, Syk translocation to the membrane and its phosphorylation increased progressively, paralleling the generation of hemichromes (Figure 5). Also in this case Syk inhibitors markedly inhibited band 3 phosphorylation with no apparent effect on band 3 oxidation and Syk translocation (Figures 3 and 5).

Interestingly, Figure 5(f) shows that phenylhydrazine treatment causes a progressive leak of hemoglobin through the membrane. Hemolysis was substantially suppressed by Syk inhibitors indicating that irreversible band 3 phosphorylation may induce a progressive destabilization of the membrane plausibly through the weakening of the linkages between band 3 and cytoskeleton [23].

4. Discussion

RBCs rapidly react to oxidative stress through very intense Tyr phosphorylation of band 3, their major integral membrane protein. We previously found that the phosphorylation of band 3 affects its interactions with the cytoskeleton inducing membrane destabilization [23]. This phenomenon appears to play a central role in the release of membrane microparticles containing hemichromes from thalassemic

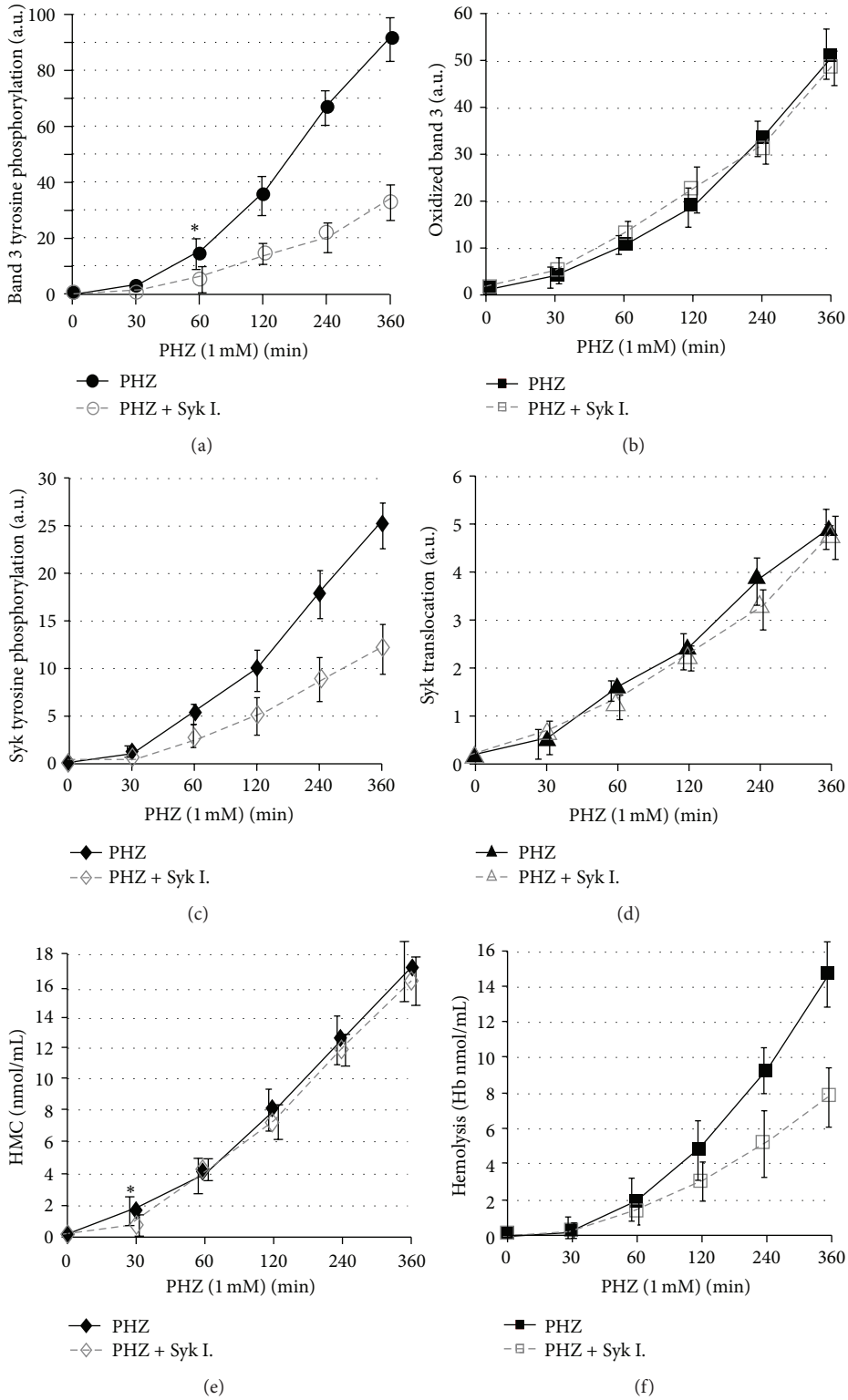

Figure 5: Band 3 modifications, hemichrome formation, and hemolysis after phenylhydrazine treatment. Erythrocytes were treated with 1 mM phenylhydrazine (PHZ) at different incubation times in the presence or in the absence of Syk inhibitor II (Syk I.). Band 3 tyrosine phosphorylation (panel (a)), oxidized band 3 (panel (b)), Syk phosphorylation (panel (c)), and Syk translocation (panel (d)) were quantified acquiring anti-phosphotyrosine, anti-band 3, and anti-Syk western blots using an IR fluorescence detection scanner (Odyssey, Licor, USA) and analyzing images with Odyssey V3.0 software. Values are the average of 5 separated experiments and are expressed as arbitrary units (au); the bars represent the standard deviations. Hemichromes (HMC) were quantified by vis spectrometry (panel (e)), hemolysisby measuring hemoglobin absorbance at 405 nm (panel (f)) and are expressed in nmoles/mL. $*$ indicates the minimal concentration or shorter incubation time that determines a statistically significant change by Student's t-test in comparison to the control sample ($p < 0.01$).

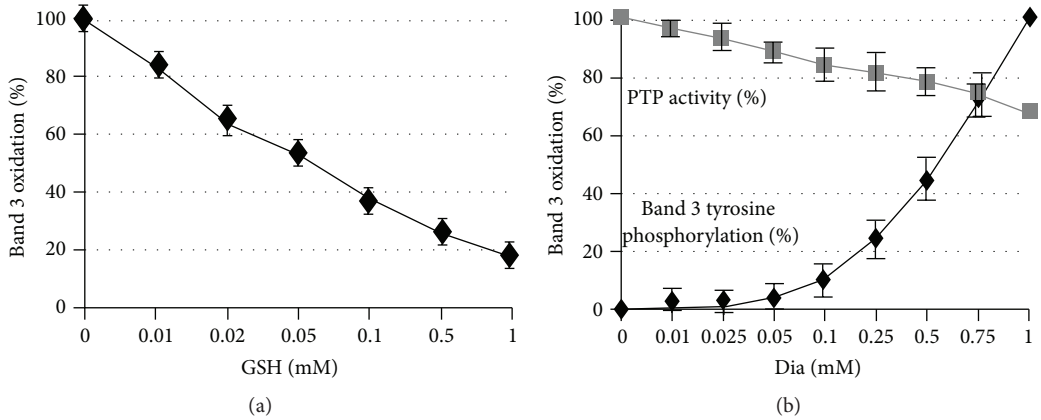

Figure 6: Comparative analysis of Syk kinase and protein Tyr phosphatase activities following diamide treatment. Erythrocyte membranes were treated with 0.1 mM diamide in the presence of increasing concentrations of GSH. Band 3 oxidation (percentage of maximal oxidation, in the absence of GSH) is showed in panel (a). Tyr kinase activity was measured as band 3 tyrosine phosphorylation at different diamide concentrations and expressed as percentage of maximal phosphorylation. PTP activity was measured as dephosphorylation of Tyr phosphorylated band 3 (obtained treating RBCs with 1 mM diamide) and expressed as percentage of maximal PTP activity (panel (b)). Band 3 oxidation and phosphorylation were quantified by Odyssey V3.0 software. Values are average of 3 separated experiments; the bars represent the standard deviations.

erythrocytes and appears to be involved in the action mechanism of some prooxidant antimalarial compounds [18, 41]. Denatured hemoglobin products (hemichromes) are generated in senescent erythrocytes in numerous hemolytic diseases [42] and in malaria [16]. They bind to the cdbd3 and release iron in the membrane generating free radical species. Therefore, hemichromes appear to be a sensible source of redox stress under physiological conditions and in prooxidant pathological conditions. The crystal structure of cdbd3 indicates a tight dimer formed by interlocking dimerization arms of the two monomers. The Cys 201 residue in one subunit and the Cys 317 residue of the paired subunit are at close distance and can easily form intermolecular disulfide bonds following moderate oxidative stress [36, 43] and following the binding of hemichromes [18, 35]. No data are available on the structural changes induced by this modification but previous findings demonstrated an increased accessibility of extracellular band 3 epitopes following the exposure to very low concentration of oxidants and in senescent red cells [44–46], suggesting that conformational modification may occur following disulfide crosslinking of band 3.

Anyway, the involvement of Syk kinase of band 3 oxidation and of all the major steps of the pathway such as the mechanism of redox sensing, its transduction, the regulation of Syk activation, and docking to band 3 need to be clarified to envisage the physiological role of this intense redox response characteristic of erythrocytes. To address those issues, the present work has been performed to obtain a series of quantitative data to study (i) the temporal and dose effects of different physiological and nonphysiological oxidants in eliciting the minimal band 3 and Syk modifications, (ii) the role of disulfide cross-linked band 3 in docking Syk, (iii) the buffering effect of GSH on the oxidation of band 3 Cys residues to rule out if band 3 could display activity as redox sensor in intact erythrocytes, and (iv) the relative roles of Syk activation and docking versus PTPs inhibition in the phosphorylation of band 3.

The presented results indicate that band 3 possess highly reactive Cys residues capable of being easily oxidized in the presence of physiological concentration of GSH and that disulfide cross-linked band 3 docks Syk and acts as competitive inhibitor of band 3 phosphorylation. Those results support the observed changes in whole RBCs with very low concentration of a specific sulfhydryl reagent (diamide) or following the formation of minute amounts of hemichromes. In both models band 3 phosphorylation exactly parallels its oxidation. On the other hand erythrocytes seem to be fairly protected by H_2O_2. The comparative measurement of band 3 phosphorylation and of PTPs inhibition at different concentrations of diamide revealed that intense phosphorylation can occur at concentrations that minimally inhibit erythrocytes PTPs acting on phospho-band 3. This finding is in accordance with the relatively low reactivity of the Cys residue located in the catalytic site of PTPs [30] and with the much higher reactivity of the Cys residues located in the cdbd3.

In the present report, we observed that Syk inhibitors are potent inhibitors of the hemolysis that follows to the generation of hemichromes. Considering that treatment with diamide or H_2O_2 which induces a transient phosphorylation of band 3 does not cause hemolysis, a persistent phosphorylation of band 3 induced by irreversible hemichromes apparently leads to a severe membrane destabilization. It should be anyway noticed that hemichromes formation was also accompanied by serine phosphorylative changes involving some membrane protein; those phosphorylation changes have been usually considered to cause a decrease of the affinity between some components of the RBC membrane junctional complexes [47–50] and may therefore contribute to an alteration of the membrane structure.

Considering that band 3 phosphorylation may have a function in remodeling the RBC membrane to remove noxious hemichromes [18], the present findings support the hypothesis that erythrocytes may possess a very straight and effective mechanism to sense the oxidative stress exerted by low amounts of hemichromes through band 3 oxidation, selective docking of Syk, and phosphorylation of two band 3 Tyr residues critical for assuring the local stability of the membrane.

Abbreviations

ROS: Reactive oxygen species
GSH: Glutathione
G6PD: Glucose 6 phosphate dehydrogenase
PTKs: Protein tyrosine kinases
PTPs: Protein tyrosine phosphatases
cdbd3: Cytoplasmic domain of band 3.

Conflict of Interests

The authors declare no competing financial interests.

Acknowledgment

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Review Article

Redox Modulations, Antioxidants, and Neuropsychiatric Disorders

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Although antioxidants, redox modulations, and neuropsychiatric disorders have been widely studied for many years, the field would benefit from an integrative and corroborative review. Our primary objective is to delineate the biological significance of compounds that modulate our redox status (i.e., reactive species and antioxidants) as well as outline their current role in brain health and the impact of redox modulations on the severity of illnesses. Therefore, this review will not enter into the debate regarding the perceived medical legitimacy of antioxidants but rather seek to clarify their abilities and limitations. With this in mind, antioxidants may be interpreted as natural products with significant pharmacological actions in the body. A renewed understanding of these often overlooked compounds will allow us to critically appraise the current literature and provide an informed, novel perspective on an important healthcare issue. In this review, we will introduce the complex topics of redox modulations and their role in the development of select neuropsychiatric disorders.

1. What Are Redox Modulations?

As a dynamic environment, a variety of chemical reactions are constantly occurring within our cells at all times. A common type of reaction, the reduction-oxidation (redox) reaction, plays a vital role in maintaining cellular functions (Table 1) including metabolic cycles (e.g., NAD^+ and NADH recycling) and detoxification of harmful substances [1]. In these reactions, usually facilitated by an enzyme, one reactant loses electrons (becomes oxidized) and another gains those same electrons (becomes reduced) [1, 2]. As a result, our cells must maintain a delicate electrical balance between the various macromolecules that comprise them. This balance between oxidized and reduced compounds within the cell is known as the redox status [1, 2]. In a healthy cell, this balance is maintained as a result of our natural, endogenous antioxidant defences counteracting the continuous production of reactive species. Under normal conditions, reactive species are commonly produced as by-products of metabolism [3]. Over time, however, our bodies have evolved adaptations to not only detoxify these reactive species but use them to fulfill useful biological functions [4] (Table 1). In cases where this balance of antioxidants and reactive species is disrupted by an *excess* or *deficiency* of either one, our body experiences a strong modulation of its redox status, commonly referred to as oxidative stress [5].

Redox modulation is defined as an imbalance in the redox status. If this imbalance is a shift towards a drastically more oxidized environment, it is characterized by alterations in cellular dynamics and varying degrees of DNA, RNA, protein, and lipid damage [6]. While there are many compounds such as reactive carbon and bromine species that can cause damage to our cells, the focus of this review will be on reactive oxygen species (ROS) and reactive nitrogen species (RNS) due to their high prevalence within our body and the surrounding environment [7].

As shown in Figure 1, the majority of ROS and RNS species originate from the metabolism of oxygen in the

Table 1: Biological roles of reactive species.

Neurological	Cardiovascular	Immune response	Cell biology
Mediation of learning and memory Involved in the regulation of striatal dopamine release via glutamate	Regulation of cardiac contractility Regulation of vascular tone (e.g., penile erection) via NO production Signalling involving carotid bodies (monitor arterial oxygen levels)	Response to foreign pathogens (oxidative burst) Production of cytokines Wound repair	Embryogenesis Prevent overpopulation of cells and destroys malfunctioning cells Cellular differentiation

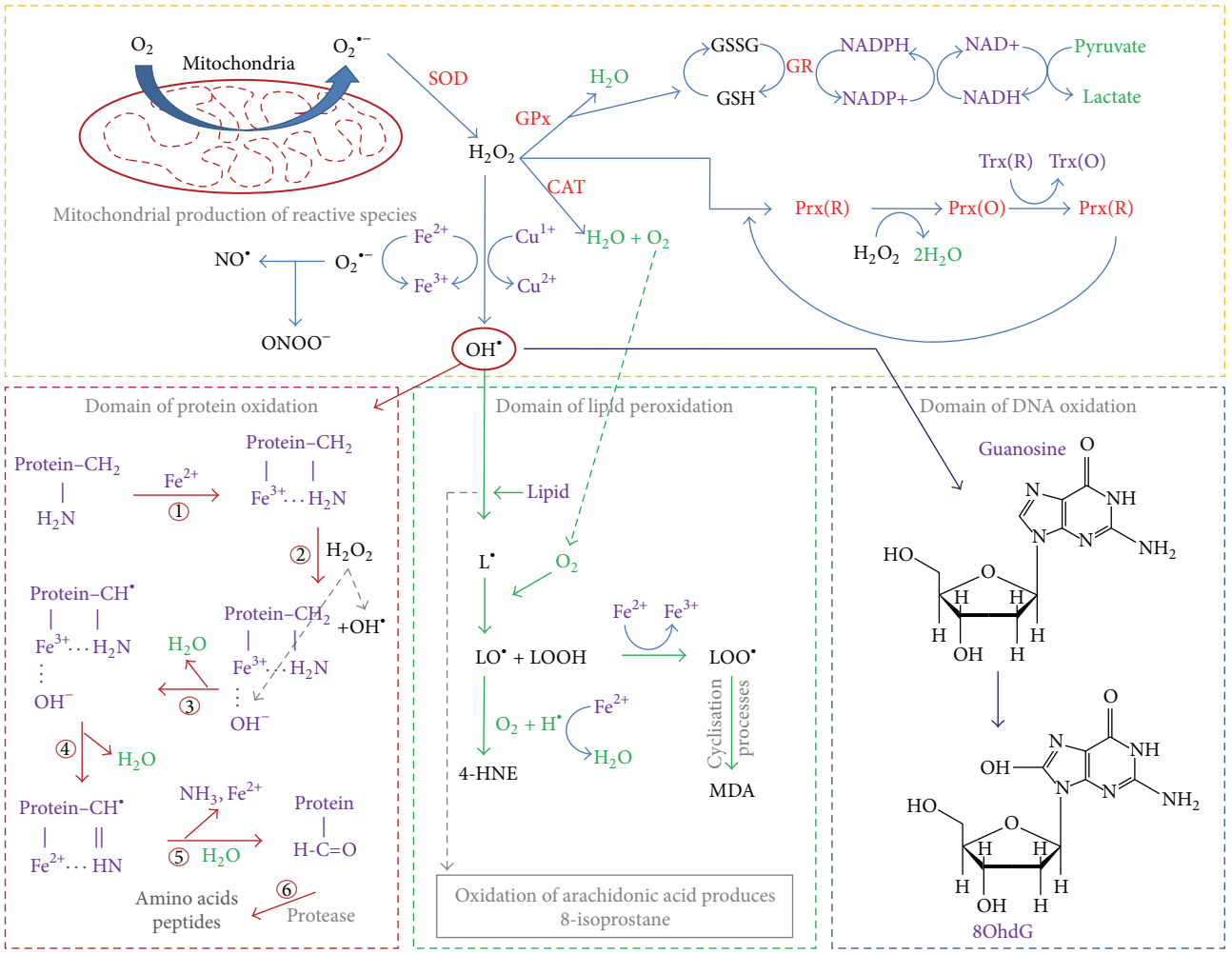

Figure 1: Production of reactive species and the endogenous antioxidant system. Red: enzymes; green: other products; purple: cofactor/substrate; black: reactive species.

mitochondria [8] . The primary reactive by-product, the superoxide anion ($O_2^{\bullet -}$), is exported from the mitochondria into the cytosol, via an anion channel, where it proceeds through numerous chemical reactions in our body's attempt to reduce its toxicity. Unfortunately, at the same time and under the correct environmental conditions, the superoxide anion can be converted into additional reactive species either directly or indirectly through catalysis [9]. A common example within the human body is the reduction of hydrogen peroxide into hydroxyl radicals via transition metals, usually iron (Fenton and Haber-Weiss reactions) [10] (following equations):

$$Fe^{3+} + {}^{\bullet}O_2^{-} \longrightarrow Fe^{2+} + O_2 \quad \text{(Reduction of ferric iron)} \tag{1}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{-} + {}^{\bullet}OH \quad \text{(Fenton Reaction)} \tag{2}$$

$${}^{\bullet}O_2^{-} + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^{-} + O_2 \quad \text{(Net Reaction)} \tag{3}$$

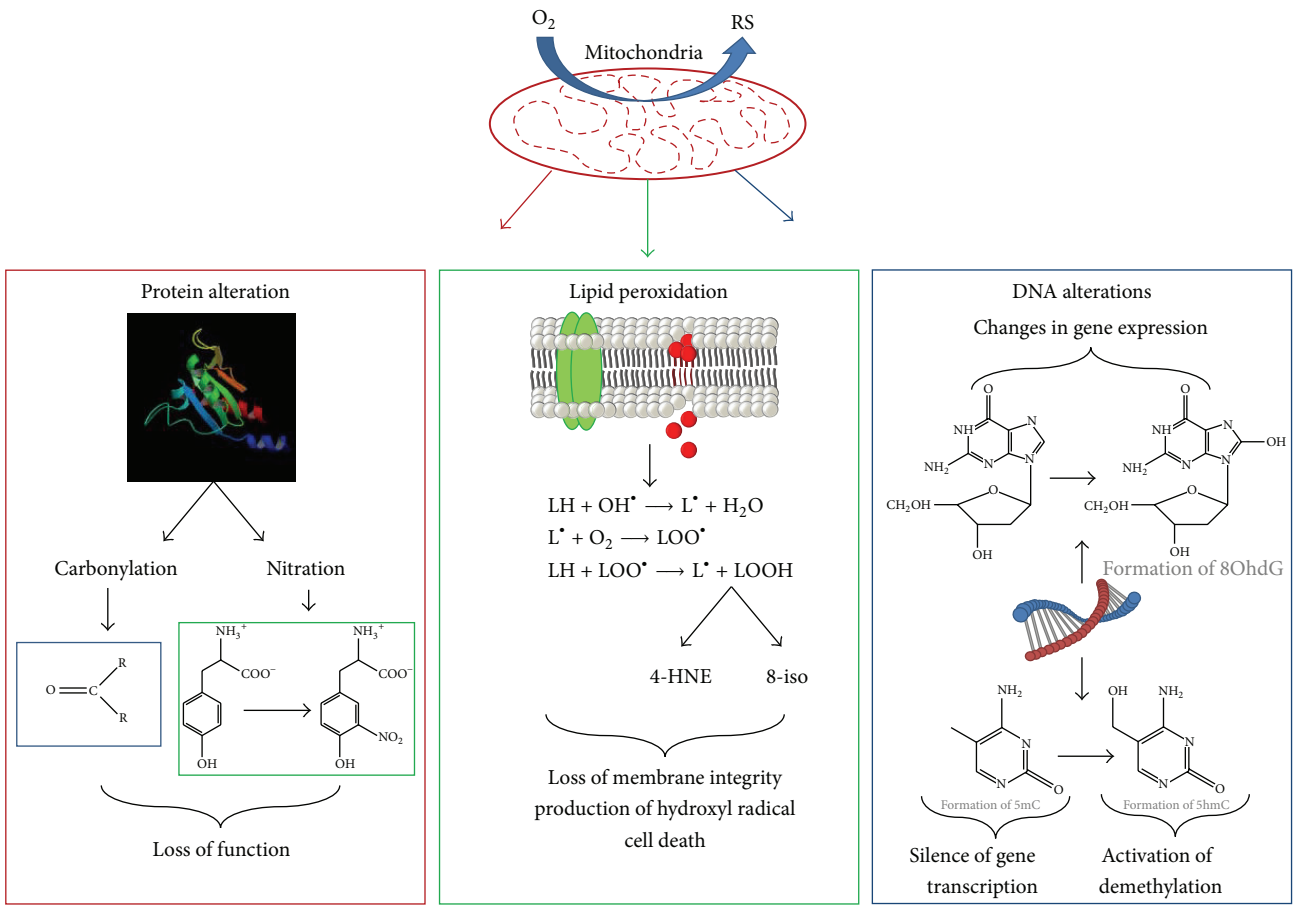

Figure 2: Examples of the effects of reactive species in the cell.

2. Macromolecular Changes Caused by Reactive Species

Once these toxic molecules are produced in the body, they begin to interact with DNA, lipids, and proteins to cause damage, leading to an alteration in cellular function (Figure 2). It is important to remember that although the effects shown in Figure 2 are negative, not all changes caused by reactive species are detrimental to the body [4]. In fact, recent evidence has provided support for the hypothesis that posttranslational modifications, such as carbonylation, S-nitrosylation, and nitration, play a vital role in the degradation of unnecessary or damaged proteins, maintaining cellular health [11, 12]. A second example is the regulation of cellular development by H_2O_2, considered to be a key component in mediating the cell cycle and the aging process [13]. At different concentrations, hydrogen peroxide influences the cell to advance or halt the cell cycle. For example, at *in vivo* concentrations of 10^{-8}, 10^{-6}, and 10^{-4} M, H_2O_2 causes the cell to proliferate, cease its growth, or initiate apoptosis, respectively [13].

An important issue to address is the point at which the oxidative damage that is initially beneficial becomes harmful to the cell. In order to differentiate between negative and positive effects of reactive species on the body, we must analyze several determining factors including the concentration, half-life, and diffusibility of the reactive species produced. When the cell is utilizing these molecules for signalling purposes, they usually possess very short half-lives and very limited diffusibility or are present in low concentrations [14]. For example, monocytes and neutrophils use NADPH oxidase to produce the superoxide anion as a defence against bacterial or fungal infection [15]. Considering that the superoxide anion has a very short half-life (10^{-6} s), a very limited ability to diffuse throughout the cell, and is generated in a small concentration onto a focused target (i.e., bacteria or fungi) [14], it is generally viewed as beneficial in this context. Numerous other examples, indeed derivatives from reactions involving the superoxide anion, including H_2O_2 and hypochlorite, also play an important role in the neutralization of harmful pathogens and maintenance of a healthy cell. Similarly, antioxidants also play an important role in maintaining cellular functions in the face of redox modulations through a variety of mechanisms.

3. What Is an Antioxidant?

As the name implies, antioxidants are compounds that neutralize reactive species by decreasing their reactivity in

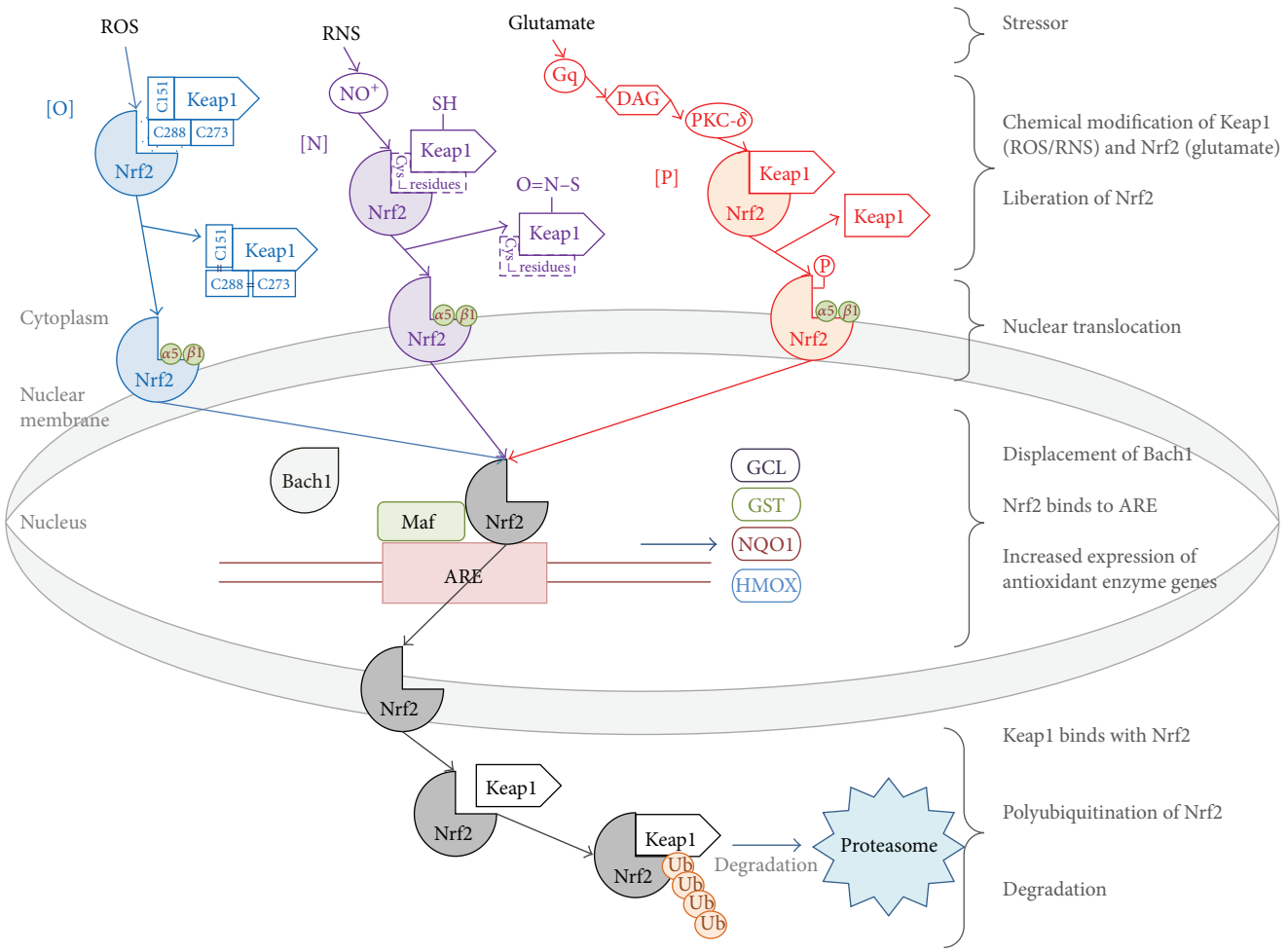

Figure 3: Mechanisms of Nrf2 activation and degradation. ROS, reactive oxygen species; RNS, reactive nitrogen species; Nrf2, nuclear factor- (erythroid-derived-2-) like 2; Keap1, Kelch-like ECH-associated protein 1; DAG, diacylglycerol; PKC, protein kinase C; Bach1, transcription regulator protein BACH1; Maf, transcription factor Maf; ARE, antioxidant response elements; GCL, glutamate-cysteine ligase; GST, glutathione-S-transferase; NQO1, NADPH:quinone oxidoreductase 1; HMOX, heme oxygenase; Ub, ubiquitin.

the body [2]. We can divide antioxidants into two broad categories: endogenous and exogenous. The antioxidants within the body are composed of antioxidant enzyme defenses (Table 2) and additional antioxidant compounds such as melatonin and glutathione that are internally synthesized.

Outside of the body, antioxidants can be supplied by the diet with a wide variety of natural and synthetic compounds found in complex mixtures (such as chocolate or olive oil) or isolated to be taken as a supplement [16]. The mechanism of action of each antioxidant will vary depending upon location, chemical structure, and bioavailability within the body as well as the degree of redox modulation experienced by the cell.

4. The Endogenous Antioxidant Response System

Under conditions of oxidative/nitrosative stress, the antioxidant response system (ARS) becomes active in order to ensure cellular survival and restoration of a balanced redox status [17]. In our body, Nrf2 acts as a master control for most of our antioxidant defenses, including the ones in the brain. As shown in Figure 3, a stressor can act directly or indirectly to influence the activation of the Nrf2 signal transduction pathway. In fact, Habas et al. report that neuronal activity at the tripartite synapse regulates Nrf2 activity in astrocytes [18]. Following an increase in neuronal activity signalled through neurotransmitters such as glutamate, the astrocytic Nrf2 signalling cascade is triggered via stimulation of group I metabotropic glutamate receptors and $Ca^{2+}{}_i$. Regardless of the stressor in question, the translocation of Nrf2 into the nucleus can be accomplished in two primary ways: chemical modification of cysteine residues on Keap1 and/or phosphorylation of Nrf2 [19].

In the kinase-independent mechanism of Nrf2 dissociation, reactive species directly oxidize (C151, C273, and C288) [20] or nitrosylate [21] key cysteine residues on Keap1, a protein bound to Nrf2 that facilitates its polyubiquitination and subsequent degradation under normal conditions. This process creates chemically modified cysteine residues (oxidized disulphide bridges or S-nitrosothiol groups) that allow for Nrf2 to become free within the cytosol.

Table 2: Endogenous system of antioxidant enzymes.

Antioxidant enzyme	Cofactor/substrate	Reaction catalyzed	Location	Biochemical function
Copper-zinc-SOD (Cu, Zn-SOD, or SOD1) [93]	Copper and zinc*	$^{\bullet}O_2^- + {}^{\bullet}O_2^- + 2H^+ \rightarrow H_2O_2$	Cytosol, nucleus, mitochondria (intermembrane space)	Catalyzes the dismutation reaction of superoxide to H_2O_2 to decrease its reduction potential
Manganese SOD (MnSOD or SOD2) [93]	Manganese*	$^{\bullet}O_2^- + {}^{\bullet}O_2^- + 2H^+ \rightarrow H_2O_2$	Mitochondrial matrix	Same as above
Extracellular SOD (ecSOD or SOD3) [93]	Copper and zinc*	$^{\bullet}O_2^- + {}^{\bullet}O_2^- + 2H^+ \rightarrow H_2O_2$	Isoform secreted extracellularly	Same as above
Glutathione peroxidase (GPx) [93]	GSH** Selenium*	(1) $R\text{-}Se^-H^+ + ROOH/ONOO^- \rightarrow ROH/ONO^- + R\text{-}SeOH$ (2) $2GSH + R\text{-}SeOH \rightarrow GS\text{-}SG + R\text{-}Se^-H^+$	Throughout the body	Reduce lipid hydroperoxides to alcohols and H_2O_2 to water
Glutathione-S-transferase (GST) [94]	GSH**	$GSH + RX \rightarrow GSR + HX$ X = leaving group R = electrophilic group	Cytosol, mitochondria, peroxisome	Detoxification of xenobiotics
Glutathione reductase (GR) [94]	FAD* NADPH** GS-SG**	$GSSG + NADPH \rightarrow 2GSH + NADP^+$	Cytosol, mitochondrial matrix	Maintenance of GSH levels
Catalase (CAT) [93]	Fe^{2+} and Fe^{3+}*	$H_2O_2 \rightarrow H_2O + O_2$	Throughout the body; lowest in the brain	Reduces H_2O_2 to water and oxygen
Peroxiredoxins (Prx) [95]	Thioredoxin (Trx)**	(1) $Prx^{red} + H_2O_2 \rightarrow Prx^{ox} + 2H_2O$ (2) $Prx^{ox} + Trx^{red} \rightarrow Prx^{red} + Trx^{ox}$	Throughout the body (intracellular)	Reduces H_2O_2 to water Prx is reduced by Trx to be used in subsequent reactions

*Cofactor; **substrate; SOD, superoxide dismutase.

In the kinase-dependent mechanism of Nrf2 dissociation, a stressor, such as glutamate, activates the G_q pathway, leading to the phospholipase C (PLC) catalyzed breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) [22]. The membrane bound DAG acts as a physiological activator of PKC which proceeds to subsequently phosphorylate S40 on Nrf2 [23]. According to recent studies, although PKC-β is the most abundant isoform in astrocytes [24], the predominant PKC isoform that participates in the phosphorylation of Nrf2 is PKC-δ [25]. Since the delta isoform of PKC is novel, it only requires DAG alone to become active [25] and, as such, the mechanism of increased [26] $Ca^{2+}{}_i$ by IP_3 is discussed elsewhere [22].

As a result of one or both of the mechanisms above, numerous importins, including α5 and β1, bind to the newly exposed NLS on Nrf2 to facilitate nuclear translocation [27]. Once inside the nucleus, Nrf2 displaces Bach1, a transcriptional repressor of antioxidant response elements (ARE), and heterodimerizes with transcription factor Maf to bind to ARE on the DNA [28]. Consequently, increased expression of endogenous antioxidant enzyme genes such as *NQO1*, *HMOX-1*, *GCL*, and *GST* occurs, increasing cellular defenses against detrimental redox modulations [29].

Once the cell has effectively compensated for the redox modulation, a deactivation cascade commences involving the phosphorylation of glycogen synthase kinase 3β (GSK3β) via unknown tyrosine kinases [28]. GSK3β proceeds to phosphorylate Fyn, a nonreceptor protein-tyrosine kinase, to facilitate its translocation into the nucleus. Once inside the nucleus, Fyn phosphorylates Y568 on Nrf2 to facilitate nuclear export which is immediately followed by Keap1 association, polyubiquitination, and proteolysis.

Considering that our endogenous antioxidant response system is able to tightly regulate the amount of reactive species and minimize related cellular damage, the role of exogenous antioxidants seems, on the surface, superfluous. However, Kaspar et al. [28] found that exogenous antioxidants have a priming effect on the antioxidant response system [19]. Following approximately 0.5–1 hour after exposure, antioxidants were found to induce the phosphorylation of Keap1 (Y85), Fyn (Y213), and Bach1 (Y486) via unknown tyrosine kinases to facilitate their export out of the nucleus. The overall effect of nuclear exportation of negative regulators of Nrf2 is reduced competition for ARE (with Bach1) and decreased nuclear export and degradation of Nrf2 via Fyn and Keap1. Working together with our endogenous antioxidant response system, exogenous antioxidants allow for a more enhanced and efficient defense against detrimental redox modulations.

5. Mechanisms of Action of Exogenous Antioxidants

Aside from enhancing the efficiency of antioxidant gene regulation, exogenous antioxidants also exert their effects through additional mechanisms of action. In cases such as tocopherols and resveratrol, 2 or 3 different actions can be simultaneously carried out to counter the effects of detrimental redox modulations [30, 31]. Shown in Figure 4 below are several examples of antioxidant reactions that take place in the body. In general, there are several common antioxidant mechanisms of action as described in Figure 4.

5.1. Hydrogen Atom Transfer, Electron Donation, and Direct Radical Scavenging. In free radical scavenging there are three known primary mechanisms of action: hydrogen atom transfer (Reaction 1A), electron donation (Reaction 1B), and direct radical scavenging (Reaction 1C). In H atom transfer, a reactive hydrogen-containing group on the antioxidant compound undergoes homolytic fission, generating a hydrogen radical and antioxidant radical [32]. The hydrogen radical is then able to interact with the free radical, creating a less reactive species. The antioxidant radical, while still reactive, is relatively less dangerous and can bind with another antioxidant radical to form a nonreactive dimer. In electron donation, the antioxidant compound, containing a conjugated system, donates an electron to the reactive species, producing an anion [32–35]. Using its conjugated system, the antioxidant is able to electronically redistribute the positive charge throughout its chemical structure or adopt an alternative, stable conformation as is the case for catechol containing compounds such as catechins [36] or caffeic acid [37]. In the particular case of caffeic acid, the compound initially undergoes deprotonation under physiological pH conditions, allowing for electron donation to occur from the catechol-like moiety, effectively reducing the nitronium ion [37]. In direct radical scavenging, the antioxidant absorbs a radical into its structure, producing a less reactive final product that possesses reduced cytotoxicity [33, 38, 39].

5.2. Metal Chelation. In order to chelate metals, the antioxidant must contain free electron pairs with which to form coordinate or normal covalent bonds with the free metal ion [40]. Common examples of antioxidant ligands include polyphenols [41] and various flavonoids [42]. However, it is also possible to have other antioxidants using sulfur or nitrogen atoms to chelate metal ions with or without a resulting de-protonation [40]. Once the metal ion and antioxidant interact, the antioxidant donates electrons to the metal ion (the number is dependent upon the nature of the covalent bond as described above), reducing it to its ground electronic state and inhibiting its ability to participate in RS generating reactions.

5.3. Restoration of Antioxidant Levels. As our body works to maintain the redox status, our endogenous supply of antioxidants begins to diminish, effectively reducing our capacity to fight excessive amounts of reactive species. In order to supplement our antioxidant defenses, we can ingest food or supplements containing natural or synthetic compounds that either get directly converted into the endogenous antioxidant or aid in its replenishment. Two representative examples of lipoic acid and N-acetylcysteine and its amide are explained below.

5.3.1. N-Acetylcysteine (NAC) and N-Acetylcysteine Amide (NACA). Once NAC or NACA reaches the cells, it is absorbed

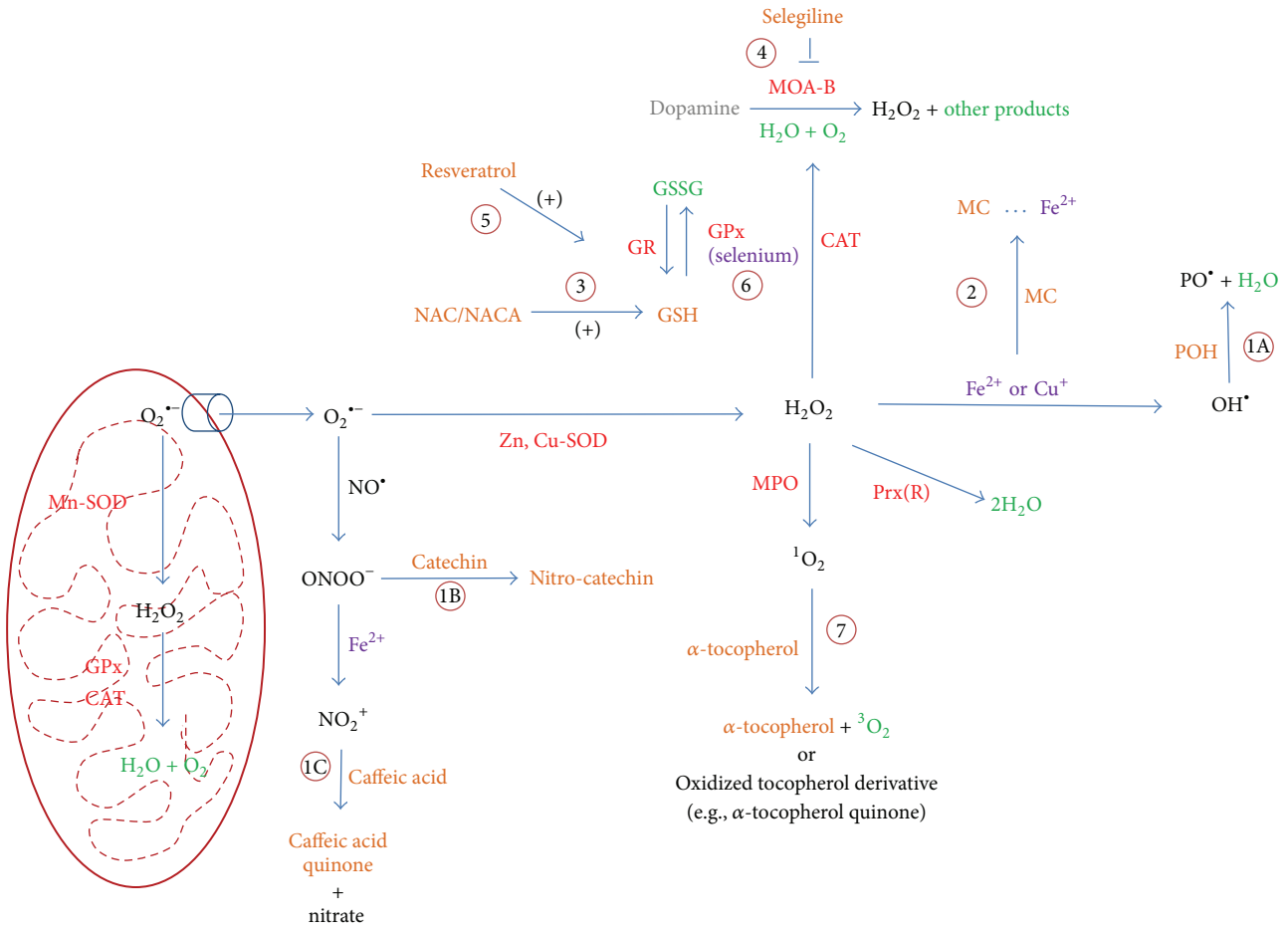

Figure 4: Mechanisms of action of exogenous antioxidants. Red: enzymes; green: other products; purple: cofactor/substrate; black: reactive species; CAT, catalase; MC, metal chelator; POH, polyphenol; GSSG, oxidized glutathione; MPO, myeloperoxidase. Reaction legend 1A: H-atom transfer, 1B: electron donation, 1C: direct scavenging, 2: metal chelation, 3: restoration of endogenous antioxidants, 4: inhibition of RS generating species and reactions, 5: support of endogenous antioxidant enzymes, 6: cofactor in antioxidant enzymes, 7: singlet oxygen quenching.

into the cytosol where it is hydrolyzed to release cysteine, the limiting reagent in the formation of GSH [43]. Using γ-glutamylcysteine synthetase, glutamine and cysteine are combined into γ-glutamylcysteine where a further addition of glutamine produces glutathione.

5.3.2. Reduced Lipoic Acid. In its reduced form, dihydrolipoic acid (DHLA) can aid in the restoration of endogenous antioxidants including vitamin C, vitamin E, and GSH by acting as a reducing agent [44].

5.4. Inhibition of RS Generating Enzymes and Reactions. Commonly used as an adjunct therapy for Parkinson's disease (PD), selegiline acts as a selective irreversible inhibitor of monoamine oxidase B (MAO-B) [45]. By doing so, selegiline increases dopamine availability and reduces the required dosage of L-DOPA, minimizing side effects. A secondary effect of this drug is to reduce the amount of hydrogen peroxide, a natural by-product of dopamine metabolism, in the neuron [45].

5.5. Promote Activities of Antioxidant Enzymes. Certain antioxidants play an indirect role in the protection of cells against oxidative stress by modulating the expression of some endogenous antioxidant enzymes. Two examples of such a mechanism of action involve lipoic acid and resveratrol.

5.5.1. Lipoic Acid. This exogenous antioxidant has the ability to alter the expression of phase II metabolic enzyme genes (conjugating enzymes such as UDP-glucosyltransferase, sulfotransferases, and glutathione-S-transferases) through Nrf2 dependent pathway [44].

5.5.2. Resveratrol. Among its many other mechanisms of action, resveratrol has been shown to induce sirtuin activity [46] leading to nuclear translocation of the FOXO transcription factor [47], an increase in FOXO3a transcription and upregulation of mitochondrial Mn-SOD [48].

5.6. Cofactor in Antioxidant Enzymes. In order for the endogenous antioxidant enzymes to work properly, they

require numerous cofactors from organic (heme and flavin) and inorganic (metal ions) sources. A common enzyme participating in the detoxification of reactive species is cytosolic glutathione peroxidase which requires a selenium cofactor bound to a cysteine residue to act as a catalytic site for the enzyme. The mechanism involves hydroperoxides or peroxynitrites oxidizing the selenol on the selenocysteine active site on GPx to create less reactive alcohols and nitrites, respectively [49]. The oxidized selenocysteine is reduced via two units of GSH into its corresponding selenic acid. It has also been proposed that thioredoxin reductase may reduce oxidized selenocompounds at the expense of NADPH [50].

5.7. Singlet Oxygen Quenching. Certain antioxidants, such as the tocopherols (Vitamin E), exhibit a potent quenching effect when reacting with singlet oxygen. The two known methods by which singlet oxygen is neutralized involve physical or chemical quenching of the excited electronic state. While each of these processes is not mutually exclusive in solution (or in our case, *in vivo*), physical quenching is usually the predominant mechanism [30].

In physical quenching, a charge transfer occurs following an electronic interaction between the singlet oxygen and the tocopherol that results in the singlet oxygen molecule being deactivated to its triplet configuration [30]. It is hypothesized by Gorman et al. (1984) that this occurs via intersystem crossing induced by spin-orbit coupling [51]. Chemical quenching has the same net effect as physical quenching, resulting in a deactivation of a singlet oxygen molecule. However this mechanism of quenching involves the incorporation of the singlet oxygen molecule into the tocol structure to create a quinone and/or quinone-epoxide as well as other oxidized products [30].

Through our understanding of antioxidant mechanisms of action, it becomes possible to hypothesize which compound would be best suited to counteract a neuropsychiatric disease. For example, a hallmark of PD pathology is excess iron in the substantia nigra pars compacta that subsequently generates reactive species via the Fenton and Haber-Weiss reactions [52]. Therefore, a possible strategy to combat PD would be to utilize a compound with iron chelation properties [53] such as flavonoids or DHLA. However, although antioxidants possess many positive functions within our body, like any pharmacologically active compound, they also have side effects and in some cases detrimental effects. Some potential problems surrounding antioxidants will be covered in the following section.

6. Limitations of Antioxidants

At first glance, antioxidants appear to be a panacea. However, as with any pharmacologically active compound, there are limitations to their usage and effectiveness within the body. These limitations are mostly concerned with the dosage/concentration, route of administration, possible drug interactions, and negative side effects of the antioxidants.

6.1. Dosage/Concentration. In order to demonstrate this point effectively, we will examine the case of the amyloid-β peptide, one of the major contributing factors in the pathophysiology of Alzheimer's disease (AD) [54]. In a patient that is not exhibiting symptoms of AD, there is a very small concentration (0.1–1.0 nM) of the amyloid-β peptide present in the CSF and plasma [55]. At these low physiological concentrations, amyloid-β exhibits antioxidant effects using a hydrophilic moiety to chelate transition metals (Cu and Fe ions) as well as a cysteine residue on Met35 as a free radical scavenger to prevent lipoprotein oxidation [55]. In fact, in comparison to the well-known antioxidant ascorbate, amyloid-β levels correlate better with oxidative resistance in the CSF [56]. However, at higher physiological (amyloid-β), usually in the μM range, and in the presence of transition metals, the peptide demonstrates prooxidant activity [55]. This general principle of toxicity in proportion to the administered dose can be widely applied to almost every pharmaceutical including exogenous antioxidants.

6.2. Route of Administration. In the context of antioxidants, the most common method of administration is oral due to its high compliance among patients. Considering that oral intake of antioxidants is most relevant, it is worth noting that first-pass metabolism, dietary intake, and BBB permeability have dramatic effects on the cerebral absorption and bioavailability of the ingested antioxidant [57]. A prominent example would be the antioxidant selegiline. When administered as an adjunct therapy with L-DOPA for the treatment of PD, it is recommended that the patient ingests a high-fat meal to increase the absorption of the drug due to its hydrophobic properties [45].

6.3. Drug Interactions. As a consequence of the combination of aging populations and a rise in popularity of nutritional supplements, interactions between antioxidants and pharmaceuticals constitute an emerging area of research and inquiry. From a pharmacodynamics perspective, antioxidants could act as competitive or noncompetitive antagonists (reversible or irreversible) with medication, effectively reducing the therapeutic window of the medication. A prominent example is the possible physiological antagonism of nifedipine, an antihypertensive agent, by melatonin [58]. Melatonin is an endogenous antioxidant that plays an important role in protecting against free radical-induced oxidative damage [59]. While the exact mechanism is unknown, melatonin is thought to interfere with nifedipine's mechanism of action through directly interacting with several enzymes involved in calcium signalling including calmodulin or adenylate cyclase.

From a pharmacokinetic perspective, in contrast to a pharmacodynamic interaction, the antioxidant would affect the concentration of the medication at several sites including the gastrointestinal tract, binding to plasma proteins, metabolism by CYP enzymes, and renal clearance. A popular example involves the inhibitory interactions between components of certain fruits such as grapefruit, known as furanocoumarins, and intestinal CYP3A4 [60]. Upon ingestion of the furanocoumarins, intestinal CYP3A4 is inhibited, leading to increased oral bioavailability of a drug [60]. Considering that the half-life of the drug is unchanged, this can lead to an unsafe peak plasma concentration within

the patient. A similar effect can be found following ingestion of curcumin, an antioxidant component of turmeric [61]. In a study by Burgos-Morón et al., curcumin has been shown to inhibit cytochrome P450 enzymes, glutathione-S-transferase, and UDP-glucuronosyltransferase, leading to a potentially toxic increase in the concentration of any medications that a patient may be taking.

6.4. Negative Side Effects. One of the most important issues to address in this context is the false equivalency between "natural" and "safe" often made by those who are wary of the side effects and sceptical of the efficacy of modern pharmaceuticals. As mentioned earlier, the dosage of any pharmacologically active compound must be carefully regulated in order to stay within the experimentally determined therapeutic window. Once the given intake exceeds the median toxic dose (TD_{50}), negative side effects can begin to manifest themselves as distressing physical symptoms. One example of negative side effects can be seen in the mechanism of action of a key ingredient in green and black teas, epigallocatechin gallate (EGCG) [62]. Purported as a strong antioxidant, EGCG also displays cytotoxicity *in vitro* in both cancerous and primary human cell lines. Whether these effects can be translated into an *in vivo* context remains to be seen.

Overall, these examples highlight the desperate need for more peer-reviewed research into the efficacy and toxicity of antioxidant compounds that are currently being ingested by the public.

7. How Are Oxidative Stress and Antioxidants Relevant to Brain Health?

Thus far, we have considered oxidative stress in a cellular context. However, considering that the body is much more than the sum of its parts, it is important to apply our mechanistic and cellular understanding of oxidative stress to the general concept of brain health. According to Halliwell and Emerit et al., the brain possesses several key physiological features that make it susceptible to oxidative stress (Figure 5) [7, 63]. (1) *High* O_2 *Utilization*. Relative to the rest of the body, the brain accounts for a small fraction of body weight. However, since it uses a high supply (up to 20%) of available oxygen, toxic by-products such as hydrogen peroxide and superoxide are inevitably produced and begin to cause damage. (2) *High PUFA Content*. The neuronal membrane consists of numerous polyunsaturated fatty acids (PUFA), notably docosahexaenoic acid (DHA). Vulnerable to oxidation by reactive species, PUFA can be oxidized into radicals and 4-hydroxynonenal (4-HNE), a cytotoxic compound that interferes with neuronal metabolism. (3) *Presence of Redox-Active Metals*. In the average adult brain, there is approximately 60 mg of nonheme iron usually bound to ferritin and hemosiderin. In a normal, healthy brain, the movement of iron into the brain is controlled via transferrin and its associated receptors. However, if there is damage to the brain, especially in areas with high iron content (substantia nigra, caudate nucleus, putamen, and globus pallidus), iron is released from ferritin or diffuses through damaged microvasculature. Once inside the brain, this catalytic iron causes extensive amounts of damage due to the negligible iron-binding ability of the CSF. (4) *High* Ca^{2+} *Flux across Neuronal Membranes*. In the presence of reactive species such as H_2O_2, disruptions in mitochondrial and endoplasmic reticulum function, specifically to their calcium sequestration abilities, can cause a rise in intracellular Ca^{2+}. This causes the production of reactive species by mitochondria to increase and cause further damage. It has additionally been reported by Fonfria et al. that, in the presence of reactive species such as H_2O_2, some neurons and glial cells allow for Ca^{2+} influx via specific cation channels, initiating a detrimental cascade that culminates in cytoskeletal damage [64]. (5) *Excitotoxic Amino Acids*. Once reactive species have induced a state of oxidative stress in neurons, there is a release of glutamate following cell death. This excitatory neurotransmitter proceeds to bind to glutamate receptors on neighbouring neurons, causing cation (Ca^{2+} and Na^{+}) influx and eventually necrosis. This initiates an excitotoxic "chain reaction" in which neurons continually experience excessive extracellular glutamate levels. The problem is further compounded by disruptions in glutamate transporters and glutamine synthetase activity. (6) *Autoxidizable Neurotransmitters*. Catecholamine neurotransmitters (dopamine, epinephrine, and norepinephrine) can react with O_2 to produce superoxide and quinones/semiquinones that readily bind to sulfhydryl side chains and deplete the already low cerebral GSH reserves. (7) *Low Antioxidant Defenses*. Throughout the brain there are lower levels of antioxidant defenses relative to the rest of the body. The only substantial antioxidant enzyme in the brain is catalase, which is very limited in its ability to detoxify H_2O_2 since it is localized to microperoxisomes.

As a result, various neuropsychiatric diseases manifest themselves as exploitations of these substrates and cofactors that usually contribute to normal brain health. The main sources of reactive species in the brain are, as in the rest of the body, by-products of normal homeostatic functions such as protein degradation and energy production (Table 3).

8. Antioxidants and Neuropsychiatric Disorders

Despite these theoretical and practical difficulties, antioxidants have the potential to act as effective treatments for a variety of neuropsychiatric disorders. It has been previously established in patients with these neuropsychiatric disorders that there is an imbalance in the levels of antioxidants in the brain and blood plasma as well as some elements of mitochondrial dysfunction. For example, patients with AD were found to have decreased plasma levels of well-known antioxidants lycopene, vitamin A, vitamin C, and vitamin E [65]. Unfortunately, clinical trials directly treating the disorder with supplementation have not displayed positive results with some cases demonstrating a progressive decline in cognitive function in participants [66]. While these results are negative and do not support antioxidant therapy, a variety of factors such as the prooxidant effects of antioxidants

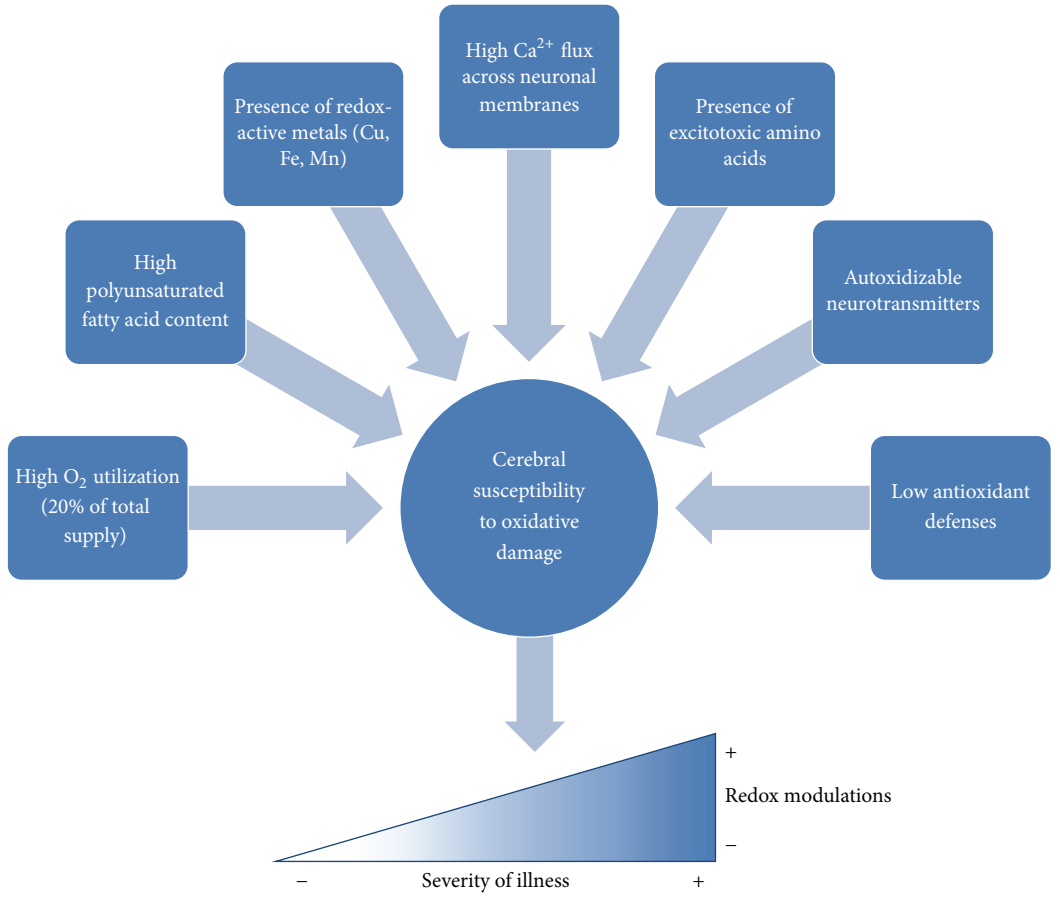

Figure 5: Contributing factors to the cerebral susceptibility to oxidative damage.

Table 3: Various biological sources of reactive species in the brain.

	Source of reactive species in the brain	Function in the brain	General role in neuropathology
Organelle	Mitochondria [7]	Generates ATP	Defect or reduction in mitochondrial complex I/II/III/IV activity
Enzyme	Monoamine oxidases (MOA-A and MOA-B) [96]	Degrades neurotransmitters	Increased or decreased activity can lead to neurotransmitter imbalances as well as excess reactive species
	Nitric oxide synthase [97]	Synthesis of nitric oxide	Production of superoxide anion during normal $NO^{\bullet}$ production
	Xanthine oxidase [98]	Catabolism of purines	Produces superoxide anions during normal metabolism
	Cytochrome P450 enzymes [99]	Drug metabolism Bioactivation of neurosteroids such as dehydroepiandrosterone (DHEA) Metabolism of retinoic acid (regulates gene expression) Cholesterol turnover in the brain	Reduced DHEA levels correlated with memory impairment Altered gene expression Reduced cholesterol turnover leading to accumulation in the brain
Metabolism	Arachidonic acid (AA) metabolism [97]	Maintains membrane fluidity Aids in the growth and repair of neurons Participates in activation of enzymes to store free fatty acids in the brain (prevents oxidative damage)	Elevated AA metabolism and/or overexpression of metabolizing enzymes Increased amounts of free fatty acids

and timing of administration can influence the outcome of the trial. Considering that reduced antioxidant enzyme activity, specifically superoxide dismutase, glutathione peroxidase, and glutathione reductase, and increased levels of 8-isoprostane were found in the CSF, plasma, and urine of patients with mild cognitive impairment (MCI) [65], a condition commonly seen in pre-AD patients, it is likely that the failure of antioxidant therapy in the treatment of AD can be ameliorated through earlier intervention. The concept of early intervention with antioxidant therapy still shows promise and should be investigated further in different contexts as the potential for an effective treatment across multiple neuropsychiatric disorders is high considering their common pathophysiological origins and mechanisms of progression.

9. Redox Modulations and Neuropsychiatric Disorders

Redox modulations play a major role in the development and progression of neuropsychiatric disorders [67]. Processes such as lipid peroxidation, protein and DNA oxidation, and mitochondrial dysfunction in the brain and periphery are indicative of neuropsychiatric disease, among other things. For example, mitochondrial dysfunction in PD [68] specifically complex I dysfunction [69] is linked to increased oxidative damage to the macromolecules and toxic products such as 4-hydroxynonenal (4HNE) found in PD. Moreover, 4HNE is correlated to damages to the 26/20S proteasome system [70] in PD.

In response to these toxic insults and enzymatic dysregulation, a broad-spectrum neuroprotective response is elicited that includes the increased expression of GSH peroxidase, succinic semialdehyde reductase, heme oxygenase-1, and NADPH dehydrogenase-1 enzymes. Considering that the degeneration of the SNpc is at least correlated with an increase in neuronal and astroglial NADPH dehydrogenase-1 expression, this constitutes a potential intervention point for therapeutics, including antioxidants. Whether artificial or natural inducers of endogenous antioxidant enzyme activity and the neuronal Nrf2 system could hypothetically lead to an amelioration of any neuropsychiatric pathology remains an open and challenging question for basic and translational research.

Furthermore, mitochondrial dysfunction leading to oxidative damage has long been linked with many neuropsychiatric disorders such as AD [71–75], bipolar disorder (BD) [76–81], major depressive disorder (MDD) [82–84], schizophrenia (SCZ) [85], Huntington's disease (HD) [86–88], and amyotrophic lateral sclerosis (ALS) [89–91]. In fact, there are common pathophysiological points between these neuropsychiatric disorders, emphasizing the possibility of common pharmacological intervention through synthetic or natural antioxidant compounds.

10. Future Directions

In light of the available evidence regarding antioxidants, it is clear that more studies are needed to explore their potential pharmacological properties. While there are many published and peer-reviewed studies regarding the mechanism of action and biological effects of antioxidants, there are few that seek to address the underlying issue of drug interactions, specifically with respect to medication prescribed for neuropsychiatric disorders. In order to supplement this growing body of research, clinical trials regarding the efficacy of antioxidants as potential stand-alone or adjunctive treatments need to be conducted. In addition, more studies are required to assess the long-term safety of antioxidants in healthy and nonhealthy individuals. From here, it becomes possible to closely examine the physicochemical properties of each antioxidant and use these as a basis for future drug development in the treatment of neuropsychiatric disorders and other various illnesses in accordance with previously established CNS drug characteristics [92].

Abbreviations

ROS: Reactive oxygen species
RNS: Reactive nitrogen species
Nrf2: Nuclear factor (erythroid-derived 2)-like 2
Keap1: Kelch-like ECH-associated protein 1
PLC: Phospholipase C
PIP2: Phosphatidylinositol 4,5-bisphosphate
DAG: Diacylglycerol
IP3: Inositol triphosphate
PKC: Protein kinase C
Bach1: Transcription regulator protein BACH1
Maf: Transcription factor Maf
NQO1: NADH quinone oxidoreductase 1
HMOX-1: Heme oxygenase 1
GCL: Glutamate cysteine ligase
GST: Glutathione S-transferase
GSK3β: Glycogen synthase kinase 3 beta
GSH: Glutathione
DHLA: Dihydrolipoic acid.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Pulmonary Protection Strategies in Cardiac Surgery: Are We Making Any Progress?

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Pulmonary dysfunction is a common complication of cardiac surgery. The mechanisms involved in the development of pulmonary dysfunction are multifactorial and can be related to the activation of inflammatory and oxidative stress pathways. Clinical manifestation varies from mild atelectasis to severe respiratory failure. Managing pulmonary dysfunction postcardiac surgery is a multistep process that starts before surgery and continues during both the operative and postoperative phases. Different pulmonary protection strategies have evolved over the years; however, the wide acceptance and clinical application of such techniques remain hindered by the poor level of evidence or the sample size of the studies. A better understanding of available modalities and/or combinations can result in the development of customised strategies for the different cohorts of patients with the potential to hence maximise patients and institutes benefits.

1. Introduction

Pulmonary dysfunction is a common complication of cardiac surgery that can impact patient's outcomes and health economics. It is recognised that many patients will have altered pulmonary mechanics after surgery which may appear in a wide range of clinical presentations, from mild atelectasis to life threatening acute lung injury (ALI) or adult respiratory distress syndrome (ARDS) [1–3].

Surgical incisions by abolishing the integrity of the chest wall affect respiratory mechanics leading to impaired respiratory effort. Postoperative pain has been shown to be associated with decreased lung function by precluding deep inspirations. Furthermore, patients undergoing surgical procedures associated with opening the pleura will have increased rates of atelectasis, pleural effusions, and postoperative pain especially in the early postoperative period [4–6].

Cardiopulmonary bypass (CPB) can lead to the activation of different inflammatory and coagulation pathways and alters redox balance due to the passage of blood through the circuit (contact activation) and ischaemia and reperfusion injury [7–10].

Vascular endothelial cells (EC) dysfunction during CPB due to changes in blood flow patterns, shear stress, ischaemia, and reperfusion and circulating cytokines will result in the activation of multiple proinflammatory and proapoptosis pathways [11–14] while suppressing its ability to produce vasoprotective mediators [14–17]. EC activation is known to initiate leukocytes adhesion cascade by the expression of members of the selectin family which are responsible for the initial attachment of leukocytes from circulation. Transmigration of leukocyte through EC (crucial step for leukocytes recruitment to tissue) follows leukocytes attachment and is mediated by the upregulation of different adhesion molecules such as platelet endothelial cell adhesion molecule-(PECAM-) 1, ICAM-1, and very late antigen-4 (VLA-4) [18–21].

CPB is traditionally associated with inadequate lung perfusion as there is no flow to the pulmonary artery during periods of cross clamping and when the heart is not ejecting blood, and thus blood supply is limited to the bronchial arteries [22]. Pulmonary physiology alteration during CPB can result in disturbing the balance in the blood gas barrier due to the alteration of the different force

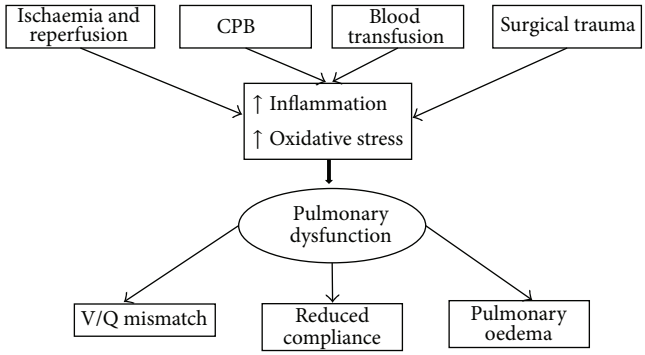

Figure 1: Mechanisms involved in pulmonary dysfunction during cardiac surgery and its consequences.

affecting the parenchyma thus abolishing gas exchange by passive diffusion at the blood-gas barrier level and leading to ventilation/perfusion mismatch and impaired pulmonary compliance [23, 24]. Moreover, ischaemia during CPB is associated with reduced alveolar blood supply resulting in alveolar ischaemia and hypoxic pulmonary vasoconstriction [25]. Pulmonary vascular endothelial cells dysfunction and activated neutrophils sequestration into lungs parenchyma during period of reperfusion can increase lung tissue permeability and elevate vascular resistance and pulmonary surfactant changes leading to alveolar protein accumulation and pulmonary oedema and driving more ROS and cytokines production [26–28]. This will be manifested as abnormal gas exchange, poor lung mechanics, increased pulmonary shunt fraction and reduced functional residual capacity, and carbon monoxide transfer factor [28–30] (Figure 1).

Blood and blood products usage after surgery can be associated with the production of excessive amount of ROS and systemic and pulmonary inflammation. It has been previously shown that the duration of blood storage before transfusion can influence adverse effect associated with transfusion as there are an increased risk of respiratory insufficiency and the need for prolonged ventilation in patients receiving blood stored for longer than two weeks [10, 31–34].

Here we review the most commonly used strategies to minimise pulmonary dysfunction after cardiac surgery.

2. Pulmonary Protection Strategies

2.1. Pharmacological Interventions. The fact that inflammation and oxidative stress play a pivotal role in the development of pulmonary dysfunction resulted in multiple studies aimed toward modulating such events by the administration of vasodilators and antioxidant and anti-inflammatory medications in experimental animal models or patients undergoing cardiac surgery.

Prostacyclins can induce vascular smooth muscle cells relaxation by the release of intracellular adenosine $3',5'$ cyclic monophosphate (cAMP) resulting in pulmonary and systemic vasodilation [35–38]. Moreover, they can have inhibitory effect on platelet aggregation and leukocytes and monocytes activity [39]. The use of inhaled prostacyclins (Epoprostenol or Flolan) has been shown to decrease pulmonary arterial endothelial dysfunction induced by CPB in experimental studies [40–42]. Furthermore, when administered prior to CPB, they can have beneficial effect in the presence of pulmonary hypertension and may result in lower rate of reintubation in high risk postoperative cardiac patients [43, 44].

Phosphodiesterase inhibitors such as pentoxifylline (PTX) which is a known nonselective phosphodiesterase (PDE) inhibitor can result in elevated levels of intracellular cAMP and vasodilatation. Although PTX has been used classically for claudication symptoms in peripheral vascular disease [45], it has been shown to exert anti-inflammatory and antioxidative properties resulting in modulation of ALI [46–48]. More selective PDE inhibitors such as milrinone can be an advantageous therapeutic strategy for cardiac surgical patients with increased pulmonary vascular resistance (PVR) and right ventricular failure when nebulized and inhaled. It can cause selective pulmonary vasodilation and potentiate the vasodilation effects of inhaled prostacyclin [49, 50].

Nitric oxide (NO) is known to play a pivotal role in vascular endothelial cells homeostasis and regulation of oxidative stress and inflammatory responses [51]. Ischaemia and reperfusion injury during surgery is associated with significant loss in NO; thus NO preconditioning has been suggested to reduce perioperative pulmonary dysfunction and its sequels [52–54]. The protective effects may be due to reversal of postischemic lung hypoperfusion and reduction of lung neutrophil sequestration. The administration of NO in patients with severe left ventricular dysfunction can lead to pulmonary vasodilatation and may augment left ventricular filling [55–57]. The timing of administration and/or concentration of inhaled NO during ischaemia or reperfusion periods is a very important determinant of its effect as NO is toxic early in reperfusion, due to its interaction with superoxide which may lead to damage of alveolar type 2 cells [58, 59].

A large number of other drugs have been used with various degrees of success. Aprotinin (serine proteases) had been shown to reduce neutrophil elastase, malondialdehyde, and proinflammatory cytokines levels in bronchoalveolar lavage fluids of patients undergoing cardiac surgery [60]. The use of aprotinin can result in improving lung function and reducing reperfusion lung injury [61]. The administration of corticosteroids before CPB may reduce the activation of multiple proinflammatory mediators. The translation of proinflammatory mediator's changes into clinical outcomes remains controversial and most of the evidence in the literature originates from small RCTs or observational studies with biomarkers as primary end points [62, 63].

2.2. CPB Modification. Different strategies have been attempted over the years to minimise proinflammatory activation and oxidative stress when using CPB such as coating the circuit with biocompatible material (heparin, poly-2-methoxyethyl acrylate, synthetic protein, and phosphorylcholine), removal of leukocytes (by adding special filters to the CPB), ultrafiltration, and reduced haemodilution.

Heparin is thought to reduce the inflammatory responses linked to platelets and leukocytes by reducing the release of IL-6, IL-8, E-selectin, lactoferrin, myeloperoxidase, integrin, selectin, and platelet thromboglobulin and decreasing the production of oxygen free radicals [64–66]. It has been suggested that compared with conventional circuits (poly-2-methoxyethyl acrylate, synthetic protein, and phosphorylcholine), the heparin-coated circuit may improve lung compliance and pulmonary vascular resistance and thus reduce intrapulmonary shunt although intubation time and ICU stay were not affected [67, 68].

The use of leukocyte filtration mechanisms can modulate proinflammatory cytokines and oxidative stress [69–71]. A clinical study compared the effectiveness of leukocyte filter depletion with a common arterial filter in CABG patients who reported better oxygenation indices and less duration of postoperative mechanical ventilation in the leukocyte depletion filter group [71]. Another study suggested that leukocyte depletion filters preferentially remove activated leukocytes. Improvement in lung function was evident only in the early postoperative phase, but this did not lead to decreasing mortality or better clinical outcomes [72]. The use of ultrafiltration or modified ultrafiltration techniques at the end of surgery may reduce postoperative oedema specifically that of lungs resulting in better oxygenation and improved lung compliance postoperatively. Furthermore, ultrafiltration may remove proinflammatory mediators from the circulation such as IL-6 and IL-8 but it did not result in significant improvement of clinical outcomes [73]. Similarly, controlled haemodilution to regulate oncotic pressure can reduce priming volumes and result in better haemodynamic parameters such as vascular resistance and higher oxygen delivery and affect hospital stay significantly [74, 75].

Understanding problems associated with pulmonary ischaemia and reperfusion results in attempts to provide continuous pulmonary perfusion during CPB. Experimental animal models of pulmonary perfusion demonstrated reduced inflammatory and apoptosis pathways activation with such strategy [25, 76, 77]. Moreover, pulmonary perfusion was found to have favorable effect on lung compliance, oxygenation, and vascular resistance in patients undergoing CABG [78, 79]. Furthermore, pulmonary artery perfusion during CPB can be effective in reducing postoperative right ventricular dysfunction in high-risk patients undergoing LVAD placement [80]. The translation of such changes into better clinical outcomes remains unclear and may be restricted to selective group of patients as demonstrated by a recent trial in patients with COPD undergoing cardiac surgery using CPB where no significant protective effect on lungs was documented [81].

The deleterious effects of surface contact activation as discussed previously have led to the development of minimised cardiopulmonary bypass circuit (mini-CPB). This is characterised by reduced surface area and thus priming volume and prevention of air-blood contact. The utilisation of mini-CPB has been shown to be associated with attenuated production of proinflammatory cytokines and complement activation and blunted leukocytes activation compared to conventional circuit. Markers of oxidative stress tend to be reduced in patients undergoing surgery using mini-CPB compared to conventional circuit [82, 83]. Additionally, mini-CPB reduces organ damage and results in better postoperative gas exchange and lower lung injury scores [84, 85]. Unfortunately most of the clinical trials investigating the role of mini-CPB have evaluated diverse technologies of varying complexity and degree of miniaturisation, which would be expected to give rise to heterogeneity in findings.

2.3. Surgical Strategies. It has been suggested that eliminating the usual standard of no lung inflation during CPB by maintaining a degree of lung ventilation may be beneficial. The use of continuous positive airway pressure (CPAP) during CPB may result in less shunt and better gas exchange [86]; however, it seems that such effect is dependent on the airway pressure used. Using low frequency ventilation (LFV) along with CPAP during CPB to reduce post-CPB lung injury has been evaluated in an experimental pig model [87]. This study showed that the use of LFV is associated with significantly better pulmonary gas exchange, higher adenine nucleotide, lower LDH levels, and reduced histological damage in lung biopsies as well as lower DNA levels in bronchoalveolar lavage (BAL) compared to the collapsed lungs control group. However, a clinical study in patients undergoing cardiac surgery compared the effect of low volume ventilation to conventional strategy of no ventilation and demonstrated no significant changes in PVRI, PaO(2)/FiO(2) ratio, postoperative length of stay, and postoperative pulmonary complications [88]. Furthermore, a meta-analysis of 814 cases in 16 RCTs looking at three lung protective strategies in patients during CPB including CPAP, low-volume ventilation, and vital capacity manoeuvres during CPB showed that the effects of the designated techniques are probably short lived with a questionable impact on the long term clinical outcome of the treated patients [89].

Off pump coronary artery bypass (OPCAB) surgery seems to provide better lung protection by eliminating ischemia-reperfusion injury through maintaining lung ventilation and avoiding CPB. Many studies consistently reported better early and midterm outcomes in OPCAB when compared with conventional on-pump CABG: fewer respiratory complications, shorter intubation time and ITU stay, reduced incidence of pneumonia, and overall shorter hospital stay [90–92].

2.4. Physiotherapy. Preoperative prophylactic physiotherapy with inspiratory or expiratory muscle training can be used as a preventative measure for lung protection [93, 94]. Postoperative physiotherapy is used prophylactically in patients undergoing cardiac surgery. Different techniques can be utilised during this period to improve ventilation-perfusion inequalities, increase pulmonary compliance, and help reinflate collapsed alveoli.

These techniques include deep breathing exercises, slow maximal inspirations with an inspiratory hold, intermittent deep breathing exercises with and without the use of incentive spirometer, and deep breathing exercises with expiratory resistance [95–98].

2.5. Postoperative Noninvasive Ventilation (NIV). NIV refers to the administration of ventilatory support without using an invasive artificial airway (endotracheal tube or tracheostomy tube). NIV exerts its main effects on the pulmonary and on the cardiovascular systems through the application of a positive end-expiratory pressure (PEEP); with or without a pressure support during inspiration, NIV restores lung volumes by opening atelectatic areas, increases alveolar ventilation, and reduces the work of breathing [99–101].

Continuous positive airway pressure (CPAP) aims to maintain a level of positive airway pressure in a spontaneously breathing patient. It is functionally similar to positive end-expiratory pressure (PEEP), except that PEEP is an applied pressure against exhalation and CPAP is a pressure applied by a constant flow. The ventilator does not cycle during CPAP, no additional pressure above the level of CPAP is provided, and patients must initiate all of their breaths. To avoid drying of the respiratory mucosa, there has been general agreement that the application of humidified CPAP helps to recruit the lungs by increasing functional residual capacity (FRC), increase the surface area of lung, decrease intrapulmonary shunt, and improve oxygenation [102–104].

Bilevel positive airway pressure (BLPAP) is a continuous positive airway pressure with pressure support breaths. It delivers a preset inspiratory positive airway pressure (IPAP) during inspiration and expiratory positive airway pressure (EPAP). BLPAP can be described as CPAP with a time-cycled or flow-cycled change of the applied pressure level [105]. BLPAP senses patients breathing efforts by monitoring air flow in the patient's circuit and adjusts its output by assisting inspiration. Therefore, its physiological effects can benefit the patient in both phases of respiration [106–108]. BLPAP application can only be commenced on conscious, cooperative, and hemodynamically stable patients who can breathe spontaneously, have an adequate gag and cough reflex, and are able to remove the mask when required. Several studies have demonstrated beneficial effects of BLPAP in reducing pulmonary complications and overall length of hospital stay after cardiac surgery [109–111]. Furthermore, the prophylactic use of BLPAP after early extubation has been shown to be safe and effective [111, 112]. A better tolerance was noted when BLPAP settings were commenced on low level and gradually adjusted to achieve the therapeutic target. Radiological improvement of atelectasis after cardiac surgery has been achieved on maintaining 8–10 mL/kg of tidal volume with BLPAP [113].

3. Conclusions

Pulmonary dysfunction is one of the most common and serious complications after cardiac surgery and can significantly impact on patient outcomes and health economics. The mechanisms involved in the development of pulmonary dysfunction are multifactorial and are related to the activation of different inflammatory and oxidative stress pathways. Clinical manifestation varies from mild atelectasis to severe respiratory failure. Managing pulmonary dysfunction postcardiac surgery is a multistep process that starts before surgery and continues during both the operative and postoperative phases. Pulmonary protection strategies have evolved over the years with various degrees of success. The main weakness of the majority of studies is often being observational in nature, small sample size, or being concentrated on a single intervention. Managing pulmonary dysfunction needs to be a multistep process involving more than one modality for each step of the surgical pathway. A better understanding of available modalities and/or combinations will result in the development of customised strategies for the different cohorts of patients. This in turn will help reduce pulmonary dysfunction and hence improve early outcome and costs after cardiac surgery.

Conflict of Interests

No conflict of interests is declared by any of the authors.

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