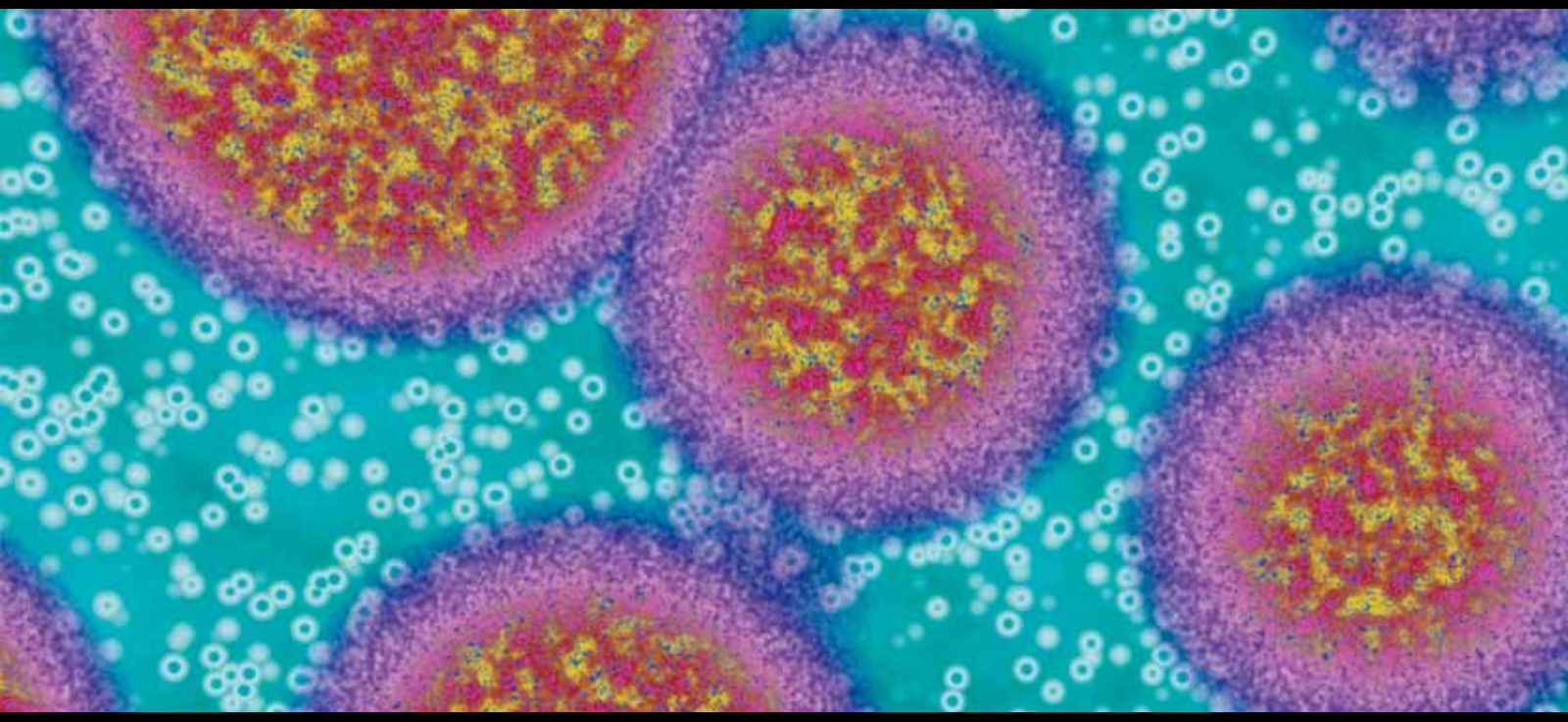
International Journal of Cell Biology

Cell Stress and Cell Death

Guest Editors: Afshin Samali, Simone Fulda, Adrienne M. Gorman, Osamu Hori, and Srinivasa M. Sirinvasula



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Editorial Board

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Editorial

Cell Stress and Cell Death

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This special issue on Cell Stress and Cell Death is aimed at bringing together recent developments in the fields of cellular stress and cell death and, in particular, the interplay between cell stress responses and cell death. The special issue opens with a review by S. Fulda et al. which provides an overview of how cells can respond to stress in a variety of ways ranging from the activation of survival pathways to the initiation of cell death that eventually eliminates damaged cells. Whether cells mount a protective response or succumb to death depends to a large extent on the nature and duration of the stress as well as the cell type. For example, milder stresses can lead to protection through activation of the heat shock response or the unfolded protein response (UPR). This review also describes several types of cell death (e.g., apoptosis, necrosis, pyroptosis, or autophagic cell death) and the mechanism by which a cell dies often depends on various exogenous factors as well as the cell's ability to handle the stress to which it is exposed. The implications of cellular stress responses for human physiology and disease are multifold and are discussed in this review in the context of some major world health issues such as diabetes, Parkinson's disease, myocardial infarction, and cancer.

There are many molecules and cellular processes that play critical roles in normal cell signaling and survival responses, while also having a dual role in inducing cell death. A number of papers in this special issue, covering endoplasmic reticulum (ER) stress and Ca^{2+}, address this topic. In recent years there has been a significant increase in the number of papers in the field dealing with ER stress and ER stress-induced cell death. This reflects the growing recognition of the importance of the ER in cell stress and in different modes of cell death. The ER is the site of folding of membrane and secreted proteins in the cell. Physiological or pathological processes that disturb protein folding in the ER initiate a complex intracellular signal transduction pathway, known as the UPR. This response is an attempt to reestablish ER homeostasis, although it can also lead to cell death. The review by A. Samali et al. provides a comprehensive overview of current methodologies for monitoring the UPR and ER stress, and it puts together a set of criteria to assess this response, which will be useful for researchers who wish to examine these phenomena in different model systems. The UPR is essentially tailored to reestablish ER homeostasis and it can also signal through other adaptive mechanisms involving the stimulation of autophagy. However, when ER stress is persistent, the cytoprotective functions of the UPR and autophagy can switch to cell death-promoting mechanisms. A review by T. Verfaillie et al. discusses the relationship between ER stress and autophagy and the implications for cancer therapy. Recently, a variety of anticancer therapies have been linked to the induction of ER stress in cancer cells, envisaging strategies that stimulate prodeath function or block its prosurvival function, to improve tumoricidial action. A better understanding of the molecular mechanisms that determine the final outcome of UPR and autophagy activation by chemotherapeutic agents will offer new opportunities to improve existing cancer therapies as well as revealing novel targets for cancer treatment. Prolonged or severe ER stress has been linked to induction of apoptosis. Caspase activation is one of the key steps in commitment of cells to apoptosis and is dependent on mitochondrial outer membrane permeabilization (MOMP) and the release

of cytochrome *c* from the mitochondria. The research article by S. Gupta et al. investigates the mechanism of ER stress-induced MOMP using thapsigargin as an inducer of ER stress. They genetically dissected the role of caspase-9, -3, and -2 in the induction of MOMP by ER stress using embryonic fibroblasts derived from knockout mice and also treated cells with chemical and molecular inhibitors of the mitochondrial permeability transition. Their results suggest that caspase-9 and -2, Bcl-2 family members, and the mitochondrial permeability transition pore all play a role in MOMP during ER stress-induced apoptosis.

Ca^{2+} is an important second messenger which is also poised at the intersection between cell survival and cell death. The review by C. Cerella et al. examines the events that occur during Ca^{2+} toxicity and how reparative or death pathways can be activated. They also discuss the observations that while Ca^{2+} can elicit these opposing responses, it also plays a role as a second messenger in signal transduction associated with cell death and survival.

Knowledge of cell stress and cell death pathways, and the interplay between these, beneficially provides us with new ways to tackle diseases, particularly cancers and degenerative disease. Resistance to apoptosis is a feature of many cancer cells and this is the subject of the review by S. Fulda which summarizes the main mechanisms by which cancer cells evade the intrinsic and extrinsic apoptosis pathways. Generally, altered ratios of key pro- and antiapoptotic proteins are responsible for resistance to cell death; for example, altered ratios of Bcl-2 family proteins as well as increased expression of caspase inhibitors (IAPs) regulate the intrinsic pathway, while reduced sensitivity of extrinsic pathways occurs due to decreased expression of death receptors on the plasma membrane and increased expression of intracellular decoy proteins. Given this increased resistance to cell death pathways, the search for novel molecules to induce cell death in cancer cells is an important goal. Histone deacetylase inhibitors have become a promising new avenue for cancer therapy, and many are currently in clinical trials for various tumor types. The research article by N. Rivera-Del Valle et al. demonstrates that a novel hydroxamic acid histone deacetylase inhibitor, PCI-24781, exerts cytotoxicity and histone alterations in leukemia cells, via a mechanism that is dependent on caspase-8 and Fas-associated death domain. Another avenue for identifying novel therapeutic targets for cancers is study of inflammation, since many human malignancies have been strongly linked with chronic inflammation. The review by C. Sobolewski et al. describes the role of cyclooxygenase-2 in these diseases. This enzyme is a member of a family, which catalyzes the rate-limiting step of prostaglandin biosynthesis. Cyclooxygenase-2 is upregulated during both inflammation and cancer, and has been described to modulate cell proliferation and apoptosis mainly in solid tumors and more recently in hematological malignancies. Thus, the use of cyclooxygenase-2 inhibitors, together with other therapeutic strategies, may further improve the efficiency of anticancer treatments in the clinic.

In contrast to cancers, an important aim of research into degenerative diseases is to discover novel ways to *inhibit* cell death. One such disease is Parkinson's disease, where there are currently no therapies which halt the degeneration of dopaminergic neurons. In the research article by K. Mnich et al. the ability of the endogenous cannabinoid, anandamide, to inhibit apoptotic cell death by the Parkinson mimetic, 6-hydroxydopamine, was shown. The protection provided by anandamide involved activation of phosphatidylinositol 3-kinase and prevention of 6-hydroxydopamine induced activation of c-Jun-NH2-terminal kinase (JNK). These data add to the growing body of literature concerning cannabinoids and Parkinson's disease. Certain other neurodegenerative diseases, termed tauopathies, feature filamentous tau-positive protein inclusions in neurons and glia, which are characterized by the expression of stress response proteins, particularly heat shock proteins (Hsps), in these inclusions. The article by L. Schwarz et al. investigated the contribution of small Hsps, Hsp27, and αB-crystallin, to neurodegenerative diseases by analyzing the association of Hsp27 with pathological lesions of tauopathies. Their results suggest distinct mechanisms for Hsp27 action in glial and neuronal cells, with prominent expression in unstressed astrocytes but with low expression observed in neurons even after stress situations.

We hope that this special issue will alert researchers to some new developments in the fields of cell stress and cell death, particularly the interplay between prosurvival and prodeath responses, and how our knowledge of these can direct our efforts in discovering new therapeutic strategies for the treatment of cancers and degenerative diseases.

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Review Article

Cellular Stress Responses: Cell Survival and Cell Death

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Cells can respond to stress in various ways ranging from the activation of survival pathways to the initiation of cell death that eventually eliminates damaged cells. Whether cells mount a protective or destructive stress response depends to a large extent on the nature and duration of the stress as well as the cell type. Also, there is often the interplay between these responses that ultimately determines the fate of the stressed cell. The mechanism by which a cell dies (i.e., apoptosis, necrosis, pyroptosis, or autophagic cell death) depends on various exogenous factors as well as the cell's ability to handle the stress to which it is exposed. The implications of cellular stress responses to human physiology and diseases are manifold and will be discussed in this review in the context of some major world health issues such as diabetes, Parkinson's disease, myocardial infarction, and cancer.

1. Overview of Cellular Stress Responses

Cells respond to stress in a variety of ways ranging from activation of pathways that promote survival to eliciting programmed cell death that eliminates damaged cells. The cell's initial response to a stressful stimulus is geared towards helping the cell to defend against and recover from the insult. However, if the noxious stimulus is unresolved, then cells activate death signaling pathways. The fact that the cell's survival critically depends on the ability to mount an appropriate response towards environmental or intracellular stress stimuli can explain why this reaction is highly conserved in evolution. For example, antioxidant defence mechanisms against oxidative injury and stress proteins such as heat shock proteins occur in lower organisms as well as the mammals.

There are many different types of stress and the response a cell mounts to deal with these conditions will depend on the type and level of the insult. For example, protective responses such as the heat shock response or the unfolded protein response mediate an increase in chaperone protein activity which enhances the protein folding capacity of the cell, thus counteracting the stress and promoting cell survival. The adaptive capacity of a cell ultimately determines its fate.

Therefore, depending on the level and mode of stress, different defense mechanisms and prosurvival strategies are mounted; however, if these are unsuccessful, then the cell death programs are activated to eliminate these damaged cells from the organism. The mechanism by which a cell dies, that is, apoptosis, necrosis, pyroptosis, or autophagic cell death, often depends on its ability to cope with the conditions to which it is exposed. In this review we initially discuss the different forms of cell death that can be activated by adaptive responses because activation of death signaling pathways is the ultimate response to all types of persistent irresolvable stress. In Section 3 we will discuss the many types of stress a cell can encounter and the different responses that are activated to survive adverse conditions. Finally, we will discuss the involvement or contribution of cellular stress responses to disease states.

2. Stress-Induced Cell Death

Cell death has many forms and shapes. Cell death research encompasses not only the study of programmed forms of cell death (both apoptosis and autophagic cell death), necrosis and other modes of cellular demise but also the role these

phenomena play in physiological and pathological processes including development, aging, and disease.

The cell death field has attracted much attention in the last two decades, mainly because of its relevance to development, degenerative diseases, and cancer. However, the field of cell death research is by no means new [1]. The concepts of cellular demise and associated terminology have been evolving since the 19th century. The term *programmed cell death* refers to controlled or regulated forms of death associated with a series of biochemical and morphological changes [2–4]. The realization that some forms of cell death were biologically controlled or programmed has led to exploitation of these processes and has made profound impact in various fields of biology and medicine [5–7].

Nowadays, programmed cell death is synonymous with apoptosis; however, based on the original definition it also refers to autophagic cell death [8]. The term *apoptosis* was first used to describe a particular morphology of cell death [9] common to the vast majority of physiological cell deaths. This morphology includes shrinkage and blebbing of cells, rounding and fragmentation of nuclei with condensation, and margination of chromatin, shrinkage, and phagocytosis of cell fragments without accompanying inflammatory responses (in most cases) [9–11]. The morphology of cells undergoing apoptosis appeared dissimilar and distinct from the morphology associated with necrosis [9, 10]. *Necrosis*, a term commonly used by pathologists, refers to any deaths associated with the loss of control of ionic balance, uptake of water, swelling, and cellular lysis [12, 13]. This lysis releases many intracellular constituents, attracting immune cells and provoking an inflammatory response.

2.1. Apoptosis. During the 1980s, apoptosis became the focus of attention, primarily because of the relative ease with which it could be distinguished morphologically from other types of cell death. Within a few years apoptosis and delineation of the underlying biochemical and molecular pathways dominated cell death research. The discoveries of the Bcl-2 family of proteins [14–16], death receptors [17], caspases [18], mitochondrial cytochrome *c* release [19], and a role for the endoplasmic reticulum [20] in apoptosis were just a few major milestones in the history of the field. Today the morphological and biochemical changes associated with apoptosis are largely explained by activation of caspases, and apoptosis has become generally accepted as caspase-dependent programmed cell death [21].

Of all the forms of cell death apoptosis is the best characterized and its highly regulated nature makes it an attractive target for therapeutic intervention. Apoptosis is highly conserved throughout evolution [22, 23] and plays a major physiological role in both embryonic development and aging [22, 24]. Various types of cellular stress stimuli have been shown to trigger apoptosis, including chemotherapeutic agents, irradiation, oxidative stress, and ER stress. Caspases, a family of cysteine proteases, act as common death effector molecules in various forms of apoptosis [25]. Caspases are synthesized as inactive proenzymes, which upon activation cleave various substrates in the cytoplasm or nucleus. This leads to many of the morphologic features of apoptotic cell death, for example, polynucleosomal DNA fragmentation, loss of overall cell shape, and nuclear shrinking [22, 25–27].

During apoptosis caspases are activated by different mechanisms. Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) or TNF-related apoptosis inducing ligand (TRAIL) receptors by their respective ligands or agonistic antibodies results in receptor aggregation and recruitment of the adaptor molecule Fas-associated death domain (FADD) and procaspase-8 to form the death inducing signaling complex (DISC) [26]. Upon recruitment caspase-8 becomes activated and initiates apoptosis by direct cleavage of downstream effector caspases [26]. The mitochondrial pathway to caspase activation is initiated by the release from the mitochondrial intermembrane space of apoptogenic factors such as cytochrome *c*, apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac)/direct IAP binding protein with low pI (DIABLO) or Omi/high-temperature requirement protein A2 (HtrA2) [28]. The release of cytochrome c into the cytosol results in caspase-3 activation through formation of the cytochrome *c*/Apaf-1/caspase-9-containing apoptosome complex [29]. Smac/DIABLO or Omi/HtrA2 promotes caspase activation through neutralizing the inhibitory effects of Inhibitor of Apoptosis Proteins (IAPs) [30]. Activation of caspases has to be tightly controlled because of the potential detrimental effects on cell survival if they are inappropriately activated. For example, resistance to apoptosis can be caused by aberrant function or expression of IAPs [30]. IAPs present a group of endogenous inhibitors of caspases with eight members in human cells, that is, XIAP, cIAP1, cIAP2, survivin, livin (ML-IAP), NAIP, Bruce (apollon), and ILP-2 [30]. All IAP proteins harbor one or more baculovirus IAP repeat (BIR) domains that mediate their inhibitory interaction with caspases [30]. Among the IAP family proteins, XIAP is the most potent inhibitor of caspases and blocks apoptosis by binding to active caspase-3 and -7 and by interfering with caspase-9 activation [30].

In addition, the ratio of antiapoptotic versus pro-apoptotic Bcl-2 family proteins regulates apoptosis sensitivity. The Bcl-2 proteins comprise both anti-apoptotic family members, for example, Bcl-2, Bcl-X_L, and Mcl-1, and pro-apoptotic molecules such as Bax, Bak, and BH3 domain only molecules [31]. According to the direct activation model of Bcl-2 protein activation, BH3-only proteins that function as direct activators (such as Bim and the cleaved form of Bid (tBid)), directly bind to Bax and Bak to stimulate their activation [32]. In this model, BH3-only proteins that act as sensitizers such as Bad promote apoptosis by binding to the prosurvival Bcl-2 proteins [32]. In contrast, the indirect activation model proposes that BH3-only proteins activate Bax and Bak in an indirect manner by binding to the multiple anti-apoptotic Bcl-2 proteins that inhibit Bax and Bak, which in turn leads to the release of Bax and Bak [33, 34]. Moreover, apoptosis sensitivity may be controlled by IAPs, through the regulation of additional signaling cascades, for example, the NF-κB, JNK, TNFR, and the ubiquitin/proteasome pathway [30, 35]. The anti-apoptotic mechanisms regulating cell

death have also been implicated in conferring drug resistance to tumor cells.

2.2. Autophagic Cell Death. Autophagy (self-eating) is a multistep process that is characterized by the vesicular sequestration and degradation of long-lived cytoplasmic proteins and organelles, for example, mitochondria [36]. The resulting double-membrane vesicle is termed an autophagosome [36]. The discovery of autophagy-related (*atg*) genes, first in yeast and subsequently in humans, has greatly enhanced the molecular understanding of the mechanisms that are involved in the control of autophagy [36]. The protein product of the tumor suppressor gene Beclin 1 is the mammalian homolog of Atg6 and forms a multiprotein complex together with Vps34, a class III phosphatidylinositol 3-kinase, UVRAG (UV irradiation resistance-associated tumor suppressor gene), and a myristylated kinase (Vps15, or p150 in humans) [36, 37]. This complex is required for the initiation of the formation of the autophagosome. Once this complex forms, Vps34 becomes activated and catalyzes the generation of phosphatidylinositol-3-phosphate, which is required for vesicle nucleation.

Two major protein conjugation systems exist that are required for autophagosome formation, that is, the Atg12–Atg5 conjugation and Atg8-phosphatidylethanolamine conjugation systems [38]. Mechanistically, both conjugation systems function in a manner that is closely related to ubiquitin conjugation to proteins, with corresponding conjugation-assisting enzymes that resemble the E1 and E2 enzymes in ubiquitin conjugation [38]. In the Atg12–Atg5 conjugation pathway, Atg12 is covalently conjugated to Atg5 with the help of the E1-like enzyme Atg7 and the E2-like enzyme Atg10 [36]. In the other conjugation pathway, phosphatidylethanolamine (PE) is conjugated to LC3, one of the mammalian homologues of Atg8 [36]. This process involves the sequential action of the protease Atg4, the E1-like enzyme Atg7 and the E2-like enzyme Atg3. Subsequently, lipid conjugation results in the conversion of the soluble form of LC3, that is, LC3-I, to the autophagic-vesicle-associated form that is termed LC3-II [39]. Thus, LC3 is soluble under unstressed conditions and undergoes association with peripheral membranes of autophagosomes during the induction of autophagy. Via the fusion with lysosomes, the content of autophagosomes is degraded by the action of acid-dependent enzymes [36].

Autophagy is typically observed in cells that are exposed to a variety of metabolic and therapeutic stresses, including growth factor deprivation, inhibition of the receptor tyrosine kinase/Akt/ mammalian target of rapamycin (mTOR) signaling, shortage of nutrients, ischemia/reperfusion, inhibition of proteasomal degradation, the accumulation of intracellular calcium, and endoplasmic reticulum (ER) stress [40–43]. Reactive oxygen species (ROS) may provide a common link between cellular stress signals and the initiation of autophagy, as ROS accumulation has been reported to result in inactivation of the cysteine protease ATG4, which in turn causes accumulation of the ATG8-phosphoethanolamine precursor that is required for the initiation of autophagosome formation [44]. The functional relationship between autophagy and cell death is complex in the sense that, under most cellular settings, autophagy functions as a stress adaptation that prevents cell death, whereas in some circumstances, it constitutes an alternative route to cell death. This complex interrelationship between autophagy and cell death implies that these responses are somewhat linked at the molecular level. However, the key molecular events that eventually determine whether autophagy is protective or destructive are still poorly understood.

Although it is still controversial whether autophagy is protective or toxic for the cells, accumulating evidence suggests that it has beneficial roles in the heart under both physiological and pathological conditions [45, 46]. Autophagy was shown to mediate turnover of intracellular proteins and organelles in the heart and protect against hemodynamic stress [45]. Consistent with this, rapamycin, which induces autophagy by inhibiting mTOR, can protect myocardium against ischemia/reperfusion injury [47]. In contrast, recent studies also demonstrated that downregulation of the transcription factors, activating transcription factor 5 or 7 (ATF5 or ATF7), using siRNA prevented stress-induced cell death [48, 49], suggesting that the level or timing of autophagy may be critical for deciding the fate of the cells. Autophagic cell death has mainly been shown during development. However, during recent years accumulating evidences suggest that inhibition of apoptosis induces cell death that is either associated with or dependent on autophagy [48–50]. There is evidence of cross-talk between apoptosis and autophagy at the molecular level, particularly with regard to the Bcl-2 family. In addition to its role in inhibiting apoptosis, Bcl-2 has also been shown to inhibit autophagy [51, 52] and autophagic cell death [53]. This effect is mediated through the ability of Bcl-2 to interact with Beclin 1, a key protein in autophagosome formation [52]. In fact, Beclin 1 has been shown to be a novel BH3-only protein and to interact with a number of anti-apoptotic Bcl-2 family members including Bcl-2, Bcl-xL, Bcl-w, and Mcl-1 [54–57].

2.3. Necrosis. Necrosis has been considered as an accidental mode of cell death for many years, implying that within a multicellular organism it is an unregulated process. However, there is now mounting evidence that the execution of necrotic cell death is also regulated by a set of signaling pathways [58–60]. For instance, death domain receptors, for example, TNFR1, and Toll-like receptors have been reported to trigger necrosis, in particular in the presence of caspase inhibitors [58]. In addition, necrotic cell death has been reported in response to cellular stress stimuli, including ischemia or glutamate excitotoxicity in neurons or cancer cells exposed to alkylating DNA damaging agents [61–63]. Morphologically, necrosis is characterized by a gain in cell volume, swelling of organelles and plasma membrane rupture, which results in the loss of intracellular contents. Several signal transduction cascades have been described that are involved in the propagation of necrotic cell death. There

is mounting evidence that the serine/threonine kinase RIP1 is one of the key mediators of necrotic cell death, at least in the case of death receptors or Toll-like receptors [64, 65]. Studies in RIP1-deficient leukemia cells revealed that RIP1 is required for death receptor-induced necrosis [66, 67]. Furthermore, RIP1 has been described to be required for lipopolysaccharide-induced cell death of macrophages [68]. In line with a central role of RIP1 in necrotic cell death, small molecule inhibitors of RIP1 kinase were reported to protect against ischaemic brain injury in an in vivo model of necrosis [69–71]. In addition to RIP1, there is very recent evidence that RIP3 is also critical for necrotic cell death [72–74]. To this end, RIP3 was identified in an RNA interference screen to be essential for necrosis in response to TNFα stimulation and during virus infection [72, 73]. RIP3 interacts with RIP1 and regulates RIP1 phosphorylation and the generation of ROS [72–74].

Moreover, ROS and calcium constitute important mediators that are involved in the propagation of the necrotic signal in various forms of necrosis, for example, upon stimulation with TNFα or exposure to double-stranded DNA [75, 76]. ROS may be generated intracellularly by mitochondria and glycolysis [75, 77]. While the ER is the main intracellular calcium store, mitochondrial calcium has been described to stimulate oxidative phosphorylation, thereby promoting ROS generation [78]. Both ROS and calcium can cause damage to organelles and macromolecules, which contributes to the loss of cell integrity. In addition calcium-mediated activation of calpain can lead to cleavage and inactivation of caspases [79], whereas the ROS can target the active site of caspases and render them inactive [80]. Many stimuli that drive necrosis can inhibit the apoptotic machinery.

3. Cellular Stress Responses

During tissue homeostasis there is an equilibrium between the net growth rate and the net rate of cell death [22]. Upon exposure to cellular stress this physiological homeostasis is in danger. Depending on the type of cellular stress and its severity, the cell's response can be manifold. In essence, if the stress stimulus does not go beyond a certain threshold, the cell can cope with it by mounting an appropriate protective cellular response, which ensures the cell's survival. Conversely, the failure to activate or maintain a protective response, for example, if the stressful agent is too strong, results in activation of stress signaling cascades that eventually fuel into cell death pathways [81, 82].

3.1. The Heat Shock Response. One of the main prosurvival activities of cells, the heat shock response, was originally described as the biochemical response of cells to mild heat stress (i.e., elevations in temperature of 3–5°C above normal) [83, 84]. It has since been recognized that many stimuli can activate this response, including oxidative stress and heavy metals. One of the main cellular consequences of these stresses is protein damage leading to the aggregation of unfolded proteins. In order to counteract this, cells increase the expression of chaperone proteins that help in the refolding of misfolded proteins and alleviate protein aggregation. This confers a transient protection, leading to a state that is known as thermotolerance, whereby cells become more resistant to various toxic insults, including otherwise lethal temperature elevations, oxidative stress, various anticancer drugs, and trophic factor withdrawal [85–88].

During initiation of the heat shock response general protein transcription and translation is halted, presumably to alleviate the burden of misfolded proteins in the cell. However, transcription factors that enhance expression of a specific subset of protective genes are selectively activated under these conditions; these are the heat shock factors (HSFs) [89]. Vertebrate cells have three different HSFs: HSF1 is essential for the heat shock response and is also required for developmental processes, HSF2 and HSF4 are important for differentiation and development, while HSF3 is only found in avian cells and is probably redundant with HSF1 [90, 91]. Cells derived from mice lacking HSF1 are sensitive to stress and are unable to develop thermotolerance or induce heat responsive genes upon heat shock [92–94], which has confirmed that HSF1 in particular is responsible for the heat shock response. More recent work has shown that HSF2 can modulate HSF1-mediated expression of heat-responsive genes [95], suggesting that HSF2 also participates in transcriptional regulation of the heat shock response.

Inactive HSF1 is maintained in a monomeric form in the cytoplasm through interaction with Hsp90 and cochaperones [96, 97] (Figure 1). When the cell is exposed to stressful conditions, there is accumulation of unfolded proteins which compete with HSF1 for Hsp90 binding. Thus, HSF1 is released from the complex stimulating its transition from a monomer to a homotrimer that can translocate to the nucleus and bind to DNA (Figure 1). HSFs bind to upstream sequences (heat shock elements) in the promoters of target genes, leading to the expression of heat shock proteins (Hsps).

Hsps are a set of evolutionary conserved proteins that are grouped into subfamilies with molecular weights of approximately 110, 90, 70, 60, 40, and 15–30 kDa [85, 98]. Some of these, for example, Hsp90, are constitutively expressed and act intracellularly as molecular chaperones, preventing premature folding of nascent polypeptides [99]. Others, particularly Hsp27 and Hsp70, are usually expressed at low basal levels and increase in response to environmental and physiological stressors, and as such they are termed inducible Hsps and are part of the heat shock response [85]. Hsp27 belongs to a subfamily of stress proteins, the small Hsps, which are detectable in virtually all organisms. Hsp27 is also regulated by phosphorylation and dynamic association/dissociation into multimers ranging from dimers to large oligomers [100]. Hsp70 is the inducible member of the 70 kDa family of Hsps. Both Hsp27 and Hsp70 have been shown to protect cells against the induction of cell death by a variety of stresses and by different modes of cell death, including apoptosis [86, 101] and necrosis [102–104]. They achieve these effects directly, through inhibition of cell death pathways, and indirectly, through general prosurvival activities. For example, in their capacity as

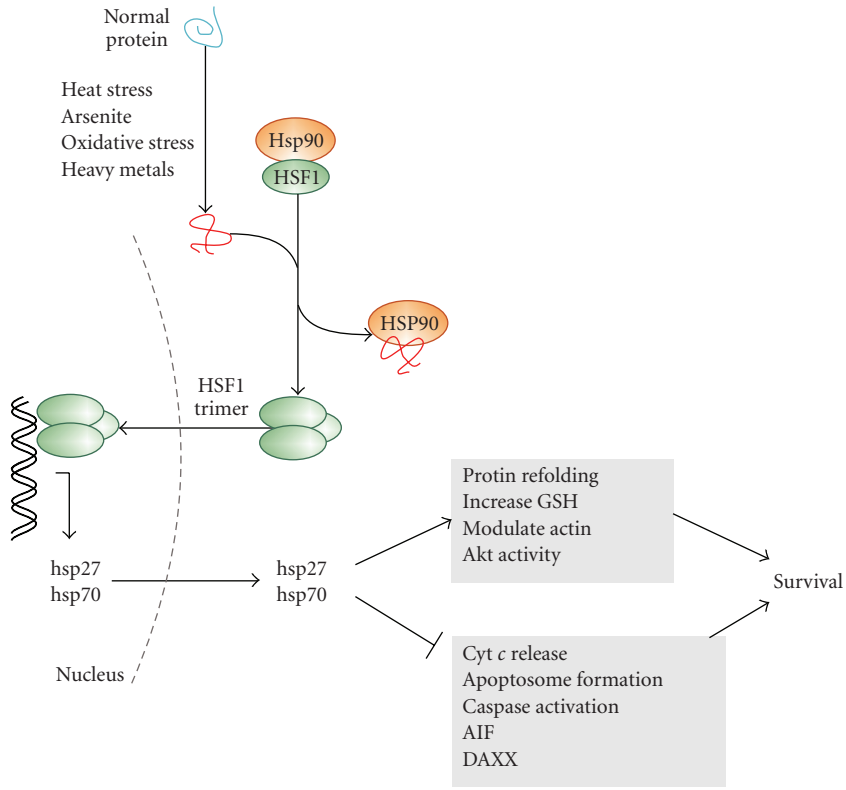

Figure 1: Induction of heat shock proteins inhibits apoptosis and promotes cell survival. Exposure of cells to elevated temperatures, oxidative stress, and heavy metals causes accumulation of unfolded proteins, which through activation of HSF1 leads to induction of Hsp27 and Hsp70. These Hsps inhibit apoptosis and promote survival.

molecular chaperones, inducible Hsps bind to and aid the refolding of unfolded proteins, thereby preventing protein aggregation [105]. Hsp27 can interact with actin and is thus important for maintaining the integrity of the cytoskeleton which may play a role in promoting survival [106].

Apart from these indirect mechanisms, Hsp27 and Hsp70 can directly inhibit apoptosis by modulating both the intrinsic and the extrinsic apoptosis pathways and by interfering with caspase activation at several different levels [107–109]. Both Hsp27 and Hsp70 have been reported to directly block release of pro-apoptotic factors, including cytochrome *c*, from the mitochondria [110–112]. In the cytosol, these Hsps can block apoptosome formation and activation of downstream caspases through their ability to bind to cytochrome *c* and procaspase-3 (in the case of Hsp27) [107, 108] and procaspases -3, -7 and Apaf-1 (in the case of Hsp70) [101, 113–115]. Hsp70 can also interact with and inhibit apoptosis-inducing factor (AIF) thus inhibiting apoptotic nuclear changes [116, 117]. Hsps can also modulate the death receptor pathway. Hsp27 is reported to inhibit DAXX, an adaptor protein that links the Fas death receptor and the ER stress sensor IRE1 to ASK-1 and downstream JNK pro-apoptotic signaling [118]. Hsp70 also inhibits JNK activity [119–121], although this is not observed in all systems [101]. Hsp27 and 70 can also interact with other proteins that regulate cell survival. For example, Hsp27 can interact with the prosurvival Ser/Thr kinase Akt which is suggested to be important for sustained Akt activity [122–124]. Hsp70 can exist in complex with cochaperones, including DnaJ/Hsp40 and BAG-1 which affect its ability to modulate apoptosis [125, 126]. Overall, Hsps can be activated or induced by a number of stresses and they act to protect the cell by influencing a variety of cellular processes which determine cellular fate. Hsps are, in general, prosurvival and anti-apoptotic molecules.

3.2. The Unfolded Protein Response (UPR). Secretory and membrane proteins undergo posttranslational processing, including glycosylation, disulfide bond formation, correct folding, and oligomerization, in the ER. In order to effectively produce and secrete mature proteins, cellular mechanisms for monitoring the ER environment are essential. Exposure of cells to conditions such as glucose starvation, inhibition of protein glycosylation, disturbance of Ca^{2+} homeostasis and oxygen deprivation causes accumulation of unfolded proteins in the ER (ER stress) and results in the activation of a well orchestrated set of pathways during a phenomenon known as the unfolded protein response (UPR) [127, 128] (Figure 2). The UPR is generally transmitted through activation of ER resident proteins, most notably inositol-requiring protein-1 (IRE1), protein kinase RNA (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6). In some cells/tissues, additional ATF6-like

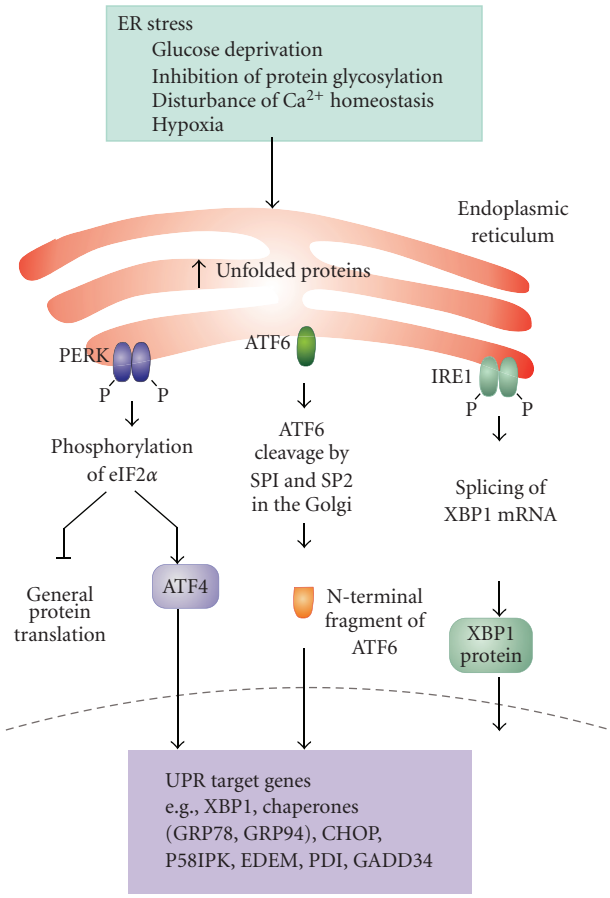

Figure 2: *ER stress and the unfolded protein response.* Stress to the ER stimulates the activation of the three endoplasmic reticulum (ER) stress receptors, PKR-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (Ire1) that are involved in the unfolded protein response (UPR). PERK phosphorylates eukaryotic initiation factor 2 (eIF2α) which inhibits general protein translation, allowing eIF2α-independent translation of ATF4, which activates transcription of chaperones such as GRP78. ATF6 undergoes specific proteolysis in the Golgi apparatus which leads to activation. One of the ATF6 target genes is XBP1. IRE1 catalyzes the alternative splicing of XBP1 mRNA leading to expression of the active XBP1 transcription factor. Together the three arms of the UPR block protein translation, increase chaperone expression and enhance ER-associated protein degradative pathways.

bZip type transcription factors such as OASIS, CREB-H, Tisp40, and Luman also transmit the UPR signaling [129–132]. The UPR target genes include molecular chaperones in the ER, folding catalysts, subunits of translocation machinery (Sec61 complex), ER-associated degradation (ERAD) molecules and anti-oxidant genes [127].

Among the UPR transmitters so far identified, IRE1 and PERK are both type I transmembrane protein kinases which dimerize to promote autophosphorylation and activation in response to ER stress. Activated IRE1 endonucleolytically cleaves mRNA that encodes a transcription factor named homologous to ATF/CREB1 (Hac1) in yeast [133, 134] and X-box binding protein-1 (XBP1) in higher species [135, 136]. The spliced forms of Hac1 and/or XBP1 in turn activate the transcription of the UPR target genes. In contrast, activated PERK phosphorylates the α-subunit of eukaryotic translation initiation factor-2 (eIF2α) which leads to lower levels of eIF2 and translational suppression [137]. The PERK-eIF2α signaling pathway also activates the transcription of the UPR target genes through CAP-independent upregulation of the translation of a transcription factor ATF4 [138]. PERK can also directly phosphorylate and activate the transcription factor, NF-E2-related factor-2 (Nrf2), which contributes to cellular redox homeostatis by inducing the expression of anti-oxidant genes [139, 140]. ATF6 is a type II transmembrane protein which is cleaved by Golgi apparatus-resident proteases site-1 protease (SP1) and site-2 protease (SP-2) in response to ER stress [141, 142]. The cleaved N-terminal fragment of ATF6 acts as a transcription factor to increase the transcription of the UPR target genes together with XBP1 and ATF4.

UPR signaling generally promotes cell survival by improving the balance between the protein load and the folding capacity in the ER and/or by improving the secretion of trophic factors/growth factors [143, 144]. However, if the protein load in the ER exceeds its folding capacity, or some defects in the UPR exist, cells tend to die, typically, with apoptotic features (ER stress-induced cell death). Although the exact molecular mechanisms that regulate this type of cell death remain to be elucidated, at least three pathways have been identified as being involved: the caspase-12/caspase-4 pathway and CHOP and IRE1-JNK pathways. Caspase-12 [145] in mice and caspase-4 in human [146] have been proposed as caspases that initiate ER stress-induced cell death. Caspase-12 null mice are reported to be relatively resistant to ER stress and amyloid-beta toxicity [145]. Caspase-12 is reported to directly cleave procaspase-9 without involvement of the cytochrome *c*/Apaf-1 pathway [147]. C/EBP homologous protein (CHOP), a transcription factor that is induced downstream of PERK and ATF6 pathways, induces ER stress-induced cell death at least in part by suppressing the expression of Bcl-2 [148] and inducing Bim expression [149]. IRE1 also participates in ER stress-induced cell death by activating JNK through the binding with ASK1 and Traf2 [150, 151].

Important roles for ER stress and ER stress-induced cell death have also been demonstrated in a broad spectrum of pathophysiological situations, including ischemia, diabetes, atherosclerosis, endocrine defects, development, neurodegenerative disorders, and cancer as described below [143, 144, 152–155].

Among the UPR targets, glucose-regulated proteins (GRPs) are the most studied and best characterized. GRPs were originally identified as proteins induced by glucose starvation [156]. Later, it was found that these molecules were transcriptionally induced by ER stress through the *cis*-acting element termed ER stress response element (ERSE) [157]. GRPs include molecular chaperones in the ER such as GRP78/Bip, GRP94, ORP150/GRP170, and oxidoreductases in the ER such as PDI, ERp72, and GRP58/ERp57. Accumulating evidence suggests that GRPs promote cell survival

when exposed to stresses such as hypoxia/ischemia [143, 158], glutamate excitotoxicity [159], and neurodegeneration [160–162]. GRP78 could be a potential factor to inhibit atherosclerosis by preventing ER stress-induced cell death in endothelial cells [163]. This involves the inhibition of the activation of SREBPs, a molecule that induces cholesterol and triglyceride biosynthesis, or by inhibiting tissue factor procoagulant activity [164–166]. ORP150/GRP170 was found to be associated with insulin sensitivity in both human and mice as described below. Furthermore, GRPs also play important roles in survival during early mammalian development [159, 167–169].

Interestingly, recent studies have revealed that small compounds that mimic the functions of GRPs (chemical chaperones) and those that induce endogenous GRPs (molecular chaperone inducers) can prevent protein aggregation [170], improve protein secretion [171], and protect cells against brain ischemia [172] or neurodegeneration [173]. These results suggest that the regulation of ER stress can be a novel therapeutic target in a variety of diseases.

3.3. The DNA Damage Response. Upon cellular stress conditions that are caused by exposure to chemotherapeutic agents, irradiation, or environmental genotoxic agents such as polycyclic hydrocarbons or ultraviolet (UV) light, damage to DNA is a common initial event [174, 175]. DNA double strand breaks (DSBs) and single strand breaks (SSBs) are considered as key lesions that initiate the activation of the DNA damage response [174]. Since the DNA duplex is more vulnerable to chemical attack or nucleases when it is separated into two single-stranded DNA strands, for example, during DNA replication and transcription, SSBs are preferentially generated under these conditions [176]. Defined SSBs are also generated during distinct pathways of DNA repair, for example, in the course of nucleotide excision repair (NER). After DNA damage recognition, dual incision 5′ to the DNA lesion by ERCC1-XPF and 3′ to the damage by XPG results in the removal of the lesion-containing oligonucleotide [177]. DSBs are produced directly or indirectly by many anticancer drugs, including DNA intercalating, alkylating or crosslinking agents, topoisomerase inhibitors, and nucleotide analogs [174]. Once DSBs are generated, ataxia telangiectasia mutated (ATM) is recruited by the MRE-11-Rad50-NBS1 (MRN) complex to sites of broken DNA and phosphorylates downstream substrates such as checkpoint kinase 2 (Chk2) and p53 [175, 178] (Figure 3). p53 induces transcriptional activation of different functional programs, for example, cell cycle regulatory proteins such as p21 and pro-apoptotic factors such as CD95, PUMA, and BAX [179]. In addition, recent studies have also defined a nontranscriptional pro-apoptotic activity of p53 that regulates the intrinsic mitochondria-mediated pathway of apoptosis [180]. Damage to DNA engages DNA repair processes to ensure the cell's survival in the case of sublethal damage [174]. Alternatively, if the damage is too severe to be repaired—the DNA-damaging insult is transmitted by the cellular stress response to the activation of effector systems to mediate cell death [174]. In the latter case, various stress-inducible molecules, including NF-κB, p53, JNK, or MAPK/ERK, have been implicated in propagating and modulating the cell death signal [81, 82].

Depending on the type of lesion, DNA damage initiates one of several mammalian DNA repair pathways, which eventually restore the continuity of the DNA double strand. There are two main pathways for the repair of DSBs, that is, nonhomologous end-joining and homologous recombination [181, 182]. The former constitutes the predominant DNA repair pathway in humans and involves DNA repair proteins such as DNA-PK, Ku70, and Ku80 [181, 182]. Base damage can be repaired either by enzyme-catalyzed reversal or alternatively via excision repair [183]. Mismatch repair is responsible for the removal of incorrectly paired nucleotides [184]. It is important to note that DNA repair can, in principle, be error-free and error-prone. Several proteins have been discovered recently that exert a specific function in error-free repair processes to guarantee high-fidelity reconstitution of the DNA [185]. Faithful genome transmission requires the coordination of this highly complex network of DNA repair pathways and repair surveillance mechanisms linked to cell cycle checkpoints as well as cell death mechanisms [185]. Error-prone repair or complete failure of DNA repair cannot only lead to mutations but can also lead to the initiation of cell death pathways [185].

3.4. The Response to Oxidative Stress. Cell survival requires appropriate proportions of molecular oxygen and various antioxidants. Reactive products of oxygen are amongst the most potent and omnipresent threats faced by cells. These include ROS such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), singlet oxygen, hydroxyl radical ($OH^{\bullet}$), peroxy radical, as well as the second messenger nitric oxide ($NO^{\bullet}$) which can react with $O_2^{\bullet -}$ to form peroxynitrite ($ONOO^-$). Normally in cells there exists equilibrium between pro-oxidant species and antioxidant defense mechanisms such as ROS-metabolizing enzymes including catalase, glutathione peroxidase, and superoxide dismutases (SODs) and other antioxidant proteins such as glutathione (GSH) (Figure 4). Oxidative stress occurs when there is a disturbance in this pro-oxidant:antioxidant balance and it has been implicated in several biological and pathological processes [186]. Although most oxidative insults can be overcome by the cell's natural defenses, sustained perturbation of this balance may result in either apoptotic or necrotic cell death [186–190].

ROS can emanate from intracellular or extracellular sources. Auto-oxidation of reduced respiratory components of the mitochondrial electron transport chain causes the production of free radical intermediates, $O_2^{\bullet -}$ and H_2O_2, which in the presence of iron can produce highly reactive $OH^{\bullet}$ radical via the Fenton reaction. These ROSs are dealt with by SODs, enzymes considered to be the first line of defense against oxygen toxicity. ROS can also be produced in the cytosol. For example, the arachidonic acid cascade, yielding prostaglandins, and leukotrienes may generate ROS when the released lipid is metabolized [191], and some cytochrome P-450 isozymes are well-known ROS producers

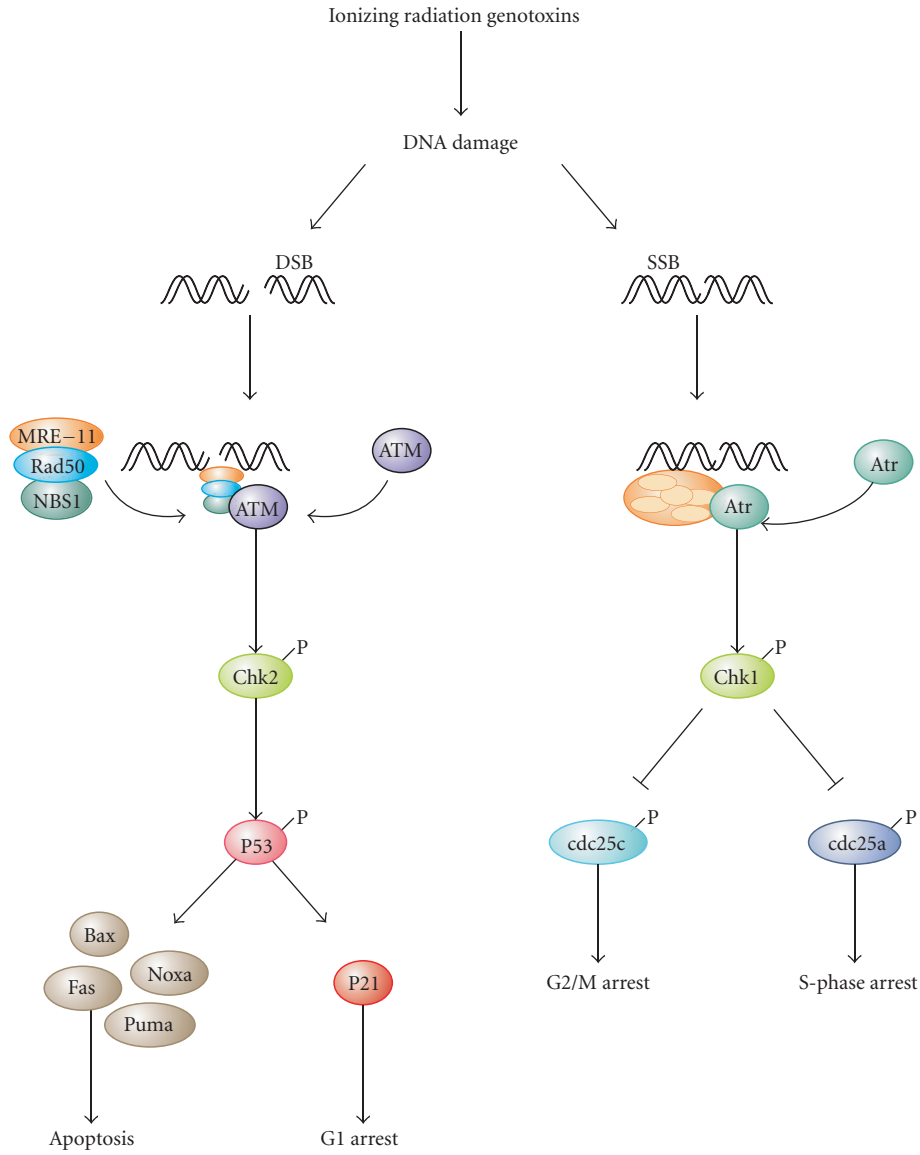

Figure 3: *DNA damage responses and cell death.* Upon exposure to ionizing radiation or genotoxins, the damage to DNA is a common initial event. DNA double strand breaks (DSBs) or single strand breaks (SSBs) are considered to be key lesions that initiate activation of the DNA damage response. Upon DSBs, ataxia telangiectasia mutated (ATM) is recruited by the MRE-11-Rad50-NBS1 (MRN) complex to sites of broken DNA and phosphorylates downstream substrates such as checkpoint kinase 2 (Chk2), which subsequently phosphorylates p53. Sublethal damage to DNA can engage survival pathways via p21-mediated cell cycle arrest. Alternatively—if the damage is too severe to be repaired—pro-apoptotic p53 target genes are activated including Bax, Puma, Noxa, and Fas, which promote apoptosis. Upon SSBs, it is ataxia telangiectasia and Rad3 related (ATR) that gets activated and phosphorylates Chk1. Chk1 in turn phosphorylates and inhibits cdc25c to mediated G2/M arrest or alternatively cdc25a to promote S-phase arrest.

[192]. Also, the auto-oxidation reactions of ascorbic acid, low molecular weight thiols, adrenalin, and flavin coenzymes can cause ROS production. In many of these cases, cytosolic GSH neutralizes the offenders. In addition to physiological sources of ROS, diverse exogenous agents can contribute to the intracellular production of free radicals. Most of these compounds cause the generation of $O_2^{\bullet -}$ and H_2O_2 [80, 193, 194]. The mechanism of action of many exogenous agents involves redox cycling whereby an electron is accepted to form a free radical and it is then transferred to oxygen.

Interestingly, there is evidence of cross-talk between oxidative stress and other stress response pathways. For example, oxidative stress is known to cause an increase in the expression of certain inducible Hsps, particularly Hsp27 [195–197]. Hsps have been reported to protect against many stresses apart from heat shock, including heavy metals, radiation, nitric oxide, and other oxidants. In addition, activation of the UPR stimulates upregulation of antioxidant genes through PERK-dependent phosphorylation of the Nrf2 transcription factor, whose target genes include

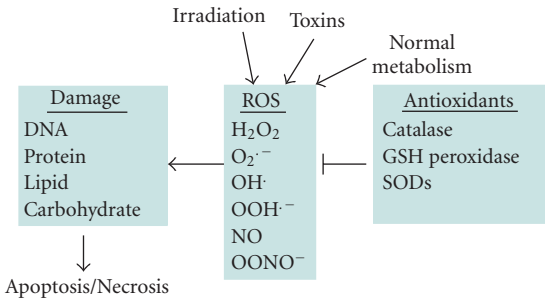

Figure 4: *Oxidative stress and cell death.* There is a plethora of stimuli that can trigger the generation of reactive oxygen species (ROS), among them irradiation, toxins, and also normal metabolic processes. A range of different ROS species have been identified, which are kept in check by antioxidant defenses. These include several detoxifying enzymes, for example, catalase, GSH peroxidase, and superoxide dismutase (SOD). If these antioxidants defense mechanisms are too weak, ROS-mediated damage to cellular macromolecules will eventually lead to cell death.

enzymes involved in GSH biosynthesis, and heme oxygenase-1 [198]. Moreover, perturbations in cellular redox status sensitize cells to the harmful effects of ER stress [199]. Similarly, accumulating evidence suggests a role for $O_2^{\bullet -}$ in the activation of autophagy [200].

ROS can cause damage to all of the major classes of biological macromolecules, including nucleic acids, proteins, carbohydrates, and lipids. When the cell's antioxidant defenses are overwhelmed, ROS can induce cell death. Numerous, recent studies have shown that the mode of cell death that occurs depends on the severity of the insult [187–189]. In fact, oxidants and antioxidants not only determine cell fate, but can also modulate the mode of cell death [186, 190].

Many cytotoxic agents induce ROS, including peroxide and $O_2^{\bullet -}$, which are involved in the induction of apoptotic cell death [201]. H_2O_2 can cause the release of cytochrome *c* from mitochondria into the cytosol and H_2O_2 may also activate nuclear transcription factors, like NF-κB, AP-1, and p53 [202], which may upregulate death proteins or produce inhibitors of survival proteins. One model proposed for H_2O_2 induction of apoptosis is upregulation of the Fas-FasL system, leading to activation of caspase-8 and downstream caspases [203, 204]. It is also possible that $NO^{\bullet}$ may also inactivate several antioxidant enzymes, including catalase, glutathione peroxidase, and superoxide dismutases [205, 206]. Also, $NO^{\bullet}$ has been reported to induce apoptosis by increasing ceramide generation through caspase-3 activation, induction of mitochondrial permeability transition, and activation of the Fas system [207].

Certain anti-apoptotic proteins have also been reported to have antioxidant roles. An early suggestion regarding the mechanism of action of Bcl-2 was that it inhibited cell death by reducing the generation of reactive oxidants, thus preventing critical intracellular oxidations that are requisite for the completion of the apoptotic program [208]. However, it is now understood that the reduction in ROS observed with Bcl-2 overexpression is probably the result of its ability to prevent loss of cytochrome *c* from mitochondria. Yet it is interesting to note that separate studies illustrate that Bcl-2-overexpressing cells have higher levels of total cellular GSH [209]. The product of the baculovirus p35 gene, a potent anti-apoptotic protein, is thought to have antioxidant role and is protective against many apoptotic stimuli including growth factor withdrawal, staurosporine, glucocorticoid, and actinomycin-D treatment, and is a broad-spectrum caspase inhibitor [210]. However, caspase inhibition may not be p35's sole mechanism of cytoprotection. Expression of the p35 gene inhibits H_2O_2-induced apoptosis in insect cells and may be acting as a sink for free radicals [211].

However, ROS are also reported to interfere with the apoptosis death program, compelling cells to adopt an alternative mode of cell death. Apoptotic cell death can be switched to necrosis during oxidative stress by two possible mechanisms: inactivation of caspases or a drop in cellular levels of ATP levels. Caspases contain an active site cysteine nucleophile [212] which is prone to oxidation or thiol alkylation as well as S-nitrosylation [80, 213, 214]. This leads to their inactivation, switching the mode of cell death to necrosis [80, 214]. $NO^{\bullet}$ may act as a molecular switch to control protein function via reactive thiol groups. For example, $NO^{\bullet}$-mediated inhibition of apoptosis in most cases is due to direct inhibition of caspase activity through S-nitrosylation of the active site cysteine conserved in all caspases although indirect effects on caspases can also be a component of toxicity in certain systems [214]. A switch from apoptosis to necrosis can also occur due to a drop in cellular levels of ATP caused by the failure of mitochondrial energy production by oxidants [215, 216]. As mentioned previously ROS may provide a common link between cellular stress signals and the initiation of autophagy, and ROS accumulation has been reported to result in inactivation of the cysteine protease ATG4, which in turn causes accumulation of the ATG8-phosphoethanolamine precursor that is required for the initiation of autophagosome formation [44]. In most circumstances, the induction of an autophagic response serves as a strategy that should ensure the cell's survival [217]. Under certain conditions, however, it may also bring about cell death, although the molecular determinants that may control the switch from survival to death are still poorly defined. In fact, in response to several anticancer drugs ROS can induce autophagic cell death.

4. Switch from Prosurvival Signaling to Cell Death Signaling

While conditions of stress stimulate cells to mount protective responses to counteract the effect of the stress on cellular processes, if the stress remains unresolved, eventual death of the cell ensues. This raises key questions about the molecular mechanisms involved in this switch from prosurvival signaling to prodeath signaling. For example, is there a particular molecule that acts as a molecular switch? How do the duration and severity of the stress contribute to activation of this switch? As described above, in the face of exposure to

cell stress, the cell mounts protective responses such as the heat shock response, or the unfolded protein response, in order to relieve the stress and promote survival. However, it is known that if the stress is very severe or if it is prolonged, the cell will die in spite of the activation of prosurvival signaling.

In the case of the response of cells to heat stress, the induction of Hsps does not occur if the stress is too severe and it has previously been suggested that the induction of thermotolerance, that is, Hsp expression, and of cell death is mutually exclusive events within the same cell [87, 195]. In support of this, we have observed that in a culture exhibiting mixed responses to a stressor, that is, expression of Hsps, induction of apoptosis, and induction of necrosis, the expression of Hsps was mainly observed in the surviving cells [196]. However, a recent report suggests that this may not always be the case, as at least one agent, which induces expression of Hsps through direct activation of HSF1, induces apoptosis rather than being protective [218].

During ER stress, IRE1 may be involved in the switch between the prosurvival UPR and initiation of cell death pathways [219]. Interestingly, the three arms of the UPR are thought to be activated sequentially, with PERK being activated most rapidly, followed by ATF6 and then IRE1. This suggests that time is allowed so that PERK and ATF6 may resolve the stress, and although IRE1 also contributes to the prosurvival UPR, it ultimately terminates it by relieving the translational inhibition by inducing $p58^{IPK}$ [20]. If the stress has been resolved, the cell returns to normal, but if not, then apoptosis is initiated, possibly by IRE1-dependent activation of ASK1 and its downstream target JNK. However, recently it has been shown that attenuation of IRE1 can switch the adaptive UPR to apoptosis and that persistent activation of IRE1 increases cell viability upon ER stress, suggesting that the duration of IRE1 signaling may act as a switch [219].

5. Stress Responses in Disease States

It is currently understood that a pathological stress response is a hallmark of many common human diseases for a number of reasons. Firstly, the stress stimulus may be too strong and/or prolonged, thereby allowing insufficient time for recovery to the normal status. Secondly, a cell's ability to handle even physiological levels of stress may be altered in disease states, similarly resulting in detrimental outcomes. In the following section, we will provide some selected examples of how pathological handling of stress is one of the major underlying causes of the pathophysiological state in very different types of human diseases.

5.1. Diabetes. Loss of function or death of the pancreatic β-cells in the Islets of Langerhans in the pancreas is the major pathological feature of diabetes mellitus. The pancreatic β-cells have a highly developed secretory system, in which the ER has an integral role, enabling a rapid response to glucose stimulation by producing and releasing large amounts of insulin. Both oxidative stress and ER stress are involved in the failure of pancreatic β-cells and development of diabetes.

The reactive species which play an important role in the pathogenesis of pancreatic β-cell loss in diabetes are generated intracellularly when the β-cells are targeted by proinflammatory cytokines in autoimmune Type 1 diabetes or when exposed to a hyperglycaemic and hyperlipidaemic milieu in Type 2 diabetes. There is evidence for the participation of both $NO^{\bullet}$ and ROS in the pathogenesis of β-cell death in Type 1 diabetes, whereas, for β-cell dysfunction in Type 2 diabetes, ROS are the main culprits.

Proinflammatory cytokines, including IL-1β (interleukin 1β), TNFα (tumor necrosis factor α), and IFNγ (interferon γ), released from immune cells infiltrating the pancreas in Type 1 diabetes, target the β-cells via their respective receptors [220]. They activate a multitude of signaling cascades, culminating in apoptosis of β-cells [221]. A number of steps in this chain of events affect the rate of generation of $NO^{\bullet}$ and ROS. It is evident from studies in patients with diabetes and in animal models of Type 1 diabetes, that IL-1β is the key proinflammatory cytokine which significantly contributes to β-cell dysfunction and apoptosis in the pathogenesis of Type 1 diabetes. It does so through activation of the transcription factor NF-κB which is responsible for the induction of iNOS and subsequent production of $NO^{\bullet}$ [155, 221]. The production and release of IFNγ acts synergistically with IL-1β. High concentrations of IFNγ are required to potentiate the effects of IL-1β on iNOS and $NO^{\bullet}$ production [222]. $NO^{\bullet}$ and ROS seem to also cross-talk with ER stress and UPR [223].

IL-1β also induces MnSOD (a manganese-dependent SOD isoenzyme) and this results in an increased rate of conversion of $O_2^{\bullet -}$ into H_2O_2 in the mitochondria [224]. Cu/ZnSOD, the cytoplasmic isoenzyme, is unaffected by IL-1β. The profile of the effects of TNFα and IFNγ alone, or in combination with IL-1β, on MnSOD is comparable with that of their regulation of iNOS. The effects on the generation of both radicals are not only important in themselves but also affect the balance between $NO^{\bullet}$ and $O_2^{\bullet -}$, and this can have significant effects on β-cell toxicity. A decrease in $O_2^{\bullet -}$ through MnSOD may present as a protective signal through a reduction of NF-κB activation and other components of the IL-1β signaling pathway [225]. On the other hand, an increased conversion rate of $O_2^{\bullet -}$ into H_2O_2 by SOD is likely to increase toxicity to the β-cell with its poor enzymatic capacity for H_2O_2 inactivation [226, 227].

Another major proinflammatory cytokine, TNFα, is released from the infiltrating immune cells speeds up β-cell loss significantly, resulting in an accelerated progression of the disease with rapid loss of the entire pancreatic β-cell population and Islet mass. Ceramide is likely to play a significant role as a mediator of $O_2^{\bullet -}$ formation in TNFα-mediated toxicity [228], thereby explaining the dominance of ROS in the case of TNFα when compared with IL-1β. Thus, with a significant contribution of TNFα produced by the infiltrating immune cells in Type 1 diabetes the resulting greater cytotoxicity is the result of the more pronounced ROS component of TNFα toxicity.

That the ROS-mediated component of cytokine toxicity primarily targets the mitochondria is shown by the fact that exposure of insulin-producing cells to IL-1β, or to a

cytokine mixture containing both IFNγ and TNFα, causes mitochondrial damage, while other subcellular structures remain intact. This damage can be prevented by expression of high levels of catalase or GSH in the mitochondria, but not in the cytosol [228]. IL-1β toxicity, mediated through $NO^{\bullet}$ and potentiated by IFNγ and TNFα, is likely to focus its effects in the cytoplasm. This component will presumably contribute to ER stress, which plays a significant role in dysfunction of β-cells under cytokine attack [229].

β-Cell loss in Type 2 diabetes is slower than in Type 1 diabetes, typically with a long phase of β-cell dysfunction, characterized by defective insulin secretion in response to glucose. In Type 2 diabetes, glucolipotoxicity, rather than proinflammatory cytokines, is considered to be an important contributing factor to β-cell dysfunction [230–234]. It is evident from studies on β-cells exposed to a combination of high glucose and a saturated fatty acid that $NO^{\bullet}$ generation through iNOS induction does not contribute to β-cell dysfunction [235].

Increased mitochondrial metabolic flux is required in the β-cell for generation of the ATP signal for glucose-induced insulin secretion [236] and its potentiation through fatty acids [231]. On the other hand, increased metabolic flux through the respiratory chain at high glucose and lipid concentrations should increase $O_2^{\bullet -}$ formation, thereby reducing the mitochondrial membrane potential via uncoupling protein 2 [230, 237]. This should decrease metabolic flux through the respiratory chain and thus reduce $O_2^{\bullet -}$ production, thereby acting in a protective manner against ROS-induced damage, but, at the same time, attenuating nutrient-induced insulin secretion. This casts doubt on the concept that increased intra-mitochondrial generation of ROS crucially contributes to β-cell damage in Type 2 diabetes.

This interpretation is supported by the results of morphological analyses showing that insulin-producing cells exposed to the fatty acid palmitate show no signs of mitochondrial damage, but very pronounced defects of the ER [238], confirming observations of increased ER stress in response to glucolipotoxicity [235]. Thus one of the prominent targets of this free-radical-mediated toxicity might indeed be the ER.

Defects in PERK-eIF2α pathways cause Wolcott-Rallison syndrome, a rare infantile-onset insulin-requiring diabetes [239] and PERK-null mice developed similar phenotypes [240]. Mice with mutated proinsulin (proinsulin-2) that cannot form a disulfide bond (Akita mice) also develop severe diabetes which is associated with the ER stress-induced cell death in pancreatic β-cells [241, 242]. Mice deficient for $p58^{IPK}$, which suppresses PERK-mediated phosphorylation of eIF2α, exhibit apoptosis of pancreatic β-cells and diabetes [243]. This suggests that the tight regulation of PERK-eIF2α pathway is required for the maintenance of pancreatic β-cells. In contrast, some single nucleotide polymorphisms (SNPs) in the ORP150/GRP170 genome of Pima Indians are associated with insulin sensitivity in peripheral tissues [244]. Accordingly, overexpression of ORP150 enhances insulin sensitivity and suppresses oxidative stress but does not improve insulin secretion [245]. These findings suggest that proper functioning of the ER is important for both insulin synthesis in pancreatic β-cells and insulin sensitivity in peripheral tissues. Consistent with this hypothesis, chemical chaperones such as 4-phenylbutryic acid and tauroursodeoxycholic acid improved both insulin resistance and insulin synthesis [171, 246].

5.2. Parkinson's Disease. Neurodegenerative diseases are characterized by the loss of subsets of neurons. The course of these diseases can last decades, with the accumulation of neuronal loss causing progressively worse symptoms. Post-mortem tissue is usually obtained from end-stage patients, at which time many of the evidences regarding the events preceding cell death are long gone. However, there is substantial and growing evidence for the activation of stress responses in neurons in all of the common neurodegenerative diseases. This suggests that when neurons are exposed to stress, they counteract with activation of one or more protective stress responses; however, eventually the neurons are unable to cope and one-by-one they are lost as the disease progresses. There is a growing recognition that protein misfolding and impairment of protein handling play a key role in neuronal cell death in neurodegenerative diseases [153].

As an example of stress responses and stress-induced cell death in neurodegenerative disease, we will describe the evidence pertaining to Parkinson's disease. Parkinson's disease is the second most common neurodegenerative disease, affecting mainly people over 55 years and causing progressively worsening motor impairment. It is characterized pathologically by the degeneration of midbrain dopaminergic neurons in the substantia nigra pars compacta and the presence of proteinaceous intracytoplasmic inclusions (Lewy bodies) within the surviving neurons.

The molecular mechanisms that initiate dopaminergic neuron loss in Parkinson's disease are not known. Evidence from various sources suggest that environmental toxins, genetic predisposition, and aging are important factors in the onset and progression of the disease [247–249]. Insecticides such as rotenone and the mitochondrial toxin 1-Methyl-4-phenyl-1, 2,3,6-tetrahydropyridine (MPTP) cause dopaminergic neuronal loss in animal models and have been implicated in Parkinson's disease itself [250, 251]. To date, mutations in at least 13 PARK genes have been linked to the pathogenesis of familial Parkinson's disease which include mutations in genes that encode the proteins α-synuclein, parkin, PTEN-induced kinase 1 (PINK1), DJ-1, leucine-rich repeat kinase2 (LRRK2), Omi/Htra2, and ubiquitin carboxy-terminal hydrolase L1 (UCHL1) [252]. Of these, α-synuclein (along with chaperone proteins and ubiquitin) is a major component of Lewy bodies. Parkin and UCHL1 are linked to the ubiquitin-proteasome system that degrades damaged or misfolded proteins [253]. In addition, several of these genes, including parkin, PINK1, DJ-1, and Omi/Htra2 are linked to the mitochondria and may have roles in mitochondrial function and resistance to oxidative stress [254].

Mutations in PARK genes, as well as toxins that specifically target dopaminergic neurons, have been strongly

linked to the activation of stress responses in dopaminergic neurons. For example, mitochondrial dysfunction due to mutations in certain PARK genes or to environmental toxins is linked with impairment of mitochondrial complex I which causes oxidative stress in affected cells. It has long been known that oxidative stress is a feature of Parkinson's disease and it is observed in experimental models of Parkinson's disease and in tissues from individuals with sporadic forms of the disease [255].

Most of the evidence regarding activation of the heat shock response in Parkinson's disease come from models. Targeted overexpression of α-synuclein in mouse substantia nigra causes an increase in the expression of Hsp27, Hsp40, and Hsp70 [256, 257] and elevations in Hsp27 are observed in in vitro models of Parkinson's disease using the neurotoxin 6-hydroxdopamine [196]. Recent findings from Parkinsonian patients have described that DnaJB6 is present in the core of Lewy bodies and is also upregulated in astrocytes [258]. DnaJB6 is one of the Hsp40 chaperones, which stabilizes the interactions of Hsp70s with their substrate proteins. In vitro and in vivo models of Parkinson's disease demonstrate that overexpression of Hsps prevents α-synuclein aggregation as well as dopaminergic neuronal cell death due to α-synuclein and Parkinson mimetic toxins [216, 237, 239–242]. Interestingly, the inducibility of Hsps decreases with aging, which may contribute to the inability of aged neurons to fully protect themselves from stresses such as protein misfolding, aggregation, and oxidative stress [259].

Activation of the UPR has been reported in postmortem brain tissue from patients with Parkinson's disease. Specifically, phosphorylated PERK and phosphorylated eIF2α have been detected in dopaminergic neurons in the substantia nigra of Parkinson's disease patients [260]. Phospho-PERK immunoreactivity was colocalized with increased α-synuclein immunoreactivity in dopaminergic neurons [260]. Supporting evidences from in vitro models of Parkinson's disease show that 6-hydroxydopamine and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPP^+) (Parkinson mimetic drugs) trigger ER stress in dopaminergic neurons [261, 262]. Furthermore, neuronal cultures from PERK knockout mice display an increased sensitivity to 6-hydroxydopamine [262], while a null mutation in CHOP results in a reduction in 6-hydroxydopamine-induced apoptosis in vivo [263]. However, protection was not observed in the chronic MPTP model, despite robust expression of CHOP [263].

The information from models, the genetic information, as well as analysis of postmortem tissue, when taken together, strongly connects the induction of stress responses with the loss of dopaminergic neurons in Parkinson's disease. It is likely that the induction of stress responses is the neurons attempts at protection, which eventually fail with neuronal cell death being the inevitable outcome. Interestingly, these observations are mirrored in research findings of other common neurodegenerative diseases, including Alzheimer's disease and Huntington's disease, indicating the important role for protein misfolding, aggregation and formation of protein inclusions in these chronic diseases [153].

5.3. Myocardial Infarction. Cardiovascular disease (CVD), a group of disorders of the heart and the vasculature, includes high blood pressure, coronary heart disease, congestive heart failure, stroke, and congenital heart defects. Apoptotic cell death is a fundamental process in the morphogenesis of the developing heart [264, 265]. Until recently the classical view was that necrosis was the major mode of cardiomyocyte death during CVD. However, accumulating in vitro and in vivo studies provides compelling evidence that terminally differentiated cardiomyocytes, can and do undergo apoptosis [266]. Apoptosis has important pathophysiological consequences, contributing to the loss and functional abnormalities of the myocardium. Cardiomyocyte apoptosis has been reported in a variety of cardiovascular diseases, including myocardial infarction, end-stage heart failure, arrhythmogenic right ventricular dysplasia, and adriamycin-induced cardiomyopathy [267]. Animal models have been instrumental in establishing the occurrence of cardiomyocyte apoptosis and in the elucidation of the apoptotic mechanisms. Features of myocyte apoptosis were first reported in rabbit and rat heart models of MI or ischemia/reperfusion injury [268, 269]. Since these pioneering studies, apoptosis has been repeatedly observed in the injured human heart [270–274]. Due to its sporadic occurrence and the prompt clearance of apoptotic cells by phagocytosis, apoptosis in diseased tissue is grossly underestimated.

Oxidative damage mediated by free radicals is a contributing factor to ischemia/reperfusion-induced injury in cardiomyocytes [275–278]. Plasma and pericardial fluid obtained from patients with end stage heart failure have increased levels of thiobarbituric acid reactive substances, a commonly used marker of ROS production [279, 280]. Reperfusion is associated with a burst of ROS generated via the mitochondrial respiratory chain, where partial reduction of ubiquinone forms ubisemiquinone combine with oxygen to form $O_2^{\bullet -}$ radicals [281]. High levels of ROS can lead to mitochondrial damage and dysfunction [282] and can induce apoptosis in cardiac myocytes [275, 276].

In addition, enhanced levels of the heat shock response and UPR have been demonstrated in animal models of myocardial infarction, and overexpression of either Hsps or GRPs enhanced tolerance against ischemia/reperfusion injury in these models [283, 284]. Although Hsps may work upstream of caspase-3 but downstream of cytochrome *c* release [285], GRPs likely contribute to the maintenance of intracellular Ca^{2+} homeostasis [284]. Similarly, overexpression of sarco (endo) plasmic reticulum Ca^{2+-}ATPase (SERCA), which regulates intracellular Ca^{2+} homeostasis, improved postischemic cardiac function and decreased myocardial infarction [286].

5.4. Cancer. Since tissue homeostasis is the result of a subtle balance between proliferation on one side and cell death on the other side, changes in the rate of cell death can contribute to either the loss or gain of tissue [22]. For example, too little cell death can contribute to tumor formation and is considered to be one of the hallmarks of human cancers [287, 288]. Some oncogenic mutations

block cell death pathways creating a permissive environment for genetic instability and resulting in the accumulation of gene mutations leading to tumor initiation and progression [289]. Also, evasion of cell death promotes resistance to immune-based destruction, facilitates growth factor- or hormone-independent survival, and supports anchorage-independent survival during metastasis [288]. In addition, defects in cell death programs may confer resistance to cytotoxic therapies that are currently used in the clinic for the treatment of cancer such as chemotherapy, irradiation, or immunotherapy, since the response of cancer cells to these treatment approaches is, to a large extent, due to their ability to undergo cell death in response to cytotoxic stimuli [290–292].

In principle, the signaling to apoptosis can be blocked in cancers by loss or defective function of proapoptotic molecules, aberrantly high expression of antiapoptotic proteins, and/or by the relative dominance of cell survival signaling pathways. For example, impaired death receptor expression or function has been reported in a variety of human cancers. Reduced expression of CD95 was found in drug-resistant leukemia or neuroblastoma cells, indicating that intact signaling via CD95 is linked to drug response [293, 294]. CD95 mutations have been detected in both hematological malignancies and various solid tumors [295–300]. It is interesting to note that both agonistic TRAIL receptors, that is, TRAIl-R1 and TRAIL-R2, are located on chromosome 8p, a region that is frequently lost in cancers due to heterozygosity [301, 302]. Further, a larger range of antiapoptotic proteins are reported to be expressed at high levels in malignant versus nonmalignant tissue, including death domain-containing proteins that interfere with activation of caspase-8 at the death receptor level such as cellular FLICE-Inhibitory Protein (cFLIP) and phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes-15kDa (PED/PEA-15) [303], anti-apoptotic Bcl-2 family proteins such as Bcl-2, Bcl-X_L, and Mcl-1 [31] and IAPs, including XIAP, cIAP1, cIAP2, survivin and livin [304]. Alternatively, apoptosis regulators with proapoptotic functions have been reported to be lost, mutated or epigenetically silenced in cancers. Examples include epigenetic loss or homo- or heterozygous genomic deletions of caspase-8 [305], single nucleotide substitution or frameshift mutations of the *bax* gene in mismatch repair-deficient colon cancer or hematopoetic malignancies [306, 307], and deletion or epigenetic silencing of the *bim gene* [308–310].

It is also now generally accepted that the majority of tumors, due to poor vascularisation of the tumor mass, experience stressful conditions in the tumor microenvironment, including low oxygen supply, nutrient deprivation, and pH changes. These conditions activate a range of cellular stress-response pathways, including the UPR. Recent studies have shown that the UPR plays an important role in tumorigenesis [311–315]. Activation of at least one branch of the UPR has been reported in a number of cancers and many ER chaperones and UPR target genes show increased expression in human tumor samples. Although activation of the UPR has been reported in a variety of human cancers, the role of UPR in different forms of cancer is not yet fully characterized.

At present it is unclear how tumor cells adapt to long-term ER stress in vivo—whether the protective elements of the response are enhanced, the destructive components suppressed, or if the compromised apoptotic machinery is sufficient to protect them from UPR-induced apoptosis. Given that the UPR can trigger prosurvival and pro-apoptotic signals, it is important to understand how modulation of the UPR alters the balance between these processes and contributes to carcinogenesis in different cell types. The upregulation of UPR in cancers may be beneficial for the tumor cells by increasing the protein folding capacity and prolonging life.

Moreover, altered redox status can promote tumor initiation and progression by blunting cell death pathways. For example, a pro-oxidant intracellular milieu has been linked to carcinogenesis and tumor promotion. To this end, increased signaling via the PI3K/Akt pathway has been shown to result in enhanced intracellular ROS generation [316]. Similarly, cancer cells that constitutively express oncogenic Ras have been reported to harbor higher intracellular levels of $O_2^{\bullet -}$ and to be resistant to drug-induced apoptosis [317].

Hsps, including Hsp90, Hsp70, and Hsp27, are expressed at increased levels in many solid tumors and haematological malignancies. Since various oncogenic proteins that are critically required for the malignant transformation of cells, for example, Ras, Akt, and HER2, are client proteins of Hsp90, elevated levels of Hsp90 favor tumor initiation and promotion [318]. Similarly, the expression of Hsp27 and Hsp70 is abnormally high in cancers [319]. These chaperones participate in carcinogenesis and in cell death resistance by blocking key effector molecules of the apoptotic machinery at the pre- and post-mitochondrial level [319]. Thus, targeting Hsps, for example, with chemical inhibitors, is currently under investigation as anticancer strategy [318].

Error-prone repair or complete failure to repair DNA damage as well as inherited or acquired defects in maintenance systems of the mammalian genome can lead to mutations [185]. In addition, such deficiencies in the DNA damage response contribute substantially to carcinogenesis and promote the progression and treatment resistance of cancer [185].

6. Summary and Future Perspectives

Cellular stress responses are an integral part of normal physiology to either ensure the cell's survival or alternatively to eliminate damaged or unwanted cells. Several distinct stress responses can be distinguished, among them the heat shock, unfolded protein, DNA damage, and oxidative stress responses. Despite individual signaling components, these different stress responses can eventually fuel into common cell death effector mechanisms, if the cell is unable to cope with the stress. Whether or not cellular stress triggers cell death or cell survival programs is determined by a set of different factors, among them the initial stress stimulus, cell

type, and environmental factors. Because aberrant cellular stress responses are tightly linked to many common human diseases, a better understanding of the underlying molecular mechanisms is expected to enable us to interfere with these processes, for example, to switch such response from cell death into survival programs or vice versa, depending on the desired outcome. In addition, new insights into the mechanistic basis of stress responses will open new perspectives for the development of molecular targeted treatment approaches and thus have a great potential for drug discovery.

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Review Article

Methods for Monitoring Endoplasmic Reticulum Stress and the Unfolded Protein Response

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The endoplasmic reticulum (ER) is the site of folding of membrane and secreted proteins in the cell. Physiological or pathological processes that disturb protein folding in the endoplasmic reticulum cause ER stress and activate a set of signaling pathways termed the Unfolded Protein Response (UPR). The UPR can promote cellular repair and sustained survival by reducing the load of unfolded proteins through upregulation of chaperones and global attenuation of protein synthesis. Research into ER stress and the UPR continues to grow at a rapid rate as many new investigators are entering the field. There are also many researchers not working directly on ER stress, but who wish to determine whether this response is activated in the system they are studying: thus, it is important to list a standard set of criteria for monitoring UPR in different model systems. Here, we discuss approaches that can be used by researchers to plan and interpret experiments aimed at evaluating whether the UPR and related processes are activated. We would like to emphasize that no individual assay is guaranteed to be the most appropriate one in every situation and strongly recommend the use of multiple assays to verify UPR activation.

1. Introduction

The endoplasmic reticulum (ER) is the cellular site for Ca^{2+} storage and for synthesis, folding, and maturation of most secreted and transmembrane proteins. Physiological or pathological processes that disturb protein folding in the endoplasmic reticulum cause ER stress and activate a set of signaling pathways termed the Unfolded Protein Response (UPR) [1]. This concerted and complex cellular response is mediated initially by three molecules, PKR-like ER kinase (PERK), activated transcription factor 6 (ATF6), and Inositol-requiring enzyme 1 (IRE1) [2]. The ER luminal domain of PERK, IRE1, and ATF6 interacts with the ER chaperone GRP78 (glucose-regulated protein); however, upon accumulation of unfolded proteins, GRP78 dissociates from these molecules, leading to their activation [3]. Notably, activation of ER stress sensors is modulated by other cellular factors, in addition to the dissociation of GRP78. A mutant of yeast IRE1, having deletion of GRP78 binding site in the ER luminal domain, is not constitutively active. Furthermore activation of this mutant (GRP78 binding site deleted) is regulated by accumulation of unfolded proteins in the ER [4, 5]. Dimerization of core stress-sensing region (CSSR) of the ER luminal domain of IRE1 creates a shared central groove similar to the peptide binding domains of major histocompatibility complexes (MHCs) [6–8]. It is proposed that MHC-like groove binds portions of unfolded polypeptide chain to promote formation of higher-order oligomers necessary for UPR activation [6–8]. Indeed luminal domain of yeast IRE1 interacts with unfolded proteins and inhibits aggregation of denatured proteins in vitro [7]. However, the ER luminal domain fragments of mammalian IRE1α did not interact with unfolded proteins in vitro [9]. IRE1 and PERK have conserved essential structural motifs in their ER luminal domains required for their dimerization. Similar to IRE1, ER luminal domain of PERK can also inhibit aggregation of denatured proteins in vitro [7]. Thus IRE1 and PERK appear to be regulated both by GRP78 and by direct binding of unfolded proteins. Activation of ATF6 is also regulated by combination of two discrete events: firstly by interaction with GRP78 and secondly by intra- and intermolecular disulfide bridges [10, 11]. The ER luminal

region of ATF6 has two Golgi localization signals: GLS1 and GLS2. Binding of GRP78 masks the GLSs in the luminal domain of ATF6, and dissociation of GRP78 allows ATF6 to be transported to the Golgi body [11]. Further ER luminal domain of ATF6 is disulfide bonded and ER stress-induced reduction plays important role in both translocation to Golgi body and subsequent recognition by the site-1 and site-2 proteases (S1P and S2P) [10]. These differences may explain the different kinetics in the activation of IRE1, PERK, and ATF6 to various ER stress inducers.

Activated PERK phosphorylates translation initiation factor 2α (eIF2α), thereby reducing the rate of translation and the protein load on the ER [12, 13]. Phosphorylation of eIF2α paradoxically increases translation of ATF4 mRNA to produce a transcription factor that activates expression of several UPR target genes [12, 14]. Activation of the ER protein kinase IRE1 triggers its endoribonuclease activity to induce cleavage of X box-binding protein 1 (XBP1) mRNA. XBP1 mRNA is then ligated by an uncharacterized RNA ligase and translated to produce spliced XBP1 protein [15]. Spliced XBP1 protein is a highly active transcription factor and one of the key regulators of ER folding capacity [16]. Concurrently, ATF6 is released from GRP78 and transits to the Golgi body where it is cleaved to release a transcriptionally active fragment [17]. Cleaved ATF6 acts in concert with spliced XBP1 protein to induce expression of genes encoding protein chaperones and components of the ER-associated degradation (ERAD) machinery [18, 19]. Moreover, ER stress can also induce autophagy [20], a catabolic cellular program that promotes cell survival in many contexts but which has been associated with induction of nonapoptotic cell death in others [21].

As discussed above the three proximal sensors of ER stress are PERK, ATF6, and IRE1. Exposure to ER stress activation of these proximal sensors leads to autophosphorylation of IRE1 at serine 724, autophosphorylation of PERK at threonine 980, and proteolytic processing of full-length ATF6 [1, 2]. The 90 kDa full-length ATF6 is processed within the Golgi body to its active 50 kDa form through sequential cleavage by site-1 and site-2 proteases (S1P and S2P) [17]. Therefore, proteolytic processing of ATF6 and phosphorylation of PERK and IRE1 can serve as markers of their activation status. However, detection of cleaved ATF6, phospho-PERK and phosho-IRE1 is quite difficult as these are expressed at very low levels and there is currently a lack of good commercial antibodies to detect them. Over the last 10 years, rapid progress has been made in understanding the molecular mechanisms of the UPR, and a number of genes modulated by the UPR have been identified. Most of these genes function in restoring ER homeostasis and alleviating ER stress. Therefore, these genes can be used as specific markers for the UPR. In our experience detection of the proteolytic processing of ATF6 or the phosphorylation of PERK and IRE1 is not advisable. Instead we suggest that detection of downstream protein targets of ER stress such as CHOP, HERP, XBP1, GRP78, and ATF4 (http://saturn.med.nyu.edu/research/mp/ronlab/Postings/UPR.detect.html) be a more robust approach for detecting activation of the UPR. One of the most commonly used indicators of ER stress is an increase in the expression level and the nuclear translocation of the transcription factor C/EBP homologous protein (CHOP) [22, 23]. However, it was recently reported that three out of seven commercially available CHOP antibodies gave false results by western blotting and immunocytochemistry for detection of CHOP [24]. Furthermore, there was a lot-to-lot variance in specificity from the same commercial source [24]. Therefore, we advise first validating the specificity of the antibody used for detecting CHOP protein expression to establish the presence or absence of ER stress.

UPR pathways are important for normal cellular homeostasis and development and also play key roles in the pathogenesis of many diseases [25, 26]. Examples of pathophysiological conditions that can perturb the ER homeostasis include stroke, ischemia, diabetes, viral infections, and mutations that impair protein folding [25, 26]. Although the importance of ER stress and the UPR is being increasingly recognized, we still have only a limited number of good diagnostic methods to monitor the UPR. This limitation impedes our complete understanding and monitoring of the UPR, and in some cases, it may result in confusion. Importantly, there are no absolute criteria for determining the UPR signaling that can apply to every situation. This is because some assays are inappropriate, problematic, or may not work at all in particular cells, tissues, or model systems.

2. Experimental Approaches for the Detection of ER Stress

2.1. Splicing of XBP1 mRNA. In response to accumulation of unfolded proteins in the ER, IRE1 oligomerizes in the plane of the membrane, allowing for transautophosphorylation of juxtaposed kinase domains. The transautophosphorylation of the kinase domain of IRE1 activates its unusual effector function, which causes the unconventional splicing of the mRNA that encodes a transcription factor named XBP1 [15]. In metazoans, a 26-nucleotide intron is spliced out by activated IRE1, leading to a shift in the codon reading frame (Figure 1(a)). The XBP1 protein encoded by the spliced mRNA is more stable and is a potent transcription factor of the basic-leucine zipper (bZIP) family and one of the key regulators of ER folding capacity [15, 16]. The splicing of XBP1 mRNA can be detected by semiquantitative RT-PCR using primers specific for *XBP1* which will detect both unspliced and spliced isoforms. The 5′ to 3′ sequences of primers used to detect unspliced and spliced XBP1 mRNA are as indicated below.

Rat XBP1

Forward primer: TTACGAGAGAAAACTCATGGGC
Reverse primer: GGGTCCAACTTGTCCAGAATGC
Size of PCR products: unspliced XBP1 = 289 bp, spliced XBP1 = 263 bp.

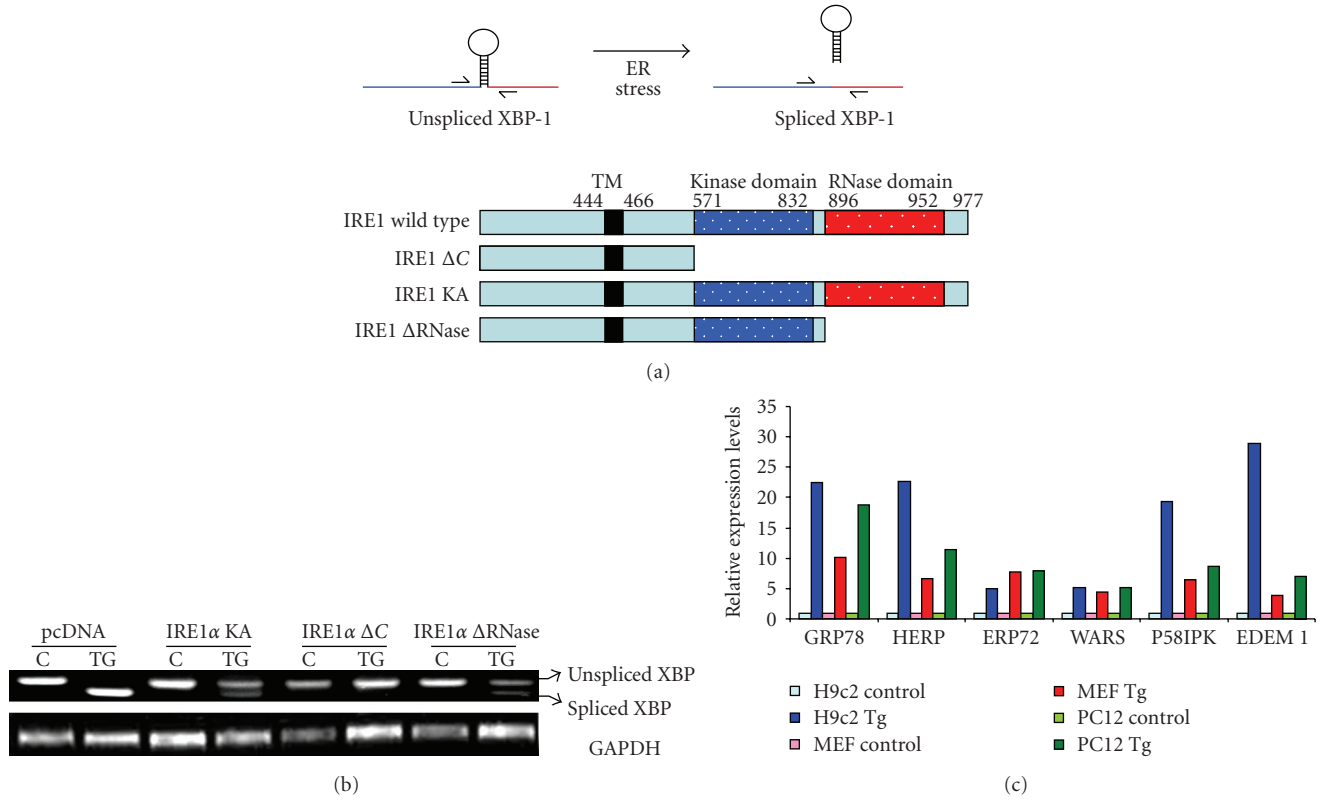

Figure 1: *Detection of transcript levels of UPR target genes by RT-PCR*. (a) Upper panel, cartoon of XBP1 splicing during ER stress. Lower panel, schematic representation of various mutant constructs of IRE1. (b) Modulation of XBP1 splicing by mutant IRE1. Total RNA was isolated from HEK 293 cells that were transfected with IRE1 mutants, either untreated or treated with thapsigargin (0.5 μM) 6 hours, and RT-PCR analysis of total RNA was performed to simultaneously detect both spliced and unspliced XBP1 mRNA and GADPH. (c) Induction of UPR target genes upon exposure to thapsigargin. Total RNA was isolated from indicated cells after treatment thapsigargin (Tg), and the expression levels of the indicated genes were determined by real-time RT-PCR, normalizing against GAPDH expression.

Human XBP1

Forward primer: TTACGAGAGAAAACTCATGGCC
Reverse primer: GGGTCCAAGTTGTCCAGAATGC
Size of PCR products: unspliced XBP1 = 289 bp, spliced XBP1 = 263 bp.

Mouse XBP1

Forward primer: GAACCAGGAGTTAAGAACACG
Reverse primer: AGGCAACAGTGTCAGAGTCC
Size of PCR products: unspliced XBP1 = 205 bp, spliced XBP1 = 179 bp.

We have detected IRE1-dependent splicing of XBP1 mRNA under conditions of ER stress by using various mutants of IRE1 (Figures 1(a) and 1(b)). A variety of mammalian cell lines can be used to determine the splicing of XBP1. To follow this method, cells should be seeded on six-well plates and transfected with indicated IRE1 mutants. Twenty-four hours post transfection, cells are subjected to ER stress stimuli, for example, tunicamycin, thapsigargin, or Brefeldin A for different time points ranging from 6–48 hours. Three chemicals are generally used to experimentally induce ER stress: tunicamycin (Sigma), thapsigargin (Sigma), and Brefeldin A (BFA) (Sigma). Although these chemicals target different components of the ER, their common effect is to interfere with ER functions and thereby lead to ER protein misfolding. Tunicamycin inhibits N-linked glycosylation, while thapsigargin blocks the ER calcium ATPase pump, leading to the depletion of ER calcium stores. Brefeldin A interferes with protein transport from the endoplasmic reticulum to the Golgi apparatus by inhibiting transport in the Golgi, which leads to proteins accumulating inside the ER. The concentration and time of treatment depend on system being studied and need to be determined individually for each system. Cells are harvested and total RNA is isolated using RNeasy kit (Qiagen) or TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription (RT) is carried out with 2 μg RNA and Oligo dT (Invitrogen) using 20 U Superscript II reverse transcriptase (Invitrogen). Then standard conditions of RT-PCR can be used to determine the unspliced and spliced isoforms of XBP1 (Figure 1(b)). The ER stress-mediated splicing of XBP1 requires activation of IRE1, and if the function of IRE1 is compromised, ER stress-mediated splicing of XBP1 is attenuated (Figure 1(b)).

Table 1: List of TaqMan assays that reproducibly detect markers of UPR.

	Rattus norvegicus		Mus musculus	
Target gene	Accession number	Assay number	Accession number	Assay number
GRP78	NM_013083.1	Rn01435771_g1	NM_022310.2	Mm01333324_g1
HERP	NM_053523.1	Rn01536690_m1	NM_022331.1	Mm01249592_m1
ERP72	NM_053849.1	Rn01451754_m1	NM_009787.2	Mm00437958_m1
WARS	NM_001013170.2	Rn01429998_g1	NM_011710.2	Mm00457097_m1
P58IPK	NM_022232	Rn00573712_m1	NM_008929	Mm00515299_m1
EDEM1	XM_238366.4	Rn01765441_m1	NM_138677.2	Mm00551797_m1

2.2. mRNA Levels of UPR Target Genes. The ER stress response is an autoregulatory program that upregulates a large number of genes that expand the folding capacity of the ER, such as ER chaperones and ERAD components [1]. Mapping of the promoters of a number of ER stress responsive genes, such as BiP/GRP78, GRP94, calreticulin, HERP, EDEM1, and HRD1, have identified three cis-acting response elements, namely, ERSE (ER Stress Response Element), ERSE-II (ER Stress Response Element II), and UPRE (Unfolded Protein Response Element) [27–31]. ERSE has a consensus sequence CCAAT-N9-CCACG, which is necessary and sufficient for the induction of at least three major ER chaperones (GRP78, GRP94, and calreticulin) [28, 31]. HERP, one of the most highly inducible genes during the UPR, has a promoter that contains not only ERSE but also a cis-acting element with a sequence of ATTGG-N_1-CCACG termed ESRE-II [27]. UPRE which contains the consensus sequence TGACGTGG/A was originally identified as a DNA sequence bound by bacterially expressed ATF6 [29]. Loss of ATF6 leads to reduced activation of UPRE containing genes such as EDEM1 and HRD1 [19]. We recommend determining the transcript levels of bona fide UPR target genes whose induction has been reported to occur during conditions of ER stress and whose promoter regions contain at least one of the three cis-acting response elements, namely, ERSE, UPRE, or ERSE-II.

In our laboratory, the induction of mRNA of UPR target genes has been detected in a variety of mammalian cell lines using real-time RT-PCR (Figure 1(c)). Cells were generally induced to undergo ER stress by incubating with tunicamycin, thapsigargin, or Brefeldin A. The concentration and time of treatment depend on system being studied and need to be determined individually for each system. In these experiments cells were treated with ER stress inducing agents such as Tg, Tm, and BFA and total RNA was isolated using RNeasy kit (Qiagen) or TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription (RT) was carried out with 2 μg RNA and Oligo dT (Invitrogen) using 20 U Superscript II Reverse Transcriptase (Invitrogen). For real-time PCR experiments, cDNA products were mixed with 2 × TaqMan master mixes and 20 × TaqMan Gene Expression Assays (Applied Biosystems) and subjected to 40 cycles of PCR in StepOnePlus instrument (Applied Biosystems). Relative expression was evaluated with ΔΔCT method. We would like to point out that other methods for detection of mRNA levels such as northern blotting, RNAse protection assays, and conventional RT-PCR can also be used. We prefer real-time RT-PCR with TaqMan chemistry (also known as "fluorogenic 5′ nuclease chemistry") because of its sensitivity, specificity, speed, and ease of handling. Table 1 provides a list of TaqMan Assays (Applied Biosystems) that have worked reproducibly in our experience to detect the transcripts of the several UPR markers.

2.3. Western Blotting and Immunohistochemistry for UPR Target Genes. We recommend determining the protein levels of established UPR target genes whose induction has been reported to occur during relevant conditions of ER stress. Activation of the UPR has been found in various pathological states of the brain including ischemia and degenerative diseases. Increased phosphorylation of PERK has been shown after cerebral Ischemia and reperfusion by immunohistochemical analysis [32]. Several postmortem studies of primary human Alzheimer's disease brain tissues show evidence of ER stress in the form of enhanced ER chaperone expression and immunohistochemical reactivity for specific markers of the UPR [33, 34]. Recently we found increased expression of GRP78, CHOP, and XBP1 in acute, active, and chronic multiple sclerosis (MS) lesions by immunohistochemical and dual-immunofluorescent analyses [35]. Figure 2(a) shows immunohistochemical staining of fixed frozen paraffin-embedded (FFPE) brain tissue sections from MS patient which showed upregulation of CHOP, GRP78, and XBP1. Specific antibodies used are detailed in Table 1. FFPE tissue was used in preference to frozen blocks as in our hands it yielded higher quality staining with lower background and fewer staining artifacts. Following deparaffinization, all sections were incubated for 10 minutes at room temperature in 3% hydrogen peroxide in methanol (Sigma-Aldrich, Dublin), to block endogenous peroxidases. For CHOP and GRP78 staining, antigen retrieval was achieved by incubating sections in 0.01 M Tris-EDTA pH 9 (Sigma-Aldrich, Dublin) for 2 minutes in a Tefal pressure cooker at full steam. To retrieve antigen before XBP1 staining, tissue was placed in 0.01 M citrate pH 9 (Sigma-Aldrich, Dublin) before microwaving it for 20 minutes in a 700 watt Sanyo microwave. Bound CHOP, GRP78, or XBP1 antibody was detected following incubation for 30 minutes at room temperature in peroxidase-labeled EnVision anti-mouse or anti-rabbit antibody (Dako, Ely,

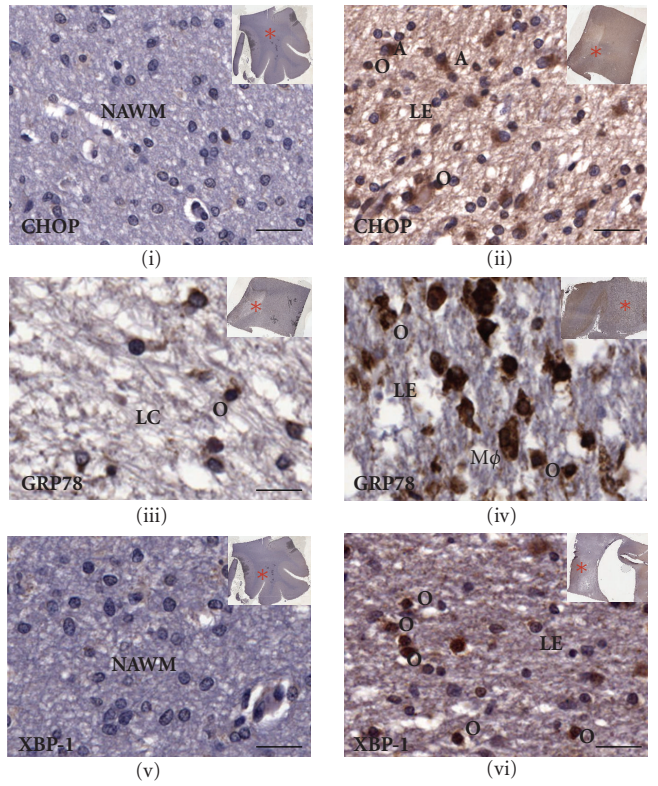

Figure 2: *Detection of protein levels of UPR target genes.* (a) Immunohistochemical detection of CHOP, GRP78, and XBP1 in Multiple Sclerosis patient postmortem brain tissue. Representative images showing upregulation of CHOP (ii) at the edge (LE) of a chronic active lesion, in comparison to (i) NAWM. GRP78 expression was downregulated in the center of a chronic active lesion (iii) when compared to the edge (iv) of actively demyelinating lesions. Sample images illustrate the variety of morphologically distinct cell types that express CHOP or GRP78 including macrophages (Mø), astrocytes (a), and oligodendrocytes (o). Increased expression of XBP1 was found at the edge of a chronic active lesion (vi), when compared to normal-appearing white matter (v). XBP1 immunostaining is also apparent in a large number of oligodendrocytes (o). All immunoperoxidase-stained cells were detected using the chromogen, DAB (brown), and counterstained with hematoxylin for nuclei (blue). Scale bars = 250 μm. Letter codes are as follows: NAWM = normal-appearing white matter; LC = lesion center; LE = lesion edge. Red astrices indicate location of lesion within brain sections analyzed. (b) PC12 cells were treated with 0.25 μM of Tg for 0, 2, 4, 6, and 8 hours. Whole cell lysates were analyzed by Western Blot for GRP78, CHOP, spliced XBP1, phospho-eIF-2α, and total- eIF-2α. β-Actin was used to determine equal loading of samples.

UK) with 3,3′- diaminobenzidine (DAB) as a chromogen (Dako, Ely, UK).

When carrying out western blotting, we suggest performing standard procedures to determine the protein levels of bona fide UPR target genes within protein samples (Figure 2(b)). Table 2 provides a list of antibodies that have worked best and most reproducibly in our experience to detect several UPR marker proteins in western blotting and immunohistochemistry.

2.4. Reporter Assays for Activity of XBP1 and ATF6. The most salient feature of the UPR is an increase in the transactivation function of a number of bZIP transcription factors, such as ATF6, ATF4, and XBP1. It has been well established that transcriptional induction of UPR target genes upon ER stress is mediated by the cis-acting response elements. There are several reporter systems which can be used to detect ATF6 and XBP1 activation. In the p5xATF6-GL3 reporter, the luciferase gene is under the control of the c-fos minimal promoter and five tandem copies of the ATF6 consensus binding site identifed by in vitro gel mobility shift assays with recombinant ATF6 [29]. In p4xXBPGL3 reporter, the luciferase gene is under the control of four tandem copies of the XBP1 consensus binding site 5′-CGCG$(TGGATGACGTGTACA)_4$-3′ [16]. In addition there are several other ERSE reporters which have promoter regions of GRP78, GRP94, Calreticulin, XBP1 [28], and an ERSE-II reporter which has the HERP promoter upstream of the luciferase reporter gene [27]. These reporters should be used in combination with the corresponding mutant promoter where the functional cis-elements have been mutated. The advantage of these reporters is that they can be used to monitor the activation of endogenous ER stress. However, there is some question as to whether these reporters respond primarily to endogenous ATF6 and/or XBP1, since XBP1's binding site is similar to the ATF6 site, and activated forms of both ATF6 and XBP1 can activate the reporter. Furthermore, ATF6 and XBP1 can heterodimerize in vivo and ATF6-XBP1 heterodimer possesses 8-fold higher affinity for the UPRE than that for XBP1 homodimer [19]. Nevertheless, luciferase-based reporters are a very sensitive method to detect ER stress whether it measures activation of ATF6, XBP1, or both.

A variety of mammalian cell lines can be used to determine the activity of XBP1/ATF6 using these reporter constructs. Cells should be seeded on six-well plates and transfected by the optimized transfection method 24 hours later. The transfection mixture for each well should contain the luciferase reporter gene and an internal control to normalize the transfection efficiencies (Renilla luciferase or β-galactosidase). The internal control plasmid is not responsive to ER stress. 24 hours post transfection, cells are induced to undergo ER stress by incubating with appropriate concentrations of tunicamycin, thapsigargin, or Brefeldin A for different time points ranging from 6–48 hours. Cells are then harvested and the firefly luciferase present in the cell lysate is measured along with the appropriate internal control (Renilla luciferase or β-galactosidase). The results should be normalized to the internal control for each point to determine the fold induction in the reporter activity.

2.5. Detection of IRE1 Activation and ATF6 Translocation from the ER to the Nucleus with Fluorescent Microscopy. ER stress-dependent splicing of XBP1 has been used to develop fluorescent reporter constructs by fusing XBP1 sequence to *venus*, a variant of green fluorescent protein which enables the activation of IRE1 to be monitored [36, 37]. The design of the XBP1-*venus* reporter is shown in Figure 3(a). In this construct, the gene encoding *venus* is cloned downstream the 26-nt ER stress-specific intron of human *XBP1* [36]. Under normal conditions, the mRNA of the fusion gene is not spliced, and its translation terminates at the stop codon near the joint between the *XBP1* and *venus* genes. However, during ER stress, the 26-nt intron is spliced out, leading to a frame shift of the chimeric XBP1-*venus* mRNA, similar to that of the endogenous XBP1 mRNA. Translation of the spliced mRNA produces an XBP1-*venus* fusion protein and cells experiencing ER stress can be detected by monitoring the fluorescence activity of *venus*. As *venus* expression can only occur from the spliced form of the XBP1-GFP mRNA, its presence signals the activation of IRE1. Upon transfection of the XBP1-GFP reporter into cells, tunicamycin treatment results in detectable fluorescence in the nucleus, whereas negligible fluorescence is detected in any compartment under normal conditions [36, 37]. Moreover, *venus* expression during tunicamycin treatment has been shown in splicing assays to correlate with the extent of splicing of the UPR intron from XBP1/GFP mRNA [36, 37]. We have used 293T cells to detect activation of IRE1 using two different XBP1-*venus* reporter plasmids: F-XBP1-*venus* and F-XBP1ΔDBD-*venus* (Figure 3(a)). In F-XBP1ΔDBD-*venus* construct, DNA-binding domain (DBD) of XBP1 is deleted. F-XBP1ΔDBD-*venus* construct is recommended for use as overexpression of F-XBP1ΔDBD-*venus* does not affect induction of UPR target genes and can be used to detect activation of IRE1 similar to F-XBP1-*venus* construct. F-XBP1ΔDBD-*venus* construct has been used to generate a transgenic mouse model for monitoring ER stress (discussed later). Twenty-four hours post transfection, cells are induced to undergo ER stress by incubating with appropriate concentrations of tunicamycin, for 24 hours. In the cells transfected with F-XBP1-*venus* construct, tunicamycin treatment leads to appearance of green fluorescence in the nucleus (Figure 3(b)). However in the cells transfected with F-XBP1ΔDBD-*venus* construct, tunicamycin treatment leads to appearance of green fluorescence in the cytosol (Figure 3(b)). One important point to note is that overexpression of F-XBP1-*venus* construct interferes with induction of UPR target genes in a dominant-negative manner [36]. The major drawback, however, is the relatively large amount of GFP that needs to be expressed in the cell for visualization by microscopy. Thus, there will be a time lag between actual IRE1 activation and its detection by the accumulation of GFP.

A key regulatory step in ATF6 activation is its transport from the ER to the Golgi body, where it is processed by

Table 2: List of antibodies that reproducibly detect markers of UPR.

Target name	Supplier	Applications
phospho-PERK	#3191; Cell Signaling	WB (1 : 2000), IHC (1 : 100)
phospho-PERK	#3179; Cell Signaling	WB
CHOP	MA1-250, Affinity bioreagents	WB
CHOP	sc-793; Santa Cruz Biotechnology	WB (1 : 1000) IHC (1 : 400–1 : 800)
spliced XBP-1	sc-7160;Santa Cruz Biotechnology	WB (1 : 2000) IHC (1 : 100)
ATF4	ARP37017_P050; Aviva Systems Biology	WB (1 : 5000)
Grp78	SPA-926; Stressgen	WB (1:1000)
	AB32618; Abcam	IHC (1 : 200)
phospho-eIF2 alpha	#9721; Cell Signaling	WB (1 : 2500), IHC (1 : 100)
total-PERK antibody	sc-9477; Santa Cruz Biotechnology	WB (1 : 1000), IP
IRE1-alpha	#3294; Cell Signaling	WB (1 : 1000)

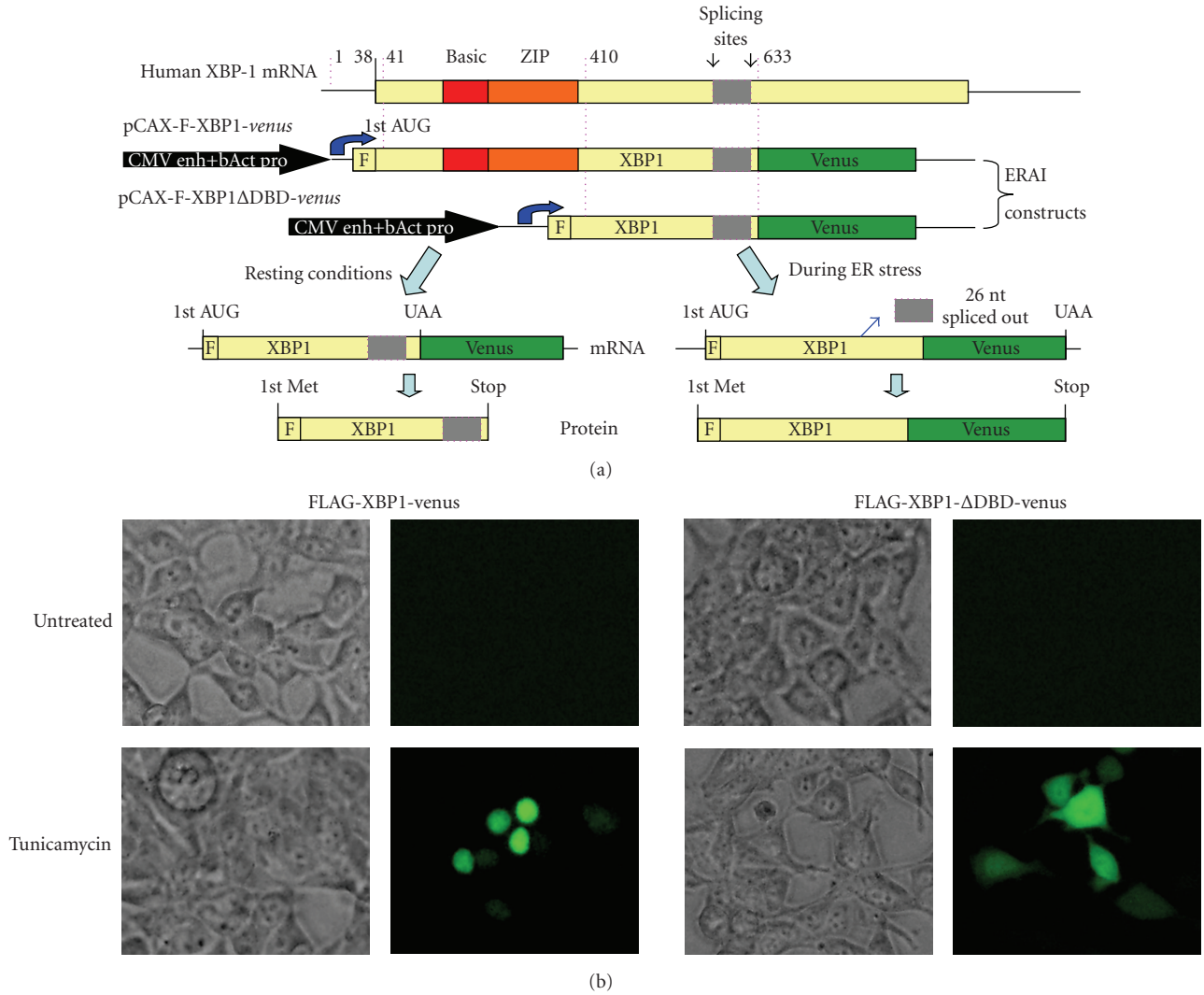

Figure 3: *Detection of IRE1 activity using "ER stress-activated indicator" (ERAI) constructs.* (a) Schematic presentation of ERAI plasmid obtained by fusing XBP1 and *venus*, a variant of the green fluorescent protein (adapted from [36] by Iwawaki et al. (2004)). (b) Twenty-four hours after transfection F-XBP1-*venus* and F-XBP1ΔDBD-*venus*, 293T cells were left untreated or treated with(1 μg/mL) tunicamycin for 24 hours and then analyzed by fluorescence microscopy.

S1P and S2P proteases [38, 39]. The cytoplasmic fragment of ATF6, thereby liberated from the membrane, translocates into the nucleus and activates transcription of its target genes [38, 39]. A GFP-ATF6 fusion protein, which relocates from the ER to the nucleus via the Golgi apparatus in response to ER stress, can be used to monitor activation of ATF6 by fluorescent microscopy [38, 39]. One limitation of this approach, however, is that overexpression can sometimes alter the subcellular localization and kinetics of protein trafficking. This problem has been addressed to some extent by expressing GFP-ATF6 from a shortened CMV promoter which has a deletion of 430 base pairs from the 5′ side. The short promoter possesses considerably lower activity than the full promoter and GFP-ATF6 expressed using the short CMV promoter is localized exclusively to the ER and translocates to the nucleus similarly to endogenous ATF6 [39]. For detection of GFP-ATF6, 293T cells were transfected with pCMVshort-EGFP-ATF6 (WT), pCMVshort-EGFP-ATF6 (S1P$^-$), and pCMVshort-EGFP-ATF6 (S2P$^-$) plasmids. pCMVshort-EGFP-ATF6 (S1P$^-$) and pCMVshort-EGFP-ATF6 (S2P$^-$) have a mutation that abrogates the cleavage by S1P or S2P, respectively. 24 hours post transfection, cells were treated with 1 μg/mL tunicamycin. As shown in Figure 4(a), the wild-type GFP-ATF6 was translocated to the nucleus via the Golgi apparatus. Both EGFP-ATF6 (S1P$^-$) and EGFP-ATF6 (S2P$^-$) were localized in 293T cells similarly to the wild-type GFP-ATF6 (Figure 4(b): a–c). In contrast to wild-type GFP-ATF6 (Figure 4(b): a, d, g), GFP-ATF6_(S1P$^-$) (Figure 4(b): b, e, h) and EGFP-ATF6 (S2P$^-$) (Figure 4(b): c, f, i) remained associated with the Golgi apparatus even 4 hours after tunicamycin treatment. These results demonstrate that cleavage by S1P and S2P is critical for the processing of GFP-ATF6 and that only the processed product, GFP-ATF6, can enter the nucleus. The advantage of GFP is that its intrinsic fluorescence allows the translocation of ATF6 to be continuously followed in single living cells and the whole process recorded over time using, for example, time-lapse photography.

2.6. Use of Transgenic Models. ER stress has been implicated in human neuronal diseases, such as Parkinson's disease, Alzheimer's disease, as well as other disorders [25]. The exact contributions to and casual effects of ER stress in the various disease processes are not known. Furthermore, components of ER stress signaling are also required during development [40, 41]. Studies of ER stress in vivo will provide information that is important and useful in pathology and developmental biology. Two different transgenic mouse models have been described for monitoring ER stress in vivo. The first model, referred to as "ER stress-activated indicator" (ERAI), was constructed by fusing XBP1 and *venus*, a variant of the green fluorescent protein (described in Section 2.4) [36]. This mouse model could serve as a specific and sensitive indicator of ER stress in vivo during development and disease, as well as for analysis of drug effects on ER function. However, this ERAI model detects activation of IRE1 only and does not reveal any information about ATF6 and PERK activation. The other limitations of this model include lack of ERAI

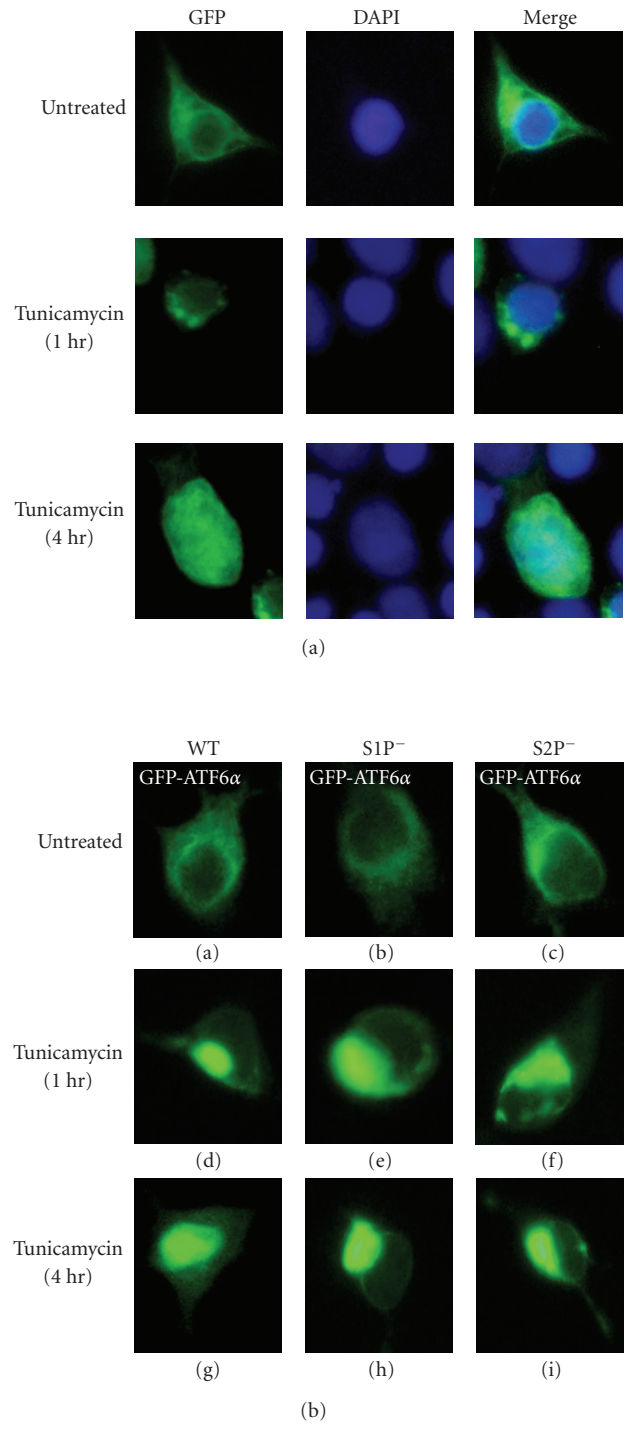

Figure 4: *ER stress-induced processing and nuclear translocation of GFP-ATF6.* (a) Twenty-four hours after transfection with pCMVshort-EGFP-ATF6 (WT), 293T cells were left untreated or treated with 1 μg/mL tunicamycin for the indicated periods. Cells were fixed in 4% paraformaldehyde, stained with DAPI, and then analyzed by fluorescence microscopy. (b) Twenty-four hours after transfection with pCMVshort-EGFP-ATF6α (WT), pCMVshort-EGFP-ATF6α (S1P$^-$), or pCMVshort-EGFP-ATF6α (S2P$^-$), 293T cells were left untreated or treated with 1 μg/mL tunicamycin for the indicated periods and then analyzed by fluorescence microscopy.

expression in some cell types and the inability to detect weak ER stress signals.

The second model, known as ERSE-LacZ model, was constructed by using a LacZ reporter gene driven by 3 kilobases of the rat GRP78 promoter [42]. Two additional transgenic lines have been reported for this model. First, the D300LacZ mouse contains a 230 bp internal deletion spanning from −300 to −70, which eliminates the known ER stress-inducible elements of the GRP78 promoter, including both the ERSE and the cAMP-response element (CRE) [42]. Second, the D170LacZ mouse has a 100 bp internal deletion spanning −170 to −70, which eliminates only the three tandem copies of the ERSE [42]. The wild-type ERSE-LacZ model recapitulates the endogenous expression profile of GRP78 with highest expression in the early embryonic heart which is dependent on the presence of ERSE in the promoter region of GRP78. When using the ERSE-LacZ model, it is recommended to use wild-type GRP78 promoter along with ERSE-deleted GRP78 promoter. ERSE-deleted GRP78 promoter serves as an important control for specificity of ERSE-mediated ER stress in vivo. However, this system does not reveal any information about the three different arms of UPR. One obvious limitation of the ERSE-LacZ model is possible interference by signals not directly related to ER stress since expression of GRP78 is regulated by the coordinated function of several other transcription factors that can act outside of ERSE. Therefore, while both ERAI and ERSE-lacZ mouse models have their unique advantages and pitfalls, they may complement each other to provide novel insights into the complexity of ER stress signaling in vivo in multicellular organisms.

3. Concluding Remarks

In addition to maintaining the homeostasis of ER function, the ER stress response is involved in a number of cellular processes. It has been shown that ER stress is induced during the differentiation of B cells into antibody-secreting plasma cells, likely due to the need to increase the secretory capacity of the cells. In addition, ER stress activation is associated with several human diseases including Alzheimer's disease, diabetes, and atherosclerosis. The experimental approaches discussed above should prove useful to those researching ER stress in vitro and in vivo. Further, these experimental strategies may evolve as new methodologies are developed and our understanding of UPR improves. Nonetheless, it is useful to establish guidelines for acceptable assays that can reliably monitor UPR in many experimental systems.

Abbreviations

ATF4: Activating transcription factor 4
ATF6: Activating transcription factor 6
BiP: Binding immunoglobulin protein
BFA: Brefeldin A
bZIP: Basic leucine zipper domain
CHOP: CAAT/enhancer binding protein (C/EBP) homologous protein
CMV: Cytomegalovirus
CRE: cAMP-response element
eIF2α: eukaryotic initiation factor 2α
ERAD: ER-associated degradation machinery
EDEM1: ER degradation enhancer, mannosidase alpha-like 1
ERSE: ER stress response element
ERAI: ER stress-activated indicator
FFPE: Fixed frozen paraffin-embedded
GRP78: glucose regulated protein 78
GRP94: glucose-related protein 94
GFP: Green fluorescent protein
HERP: homocysteine-induced ER protein
HRD1: HMG-coA reductase degradation 1
IRE1: Inositol-requiring enzyme 1
MS: Multiple sclerosis
PCR: Polymerase chain reaction
PERK: PKR-like ER kinase
RT: Reverse transcription
S1P: Site-1 protease
S2P: Site-2 protease
TG: Thapsigargin
TM: Tunicamycin
UPR: Unfolded protein response.

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Review Article

Linking ER Stress to Autophagy: Potential Implications for Cancer Therapy

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Different physiological and pathological conditions can perturb protein folding in the endoplasmic reticulum, leading to a condition known as ER stress. ER stress activates a complex intracellular signal transduction pathway, called unfolded protein response (UPR). The UPR is tailored essentially to reestablish ER homeostasis also through adaptive mechanisms involving the stimulation of autophagy. However, when persistent, ER stress can switch the cytoprotective functions of UPR and autophagy into cell death promoting mechanisms. Recently, a variety of anticancer therapies have been linked to the induction of ER stress in cancer cells, suggesting that strategies devised to stimulate its prodeath function or block its prosurvival function, could be envisaged to improve their tumoricidial action. A better understanding of the molecular mechanisms that determine the final outcome of UPR and autophagy activation by chemotherapeutic agents, will offer new opportunities to improve existing cancer therapies as well as unravel novel targets for cancer treatment.

1. Introduction

The endoplasmic reticulum (ER) is an organelle with crucial biosynthetic and signaling functions in eukaryotic cells. The ER is not only the major intracellular calcium (Ca^{2+}) storage organelle critically involved in Ca^{2+} homeostasis and Ca^{2+} mediated signaling pathways, but it also provides the environment for the synthesis, folding, and modification of proteins destined to be secreted or embedded in the plasma membrane (reviewed in [1, 2]). Moreover, the ER is the major site for the biosynthesis of steroids, cholesterol, and lipids. Proper folding, maturation, and stabilization of the nascent protein in the ER require the highly oxidizing and Ca^{2+}-rich ER environment, which is essential for the diverse posttranslational and cotranslational modifications, including glycosylation and disulfide bridge formation, to which proteins are subjected after entering the ER. These processes are assisted and monitored by several resident chaperones and Ca^{2+} binding proteins, including the glucose-regulated proteins [such as GRP78 or BiP (immunoglobulin heavy-chain binding protein)], calreticulin and calnexin, and several folding enzymes, such as the thioredoxin-like protein disulfide isomerase (PDI). PDI oxidizes cysteine residues in nascent proteins (i.e., oxidative folding) resulting in formation of intra- and intermolecular disulphide bonds, while reduced PDI is in turn oxidized by the thiol oxidoreductase ERO1. ERO1 transfers reducing equivalents to molecular oxygen, generating stoichiometric amounts of H_2O_2 per newly formed disulphide, which is coupled with a depletion of the reduced gluthatione pool [3]. Proteins that fail to adopt a correctly folded or native conformation, or a proper oligomeric assembly in case of multisubunit proteins, are retrotranslocated to the cytosol through a process known as ER-associated protein degradation (ERAD), and further degraded by the 26S proteasome.

Various physiological and pathological conditions, including hypoxia, ER-Ca^{2+} depletion, oxidative injury, high-fat diet, hypoglycemia, and viral infections may cause an imbalance between ER protein folding load and capacity, leading to the accumulation of unfolded proteins in the

ER lumen, a condition referred to as "ER stress". ER stress sets in motion an evolutionary conserved and integrated signal transduction pathway known as the Unfolded Protein Response (UPR). The UPR primarily aims at ameliorating the protein load on the ER by coordinating the temporal shut down in protein translation along with a complex program of gene transcription to increase ER folding capacity. If this transcriptional program fails to reestablish proper ER homeostasis, persistence in ER stress induces cell death.

Severe ER stress can cause cell death, usually by activating intrinsic apoptosis [4]. Moreover, in order to clear the ER from the accumulation of terminally misfolded protein aggregates that cannot be degraded by the proteasome, the UPR may upregulate the autophagy machinery [5, 6]. Macroautophagy (hereafter referred to as autophagy) is a major lysosomal pathway for the in bulk degradation of cytoplasmic materials, including proteins and damaged organelles, characterized by the sequestration of entire portions of the cytoplasm by a double-membrane bounded vacuole called the autophagosome [7, 8]. In spite of its role as a self-digestion mechanism, autophagy is mainly activated to protect against cell death [8]. However, just like in the case of the UPR, stimulation of autophagy can under certain circumstances be required to activate the cell death machinery [9]. Although both the UPR and autophagy can function independently from each other, recent reports show that they may be interlinked and share the functional duality of exerting both a cytoprotective (under basal or metabolic stress conditions) and cytocidial activity (after acute cellular damage).

Tumor cells are bathed in a hostile microenvironment and confronted with chronic metabolic stress conditions that favor the activation of adaptive mechanisms, such as the UPR and autophagy [10, 11]. Moreover, certain promising anticancer regimens have been shown to activate concomitantly ER stress and autophagy in cancer cells (see Section 4). The molecular link between the UPR and the autophagic response to ER stress, and how these stress pathways influence therapeutic outcome, remain largely undefined, making this topic a very important area for future research in cancer therapy.

Here, we review the molecular mechanisms underlying the emerging connections between the UPR and autophagy pathways, and discuss their potential implications in the context of anticancer therapy.

2. Signal Transduction in ER Stress

2.1. UPR Signaling Pathways. The unfolded protein response in mammalian cells is governed by three transmembrane ER stress sensors, namely PERK (PKR-like ER kinase), IRE1 (inositol requiring enzyme 1), and ATF6 (activating transcription factor 6), which are kept in an inactive state by binding to the ER chaperone BiP, preventing their oligomerization-induced activation. When ER homeostasis is perturbed, accumulating misfolded proteins become progressively bound to BiP, titrating away BiP from interaction with these transmembrane signaling proteins. Upon deinhibition and homodimerization these ER sensors activate a complex ER-to-nucleus signaling pathway that transmits information across the ER membrane to an extensive gene-expression program mediated by the activation of downstream transcription factors. The genetic program activated by the UPR results in upregulation of the folding machinery along with an expansion of the ER lumen and enhanced degradation of terminally misfolded proteins through ERAD. Additional mechanisms include a general translational shutdown as well as the degradation of a select group of secretory mRNAs and proteins that are delayed at the translocon, a process also known as preemptive quality control [12]. The mechanisms by which UPR induction coupled with the failure in reestablishing the ER folding capacity leads to cell death and the requirement of UPR signaling in autophagy stimulation, are still unsettled questions. Furthermore, recent studies have revealed that the ER serves as a subcellular platform for the formation of signaling complexes comprising molecular elements of the UPR, Bcl-2 family members (both pro- and antiapoptotic) (reviewed in [13]), and perhaps regulators of autophagy.

In the following sections we will discuss current knowledge on the main signaling pathways emanating by each branch of the UPR along with their downstream targets (Figure 1).

2.1.1. IRE1. Two human isoforms (paralogous) of yeast Ire1 have been identified. IREα is expressed in all cell types and tissues, whereas expression of IRE1β is primarily restricted to the epithelial cells of the gastrointestinal tract [14, 15]. IRE1 is a type I transmembrane protein with an N-terminal luminal sensor domain and a C-terminal cytosolic effector region that contains both kinase and endoribonuclease (RNase) domains [16]. In cells undergoing ER stress, oligomerization of IRE1 results in trans-autophosphorylation and activation of the RNase domains which excise a 26 nt sequence from *XBP1u* (unspliced *XBP1*), producing mature *XBP1s* mRNA (spliced *XBP1*) [17]. *XBP1s* encodes an active leucine zipper (bZIP) transcription factor XBP1s that regulates the transcription of several genes involved in ER quality control mechanisms, ER/Golgi biogenesis, as well as ERAD components [18–22] and as recently revealed, also genes involved in redox homeostasis and oxidative stress responses [23].

Consistent with this, XBP1 deficient cells were found to be more susceptible to exogenous agents causing oxidative stress, such as H_2O_2 and parthenolide, concomitant with a reduced expression of several antioxidant enzymes including catalase and thioredoxin (TRX1) [24]. Overexpression of XBP1 restored catalase expression and reduced ROS generation after H_2O_2, thus implicating a protective role for XBP1 in oxidative stress. Intriguingly, this antioxidant effect was mediated by XBP1u (i.e., the protein encoded by the unspliced XBP1 mRNA), whereas XBP1s (i.e., the product of IRE1 activation) failed to induce changes in catalase expression in response to ROS or following ER stress, thus underscoring that IRE1 activity is dispensable [24]. Although the molecular mechanism underlying the differential function of the unspliced (XBP1u) and spliced (XBP1s) products of XBP1 is still elusive- and may involve the binding and regulation of selected targets dependent

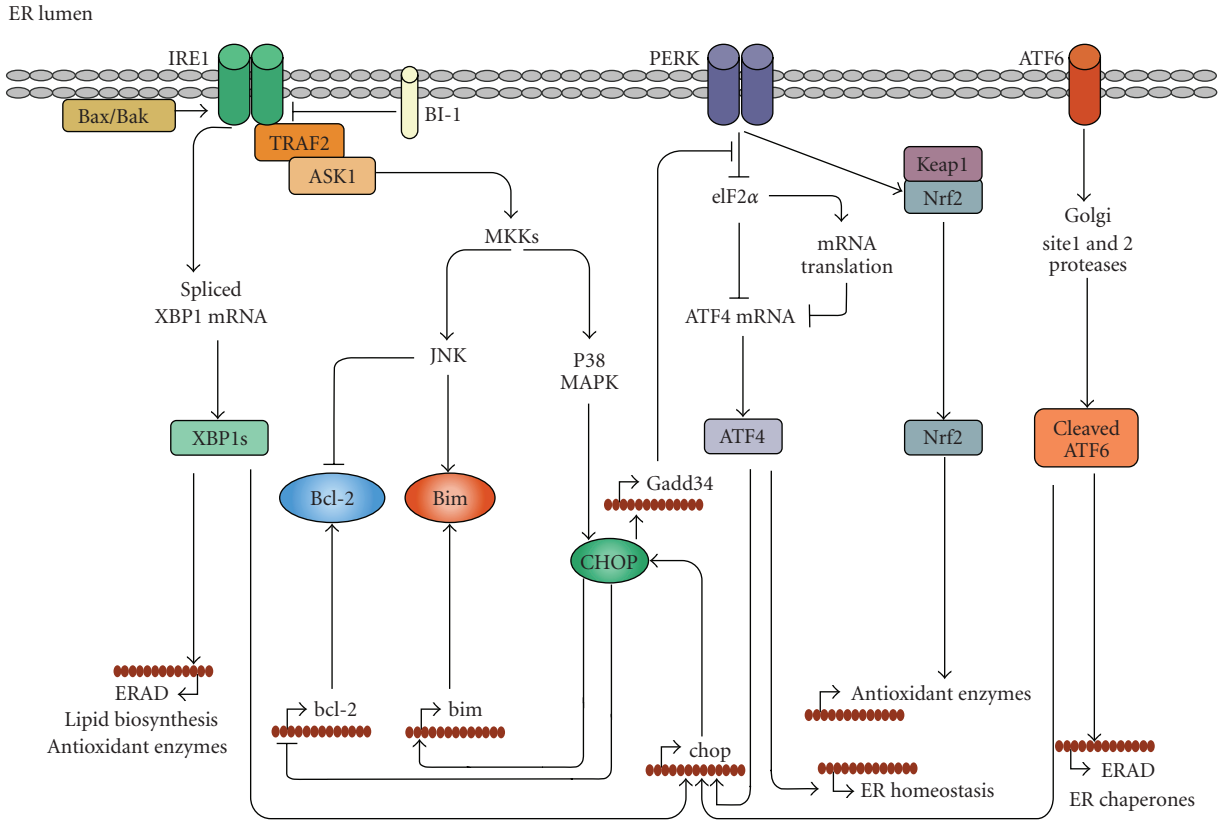

Figure 1: The unfolded protein response in adaptive, apoptotic and redox responses. Upon accumulation of misfolded proteins in the ER, the release of BiP allows IRE1 and PERK to oligomerize. Oligomerized IRE1 disposes of an intrinsic endoribonuclease activity that mediates the unconventional splicing of XBP1mRNA which is subsequently translated into XBP1s, a potent transcription factor regulating expression of genes involved in ERAD and ER quality control. IRE1 signaling is positively regulated by binding of the multidomain proapoptotics Bax and Bak, while its activity is suppressed by the transmembrane protein BI-1. The interaction of Bax/Bak with IRE1 is required for the recruitment of TRAF2 and ASK1 leading to the activation of the MAPKs JNK and p38 MAPK, through specific MKKs. Oligomerized PERK phosphorylates the translation initiating factor eIF2α, resulting in suppression of general protein translation while favoring the translation ATF4, which induces the expression of genes involved in restoring ER homeostasis. Phosphorylation of Nrf2 by PERK disrupts its association with Keap1 resulting in its nuclear accumulation and upregulation of genes associated with various antioxidant responses. In contrast to PERK and IRE1, release of BiP from ATF6 induces its translocation to the Golgi where its processing generates an active transcription factor. Cleaved ATF6 controls mainly genes involved in ERAD and ER homeostasis. Upon severe ER stress, ATF4, XBP1s, and ATF6 can upregulate the expression of the proapoptotic transcription factor CHOP, which mediates apoptosis by the upregulation of proapoptotic BH3-only protein Bim and by suppressing Bcl-2 expression. CHOP activity is enhanced through phosphorylation by p38MAPK. Phosphorylation by JNK in turn activates Bim while inhibiting Bcl-2 functions.

on their relative abundance in different cell types [23] this study highlights a new role for XBP1 in ROS signaling, independent of IRE1 RNase activity.

Although IRE1 displays intrinsic kinase activity, there are no other substrates known thus far than IRE1 itself. However, prolonged activation of IRE1 is capable to transmit a MAP kinase activation cascade. It has been shown that IRE1 can serve as a molecular platform for the recruitment of the adaptor protein TNF-receptor associated receptor 2 (TRAF2), an E3 ubiquitin ligase, which leads to the activation of apoptosis signal regulating kinase 1 (ASK1), a MAP3K of the JNK/p38 MAPK pathway [25, 26]. Depending on the cellular context, activation of JNK can either allow the cells to adapt to ER stress by initiating autophagy [5] or, as discussed further, promote apoptosis in response to persistent or irrecoverable ER stress.

2.1.2. PERK. Like IRE1, PERK is a type I transmembrane protein with a luminal sensing domain and a cytosolic kinase domain which becomes activated following dimerization. The resulting transautophosphorylation induces a conformational change that enhances the affinity of PERK for eIF2α (eukaryotic initiation factor 2 alpha) [27]. Phosphorylation of eIF2α on Ser51 by PERK results in the rapid shut down of general translation, relieving the protein burden on the stressed ER, while the concomitant loss of cyclin D1 arrests the ER stressed cells in G1. In addition, a recent study has shown that eIF2α phosphorylation also regulates translation via inhibition of rRNA synthesis, coordinately regulating translation and ribosome biogenesis during cellular stress [28]. Paradoxically, this translational shutdown leads to the selective translation of the transcription factor ATF4, a member of the bZIP family of transcription factors [29].

The PERK-eIF2α-ATF4 axis regulates the expression of genes involved in amino acid biosynthesis and transport functions, antioxidant stress responses, and apoptosis.

In addition to eIF2α, PERK also phosphorylates the Nuclear factor-E2-related factor 2 (Nrf2) [30]. Nrf2 is a bZIP Cap 'n Collar transcription factor that integrates a variety of cellular responses to oxidative stress. Nrf2 is maintained inactive in a cytoplasmic complex with the microtubule associated protein KEAP1 (Kelch-like Ech-associated protein 1). Nrf2 phosphorylation promotes its dissociation from KEAP1, leading to the nuclear accumulation of Nrf2, binding the antioxidant response element (ARE) in the promotor of genes encoding detoxifying enzymes such as heme oxygenase 1 (HO-1) [31]. In line with these results, it was shown that $Nrf2^{-/-}$ cells are more prone to ER stress induced apoptosis [30]. Likewise, $PERK^{-/-}$ cells, along with an impaired attenuation of protein synthesis, were found to mount a high amount of endogenous peroxides preceding apoptotic induction in response to agents causing perturbation of ER functions [32]. Interfering with ERO1 blocked the increased ROS production, thus providing a link between protein oxidation in the ER and ROS production during ER stress. This PERK function was linked to the ability of ATF4 to regulate the expression of genes involved in glutathione biosynthesis and antioxidant response [32]. These studies suggest that the PERK branch of UPR bifurcates in two parallel but integrated signaling pathways, PERK-eIF2α-ATF4 and PERK-Nrf2, with a key role in adaptation to oxidative stress, a metabolic consequence of biosynthesis and posttranslational oxidative processing in the ER.

2.1.3. ATF6. Both isoforms of ATF6, ATF6α and ATF6β, are present in all cell lines as type II transmembrane ER proteins. Release of BiP does not cause ATF6 oligomerization, but instead reveals a Golgi localization sequence [33]. Once translocated to the Golgi, ATF6 is cleaved at a juxtamembrane site by the site 1 and site 2 proteases (S1P and S2P) [34], which are also involved in the cleavage of the ER membrane transcription factor SREBP (sterol response element-binding protein) [35, 36]. Processed ATF6 moves to the nucleus where it forms active homodimers or dimerizes with other bZIP transcription factors like NF-Y (CAAT binding factor) as well as XBP1s [37], to regulate transcription from ATF/cAMP response elements (CREs) and ERSEs [38;39]. One of the ATF6 transcriptional targets is the IRE1 substrate *XBP1* [22]. Interestingly, Yoshida et al. found that XBP1u interacts directly with the active form of ATF6 (but not ATF4), targeting it for proteasomal degradation which may provide a negative feedback loop to decrease XBP1 expression [38]. Other transcriptional targets include proteins increasing ER chaperone activity and degrading of ER client proteins [37, 39]. Although ATF6 is neither essential for basal expression of ER chaperones nor for embryonic or postnatal development, it plays an important role in recovery from acute ER stress and adapting cells to chronic ER stress [39]. Additionally, a recent study shows that ATF6α also contributes, in an XPB1s-independent manner, to lipid biogenesis and ER expansion, an ER stress response which was thought to be predominantly mediated by the IREI pathway [40].

2.2. Regulation of the UPR by Bcl-2 Family Members. Bcl-2 family proteins, which consist of proapoptotic multidomain proteins (e.g., Bax, Bak), antiapoptotic multidomain proteins (e.g., Bcl-2), and BH3-only proteins (e.g., Bid, Bim, Bad), are key regulators of mitochondrial apoptosis [41, 42]. They function as gatekeepers (antiapoptotic; Bcl-2) or gatecrashers (proapoptotic; Bax/Bak) of the outer mitochondrial membrane [43]. While the molecular mechanism underlying their mitochondrial action is still a matter of debate, it is becoming clear that Bcl-2 family proteins can exert a tight control on apoptosis at different subcellular sites. A constellation of ER localized Bcl-2 family members, including Bax, Bak, Bik/Nbk, and Bcl-2, has been shown to be engaged in the control of ER-Ca^{2+} homeostasis [44–46] (for an extensive review see [13, 47]). Moreover, recent reports have identified Bcl-2 members as vital regulators of UPR sensor mechanisms and cellular fate following ER stress.

For instance, ATF6 negatively regulates Bad proapoptotic activity by upregulating regulator of calcineurin 1 (RCAN1) [48], an endogenous inhibitor of calcineurin (protein phosphatase B). Bad dephosphorylation allows its dimerization with antiapoptotic Bcl-2 protein family members like Bcl-X_l, thus inhibiting their activity [49]. This mechanism underscores a prosurvival role for the genetic program activated by ATF6, through the suppression of Bad proapoptotic activity [49].

The proapoptotic multidomain proteins Bax and Bak can form a protein complex with the cytosolic domain of IRE1, and this interaction has been shown to be essential for IRE1 signaling [50]. Genetic ablation of *bax/bak* in mice caused abnormal response to tunicamycin-induced ER stress in the liver along with extensive tissue damage, decreased expression of XBP1, and reduced JNK activation [50]. Furthermore, the requirement of Bax/Bak proteins for proper IRE1 signaling was confirmed in MEFs doubly deficient (DKO) in these proapoptotic proteins [50]. In a recent report Klee et al. [51] showed that reconstituting Bak expression at the ER membranes in DKO cells is sufficient to reestablish IRE1-TRAF2 activation and mitochondrial apoptosis (as discussed further in Section 2.3) instigated by reticular forms of the BH3-only proteins Bim and Puma. Interestingly, the IRE1 pathway activated by reticular BH3-only effectors was atypical as it did not lead to XBP1 splicing, likely because other arms of the UPR required for the upregulation of *XBP1* mRNA levels, such as ATF6, were not sufficiently activated [51]. However, an alternative and intriguing possibility could involve a differential regulation of the IRE1 RNase activity (required for *XBP1* mRNA splicing) and IRE1-TRAF2 complex formation (required to activate proapoptotic JNK signaling) by a different subset of proapoptotic proteins at the ER membrane. Clearly, further studies are required to shed more light into the mechanisms regulating IRE1 signal transduction.

Recently, the ER associated Bax inhibitor-1 (BI-1), an evolutionary conserved antiapoptotic protein, has been identified as a new player in the regulation of IRE1 by Bcl-2

family members and their modulators. BI-1 can block Bax-mediated apoptosis following ER stress and other intrinsic stress signals by directly interacting with antiapoptotic Bcl-2 family members and enhancing their antiapoptotic function [52]. BI-1 at the ER was found to be capable to interact through its C-terminus domain with IRE1 and to inhibit IRE1 signaling, in vitro as well as in mice and flies, conferring increased resistance under conditions of mild ER stress [53]. ER stressed BI-1 deficient cells displayed IRE1 hyperactivation along with an increased *XBP1* mRNA splicing and expression of XBP1s-dependent genes, thus unraveling a paradoxical role of BI-1 as inhibitor of the cytoprotective IRE1 branch of the UPR in mildly ER stressed cells. Interestingly, in another study using human fibrosarcoma cells, overexpression of BI-1 inhibited ROS production downstream ER stress through the upregulation of HO-1, an effector of the PERK-pathway (as described previously) [54]. Although in the study of Lisbona et al. [53] BI-1 deficiency in MEFs did not affect expression of HO-1, this raises the intriguing possibility that BI-1 may affect IRE1 and possibly PERK pathway in a cell type specific manner. Whereas further mechanistic studies are required to solve these discrepancies, these findings reveal how a subtle cross-talk between molecular sensors of UPR and cell death regulators might affect the amplitude and function of the UPR. Moreover, abundant evidence claims for a critical role of proapoptotic Bcl-2 family proteins in the induction of apoptosis following ER stress.

2.3. From ER Stress to Cell Death. When the initial cellular responses fail to restore ER homeostasis, sustained ER stress causes the UPR to switch from an adaptive to a cell death pathway. However, the molecular elements of this switch are still elusive. With the exception of few components of the UPR for which a dominant prosurvival (i.e., BiP, [55]) or proapoptotic (i.e., CHOP, [56, 57]) role has been assigned by genetic studies, each apical UPR sensor holds a dualistic role in propagating adaptive as well as a toxic signals.

For example, genetic deletion of *PERK* or interference with eIF2α phosphorylation impairs cell survival [58, 59] and tumor growth under hypoxia [60], while artificially increasing PERK activity increases cell survival [61]. However, Lin et al. [62] have shown that sustained PERK signaling is lethal, whereas the equivalent duration of IRE1 signaling is not, suggesting that transition from protective to proapoptotic UPR function involves a switch in IRE1 signaling along with enduring PERK activity [62].

The main effector of PERK-mediated apoptosis is the proapoptotic transcription factor CHOP (C/EBP homologous protein; GADD153) which can be induced by ATF4, ATF6, as well as XBP1s. However, the PERK-eIF2α branch appeared to be essential for CHOP upregulation as both PERK$^{-/-}$, ATF4$^{-/-}$ and eIF2α Ser51Ala knock-in cells failed to induce CHOP during ER stress [32, 58, 59]. CHOP activity is also regulated translationally by the limited CHOP mRNA lifetime [63] and posttranslationally by p38MAPK phosphorylation, which enhances its proapoptotic activity [57, 64]. The latter mechanism may provide a point of convergence between the PERK and IRE1 signaling pathways since p38MAPK is a downstream target of the IRE1-TRAF2-ASK1 signaling complex [25, 26]. Genetic studies have shown that *CHOP* loss-of-function results in cytoprotection, whereas *CHOP* gain-of-function enhances sensitivity to a variety of stresses perturbing ER function [56, 65].

CHOP mediated cell death entails the induction of a variety of genes that may potentiate apoptosis, including *GADD34*, *ERO1α*, *Bim*, and *TRB3* (Tribbles homologue 3). GADD34 is a regulatory subunit of protein phosphatase 1 (PP1) that targets PP1 to dephoshorylate eIF2α, which promotes the resumption of protein synthesis [66]. If the protein folding capacity of the ER has not been reestablished, a premature deinhibition of translation will increase client protein load in the ER and may favor improper disulphide bond formation of unfolded proteins, thus amplifying the damage. In addition, elevated expression of ERO1α by CHOP is thought to instigate hyperoxidizing conditions in the ER [67, 68]. Thus the PERK-axis, which is involved in maintaining the redox state during ER stress, as discussed before, has also the ability to turn into a prooxidant signal when the transcriptional program of CHOP is efficiently set in motion.

As suggested by a recent study wherein the stability of prosurvival and prodeath mRNAs and proteins was studied under conditions of mild or severe ER stress [63], ATF4-dependent prosurvival gene expression is likely to be more sustained when PERK is activated transiently and to a limited extent. In contrast, as a consequence of the intrinsic instability of the proapoptotic mRNAs and proteins, the apoptotic program mediated by the ATF4 target CHOP would be activated only when protective mechanisms fail and require a more sustained PERK activation.

CHOP can also regulate the expression of a number of Bcl-2 family proteins. By a yet unidentified mechanism, it suppresses the expression of the antiapoptotic Bcl-2 [65] while directly promoting the transcription of the proapoptotic BH3-only protein Bim [69].

Although it is clear that CHOP fulfills an important role in ER stress induced apoptosis, the fact that PERK$^{-/-}$ and eIF2α Ser51Ala knock-in cells are unable to induce CHOP yet are very susceptible to ER stress [58, 59] unravels the dual role of the PERK axis in triggering both adaptive and proapoptotic processes. The increased sensitivity of PERK deficient cells could be explained, at least in part, by the impaired activation of the prosurvival PI3K (phosphatidylinositol 3 kinase)-Akt signaling pathway which has been shown to promote the expression of inhibitor of apoptosis proteins (IAPs), thus conferring cellular resistance to ER stress [70, 71].

An interesting molecular switch between the prosurvival and prodeath functions of the PERK pathway could involve the human orthologue of the *Drosophila* tribble protein (TRB3), a downstream transcriptional target of CHOP [72]. Ohoka and et al. [72] showed that TRB3 knock down sensitized the cells to cell death during tunicamycin treatment. Remarkably, TRB3 could downregulate its own induction by repressing CHOP/ATF4 functions [72, 73]. A mechanism was proposed wherein TRB3 exerts a negative feedback on CHOP during mild ER stress, allowing the cell to

adapt to ER stress [72, 73]. In contrast, during severe or persistent ER stress, induction of TRB3 would be more robust, leading to apoptosis through a mechanism involving TRB3-mediated inhibition (dephosphorylation) of Akt [74, 75]. This feedback mechanism could facilitate ER stress mediated apoptosis in severely ER stressed cells that have successfully mounted proapoptotic threshold levels of CHOP.

Similar to PERK, IRE1 signaling has also been implicated in promoting or impairing cell survival. For instance, when unfolded proteins accumulate, artificially extending IRE1's RNase function led to enhanced survival [62, 76] and the knock down of XBP1 impaired cell survival, [77, 78] pointing to a general protective role for the IRE1-XBP1 signaling during ER stress. However, in another report, IRE1 overexpression in HEK293T cells led to its activation in the absence of ER stress and subsequent cell death [14]. As discussed before, IRE1 has apparently gained signaling properties independent of XBP1 splicing, which are strongly dependent on interaction with Bcl-2 proapoptotics and Bcl2 modulators at the ER membrane. Thus, IRE1 can promote cell death by recruiting a TRAF2-ASK1 complex leading to the activation of JNK and p38 MAPK cascades [25, 26]. JNK, in turn, can exert its proapoptotic effect by activating certain BH3-only proteins, such as Bim [79, 80], or by suppressing the antiapoptotic activity of Bcl-2 [81].

The apoptotic pathway evoked after UPR is still unclear, but mounting observations indicate that the mitochondrial pathway is heavily involved, since cells lacking Bax and Bak, or Apaf-1 are resistant to apoptosis induction by different ER stressors [44, 82, 83]. Moreover, as mentioned before, several Bcl-2 family members localize at the ER and regulate both calcium levels as well as signal transduction through the UPR. In addition to Bim, other BH3-only proteins, such as Noxa and Puma, are transcriptionally activated, through p53-dependent [84] and independent mechanisms [85] depending on the type of ER stressor, thus bridging ER stress to Bax/Bak mediated mitochondrial membrane permeabilization. Recently Klee and coworkers using $Bax^{-/-}/Bak^{-/-}$ cells showed that Bak targeted at the ER membrane is sufficient to engage mitochondrial apoptosis when activated by BH3-only molecules Puma and Bim at the ER, thus bypassing the need to be localized to the mitochondria [51]. Reticular Bak engaged an atypical IREα-TRAF2 activation pathway, wherein the mobilization of Ca^{2+} facilitated persistent JNK activation [51]. Intriguingly, ER Ca^{2+} release *per se* was not able to incite mitochondrial apoptosis unless Bak was expressed at the reticulum [51], whereas it favored nonapoptotic cell death, as shown also in our previous study [82]. Whether this pathway has any role in normal cells expressing both mitochondrial and ER Bax/Bak still needs to be proven, however it can already be argued that JNK functions as a master regulator of both apoptosis and perhaps autophagy pathways after ER stress.

Thus all together the emerging consensus is that the amplitude and the temporal activation of specific arms of the UPR, along with the repertoire of signaling platforms formed at the ER membrane (UPR interactome), are crucial elements determining cellular fate following ER stress.

3. ER Stress and Autophagy

3.1. Autophagy. Proteasomal degradation and autophagy are the two main mechanisms that are in charge of protein clearance in the cell. Unlike proteasomal degradation (that digests soluble ubiquitin-conjugated proteins in a specific way), autophagy can degrade both soluble and aggregated proteins [8, 86]. Thus, during the autophagic process, entire cytoplasmic portions—including organelles and other cytoplasmic components—are engulfed within a double membrane vesicle designated autophagosome. The maturation of these vesicles involves their fusion with lysosomes, which leads in turn to the degradation of the autophagosome components by the lysosomal degradative enzymes [8, 86]. As discussed below, a variety of stress signals such as nutrient starvation or treatment with different anticancer agents (including those that induce ER stress) stimulate the autophagy process—which is nowadays considered as an essential cellular process participating in a number of physiological functions within the cell.

The molecular mechanisms responsible for the regulation of autophagy have not been completely elucidated yet, although genetic and biochemical analyses performed during the last few years have identified several autophagy genes (Atg) that participate in the regulation of this cellular process. Researchers working in the autophagy field have formally divided the autophagic process in several steps. Initiation of autophagy relies on the formation of an isolation membrane (IM) at the so-called preautophagosomal site. Elongation of this isolation membrane leads to the formation of the autophagosome. The autophagy process ends with the fusion of the autophagosome and the lysosome, the digestion of the autophagosome content, and the release of the digested components back to the cytosol [8, 86]. In these sections, we will briefly summarize the mechanisms by which the different stages of autophagy are regulated.

The normal rate of autophagy in the cell is low and therefore this cellular process only becomes activated in response to certain situations. Thus, exposure of the cell to an autophagic stimulus triggers a series of modifications in the autophagic machinery that allow the formation and elongation of the IM. The precise origin of the IM in mammalian cells is still unknown, although it has been proposed that it could be either derived from *de novo* synthesized lipids or generated by vesicle budding from ER, Golgi apparatus, or endosomes [87]. The transmembrane proteins Atg9 and VMP-1 [88, 89] are required for autophagosome formation and it has been suggested that they could play a role in the transport of lipids to the IM as well as in the recruitment of additional proteins involved in the initiation of autophagy. Thus, the movement of Atg9 from the trans-Golgi location to the preautophagosomal site seems to be a crucial event in the initiation of autophagy [87, 90].

The relocation of the transmembrane protein Atg9 to the autophagosome is thought to require activation of the complex formed by the proteins Atg1, Atg13, and Atg17/FIP200.[88].The activity of the Atg1 complex is modulated by the mammalian target of rapamycin complex 1 (mTORC1). mTORC1 is a protein complex formed by

mTOR, RAPTOR (regulatory associated protein of mTOR), mLST8, and PRAS40 (proline-rich AKT substrate 40 kDa) [91] that plays a central role in the control of protein synthesis, cell growth, and cell proliferation through the regulation of several downstream targets [91]. In addition, mTORC1 has been proposed to regulate autophagy by repressing the activity of the Atg1-Atg13-Atg17/FIP200 complex [92–95]. Thus, inhibition of mTORC1 facilitates the initiation of autophagy. Regulation of mTORC1 relies on the small G protein Rheb (ras homologue enriched in brain) which (through a still not completely elucidated mechanism) activates mTORC1. The tuberous sclerosis proteins (TSC1 and TSC2) have GTPase activating protein (GAP) activity on Rheb and therefore promote its inhibition. Hence, inactivation of TSC1/2 stimulates Rheb and mTORC1 and inhibits autophagy [91].

As a result of its central position in the control of cellular homeostasis, mTORC1 integrates signals from different inputs. One of the most important upstream regulators of mTORC1 is the prosurvival kinase Akt, which phosphorylates and inactivates TSC2 as well as PRAS40 [91]. Thus, Akt activation stimulates mTORC1 and inhibits autophagy. Another important regulator of TSC2 is the AMP-activated protein kinase (AMPK) which phosphorylates TSC2 in a different residue than Akt leading to activation of TSC1/2, inactivation of Rheb, and inhibition of mTORC1 [96]. As discussed in the following sections, modulation of mTORC1 activity is one of the mechanisms by which ER stress and autophagy become connected.

Another important step in the initiation of autophagy is the generation of a specific pool of phosphatidylinositol-3-P (PIP3) at the autophagosome. In mammals, this event is catalyzed by the class III phosphatidylinositol 3 kinase (PI3K) complex [which consists of Vps34 (vacuolar protein sorting 34) and its regulatory protein p150 (homolog to the yeast Vps15 protein)] [97]. Accumulation of PIP3 seems to be crucial for the recruitment of autophagy proteins such as Atg18/WIPI-1 to the IM which is important for Atg9 trafficking and therefore for the initiation of the autophagic process [90]. In addition, other proteins such as mAtg2 and DFCP1 (double FYVE domain-containing protein 1) may also be regulated by PIP3 and play a role in the regulation of the formation and elongation of the autophagosome [90]. Underlining the importance of PIP3 in the early stage of autophagy, a specific phosphoinositide 3-phosphatase (Jumpy) has been very recently identified as a new modulator of this cellular process [98].

Importantly, other Vps34-interacting proteins are required for autophagy including, Vps30/Atg6/Beclin1, Atg14 and autophagy/beclin-1 regulator 1 (Ambra-1), and UVRAG [86]. Among the different partners of Vps34, particular attention has been focused on Beclin-1. Beclin-1 has a BH3-only domain that permits the interaction of this protein with the antiapoptotic proteins Bcl-2 and Bcl-X_L. This interaction abrogates Beclin-1 ability to induce autophagy [99–102]. Different stimuli, including ER stress, modulate the interaction between Beclin-1 and Bcl-2 family members (see also the following sections) which is considered an important mechanism of autophagy regulation.

Atg14 and UVRAG are also interactors of Vps34 although their presence in the class III PI3K complex seems to be mutually exclusive [87, 103, 104]. Recent findings support that Atg14 plays an important role in the early stages of autophagy activation in response to starvation [87]. In any case, further research is still necessary to understand the complex lipid-protein and protein-protein interactions that regulate the formation of the IM.

The elongation of the initial autophagic membrane requires the participation of two ubiquitin-like protein conjugation systems which modify the autophagy proteins Atg5 and Atg8/LC3. Thus, upon autophagy stimulation, Atg5 is conjugated to Atg12. In this process Atg12 is activated by the E1 activating enzyme Atg7 and transferred to the E2-like protein Atg10. Finally Atg12 is attached to an internal lysine of Atg5 in a process that does not seem to require an E3 ligase protein. The Atg5-Atg12 conjugation complex interacts with Atg16L to form the Atg16L complex [87]. The other conjugation system involves the modification of Atg8/LC3. Initially Atg8/LC3 is cleaved by the protease Atg4 (which generates a glycine Ct residue in Atg8/LC3). Then, the E1 enzyme Atg7 activates Atg8, which is transferred to the E2-like protein Atg3. The last step in Atg8/LC3 modification involves the conjugation of this protein to phosphatidylethanolamine (PE), a process that is facilitated by the E3-like activity of the Atg12-Atg5 conjugate [105, 106]. Upon autophagy induction, most of Atg8/LC3 becomes lipidated and associates with the autophagosome, which is widely used to monitor activation of autophagy by immunofluorescence [8, 86, 107]. The Atg16L and Atg8/LC3 complexes play a crucial role on the modification of the autophagosomal membrane and therefore in the elongation and closure of the autophagosome

The last step in the autophagic process is the fusion of the autophagosome with lysosomes. The canonical machinery of vacuole membrane fusion seems to participate in the regulation of this process [87, 90]. Thus, the lysosomal protein LAMP2 and the small GTPase Rab7 have been implicated in autophagosome-lysosome fusion in mammalian cells. Nevertheless, many additional proteins including those belonging to the Rab and soluble N-ethylmaleimide sensitive factor attachment protein receptors family (SNARE) are believed to play an important role in the autophagosome-lysosome fusion process [8, 86]. The lysosomal degradation of the autophagosomal content relies on several lysosomal hydrolases including cathepsins B, D, and L.

The final outcome of the activation of the autophagy program is highly dependent on the cellular context and the strength and duration of the stress-inducing signals. Thus, besides its role in cellular homeostasis, autophagy can be a form of programmed cell death or play a cytoprotective role, for example in situations of nutrient starvation [108, 109]. Accordingly, autophagy plays a dual role in cancer. On one hand, this cellular process may help to overcome the stress evoked by the lack of nutrients and oxygen at the initial steps of tumorigenesis. On the other hand, autophagy has been proposed to play a tumor suppressor function by providing the minimal supply of ATP required for DNA repair, preventing oxidative stress and reducing

intratumoral necrosis and local inflammation [110–113]. Moreover, different anticancer treatments activate autophagy in tumor cells, which has been proposed to either enhance cancer cell death or act as a mechanism of resistance to chemotherapy [110–115].

3.2. Connecting ER Stress Responses and Autophagy. Different situations that induce ER stress also lead to induction of autophagy. As discussed above, the ER stress response is activated to protect the cells from different alterations affecting this organelle. However, when the intensity or duration of the ER damage cannot be restored by this response, ER stress can also lead to cell death [116]. Likewise, autophagy can help cells to cope with ER stress (for instance contributing to the elimination of unfolded or aggregated proteins) or participate in the mechanism of ER stress-induced cell death [115, 117–119]. In this section we will try to delineate some of the proposed mechanisms by which ER stress and autophagy become connected under certain cellular situations (Figure 2).

3.2.1. UPR and Autophagy. As described above, the accumulation of unfolded proteins triggers the UPR thus promoting the inhibition of general protein synthesis as well as the increased translation of several transcription factors that enhance the expression of ER stress genes [117] (see the previous sections for further details). Evidence for a link between UPR and autophagy was obtained from ectopic expression of polyglutamine (polyQ) proteins [6]. In these experiments, a dominant-negative form of PERK or genetic substitution of Serine 51 of eIF2α by Ala (which prevents the phosphorylation of this protein) prevented polyQ protein-induced autophagy [6], strongly suggesting that PERK-dependent eIF2α phosphorylation plays an important role in the activation of autophagy in response to the accumulation of unfolded proteins. On the other hand, eIF2α phosphorylation seems to be also important for autophagy as induced by other ER stress-related or unrelated stimuli [75, 120, 121]. It is important to bear in mind that PERK is not the only protein kinase regulating eIF2α phosphorylation (see reference [116] for a review) as double-stranded RNA-activated protein kinase (PKR; activated in viral responses), general control nonderepressible 2 (GCN2; activated upon aminoacid starvation), and heme-regulated inhibitor (HRI; activated in heme depletion) also phosphorylate eIF2α. Accordingly, PKR-dependent eIF2α phosphorylation modulates autophagy in response to viral infection [120]. Likewise, the small heat shock 22 KDa protein 8 (HspB8) and its cochaperone Bcl-2-associated athanogene 3 (Bag3) have been proposed to mediate mutated hungtingtin-induced eIF2α phosphorylation and autophagy via GCN2 activation [121].

Regarding the signalling pathways by which eIF2α phosphorylation can modulate autophagy, Kouroku and et al. showed that PERK-eIF2α-dependent Atg12 upregulation is required for induction of autophagy in response to polyQ protein accumulation [6]—which suggests that controlling the expression of autophagy-related genes by eIF2α downstream targets could be one of the mechanisms connecting both events. On the other hand, we have recently found that treatment of cancer cells with Δ^9-tetrahydrocannabinol (THC), the active component of marihuana, activates autophagy via ER stress and eIF2α phosphorylation [75] (an effect that is not mediated by PERK, PKR, or GCN2, Salazar, M. and Velasco, G. unpublished observations). Our data indicate that induction of autophagy in response to THC treatment relies on the eIF2α phosphorylation-dependent upregulation of the transcription factors p8, ATF-4, and CHOP as well as of the pseudokinase TRB3 (four genes that had been previously identified as essential mediators of THC action in cancer cells [122, 123]). We also showed that an important step in the induction of autophagy is the inhibition of the Akt/mTORC1 axis by the pseudokinase TRB3 [75] (see below for additional details) (Figure 2). In any case, further research is still necessary to clarify the precise mechanisms by which eIF2α phosphorylation regulates autophagy in response to different ER stress signals.

Activation of the IRE1 arm of the ER stress response has also been shown to regulate autophagy. Thus, treatment with tunicamycin or thapsigargin [5] or treatment with proteasome inhibitors [119] induced autophagy on an IRE1-dependent manner. The proautophagic actions of IRE1 seem to rely on the ability of this protein to interact with the cytosolic adaptor TRAF-2 and activate JNK) [5]. Of interest, JNK has been proposed to regulate autophagy through Bcl-2 phosphorylation, which prevents this protein of interacting (and inhibiting) the essential autophagy regulator Beclin-1 [99, 124, 125]. In addition, JNK has been shown to control Beclin-1 expression to regulate ceramide-induced autophagy [126]. As discussed above, Beclin-1 is associated to the Vps34 and plays a very important role in the regulation of autophagy ([102]see below) (Figure 2). It is therefore conceivable that activation of the IRE1/TRAF2/JNK arm of ER stress may regulate autophagy through modulation of Beclin-1 function and expression. Intriguingly, it has been recently shown that XBP-1 ablation increases autophagy and protects from the toxicity induced by the aggregates of the enzyme superoxide dismutase 1 in a model of Amyotrophic lateral sclerosis [127]. These observations suggest that the XBP-1 may play a different role than TRAF2/JNK on the regulation of autophagy by the Ire1 arm of the UPR.

3.2.2. Ca^{2+} Signalling and Autophagy. ER stress activation is frequently accompanied by calcium release into the cytosol which leads to the activation of several Ca^{2+}-regulated signalling pathways [116]. Different agents (including ER stress inducers) have been shown to produce an increase in cytosolic calcium concentration and activate autophagy. One of the mechanisms connecting Ca^{2+} release from the ER and autophagy is the stimulation of AMPK [128]. As explained above, several kinases regulate mTORC1 including AMPK, which inhibits mTORC1 by activating TSC2 [129]. AMPK is considered an important energy sensor that becomes activated upon ATP cellular depletion or phosphorylation by different kinases [96]. Three AMPK upstream kinases have been identified to date: LKB1, Ca^{2+}/calmodulin-dependent kinase kinase β (CaCMKKβ), and transforming growth factor-beta-activating kinase 1 (TAK1) [96]. Jäättelä and

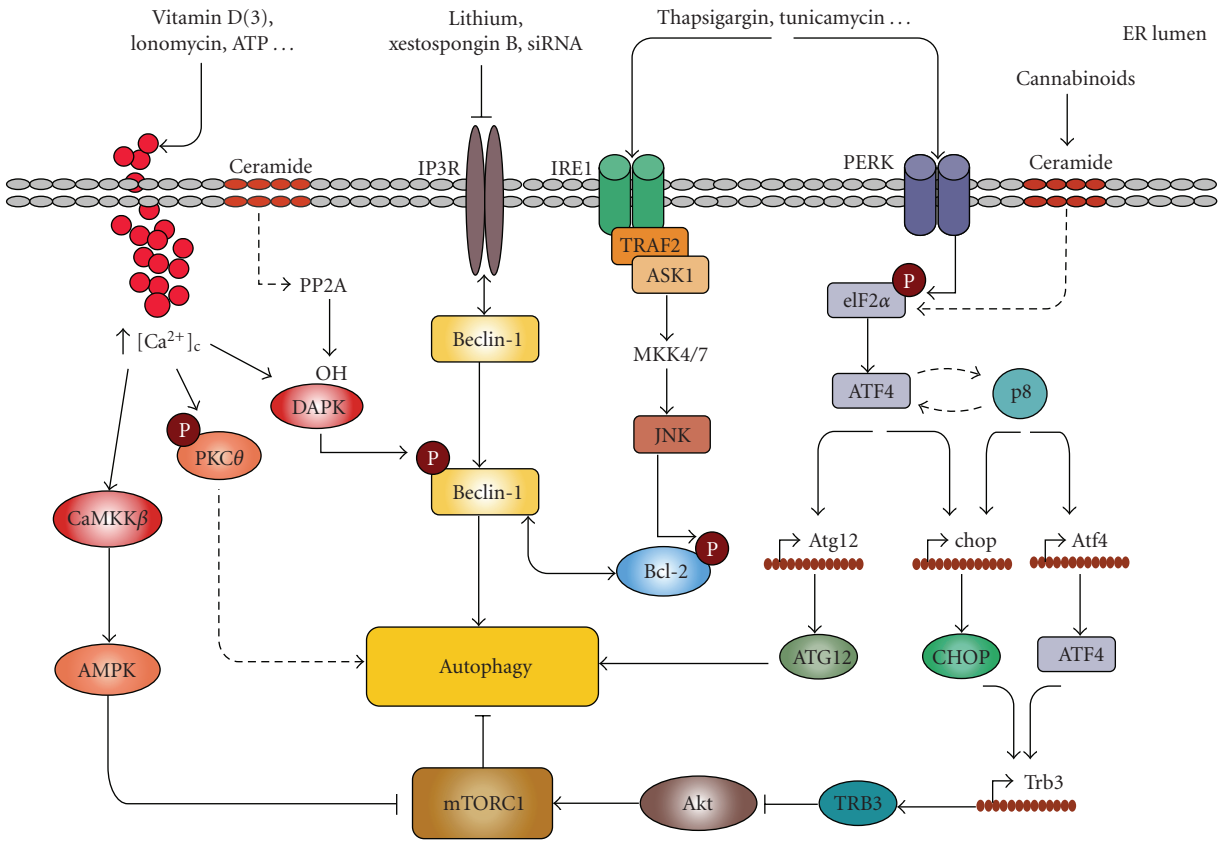

Figure 2: Mechanisms connecting ER stress and autophagy. Different ER stresses lead to autophagy activation. Ca^{2+} release from the ER can stimulate different kinases that regulate autophagy. CaCMKKβ phosphorylates and activates AMPK which leads to mTORC1 inhibition; DAPK phosphorylates Beclin-1 promoting its dissociation from Bcl-2; PKCθ activation may also promote autophagy independently of mTORC1. Inositol 1,4,5-trisphosphate receptor (IP3R) interacts with Beclin-1. Pharmacological inhibition of IP3R may lead to autophagy in a Ca^{2+}-independent manner by stimulating its dissociation from Beclin-1. The IRE1 arm of ER stress leads to JNK activation and increased phosphorylation of Bcl-2 which promotes its dissociation from Beclin-1. Increased phosphorylation of eIF2α in response to different ER stress stimuli can lead to autophagy through ATF4-dependent increased expression of Atg12. Alternatively, ATF4 and the stress-regulated protein p8 promote the up-regulation of the pseudokinase TRB3 which leads to inhibition of the Akt/mTORC1 axis to stimulate autophagy.

coworkers showed that increases in cytosolic Ca^{2+} concentration upon treatment with different ER stress inducers stimulate CaMKKβ, leading in turn to AMPK activation, inhibition of mTORC1, and autophagy stimulation [128]. The same group has recently shown that TRAIL-induced autophagy is also mediated by AMPK, in this case through a mechanism that involves phosphorylation of AMPK by TAK1 and not by LKB1 or CaM-KKβ [130]. These observations suggest that AMPK may play an important role in the regulation of autophagy in response to different Ca^{2+}-dependent and independent stress signals.

Another Ca^{2+}-activated kinase that regulates autophagy in response to ER stress is the death associated protein kinase 1 (DAPK). DAPK is a Ser/Thr kinase that plays an important role as tumor suppressor due to its ability to promote apoptosis and autophagy [131]. Thus, DAPK-deficient MEFs are less sensitive to ER stress-induced autophagy than their wild-type counterparts [132]. Activation of DAPK upon ER stress relies on the dephosphorylation of an inhibitory autophosphorylation site of the kinase by a PP2A phosphatase [132], which suggests that additional ER stress-activated signals (apart from Ca^{2+} release) are required to stimulate the proautophagic activity of the kinase. Regarding the mechanisms by which DAPK regulates autophagy, it has been recently shown that DAPK phosphorylates Beclin-1 on the BH3-only domain preventing thus the interaction of this protein with Bcl-2 [133, 134]. In addition, DAPK regulates p53 in a p19Arf-dependent manner [135]. As p53 modulates autophagy through different mechanisms [112, 136–138], this could be another way by which DAPK could regulate autophagy in response to certain ER stress stimuli.

The protein kinase C theta (PKCθ) has been also implicated in regulating autophagy in response to ER stress in a calcium-dependent manner. Thus, knock-down of PKCθ (but not inactivation of the UPR signalling routes) prevented autophagy as induced by acute ER stress [139]. In this study, inactivation of mTORC1, under the used concentrations of thapsigargin, thapsigargin were not observed, which

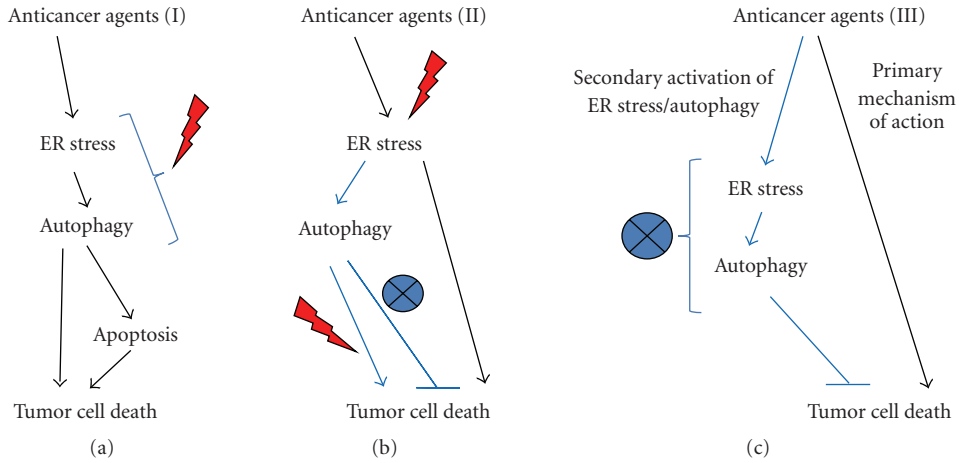

Figure 3: Hypothetic therapeutic strategies based on the modulation of ER stress and autophagy. Different strategies involving modulation of ER stress and autophagy could be potentially used in antitumoral therapies. A. One type of antitumoral agents (e.g., cannabinoids) activates ER stress and autophagy as a mechanism to promote cancer cell death. In these cases, strategies aimed at increasing the stimulation of ER stress and autophagy might be beneficial; B. Other anticancer agents (e.g., PDT) activate ER stress as part of the mechanisms by which they promote cancer cell death. Secondary ER stress-induced activation of autophagy may contribute to cell death (in apoptosis-deficient cells) or to cell survival (in apoptosis competent cells). Thus, depending on the tumor features, autophagy inhibitors or inducers might be administered to improve the response to these anticancer agents. C. A third type of antitumoral agents (e.g., Imatinib mesilate) activates a protective ER stress/autophagy response secondarily to its primary antitumoral mechanism. Inhibition of ER stress and/or autophagy would help to reduce the resistance to this type of therapy.

suggests that different signalling routes may converge in the regulation of autophagy under ER stress situations involving calcium mobilization [140].

Another link between Ca^{2+}, ER stress, and autophagy relies on the modulation of the inositol 1,4,5-trisphosphate receptor (IP3R). This receptor releases Ca^{2+} from ER stores in response to different cellular signals, although it could also play additional functions derived from its ability to interact with different proteins, including members of the Bcl-2 family [141]. Inhibition of the IP3R with xestospongin B [108] or lithium-induced decrease of myo-inositol-1,4,5-triphosphate (IP3) levels [142] promotes autophagy. Intriguingly, these effects seem to be independent of the Ca^{2+} mobilization function of IP3R [143]. Thus, it has been recently shown that use of pharmacological inhibitors of the IP3R disrupts the interaction of this protein with Beclin-1 [144] which could be an additional way of regulating the pro-autophagic function of this protein. Further investigation is nevertheless necessary to clarify whether this mechanism participates in the activation of autophagy in response to ER stress.

3.3. Survival or Death After Autophagy Stimulation by ER Stress. As discussed for the case of ER stress, autophagy is currently considered a cell survival mechanism that, under certain cellular settings, can also promote cell death. Consequently, depending on whether pharmacological or genetic inhibition of autophagy enhances or prevents cell death, activation of autophagy after ER stress has been assigned respectively a cytotoxic [75, 115, 119, 132, 145–147] or a protective [5, 6, 115, 119, 128] role. It is worth noting that depending on the intensity of the stimulus, the cell type (normal versus cancer cells), and the cellular context, (hypoxia, starvation, treatment with antitumoral agents, or presence of mutations) the final outcome of autophagy activation could be different.

An important problem at the time of predicting whether induction of ER stress will activate autophagy in a protective or cytotoxic way is our relative lack of understanding of the molecular mechanisms through which autophagy regulates cell death. Thus, autophagy has been proposed to protect from apoptosis, operate as an alternative cell death mechanism (e.g., in cells that are defective in apoptosis), or act upstream of apoptosis to activate this cellular process, (reviewed in [133, 148]). As discussed in the previous section, some of the key regulatory steps in the activation of autophagy upon stimulation of ER stress (such as mTORC1 inhibition or the interaction of Beclin-1 with Bcl-2) can also receive signals derived from different inputs including those not directly related with ER stress. Moreover, some of the regulatory proteins transmitting these signals such as Akt, AMPK, DAPK, or JNK play also a major role in the modulation of cell survival independently of autophagy. It is therefore essential to consider the cellular context in order to understand how the different ER stress signals are integrated to yield a protective or cytotoxic autophagic response.

4. ER Stress and Autophagy in Anticancer Therapy: A Double-Edged Sword

From the above discussion, it is clear that ER stress and autophagy can activate both prosurvival mechanisms as well as lethal programs, especially under conditions of

enduring ER stress and organellar damage. Thus activation of the UPR and autophagy may either impede or facilitate drug-mediated cell killing, and it is plausible that this will depend on the type of cancer and cytotoxic agents used. While a growing number of reports have started to identify molecular elements of the cross-talk between ER stress and autophagy (see Section 3.2), thus unraveling potential druggable targets, knowledge of the functional outcome of the activation of these pathways in cancer cells responding to chemotherapeutics is still very limited. In terms of therapeutic outcome, drugs (or a combination thereof) capable of activating the proapoptotic branch of the UPR while simultaneously inhibiting its prosurvival function should provide the highest therapeutic benefit. Moreover, if autophagy activated following ER stress is a survival response restoring ER homeostasis (e.g., by the removal of protein aggregates), its pharmacological blockage could protract UPR activation until a critical threshold is reached, which may precipitate its proapoptotic function. On the other hand, autophagy may endorse the proapoptotic functions of certain ER stress pathways (see also Section 4.1) or become a lethal backup pathway in cancer cells with defect on apoptotic signaling [133, 148]. (Figure 3).

A wide array of conventional and experimental chemotherapeutic agents has been shown to stimulate ER stress and activation of UPR along with autophagy in cancer cells. For example, tunicamycin, thapsigargin, and brefeldin A activate autophagy in colon and prostate cancer cells thus mitigating ER stress and protecting against cell death. However, autophagy induced by the same chemicals does not confer protection in a normal human colon cell line and in the nontransformed murine embryonic fibroblasts but rather contributes to cell death [115]. The combined administration of Vorinostat (a histone deacetylase inhibitor) and Sorafenib (a tyrosine kinase inhibitor) to carcinoma cells promotes cell death although activates at the same time a protective ER stress-driven autophagic response [149]. Similarly, the resistance to Imatinib mesylate (a BCR/ABL tyrosine kinase inhibitor used for the treatment of chronic myeloid leukaemia) might also rely—at least in part—on the secondary activation of ER stress-induced autophagy [150]. By contrast, cannabinoid treatment activates ER stress and autophagy leading to apoptotic cell death of glioma and pancreatic cancer cells but not of nontransformed embryonic fribroblasts or primary astrocytes (in which neither ER stress nor autophagy is activated in response to the treatment with these compounds) [75]. Likewise, other agents such as Nelfinavir (an HIV protease inhibitor with anticancer activity) [151, 152] or Melanoma differentiation associated gene-7/interleukin 24 (mda-7/IL-24) [145, 153] activate an ER stress response that promotes autophagy and apoptosis of cancer cells. Increased expression of Tetraspanins (a family of proteins that facilitate the spatial organisation and localisation of multiprotein complexes in distinct membranal microdomains) has also been shown to activate ER stress and autophagic cell death [154].

Understanding the precise molecular mechanisms that regulate the extent of autophagy activation in response to different triggering signals as well as the ones that control the interplay of this cellular process with apoptosis is therefore crucial to design new antitumoral therapies based on the modulation of the ER stress-autophagy response. Here we discuss further a selected group of clinically used or promising cytotoxic drugs with a demonstrated ability of inducing both UPR and autophagy, as paradigms to discuss the potential of targeting these pathways in cancer therapy.

4.1. Cannabinoids. Cannabinoids, the active components of marijuana, of which THC is the most important owing to its high abundance and potency [155], exert a wide variety of biological effects by mimicking endogenous substances, the endocannabinoids, that bind to and activate specific cannabinoid receptors [156]. Cannabinoids are currently being investigated as potential antitumoral agents. Thus, treatment with these agents has been shown to curb tumor growth in various animal models of cancer [157–159]. The antitumoral action of cannabinoids is based on the ability of these agents to inhibit tumor angiogenesis and activate apoptosis of cancer cells [157, 158].

Our recent findings have unravelled that cannabinoids induce autophagy in different types of tumor cells, including glioma/astrocytoma and pancreatic cancer cells, whereas they do not activate this cellular process in nontransformed cells (which are resistant to the cell death-promoting activity of cannabinoids) [75]. Of interest, pharmacological or genetic inhibition of autophagy prevented cannabinoid-induced cell death as well as apoptosis, whereas abrogation of apoptosis prevented cell death but not autophagy as induced by these agents. These observations led us to conclude that induction of autophagy is part of the mechanism by which cannabinoids promote the apoptotic death of cancer cells. The in vivo relevance of these findings was demonstrated by the observation that cannabinoid treatment reduced tumor growth and activated autophagy and apoptosis in subcutaneous tumor xenografts derived from human U87MG astrocytoma cells and transformed mouse embryonic fibroblasts (MEFs). Likewise, similar results have been obtained in an orthotopic model of pancreatic cancer, in which we had previously shown a proapoptotic and an antitumoral action of cannabinoids ([122, 123], Salazar, M. and Velasco G., unpublished observations). Furthermore, autophagy-deficient tumors (generated by subcutaneous injection of transformed $Atg5^{-/-}$ MEFs) were resistant to THC antitumoral action, strongly supporting that autophagy is essential for the antineoplastic activity of cannabinoids. In addition, analysis of samples obtained from two glioblastoma multiforme patients indicated that THC administration might also trigger autophagy-mediated cell death in human tumors [75].

As discussed in the previous section, the mechanism responsible for the activation of autophagy upon THC administration relies on a cannabinoid receptors-induced early accumulation of de novo-synthesized ceramide [an event that takes place in the ER [160]], which leads in turn to ER dilation and increased eIF2α phosphorylation [75]. Activation of this ER stress response induces the up-regulation of several genes, including the stress-regulated protein p8 and its downstream targets ATF-4 and CHOP

and the pseudokinase TRB3, which are required for the stimulation of autophagy in response to cannabinoid action. TRB3 plays a crucial role in the induction of autophagy upon THC administration through its inhibitory interaction with Akt, which leads in turn to mTORC1 inhibition. In agreement with these observations, treatment of mice with THC decreased mTORC1 activity, stimulated autophagy and apoptosis, and reduced tumor growth in xenografts generated with $p8^{+/+}$ cells but not in those generated with $p8^{-/-}$ cells (in which TRB3 is not up-regulated in response to THC [122]), further confirming that the p8/TRB3 pathway plays an essential role in the activation of autophagy and cell death by cannabinoids also in vivo.

Cannabinoids activate therefore a cell death-promoting signalling route that involves the stimulation of ER stress, autophagy, and apoptosis in cancer cells. Thus, cannabinoids constitute an interesting tool to investigate the differential molecular mechanisms that are responsible for the stimulation of autophagy-mediated cell death. On the other hand, the selectivity of cannabinoids (which only stimulate the above-described cell death promoting pathway in cancer cells) together with a low toxicity and good safety profile makes of these agents promising antineoplastic tools.

4.2. Photodynamic Therapy. Photodynamic therapy (PDT) is an anticancer therapy involving the selective photosensitization of malignant cell types, usually involving porphyrins, porphyrin analogs or other agents with suitable photophysical properties. The initial step in the photodynamic process involves localization of the photosensitizing agent at subcellular loci, followed by irradiation with visible light of the appropriate wavelength [161, 162]. This results in formation of singlet oxygen and other ROS that can cause photodamage at sites where the photosensitizing agent has localized. Since singlet oxygen will not migrate more than a fraction of a micron from the site of formation, as a result, photodamage can be quite specific. Thus, a distinguished property of PDT is that ROS formation is mainly targeted to a particular subcellular site and affects a rather specific subset of molecular targets. PDT with various agents has been shown to induce apoptosis along with autophagy and, in most cases, autophagy is activated as a mean to protect cells from killing [8]. Agents found to be clinically useful were reported to show affinity for the ER, mitochondria, lysosomes, or combinations of these sites [163]. A well-studied paradigm of ER-localizing dye is hypericin, a naturally occurring phototoxin with promising applications in bladder cancer [164]. Consistent with its predominant reticular localization in cultured cells [82], light activation of hypericin is coupled with massive ER expansion, preceding ultrastructural features of apoptosis, both in vitro and in bladder cancer bearing rats (Verfaillie, T. and Agostinis, P. unpublished observations), and stimulation of UPR [165]. UPR activation is likely the result of immediate ROS-damage to the SERCA pump, depleting ER-Ca^{2+} store, which is followed by the concomitant activation of autophagy and mitochondrial apoptosis [82]. This ROS paradigm of ER stress is linked to a persistent activation of the PERK-eIF2α-CHOP axis, with proapoptotic function (Verfaillie, T. and Agostinis, P. unpublished observations). Induction of autophagy in ER stressed cells unable to mount an apoptotic response (because of *bax/bak* deficiency) results in increased photokilling, suggesting the activation of an "autophagic cell death" pathway [82]. Conversely, in apoptosis-competent cells, blocking autophagy stimulation following ER stress by siRNAs that target essential modulators of the autophagic machinery, sensitizes to cell death, thus revealing a cytoprotective role for this pathway (Dewaele, M. and Agostinis, P. unpublished results). Hence, it is tempting to speculate that autophagy inhibition may potentiate the proapoptotic PERK pathway resulting in a better therapeutic opportunity, only when the cancer cell's apoptotic machinery has not been fully disabled. Further studies are required to establish whether suppression of the autophagic pathway along with UPR stimulation may represent a valuable therapeutic strategy in hypericin-based PDT.

4.3. Proteasome Inhibitors. Targeting proteasomal degradation has proven to be a valuable approach in various cancer treatments, and proteasome inhibitors have emerged as a new class of ER stress agents. Moreover, recent evidence suggests that when used in combination with certain cytotoxic drugs, such as PDT, proteasomal inhibitors are capable of enhancing their anticancer efficacy, making these agents a very promising class of pharmacological agents in combinatorial therapy.

4.3.1. Bortezomib. Bortezomib (PS-341 or Velcade) distinguishes itself from other proteasome inhibitors as it specifically inhibits the 26S proteasome by selectively blocking its chymotryptic activity. Velcade has been clinically approved for treatment of multiple myeloma and mantle cell lymphoma [166, 167] and has been shown to successfully induce apoptosis in various human cancer cell lines including myeloma, prostate, and breast cancers as well as squamous cell carcinoma [168–170]. Moreover, preclinical studies indicate that bortezomib displays anticancer activity against pancreatic cancers [171], one of the most aggressive human diseases. One of the potential mechanisms underlying the apoptotic effects of bortezomib in cancer cells relies on its ability to inhibit the NF-κB pathway by blocking the degradation of its cytoplasmic inhibitor IκBα [172]. However, inhibition of NF-κB alone could not fully account for the antitumor effect by bortezomib, suggesting additional pathways being involved [173]. This additional pathway turned out to be dependent on ER stress. Since proteasomal degradation of misfolded proteins retrotranslocated from the ER to the cytosol represents the final step in ERAD, proteasomal inhibition causes an additional burden of unfolded proteins on the ER. This explains the high efficacy of bortezomib treatment against types of cancer cells in which the ER is already predisposed with a considerable load. For instance, hypoxic cancer cells that otherwise show increased resistance to genotoxic agents as well as myeloma cells producing high amounts of immunoglobulins are hypersensitive to treatment with proteasome inhibitors [174, 175]. Therefore, therapies that

target the ER response in combination with bortezomib ought to be more successful. Indeed, it was shown that bortezomib sensitized pancreatic cancer cells to ER stress mediated apoptosis [176]. Additionally, we recently found a significant retardation of tumor growth in vivo in two different murine tumor models when photofrin-based PDT, a PDT approach stimulating the UPR, was combined with bortezomib, or other clinically used proteasome inhibitors [177]. This suggests that blocking the proteasome might offer a new therapeutic avenue to potentiate the antitumor effect of PDT.

Interestingly, Schewe and Aguirre-Ghiso [178] found that myeloma cells surviving bortezomib treatment attenuated eIF2α phosphorylation and induction of CHOP. Combined treatment with the GADD34-PP1 complex inhibitor salubrinal restored eIF2α phosphorylation and CHOP induction, maximizing bortezomib induced apoptosis, thus suggesting that strategies capable of sustaining CHOP expression might be required to successfully eradicate tumors.

Aside from proteasomal degradation, autophagy represents another important mechanism for degrading intracellular material. Furthermore, these processes are functionally coupled and proteasomal inhibition has been shown to stimulate autophagy, likely as a compensatory mechanism [119]. Surprisingly, whether autophagy enhances apoptosis induced by proteasomal inhibitors or not seems to depend on whether the treated cells are transformed or not [179]. These findings suggest that a combined inhibition of both cellular degradation systems would enhance the antitumoral efficacy. Indeed, autophagy was shown to be activated in MCF-7 cells treated with bortezomib, by a mechanism which involved proteasomal stabilization of ATF4 and ATF4 dependent upregulation of LC3B. This mechanism was suggested to contribute to the resistance of breast cancer cells towards bortezomib [180]. However, a recent study wherein myeloma cells where treated with bortezomib in combination with the autophagy inhibitor 3-methyl adenine (3-MA) resulted in an antagonistic response instead of the expected synergizing effect [181].

5. Conclusions

Research during the last decade has contributed to highlight the important role of ER stress and autophagy in the maintenance of the cellular homeostasis. The last few years have also evidenced that both processes are closely related as some of the signalling routes activated during the ER stress response are involved in stimulating autophagy. Intriguingly, activation of autophagy after ER stress can be either protective of cytotoxic. For example, accumulation of unfolded proteins in neurodegenerative diseases may activate a protective autophagy response. By contrast induction of ER stress in cancer cells may promote the stimulation of autophagy-mediated cell death or the activation of a protective autophagy that may contribute to the resistance to certain antitumoral therapies. Thus, different factors such as the intensity of the ER stress signal, the simultaneous activation of additional pathways, the cell type, and so forth, must be integrated to yield a specific autophagic response. Considering that escape from drug-mediated cell killing is one of the major obstacles of current cancer therapy, a better understanding of the role played by these processes in cancer cells in response to chemotherapy would help us to devise new and more efficient therapeutic opportunities utilizing inhibitors or activators of these ER stress pathways.

Abbreviations

ARE:	Antioxidant Response Element
ASK-1:	Apoptosis Signal-regulating Kinase 1
ATF4:	Activating Transription Factor 4
ATF6:	Activating Transription Factor 6
Atg:	Autophagy Gene
Bad:	Bcl-2 Antagonist of Cell Death
Bak:	Bcl-2 antagonist/killer
Bax:	Bcl2-Associated X Protein
Bcl-2:	B-cell lymphoma 2
BH3:	Bcl-2 Homology 3
BI-1:	Bax Inhibitor 1
Bim:	Bcl2-interacting mediator of cell death
BiP:	Immunoglobulin heavy chain-binding protein
CHOP:	CAAT/enhancer binding protein (C/EBP) homologous protein
DAPK:	Death Associated Protein Kinase
EDEM-1:	ER Degradation-Enhancing α-Mannosidase-Like Protein
eIF2α:	eukaryotic Initiation Factor-2α
ER:	Endoplasmic Reticulum
ERO1:	ER Oxidase1
ERSE:	ER Stress Response Element
GADD34:	Growth Arrest and DNA Damage-Inducible Gene 34
HO-1:	Heme Oxygenase 1
Hsp:	Heat shock protein
IP3R:	Inositol 1,4,5- Trisphosphate Receptor
IRE1:	Iiositol Requiring Enzyme 1
JNK:	c-Jun N-terminal Kinase
LC3:	Microtubule-associated protein light chain 3
MAPK:	Mitogen Activated Protein Kinase
NF-κB:	Nuclear Factor κB
PERK:	RNA dependent protein kinase (PKR)-like ER kinase
PP1:	Protein Phosphatase 1
PP2A:	protein phosphatase 2A
Puma:	P53 upregulated mediator of apoptosis
ROS:	Reactive Oxygen Species
SERCA:	Sarco/Endoplasmic Reticulum Ca^{2+} ATPase
TRAF2:	TNF (Tumor Necrosis Factor) receptor associated factor 2
TRB3:	Tribbles homologue 3
UPR:	Unfolded Protein Response
UPRE:	UPR Response Element
XBP1(u/s):	X-box Binding protein 1(unspliced/spliced).

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Research Article

Mechanisms of ER Stress-Mediated Mitochondrial Membrane Permeabilization

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During apoptosis, the process of mitochondrial outer membrane permeabilization (MOMP) represents a point-of-no-return as it commits the cell to death. Here we have assessed the role of caspases, Bcl-2 family members and the mitochondrial permeability transition pore on ER stress-induced MOMP and subsequent cell death. Induction of ER stress leads to upregulation of several genes such as Grp78, Edem1, Erp72, Atf4, Wars, Herp, p58ipk, and ERdj4 and leads to caspase activation, release of mitochondrial intermembrane proteins and dissipation of mitochondrial transmembrane potential ($\Delta\Psi$m). Mouse embryonic fibroblasts (MEFs) from caspase-9, -2 and, -3 knock-out mice were resistant to ER stress-induced apoptosis which correlated with decreased processing of pro-caspase-3 and -9. Furthermore, pretreatment of cells with caspase inhibitors (Boc-D.fmk and DEVD.fmk) attenuated ER stress-induced loss of $\Delta\Psi$m. However, only deficiency of caspase-9 and -2 could prevent ER stress-mediated loss of $\Delta\Psi$m. Bcl-2 overexpression or pretreatment of cells with the cell permeable BH4 domain (BH4-Tat) or the mitochondrial permeability transition pore inhibitors, bongkrekic acid or cyclosporine A, attenuated the ER stress-induced loss of $\Delta\Psi$m. These data suggest a role for caspase-9 and -2, Bcl-2 family members and the mitochondrial permeability transition pore in loss of mitochondrial membrane potential during ER stress-induced apoptosis.

1. Introduction

The endoplasmic reticulum (ER) is a cytosolic membrane bound network connected to the nucleus, mitochondria, and the plasma membrane. Membrane and secreted proteins are targeted to the ER for folding and posttranslational modification [1, 2]. In addition, the ER is the primary storage organelle for intracellular Ca^{2+}, thereby the main regulator of cellular Ca^{2+} homeostasis. Given its central role in protein folding and its influence on Ca^{2+}-mediated signaling pathways, disruption of the ER homeostasis, also called ER stress, has severe consequences for the cell [1, 2]. A number of pathophysiological conditions are associated with ER stress, including stroke, ischemia, hyperhomocystinemia, diabetes, viral infections, and mutations that impair protein folding [3, 4]. To combat the deleterious effects of ER stress, the cell has evolved a variety of protective strategies collectively known as the Unfolded Protein Response (UPR). This concerted and complex cellular response is initiated by three molecules, PERK (PKR-like ER kinase), ATF6 (activated transcription factor-6), and IRE1 (Inositol requiring enzyme 1) [1].

The UPR attempts to reduce the protein load on the ER and increase the folding capacity of the ER [5]. However, unresolved ER stress results in the activation of apoptosis. The exact mechanism involved in transition of the UPR from a protective to an apoptotic response is not clearly understood, but it does appear to be dependent on cysteinyl aspartate proteases of the caspase family and the proteins of Bcl-2 family [6, 7]. Several studies have reported the involvement of initiator caspase-2, -8, and -9 [8, 9] and effector caspase-3 and -7 in ER stress-induced apoptosis [10]. It has also been suggested that caspase-12 acts as an initiator caspase during ER stress-induced apoptosis [11, 12]. However, a significant role for caspase-12 in ER stress-induced apoptosis has not been supported by the majority of the literature (reviewed in [13]). For example, caspase-12-deficient murine P19 embryonic carcinoma cells

do not exhibit altered levels of tunicamycin-induced DNA fragmentation [8]. Also ER stress-induced cell death is unaffected by an absence of caspase-12 in B16/B16 melanoma cells [14] or in MEFs isolated from caspase-12 deficient mice [15]. Furthermore, in humans, a single nucleotide polymorphism in caspase-12 results in the synthesis of a truncated protein, lacking enzymatic activity [16]. Recent reports implicate the involvement of mitochondria in ER stress-induced apoptosis [10]. Release of cytochrome *c* from mitochondria during ER stress-induced apoptosis has been suggested to be mediated by mitochondrial permeability transition (MPT) [17, 18]. The molecular mechanism of the mitochondrial membrane depolarisation and the release of cytochrome *c* are well studied in various types of cellular stresses, and two mechanistically different models have been proposed [19]. The first one is controlled by proteins of the Bcl-2 family, while the second one involves a high conductance ion channel, the permeability transition pore (PTP) [20]. The role of the Bcl-2 family in ER stress-induced apoptosis is emphasized by concurrent repression of Bcl-2 and upregulation of Bim by the transcription factor, CHOP, a key determinant of ER stress-induced apoptosis [21, 22]. Furthermore, expression of the BH3 only proteins, Noxa and Puma, has been reported to be upregulated in MEFs undergoing ER stress-induced apoptosis [23]. Bcl-2 family members are known to localize both to the ER and the mitochondria, where they may act to regulate the signaling pathways that promote the opening of the PTP [19, 24]. For example, Bax and Bak can directly bind to the PTP and may act to induce MPT. They may also cause MPT by enhancing Ca^{2+} release from the ER [25]. On the other hand, when the death antagonists, such as Bcl-2 and Bcl-x_L bind to the PTP, they prevent the opening of the channel in response to many apoptotic signals [19]. The two models of cytochrome *c* release are not independent. The cytochrome *c*-dependent apoptotic pathway activated by ER-mitochondria crosstalk seems to play an essential role in the ER stress-mediated cell death [26].

The ER and mitochondria are in close contact which supports communication between these two organelles, including synthesis and transfer of lipids, and the exchange of Ca^{2+}, that regulates ER chaperones, mitochondrial ATP production and apoptosis [27]. Here we have determined the role of caspases, Bcl-2 family members, and PTP on the mitochondrial changes associated with ER-induced apoptosis. Our results show that ER stress-induced apoptosis involves loss of $\Delta\Psi m$ that is dependent on caspases and regulated by Bcl-2 family members and the mitochondrial PTP.

2. Materials and Methods

2.1. Cell Culture and Treatments. The rat neonatal cardiomyocyte-derived cell line H9c2 (ATCC) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 5 mg/ml streptomycin. To induce ER stress, cells were treated with 2 μM thapsigargin (Tg), 2 μg/ml Tm, or 2 μg/ml BFA for the indicated time periods. The broad range caspase inhibitors Boc-D.fmk and DEVD.fmk (Enzyme Systems Products) were used at a concentration of 20 μM, while BH4-Tat peptide (Calbiochem) was used at 200 nM. To study MPT, bongkrekic acid (BA, Calbiochem) was dissolved in 2N NH_4OH and used at 10 μM final concentration, cyclosporine A (CsA) at 2 μM, aristolochic acid (ArA, Calbiochem) at 25 μM, dissolved in DMSO. All reagents were from Sigma-Aldrich unless otherwise stated.

2.2. RNA Extraction and Real Time RT-PCR. Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. Reverse transcription (RT) was carried out with 2 μg RNA and Oligo dT (Invitrogen) using 20 U Superscript II Reverse Transcriptase (Invitrogen). For real-time PCR experiments, cDNA products were mixed with 2 ×TaqMan master mix and 20 ×TaqMan Gene Expression Assays (Applied Biosystems) and subjected to 40 cycles of PCR in StepOnePlus instrument (Applied Biosystems). Relative expression was evaluated with $\Delta\Delta C_T$ method.

2.3. Plasmid Transfections and Generation of Stable Clones. H9c2 cells grown to 85% confluency in 6 well plates were cotransfected with 1.5 μg of Bcl-2 (a kind gift from Prof., Stanley Korsmeyer, Howard Hughes Medical Institute, Boston, Massachusetts, USA) and 0.15 μg of pPUR puromycin resistance vector (Clontech) using Effectene transfection reagent (Qiagen) as per the manufacturer's instructions. Puromycin (5 μg/ml) was added 48 hours posttransfection to select stably transfected cells. Cells were cultured for three weeks to generate pooled transfectants.

2.4. Cell Viability Assay. Viability of cells after treatment was analysed by MTT assay. After treatment of cells with appropriate drugs, 1 mg/ml concentration of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) was added to the wells and incubated at 37°C for 3 hours. The reaction was stopped with a stop mix containing 20% SDS in 40% dimethylformamide. The color intensity is measured at 550 nm, and percentage cell viability is calculated using the untreated samples as 100%.

2.5. Annexin V Staining. Externalization of phosphatidylserine (PS) to the outer leaflet of the plasma membrane of apoptotic cells was assessed with annexin V-FITC. Briefly, cells were collected by centrifugation at 350 g, washed once in ice-cold calcium buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), and incubated with annexin V-FITC or with annexin V-PE for 15 minutes on ice. A wash step in calcium buffer was carried out prior to acquisition on a FACSCalibur flow cytometer (Becton Dickinson).

2.6. Western Blot Analysis. Protein samples (15–20 μg protein per lane) were resolved on 10% SDS-PAGE gels and electrophoretically transferred to nitrocellulose membranes. After blocking in 5% nonfat milk and 0.05% Tween 20 in PBS, blots were incubated with antibodies to KDEL (1:1,000, StressGen), caspase 3 (1:1,000, Cell Signaling Technology),

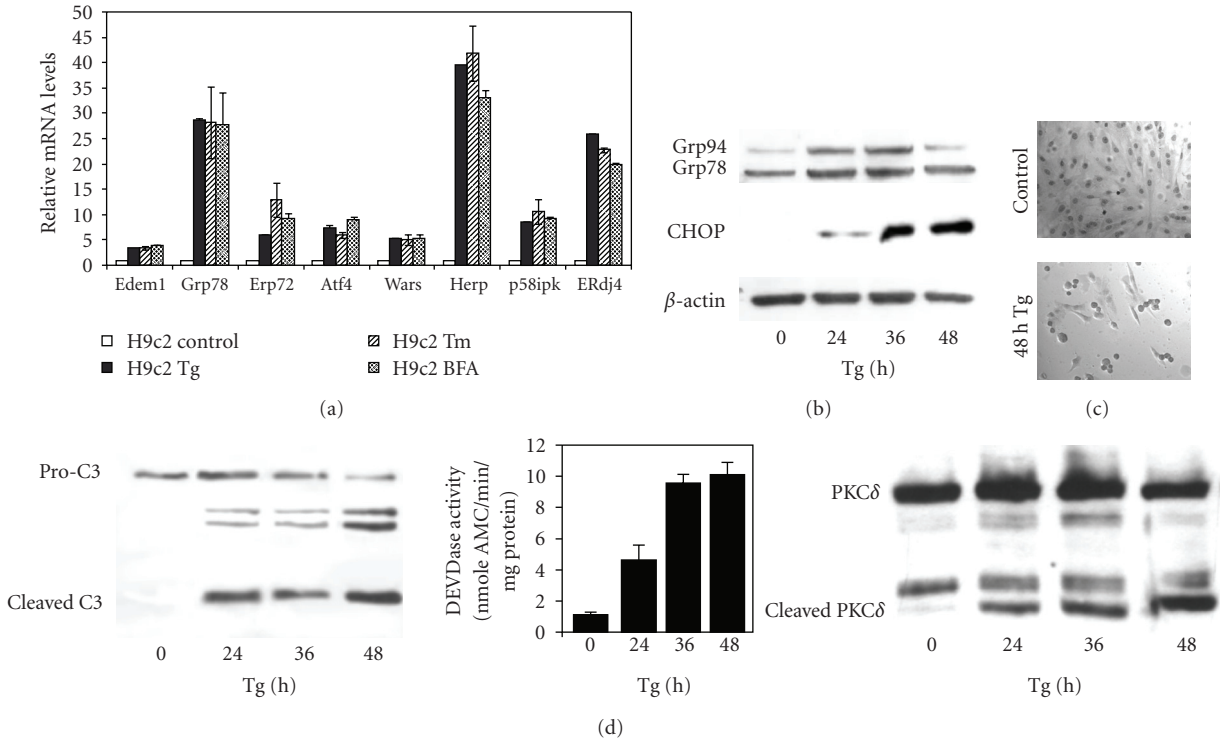

Figure 1: *ER stress-induced apoptosis in H9c2 cells.* (a) H9c2 cells were left untreated or treated with (2 μM) Tg, (2 μg/ml) Tm or (2 μg/ml) BFA for 24 hours. The change in expression levels of ER stress markers was measured by real-time RT-PCR normalizing against *GAPDH* expression and plotting expression levels relative to the control. Error bars represent mean ± SD from an experiment performed in duplicate and reproduced twice. (b) H9c2 cells were left untreated or treated with (2 μM) Tg for the indicated times. The induction of ER stress markers, Grp78, Grp94 and CHOP was determined by Western blot analysis. β-actin was used to determine equal loading of samples. (c) H9c2 cells were left untreated or treated with (2 μM) Tg for the indicated times. After 48 hours of Tg-treatment, cells were stained with haematoxylin-eosin-stain and photographed at 200 × magnification. (d) H9c2 cells were left untreated or treated with (2 μM) Tg for the indicated times. The processing of procaspase-3 and cleavage of PKCδ were determined by Western blot analysis. The caspase activity was determined using DEVD-AMC. The figure is a representative of three independent experiments.

CHOP (Santa Cruz, 1:2,000), PKCδ (Santa Cruz, 1:1,000), caspase 7, and 9 (1:1,000; Cell Signaling Technologies) and caspase 12 (1:1,500; Cell Signaling Technologies). The appropriate HRP-conjugated secondary antibodies (Pierce) were used at a 1:2,000 for antibodies from Cell Signaling technologies and at a 1:10,000 dilution for all other antibodies. Protein bands were detected with Super Signal Ultra Chemilumiescent Substrate (Pierce) on X-ray film (Agfa).

2.7. Cell Morphology . Cells were seeded onto 18 mm coverslips at a density of 4×10^4 cells/ml. After treatment with thapsigargin, cells were fixed in methanol for 5 minutes at room temperature and stained with Harris hematoxylin and Eosin Y as previously described [28].

2.8. Rapid Preparation of Cytosolic Fraction. Cell fractions were prepared as described previously [29]. Briefly, cells were washed in ice-cold PBS and pelleted by centrifugation at 400 × g for 5 minutes. The pellet was resuspended in 100 μl of lysis buffer (250 mM sucrose, 70 mM KCl, 0.5 mM DTT, 100 μM PMSF, 2 μg/ml pepstatin, 25 μM ALLN, 2.5 μg/ml aprotinin, 10 μM leupeptin, and 2 mg/ml digitonin). After 5 minutes incubation on ice, the samples were centrifuged for 5 minutes at 20,000 × g. The supernatant (cytosolic fraction) was carefully removed, and the pellet (mitochondrial fraction) was resuspended in lysis buffer.

2.9. Measurement of Mitochondrial Transmembrane Potential ($\triangle \Psi m$). Changes in $\triangle \Psi$m were detected using tetramthylrhoda mine ethyl ester perchlorate (TMRE) (Molecular Probes). Trypsinized cells were combined with supernatant medium and incubated with TMRE (100 nM) for 30 minutes at room temperature in the dark. TMRE fluorescence was measured using the FL2 channel (582 nm) of FacsCalibur flow cytometer (Becton Dickinson). A 45-minute CCCP (10 μM) treatment was used to uncouple mitochondria, as a positive control.

3. Results and Discussion

3.1. Prolonged ER Stress Induces Apoptosis and Mitochondrial Membrane Depolarization. To induce ER stress, H9c2 cells, a neonatal rat cardiomyocyte-derived cell line, were treated

with three different ER stress-inducing agents: thapsigargin (Tg) an inhibitor of the Sacroplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump, tunicamycin (Tm) an inhibitor of N-linked glycosylation, and brefeldin A (BFA) an inhibitor of protein transfer from the ER to the Golgi. Treatment of H9c2 cells with any of these ER stress inducing agents caused an increase in the mRNA levels of many genes associated with the ER stress response (Figure 1(a)). We also examined protein levels by Western blot analysis for a subset of these genes and found them to reflect the changes observed in mRNA expression with Grp78, Grp90, and the proapoptotic transcription factor CHOP/GADD153 being significantly upregulated after treating cells with Tg (Figure 1(b)). Conditions of prolonged (48 hours) ER stress induced morphological changes associated with apoptosis, including cellular shrinkage, nuclear condensation, and membrane blebbing (Figure 1(c)). ER stress-induced apoptosis was associated with activation of caspases as detected by the processing of caspase-3, an increase in DEVDase activity, and the cleavage of protein kinase C delta (PKCδ), a cellular substrate of caspases, which was detectable as early as 24 hours post Tg treatment (Figure 1(d)). Collectively, these data demonstrate that ER stress induces apoptotic cell death in H9c2 cardiomyocytes.

Several reports have indicated a possible role for mitochondria in ER stress-induced apoptosis [10, 27]. To determine the exact contribution of the mitochondria to ER stress-induced apoptosis, alterations of $\Delta\Psi$m and release of mitochondrial intermembrane space proteins into the cytosol were analyzed. Alterations in $\Delta\Psi$m were studied using tetramethyl rhodamine ethyl ester (TMRE). The loss of $\Delta\Psi$m was detectable by flow cytometry starting after 24 hours of induction of ER stress and increasing over time (Figure 2(a)). Consistent with a drop in $\Delta\Psi$m, Western blot analysis demonstrated increased cytosolic levels of cytochrome *c* and Smac, when compared to untreated controls, at 36 and 48 hours post Tg treatment (Figure 2(b)). The slight differences observed in the kinetics of release from mitochondria between cytochrome *c* and SMAC may be due the differences in the affinity of cytochrome *c* and SMAC antibodies used for western blotting. These results show that ER stress induces MOMP and the release of proapoptotic proteins from the intermembrane space into the cytosol. These observations suggest that the loss of $\Delta\Psi$m and release of mitochondrial intermembrane space proteins into the cytosol are coupled and a component of ER stress-induced apoptosis.

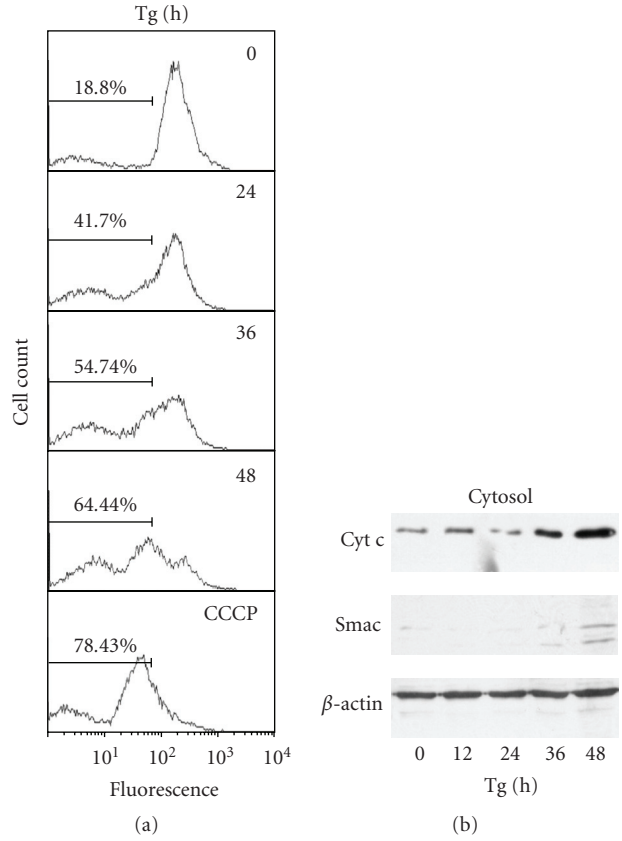

Figure 2: *Loss of mitochondrial membrane potential and release of cytochrome c during ER stress-induced apoptosis.* (a) H9c2 cells were treated with (2 μM) Tg for the indicated times. Following treatment, cells were incubated with (100 nM) TMRE. Mitochondrial membrane potential was monitored by measuring the fluorescence intensity at 582 nm (FL2). As a positive control for depletion of membrane potential, cells were treated with (10 μM) CCCP for 45 minutes. The data is a representative of at least three independent experiments. (b) Western blot analysis of cytochrome *c* and Smac in cytosolic fractions. The data is a representative of at least three independent experiments.

3.2. Effect of Caspases on ER Stress-Induced Drop in $\Delta\Psi$m. In Tg-treated H9c2 cells, we detected caspase-3 activation starting as early as 12–18 hours, which preceded detectable changes in the mitochondria. This suggested a possible involvement of caspases in inducing $\Delta\Psi$m depletion. Caspase-3 and -9 are important in both the intrinsic and extrinsic pathways of apoptosis. To determine the role of these caspases in ER stress-induced cell death, we have used mouse embryonic fibroblasts (MEFs) deficient in caspase-3, -2, and -9. Fibroblasts from wild-type and homozygous knock-out embryos were treated with 2 μM Tg for 24 hours, and MEFs were assayed for viability using annexin V staining. As shown in Figures 3(a)–3(c), caspase-9, -2, and -3 knock-out MEFs were protected against apoptosis induced by Tg. Next we tested the effect of broad range caspase inhibitors on the ER stress-mediated drop in $\triangle$ Ψm. H9c2 cells were treated with Tg in the presence or absence of the caspase inhibitors Boc-D.fmk and DEVD.fmk (Figure 3(d)). Inhibition of caspases reduced Tg-induced loss of $\Delta\Psi$m up to 36 hours, but loses effectiveness at 48 hours. To further confirm the role of caspases in ER stress-mediated $\Delta\Psi$m loss, we determined mitochondrial membrane potential in caspase-9, -2, and -3 deficient and wild-type MEFs upon exposure to ER stress. We observed that caspase-9 and caspase-2 deficient MEFs showed resistance to ER stress-mediated loss of $\Delta\Psi$m as compared to wild-type MEFs (Figure 3(e)). In contrast, caspase-3 deficient MEFs showed loss in $\Delta\Psi$m comparable to wild-type MEFs (Figure 3(f)). These results

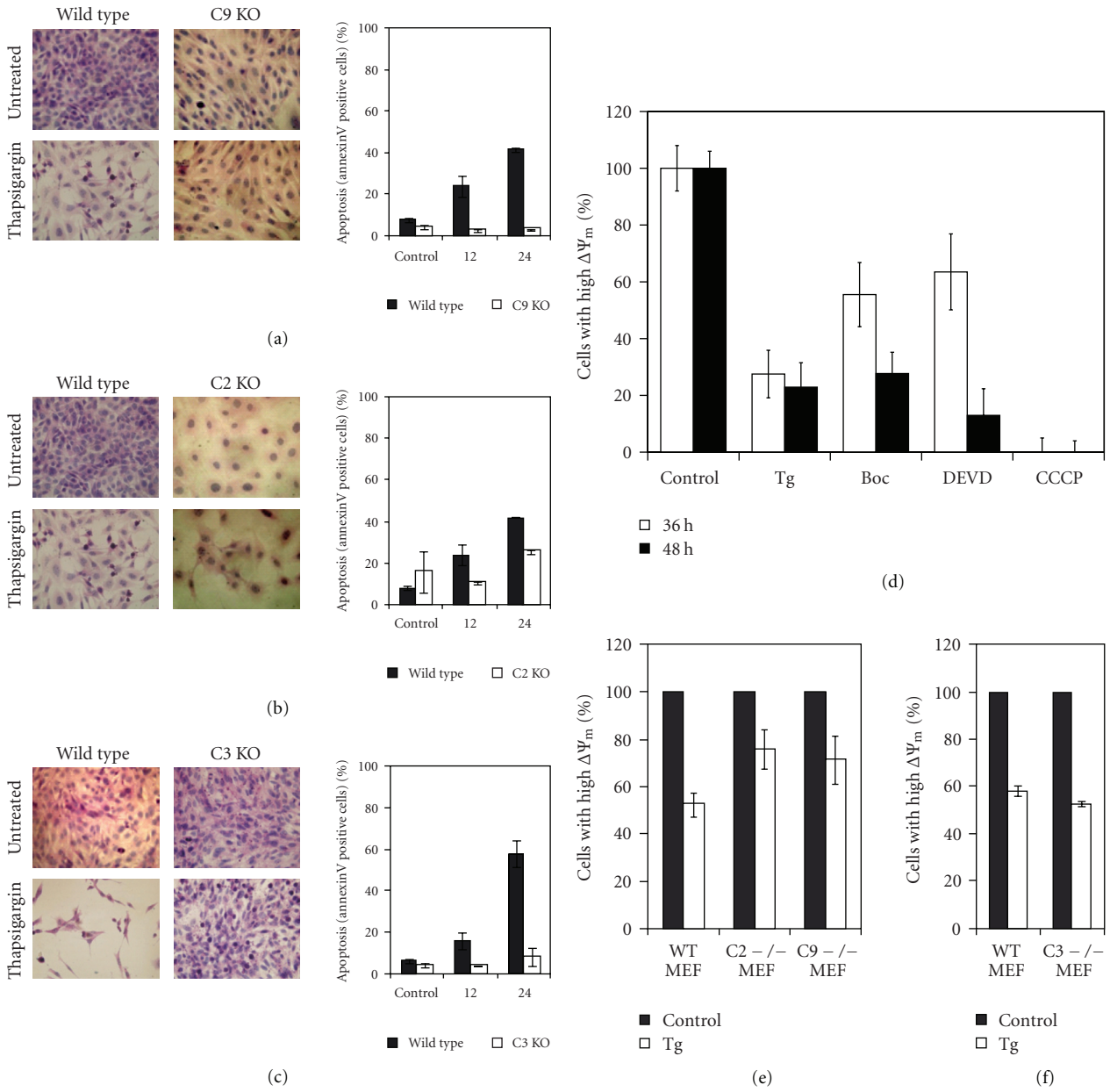

Figure 3: *Resistance to ER stress-induced death and loss of mitochondrial membrane potential in absence of caspases.* (a)–(c) *Left panel*, Indicated MEFs were treated with (2 μM) Tg for 24 hours. Cells were stained with haematoxylin-eosin-stain and visualised using an Olympus IX71 microscope at 40×. Images are representative of 2 independent experiments. *Right panel*, indicated MEFs were treated with (2 μM) Tg for the indicated times. Increase in cell death was measured by annexin V staining. The data is representative of at least 2 independent experiments. (d) H9c2 cells were treated with (2 μM) Tg alone or pretreated for 30 minutes with Boc-D.fmk (20 μM) and DEVD.fmk (20 μM) prior to treatment with Tg, for the indicated time periods. Following treatment cells were incubated with (100 nM) TMRE. Mitochondrial membrane potential was monitored by measuring the fluorescence intensity at 582 nm (FL2). As a positive control for depletion of membrane potential, cells were treated with (10 μM) CCCP for 45 minutes. The data is a representative of at least three independent experiments. (e)–(f) Indicated MEFs were treated with (2 μM) Tg for 24 hours. Following treatment cells were incubated with (100 nM) TMRE. Mitochondrial membrane potential was monitored by measuring the fluorescence intensity at 582 nm (FL2). The data is a representative of at least three independent experiments.

suggest that caspase-2 and -9 but not caspase-3 play a role in the $\Delta\Psi$m loss during ER stress-induced apoptosis. The apparent differences in ER stress-mediated loss of $\Delta\Psi$m upon DEVD.fmk pretreatment (Figure 3(d)) and in caspase-3 deficient MEFs (Figure 3(f)) may be due to inhibition of both caspase-3 and -7 by DEVD.fmk. This is in agreement with a previous study that showed that early apoptoptic events (e.g., Bax translocation and cytochrome *c* release) following mitochondria-mediated apoptosis triggered by UV irradiation were compromised by a double knock-out of caspase-3 and -7 in MEFs, but not in caspase-3 knock-out cells [30].

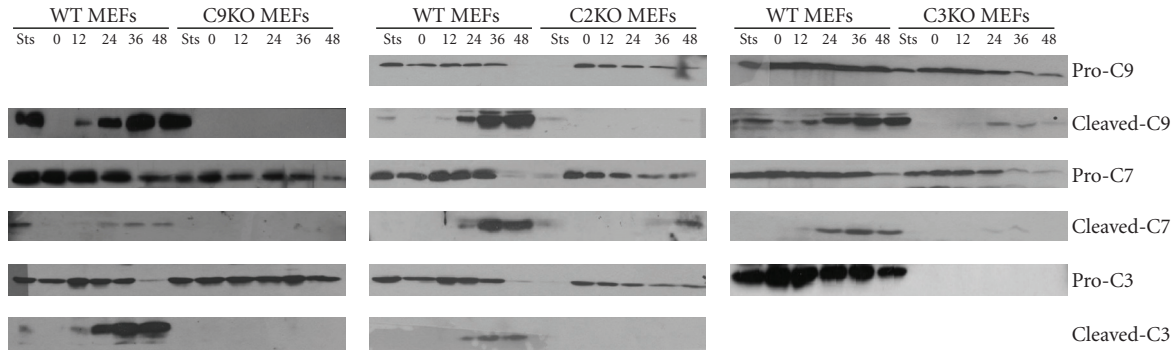

Figure 4: *Altered caspase processing in caspase-9, -2, or -3 deficient MEFs upon exposure to ER stress.* Indicated wild-type and caspase deficient MEFs were treated with thapsigargin (2 μM) for 12, 24, 36, and 48 hours. Protein extracts were isolated and subjected to western blot analysis using the indicated antibodies. Staurosporine (Sts) 100 nM for 12 hours was used as a positive control for caspase processing. Images are representative of a least 2 independent experiments.

To determine the functional consequences of loss of caspase-9, -2, and -3 in ER stress-induced apoptosis, we characterized the processing of pro-caspase-3, -7, and -9 in wild-type and corresponding knock-out MEFs by Western blotting of whole cell lysates after treatment with Tg (Figure 4). In wild-type cells, processing of pro-caspase-3, -7, and -9 was observed upon Tg treatment as compared with untreated controls (Figure 4). Processing of pro-caspase-3 and -7 was, however, completely inhibited in caspase-9 knock-out MEFs. This indicates that proteolytic activation of pro-caspase-3 and -7 during ER stress-induced apoptosis is dependent on caspase-9. Further, pro-caspase-9 processing was strongly reduced in the caspase-3 knock out MEFs (Figure 4). The activated effector caspase-3 acts on caspase-9 processing in a feedback amplification loop that results in complete activation of caspase-9, and consequently loss of caspase-3 may prevent complete activation of pro-caspase-9 [31]. Recently, it has been shown that caspase-2 can serve as a proximal caspase that functions upstream of mitochondria during ER stress-induced apoptosis, cleaving the BH3-only protein Bid which then functions as a critical apoptotic switch [9]. In this study the cleavage of caspase-3 and -9 was inhibited in caspase-2 knock-out MEFs, which corroborates the importance of this caspase in ER stress-induced apoptosis. However, we observed that there was some processing of caspase-7 in caspase-2 deficient MEFs. In agreement with these results, we observed that resistance to ER stress-induced cell death in caspase-2 deficient MEFs was not as pronounced as in caspase-9 deficient MEFs (Figures 3(a) and 3(b)). However, at present the mechanism for processing of caspase-7 in caspase-2 deficient MEFs is not clear. Taken together, the data from whole cell lysates indicates that caspase-2 and -9 play an important role in Tg-induced apoptosis.

3.3. The Role of Bcl-2 Family Proteins in ER Stress-Induced Drop in $\Delta\Psi m$. The mitochondrial apoptotic signalling pathway involves activation of the proapoptotic Bcl-2 family members Bax and Bak, that induce permeabilization of the mitochondrial outer membrane and release of cytochrome *c*

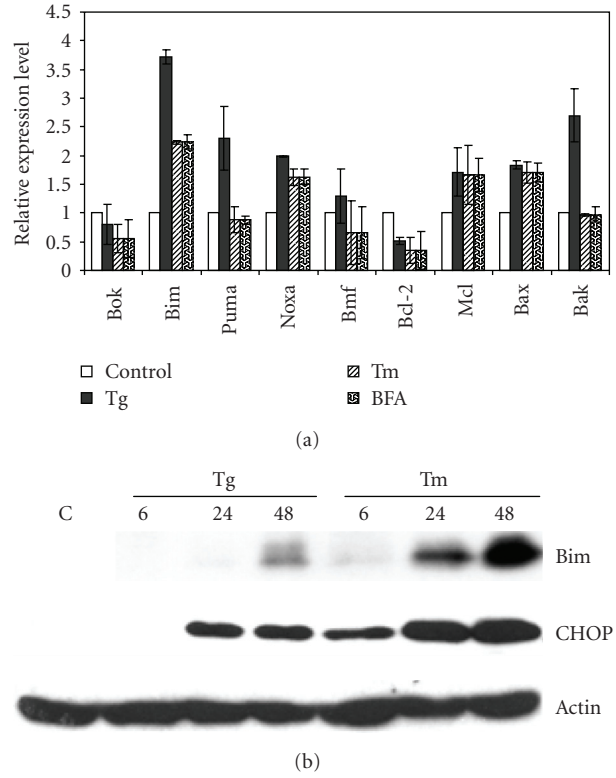

Figure 5: *ER stress-mediated induction of Bcl-2 family members in H9c2 cells.* (a) H9c2 cells were left untreated or treated with (2 μM) Tg, (2 μg/ml) Tm, or (2 μg/ml) BFA for 24 hours. The change in expression levels of ER stress markers was measured by real-time RT-PCR normalized against *GAPDH* expression and plotting expression levels relative to the control. Error bars represent mean $\pm$ SD from an experiment performed in duplicate and reproduced twice. (b) H9c2 cells were left untreated or treated with (2 μM) Tg, (2 μg/ml) Tm for indicated time points, and induction of Bim, and CHOP was determined by western blot analysis. β-actin was used to determine equal loading of samples.

[18]. To investigate the involvement of Bcl-2 family proteins in ER stress-induced cell death, we determined the effect of

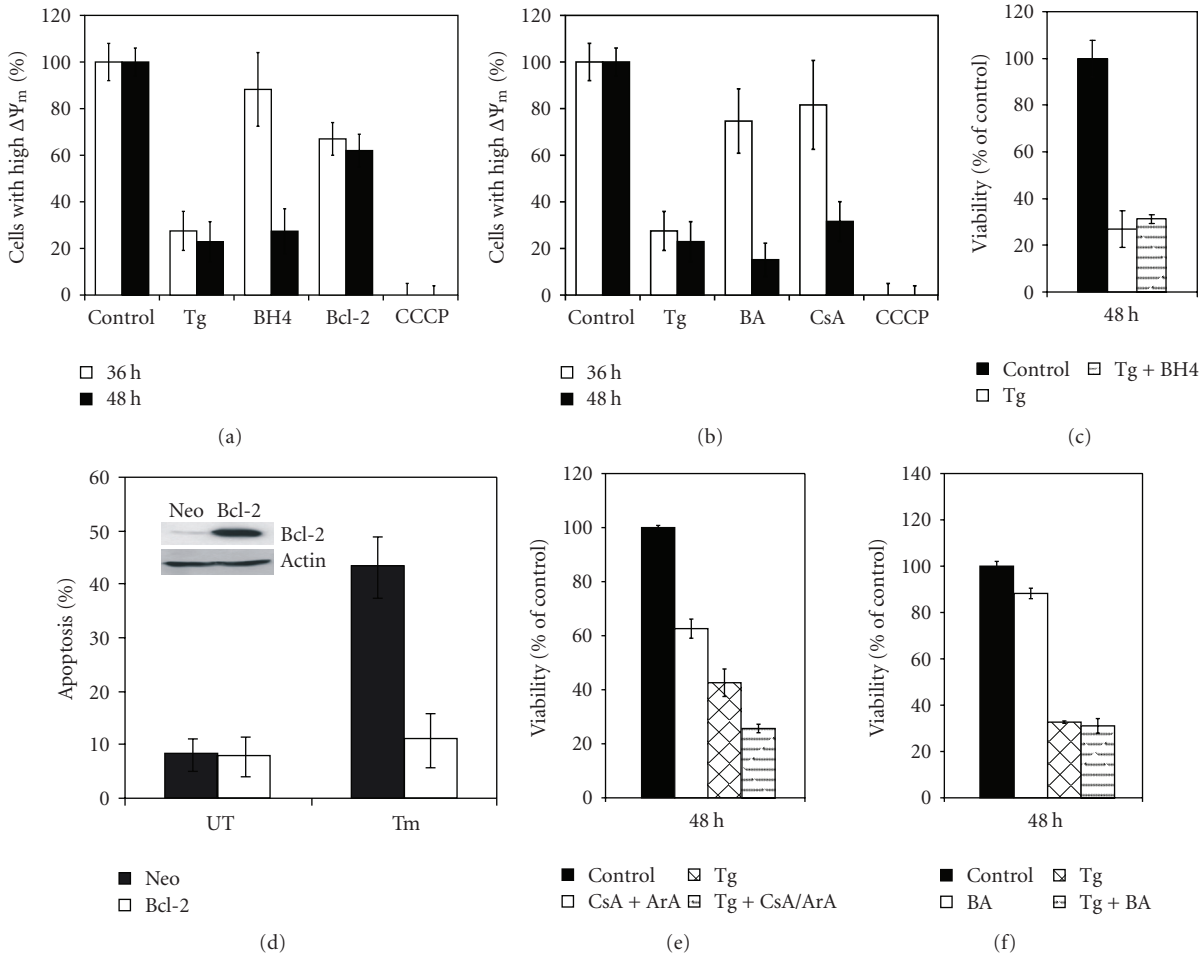

Figure 6: *Regulation of ER stress-induced mitochondrial changes by Bcl-2 family proteins.* (a) H9c2 cells were treated with (2 μM) Tg alone or pretreated for 30 minutes with BH4-Tat peptide (200 nM) prior to treatment with Tg, for the indicated time periods. Bcl-2 overexpressing H9c2 cells were treated with Tg (2 μM) for the indicated time periods. (b) H9c2 cells were treated with Tg (2 μM) alone or pretreated for 30 minutes with bongkrekic acid (BA) (10 μM); cyclosporine A (CsA) (2 μM) and aristolochic acid (ArA, Calbiochem) at 25 μM prior to treatment with Tg, for the indicated time periods. (a)-(b) Following treatment, cells were incubated with TMRE (100 nM). Mitochondrial membrane potential was monitored by measuring the fluorescence intensity at 582 nm (FL2). As a positive control for depletion of membrane potential, cells were treated with 10 μM CCCP for 45 minutes. The data is a representative of at least three independent experiments. (c) H9c2 cells were treated with (2 μM) Tg alone or pretreated for 30 minutes with BH4-Tat peptide (200 nM) prior to treatment with Tg, for 48 hours and reduction in cell viability was determined by MTT assay. Error bars represent mean ± SD from three independent experiments performed in triplicates. (d) Bcl-2 overexpressing H9c2 cells (Bcl-2) and control cells (neo) were treated with Tm (2 μg/ml) for 48 hours. Increase in cell death was measured by annexin V staining. The data is representative of at least 3 independent experiments. Protein extracts from Bcl-2 overexpressing H9c2 cells (Bcl-2) and control cells (neo) were subjected to western blot analysis using the indicated antibodies. (e)-(f) H9c2 cells were treated with Tg (2 μM) alone or pretreated for 30 minutes with (e) cyclosporine A (CsA) (2 μM) and aristolochic acid (ArA) at 25 μM; (f) bongkrekic acid (BA) (10 μM); prior to treatment with Tg, for 48 hours and reduction in cell viability was determined by MTT assay. Error bars represent mean±SD from three independent experiments performed in triplicates.

ER stress on the expression levels of Bcl-2 family members in H9c2 cells (Figure 5(a)). Our studies demonstrated that while Noxa, Mcl1, and Bax were upregulated by all three ER stress-inducing agents (Tg, Tm and BFA), Bim showed the greatest fold changes in response to any type of ER stress. The upregulation of Bim protein upon ER stress was confirmed by Western blotting (Figure 5(b)). We observed that Tg was most effective in inducing Bim mRNA levels; however, Tm was more potent in inducing Bim protein levels. This could be due to the posttranslational modifications regulating BIM protein stability upon exposure to ER stress [22]. In order to test the function of Bcl-2 on the mitochondria, we used the cell permeable BH4-Tat peptide. The BH4 domain of antiapoptotic Bcl-2 family members accumulates on the mitochondria and inhibits cell death [32]. Pretreatment of cells with BH4-Tat protected the mitochondria against the effect of Tg, delaying membrane depolarisation by at least 12 hours (Figure 6(a)). Next we generated a Bcl-2 overexpressing H9c2 clone and examined the protective potential of Bcl-2 in these cells. Bcl-2 overexpression was able to

prevent loss of ΔΨm upon Tg treatment, for at least 48 hours (Figure 6(a)). Furthermore, Bcl-2 overexpression efficiently protected cells against Tm induced cell death (Figure 6(d)), whereas pretreatment of cells with BH4-Tat was not able to inhibit ER stress-induced apoptosis (Figure 6(c)).

3.4. The Role for Mitochondrial Permeability Transition Pore in the ER Stress-Induced Drop in ΔΨm. Next we evaluated whether MPT was involved in ER stress-induced loss of mitochondrial membrane potential. For this purpose, we used two PTP inhibitors, bongkrekic acid and a combination of cyclosporine A and aristolochic acid. A 30-minute pretreatment with either the combination of 2 μM cyclosporine A (inhibitor of cyclophylin D) and aristolochic acid (25 μM) or 10 μM bongkrekic acid (an inhibitor of the adenine nucleotide transporter (ANT)), prior to Tg treatment prevented loss of ΔΨm at 36 hours (Figure 6(b)). This protective effect was lost by 48 hours, suggesting limited efficacy of the drugs or a contribution of PTP independent processes. In line with the transient effect on ΔΨm, pretreatment of the cells with either the combination of 2 μM cyclosporine A and aristolochic acid (25 μM) or 10 μM bongkrekic acid was not able to inhibit ER stress-induced apoptosis (Figures 6(e) and 6(f)).

In this study, we have investigated the factors regulating the loss of mitochondrial membrane potential during ER stress-induced apoptosis. Involvement of the mitochondria during ER stress-induced apoptosis seems to be a central amplification step and probably a point of no return [18]. The majority of cells are committed to die following MOMP because it leads not only to the activation of the well-established caspase-mediated apoptotic pathway, but, should there be a failure of its execution through insufficient caspase activation, a parallel, caspase-independent cell death pathway is set in motion that is controlled by HtrA2/Omi, AIF and Endo G [33, 34]. Although a number of mechanisms may be responsible for ER stress-induced mitochondrial changes, caspase activation upstream of the mitochondria has been linked to ΔΨm depolarization [7, 30]. We used MEFs deficient in caspase-3, -2, or -9 in order to determine the roles of these proteases in the ER stress apoptotic program, and subsequently established a role for these proteases in ER stress-induced apoptosis. Our results show that caspases are activated during ER stress, with caspase-2 and -9 acting upstream of caspase-3 and -7.

The Bcl-2 protein family governs mitochondrial homeostasis. Besides the mitochondria, Bcl-2 proteins are also localised at the ER [6]. However, the primary site of their action and the exact mechanism by which they control cell fate during ER stress was not fully understood. Here we show that overexpression of wild type Bcl-2 is able to protect mitochondria from the effect of Tg. A restricted, but similar protective effect was seen by transducing the BH4 domain of Bcl-2 into H9c2 cells. The BH4 peptide has been shown to localise at the mitochondria, suggesting that mitochondrial localised antiapoptotic Bcl-2 proteins are able to prevent ER stress-induced mitochondrial damage [32]. Besides their effect on the PTP, multidomain proapoptotic Bcl-2 proteins in the outer mitochondrial membrane can oligomerize to form nonspecific conducting channels through which cytochrome *c* can be released [6]. During conditions of ER stress, BH3-only proteins are activated either by transcriptional upregulation or through posttranslational modifications. Once activated, the BH3-only proteins converge on the activation of multidomain proapoptotic proteins Bax or Bak, which act as a gateway to the intrinsic apoptotic pathways operating at the mitochondria [18]. Recently, ER stress was shown to upregulate Bim through CHOP-C/EBPα-mediated direct transcriptional induction [22]. Therefore, upregulation of BH3-only proteins, such as Bim, at the transcriptional level may result in activation of Bax/Bak at the mitochondria, triggering cell death. The protective effect of Bcl-2 supports a role for proapoptotic Bcl-2 family members in targeting the mitochondria upon ER stress. In conclusion, our results show that multiple signals such as caspase activation and induction of BH3-only proteins converge on the mitochondria upon induction of ER stress and these signals trigger MOMP and loss of mitochondrial membrane potential.

Acknowledgments

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Review Article

The Dual Role of Calcium as Messenger and Stressor in Cell Damage, Death, and Survival

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Ca^{2+} is an important second messenger participating in many cellular activities; when physicochemical insults deregulate its delicate homeostasis, it acts as an intrinsic stressor, producing/increasing cell damage. Damage elicits both repair and death responses; intriguingly, in those responses Ca^{2+} also participates as second messenger. This delineates a dual role for Ca^{2+} in cell stress, making difficult to separate the different and multiple mechanisms required for Ca^{2+}-mediated control of cell survival and apoptosis. Here we attempt to disentangle the two scenarios, examining on the one side, the events implicated in deregulated Ca^{2+} toxicity and the mechanisms through which this elicits reparative or death pathways; on the other, reviewing the role of Ca^{2+} as a messenger in the transduction of these same signaling events.

1. Introduction: Ca^{2+} Signaling versus Deregulation in Life and Death

Ca^{2+} is an ion involved in living processes in an atypical way: if other cations participate to enzyme activity without performing essential regulatory functions due to their abundance in all cell compartments, Ca^{2+} has a peculiar distribution, being present at very low levels in the cytosol of eukaryotic cells; this enables it to act as a messenger regulating cytosolic Ca^{2+}-dependent enzymes and functions, when and where its local concentration raises above the steady-state level. For many decades the research on ion (and specifically Ca^{2+}) roles in cell physiopathology was hampered by intrinsic technical difficulties. A big impulse came with the development of Ca^{2+}-sensitive fluorescent probes [1, 2], that localize in specific cell compartments (cytosol, ER, and mitochondria) allowing separately and specifically evaluating and quantifying Ca^{2+} compartmentalization; and with the diffusion of instrumentations performing kinetic analyses, which allowed performing accurate and quantitative analysis of Ca^{2+} dynamics.

An efficient Ca^{2+} signaling implies maintenance of Ca^{2+} homeostasis, which requires mechanisms keeping cytosolic Ca^{2+} concentration ($[Ca^{2+}]c$) low and stable. These include active pumping against gradient by Ca^{2+} ATPases, enzymes present on the cytosolic side of plasma membrane and endoplasmic reticulum (ER) performing the high energy-expensive task of pumping Ca^{2+} out of the cytosol against gradient, or by ion exchangers (e.g., the Na^+/Ca^{2+}) [3]. Instead, signaling is exerted by discrete and highly controlled ER membrane channels, originating local Ca^{2+} rises possessing specific signaling roles, such as those regulated by the phospholipase C-inositol-3-phosphate pathway [3], or by cyclic (ADP-ribose) [4]. These local increases activate/deactivate Ca^{2+}-sensitive enzymes, eliciting signal transduction chains aimed at controlling many diverse cell functions such as mitosis, or activation, or motility or apoptosis. As a feedback mechanism, the channels rapidly close due to local high $[Ca^{2+}]$, which is rapidly extinguished by cytosolic buffering proteins, by the Ca^{2+}-ATPases and by mitochondria, thus being in fact Ca^{2+} transients. To restore ER homeostasis, the partially emptied ER is replenished by Ca^{2+} entry from the extracellular space through controlled opening of plasma membrane channels (capacitative Ca^{2+} entry, see below [5]), or by Ca^{2+} released from mitochondria present in the vicinity of inositol-3-phosphate

(IP_3) channels [6]. In the category of excitable cells, mainly neurons and myocytes, plasma membrane channels open following specific hormonal or physico-chemical stimulations, even in the absence of previous ER emptying, and possess signaling meaning of their own [7].

Ca^{2+} signaling requires the strict cooperation among the different cellular compartments and organelles, being in fact a highly sophisticated way of communication to maintain homeostasis and functionality of the whole cell. In particular, much attention is being given to the cooperation between ER and mitochondria, which interact through highly dynamic physical connections [8] containing abundant Ca^{2+}-mediating transport systems [9]. These mediate the controlled reciprocal exchange of Ca^{2+} [10] aimed at modulating and supporting each other functions in the guise of an interorganellar symbiotic relationship [11–13].

The important implication of the low cytosolic Ca^{2+} concentration ($[Ca^{2+}]c$) for cell homeostasis is that it must be maintained low against gradient (extracellular space and the internal Ca^{2+} stores such as ER, have 10,000 times as much Ca^{2+}), since excess or deregulated $[Ca^{2+}]c$ pose cells in such a dramatic asset to be in fact cell-toxic. To this purpose, a wide range of mechanisms are displayed, including high capacity binding proteins, pumps and exchangers, and mitochondria, which possess a low affinity Ca^{2+} uniporter that sequesters cytosolic Ca^{2+} when it reaches the dangerous threshold of 500 nM, thus being a major detoxifying mechanism against Ca^{2+} overload [14].

Failure of the mechanisms devoted to maintain Ca^{2+} homeostasis produces generalized Ca^{2+} alterations, in turn producing rough cell damage, without involving specific signaling meaning; when strong, Ca^{2+} alterations cause cell death by necrosis [15–19].

What becomes clear after many years of intense studies about the role of Ca^{2+} in cell stress response and death, is that pathological Ca^{2+} alterations resulting from failure of homeostatic controls coexist with regulated Ca^{2+} signaling. The former produces rough damage that leads to cell repair, or apoptosis, or necrotic cell death, according to the intensity of the damage, whereas the latter constitutes a controlled cell response participating in survival or apoptotic pathway. This recommends careful analyses to separate active responses from passive changes.

2. Ca^{2+} and Cell Damage

2.1. Ca^{2+} as an Intrinsic Stressor. Ca^{2+} deregulation is a consequence of many different insults that end up altering Ca^{2+}homeostasis, causing and increasing damage to cells; for this reason, it may be defined as an "intrinsic stress," meaning that it is autoinduced by the cells as a consequence of an extrinsic stress of a different nature. The proteins controlling Ca^{2+} homeostasis are so many and so diverse that it is quite likely that any insult or physico-chemical alterations end up deregulating some of them, producing a set of reactions that may not properly be defined as signaling (since it has no physiological purposes), but it is nonetheless obliged by the presence of Ca^{2+}-sensitive determinants. What is lacking in such instances is the coordination between the multiple pathways, which are instead casually activated, overlapping and superimposing one another, leading to cell collapse. The intrinsic Ca^{2+} stress may consist in either depletion of ER Ca^{2+}, or increase of cytosolic (or mitochondrial, or nuclear) Ca^{2+}, or both.

2.2. Damage by Ca^{2+} Overload. When stress leads to Ca^{2+} overload, Ca^{2+}-produced damage may reach levels sufficient to cause necrotic cell death [15–19]. Damage and death are due to excess stimulation of Ca^{2+}-sensitive targets, which are numerous and concern key cellular functions: many enzymes that control supramolecular assembly, or degrading nucleic acids, lipids or proteins are Ca^{2+}-sensitive. Among them are m-calpains, activated by high Ca^{2+} levels and implicated in cell death and in many neurological disturbances [20]; lipoxygenases, and a set of Ca^{2+}-activated enzymes modifying arachidonic acid (AA) that are major actors of the inflammatory response, and also involved in apoptotic pathways [21]; phospholipases A2, which liberate AA from phospholipids, thus favoring, in the presence of high $[Ca^{2+}]mt$ mitochondria stress or collapse [22]; a set of DNAses, one of which historical interest for being the enzyme responsible for apoptotic laddering [23].

A major form of damage is caused by the intervention of mitochondria that, taking up the excess of cytosolic Ca^{2+} for scavenging purposes, may be subjected to stress and even collapse if it exceeds a physiological threshold, therefore increasing cell damage (see below) [14].

Another form of damage comes from energy failure, which starves Ca^{2+}-ATPases that stop pumping Ca^{2+} against gradient from cytosol to ER, or to the extra-cellular environment, thus simultaneously producing cytosolic Ca^{2+} overload and ER Ca^{2+} depletion.

Cytosolic Ca^{2+} overload is implicated in many serious human pathologies.

Excitotoxicity is a major cause of neuronal cell death; it develops as a consequence of problems occurring during neurotransmission, in instances of excess of excitatory signals, such as those from the neurotransmitter glutamate [24], or of deregulated signaling; this ends up impairing the tight control of Ca^{2+} channels, leading to Ca^{2+} overload [25] and eventually cell death and neurodegeneration.

Ischemic and anoxic stress produce deep changes in cell metabolism that, upon reoxygenation/reperfusion converge into a dramatic, toxic increase of cytosolic Ca^{2+} [26, 27]. Such changes include plasma membrane depolarization, which favors the opening of the plasma membrane Ca^{2+} channels thus promoting Ca^{2+} influx [28]; and acidification, which causes the inversion of the Na^+/Ca^{2+} plasma membrane exchanger, which begins pumping Ca^{2+} within cells [29]. Mitochondria may buffer Ca^{2+} and rescue cells in instances of mild reperfusion stress; however, they paradoxically are the major cause of cell death in strong reperfusion stress [30], since the huge Ca^{2+} overload stimulate Ca^{2+} overcharging and collapse through phenomena of Ca^{2+} cycling (see below [31]).

Due to the increasing evidence that most (if not all) pathologies involve, as etiological or concurrent agents, alterations of oxidative metabolism leading to oxidative stress, much attention was paid in the 80s and 90s to the mechanisms through which oxidative stress causes Ca^{2+} derangements. Although no definite picture is still delineated, some key points have been clarified. Oxidation and redox imbalance cause ER and plasma membrane Ca^{2+} channels malfunctions, since their oligomeric active form is controlled by disulfide bridges [32]; this increases $[Ca^{2+}]$c and depletes $[Ca^{2+}]$er. Moreover, oxidative stress impairs the buffering capacity of mitochondria, lowering the internal Ca^{2+} threshold level of PTP opening [9, 33], thus depriving the cells of one of the major Ca^{2+} detoxifying mechanisms.

All of these derangements are especially critical for neurons, where Ca^{2+} is crucial to neuronal functions [34], implying that they possess more controlling steps that can be altered. Moreover, cell death is most devastating for tissues rich in post-mitotic cells, such as cardiomyocytes or neurons, which are difficult to replace; indeed, most neurodegenerative conditions are characterized by neuronal death caused by Ca^{2+} overload [34]. The scenario is even more dramatic considering that the organs that mostly depend on post-mitotic, Ca^{2+}-sensitive cells are heart and brain, whose failure causes immediate death of the organism.

2.3. Ca^{2+} Overload in Mitochondria. Mitochondria are very important for intracellular Ca^{2+} homeostasis and signaling, acting in fact as pivot of intracellular Ca^{2+} communications. Any Ca^{2+} overload exceeding the cytosolic threshold of 500 nM involve mitochondrial participation [14]. Mitochondria possess low affinity (500 nM) Ca^{2+} uniporters that allow the accumulation of large amount of Ca^{2+} within the mitochondrial matrix, which constitutes a high capacity Ca^{2+} reservoir, allowing buffering $[Ca^{2+}]$c increases over 500 nM [9, 14]. This mitochondria ability plays an important role in cell homeostasis and cell signaling, because it help extinguishing cytosolic Ca^{2+} signals [35]. The resulting $[Ca^{2+}]$mt increase modulates mitochondrial activity (i.e., increases ATP production [9, 11, 12]); moreover, overcharged mitochondria helps refilling ER after physiological Ca^{2+} emptying (e.g., after IP_3-mediated signalling [13]). In instances of mild $[Ca^{2+}]$c increases originating from stressing events, potentially toxic Ca^{2+} is sequestered within mitochondria, and then released after the stress is over: in this instance mitochondria play a prosurvival role. However, if the amount of sequestered Ca^{2+} exceeds mitochondrial capacity, it leads to collapse through opening of the permeability transition pores (PTP, formerly referred to as megachannel) [18, 19]. Since PTP is a multi-ion channel, the consequence is that the captured Ca^{2+} ions are dissipated, creating new cytosolic Ca^{2+} increase, which can be in turn taken up by new intact mitochondria [18]. This creates cycles of Ca^{2+} uptake and dissipation, recruiting more mitochondria, up to a sort of mitochondrial suicide cascade. This phenomenon was named Ca^{2+} cycling [18], and raised much interest in the 80s; the interest then declined because it did not support a clear physiological role, being rather considered a futile cycle, because it does not help cells to survive. Nowadays, a re-evaluation of this mechanism suggests that Ca^{2+} cycling provides a physiological advantage [10]: PTP opening by itself causes release of cytochrome *c* (even in the absence of an upstream canonical apoptotic signaling such as Bax translocation [19, 36, 37]), which in turn may activate caspases and promote apoptosis [18], thus transforming a necrotic cell death into a more physiocompatible apoptosis. It seems worth mentioning here that localized phenomena of mitochondria Ca^{2+} cycling may have a pro-apoptotic signaling meaning since local and controlled small episodes of cytochrome *c* release act as initiators of the intrinsic pathway of apoptosis [38] (see below).

Mitochondria can adjust their cellular localization by moving around the microtubular network [39]; it is tempting to hypothesize that they reach positions required to perform Ca^{2+} detoxification, or to modulate specific signaling events, that is, extinguish some and exacerbating others, according to need. As an example, acute oxidative stress induces the reorganization of mitochondrial pattern from pan-cytoplasmatic, to peri-nuclear (Ghibelli, unpublished observation), possibly buffering excess ER Ca^{2+} leakage due to oxidations. This scenario suggests that local Ca^{2+} increases of a stress nature, even of a small extent, may trigger an apoptotic signaling *via* recruitment of mitochondria.

2.4. Damage by Ca^{2+} Depletion (ER Stress). When referring to intracellular Ca^{2+} depletion, the emphasis goes to emptying of ER, which elicits what was recently recognized as ER stress [40]. ER stress is caused by different disturbances affecting ER homeostasis, such as protein malfolding, glucose starvation, disturbance of membrane turnover/synthesis, or of protein trafficking, which all lead to ER vesiculation and loss of function [40]. Ca^{2+} plays a key role in maintaining ER structure, since the flat shape of the cisternae is actively kept by bridges constituted by high capacity Ca^{2+} binding proteins such as calreticulin, calsequestrin, and calnexin [41]; upon ER Ca^{2+} emptying, Ca^{2+} binding is lost, the bridges weaken and ER resumes the low energy spherical shape of lipids droplets in acqueous solution, thus losing function. ER stress, as any other stress, can evolve into repair or apoptosis.

In the first instance, the stress response implies upregulation of stress proteins such as GRP78, a major luminal ER protein [42] that plays a central role as ER stress sensor displaying multiple functions. It coordinates the activation of other proteins implicated in ER stress, such as ATF6 [43], a transcription factor transactivating prosurvival genes whose promoters containing ER stress response elements (ERSEs) [44]. GRP78 also promotes removal by autophagy of the altered portions of ER by controlling the correct formation of autophagosome [45]. Multifaceted is its ability to prevent apoptosis [42, 46]: a fraction of GRP78 is present as a transmembrane ER protein, exposing a cytoplasmic domain able to directly interact and form an inhibitory complex with caspase-7 and/or caspase-12 [42, 47]; GRP78 limits the proapoptotic activation of c-Jun N-terminal kinase (JNK) [35], normally acting as a transducer of ER stress [48]; in addition, cell-free studies suggest a direct ability of GRP78 to control mitochondria, by inhibiting cytochrome *c* release [47].

If damage is severe, it triggers apoptosis. The mechanism for ER stress-induced apoptosis is still not completely clarified. Big emphasis was given to caspase-12, which is activated in ER membranes in instances of disruption of ER Ca^{2+} homeostasis or accumulation of unfolded proteins in the ER lumen of mice cell models. Caspase-12 initiates apoptosis either in a mitochondrial-independent fashion [49] or recruiting and activating mediators of the intrinsic pathway of apoptosis [50]. This led to consider caspase-12 as the general transducer of ER damage. However, caspase 12 is present only in rodents, and to-date no functional caspase-12 was identified in human cells (which in fact possess only a pseudo-gene), nor a functionally equivalent protein. While the search for a human equivalent of caspase 12 is still active (especially concerning a possible role for caspase-4 [51]), other caspase-independent scenarios, have been explored to describe the transduction of the ER stress to apoptosis via mitochondria. Recently, Klee et al. [52] provided evidence that Ca^{2+} mobilization from the ER is required to initiate the mitochondrial death pathway, by cooperating with the effectors of ER stress surveillance machinery IREa/TRAF2; according to this model, Ca^{2+} promotes the JNK pro-apoptotic pathway through a complex set of steps involving the Bcl-2 family.

3. Ca^{2+} and Cell Death

3.1. Historical Perspective. When the regulated, physiological mode of cell death, apoptosis, came into the general interest, the involvement of de-regulated Ca^{2+} rises as causative agent of apoptosis was sought for, in the view that apoptosis was a sort of "petit necrosis;" in particular, a Ca^{2+}/Mg^{2+}-dependent DNAse was hypothesized, considering that DNA laddering was the earliest apoptotic marker of a biochemical nature to be widely accepted. The issue however never came to a definite picture, because if an increase of $[Ca^{2+}]c$ was indeed occurring in some examples of apoptosis, such as the paradigmatic model of rat thymocytes treated with corticosteroids [53], in many other instances it was a drop in overall Ca^{2+} level the event that promotes apoptosis [54].

The paradox of this dual, opposite role of Ca^{2+} in apoptosis occupied several years of research. Thapsigargin (THG), a (still) popular inducer of apoptosis, is an irreversible poison of ER Ca^{2+}-ATPases (SERCA), thereby inducing a transient increase in cytosolic Ca^{2+} and a sustained depletion of the ER Ca^{2+} pool [55]; since the two events occur simultaneously, it is quite difficult to assess the specific contribution of one or the other to apoptosis. If the early studies were taking for granted that the apoptogenic event was the increase of $[Ca^{2+}]c$, it was later shown that in many instances intracellular Ca^{2+} buffering did not abrogate, but even increased, THG-induced apoptosis, demonstrating that also ER Ca^{2+} depletion was an apoptogenic event [56, 57].

In fact, in most instances $[Ca^{2+}]c$ rises in apoptosis are not rough event, but precise signaling steps, such as, for example, the Ca^{2+}-dependent proteases calpains [58] or calmodulin [59]. The notion of apoptotic Ca^{2+} signaling evolved, with not small effort, together with the awareness that apoptosis is not a "petit necrosis" but a regular signal transduction chain, occurring in functioning cells, ending up with coordinate cell demise instead of activation, or mitosis, or transcription.

To-date, the issue of the actual role of Ca^{2+} in cell death is still debated; the different contributions of the different cells (e.g., excitable versus non-excitable; dividing versus post-mitotic; tumor versus normal) and of the different apoptogenic treatments (stress or physiologic; allowing or not protein neosynthesis, etc.) produce a whole continuum of variations, and render it hard to interpret the thousands of studies on the topic. However, some generalizations can be attempted: ER Ca^{2+} depletion may elicit apoptosis through the ER stress pathway [57, 60]; rough, stress-induced Ca^{2+} overloads produce necrosis [17, 19, 61]; regulated Ca^{2+} increases play a role as signaling events in the intrinsic apoptotic pathway [62–65].

The poor knowledge of the role of Ca^{2+} in apoptosis, which is perhaps surprising considering that Ca^{2+} dynamics were among the first alterations proposed as causative of apoptosis, is also due to other, more intrinsic problems. Among them the fact that Ca^{2+} transients are very much localized in terms of space (i.e., cytosolic micro-domains) and time (seconds), and it is very easy to miss them even with sophisticated technologies. Also the asynchrony of the apoptotic process hampers the analyses: even homogeneous cultured cells initiate apoptosis at different moments after stimulation, overlapping different phases and rendering inappropriate any biochemical analysis performed in bulk. These problems were overcome with technological approaches allowing analysis at the single cell level [1, 2], that is, living cell imaging and flow cytometry, which are beginning to shed light on the process, helping to separate different phases and different subregions of Ca^{2+} signaling [1, 2, 64, 65].

3.2. Stress-Induced Apoptosis: A General View. It is now well established that the intracellular apoptotic signaling evolves through at least two different pathways, triggered by ligand stimulation of death receptor (extrinsic pathway), or by cell damage (intrinsic pathway) [66]. The extrinsic pathway is a typical signal transduction consisting of protein-protein interaction and conformational changes from the very beginning, being induced by a molecular event such as ligand-receptor interaction and culminating with caspase activation and cell dismantling. The intrinsic pathway is instead induced not by molecular, but by physicochemical events, implying that (a) sensor(s) of micro-environmental alterations or cell damage must be activated to promote the apoptotic signal [67]; afterwards, a molecular signal transduction chain of events similar to the extrinsic pathway is activated, also culminating with caspase activation. Sensors are proteins that are modified by physico-chemical alterations such as pH, redox equilibrium, or Ca^{2+} levels, thus acquiring the ability to trigger a molecular signal cascade. The most upstream molecular event of the intrinsic pathway is the translocation of Bax, which moves to mitochondria and induces mitochondrial outer membrane permeabilization (MOMP). The difficulty of finding molecular events upstream of Bax activation suggested that Bax itself might

be a sensor of physico-chemical alterations. Indeed, recent reports indicate that Bax activation can occur via direct oxidation of cysteines [68, 69], or via proteolytical activation by calpains [70].

3.3. Ca^{2+} Control of Cytochrome c Release. It is emerging a pre- or early-commitment phase of the intrinsic apoptotic pathway, occurring before MOMP, during which potential apoptotic signals, mostly relying on Ca^{2+} messages, are selected and amplified by cross-talk between ER and mitochondria [61] (Figure 1). MOMP is a set of different phenomena allowing release (or leakage, see below) of apoptogenic factors such as cytochrome *c*, SMAC/diablo, AIF, through mitochondrial membrane pores. Cytochrome *c* received most attention for its ability to nucleate the apoptosome and to initiate the caspase cascade; its release occurs through at least two different mechanisms, the apoptosis-specific Bax-based pore, and the PTP channel, both of which can be modulated by Ca^{2+} in a very different way.

The relation between Ca^{2+} and cytochrome *c* release via Bax consists of a feed-forward amplification loop between ER and mitochondria: local high concentrations of Ca^{2+} (such as those created by the Ca^{2+} efflux from IP_3 channels) favor the release of cytochrome *c* from mitochondria through Bax pores [62, 71] on the one side; on the other, cytosolic cytochrome *c* increases Ca^{2+} levels in the vicinity of IP_3 channels on ER [38] by fixing them in the open configuration after a signaling stimulus, thus transforming a transient flux into a sustained one [38]. As a result of this interplay, small cytosolic cytochrome *c* leakage may promote secondary and massive releases (i.e., that required for apoptosome nucleation), via local Ca^{2+} messages [38]. This provides a rationale to previous reports indicating that small amounts of cytochrome *c* are released from mitochondria as a very early step of apoptosis, with the goal of expanding the signal [72]. Intriguingly, the Ca^{2+} sensitivity of Bax mitochondrial pores seems to be limited to the intrinsic pathway: when Bax is activated by the extrinsic pathway via t-Bid [73] (i.e., death receptor stimulation in type 2 cell), the Bax pores are insensitive to Ca^{2+} modulation [62]. This indicates that active Bax is different according to the route of activation (i.e., damage versus t-Bid), suggesting that multiple, alternative mechanisms for Bax activation may exist, possibly leading to different effects on the folding and functions of the protein [68, 70, 73].

PTP opening is an automatic response to excess $[Ca^{2+}]$mt, which causes the interaction between the inner mitochondrial membrane complex adenine nucleotide translocator (ANT) and the outer mitochondrial membrane complex voltage-dependent anion channels (VDAC), leading to the formation of the membrane-spanning PTP pore. Cyclophilin D (Cyp-D) is a component of PTP resident in the mitochondrial matrix, which is activated by high $[Ca^{2+}]$mt, favoring PTP opening by lowering the Ca^{2+} threshold required for ANT-VDAC interaction [74–76]. In instances of mitochondrial Ca^{2+} overload, inhibitors of Cyp-D activation, such as cyclosporins, contrast PTP opening and the eventual cell death, therefore exerting a net cell protective effect, which is often used in therapies to limit immune deficiencies or neurodegenerations [76, 77].

The mechanism of cytochrome *c* release via PTP opening, which was historically the first mechanism proposed, is still unclear from the molecular and functional point of view (Figure 1). In fact, there is a topological problem. In apoptosis cytochrome *c* is liberated from its natural position on the outer side of the internal mitochondrial membrane to the intermembrane space; thus, it requires pores in the outer membrane to be released, whereas pores that span the two membranes, such as PTP, would lead to the release of molecules residing in the mitochondrial matrix. To explain cytochrome *c* release via PTP, it may be hypothesized that PTP may cause mitochondrial membrane perturbations that allow cytochrome *c* (and other factors) to leak rather than be specifically released. In such instances, the gross alterations caused by PTP-mediated ionic redistribution will be necrogenic [78], even if cytosolic relocalization of cytochrome *c* may circumstantially activate caspases. Conceivably, the extent of PTP may determine the final outcome, and apoptosis or necrosis may follow according to the strength of PTP. Indeed, Cyp-D inhibitors are often reported to prevent cell death by necrosis. As an alternative mechanism of cytochrome *c* relase via PTP, it was suggested that a VDAC-only channel may form on the outer membrane, with the help, but without the physical participation, of ANT, thus connecting the cytosol not with the matrix, as in the canonical PTP, but with the intermembrane space, thus allowing cytochrome *c* release. This model is supported by experiments performed in liver mitochondria from mice knock out for ANT isoforms [79], where the release of cytochrome *c* following an apoptogenic stimulus still occurred, but the susceptibility to Ca^{2+} alterations in the mitochondrial matrix was reduced. In this instance ANT, a sensor of Ca^{2+} through its interaction with Cyp-D, plays the regulatory function to transduce Ca^{2+} alterations to VDAC, promoting its oligomerization and the formation of pores mediating release of cytochrome *c*.

Bax pores and PTP are different in molecular, mechanistic and functional term. However, they cooperate in some examples of apoptosis to achieve cytochrome *c* release. This implies physical interaction between Bax and PTP components [80], such as Cyp-D or ANT [81]. The two mechanisms of cytochrome *c* release also coparticipates in the same induction pathway in a different temporal relationship, that is, a mild stress-induced PTP opening first causes a small cytochrome *c* leakage, which stimulates via Ca^{2+} modulation (see above) a second intense Bax-mediated release sufficient for caspase activation. The cooperation between the two pores (and the two pathways) also provides a mechanistic explanation to the established but still unexplained finding that Bcl-2, though not modulating PTP directly [74], all the same provides protection to cells against necrosis [82].

3.4. Control of Ca^{2+} by the Bcl-2 Family. The pro-apoptotic protein Bax exerts its functions by inserting into membranes and forming pores. Very well described is the anchoring to mitochondrial membrane, where Bax forms, perhaps

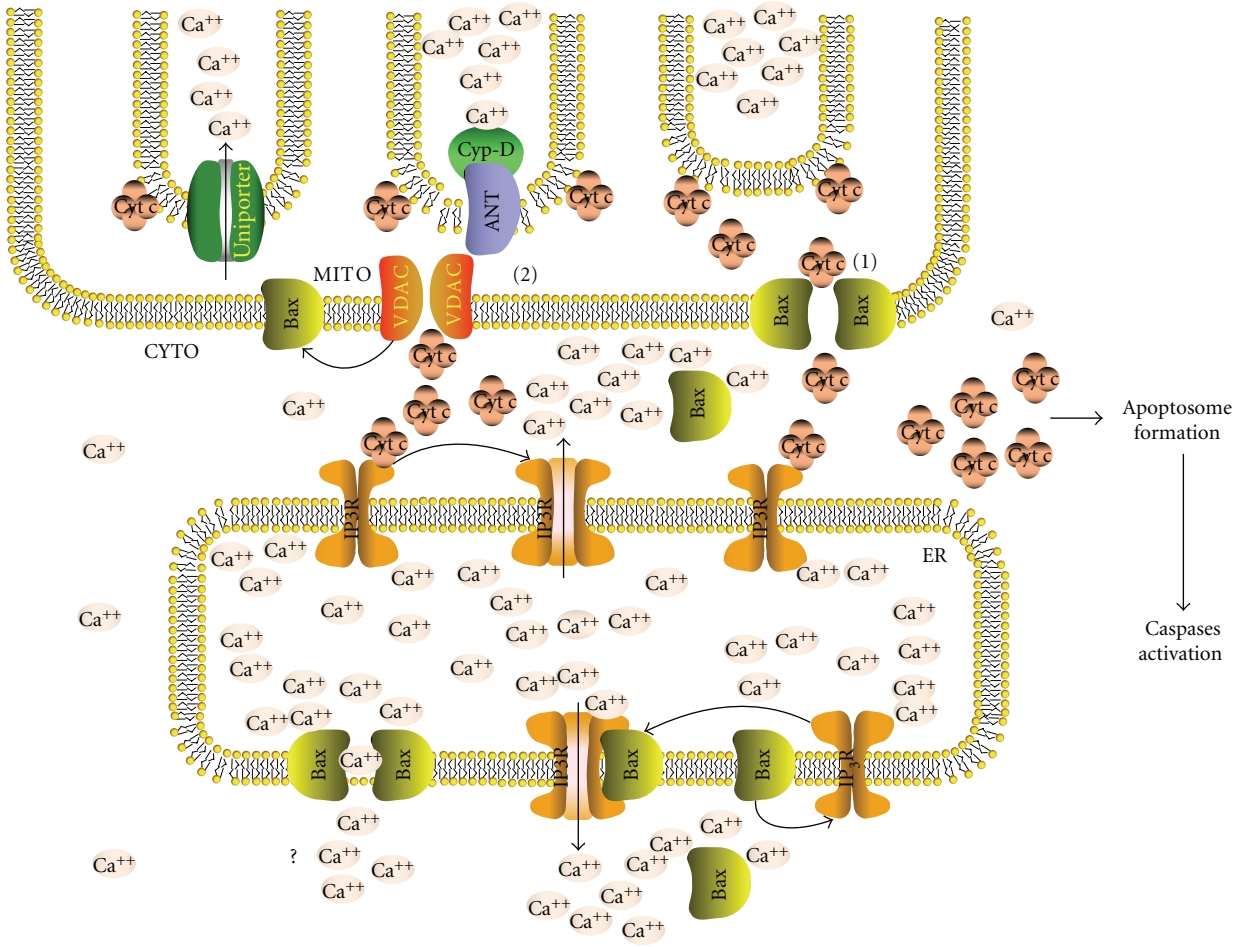

FIGURE 1: *Ca^{2+} signals between ER and mitochondria coordinate the precommitment phase of apoptosis:* amplification loops between Bax activation and Ca^{2+} release from ER amplify cytochrome *c* release to a level sufficient for apoptosome nucleation and caspase activation. Cytochrome *c* released by Bax (1) or VDAC (2) mitochondrial pores magnifies Ca^{2+} efflux from IP_3 channels; the consequent local high cytosolic Ca^{2+} recruits Bax (*via* calpain?) to mitochondria or ER membrane, stimulating more cytochrome *c* release and more Ca^{2+} efflux, respectively.

with adjuvant proteins, pores of a size large enough to allow passage of diffusible pro-apoptotic proteins such as cytochrome *c* or SMAC [83–85]; the anti-apoptotic role of the cognate Bcl-2, which integrates into mitochondrial membranes also in healthy cells, is believed to be the prevention of Bax pore forming, perhaps due to the extra protein domain (BH4) shared by all anti-apoptotic members of the family. It is now emerging that Bax and Bcl-2 play a similar role also in the ER membranes, where they would prevent or favor, respectively, Ca^{2+} leakage to the cytosol.

Bcl-2 is found within ER membranes of healthy cells, where it prevents Ca^{2+} leaking from ER [63, 86]; as the mechanism involved, it was proposed that Bcl-2 may work as a pump additional to the SERCAs [56], and/or to prevent IP_3 channels opening [87].

In apoptosis, Bax translocates not only to mitochondria, but also to ER membranes [63, 88], where it favors Ca^{2+} release from the ER lumen [62, 63], possibly after oligomerization [71]; similar evidence was shown for the cognate pro-apoptotic Bak [63, 88]. The mechanism through which Bax (and Bak) favors ER Ca^{2+} release is currently debated. Recent cell-free studies have shown that Bax forms small pores, compatible with multi-ion passage, on membranes [89], thus possibly directly allowing Ca^{2+} leakage. Other studies indicate an indirect role, that is, favoring IP_3 channels opening [90, 91]. Ca^{2+} release from ER in turn favors the recruitment of more Bax molecules from the cytosol to ER membranes [88], thus amplifying the Ca^{2+}-dependent apoptotic signal [71]. Thus, the pro-apoptotic functions of Bax and Bak are not limited to mitochondria, but consist of a dual concerted role played at an earlier pre-commitment step at the level of ER membranes, promoting Ca^{2+} movements; and at a later step in mitochondria to promote MOMP and the release of the apoptotic factors. It remains to be clarified if the mechanism of Bax translocation to ER in pre-apoptosis occurs with the same mechanism as mitochondrial translocation, that is, if there is a role for t-Bid or other BH3-only proteins of the Bcl-2 family; moreover, it is still unclear

if Bax domains involved in promoting ER Ca^{2+} leakage coincide with those required for releasing the apoptotic factors from mitochondria.

3.5. Calpain and Apoptosis. A role for the cysteine proteases calpains, which are activated by Ca^{2+} increase, was investigated since the earlier studies of apoptotic signaling, considering that (a) cytosolic Ca^{2+} overload was then considered as the main mediator of apoptosis [92], and (b) apoptosis might be conceivably executed by coordinate protein dismantling (which was later demonstrated for caspases) [58, 93]. The focus was placed on the known calpain target fodrin [94], the protein bridging plasma membrane with the cortical actin cytoskeleton; it was hypothesized that fodrin degradation might destabilize the cytoskeleton-membrane asset and promote plasma membrane blebbing [95], one of the earliest apoptotic features described [96]; however, such evidences have not been confirmed; actually, calpain activation seems rather inhibiting plasma membrane blebbing (De Nicola et. al, in preparation).

Nowadays, many pieces of evidence show that calpains are required for apoptosis in some systems [97–100], being dispensable (or not involved) in others [101]; when calpains are involved, they act at a very early step, upstream of caspases [100, 102], thus participating to the commitment phase of signaling rather than to the execution. The notion that the form of calpain involved in apoptosis is m-calpain [103], the one also involved in cell stress [20] and that requires high (mM) Ca^{2+} levels (as opposed to μ-calpain, involved in cell signaling, and requiring lower, μM doses) was very important because it allowed linking environmental alterations to apoptosis via Ca^{2+} overload. The molecular role for calpain in promoting apoptosis is still under investigation. Perhaps the most clear-cut hint is the calpain-mediated proteolytic Bax activation [70, 104], one of the few mechanisms so far proposed for direct Bax activation by cell damage [104–106]. Two mitochondrial calpains cooperate in the release of a truncated active form of AIF (tAIF) thus promoting apoptosis: a matrix m-calpain cleaves AIF [107]; and a transmembrane μ-calpain cleaves VDAC, promoting the formation of Bax-VDAC pores on the outer mitochondrial membrane and the release of tAIF [108].

A complex interplay between calpain and caspases occurs in apoptosis. Calpain have been proposed to proteolytically activate some caspases [100, 109–112]; paradoxically, caspases may also be degraded by calpains [113], which in such instances would act to prevent, rather than promote, apoptosis; the factors influencing this discrepant behavior have not been clarified.

4. Ca^{2+} and the Stress Response

4.1. Generalities. Stress consists of any physico-chemical alteration of cell environment that interferes with cell functioning, potentially or actually producing damage. Stress elicits active cell responses that, according to cell type, and to type and extent of damage, aim at cell survival (cell-protective and/or cell-reparative stress response, such as the heat shock response) or cell death (apoptosis).

Stress responses are specific for a given type of alteration/damage: heat, oxidation, hypoxia, starvation, all trigger the synthesis or activation of molecular determinants adequate to cope with the specific type of damage; for example, heat shock will induce synthesis of molecular chaperones to cope with exposure of hydrophobic residues of proteins [114]; oxidative stress will induce the synthesis/activation of anti-oxidant enzymes or molecules [115]; hypoxia promotes anaerobic metabolism [116]; starvation promotes the disassembly of whole cell areas that are digested by autophagy [117], in order to recycle the building blocks for housekeeping purposes. This specificity of response limits the cross-resistance between different stress, even though a partial overlapping exist.

A brief/mild insult is often sufficient to trigger protective responses but not to produce damage. This protects the cells from a second, more severe insult of the same type, thus producing transient tolerance to further stress, as occurring, for example, during thermotolerance [118]. Treatments with important clinical relevance, such as ischemia preconditioning, that is a short anoxic treatment that is protective towards a more severe ischemia, and whose mechanisms are still to be elucidated at the molecular level, seem to depend on Ca^{2+} signaling [119]. Ca^{2+} participates as second messenger to such defensive, reparative, or survival pathways, propagating the cell-protective signals.

High $[Ca^{2+}]c$ is involved in the stimulation of the autophagic response through the activation of calcium/calmodulin-dependent kinase-b that inhibits mTOR [120], the main negative regulator of authophagic processes in mammals, with the goal of eliminating cellular areas that may be damaged by Ca^{2+} overload.

4.2. Ca^{2+} in Cell Survival. In addition to these specific stress responses, cells are capable to build up survival pathways that render them less prone to apoptosis, thus promoting cell survival whatever the type of damage; this especially occurs in cells that reside in highly stressing environments, such as inflammatory or immune cells while exerting their functions [121], or transformed cells undergoing tumor progression [122], process in which cells carrying apoptosis-resistant mutations are favored by natural selection.

Ca^{2+} is involved in pro-survival or anti-apoptotic pathways, such as the activation of protein kinase C, whose many isoforms play pivot roles in coordinating survival cell responses [123].

Capacitative Ca^{2+} entry (CCE) is Ca^{2+} influx from the extracellular environment through specific and tightly controlled plasma membrane channels [4, 5]. CCE only transiently crosses the cytosol, its aims being rather the replenishment of ER, after it was partially emptied by signaling events such as cyclic ADP-ribose- [4] or IP_3-mediated opening of ER Ca^{2+} channels [5]. CCE poorly alters cytosolic homeostasis, but prevents ER vesiculation due to Ca^{2+} emptying, thus being a net cell-protective event.

Recently, another mechanism of Ca^{2+} influx is being considered, namely the noncapacitative Ca^{2+} influx (NCCE) [124], a non-store-operated mechanism that allows Ca^{2+} entry through plasma membrane channels that are different

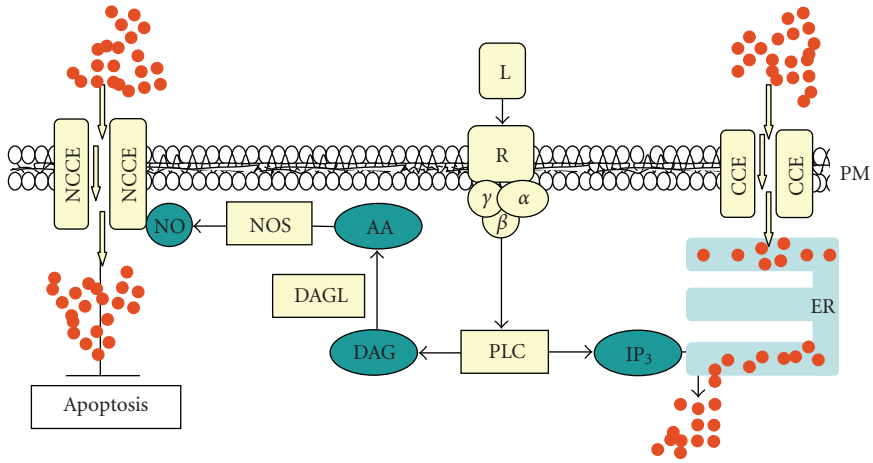

FIGURE 2: *Capacitative and noncapacitative Ca^{2+} entry.* Ligand (L) stimulation of G-protein ($\alpha\beta\gamma$)-coupled receptor (R) activates phospholipase C (PLC) to produce diacylglycerol (DAG) and inositol-3-phosphate (IP_3). IP_3 (right side) causes ER Ca^{2+} emptying, eliciting a capacitative Ca^{2+} entry (CCE) through plasma membrane (PM), aimed at refilling ER of Ca^{2+} restoring ER homeostasis. DAG (left side) is processed to arachidonic acid (AA) by DAG lipase (DAGL), stimulating NOS to produce NO, which activates Ca^{2+} entry through PM by a noncapacitative Ca^{2+} entry (NCCE), priming specific signaling including anti-apoptotic pathways. CCE and NCCE differ in protein composition [124]. Red dots symbolize Ca^{2+}.

from those of CCE from the molecular and regulative point of view. As depicted in Figure 2, NCCE, as CCE, occurs as a response to receptor stimulation implying G-protein and phospholipase C (PLC) but, unlike CCE, it does not respond to IP_3-induced ER emptying; instead, it results from the processing of diacylglycerol (the other product of inositol-bis-phosphate cleavage by PLC), which promotes a signal transduction chain culminating with NCCE assembly. Interestingly, NCCE occurs also in non-excitable cells. Even though mechanisms and functions are still poorly characterized, it is clear that Ca^{2+} entry via NCCE has a signaling function, possibly implying the control of survival pathways. Interestingly, it requires production of NO, a molecule that is involved in many survival pathways [125], including a strict interrelationship with protein kinase C [124]. Agents promoting cell survival such as magnetic fields reduce stress-induced apoptosis by increasing Ca^{2+} influx [126, 127], involving NCCE rather than CCE (Cerella and Ghibelli, in preparation).

4.3. Stand-By Mechanisms. The decision between cell repair or demise of damaged cells is a choice between the risk of mal-repair, leading to stabilize mutations and potentially preserve precancerous cells, versus loss of viable cells performing useful functions, which must be expensively replaced. Even though the former risk is definitely worse than the latter, mechanisms aiming at avoiding unnecessary loss of precious cells have evolved. To this purpose, it is important that damaged cells do not initiate apoptosis before attempting to repair the damage: this is actively achieved by damaged cells via the set-up of reversible standby scenarios, during which apoptotic signaling is transiently kept at bay. One of such standby mechanisms implies that potentially apoptogenic stress conditions such as H_2O_2 treatment cause the transient inhibition of glycolysis mediated by the reversible ADP-ribosylation of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) [128]; this inactivates ER Ca^{2+}-ATPases, which are fed by glycolytic ATP [129], thus decreasing ER Ca^{2+} while increasing $[Ca^{2+}]c$ and impairing Ca^{2+}-mediated cell signaling. Many pieces of evidence from the literature show that cells with partial Ca^{2+}-depleted ER cannot initiate apoptosis [130–132], and indeed, during the standby period of glycolysis block, apoptosis cannot initiate [128]. Starting at around 90 minutes after recovering from H_2O_2 stress, glycolysis resumes [128], ER Ca^{2+} increases, and $[Ca^{2+}]c$ is reduced [64]; only then H_2O_2-induced apoptosis begins [128]; (Cerella et al., in preparation). Similar findings were reported also for other, oxidation-unrelated apoptogenic agents, strongly supporting the scenario according to which (a) Ca^{2+} signaling is required for stress-induced apoptosis [64], and (b) ER is the initiator of the apoptotic signaling, since the standby phase seems to prevent ER from amplifying apoptotic signal and mitochondrial recruitment. A model representing the relationship between apoptogenic, repair and standby signals in damaged cells is shown in Figure 3.

5. Conclusions

The relationship between Ca^{2+} and cell death has a long and complex story. It was a reasonably simple task when the goal was describing how strongly deregulated intracellular Ca^{2+} may cause the passive cell death by necrosis. The scenario became very much complex when the increasing information of the mechanisms of Ca^{2+}-mediated cell signaling in general, and apoptotic signaling in particular, begun to merge. Figure 4 depicts the different roles that Ca^{2+} alterations, as an intrinsic stressor, play in the survival or death of damaged cells, aiming at separating Ca^{2+} deregulation from pro-apoptotic Ca^{2+} signaling. Perhaps

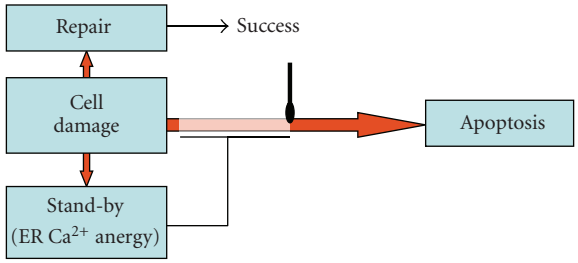

Figure 3: *Temporary ER Ca^{2+} anergy avoid loss of repairable cells.* Cell damage elicits repair and apoptosis as well as standby periods (red arrows). ER Ca^{2+} anergy is temporarily achieved via ADP-ribosylation of GAPDH, glycolysis block and starvation of Ca^{2+}-ATPases, and hampers apoptotic signal transduction at the ER signaling stage. After resumption of glycolysis and ER Ca^{2+} activity, the apoptotic signal is allowed to proceed, unless successful repair has occurred in the meantime.

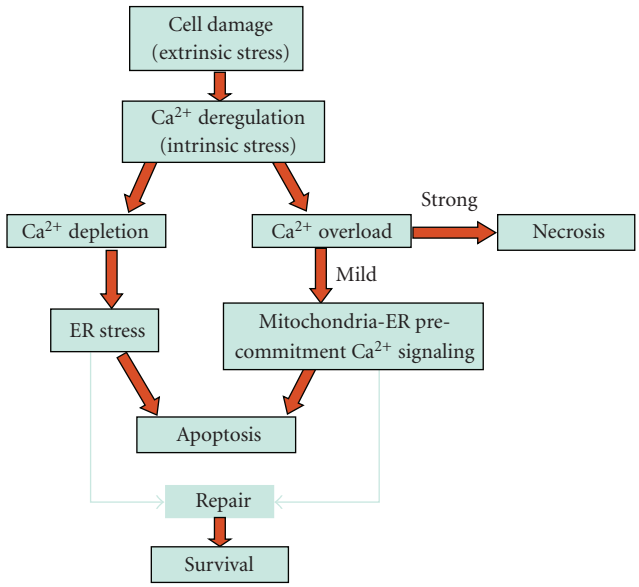

Figure 4: Ca^{2+} signaling in survival and apoptosis versus deregulation of Ca^{2+} homeostasis as necrogenic event.

the hottest topic to-date in this field is the role that ER, and the Ca^{2+} messages it exchanges with mitochondria, plays in the amplification of the apoptotic signal, ending up with the promotion of MOMP and the trigger of the commitment phase of the intrinsic apoptotic signaling. The amplification loops created by the concerted action of ER, Bax, IP_3 channels and cytochrome *c* with Ca^{2+} signals, spanning ER and mitochondria via cytosol, as depicted in Figure 1, are beginning to define a novel precommitment phase of apoptosis. This is a very important issue because, unlike the extrinsic apoptotic pathway, which has been very well characterized at the molecular level since many years, the molecular events of the intrinsic pathway upstream of MOMP are poorly understood. From the functional point of view, a precommitment phase might have the role of selecting, among the pro-apoptotic signals deriving from cell damage, the ones that have to be finalized in cell death, thus avoiding unnecessary cell loss. Unlike receptor-induced apoptosis, before commitment to damage-induced apoptosis cells must check the extent of the damage, and the possibility to repair it, before engaging the apoptotic signaling and commit suicide. To this purpose, different signals reporting the nature and the extent of the damage must merge into a mainstream signal that actually allows the onset of apoptosis, which in molecular terms coincides with MOMP. Many evidences allow proposing the fascinating scenario according to which ER plays as a pivot that receives the damage signals and select those that actually deserve ending up in apoptosis.

The acknowledgement of a Ca^{2+}-dependent pre-commitment apoptotic phase would place Ca^{2+}-related events among the earliest of apoptosis, which would make the closing of a circle that begun almost 20 years ago, when Ca^{2+} as an intrinsic stressor was considered as "the" mediator of apoptosis.

Abbreviations

AA: Arachidonic acid
AIF: Apoptosis inducing factor
ANT: Adenine nucleotide translocator
CCE: Capacitative calcium entry
ER: Endoplasmic reticulum
GAPDH: Glycerhaldehyde-3-phospahate dehydrogenase
IP_3: Inositol-3-phosphate
MOMP: Mitochondrial outer membrane permeabilization
NCCE: Non-capacitative calcium entry
PLC: Phospholipase C
PTP: Permeability transition pore
SERCA: Sarcoplasmic/endoplasmic reticulum calcium ATPases
THG: Thapsigargin
VDAC: Voltage-dependent anion channels.

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Review Article

Evasion of Apoptosis as a Cellular Stress Response in Cancer

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One of the hallmarks of human cancers is the intrinsic or acquired resistance to apoptosis. Evasion of apoptosis can be part of a cellular stress response to ensure the cell's survival upon exposure to stressful stimuli. Apoptosis resistance may contribute to carcinogenesis, tumor progression, and also treatment resistance, since most current anticancer therapies including chemotherapy as well as radio- and immunotherapies primarily act by activating cell death pathways including apoptosis in cancer cells. Hence, a better understanding of the molecular mechanisms regarding how cellular stress stimuli trigger antiapoptotic mechanisms and how this contributes to tumor resistance to apoptotic cell death is expected to provide the basis for a rational approach to overcome apoptosis resistance mechanisms in cancers.

1. Introduction

Tissue homeostasis is characterized by the balance between proliferation and cell growth on one side and cell death on the other side [1]. In response to stressful stimuli, cells usually mount a cellular stress response to ensure survival [2]. Under physiological conditions, such a stress response limits tissue damage. However, in cancer cells activation of pathways that favor cell survival instead of cell death under stressful conditions may contribute to tumorigenesis. In addition, this adaptive stress response promotes the development of acquired resistance, since current treatment approaches such as chemotherapy and irradiation trigger cellular stress pathways, and thus, initiate the activation of survival cascades and anti-apoptotic mechanisms [3]. Apoptosis or programmed cell death is the cell's intrinsic death program that regulates various physiological as well as pathological processes and that is evolutionary highly conserved [1]. Hence, further insights into the molecular mechanisms of how cellular stress signals trigger anti-apoptotic mechanisms and how this contributes to tumor resistance to apoptotic cell death are expected to provide the basis for a rational approach for the development of new molecular targeted therapies.

2. Signaling to Apoptotic Cell Death and Cellular Stress

There are two major apoptosis signaling pathways, that is, the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway [4]. Under most circumstances, activation of either pathway eventually leads to proteolytic cleavage and thus activation of caspases, a family of cysteine proteases that act as common death effector molecules [5]. Accordingly, caspases are responsible for many of the biochemical and morphological hallmarks of apoptotic cell death by cleaving a range of substrates in the cytoplasm or nucleus [5]. Ligation of death receptors of the tumor necrosis factor (TNF) receptor superfamily such as CD95 (APO-1/Fas) or TRAIL receptors by their corresponding natural ligands, that is, CD95 ligand or TRAIL, results in the recruitment of caspase-8 into a multimeric complex at the plasma membrane, the death-inducing signaling complex (DISC) [6, 7]. This in turn leads to caspase-8 activation, which can then directly cleave downstream effector caspases such as caspase-3 [7]. Alternatively, caspase-8 can promote outer mitochondrial membrane permeabilization by cleaving Bid, a BH3-only protein that translocates to mitochondria upon cleavage and causes cytochrome c release [8]. The mitochondrial pathway is initiated by the release

of apoptogenic factors such as cytochrome c, apoptosis-inducing factor (AIF) second mitochondria-derived activator of caspase (Smac)/direct IAP Binding protein with Low pI (DIABLO) or Omi/high temperature requirement protein A (HtrA2) from the mitochondrial intermembrane space into the cytosol [9]. The release of cytochrome c into the cytosol triggers activation of caspase-3 via the formation of a large cytosolic complex, which is called the apoptosome and consists of cytochrome c, Apaf-1, and caspase-9 [9]. Smac/DIABLO or Omi/HtrA2 promotes caspase activation by binding to Inhibitor of Apoptosis (IAP) proteins and thereby disrupts the interaction of IAPs with caspase-3 or -9 [9, 10]. Accidental stimulation of the apoptotic machinery can have detrimental effects on cell survival. Therefore, cancer cells react to cellular stress signals by mounting an anti-apoptotic response, which enables cancer cells to evade apoptotic cell death and ensures cell survival [11]. A wide range of stress signals has been identified, which may evoke a cell survival program in case of sublethal damage, while cell death is usually initiated if the damage is too severe, that is, starvation, hypoxia, DNA damaging drugs, irradiation, ER stress, and reactive oxygen species just to name a few [2].

The molecular mechanisms that initiate cell death upon cellular stress stimuli have often not exactly been identified and likely depend on the individual stimulus. For example, following exposure to genotoxic substances, damage to DNA or to other critical molecules is considered to be a common initial event which is then transmitted by the cellular stress response to the activation of cellular effector systems such as the apoptotic machinery [12]. Various stress-inducible molecules, for example, JNK, MAPK/ERK, NF-κB, or ceramide have been implicated in propagating the apoptotic signal [13–15].

Besides caspase-dependent and caspase-independent apoptosis, additional non-apoptotic modes of cell death also exist and have gained increasing attention over the last years, including necrosis, autophagy, mitotic catastrophe, and lysosomal cell death [16, 17]. While resistance to these cell death modalities can also contribute to evasion of cell death under stress conditions, the discussion of these alternative modes of cell death is beyond the scope of this review.

3. Evasion of Apoptosis in Response to Cellular Stress in Cancers

A characteristic feature of human cancers is the evasion of apoptosis in response to stress stimuli, which contributes to both tumorigenesis and treatment resistance [18]. In principle, cell death pathways can be blocked at different levels of the signaling cascade by upregulation of anti-apoptotic proteins and/or by downregulation or dysfunction of proapoptotic molecules. Examples of altered apoptosis signaling pathways that contribute to stress resistance in human cancers will be discussed in the following paragraphs (Figure 1).

3.1. Evasion of the Death Receptor Pathway. Death receptors are part of the tumor necrosis factor (TNF) receptor gene superfamily, which comprises more than 20 proteins, for example, CD95 (APO − 1/Fas), TRAIL receptors, and TNF receptor 1 (TNFRI) [7, 19]. Death receptors exert many different biological functions, including the regulation of cell death and survival, differentiation, and immune regulation [7, 19]. Members of the TNF receptor family share a characteristic cytoplasmic domain called the "death domain," which is pivotal for transducing the death signal from the cell's surface to intracellular signaling pathways [7, 19].

Signaling via death receptor can be impaired in human cancers via downregulation of receptor surface expression as part of an adaptive stress response. For example, in chemotherapy-resistant leukemia or neuroblastoma cells, downregulation of CD95 expression was identified as a mechanism of acquired drug resistance [20, 21]. For the apoptosis-inducing TRAIL receptors TRAIL-R1 and TRAIL-R2, abnormal transport from intracellular stores such as the endoplasmatic reticulum to the cell surface rendered colon carcinoma cells resistant to TRAIL-induced cell death [22]. Further, membrane expression of death receptors can be reduced by epigenetic changes such as CpG-island hypermethylation of gene promoters in response to stress signals [23, 24].

Abnormal expression of decoy receptors presents an alternative mechanism of resistance to TRAIL- or CD95-induced apoptosis. To this end, the decoy receptor 3 (DcR3), which counteracts CD95-mediated apoptosis by competitively binding CD95 ligand, was shown to be overexpressed in lung carcinoma or colon carcinoma and in glioblastoma [25, 26] and TRAIL-R3; a decoy receptor for TRAIL was reported to be expressed at high levels in gastric carcinoma [27].

In addition, anti-apoptotic proteins with a death effector domain (DED) such as cellular FLICE-Inhibitory Protein (cFLIP) and phosphoprotein enriched in diabetes/ phosphoprotein enriched in astrocytes-15 kDa (PED/PEA-15) can be aberrantly expressed upon cellular stress [28, 29]. For example, high oxygen tension (hyperoxia) has been reported to lead to upregulation of cFLIP, which inhibited apoptosis during hyperoxia by suppressing both extrinsic and intrinsic apoptotic pathways, the latter via inhibition of Bax [30]. Because of their sequence homology to caspase-8, both cFLIP and PED can be recruited into the death-inducing signaling complex (DISC) upon receptor ligation instead of procaspase-8, thereby preventing caspase-8 activation [28, 29].

Moreover, the expression of caspase-8 or its function is impaired by genetic or epigenetic mechanisms in various cancers. For example, caspase-8 mutations were identified in some tumors, that is, in colorectal and head and neck carcinomas, although the overall frequency is low [31, 32]. In addition, homo- or heterozygous genomic deletions were detected in neuroblastoma [33]. Alternative splicing of intron 8 of the caspase-8 gene resulting in the generation of caspase-8L, a catalytically inactive splice variant presents another mechanism of caspase-8 inactivation [34, 35]. Epigenetic silencing secondary to hypermethylation of regulatory

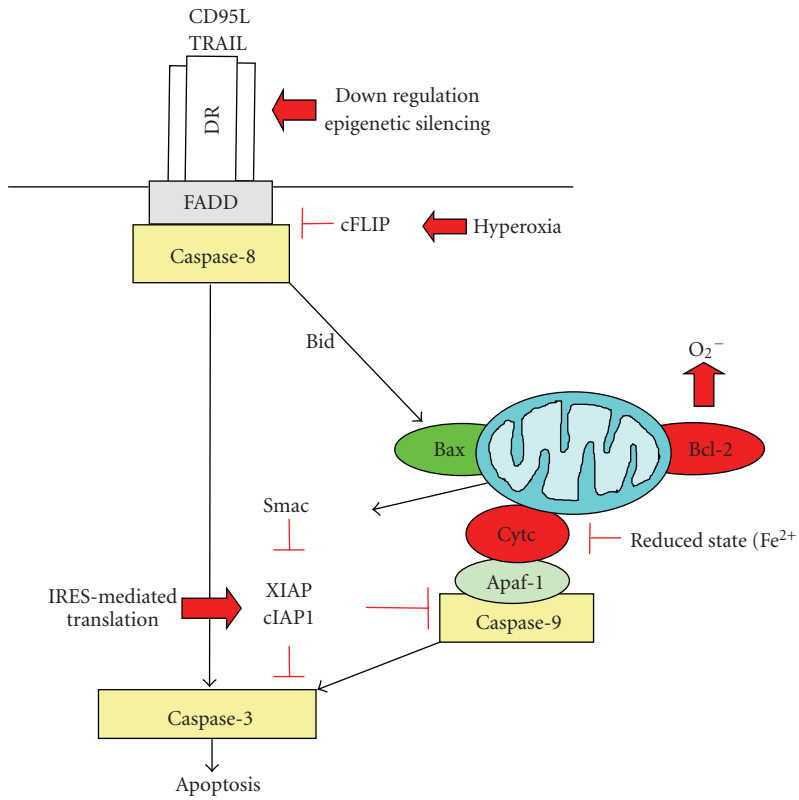

Figure 1: Interplay of cellular stress signals with apoptosis pathways. Signaling via the death receptor pathway can be inhibited by downregulation or epigenetic silencing of death receptors (DRs) or upregulation of cFLIP by hyperoxia. In the mitochondrial pathway, Bcl-2 favors a prooxidant milieu that promotes survival, while the reduced form of cytochrome c is inhibited in its activity to trigger caspase activation via the apoptosome. At the postmitochondrial level, translation of XIAP and cIAP1 is sustained via an IRES-dependent mechanism even under cellular stress conditions. See text for more details.

sequences of the caspase-8 gene occurs in various tumors, for example, neuroblastoma, malignant brain tumors, Ewing tumor, retinoblastoma, rhabdomyosarcoma, or small lung cell carcinoma [33, 36–39]. Furthermore, phosphorylation of caspase-8 on tyrosine 308 by, for example, Src has been shown to interfere with its proapoptotic activity [40].

3.2. Evasion of the Mitochondrial Pathway

3.2.1. Bcl-2 Family Proteins. The Bcl-2 family of proteins consists of both anti-apoptotic proteins, for example, Bcl-2, Bcl-X_L, and Mcl-1, as well as proapoptotic molecules such as Bax, Bak, and BH3 domain only molecules [8]. There are currently two models to explain the activation of Bax and Bak by BH3-only proteins. The direct activation model holds that BH3-only proteins, which act as direct activators such as Bim and the cleaved form of Bid (tBid), bind directly to Bax and Bak to trigger their activation, while BH3-only proteins that act as sensitizers, for example, Bad, bind to the prosurvival Bcl-2 proteins [41]. According to the indirect activation model, BH3-only proteins activate Bax and Bak in an indirect fashion by engaging the multiple anti-apoptotic Bcl-2 proteins that inhibit Bax and Bak, thereby releasing their inhibition on Bax and Bak [42, 43]. Regardless of the exact mode of Bax and Bak activation, the ratio of anti-apoptotic versus proapoptotic Bcl-2 proteins rather than the expression levels of one particular molecule of the Bcl-2 family regulates apoptosis sensitivity.

An increase in the ratio of anti- to proapoptotic Bcl-2 proteins has been detected in various cancers and has been correlated to tumor cell survival and apoptosis resistance. More recently, Bcl-2 has also been implicated in the regulation of the intracellular redox status [44]. Bcl-2 localizes to mitochondrial membranes as well as the endoplasmatic reticulum and the nuclear envelope, which are all sites of ROS production [45]. While Bcl-2 has initially been described as an anti-oxidant because of its inhibitory effect on H_2O_2-induced lipid peroxidation [46], there is also evidence that Bcl-2 may promote a prooxidant intracellular milieu. Accordingly, ectopic expression of Bcl-2 resulted in an elevated constitutive level of superoxide anion and intracellular pH in leukemia cells [47]. Conversely, reduction of intracellular superoxide sensitized Bcl-2-overexpressing tumor cells to apoptotic stimuli independent of the mitochondria [47]. These findings provide a link between oncogene-mediated alterations in the intracellular redox status and cell survival.

3.2.2. Cytochrome c. Besides Bcl-2, also cytochrome c has been implicated in the redox regulation of apoptosis. Once cytochrome c is released from mitochondria into the cytosol, it triggers formation of the cytochrome c/Apaf-1/Caspase-9-containing apoptosome, which in turn lead to activation of caspase-9 and downstream effector caspases [48]. There is recent evidence that also the redox state of cytochrome c is involved in the regulation of apoptosis. To this end, the oxidized form of cytochrome c (Fe(3+)) has been reported to induce caspase activation via the apoptosome, while the reduced form of cytochrome c (Fe(2+)) is unable to do so [49–51]. Several mechanisms have been discussed to be responsible for this redox-mediated regulation of cytochrome c activity, including different affinities of the oxidized versus the reduced form of cytochrome c for binding to Apaf-1, different abilities of these cytochrome c forms to activate Apaf-1, or, alternatively, different affinities for other factors not belonging to the apoptosome. Regardless of the exact mechanisms, this regulation of the redox state of cytochrome c opens the possibility of controlling the effector phase of apoptosis at a postmitochondrial level.

Besides these genetic alterations in Bcl-2 family proteins, impairment of mitochondrial apoptosis may also occur at the postmitochondrial level. For example, expression level or activity of Apaf-1 may be reduced due to promoter hypermethylation or loss of heterozygosity at chromosome 12q22-23, which in turn leads to impaired assembly of a functional apoptosome [52–56].

3.3. Evasion of Apoptosis via Aberrant Expression of "Inhibitor of Apoptosis" (IAP) Proteins. Moreover, tumor resistance to apoptosis may be caused by aberrant expression or function of "Inhibitor of Apoptosis" (IAP) proteins. IAP proteins are a family of endogenous caspase inhibitors with eight human members, that is, XIAP, cIAP1, cIAP2, survivin, livin (ML-IAP), NAIP, Bruce (apollon), and ILP-2 [10, 57]. All IAP proteins have at least one baculovirus IAP repeat (BIR) domain that is required for classification as IAP family protein. This domain is also the region of the protein that mediates the interaction with caspases [58]. Among the IAP family proteins, XIAP exhibits the strongest anti-apoptotic properties and inhibits apoptosis signaling by binding to active caspase-3 and -7 and by preventing caspase-9 activation [59].

The expression and function of IAP proteins are tightly regulated by several mechanisms, among them is translational regulation [60]. To this end, it is particularly interesting to note that XIAP and cIAP1 belong to the proteins, which are translated via an internal ribosome entry site (IRES). This unique property enables protein translation of these IAP proteins even under cellular stress conditions when protein synthesis is usually shut down, for example, because of caspase-dependent breakdown of eukaryotic translation initiation factors coupled with activation of the double-stranded RNA-activated protein kinase PKR [61].

Typically, mRNA molecules are translated via a cap-dependent translation mechanism [62]. However, the mRNAs encoding XIAP or cIAP1 protein contain very long 5′ untranslated regions (UTRs), which are not amenable to a ribosome-scanning translation initiation mechanism and thus, require a cap-independent translation initiation mechanism, that is, IRES-mediated translation [60]. IRES-mediated translation allows for the continued translation of XIAP and cIAP1 even under conditions where cap-dependent translation is inhibited such as cellular stress [60]. In addition, IRES-mediated translational regulation of XIAP and cIAP1 expression enables a rapid response to transient cellular stress conditions in order to delay cell death and ensure survival. Of note, cellular stress signals, including low-dose irradiation, anoxia, serum starvation and chemotherapeutic drugs, have been reported to stimulate the IRES activity of XIAP or cIAP1 [63–66]. This is in line with the concept that such stress signals promote cell survival under stress conditions, at least in part, via IRES-mediated upregulation of anti-apoptotic proteins.

4. Conclusions

Evasion of apoptosis is one of the hallmarks of human cancers that promote tumor formation and progression as well as treatment resistance. Cellular stress signals can contribute to evasion of apoptosis by activating anti-apoptotic and cell survival programs that ultimately block cell death. This interference with proper apoptosis signaling under stress conditions can occur at different points of the apoptosis signaling network, for example, within the death receptor or the mitochondrial pathway or at the postmitochondrial level. Whether or not cellular stress eventually engages cell survival or cell death programs also depends on the type and strength of the stress stimulus as well as the cell type. A better understanding of the molecular mechanisms of this interplay between the cellular stress response and anti-apoptotic programs is expected to yield novel molecular targets for therapeutic interventions. The aim is to prevent protective responses in order to maximize the antitumor activity of anticancer treatment approaches. This strategy will hopefully lead to more effective treatment options for cancer patients.

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Research Article

PCI-24781, a Novel Hydroxamic Acid HDAC Inhibitor, Exerts Cytotoxicity and Histone Alterations via Caspase-8 and FADD in Leukemia Cells

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Histone deacetylase inhibitors (HDACi) have become a promising new avenue for cancer therapy, and many are currently in Phase I/II clinical trials for various tumor types. In the present study, we show that apoptosis induction and histone alterations by PCI-24781, a novel hydroxamic acid-based HDAC inhibitor, require caspase-8 and the adaptor molecule, Fas-associated death domain (FADD), in acute leukemia cells. PCI-24781 treatment also causes an increase in superoxide levels, which has been reported for other HDACi. However, an antioxidant does not reverse histone alterations caused by PCI-24781, indicating that ROS generation is likely downstream of the effects that PCI-24781 exerts on histone H3. Taken together, these results provide insight into the mechanism of apoptosis induction by PCI-24781 in leukemia by highlighting the roles of caspase-8, FADD and increased superoxide levels.

1. Introduction

Epigenetics is currently defined as the heritable changes in gene expression without alterations in DNA sequence [1]. Epigenetic alterations include histone modification, DNA methylation, and microRNA expression. In particular, abnormal histone tail modifications such as acetylation have been linked to tumor progression [2]. Histone acetylation is modulated by two families of enzymes: histone deacetylases (HDACs) and histone acetyltransferase (HATs). Irregular patterns of histone acetylation have been hypothesized to silence tumor suppressor genes in human cancer cells [3]. Consequently, restoring the normal complement of gene expression has become a therapeutic goal. The HDAC inhibitors (HDACi) are a structurally diverse family of anticancer drugs that target these abnormal histone acetylations by inhibiting HDAC enzymes [4]. In mammalian systems, eleven HDAC enzymes are grouped into four classes based on structural and functional characteristics. Consequently, the HDACi compounds are often categorized based on their ability to inhibit various HDAC classes. The approval of vorinostat (suberoylanilide hydroxamic acid (SAHA)), a pan-HDAC inhibitor, by the U.S. Food and Drug Administration for treatment of cutaneous T-cell lymphoma [5] was a recent major milestone in validating the clinical utility of this class of compounds. This success has encouraged the preclinical and clinical developments of dozens of other HDACi.

One such compound is PCI-24781 (formerly known as CRA-024781), a novel, orally dosed HDACi. Like vorinostat, PCI-24781 is a hydroxamic acid, and can inhibit all Class I and Class II HDAC isoforms, although it is reported to be a more potent inhibitor of HDACs 1 and 3 at low concentrations [6]. Assessment of in vitro activity against tumor cell lines revealed growth inhibition of multiple solid tumor lines including colon, breast, lung, prostate, ovarian, Hodgkins lymphoma, and non-Hodgkins lymphoma [7]. Only one published study has probed the mechanism of cell death induced by PCI-24781 in a series of lymphoma lines and reported caspase activation and generation of reactive oxygen species, consistent with the mechanism of cytoxicity of other HDACi [7]. Tumor inhibition and histone acetylation were also noted *in vivo* in glioma, colon, and lung tumor xenograft models [6].

Our current study seeks to extend these mechanistic studies to acute leukemia cells and to clarify the specific role of caspase-8 and the adaptor molecule Fas-associated death domain (FADD) in the mechanism of apoptosis induced by PCI-24781. Effects on acetylation of histone H3 by PCI-24781 were also examined in acute lymphocytic leukemia (ALL) cells and in variants lacking caspase-8 or FADD, and revealed a lower degree of histone H3 acetylation in the latter lines. This surprising result highlights the importance of these two components of the Fas receptor pathway in conferring sensitivity to PCI-24781 in acute lymphocytic leukemia cells.

2. Material and Methods

2.1. Cell Lines. Jurkat, I2.1 (FADD deficient Jurkat cells) and CEM human leukemia cell lines were acquired from American Type Culture Collection (Manassas, VA). I9.2 (caspase-8 deficient Jurkat cells) were provided by Dr. Michael Andreeff (The University of Texas M. D. Anderson Cancer Center (UTMDACC), Houston, TX). All cells were grown in a humidified incubator with 5% CO_2 at 37°C and cultured in RPMI 1640 with 10% (v/v) heat-inactivation fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma St. Louis, MO).

2.2. Reagents. PCI-24781 was kindly provided by Pharmacyclics Inc. (Sunnydale, CA). Trypsin-ethylenediaminetetraacetic acid (EDTA), propidium iodide (PI), N-acetyl cysteine (NAC), Buthionine sulfoximine (BSO), and Triton X-100 were purchased from Sigma (St. Louis, MO). Dye for the detection of intracellular superoxide (dihydroethidium [HEt]) was purchased from Molecular Probes (Eugene, OR). Caspase-3 substrate, DEVD-amc, was purchased from Biomol International, LP (Plymouth Meeting, PA). The caspase inhibitors zVAD-fmk and IETD-fmk were purchased from Calbiochem (San Diego, CA). Antibodies were purchased for caspase-3 (Cell Signaling, San Diego, CA), polyclonal anti-acetyl-histone H3 (Abcam, Inc., Cambridge, MA), and actin (Sigma). Annexin V-fluorescein isothiocyanate (Annexin V-FITC) was purchased from BD Bioscience (Franklin Lakes, NJ). QVD-OPH was purchased from MBL International (Woburn, MA)

2.3. Assessment of DNA Fragmentation. Apoptosis was assessed by determining the percentage of subdiploid cells using PI staining followed by flow cytometric analysis as previously described [8]. The cells were incubated for 24 hours, centrifuged, and resuspended in 500 μL of PI solution (50 μg/mL PI, 0.1% Triton X-100, and 0.1% sodium citrate in PBS). Samples were assessed by flow cytometry on the FL-3 channel (FACSCalibur, Becton, Dickinson, Franklin Lakes NJ). CellQuest software was used for the analysis of the data (BD Bioscience, Franklin Lakes NJ).

2.4. Annexin V Staining. Phosphatidylserine was measured by Annexin V-FITC staining according to the manufacturer's protocol. CEM cells were pretreated with 5 μM QVD-OPH (a pan caspase inhibitor) and treated with 0.5 μM PCI-24781 for 30 hours, washed twice in cold PBS, resuspended in 1 X binding buffer (0.01 M HEPES, pH 7.4; 0.14 M NaCl; 2.5 mM $CaCl_2$), and incubated for 30 minutes in the dark at room temperature with 5 μL Annexin V-FITC and 10 μL of 50 μg/mL PI. Samples were analyzed by flow cytometry on the FL-1 (for FITC) and FL-3 (for PI) channels and analyzed using CellQuest software.

2.5. Detection of Intracellular Superoxide. The intracellular superoxide level was measured using the cell-permeable HEt dye as previously described [9]. Cells were centrifuged and resuspended in 1 mL of phosphate buffered saline (PBS) containing 10 μM HEt. The samples were incubated for 30 minutes in the dark at 37°C. Fluorescence intensity was assessed by flow cytometer on the FL-3 (for HEt) channel and analyzed by CellQuest software.

2.6. Caspase-3-Like Activity Assays. Cells were centrifuged, resuspended in 100 μL PBS and lysed by freezing and thawing. To each well, 50 μL of lysate and 150 μL of 50 μM DEVD-amc in DEVD buffer (10% sucrose, 0.001% IGEPAL, 0.1% CHAPS, 5 mM HEPES, pH 7.25) were added in duplicates on a 96-well plate. The release of fluorescence (amc) generated from the cleavage of DEVD-amc was measured using a spectrofluorometer (SpectraMax Gemini EM, Molecular Devices, Sunnyvale, CA) using an excitation of 355 nm and emission of 460 nm.

2.7. Western Blotting. After treatment, Jurkat cells (5×10^6) were resuspended in lysis buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 20 mM sodium phosphate, pH 7.4). Aliquots of protein lysates (30 μg) were loaded on 12% sodium dodecyl sulfate- (SDS) polyacrylamide gels, transferred to nitrocellulose membranes, and blocked overnight at 4°C with 5% nonfat dry milk in Tris-Buffered Saline 0.05% Tween-20 (TBS-T). Membranes were probed with 1 : 1000 dilution of primary antibody in 5% milk in TBS-T. The bound antibodies were detected using enhanced chemiluminescence, ECL plus Western blotting detection

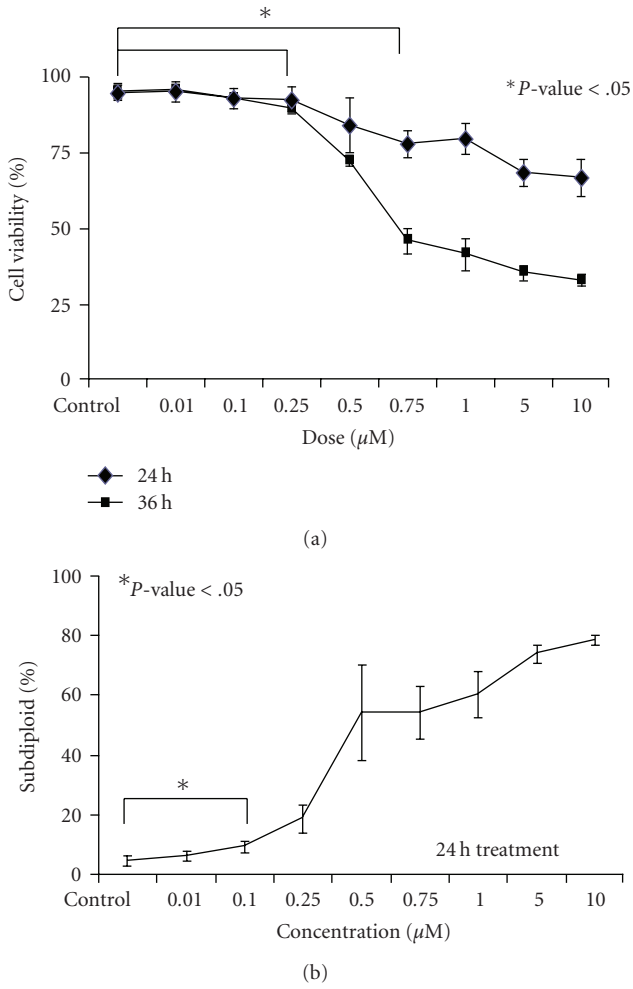


Figure 1: *The exposure of PCI-24781 increases cytotoxic effects of leukemic cells.* Jurkat cells were treated with a range of PCI-24781 doses (0.01 μM–10 μM), (a) incubated for 24 and 36 hours, and the % cell viability was quantitated by trypan blue exclusion using a Vi-Cell Coulter Counter, (b) incubated for 24 hours, dyed with PI reagent, and analyzed by flow cytometry to assess DNA fragmentation. Error bars represent the means ± S.D. of three independent experiments. $^*P < .05$ compared to control.

system (Amerham Bioscience, UK limited, Little Chalfont Buckinghamshire, England).

2.8. Statistical Analysis. For each condition, multiple experiments were performed, and the results are presented as the mean ± standard deviation (S.D). The differences between two group conditions were analyzed using independent, two-tailed *t*-tests (Microsoft Excel software, Redmond, WA).

3. Results

3.1. PCI-24781 Induces In Vitro Apoptotic Cell Death in Leukemia Cells. Previous research indicated that PCI-24781 is cytotoxic in multiple solid tumor lines [6], however the study of this drug in hematopoetic cells is limited to one study conducted in Hodgkins lymphoma and non-Hodgkins lymphoma cell lines [7]. To extend these mechanistic studies to leukemia cells, the cytotoxic effects of PCI-24781 were investigated in an ALL cell line. Jurkat cells were treated with a range of PCI-24781 doses (0.01 μM–10 μM), incubated for 24 hours and 36 hours, and percent viability was quantified by trypan blue exclusion. As seen in Figure 1(a), there was a significant reduction in Jurkat cell viability beginning at the 0.25 μM and 0.75 μM dose of PCI-24781 after exposure for 24 or 36 hours, respectively, (P-value < .05).

Having identified doses at which PCI-24781 is cytotoxic to ALL cells, the next step was to examine whether the observed cell death was due to apoptosis. DNA fragmentation is a well-defined characteristic of apoptosis and can be quantified by measuring the increase in the percentage of cells containing subdiploid amounts of DNA by staining cells with PI. Jurkat cells were treated with a range of PCI-24781 doses (0.01 μM–10 μM), incubated for 24 hours, stained with PI and assessed by flow cytometry. Figure 1(b) shows that a 24-hour exposure to PCI-24781 led to a dose-dependent increase in DNA fragmentation beginning at the 0.1 μM dose (P-value < .05).

3.2. PCI-24781 Induced Apoptosis Is Caspase Dependent. Having demonstrated that the cytotoxic effects of PCI-24781 in ALL cells involve DNA fragmentation, we next investigated if a caspase-dependent apoptotic pathway was activated. Jurkat cells were pretreated with 10 μM zVAD-fmk (a pan caspase inhibitor) for 30 minutes and then treated with 5 μM PCI-24781 for 24 hours, followed by PI staining and flow cytometry. As shown in Figure 2(a), the pan-caspase inhibitor alone had no effect on DNA fragmentation. However, apoptotic DNA fragmentation induced by PCI-24781 was significantly reduced when caspase activity was blocked (P-value < .05).

Since caspase-3 activation induces apoptotic DNA fragmentation, this end point was specifically examined in Jurkat cells in response to treatment with PCI-24781. Caspase-3-like activity was measured by monitoring fluorescence levels generated from the hydrolysis of the DEVD-amc fluorogenic substrate. Jurkat cells were pretreated with zVAD-fmk for 30 minutes, and then treated with 5 μM PCI-24781 for 16 hours. Figure 2(b) shows that 5 μM PCI-24781 increased caspase-3-like activity by 7-fold as compared with control. In addition, pretreatment with the pan caspase inhibitor, zVAD-fmk, successfully abrogated the increase of caspase-3-like activity induced by 5 μM PCI-24781 (P-value < .05). Although caspase-3-like activity was higher with the 0.5 μM dose compared to 5 μM PCI-24781, these results most likely reflect that the higher dose (5 μM) is peaking at an early time point. This idea is supported by Figure 3(d), in which a time course with 5 μM revealed that maximum levels are reached at 14 hours and begin to decline after this time point. Analysis of later time points, after 16 hours, most likely will further support this idea.

DEVD-amc has been criticized as a nonspecific substrate for caspase-3, because it can detect caspase-3 and/or caspase-7 activities. Caspase activation can also be measured by

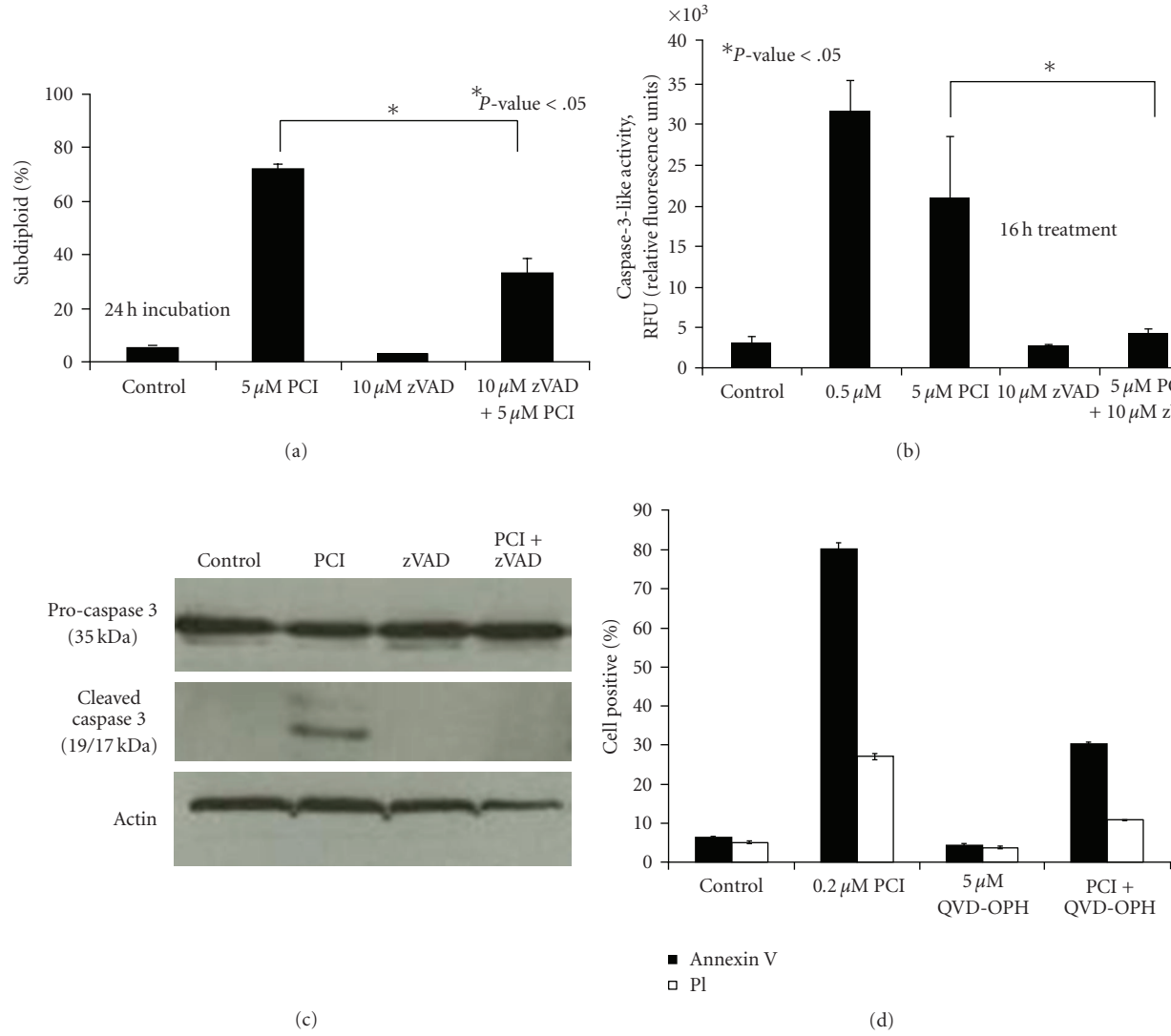
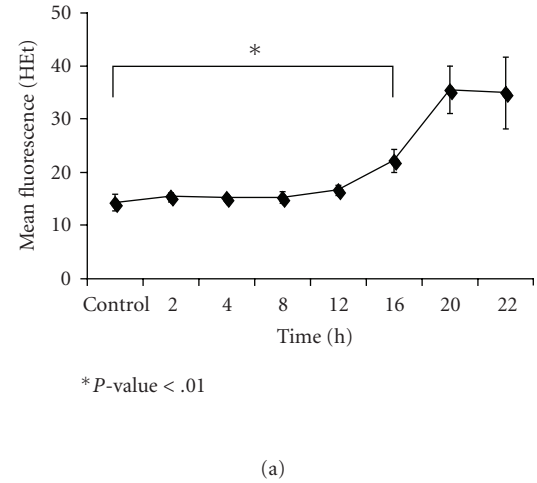


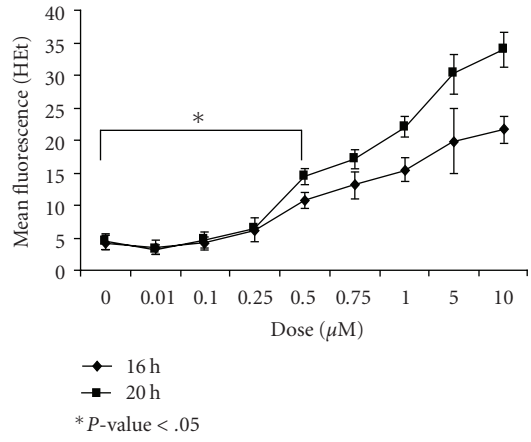
Figure 2: *The exposure of PCI-24781 induces caspase-dependent apoptosis in leukemic cells.* (a) DNA fragmentation was assessed in Jurkat cells treated with 5 μM of PCI-24781 (PCI), with or without 30 minutes of zVAD-fmk pretreatment, incubated for 24 hours, dyed with PI reagent, and assessed on the flow cytometer. (b) *Jurkat* cells were treated with 0.5 μM and 5 μM PCI-24781, with or without 30 minutes of zVAD-fmk pretreatment, and incubated for 16 hours. Incubated cells were lysed and stained with DEVD-amc to measure caspase-3-like activity. The release of amc was measured on a spectrofluorometer using an excitation of 355 nm and an emission of 460 nm. (c) In protein lysates from cells treated with 5 μM PCI-24781 for 16 hours, procaspase 3, cleaved caspase 3, and actin were measured by western blot. (d) CEM cells were pretreated with 5 μM QVD-OPH for 2 hours and treated with 0.5 μM PCI-24781 for 30 hours. Percentage cells positive with Annexin V/PI staining was measured for both. Error bars ((a), (b), and (d)) represent the means ± S.D. of three independent experiments. ((a) and (b)) $^*P < .05$ relative to 5 μM PCI-24781.

western blotting to visualize the cleavage of the large and small subunits of the caspase. To investigate if PCI-24781 specifically results in caspase-3 activation, cleaved caspase-3 was measured by western blot. The 19-kDa and 17-kDa cleaved products were evident after treatment with 5 μM PCI-24781, but there was no caspase-3 cleavage when the drug was combined with zVAD-fmk pretreatment (Figure 2(c)), verifying that caspase-3 activation is a consequence of PCI-24781 treatment.

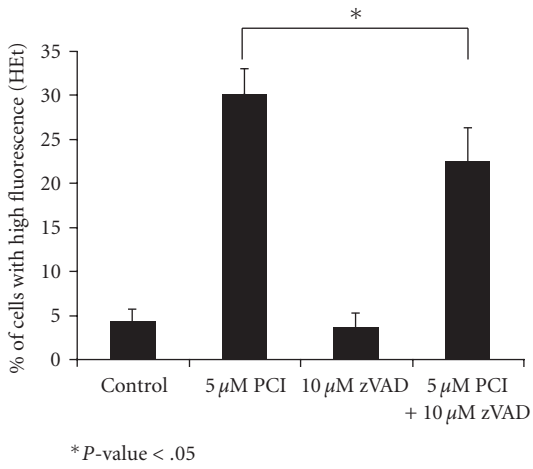
In order to further validate the results in Jurkat cells, apoptosis was measured in a different ALL cell line (CEM) and by detection of a different biochemical event that occurs during apoptotic cell death. Annexin V binds to phosphatidylserine displayed on the cell membrane, which is required for efficient disposal of the apoptotic cell. CEM cells were pretreated with 5 μM QVD-OPH and treated with 0.2 μM PCI-24781 for 30 hours. Cells were stained with Annexin V/PI and then analyzed by flow cytometry. As expected, in CEM cells, the percentage of Annexin V positive cells increases with PCI-24781 treatment and decreases when caspase activation is inhibited in PCI-24781 treated cells (Figure 2(d)).



(a)



(b)



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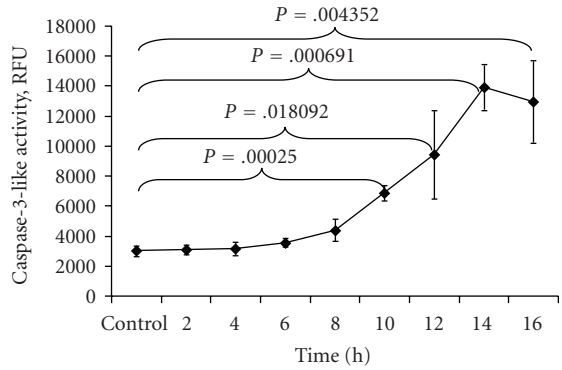
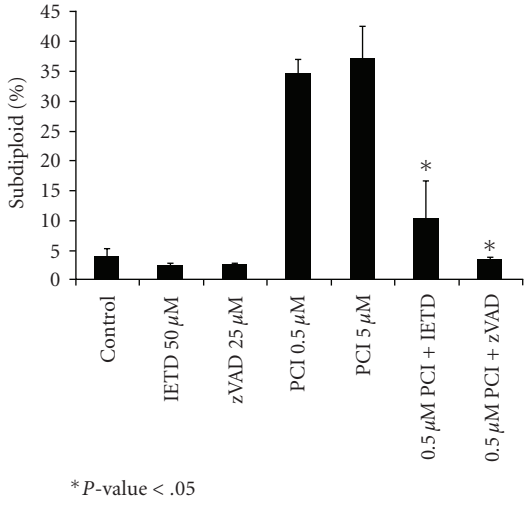
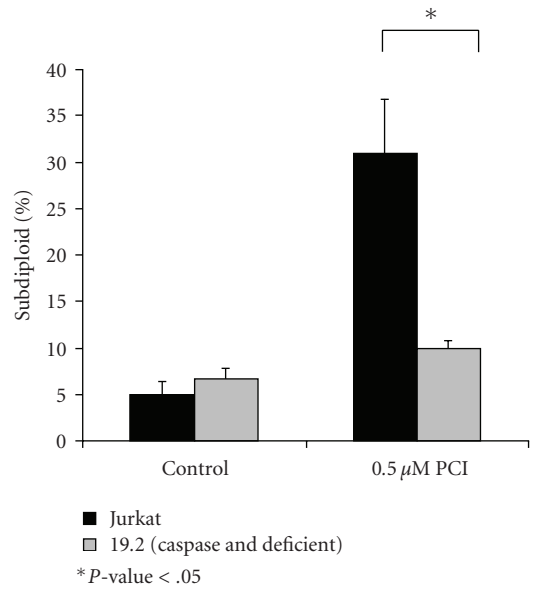


(d)

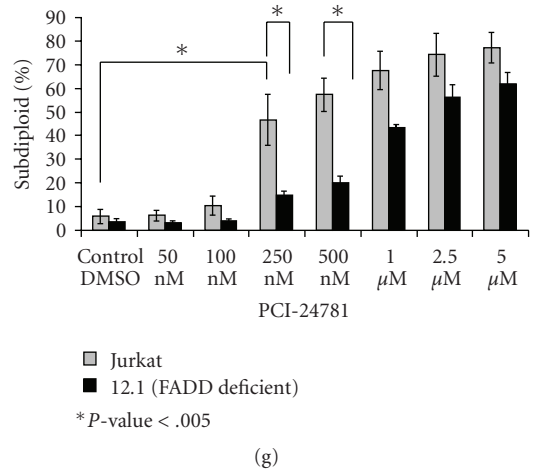


(e)



(f)

Figure 3: Continued.



(g)

Figure 3: *PCI-24781 induces ROS generation in a caspase-dependent and time-dependent manner*. (a) Jurkat cells were treated with 5 μM of PCI-24781, incubated between 2 and 22 hours. Intracellular superoxide was measured at each time point using dihydroethidium (HEt) and assessed by flow cytometry. $^*P < .01$ compared to control. (b) Jurkat cells were treated with a range of PCI-24781 doses (0.01 μM–10 μM), and incubated 16 and 20 hours. The mean HEt fluorescence at each dose was measured by flow cytometry. $^*P < .05$ compared to control. (c) Jurkat cells were treated with 5 μM PCI-24781, with or without zVAD-fmk pretreatment, and incubated for 24 hours, intracellular superoxide levels were assessed using HEt. $^*P < .05$. (d) Jurkat cells were treated with 5 μM PCI-24781, incubated between 4 and 16 hours, lysed and stained with DEVD-amc to assess caspase-3-like activity. Caspase-3 activity was measured in relative fluorescence unit (RFU). The release of amc was measured on a spectrofluorometer using an excitation of 355 nm and an emission of 460 nm. $^*P < .05$ compared to control. (e) Jurkat cells were pretreated with either inhibitor of caspase 8 (IETD-fmk) or a pan caspase inhibitor (zVAD-fmk) for 30 minutes. After pretreatment, the samples were treated with 0.5 or 5 μM PCI-24781 for 16 hours and dyed with PI reagent. DNA fragmentation (% subdiploid) was assessed by flow cytometer. (f) Jurkat and I9.2 cells were treated with or without 0.5 μM PCI-24781 and incubated by 16 hours, dyed with PI reagent, and assessed on the flow cytometer. $^*P < .05$ relative to Jurkat 0.5 μM PCI-24781. (a)–(f) Error bars represent the means ± S.D. of three independent experiments. (g) Jurkat and I2.1 cells were treated with or without 0.5 or 5 μM PCI-24781 and incubated by 16 hours, dyed with PI reagent, and assessed on the flow cytometer. $^*P < .05$ relative to Jurkat cells treated with PCI-24781.

3.3. PCI-24781 Induces ROS Generation in a Caspase-Dependent and Time-Dependent Manner. ROS have been shown to induce apoptosis by the release of cytochrome c from the mitochondria, which activates the caspase cascade. Previous studies have shown that many cancer cells have higher ROS levels compared to normal cells [10]. Therefore, one therapeutic approach is to use ROS generating anticancer agents that push intracellular ROS levels beyond a critical threshold and induce apoptosis. Various anticancer drugs like HDAC inhibitors have been shown to increase intracellular ROS levels as a single agent [11]. Since we observed caspase-3 activation in ALL cells treated with PCI-24781, we sought to determine the role of ROS by measuring intracellular superoxide levels. Jurkat cells were treated with 5 μM of PCI-24781, incubated for various times spanning 2–22 hours, stained with dihydroethidium, and analyzed by flow cytometry. As shown in Figure 3(a), ROS increased in a time-dependent manner beginning at 16 hours (P-value < .01) and peaks at 20 hours. Figure 3(b) shows that ROS also increases in a dose-dependent manner with PCI-24781 treatment, beginning at 0.5 μM at both 16 hours and 20 hours time points (P-value < .05).

Thus, so far our findings indicate that PCI-24781 induces apoptosis, which can be linked to ROS generation and/or caspase activation. The next step was to determine if ROS generation precedes or follows caspase activation. Superoxide levels were measured after exposure to 5 μM PCI-24781, with or without pretreatment of zVAD-fmk. Figure 3(c) shows that when caspase activity is blocked, PCI-24781 induced ROS generation is blunted (P-value = .03). Therefore, caspase activation plays a role in the increase of ROS levels seen with PCI-24781 treatment. Next, the kinetics of caspase activation was examined. Jurkat cells treated with 5 μM PCI-24781 were incubated between 4 and 16 hours, and caspase-3-like activity was measured using DEVD-amc as a substrate (Figure 3(d)). Since no ROS generation was observed at 8 hours of exposure to the same dose of PCI-24781 (data not shown), these results demonstrate that caspase activation occurs first, followed by ROS generation.

A single study has examined the molecular mechanism of apoptosis induction by PCI-24781 and reported that both caspase-8 and -9 are cleaved and activated by the HDACi in lymphoma lines. In order to more carefully examine the role of caspase-8 in PCI-24781 induced cell death, we used various peptide-based caspase inhibitors. Jurkat cells were treated with 0.5 μM and 5 μM PCI-24781, with or without pretreatment with either zVAD-fmk (a pan caspase inhibitor) or an inhibitor of caspase-8 (IETD-fmk). After 16 hours the cells were stained with PI reagent and DNA fragmentation was assessed by flow cytometer as shown in Figure 3(e). Results show no significant difference between 0.5 μM and 5 μM doses. Since 0.5 μM represents a potentially less toxic and more clinically relevant dose, we decided to combine the lower dose (0.5 μM) with the caspase inhibitors.

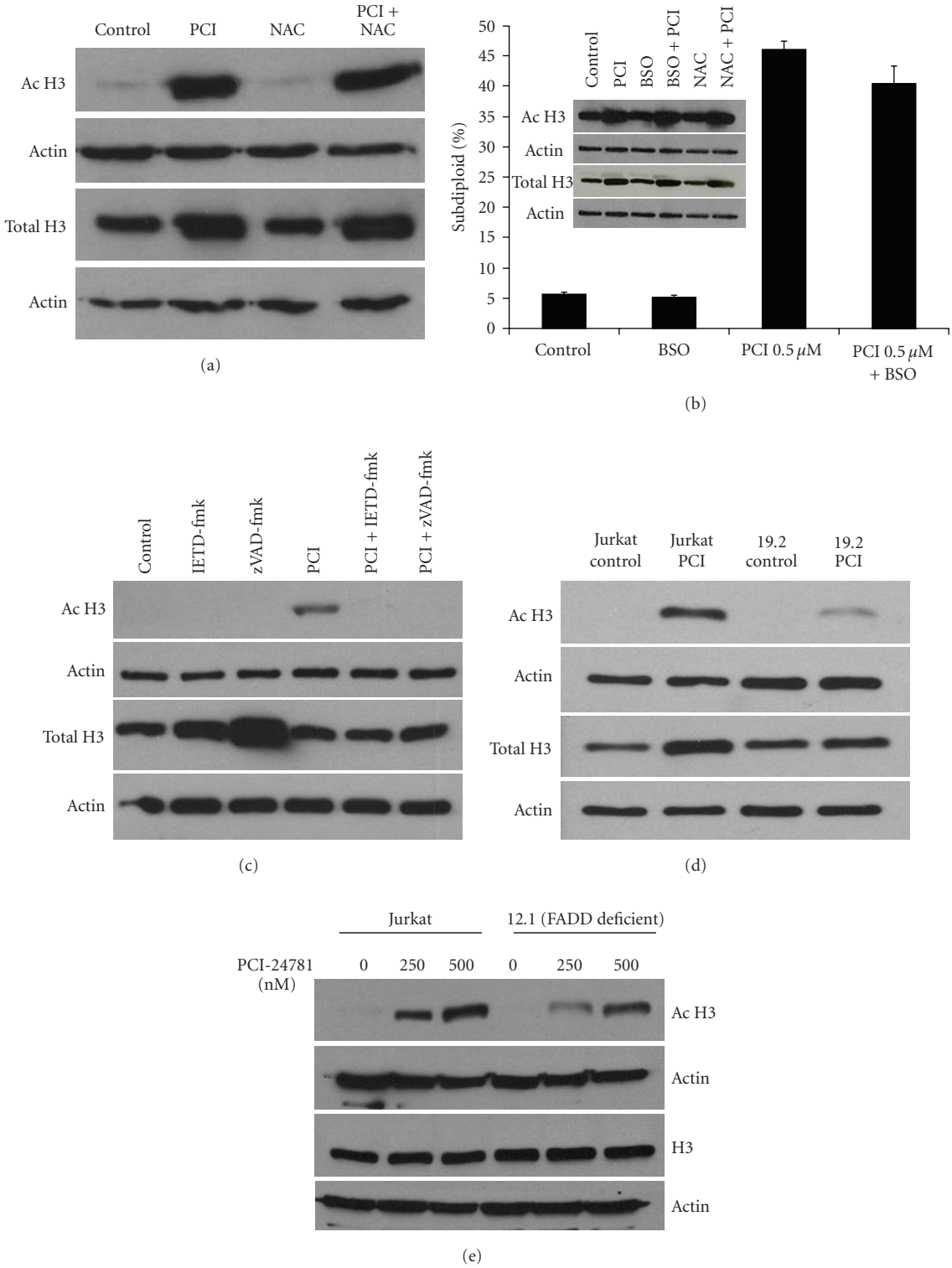


Figure 4: *PCI-24781 induces a caspase-dependent increase in both total and acetylated histone H3 protein expressions*. Jurkat cells were treated with 0.5 μM of PCI-24781, with or without pretreatment of (a) 24 mM NAC, (b) 1 mM buthionine sulfoximine (BSO), (c) 50 μM zVAD-fmk or IETD-fmk, for 30 minutes. After 16 hours of incubation, samples were either harvested to determine DNA fragmentation by PI staining or lysed for western blot. Acetylated histone H3, total histone H3, and actin were measured by western blot. (d) Jurkat and I9.2 cells were treated with or without 0.5 μM PCI-24781 and incubated for 16 hours. Lysates were harvested and lysed. Acetylated histone H3, total histone H3, and actin were measured by western blot. (e) Jurkat and I2.1 cells were treated with or without 0.25 or 0.5 μM PCI-24781 and incubated for 16 hours. Samples were harvested and lysed. Acetylated histone H3, total histone H3, and actin were measured by western blot. For Figures 4(a)–4(d), results are representative of at least two independent experiments.

DNA fragmentation by PCI-24781 was significantly reduced in the presence of IETD-fmk, suggesting that caspase-8 is involved in the induction of apoptosis by PCI-24781. To validate this result, I9.2 cells (a caspase-8 deficient Jurkat variant) [12] were treated with 0.5 μM PCI-24781 for 16 hours. Figure 3(f) shows a significant decrease of DNA fragmentation in I9.2 cells treated with the HDACi as compared with wildtype Jurkat cells, demonstrating again the importance of caspase-8 in PCI-24781 induction of apoptosis. Activation of caspase-8 requires participation of an adaptor molecule which links the death receptor (Fas in this case) with caspase activation. A second Jurkat cell variant, this one lacking FADD (I2.1), was used to further probe the functionality of the Fas pathway [12]. Figure 3(g) indicates that FADD deficiency reduces DNA fragmentation by PCI-24781.

3.4. PCI-24781 Induces Total and Acetylated H3 Protein Expression in a Caspase and FADD Dependent Manner. The primary mechanism of action of HDAC inhibitors is to prevent abnormal deacetylation of histones by antagonizing HDAC enzymes. This mechanism has been hypothesized to increase expression of tumor-suppressor genes. In order to test whether PCI-24781 induced histone acetylation is linked to the apoptotic pathway and generation of ROS, Jurkat cells were treated with 0.5 μM of PCI-24781, with or without pretreatment of NAC (an antioxidant that works by increasing levels of the most abundant intracellular antioxidant, glutathione (GSH)), BSO (an agent that depletes GSH) zVAD-fmk (the pan-caspase inhibitor), or IETD-fmk (caspase-8 inhibitor), for 30 minutes. After 16 hours of incubation, acetylated histone H3 (Ac H3) and total histone H3 (total H3) were measured by western blot. In addition DNA fragmentation was assessed for BSO pretreatment samples. Results show that, as expected, PCI-24781 exposure leads to an increase in acetylated histone H3 protein levels (Figures 4(a), 4(b) and 4(c)). Neither NAC nor BSO changed the increase in Ac H3 (Figures 4(a) and 4(b)), indicating that boosting or depleting GSH, respectively, has no effect on PCI-24781's ability to hyperacetylate histone H3. Consistent with this result, BSO depletion of GSH did not promote further DNA fragmentation induction by PCI-24781 when the two compounds were combined (Figure 4(b)). In contrast, inhibition of caspase activation with IETD-fmk or zVAD-fmk blocked the increase in Ac H3 protein levels (Figure 4(c)), implicating caspases in elevated levels of acetylated histone H3. To confirm the results obtained with the caspase-8 inhibitor, IETD-fmk, we examined the effects of PCI-24781 exposure on histone H3 acetylation in I9.2 cells. Our findings indicate that there was less of an increase in Ac H3 protein levels in caspase-8 deficient I9.2 cells as compared to Jurkat cells (Figure 4(d)). Similar results were obtained using FADD deficient I2.1 cells, supporting a role for FADD in the mechanism of PCI-24781-mediated histone H3 acetylation (Figure 4(e)). The difference between I2.1 and wildtype Jurkat cells was more apparent at the lower (250 nM) dose of PCI-24781, indicating that dose escalation could overcome the effect of FADD deficiency.

4. Discussion

The current study focuses on the cytotoxic effects of a hydroxamic acid HDACi, PCI-24781, in leukemia cells. Using Jurkat cell variants that lack caspase-8 (I9.2) or FADD (I2.1), we show that apoptosis induction and histone acetylation by this HDACi are dependent upon these two proapoptotic molecules. In particular, the effects of FADD deficiency are suggestive of a role for the extrinsic apoptotic pathway triggered by Fas/Fas ligand interactions in these cells. Data showing that inhibition of caspase activation by zVAD-fmk or a lack of FADD or caspase-8 decreases the total and acetylated protein levels of histone H3 induced by PCI-24781 was unexpected and interesting (Figures 4(c)–4(e)). Previous work in our laboratory has shown hyperacetylation of histone H3 in caspase-8 deficient cells when treated with a different HDACi, MS/SNDX-275, indicating that PCI-24781 may be unique in this regard [13]. However, a broader array of HDACi would need to be tested in order to determine if caspase-8 dependent acetylation is a feature exclusive to PCI-24781. Interestingly, this caspase-8 and FADD dependent protection against DNA fragmentation and histone alterations, however, appears to be surmountable by increased doses of PCI-24781. As shown in Figure 3(e), both the 0.5 μM and 5 μM doses of PCI-24781 induce similar amounts of DNA fragmentation as assessed by the percent subdiploid population. However, in comparing the degree of protection conferred by FADD deficiency for the two doses (Figure 3(g)), it is apparent that the higher dose (5 μM) of PCI-24781 is less protected than the lower (0.5 μM) dose. A similar pattern is observed when histone H3 acetylation by PCI-24781 is examined in FADD deficient cells (Figure 4(d)), which further supports the relationship between apoptosis induction and histone H3 acetylation. The exact mechanism linking caspase-8 or FADD to histone H3 acetylation is currently under investigation in our lab. Both HDAC dependent and independent scenarios are being considered. For HDAC dependent molecular explanations, it has been reported that caspase cleavage of HDACs can occur [14], resulting in their inactivation and histone H3 hyperacteylation. An HDAC-independent mechanism could explain increased histone H3 acetylation and total histone H3 levels if apoptotic DNA fragmentation (mediated by caspases) was causing release of histones from DNA [15]. In this case, free histone H3 (some acetylated and some unacetylated) would be detected in Triton soluble lysates to a lesser degree when caspases are inhibited.

Our results also address a role for oxidative stress in the mechanism of action of PCI-24781. Several structurally diverse HDACi are reported to heighten intracellular levels of superoxide and peroxide [11], and similar results were obtained for PCI-24781 by others in lymphoma lines [7] and by us in leukemia lines (Figures 3(a)–3(c)). This oxidative stress appears caspase dependent since zVAD-fmk has a statistically significant, albeit modest effect (Figure 3(c)). However, the antioxidant, NAC, which effectively blunts ROS production by PCI-24781 (data not shown) did not alter histone H3 acetylation by PCI-24781 (Figure 4(a)). Since NAC possesses potent antiapoptotic effects, this result

supports the notion that the histone H3 effects observed in the caspase-8 deficient and FADD deficient results are due to an HDAC dependent effect rather than a generalized apoptosis-mediated effect. Given reports from other groups that caspases can cleave specific HDAC family members rendering them inactive [14], and our results indicating that a pan-caspase inhibitor as well as a caspase-8 specific inhibitor can reverse acetylation changes by PCI-24781 (Figure 4(b)), this possibility will be further explored. The most widely cited mechanism of action for NAC's antioxidant effects is by bolstering total cellular GSH levels. We used BSO, a chemical inhibitor of the pathway critical for GSH synthesis, to determine if depletion of GSH would promote apoptosis and histone H3 acetylation by PCI-24781. It did not (Figure 4(b)), and together with the lack of effect of NAC on PCI-induced hyperacetylation, indicate that modulating GSH levels does not alter the drug's acetylation effects.

A role for death receptor induced apoptotic pathways involving TRAIL and Fas has been investigated in the mechanism of action of HDACi other than PCI-24781 such as trichostatin A [16], MS/SNDX-275 [17], valproic acid [18], and vorinostat alone and in combination with other agents such as proteasome inhibitors [12]. Caspase-8 activation has been described in a handful of these studies, however, components and regulators of the death inducing signaling complex (DISC) which ultimately result in caspase-8 activation have only been addressed in two HDAC related papers. One study proposes that depsipeptide, (also called FR901228) can upregulate FasL at the mRNA level in osteosarcoma cells resulting in caspase-8 and -3 activation [19]. The same investigators also reported in a subsequent paper that in Fas resistant osteosarcoma cells, depsipeptide causes downregulation of c-FLIP [20]. Since c-FLIP confers resistance to Fas-mediated apoptosis, lowering levels of c-FLIP is able to overcome resistance and promote caspase-8 activation. Whether these mechanisms will hold true for PCI-24781's effects in leukemia cells remains to be determined.

Despite the plethora of studies (including ours) citing caspase activation as a conserved event during HDACi induced cell death, numerous reports describe autophagic cell death as a consequence of treatment with this class of compounds. No study has as yet looked at PCI-24781's ability to induce auutophagy. However, a recent paper examined the caspase-8 and caspase-9 dependence of two hydroxamic acid HDACi: LAQ824 and LBH589 [21]. These investigators used a genetically tractable *in vivo* myc driven lymphoma model in which death receptor signaling was compromised due to overexpression of CrmA, a viral caspase-8 inhibitor, or due to deficiency of TRAIL. Caspase-9 deficiency and Apaf-1 deficiency were also incorporated into lymphoma model. Interestingly, none of these approaches to block caspase activation were able to prevent cell death by the two HDACi in the long term with morphological features of autophagy emerging. A caveat of these HDACi, though, is that unlike PCI-24781, these two compounds are reported to inhibit HDAC6, an HDAC family member which appears to regulate aggresome formation and autophagy [22]. In contrast, PCI-24781 displays the greatest potency for HDAC1 with no apparent selectivity for HDAC6 as compared to the remainder of the HDAC family.

Our work is the first to implicate the adaptor protein FADD specifically in the mechanism of action of an HDAC inhibitor. Compellingly, in addition to its function in the cytoplasm proximal to the Fas receptor, FADD is reported to be localized to the nucleus of resting cells. Using an internalization defective Fas mutant lymphoma cell line, Foger et al. found that FADD retaines its ability to translocate from nuclei to cytoplasm and suggest that a caspase-8 dependent feedback loop regulates FADD trafficking [23]. This model raises interesting possibilities regarding an endogenous role for nuclear FADD in transcriptional complexes that routinely contain HDAC family members. A molecular connection between HDACs and FADD offers insight into our novel observation that FADD deficiency is a determinant of sensitivity to PCI-24781. Thus, extrapolating our findings to other HDACi and to other cancer models may contribute to efforts to maximize the therapeutic efficacy of this interesting and versatile class of agents.

Acknowledgments

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Review Article

The Role of Cyclooxygenase-2 in Cell Proliferation and Cell Death in Human Malignancies

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It is well admitted that the link between chronic inflammation and cancer involves cytokines and mediators of inflammatory pathways, which act during the different steps of tumorigenesis. The cyclooxygenases (COXs) are a family of enzymes, which catalyze the rate-limiting step of prostaglandin biosynthesis. This family contains three members: ubiquitously expressed COX-1, which is involved in homeostasis; the inducible COX-2 isoform, which is upregulated during both inflammation and cancer; and COX-3, expressed in brain and spinal cord, whose functions remain to be elucidated. COX-2 was described to modulate cell proliferation and apoptosis mainly in solid tumors, that is, colorectal, breast, and prostate cancers, and, more recently, in hematological malignancies. These findings prompt us to analyze here the effects of a combination of COX-2 inhibitors together with different clinically used therapeutic strategies in order to further improve the efficiency of future anticancer treatments. COX-2 modulation is a promising field investigated by many research groups.

1. Introduction: Inflammation and Cancer are Linked

Inflammation is the major reaction of natural immunity with the goal to defend the organism against pathogens. It can be induced upon bacterial infections by compounds including lipopolysaccharides, as well as by viruses, which are detected by Toll-like receptors (TLRs), expressed by immune cells like macrophages. Besides, inflammation can be triggered by physical injuries (i.e., UV) or chemical compounds (i.e., reactive oxygen species) [1]. The activation of specific receptors triggers intracellular signals (i.e., NFκB, p38, or MAPKs-mediated), which regulate pro-inflammatory cytokine expression, such as interleukin 1 beta (IL1β), tumor necrosis factor alpha (TNFα), interleukin 6 (Il6), together with chemokines and cell adhesion proteins [1], in turn, leading to the recruitment and the activation of immune cells.

Several diseases are associated to chronic inflammation, such as osteoarthritis, Crohn's disease, and cancer [2]. Although the first evidence of a connection between inflammation and cancer dates back to more than a century ago [3], only recently, this link has been further investigated, thus evidencing that the incidence of several cancers is tightly associated to inflammation such as colon, breast, and prostate cancers [4–6]. This hypothesis is supported by the findings that the tumor microenvironment is characterized by the infiltration with different types of immune cells (*i.e.*, dendritic cells, lymphocytes, and macrophages) responsible for the release of cytokines [1]. The role of these cytokines in tumor incidence has been established in many studies. For example, the overexpression of TNFα in transgenic mice bearing a lung tumor is associated with an increase of the size of the tumor [7]. Moreover, a chronic intake of nonsteroidal antiinflammatory drugs (NSAIDs) leads to a significant reduction in the incidence of such tumors. Colorectal cancer (CRC), which remains an important cause of death in the industrialized world, is one of the most characterized types of tumor that benefits from treatment by NSAIDs [8]. Interestingly, chronic use of aspirin is reported to reduce the relative risk of CRC by about 50% [9]. Familial adenomatous polyposis, an inherited form of colon cancer, is characterized by the development of preneoplastic polyps.

At the molecular level, this disease is caused with a mutation of a tumor suppressor gene called Adenomatous polyposis coli (APC). It has been shown that the use of NSAIDs, like sulindac, as a chemopreventive treatment, is able to decrease the incidence of polyp formation [10]. Similar results were obtained with celecoxib [11], which is now approved by the Food and Drug Administration's Oncologic Drugs Advisory Committee as an adjuvant in FAP therapy.

A body of evidence indicates a role for inflammation in the development/modulation of different steps of cancer progression. Inflammation may play a role in tumor initiation by triggering the production of reactive oxygen species (ROS), responsible for DNA damage, thus increasing the rate of mutations [12]. It may also be implicated in tumor promotion, where inflammation triggers the secretion of growth factors, such as the epithelial (EGF) and fibroblast growth factors (FGF). These, in turn, favor the proliferation of initiated tumor cells by determining an imbalance between cell proliferation and cell death stimuli [6], due to the activation of different cell survival pathways [7].

Besides, the different cytokines produced during inflammation (*i.e.*, TNFα, IL1β, IL6, and IL8) can also activate several survival pathways, thus leading to an escape of tumor cells from cell death. Well known is the case of TNFα, produced by tumor and immune cells, which leads to the survival of cancer cells by the upregulation of antiapoptotic proteins, that is, Bcl-2 [13–15], *via* the activation of the nuclear factor kappa B (NFκB) [16]. The modulation of pro-survival pathways or anti-apoptotic proteins makes the expression/activation of such proinflammatory mediators also a determining factor in chemoresistance. A constitutive activation of such proinflammatory factors has been frequently found in many cancers, such as hepatocellular carcinoma [17], prostate cancer [18], as well as chronic and acute myeloid leukemia [19], where it is frequently associated with a bad prognosis. In these instances, the modulation of Bcl-2 anti-apoptotic family members has been frequently shown [13–15, 20].

Amongst the different mediators of inflammation, the cyclooxygenases (COXs) clearly appear to be implicated in cancer. This review focuses on COX-2, the inducible form, normally induced and implicated in inflammation, and intends to analyze what is currently known about the link between COX-2 and cancer, in terms of effects on cell proliferation and cell death. In this view, we will focus our attention on studies analyzing the effects of COX-2 inhibitors on cancer cells, when used alone as well as in combination with therapeutic approaches, including radiotherapy, chemotherapeutic agents, and photodynamic therapy. Finally, we will consider the relevance of COX-2-independent effects.

2. The Cyclooxygenase Enzyme Family

Cyclooxygenases (or prostaglandin H synthases), commonly referred to as COXs, are a family of myeloperoxidases located at the luminal side of the endoplasmic reticulum and nuclear membrane [21], which catalyze the rate-limiting step of prostaglandin biosynthesis from arachidonic acid [21]. These enzymes act by two coupled reactions. The first one is the conversion of arachidonic acid released from the plasma membrane by phospholipase A2 to prostaglandin G2 by the cyclooxygenase activity. The second reaction is mediated by the peroxidase activity and leads to the conversion of prostaglandin G2 to prostaglandin H2. Then, different synthases convert prostaglandin H2 to prostaglandin D2, F2α, E2, I2, and thromboxane A2 (Figure 1).

Prostanoids (prostaglandins and thromboxanes) are immediately released from the cells, where it is believed that they act locally in an autocrine and paracrine manner through different receptors activating different intracellular pathways still to be completely elucidated (Figure 1) [22]. Prostaglandins, specifically, are important for physiological functions like vasodilatation (PGD2, PGE2, PGI2), gastric cytoprotection (PGI2), maintenance of renal homeostasis, and platelet aggregation. Besides, prostaglandins play a major role in mediating fever (PGE2), pain sensitivity, and inflammation [21].

So far, three isoforms of COXs have been identified. Cyclooxygenase-1 (COX-1) is a glycoprotein of 71kDa, which is constitutively expressed in different tissues. COX-1 is encoded by a gene on chromosome 9 and plays a role in tissue homeostasis by modulating several cellular processes ranging from cell proliferation to angiogenesis or platelet aggregation due to thromboxane production [21].

Cyclooxygenase-2 (COX-2) is the inducible isoform, which is regulated by growth factors and different cytokines such as IL1β, IL6, or TNFα [23], therefore overexpressed during inflammation. The COX-2 gene is located on chromosome 1 and its promoter displays an NFκB response element as well as other cytokine-dependent (i.e., IL6) response elements [21]. The protein shows a 60% homology with COX-1 [24]; in addition, COX-2 presents a C-terminal extension and a different binding site for NSAIDs, which makes COX-2 a preferential target compared to COX-1, thus being specifically inhibited at lower doses [25].

Finally, COX-3 has been identified as a splice variant of COX-1, and it is present mainly in brain and spinal cord [26, 27]. Currently, the role of COX-3 is not known. Some pieces of evidence suggest a possible role in pain sensitivity, based on studies focused on the mechanism of action of acetaminophen (paracetamol), recently evoked as a selective inhibitor of COX-3 [28]. However, this hypothesis is debated because other findings argue that acetaminophen targets at the same time COX-2 [29].

3. COX-2 As a Tumor Promoter and a Good Candidate for Cancer Therapy

Overexpression of COX-2 has been detected in a number of tumors, such as colorectal breast as well as pancreatic and lung cancers [2, 30–32], where it correlates with a poor prognosis. Moreover, overexpression of COX-2 has been reported in hematological cancer models such as RAJI (Burkitt's lymphoma) and U937 (acute promonocytic leukemia) [33, 34] as well as in patient's blast cells [32, 34]. Data suggested that COX-2 may play a role in different steps of cancer progression, by increasing proliferation of mutated cells [30], thus favoring tumor promotion as well as by

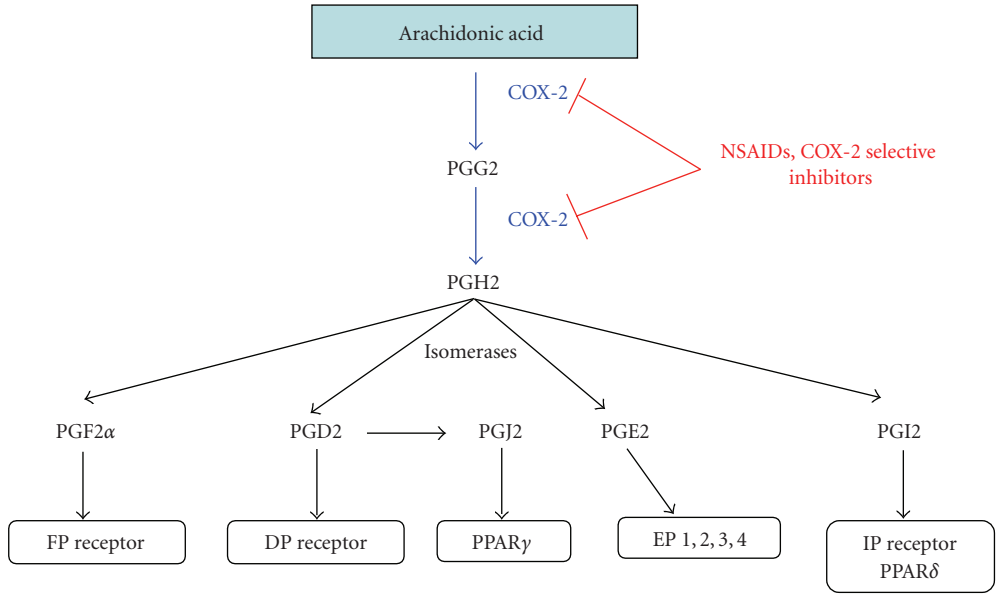

Figure 1: *Metabolism of arachidonic acid by COX-2 and receptors implicated in response to prostaglandins* (according to Chandrasekharan et al. [21]). Prostaglandins act through different receptors to mediate their effects. PGE2 is able to bind four receptors (EP1, 2, 3, and 4). These receptors do not possess the same ligand affinity and their expression is tissuedependent. The different receptors are associated with different intracellular pathways. Most of these receptors are localized in the plasma membrane but nuclear receptors PPARγ can also bind PGJ2. Abbreviation: COX-2, cyclooxygenase-2; PG, prostaglandin; FP, prostaglandin F receptor; DP, prostaglandin D receptor; EP, prostaglandin E receptor; IP, prostaglandin I receptor; PPAR, peroxisome proliferator-activated receptor; NSAIDs, nonsteroidal anti-inflammatory drugs.

affecting programmed cell death and affecting the efficacy of anticancer therapies [35–39] to be, finally, implicated in metastasis formation, for example, by affecting apoptosis induced by loss of cell anchorage (anoikis) [40].

COX-2 induction or overexpression is associated with an increased production of PGE2, one of the major products of COX-2 which is known to modulate cell proliferation, cell death, and tumor invasion in many types of cancer including colon, breast, and lung. Prostaglandin E2 acts through different membrane receptors called EP receptors (EP1, EP2, EP3, and EP4) [41]. These receptors are all located on the cell surface and characterized by seven-transmembrane domains, and rhodopsin-type G protein-coupled receptors, but trigger different signaling pathways. Thus, it is known that EP1 signaling acts through phospholipase C/inositol triphosphate signaling, leading to intracellular mobilization of calcium. EP2 and EP4 receptors are coupled with G proteins which activate adenylate cyclase, leading to an increase of intracellular cAMP [41]. cAMP is then able to activate kinases such as protein kinase A (PKA) or PI3K for example, and also GSK3 leading to an activation of β-catenin, a pathway regulating cell proliferation [42, 43]. In contrary to EP2 and EP4, EP3 is coupled with Gi protein, leading to an inhibition of adenylate cyclase, and thus a decrease of cAMP inside the cells [41]. The differential expression of these different receptors according to the cell type may explain the diverse and antagonist effects of PGE2 described in literature.

Until now, there are multiple evidences about the role of PGE2 in tumorigenesis in some cancers. These evidences are mostly described for adherent tumors while this link is poorly understood for hematopoietic malignancies such as leukemia or lymphoma. Indeed, several papers have reported that PGE2 is the most important prostaglandin produced during colorectal carcinogenesis [44]. Moreover, it is known that the level of PGE2 increases in a size-dependent manner in Familial Adenomatous Polyposis (FAP) patients [45], suggesting a correlation between tumor growth and prostaglandin biosynthesis. Tumorigenesis is characterized by a disequilibrium between cell proliferation and cell death. PGE2 is able to inhibit apoptosis in human colon cancer cells. It has been demonstrated that PGE2 can upregulate the level of the anti-apoptotic protein Bcl-2 in HCA-7 cells (adenocarcinoma), which produce significant amounts of PGE2. This paper described a modulation of the MAPK pathway that precedes the upregulation of Bcl-2 [46]. PGE2 can mediate its effect through EGF receptor, leading to MAPK activation. The ability of PGE2 to modulate tumor progression in colorectal cell has been shown in other models of colon cancer such as HT-29 cells that express EP receptors. In this cell type, PGE2 is associated with an increase of cAMP through EP4 receptor. The effect can be reversed by L-161982, an antagonist of EP4 [47]. Moreover, PGE2 transactivates EGFR by triggering the release of amphiregulin, a well-known EGFR ligand [48]. SC-236, an inhibitor of COX-2, is able to inhibit cell proliferation of HT-29 cells and this effect is greater in combination with an amphiregulin neutralizing antibody [47]. In this cell line, the expression of amphiregulin is correlated to the expression of COX-2.

The transactivation of EGFR by PGE2 can lead also to AKT activation, which is a well-known survival pathway [49]. This effect was well described in a study by Tessner et al. [50] demonstrating that 16,16-dimethyl PGE2 (dmPGE2) inhibits radiation-induced apoptosis in the mouse intestinal epithelium. Using HCT-116 cell line as a model to reflect the effect on mouse small intestine, it has been shown that the anti-apoptotic effect of dmPGE2, which is known to bind EP2, was tightly related to AKT phosphorylation through activation of EGFR and leads to an inhibition of Bax translocation in mitochondria, an important step for apoptosis [51].

PGE2 modulates also tumor growth of lung cancer. This effect has been described by Yamaki et al. [52] showing that PGE2 activates Src kinase in A549 cells, leading to an induction of cell growth. These cells express EP3 that activates Src (sarcoma) kinase. This study has demonstrated that the activation of Src leads to an activating phosphorylation of STAT3, a transcription factor known to regulate cyclin D1 transcription, an important positive regulator of cell proliferation. Apoptosis can be inhibited because STAT3 regulates the transcription of Bcl-XL, a well-known anti-apoptotic protein [53]. Moreover, Src phosphorylates p27, a protein known to inhibit cell cycle progression especially at the G1/S transition [54]. However, it has been recently shown that this protein plays a dual role as the unphosphorylated form of p27 inhibits the cell cycle, and thus cell proliferation. If phosphorylation occurs on T157 and T198 by PI3K (phosphoinositide 3-kinase), it triggers cell cycle transition by stabilizing the cyclin D1/cdk4 complex [55]. Thus phosphorylation of S10 appears to be important for other phosphorylation steps and it has been hypothesized that Src kinase can play this role [55]. Moreover, it is known that phosphorylation of p27 is responsible also for its degradation by the proteasome [56]. All together these data suggest that PGE2 increases cell proliferation via p27 phosphorylation through EP4 receptors.

Nonsmall lung cancer is characterized by a Ras mutation correlated with a poor prognosis [57]. Activation of Ras leads to an upregulation of COX-2 resulting in increased PGE2 production [58]. PGE2 increases cell proliferation of A549 cells (adenocarcinoma) and this effect is associated with an activation of Ras pathway via EP4 receptor. In this case, PGE2 mediates its effect by the release of amphiregulin, the most abundant ligand in A549 cells [59]. EGFR activation leads to activation of MAPK pathway that regulates cell proliferation by transactivating several oncogenes such as c-myc [60].

PGE2 is also important for tumor invasion. A study by Ma et al. [61] described that PGE2 can increase the number of metastasis. This effect has been demonstrated in a model in which murine mammary tumor cells 66.1 were injected in syngenic immune competent BALB/CByJ mice. All these cell lines express EP1, 2, 3, and 4. The use of EP4 antagonists (AH23848 and AH6809) decreased surface tumor colonies and reduced tumor invasion. Another study has revealed that PGE2 increases the level of VEGF in granuloma [62]. VEGF is an important factor of angiogenesis, and thus of tumor progression by enhancing the vascularization of the tumors [63].

Alltogether these data together suggest that PGE2 and, thus, COX-2 play an important role in tumor progression by enhancing cell proliferation, cell survival, and tumor invasion. The diversity of PGE2 receptors and their different signaling pathways suggest that the protumorigenic effect of PGE2 depends on the cell type and the type of receptor expressed. Until now, many signaling pathways associated with tumor progression are linked to PGE2 and this could explain why the use of COX-2 inhibitors is a good strategy in cancer therapy. However, the signaling pathways of EP receptors are not completely characterized and their precise roles in the different cancers remain to be elucidated before a clinical application.

COXs may be targets of several compounds that may inhibit their functions. Combination of such preferential or selective COX-2 inhibitors with anti-cancer agents already used in clinics were tested with the goal to improve the efficiency of anti-cancer protocols.

COX-2 is the preferential target of several NSAIDs (Figure 2) [64, 65]. Historically, NSAIDs used for clinical and anti-inflammatory purposes were represented by the nonselective COX-2 inhibitors, to which belong aspirin, sulindac acid and, more recently, agents such as nimesulide, ibuprofen and naproxen. As their definition well reflects, this first generation of NSAIDs may affect both main COXs isoforms, even if preferentially COX-2 (see above). Their mechanisms of action are not all completely elucidated, complicated by the fact that different agents seem to act in different ways. For example, different NSAIDs bind the active site of COX-2. Commonly, binding occurs by a reversible competitive inhibition (i.e., ibuprofen, naproxen, and indomethacin). In contrast, aspirin is able to acetylate the active site of COX at a serine residue, leading to an irreversible inhibition (see Figure 2, summarizing the classification of COX-2 inhibitors mentioned in this review). Considerable side effects generated by the interference with homeostatic functions modulated by COX-1 include increased incidence of gastrointestinal hemorrhage and ulceration upon chronic or long-time intake [66]. A novel generation of COX-2-selective inhibitors NSAIDs termed "Coxibs" was then developed. These compounds promised to be much less gastrotoxic. They act as competitive inhibitors of the active site of COX-2 and present indeed a higher specificity. However, concerns related to a long-time/chronic intake of these drugs raised quite soon, following some clinical reports, suggest a correlation between an increased risk of myocardial infarction and their consumption [67]. This has lead to the voluntary withdrawal of some of these agents, that is, rofecoxib and valdecoxib [68], and drastic regulatory advices regarding the use of the other ones, thus opening a discussion on the real benefits versus side effects of their use in clinics. Consequently, studies focused on the use of traditional versus COX-2-selective NSAIDs, frequently associated to the elaboration of economical models, have been performed in these latest years, with the aim to evaluate the real risks together with the costeffectiveness and, possibly, identify classes of users/patients where regular NSAIDs intake may be beneficial. Although, further analyses need to be performed, a number of reports suggest that Coxibs may really increase

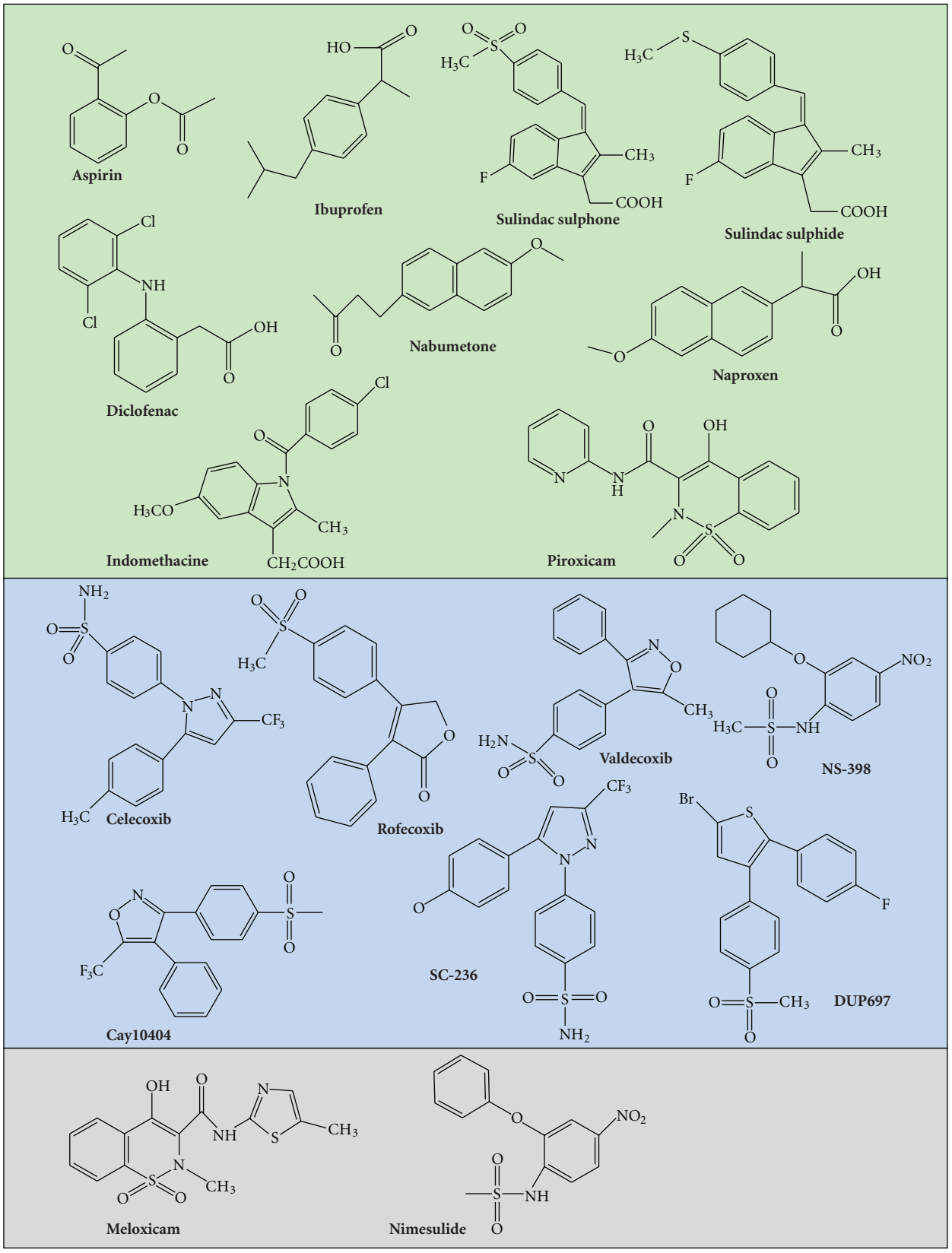

Figure 2: *COX-2 inhibitor classification.* COX-2 is the target of many compounds. COX-2 inhibitors described in this review are classified according to their ability to inhibit COX-2: nonselective (green), selective (pale blue), and preferential (grey).

cardiovascular risks only in patients presenting a positivity to other cardiovascular factor risks, as high blood pressure and altered lipid metabolism [69–73]. These results suggest that their use should be limited to patients with a low risk of cardiovascular complications after analysis of multiple biomarkers [Chaiamnuay et al., 2006, clinical reviews]. Therefore, the future perspective in the pharmacological use of preferential versus selective COX-2 inhibitors is the identification of a panel of interesting biomarkers, helping in defining individual biological risk factors and limiting the use of a specific class of COX-2 inhibitors to the appropriate responders [74, 75]. This approach will have a considerable implication in therapy as well as in chemoprevention of inherited forms of colon cancer.

It is interesting to mention that recent alternative approaches have been considered. Strillacci et al. [76] and Chan et al. suggested RNA interference using adenoviral vehicles. Moreover, other selective COX-2 inhibitors have been developed and experimentally used: SC-558 [35] , DUP-697 [77], SC-58125 [78], and NS-398 [8]. Some of them induce an irreversible inhibition. This is the case for NS-398, which acts by inducing a conformational change of COX-2 [25] (Figure 2). Another strategy discussed in literature could be the use of EP receptor antagonists. Indeed, it has been demonstrated that EP antagonists can decrease cell proliferation and cell invasion [47, 61, 79]. This could be a more specific strategy that could limit the other side effects of classic COX-2 inhibitors.

4. COX-2 As a Regulator of Cell Proliferation

Cell cycle is regulated by different serine-threonine kinase proteins called cyclin-dependent kinase (Cdk). These proteins regulate the different steps of cell cycle progression by phosphorylating many substrates (i.e., nuclear lamins) [54]. These proteins are regulated by phosphorylation and dephosphorylation. Thus, Cdks can be activated by phosphatases such as CDC25C (*cell division cycle 25 homolog C*) for CDK1 or kinase like CAK (Cdk activating kinase). The activity of cdks is also regulated by cyclins, which form heterodimers with cdks leading to an activation of Cdks by conformational change [54, 80].

Cell cycle is under the control of other factors, implicated in the regulation of cell cycle transition. These regulatory mechanisms form checkpoints where the cell cycle can be stopped after cellular damage in order to allow repair and to maintain cellular integrity or, alternatively, to eliminate mutated and potentially dangerous cells. The INK4 family (p16, p15, p18, and p19) and the Cip/Kip family (p21, p27, and p57) [54, 80, 81] are key regulators of G1/S transition. For example, after DNA damage, p53, a tumor suppressor gene, activates transcription of p21, which inhibits cyclin E phosphorylation leading to hypophosphorylation of retinoblastoma protein (pRb) [81]. INK4 family inhibits Cdk4 and Cdk6, whereas Cip/Kip family inhibits all Cdks. Retinoblastoma protein needs to be phosphorylated in order to release transcription factor E2F activating genes involved in the S phase-like PCNA (proliferating cell nuclear antigen) [82]. p53 is also important for the regulation of the G2/M transition, which requires activation of the cyclin B-cdk2 complex. This complex accumulates during the previous step of the cell cycle but is inactivated by a phosphorylation at tyrosine 15 and threonine 14 by Wee 1 and Myt 1. These phosphate groups are removed by the phosphatase CDC25A when cells enter mitosis. In the case of DNA damages, p53 is activated and increases the level of p21 that is directly inhibiting cdk2. Moreover, 14-3-3 protein, a transcriptional target of p53, leads to a sequestration of cdk2 in the cytoplasm [83]. Other mechanisms involved in the regulation of the G2 checkpoint or the mitotic spindle checkpoint are reviewed by Stewart et al. [54].

Cancer cells are characterized by deregulation of the cell cycle via alteration of cell cycle controllers (cyclins) and cell cycle regulators (p53) [54], resulting in a perturbation of cell cycle checkpoints.

Currently, there is evidence that prostaglandins produced by COX-2 intervene in tumor cell proliferation as NSAIDs and selective COX-2 inhibitors inhibit proliferation of different cancer cell types expressing COX-2 [30]. NS-398, a COX-2 specific inhibitor, was described to reduce cell proliferation of MC-26 cell line, a highly invasive mouse CRC cell model expressing constitutively COX-2 [8]. This effect was associated with a reduction of cyclin D level, a key protein involved in G1-S transition [54], and PCNA, thus increasing the processivity of DNA polymerase [82]. NS-398 and COX-2 specific inhibitor nabumetone reduced cell proliferation of U937 (acute promonocytic leukemia) and ML1 (human myeloblastic leukemia), thus leading to an accumulation in G0/G1 phase [33]. Interestingly, meloxicam was also able to downregulate PCNA and cyclin A in HepG2 cell line (hepatocellular carcinoma cells), leading to an inhibition of the cell proliferation and an accumulation of the cells in G0/G1 phase of cell cycle [84]. Alternatively, the link between COX-2 and CRC has been demonstrated by the fact that prostaglandin E2 (PGE2) derivating from COX-2-mediated arachidonic acid metabolism increased the proliferation of colorectal cancer cells [85].

The inhibitory effect of NSAIDs on cell proliferation of CRC has been also observed in ovarian cancer. Indeed, treatment of OVCAR-3 tumors xenotransplanted in nu/nu mice (nude mice) with aspirin and piroxicam (NSAIDs) and the selective COX-2 inhibitor meloxicam led to a reduction of tumor growth [86].

It has been estimated that 40% of breast cancers show an overexpression of COX-2, which is associated with a bad prognosis [5]. Indomethacin (NSAIDs), celecoxib, rofecoxib and nimesulide have been shown to able to inhibit cell proliferation of these cells [5]. Moreover, prostaglandins were able to increase cell proliferation of hormonal-dependent breast cancer by increasing transcription of CYP19 aromatase implicated in estrogen biosynthesis [87].

Several studies revealed that inhibition of COX-2 by celecoxib in Burkitt's lymphoma cell lines RAJI, BjAB, (Epstein-Barr virusnegative), and BL41 led to a reduction of cell proliferation [34]. NS-398 and celecoxib were able to reduce proliferation of pancreatic cancer cell line, Panc-1 in a dose-dependent manner [88]. Treatment with celecoxib of these cells implanted into nude mice led to a reduction of

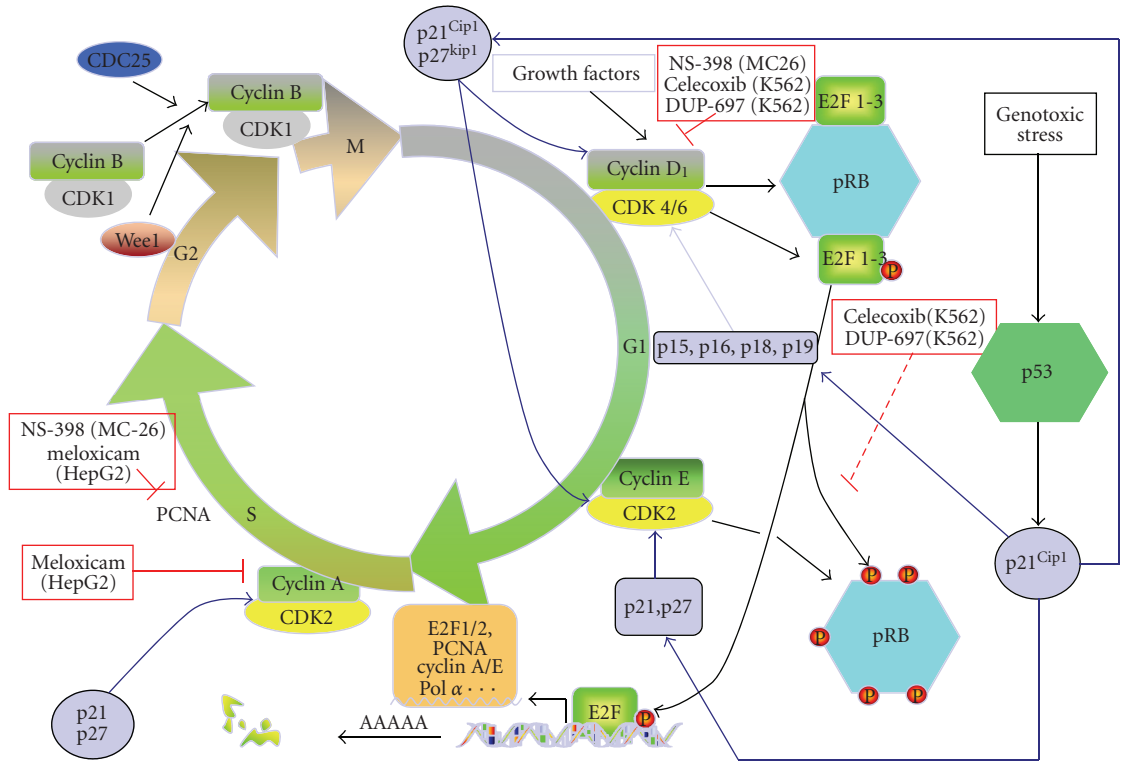

Figure 3: *Effects of COX-2 inhibitors on cell proliferation.* Cell cycle is divided into different steps: G1, S, G2, and M (mitosis). This process is regulated by cyclin proteins, which activate cyclin-dependent kinase (cdk) and phosphatase (i.e., CDC25) or kinase like cyclin-dependent kinase inhibitors such as p16, p15, p18, p19, p21, and p27 [54]. Selective COX-2 inhibitors are able to modulate some cell cycle checkpoints. In this picture, some examples of this link have been shown for different cell types: MC26, colorectal cancer; HepG2, hepatocellular carcinoma; K562, chronic myeloid leukemia. Cdk; cyclin-dependent kinase; pRb, retinoblastoma protein; PCNA, proliferating cell nuclear antigen.

the volume of the tumor [88]. Other studies have shown that celecoxib is able to reduce cell proliferation of the chronic myeloid leukemia (CML) cell line K562, which expresses COX-2 at the mRNA and protein level [89]. This effect was accompanied by an accumulation of cells in G0/G1. Moreover, the inhibition of cell proliferation was correlated to a downregulation of cyclin D1, cyclin E, and pRb and the upregulation of p16 and p27 [89]. Similar results were found on this cell type with the other selective COX-2 inhibitor DUP-697 [77]. Different effects are recapitulated in Figure 3.

5. Implication of COX-2 in Cell Death

5.1. Apoptosis. Apoptosis (type I cell death) is important for the development and maintenance of tissue homeostasis of multicellular organisms [90, 91]. This active form of cell death is characterized by the occurrence of typical cell alterations including plasma membrane blebbing, cell shrinkage, chromatin condensation and nuclear fragmentation, and, finally, formation of apoptotic bodies, which can be phagocyted by macrophages [92]. Deregulation of apoptosis is linked to several pathophysiological disorders, including autoimmune disorders, Alzheimer's disease, and cancer [93].

Two major cascades of intracellular events are commonly involved in mediating apoptosis (Figure 4). The intrinsic pathway, also called the mitochondrial or stress-induced apoptotic pathway, is activated in response to damaging stresses, such as DNA damage. Typical hallmarks of this pathway are mitochondrial outer membrane permeabilization (MOMP), accompanied by a collapse of the mitochondrial membrane potential [51]. These events lead to the release of cytochrome *c* into the cytosol, which is an indispensable component of the apoptosome, the death complex formed also by APAF-1, and procaspase-9. Once recruited, this protease is cleaved to its activated form (caspase-9) to further activate the executor caspase-3 and, finally, to finalize the apoptotic program.

Alternatively, the extrinsic, or physiological, apoptotic pathway (Figure 4) can be triggered upon binding of specific ligands to death receptors characterized by the presence of a death effector domain [94]. Ligands include cytokines, such as TNFα, tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis (TRAIL), or FAS. After binding, death inducing silencing complex (DISC) is formed. The DISC is composed by the adaptors proteins TRADD (TNF receptor-associated death domain) and FADD (Fas-associated death domain) and is able to recruit and activate pro-caspase-8. Finally, caspase-8 activates caspase-3 in order to trigger the final steps of apoptosis (Figure 4).

Cross-talks between the two pathways take place. The extrinsic apoptotic pathway can activate the intrinsic

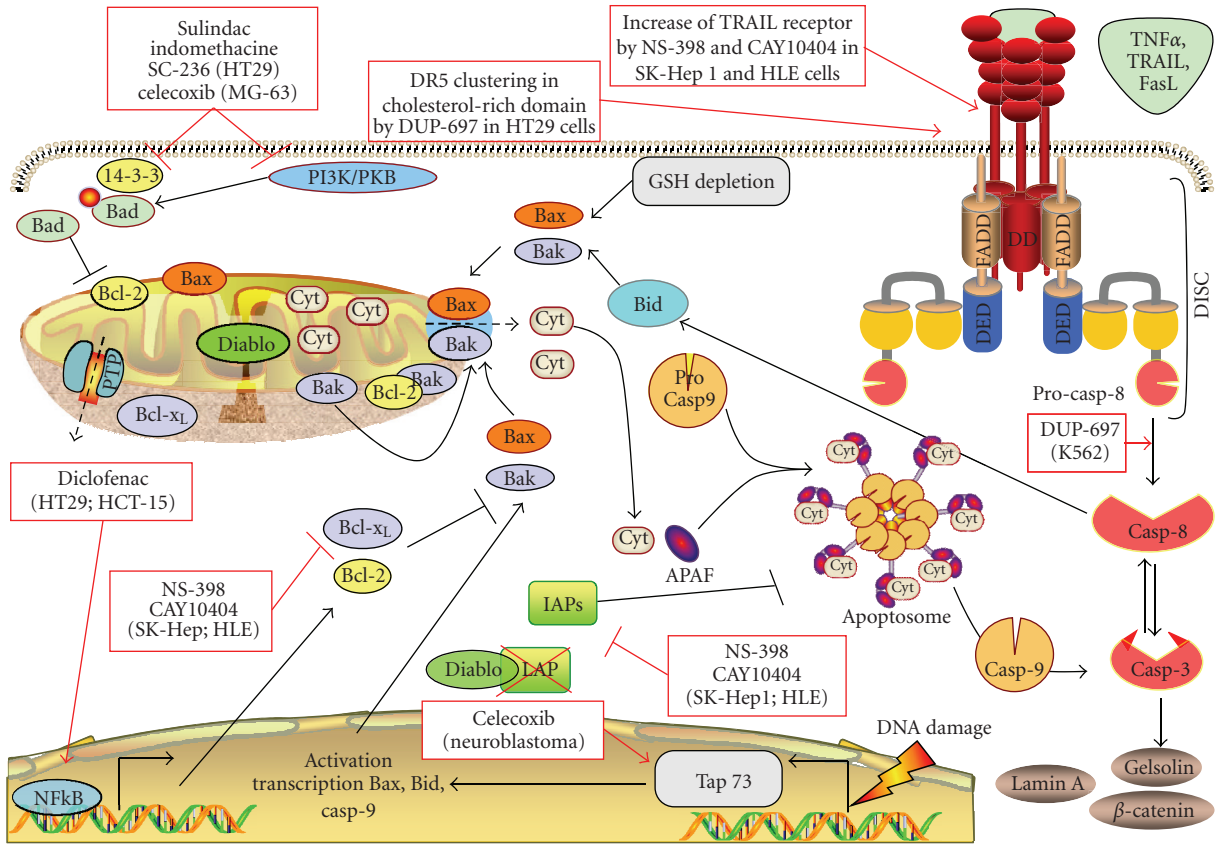

Figure 4: *Effects of COX-2 inhibitors on apoptosis.* Apoptosis can be mainly mediated by two pathways: the mitochondrial, intrinsic, or stress-induced apoptosis, which is activated in response to damaging stresses and the extrinsic pathway, triggered by the binding of ligands to specific death receptors [51]. COX-2 inhibitors are able to modulate stress-induced apoptosis as well as extrinsic apoptosis in several cell types. In this picture, some examples of these interaction discussed in the text are presented for different cell types: LNCaP, prostate cancer; K562, chronic myeloid leukemia; HT29, colorectal cancer; SK-Hep 1 and HLE, human hepatocarcinoma cells; HepG2, hepatocarcinoma; Be17402, hepatocarcinoma; SMMC-7402, hepatocarcinoma; MG-63, osteosarcoma. Abbreviation: AIF: apoptosis-inducing factor; Bcl-2, B cell lymphoma 2; Bid, Bcl-2 interacting domain; Casp, caspase; Cyt, cytochrome C; DD, death domain; DED, death effector domain; DISC, death-inducing silencing complex; PI3K/PKB, phosphatidyl inositol-3 kinase/protein kinase B; FADD, Fas-associated death domain; GSH, glutathione; PTP, transition permeability pore; TNF, tumor necrosis factor; TRAIL, TNF-related-inducing-apoptosis-ligand.

pathway via truncation of the BH3-only protein Bid (t-Bid) by caspase-8. t-Bid interacts with mitochondria, by favoring the activation of the proapoptotic Bcl-2 family members Bak and Bax, thus leading to MOMP and caspase-9 activation [51, 95] (Figure 4). The intrinsic apoptotic pathway may, in turn, activate caspase-8, downstream to caspase-3 [96] (Figure 4). Cross-talks represent an important strategy of amplification loops carried out by dying cells to ensure/potentiate cell death.

5.1.1. Involvement of COX-2 in Intrinsic Apoptosis. When cells are damaged by a variety of chemicals or physical stress (i.e., reactive oxygen species, UV, and ionizing radiation), they undergo apoptosis by triggering the intrinsic apoptotic pathway (Figure 4). This pathway may be associated with a redox disequilibrium, mediated by depletion of glutathione (GSH) [94, 97, 98], required for the activation and translocation to mitochondria of the Bcl-2 pro-apoptotic member Bax [98], which, in turn, forms complexes (oligomers) mediating MOMP and cytochrome *c* release. As Bax, Bak may play the same role [99]. In contrast to Bax, Bak is already present at the surface of mitochondria, normally sequestered in its active monomeric form by the Bcl-2 anti-apoptotic members Bcl-xL and Mcl-1 (see Burlacu for a general overview of the Bcl-2 family members modulation involved in Bax/Bak activation [51]).

Apoptosis is regulated in order to maintain tissue homeostasis. This regulation implicates protein-protein interactions, with some of them counteracting apoptosis. In this view, the interaction between Bcl-2 family pro- and antiapoptotic members represents a crucial and delicate step. Bcl-2 is the best described member of this family preventing Bax activation [51]. Bax can form also a complex with the anti-apoptotic protein Bcl-xL [53] and Mcl-1 [14]. Similarly, Bak activity is monitored by the anti-apoptotic members Bcl-xL and Mcl-1 [51]. The interaction between Bax/Bak and the Bcl-2 family anti-apoptotic members is carefully regulated by the BH-3-only proteins. Another carefully regulated downstream checkpoint of the apoptotic pathway is the activation of caspases. Inhibitor of apoptosis (IAP) family, by directly

interacting with caspases (i.e., XIAP, survivin [100]) controls and prevents their activity once cleaved. IAPs monitoring function can be, in turn, counteracted by the pro-apoptotic SMAC/DIABLO, a mitochondrial heterodimer, which is released from mitochondria when MOMP is affected [101]. This interaction favors the induction of apoptosis.

Imbalance between cell proliferation and apoptosis observed in cancer can be tightly related to an altered function of pro-apoptotic proteins as well as to an up-regulation of anti-apoptotic proteins (i.e., Bcl-2 or IAPs) or a downregulation of tumor suppressor genes (i.e., p53). In addition, the activation of prosurvival pathways (i.e., PI3K/Akt) may be implicated upstream. Inflammation can contribute to this imbalance *via* cytokines secreted in the tumor microenvironment able to activate survival pathways. For example, TNFα can induce NFκB, leading to an inhibition of apoptosis [38]. COX-2 seems also to play a role in this process because it is known that COX-2 inhibition is correlated to an increase of apoptosis in several cancer models. NS-398 downregulated Bcl-2 expression in an androgen-sensitive human prostate cancer cell line LNCaP that exhibited a high constitutive level of COX-2 [102]. Similar results have been observed in human colorectal cancer cells (HCA-7 cell line which expresses COX-2) where PGE2 was able to inhibit apoptosis induced by SC58125, a selective COX-2 inhibitor, and increase Bcl-2 expression [46]. Different mechanisms are supposed to explain how COX-2 inhibitors may trigger apoptosis. In a number of studies, COX-2 inhibition was linked to a concomitant increase of intracellular arachidonic acid. In HT-29 human colon adenocarcinoma cell this accumulation led to the induction of apoptosis [103]. The arachidonic acid-induced apoptosis was inhibited by Bcl-2 transfection, indicating a role of arachidonic acid in affecting Bcl-2 intracellular levels [103]. Accumulation of arachidonic acid can affect apoptosis by mediating an increase of pro-apoptotic intracellular ceramides caused by activation of sphingomyelinase [104, 105]. Sulindac sulphide, a metabolite of sulindac, also activates sphingomyelinase and enhances the ceramide level in the two human colorectal carcinoma cell lines HCT116 and SW480 [106].

COX-2 reduces pro-apoptotic nitric oxide (NO) levels in cancer cells downstream of prostaglandin production [30] (see Brüne et al. [107] for an overview on nitric oxide role in apoptosis). Chang et al. reported that PGE2 prevented apoptosis induced by NGF (nerve growth factor) withdrawal by increasing the level of dynein light chain, an inhibitor of neuronal NO synthase in pheochromocytoma of the rat adrenal medulla PC12 cells, thus leading to decreased intracellular NO levels [108].

More recently, connections between COX-2 inhibitors and p53 family members have been described. For example, celecoxib was shown to able to modulate different isoforms of p73, a p53 family member in neuroblastoma cell lines [109]. p73 encodes many isoforms with different roles. Tap73 is pro-apoptotic and contains a transactivation domain. This isoform is considered as a tumor suppressor gene because it seems to be involved in cell cycle regulation as well as in apoptosis induction [109, 110]. In contrast, DeltaNp73 is anti-apoptotic and lacks the transactivation domain. DeltaNp73 is overexpressed in neuroblastoma, leading to chemotherapy resistance [109]. It has been shown that celecoxib was able to upregulate Tap73 and downregulate DeltaNp73. These data suggest the use of COX-2 inhibitors as p73 modulators in order to improve efficiency of chemotherapy [110].

The apoptotic effect of COX-2 inhibitors has been also observed for other tumor cell types, such as in the chronic myeloid leukemia model K562 where DUP-697 induced apoptosis by cell cycle arrest and caspase-8 activation [77].

COX-2 inhibitors can also activate prosurvival pathways. The PI3K/Akt pathway is a survival pathway, frequently activated in cancer cells [49]. PI3K produces PIP3 (phosphatidylinositol 3,4,5 triphosphate) that activates PDK1 (pyruvate deshydrogenase kinase). This protein phosphorylates and activates PKB (protein kinase B), which, in turn, is responsible for the phosphorylation of several targets playing a modulator function in apoptosis. An anti-cancer effect of celecoxib due to the inhibition of Akt signaling [111] was observed in a gastric cancer model. Celecoxib triggered also apoptosis in osteosarcoma cells (MG-63) through down-regulation of Bcl-2, survivin and PI3K (phosphoinositide 3-kinase) pathway [112]. Similarly, Hsu et al. [113] found that inhibition of Akt phosphorylation by celecoxib in prostate cancer models (LNCaP and PC3 cell lines which express constitutively COX-2) led to apoptosis, but in this case without affecting Bcl-2 level.

The PI3K pathway is negatively regulated by PTEN (phosphatase and TENsin homolog), which converts PIP3 in PIP2, preventing PKB activation and Bad phosphorylation/sequestration. Thus PTEN is considered as a tumor suppressor gene. It has been shown that NS-398 was able to increase the level of PTEN in human gastric carcinoma cell line MKN45 [114].

One of the PKB targets is Bad, a BH3-only member [51]. The nonphosphorylated form of Bad plays a pro-apoptotic role, by binding Bcl-xL or Bcl-2 and, thus, preventing their interactions with Bak and Bax. The activation of PI3K/Akt pathway may lead to the phosphorylation of Bad, which is consequently sequestrated in the cytoplasm by 14-3-3 protein and, in this way, inhibited in its pro-apoptotic function [51]. It has been reported that sulindac sulphone, indomethacine, and SC-236 were able to induce apoptosis *via* Bad activation, by inhibiting 14-3-3 expression in a dose- and time-dependent manner in HT-29 cells [115]. This effect was tightly related to PPARδ. It is known, indeed, that 14-3-3 protein contains PPRE recognized and bound by PPARδ [115]. COX-2 can mediate the synthesis of prostaglandin I2, which can bind and activate PPARδ [41]. Thus, it has been suggested that the inhibition of COX-2, leading to a decrease of PGI2, impaired PPARδ activation, which, in turn, was responsible for a downregulation of 14-3-3 protein, thus allowing Bad to play its pro-apoptotic functions [115].

NFκB is a most important transcription factor involved in survival by enhancing transcription of anti-apoptotic proteins such as Bcl-2 [14, 15]. Sulindac inhibits NFκB in two colon cancer cell lines (human colon adenocarcinoma

HCT-15 and HT29 cell lines) [116]. Similar results were obtained with diclofenac, which was able to inhibit nuclear accumulation of NFκB [117]. In the same study, PGE2 was demonstrated to increase the transcriptional activity of NFκB p65/p50 dimer in CACO-2 cells (human epithelial colorectal adenocarcinoma cells), transfected with a luciferase construct containing NFκB response elements [117].

It is known that activation of prostaglandin receptors induces an increased cAMP level which in turn can activate protein kinase A (PKA) [58]. Studies have suggested that PKA, like PKB, phosphorylates Bad [118], leading to its sequestration and inhibition of apoptosis. Some of the pro- or anti-apoptotic mediators affected by COX-2 inhibitors are schematized in Figure 4.

5.1.2. Implication of COX-2 in Extrinsic Apoptotic Cell Signaling Mechanisms. Studies reported that COX-2 inhibitors are also associated with a sensitization of tumor cells to extrinsic apoptosis. Thus, DUP-697 sensitized HT29 colon cancer cell line to TRAIL-induced apoptosis. This effect was due to an accumulation of arachidonic acid inside the cells, which activates sphingomyelinase, triggering a clustering of death receptor (DR) 5 receptors in ceramide and cholesterol-rich domains [119]. Alternatively, the expression of COX-2 has been frequently associated with a modulation of the expression of death receptors, thus leading to an upstream control of the extrinsic apoptotic pathway. Tang et al. [120] showed that COX-2 overexpression in human colon cancers cells led to an inhibition of DR5 expression and a resistance to TRAIL-induced apoptosis. Accordingly, COX-2 specific inhibitors, NS-398 and CAY10404, are sensitizing human hepatocarcinoma cells (SK-Hep1 and HLE) to TRAIL-induced apoptosis. This effect was due to an upregulation of TRAIL receptors (TRAIL R2/DR5 and TRAIL-R1/DR4), together with an ability of the compounds to induce a down-regulation of the anti-apoptotic proteins survivin (IAP) and Bcl-xL [121]. In hepatocellular carcinoma models (HepG2, Bel7402, and SMMC-7402), Li et al. [84] showed that COX-2 inhibition with meloxicam led to an upregulation of Fas-mediated apoptosis. In vivo studies performed on transgenic mice constitutively expressing human COX-2 confirmed an increased resistance to Fas-induced apoptosis in liver, as shown by the preservation of liver architecture in COX-2-expressing mice compared to wild type [122]. Similarly, another study performed on human extrahepatic bile duct carcinoma cell line showed that COX-2 induction led to the inhibition of Fas-induced apoptosis, whereas the inhibition of COX-2 with NS-398 in cytokine-treated cells exacerbated apoptosis induced by CH-11, an agonist of Fas receptor [123].

AKT pro-survival pathway may play a role also in the modulation of extrinsic apoptosis. The human gastric carcinoma cell line MKN45, which expresses COX-2, was sensitized to Fas-induced apoptosis by NS-398. The COX-2 inhibitor, indeed, was able to increase the level of PTEN, leading to a decrease of Akt phosphorylation and activation of Bad [114]. Some effects of COX-2 inhibitors on extrinsic apoptosis are summarized in Figure 4.

Altogether, these results encourage the perspective that COX-2 inhibitors could be used in future as a therapeutic strategy to sensitize tumor cells to apoptosis induced by physiological stimuli.

5.2. Involvement of COX-2 in Other Types of Cell Death

5.2.1. Anoikis. Anoikis is a form of apoptosis mediated by the loss of cell anchorage. This pathway plays a fundamental role during development and maintenance of tissue homeostasis by killing damaged cells or detached cells in order to maintain tissue architecture. For example, the inner endodermal cells undergo anoikis after the loss of anchorage to the matrix during development [124]. It is known also that intestinal epithelial cells loose anchorage when located at the luminal surface, leading to anoikis [42]. As a form of apoptosis, anoikis is dependent on caspase activation and cytochrome *c* release by mitochondria and is regulated by Bcl-2 family members [42].

It has been shown that anoikis is prevented in cancer cells, thus favoring tumor progression with the formation of metastasis [42]. Accordingly, modulation of anoikis is considered a promising target for anti-cancer strategies.

Cell anchorage is due to cell-cell and cell-matrix interactions. Cell-cell interactions are mainly mediated by integrins which are transmembrane receptors located at the cell surface and composed of alpha and beta chains [125]. Many intracellular signals can act downstream to integrins, which, correctly switched on, can ensure cell survival. Some of them are mediated by kinases such as Focal-adhesion-kinase (Fak) or integrin-linked kinase (ILK) [42]. Fak is phosphorylated upon integrin adhesion, leading to activation of other signaling pathways like PI3K, MAPK. ILK is a serine/threonine kinase that directly phosphorylates PKB.

Together with cell-cell and cell-matrix interactions, paracrine factors could be important for the regulation of anoikis. It has been shown that E-cadherin (epithelial cadherin) can activate COX-2 [23]. It is possible that prostaglandins produced by COX-2, which act in an autocrine and a paracrine manner, favor cell survival. A study from Joseph et al. [126] showed that PGE2 inhibited anoikis in IEC-18 cells (rat intestine ileum cells). This effect was suggested to be due to cAMP signaling because prostaglandin E2 receptors are coupled to adenylate cyclase, which converts AMP to cAMP [126].

Other studies demonstrated that COX-2 inhibits anoikis via activation of PI3K/Akt pathway, as the case of a human bladder cancer cell line expressing COX-2 [40]. A link between COX-2 and anoikis has been described, furthermore, in uterine endometrial carcinoma [127]. COX-2 is over-expressed in this type of cancer and this is associated with tumor aggressiveness. In addition, a recent report based on HEC-1B and RL95-2 (two human endometrial cancer cell lines) showed that the treatment of these cells with hepatocyte growth factor (HGF) led to an up-regulation of COX-2. Hepatocyte growth factor interacts with its tyrosine kinase receptor c-Met. This interaction is responsible for tumor progression. Overexpression of HGF/c-Met has been described in different tumors such as breast cancer [128]

as well as head and neck cancer [129], also in endometrial carcinoma [130]. It has been demonstrated that HGF inhibited anoikis and treatment of HEC-1B and RL95-2 cells with the COX-2 selective inhibitor meloxicam prevented HGF-mediated anoikis resistance [127]. Similar results were obtained in head and neck squamous cell carcinoma [131].

Altogether these data suggest that COX-2 may be implicated in the inhibition of anoikis and that COX-2 inhibitors may play a role in inhibiting tumor progression (metastasis), by sensitizing tumor cell to anoikis.

5.2.2. Autophagy. Autophagy is a process triggering cells to degrade intracellular constituents, ranging from proteins up to entire organelles. It represents an important process ensuring the turnover of long-lived cellular components, which can be activated also by stress conditions like nutrient starvation in order to avoid cell death. The process starts with the formation of doubled membrane-bound vacuoles corresponding to autophagosomes that entrap parts of the cytoplasm or organelles (i.e., mitochondria). Then, these structures are fused with lysosomes (autolysosomes), thus leading to the degradation of the intracellular parts previously enclosed. Together with apoptosis, when exacerbated, autophagy contributes to the modulation of homeostasis, by eliminating damaged and potentially dangerous cells (type II cell death) [132]. However, the relationship between apoptosis and autophagy is currently still poorly understood [132] because in some cases autophagy permits an adaptation of the cells to stress (i.e., nutrient starvation), thus counteracting apoptosis, whereas, in other cases, autophagy is a process triggering downstream apoptosis [132]. Indeed, similar stimuli can induce both apoptosis or autophagy [132].

This process is implicated in pathologies such as Alzheimer's disease and cancer, suggesting a promising field in therapy. By considering that COX-2 is supposed to play a role in apoptosis and a link between apoptosis and autophagy exists, it is conceivable that COX-2 plays a role also in this process. Currently, not many studies aimed at investigating a possible link between COX-2 and autophagy have been published. Nevertheless, one study revealed that sulindac sulphide (NSAIDs) induced apoptosis of the colon cancer HT29 cell line. This effect was increased by treatment of the cells with 3 methyl-adenine, a well-known inhibitor of autophagy [133]. Moreover, the extent of apoptosis in Q204L cells (a clone of HT-29 cells in which 3 methyl-adenine-sensitive autophagic sequestration is impaired) was less than in HT29. These data suggest that autophagy can delay sulindac sulphide-induced apoptosis [133].

6. COX-2 Inhibitors in Cancer Therapy

Despite the latest progress in cancer research and the different strategies to kill cancer cells, several tumors are resistant to conventional therapeutics treatment (i.e., radiotherapy, chemotherapy, and photodynamic therapy).

COX-2 inhibitors play an important role in cancer prevention. Indeed, the chronic intake of NSAIDs is able to consistently reduce the appearance and incidence of many types of cancer as described in Familial Adenomatous Polyposis (for celecoxib) [134, 135] and also in breast cancer [136]. This property of COX-2 inhibitors could be useful for patients with a high risk to develop cancer such as people with Li-Fraumeni syndrome, for example [137]. The fact that many reports in literature suggest that COX-2 inhibitors are responsible for an inhibition of cell proliferation and apoptosis induction in a number of different cancer cell models prompts to consider a possible use of COX-2 inhibitors in future therapeutical protocols, administered alone as well as in combination with anti-cancer clinical protocols in order to improve tumor cell death.

6.1. COX-2 Inhibitors in Combination with Radiotherapy. Radiation therapy is a common treatment used for the treatment of solid tumors, such as breast, prostate, colorectal, and lung cancers. It is known that the anti-cancer properties of ionizing radiation are due to pleiotropic mechanisms. Radiation leads to the formation of DNA doubled-strand breaks in proliferating cells, which triggers the activation of DNA damage pathways (i.e., p53), followed by the induction of apoptosis [36]. The importance of Bcl-2 family members during apoptosis [51] suggests that prosurvival proteins (i.e., Bcl-2, Bcl-xL) play an important role in radioprotection of tumor cells. The NFκB pathway seems to be implicated, being required in regulating expression of the anti-apoptotic Bcl-2 family members like Bcl-xL [36]. Moreover, it is well established that NFκB regulates the level of COX-2, suggesting that COX-2 may play a role in radiotherapy resistance [21]. Similarly, nimesulide could increase radiation efficiency in nonsmall cell lung cancer in vivo (nude mice) and in vitro (A549 cell line) as shown by Grimes et al. [138]. This effect was due to a down-regulation of MnSOD (superoxide dismutase containing manganese (Mn) and localized in mitochondria), a primary antioxidant protein and survivin, an anti-apoptotic protein (IAPS family member). These two proteins are regulated by NFκB. It is well known that during radiation therapy NFκB can be upregulated due to reactive oxygen species release and inflammation (i.e., PGE2). This report suggests that nimesulide may act on NFκB to inhibit MnSOD and survivin.

Melanoma is known to be very resistant to conventional radiotherapy and chemotherapy. Irradiation of two melanoma cell lines WM35 and LU1205 in the presence of NS-398, a selective COX-2 inhibitor, strongly exacerbated the G2/M arrest as well as the induction in apoptosis. Accordingly, the down-regulation of COX-2 by RNA interference in these cell lines was followed by an upregulation of p53 and G2/M arrest [36], thus confirming that the effect of NS-398 is due to its role on COX-2 inhibition.

Other studies have shown that the radiosensitivity of PC3 (human prostate carcinoma cells) and Hela (human cervical carcinoma cells) was enhanced after silencing of COX-2 by siRNA. NS-398 was able to increase radiosensitivity of PC3 cells expressing COX-2, but not in PC3 silenced for COX-2. In contrast, NS-398 enhanced radiosensitivity of Hela cells, irrespective to the level of COX-2 [37].

However, combination of COX-2 inhibitors with radiation therapy can also lead to a reduction of efficiency of the radiotherapy. In one report, it has been shown that the

selective COX-2 inhibitor nimesulide decreased radiation efficiency of two head-and-neck cancer cells lines (SCC9 and SCC25) which are COX-2 positive [139]. This suggests that the sensitization of tumor cells to radiation might be strongly dependent on tumor cell type.

6.2. COX-2 Inhibitors in Combination with Chemotherapy. Many types of cancer are treated with chemotherapeutic agents leading to inhibition of cell proliferation or induction of apoptosis [140].

One of the major causes of chemotherapy failure is the survival and/or development of multidrug resistant cancer cells. This resistance is mediated by many mechanisms including over-expression of proteins involved in inhibition of apoptosis (i.e, Bcl-2), leading to insensitivity of tumor cells to apoptotic stimuli; an up-regulation of DNA repair; alteration of the target; up-regulation of detoxification enzymes (i.e., Glutathione S-transferases); and extrusion of chemotherapeutic drugs by overexpression of ATP-binding cassette family proteins, such as MRP (multidrug resistant-associated protein) BCRP (breast cancer resistance protein or mitoxantrone resistance protein) because these proteins regulate absorption, distribution, and excretion of various pharmacologic compounds [141]. Consequently, the chemotherapeutic agents are immediately extruded from the cells. P-gp (P-glycoprotein) is one of the best-understood mechanisms leading to multidrug resistance (MDR). Tremendous efforts have been made to find solutions to overcome MDR. Recently, COX-2 inhibitors showed an ability to sensitize tumor cells to chemotherapeutic agents in several models and also in clinical assays. Colorectal cancers are particularly affected by chemoresistance. One study revealed that the COXs inhibitors naproxen and indomethacin heptyl ester were able to downregulate P-glycoprotein in human colorectal CACO-2 cell line. [39]. Indomethacin inhibited the activity of the protein and affected COX-2 mRNA and protein level [39]. Another study showed that meloxicam was able to downregulate MDR1 in HL60 (a human promyelocytic leukemia) cell line as well as in acute myeloid leukemic blasts [142]. The regulation of MDR1 by COX-2 has been also suggested in another study [143] in which it was reported that transfection of COX-2 cDNA with adenovirus in renal rat mesangial cells led to an upregulation of MDR1 gene. The combination of COX-2 inhibitors with chemotherapy was also assayed in a study in which the sensitivity of a human gastric cancer cell line MKN45 to cisplatin (alkylating agent) resulted increased by COX-2 downregulation with siRNA [35], suggesting a possible therapeutic application of this combination. Similarly, the sensitivity to cisplatin was increased by celecoxib in a human osteosarcoma cell line (MG-63) and this effect was linked to a down-regulation of anti-apoptotic proteins survivin, Bcl-2, and an inhibition of the survival pathway PI3K/Akt [112]. It was also reported that B-CLL (B chronic lymphoid leukemia) overexpressed COX-2 and the combination of NS398 with chlorambucil, an alkylating agent, increased the level of apoptosis in B-CLL blasts coming from patients [32]. Moreover, several lymphoma cell lines overexpressed COX-2, such as RAJI, BJAB, BL41 and treatment of these cells with celecoxib led to a decrease of cell proliferation in a dose-dependent manner [34].

NS-398 was able to increase the cytotoxicity of gemcitabine, an analog of the antimetabolite nucleoside deoxycytidine, used for treatment of nonsmall cell lung carcinoma, in A549ACA cell line (lung adenocarcinoma cell line) by enhancing apoptosis [144]. The combination of NS-398 and gemcitabine is also associated with an inhibition of cell proliferation with an accumulation of the cells in G0/G1 phase of cell cycle and an increase of p21 [144].

All of these data suggest that COX-2 is implicated in anti-apoptotic and MDR pathways and that selective COX-2 inhibitors could be used to improve chemotherapy efficiency.

6.3. COX-2 Inhibitors in Combination with Photodynamic Therapy. An alternative therapeutic approach to treat cancers is photodynamic therapy. This procedure is particularly used for such solid tumors including skin, bladder, and head and neck cancers in addition to other diseases like age-related macular degeneration and psoriasis [145]. The treatment consists in the administration of a photosensitizer, a molecule that selectively accumulates in tumors and is activated by light (600–850 nm). The photosensitizers may accumulate in different compartments of tumor cells like mitochondria (i.e., porphycene monomer), nucleus, lysosomes (i.e., lysyl chlorin p6), and plasma membrane (i.e., monocationic porphyrin like Photofrin). Then, the photosensitizer is excited with a laser from a single state to a triplet state. The triplet-state photosensitizer is implicated in two oxygen-dependent reactions. In the first one, the triplet can react with cell membrane or molecules, leading to radical formation, which in combination with oxygen produce oxygenated products, cytotoxic for the cells [146, 147]. In the second reaction, the triplet-state photosensitizer can transfer its energy directly to oxygen in order to produce singlet oxygen ($^1O_2$), which is known to be a very highly reactive oxygen species and is implicated in cell damage. Therefore, this therapy leads to tumor destruction due to cell death occurring *via* apoptosis and necrosis. Vasculature damages and activation of immune response are two important effects implicated in tumor ablation.

Some parameters affect PDT efficiency, such as the distribution of the photosensitizer, photobleaching, hypoxia/anoxia, and the vascularization of the tumor [146]. The main reason of failure of this therapeutic approach is linked to an up-regulation of angiogenic and inflammatory factors in the tumor microenvironment that strongly reduces the PTD efficiency with a consequent tumor relapse. The link between inflammation and survival pathway activation, cell proliferation, and angiogenesis is well known and contributes to tumor progression [3, 6]. It has been shown that PDT leads to an increase of TNFα, IL1β, PGE2, VEGF (vascular endothelial growth factor), and MMP9 (matrix metalloproteinase 9) [147]. These molecules can counteract tumor responses to PTD by promoting cell proliferation and cell survival [38]. Interestingly, it has been demonstrated that COX-2 is upregulated during PDT treatment in different cancer models. As for radiotherapy and chemotherapy,

this suggests COX-2 as a possible target to increase PDT efficiency.

Indeed, celecoxib has been proved to affect the Photofrin-induced PDT in in vitro and in vivo studies performed on a mouse mammary carcinoma BA cell line [148]. In vitro, celecoxib and NS-398 increase PDT-induced apoptosis. These results were correlated with caspase-3 and PARP cleavage and Bcl-2 degradation. In vivo, the photosensitization by COX-2 inhibitors was not due to apoptosis exacerbation. Interestingly, celecoxib and NS-398 decrease PDT-induced apoptosis but were also able to decrease the level of angiogenic factors such as TNFα, IL1β, PGE2, VEGF, and MMP9 [148].

Upon chlorin-induced PDT, COX-2 was found up-regulated 25-fold in mouse mammary carcinoma RIF cells [149]. This up-regulation was associated with an increase of PGE2 level in the tumor microenvironment. When RIF cells were transplanted in CH3/HeJ mice, for in vivo studies, PDT similarly induced an increase of COX-2 and PGE2. These effects were prevented by NS-398. Here, it was demonstrated that PDT induced vascular endothelial growth factor expression (VEGF) and this increase was attenuated by treating mice with NS-398, meaning that COX-2 might play a role also in angiogenesis. In consequence of these effects, the combination of COX-2 inhibitors with PDT resulted in an increased efficiency of tumor treatment.

Possible correlation between COX-2 level and resistance to PDT has been also investigated in Hela (human cervix carcinoma cells) and T24 (human transitional cell carcinoma of the urinary bladder) cells [150]. It has been reported that in PDT induced by hypericin, a natural photosensitizer which accumulates in endoplasmic reticulum and Golgi apparatus, an increase of PGE2 levels occurred. Hypericin induces apoptosis by triggering the release of cytochrome *c* after light excitation through a process requiring the activation of p38 MAPK, which it is known to induce an up-regulation of COX-2 [23, 151]. The increase in PGE2 levels was prevented by the use of a p38 MAPK inhibitor (PD169316). Moreover, the impairment of p38 MAPK was associated with an increase in the susceptibility of tumor cells to PDT. However, COX-2 inhibitors did not lead to the same effect, meaning that COX-2 was not involved in PDT resistance in this model.

In contrast to the study of Ferrario et al. [148, 149], a report from Makowski et al. [152] has revealed that rofecoxib, NS-398, and nimesulide were unable to potentiate PDT in C-26 cells (poorly differentiated colon adenocarcinoma cell line) in vitro. However, chronic exposition of mice bearing C-26 cells to nimesulide potentiated PDT. These data suggest that COX-2 inhibitors may indirectly potentiate PDT.

It is known that vasculature damages are important for PDT efficiency and that COX-2 inhibitors act as anti-angiogenic factors [153]. It has been hypothesized that these antiangiogenic effects could be responsible for the anti-tumor effect.

Currently, the link between COX-2 and PDT efficiency is not well characterized. Some studies have revealed an improvement of efficiency with COX-2 inhibitors whereas other reports have demonstrated no direct effects. In any case, this effect may be cell-type dependent as for chemotherapy or radiotherapy.

7. Inhibition of COX-2 Expression by Natural Compounds

Synthetic cyclooxygenase-2 inhibitors hold promise for cancer chemoprevention; however, recent toxicity problems suggest that new strategies are needed. Natural compounds with the potential to inhibit key cell signaling pathways including COX-2 gained much attention over the last regarding years whether they are used alone or in combination with existing chemotherapeutic agents.

Recently, Bhui et al. demonstrated that Bromelain, a pharmacologically active compound present in pineapple (Ananas cosmosus), leads to a marked inhibition of COX-2 expression and inactivation of NFκB. Bromelain treatment induces up-regulation of p53 and Bax and subsequent activation of caspase-3 and caspase-9 with a decrease in Bcl-2 expression [154]. Furthermore bromelain induces apoptosis-related proteins along with inhibition of NFκB -driven COX-2 expression by blocking the MAPK and Akt/protein kinase B signaling in DMBA-TPA-induced mouse skin tumors [155].

Curcumin, a naturally occurring polyphenol from Curcuma longa, was described to act as an antiinflammatory and antiproliferative agent by causing downregulation of COX-2 in cervical cancer. Curcumin-mediated apoptosis in these cells is initiated by up-regulation of pro-apoptotic Bax, AIF, release of cytochrome *c,* and downregulation of anti-apoptotic Bcl-2, Bcl-xL in HeLa and SiHa cell lines. This onset of apoptosis was accompanied by an increase in caspase-3 and -9 activity, suggesting the role of mitochondria in curcumin-mediated apoptotic cell death as described by M. Singh and N. Singh [156]. Marín et al., furthermore, concluded that curcumin inhibits NFκB activity as well as the expression of its downstream target genes, and also selectively induces apoptosis of melanoma cells but not normal melanocytes [157]. In addition, curcumin-induced apoptosis was also associated with the activation of caspase-3 and caspase-9, and the degradation of PARP. Curcumin decreased the expression levels of COX-2 mRNA and protein without causing significant changes in COX-1 levels, which was correlated with the inhibition of prostaglandin E(2) synthesis [158]. In BV-2 microglial cells, curcumin and analogs were shown to inhibit LPS-induced COX-2 expression; analogs identified as more potent than curcumin in the screening assay were also more potent than curcumin in preventing COX-2 expression [159].

Coumarin (1,2-benzopyrone) is a naturally occurring fragrant compound found in numerous plants and spices. Results obtained with human nonsmall cell lung cancer A549 cells suggest that downregulation of Bcl-xL, COX-2, and MAP kinase pathway and up-regulation of p53, Akt, and NFκB pathway are involved in the underlying molecular mechanism of apoptosis induction as suggested by Goel et al. [160].

Suh et al. concluded that the plant flavonoid fisetin induces apoptosis and suppresses the growth of colon cancer cells

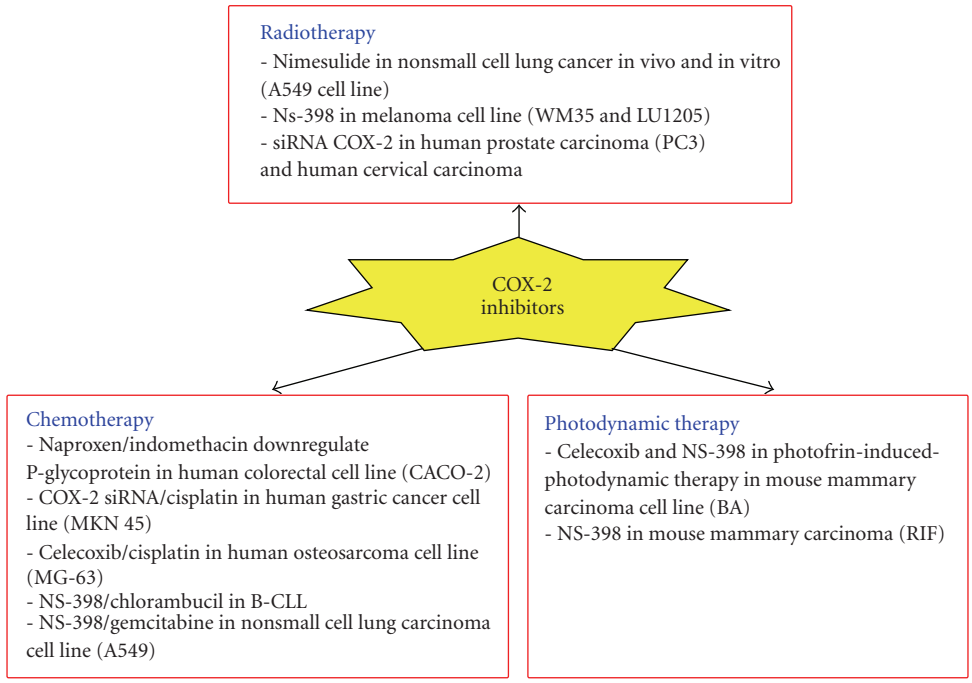

Figure 5: *COX-2 inhibition in cancer therapy.*

by inhibition of COX-2- and Wnt/EGFR/NFκB-signaling pathways [161].

Sulforaphane (SFN) is a biologically active compound extracted from cruciferous vegetables, and presents potent anti-cancer and anti-inflammatory activities by suppression of NFkB-dependent genes involved in anti-apoptotic signaling (IAP-1, IAP-2, XIAP, Bcl-2, and Bcl-xL), cell proliferation (c-Myc, COX-2, and cyclin D1), and metastasis (VEGF and MMP-9) as published by Moon et al. [162].

Nontoxic apigenin can suppress anti-apoptotic pathways involving NFκB activation including cFLIP and COX-2 expression as demonstrated by Xu et al. [88]. According to Nam et al., DA-6034, a synthetic derivative of flavonoid Eupatilin, strongly enhanced apoptosis and inhibited the expression of COX-2 and phospho-IKKalpha in inflammation-related colon cancer models [163].

EGCG from green tea was described to attenuate the AR, to downregulate IGF-1, to modulate COX-2 expression, and to decrease MAPK signaling leading to the reduction in cell proliferation and induction of apoptosis in prostate cancer without toxicity [164]. Interestingly, combination of EGCG and COX-2 inhibitor NS-398 enhanced cell growth inhibition, apoptosis induction, expression of Bax, pro-caspase-6, and pro-caspase-9, and PARP cleavage, inhibition of PPAR gamma, and inhibition of NFκB compared with the additive effects of the two agents alone, suggesting a possible synergism. In vivo, combination treatment with green tea polyphenols and COX-2 inhibitor celecoxib resulted in enhanced tumor growth inhibition, lowering of prostate-specific antigen levels, lowering of IGF-I levels, and circulating levels of serum IGF-1 binding protein-3 compared with results of single-agent treatment. Accordingly, Adhami et al. postulate the efficiency of synergistic and/or additive effects of combinatorial chemopreventive agents and further underscore the need for rational design of human clinical trials involving such natural compounds [165].

Pandey et al. published that butein inhibited the expression of the NFκB-regulated gene products involved in anti-apoptosis (IAP2, Bcl-2, and Bcl-xL), proliferation (cyclin D1 and c-Myc), and invasion (COX-2 and MMP-9). Suppression of these gene products correlated with enhancement of the apoptosis induced by TNF and chemotherapeutic agents, and inhibition of cytokine-induced cellular invasion. This group clearly demonstrates that antitumor and anti-inflammatory activities assigned to butein may be mediated in part through the direct inhibition of IKK, leading to the suppression of the NFκB activation pathway [166].

Hostanska et al. used human colon COX-2-positive HT 29 and COX-2-negative HCT 116 or lung COX-2 proficient A 549 and low COX-2 expressing SW2 cells and showed that willow bark extract BNO 1455 and its fractions inhibit the cell growth and promote apoptosis in human colon and lung cancer cell lines irrespective of their COX selectivity [167].

8. COX-2 Independent Effects

It is currently well known that several selective COX-2 inhibitors inhibit cell proliferation and induce apoptosis independently of COX-2. Celecoxib is particularly known to have these COX-2-independent effects, which were reviewed by Grosch et al. [68]. Indeed, celecoxib was able to directly bind and inhibit PKB/Akt, which plays an important role in cell proliferation and in apoptosis. Concerning cell cycle, PKB is able to phosphorylate cdk inhibitors, such as p21 and p27, leading to PCNA activation [168, 169]. Furthermore, PKB can also activate several cyclin-cdk complexes and

induce E2F factor in some cases [68], stimulating cell proliferation. Besides, PKB inhibits apoptosis, by phosphorylating the pro-apoptotic protein Bad and by inhibiting caspase-9 cleavage [51].

The COX-2-independent effects concern also the extrinsic apoptotic pathway. Indeed, we discussed that selective COX-2 inhibitors, such as NS-398, celecoxib, and meloxicam, are able to modulate the sensitivity of several tumor cells to Fas- and TRAIL-induced apoptosis. It has been discussed that this modulation could be due to COX-2-independent effects. In fact, NS-398 and nimesulide were able to promote TNF and TRAIL-induced apoptosis of D98 and H21 Hela cell lines [170]. In D98, COX-2 is inactive. Moreover, prostaglandin E2 readdition was not able to revert the sensitization effect. In the same report, it has been shown that NS-398 was able to promote apoptosis induced by TNF in MCF-7 cell line (human breast adenocarcinoma cells), which again does not express COX-2 [170].

A report from Ryan et al. [171] demonstrated that SC58125 and CAY10404, two selective COX-2 inhibitors, were able to decrease intracellular content of GSH in malignant human B-cells. This effect was accompanied by an increase of reactive oxygen species production. Indeed, GSH is the most important intracellular nonprotein thiol antioxidant defense against free radicals, meaning that it protects the cells from cellular damages. The GSH depletion was correlated in this study with a reduced survival for these cells.

The fact that many studies imply an association between COX-2 inhibition and apoptosis induction or cell proliferation inhibition, without assessing whether COX-2 activity is effectively decreased, suggests caution in the interpretation of the data. This is confirmed by the observation that different COX-2 inhibitors may trigger apoptosis in the same cancer cell model by modulating different mechanisms. For example, celecoxib [113] induced apoptosis by an inhibition of Akt phosphorylation in prostate cancer cells COX-2-positive LNCaP without affecting Bcl-2 level. In contrast, a study by Liu et al. [102] revealed that NS-398 in the same cell line was able to induce apoptosis but down-regulation of Bcl-2. These results suggest a possible COX-2-independent effect and strongly recommends considering in parallel other experimental strategies to ascertain the effective role of COX-2 in human malignancies [33], such as methodologies based on RNA interference or antisense oligonucleotides. Studies have already suggested these alternative methods. It has been shown that the sensitivity of a human gastric cancer cell line MKN45 to cisplatin (alkylating agent) was increased by COX-2 downregulation with siRNA [35].

9. Conclusion

A number of studies suggest that COX-2 inhibition may lead to an inhibition of cell proliferation in different cancer types. The fact that COX-2 inhibition may per se trigger apoptosis of tumor cells and/or sensitize them to cytotoxic treatments is an indication that COX-2 may be a good target in cancer therapy, in order to improve the efficiency of tumor cell death and to reduce tumor progression (see Figure 5 for a synthesis). Accordingly, the combination of selective COX-2 inhibitors with radiotherapy or different chemotherapeutics revealed a sensitization to apoptosis. This effect was also observed with several agents inducing apoptosis in a physiological way, thus suggesting that COX-2 inhibitors used in combination with death receptors agonists might be a novel approach to elicit apoptosis of cancer cells. However, the fact that COX-2 inhibitors can mediate their effects by COX-2-independent mechanisms suggests caution in the interpretation of the data.

Nowadays, selective COX-2 inhibitors have been included in several clinical assays. Some of them effectively increase the efficiency of radiotherapy and chemotherapy [172]. For example, celecoxib is in a clinical phase II assay in combination with Paclitaxel, carboplatin, and radiotherapy for patients with inoperable stage IIIA/B nonsmall cell lung cancer [172]. These clinical assays confirm that COX-2 inhibition may be a promising field in cancer treatment. However, the selective COX-2 inhibitors are responsible for side effects, including an increasing risk of cardiovascular complications [67, 68]. It is hoped that other methods to inhibit COX-2 will be developed. To this purpose, RNA interference using vehicle (i.e., adenovirus) as well as natural compounds were suggested by some studies [35, 76], as alternative and promising strategy.

Conflicts of Interest

The authors have no actual or potential conflict of interest.

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Research Article

Inhibition by Anandamide of 6-Hydroxydopamine-Induced Cell Death in PC12 Cells

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6-hydroxydopamine (6-OHDA) is a selective neurotoxin that is widely used to investigate cell death and protective strategies in models of Parkinson's disease. Here, we investigated the effects of the endogenous cannabinoid, anandamide, on 6-OHDA-induced toxicity in rat adrenal phaeochromocytoma PC12 cells. Morphological analysis and caspase-3 activity assay revealed that anandamide inhibited 6-OHDA-induced apoptosis. The protection was not affected by antagonists of either cannabinoid receptors (CB_1 or CB_2) or the vanilloid receptor TRPV1. Anandamide-dependent protection was reduced by pretreatment with LY294002 (inhibitor of phosphatidylinositol 3-kinase, PI3K) and unaffected by U0126 (inhibitor of extracellularly-regulated kinase). Interestingly, phosphorylation of c-Jun-NH2-terminal kinase (JNK) in cells exposed to 6-OHDA was strongly reduced by anandamide pre-treatment. Furthermore, 6-OHDA induced c-Jun activation and increased Bim expression, both of which were inhibited by anandamide. Together, these data demonstrate antiapoptotic effects of anandamide and also suggest a role for activation of PI3K and inhibition of JNK signalling in anandamide-mediated protection against 6-OHDA.

1. Introduction

In recent years, the endogenous cannabinoid (endocannabinoid) system has emerged as a potential therapeutic target for the treatment of Parkinson's disease [1–5]. These studies suggest that the potential therapeutic benefits of cannabinoid drugs may include neuroprotection of nigrostriatal dopaminergic neurons. This is of particular interest since neuroprotective therapies for Parkinson's disease are notably lacking, and current therapies are generally dopamine-enhancing strategies, that neither halt nor delay ongoing neurodegeneration. Anandamide (also known as arachidonylethanolamide), was the first endocannabinoid to be discovered, is derived from arachidonic acid and found principally in brain tissue [6]. Anandamide binds and activates the cannabinoid receptors (CB_1 and CB_2) and also the vanilloid receptor, TRPV1 [7, 8].

Mounting evidence supports a role for anandamide in the modulation of cell fate, including cell death and survival. Anandamide can protect neurons from toxic insults such as glutamatergic excitotoxicity, nutrient deprivation, hypoxia and ischemia [9–12]. These protective effects of anandamide have been reported to be mediated by CB_1 and CB_2 cannabinoid receptors, whereas activation of TRPV1 has been suggested to mediate anandamide-induced apoptosis in rat C6 glioma cells, human DAUDI leukemia cells, and cervical carcinoma cell lines [13–15].

The present study was undertaken to examine the ability of anandamide to protect PC12 cells against 6-hydroxydopamine (6-OHDA) toxicity. 6-OHDA is a hydroxylated analogue of dopamine that is commonly used in model systems to mimic Parkinson's disease [16, 17]. 6-OHDA induces apoptosis of primary mesencephalic dopaminergic neurons [18, 19], MN9D [20] and dopaminergic cell lines including PC12 [17, 21, 22]. Apoptosis is a highly regulated form of cell death that occurs under physiological and pathological conditions. It is characterised morphologically by cell shrinkage and nuclear condensation. These changes are mediated by activation of caspase proteases, and in the case of 6-OHDA this occurs as

a result of release of cytochrome *c* from the mitochondria [22].

Here we examine the effect of anandamide on 6-OHDA-induced toxicity in PC12 cells. In particular, the mechanism of anandamide action against 6-OHDA was tested by examining the possible role of signalling pathways, which are well known to be involved in regulation of cell fate, including phoshpatidylinositol 3-kinase (PI3K)/Akt, mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase1/2 (ERK1/2) and c-Jun-NH2-terminal kinase (JNK)/c-Jun pathways.

2. Experimental Procedures

2.1. Materials. Rat phaeochromocytoma PC12 cells were obtained from European Collection of Cell Cultures (ECACC). All chemicals were supplied by Sigma–Aldrich unless stated otherwise. Anandamide and SB366791 were obtained from Tocris Bioscience. SR141716A and SR144528 were from NIMH Chemical Synthesis and Drug Supply Program. U0126 and SP600125 were supplied by Calbiochem. Rabbit polyclonal antibody against Bim was from StressGen Biotechnologies. Mouse monoclonal antibody against p-JNK, rabbit polyclonal anti-caspase-3 antibody and rabbit monoclonal antibody against p-ERK1/2 were obtained from Cell Signalling Technology. Mouse monoclonal antibody against p-c-Jun was from Santa Cruz Biotechnology. Anti-Actin rabbit polyclonal antibody was from Sigma-Aldrich. Goat secondary antibodies conjugated to horseradish peroxidase were from Pierce. Ac-Asp-Glu-Val-Asp-a-(4-methyl-coumaryl-7-amide) (DEVD-MCA) was from the Peptide Institute, Osaka, Japan. Protein molecular weight marker was obtained from New England Biolabs.

2.2. Cell Culture and Treatments. Rat adrenal phaeochromocytoma PC12 cells were cultured in DMEM supplemented with 10% horse serum, 5% fetal calf serum, 50 U/ml penicillin and 50 μg/ml streptomycin, at 37°C in a humidified 5% CO_2 atmosphere. For experiments, cells were seeded at a density of 7×10^4 cells/cm^2 in plates coated with 10 μg/ml poly-L-lysine. Cells were left overnight before commencing experimental treatments. Stock solutions of 6-OHDA were made freshly in sodium metabisulfite (1 M) prior to each experiment. Unless otherwise stated, PC12 cells were incubated with 25 μM anandamide for 24 hours followed by treatment with 100 μM 6-OHDA for further 24 hours before analysis.

2.3. Cell Viability and Proliferation Assay. Cell survival was determined by using colorimetric MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide) tetrazolium salt assay [23]. PC12 cells were plated into 96-well plates at 1.7×10^4 cells/well in 100 μl of medium. MTT tetrazolium salt was dissolved in Hank's balanced salt solution to concentration of 5 mg/ml. After experimental treatments, 10 μl of MTT was added to the culture for 3 hours at 37°C. To stop the reaction and solubilize the formazan crystals 100 μl of 20% SDS in 50% dimethyl formamide was added and the absorbance was measured at 550 nm by a Wallac 1420 plate-reader with a reference wavelength of 650 nm. Cell viability was expressed as percent of the control culture.

2.4. DAPI Staining of Nuclei. Cells were washed in PBS and fixed with 3.7% paraformaldehyde for 10 minutes at room temperature. Nuclei were stained with 4′-6-diamidino-2-phenylindole (DAPI) and placed in mounting medium (Vectashield, Vector, Burlingame, CA). 5 μl aliquot of the cell suspensions were applied to glass slides. Morphological changes in chromatin of cells undergoing apoptosis were analysed by fluorescence microscopy (excitation 350 nm and emission at 460 nm). Cells were scored by counting at least 300 cells from each sample.

2.5. DEVDase Activity Assay. The activity of caspase-3-like enzymes (DEVDases) was determined fluorometrically as reported previously [24] with some modifications [22, 25]. Briefly, cells were scraped and spun down at 300 × g at 4°C for 5 min. Pellets were washed in ice-cold phosphate-buffered saline (PBS) and spun down again at 20 000 × g for 10 seconds. The pellets were re-suspended in 25 μl PBS and snap-frozen in liquid nitrogen. 50 μM of DEVDase-substrate (DEVD-MCA) in reaction buffer (100 mM N-2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid (HEPES) pH 7.5, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM dithiothreitol (DTT), 10^{-4}% Nonidet-P-40) was added into lysates and the release of free AMC was monitored at 37°C at 60 seconds intervals over a 30 minute period using a Wallac Victor multilabel counter (excitation 355 nm, emission 460 nm). Fluorescent units were converted to nanomoles of AMC released using a standard curve generated with free AMC and subsequently related to protein concentration.

2.6. Preparation of Whole Cell Extracts. Following experimental treatments cells were scraped from the culture flasks and centrifuged at 150 × g for 5 minutes at 4°C. After washing in PBS cells were lysed using whole cell lysis buffer (20 mM HEPES pH 7.5, 350 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 0.5 mM DTT, 0.1% phenylmethylsulphonyl fluoride (PMSF), 1% aprotinin, 5 mM NaF, and 1 mM Na_3VO_4). Cellular debris was spun down at 20, 000 × g for 1 minute and supernatant was taken to determine the protein content using Bradford reagent with bovine serum albumin (BSA) as the standard.

2.7. Western Blotting. 40 μg of proteins were denatured using Laemmli's sample buffer (62 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulphate (SDS), 5% β-mercaptoethanol, 4% glycerol, 1 mM PMSF, 0.01% bromophenol blue) and boiled at 95°C for 5 minutes. Proteins were separated by 10%–15% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. Membranes were blocked for 1 hour in PBS containing 0.05% Tween 20 and 5% (w/v)

non-fat dried milk. Membranes were probed with antibodies (1 : 1000) overnight at 4°C followed by appropriate horseradish peroxidise-conjugated goat secondary antibody at 1 : 10,000 (or 1 : 2,000 for detection of caspase-3) for 2 hours at room temperature. Protein bands were visualized using Supersignal West pico system (Pierce).

2.8. Statistical Analysis. Values are expressed as means ± SEM of 3 separate experiments unless otherwise indicated. Statistical analysis was performed using repeated-measures ANOVA followed by Bonferroni multiple comparisons *post hoc* test, for which levels of $P < .05$ were considered to be significant.

3. Results

3.1. Anandamide Pre-Treatment Protects PC12 Cells against 6-OHDA Toxicity. We have previously shown that 6-OHDA causes a concentration-dependent induction of apoptosis in PC12 cells [22]. In order to examine the effect of anandamide against 6-OHDA, PC12 cells were incubated with 25 μM anandamide for 24 hour followed by exposure to 100 μM 6-OHDA for a further 24 hours. Visualisation of nuclear morphology by DAPI staining demonstrated that pre-treatment of PC12 cells with anandamide inhibited 6-OHDA-induced apoptosis (Figure 1(a)). The level of cell death due to 6-OHDA with and without prior anandamide treatment was quantified and expressed as a percentage of the total number of cells. Anandamide alleviated the morphological manifestation of cell damage and reduced cell death from 19.2 ± 2.8% to 4.7 ± 4.6% (Figure 1(b)).

Next the effect of anandamide on caspase 3-like activity (DEVD-MCA cleavage activity) was examined. PC12 cells were treated with 0–50 μM anandamide for 24 hours prior to treatment with 6-OHDA for a further 24 hours. Anandamide effectively inhibited DEVD-MCA-cleavage activity in a concentration-dependent manner, causing approximately 50% inhibition at 10 μM anandamide (Figure 1(c)). This was accompanied by a reduction in the processing of procaspase-3 into its active 17 kDa form, as detected by Western blotting (Figure 1(d)). These data indicate that anandamide can protect against 6-OHDA-induced apoptosis at the level of, or upstream of, caspase-3 activation.

3.2. Anandamide-Mediated Protection Is Cannabinoid and Vanilloid Receptor-Independent. In order to determine whether the effect of anandamide is receptor-mediated, PC12 cells, which express the CB_1 receptor ([26], and our own data not shown) were incubated with selective CB_1 or CB_2 receptor antagonists, SR141716A or SR144528, respectively, prior to treatment with 25 μM anandamide and 6-OHDA. The receptor antagonists did not inhibit the protective effects of anandamide, indicating that neither CB_1 nor CB_2 receptors are involved in anandamide protection against 6-OHDA toxicity (Figures 2(a) and 2(b)). Application of the antagonists at excess concentrations (25 μM for SR141716A; 20 μM for SR144528) also did not reverse the protection (Figures 2(a) and 2(b)). Since anandamide also has activity at the TRPV1 vanilloid receptor [8], and since this receptor is expressed in PC12 cells [26, 27], the effect of a TRPV1 selective antagonist, SB366791, on anandamide protection was examined. SB366791 had no effect on the protective abilities of anandamide against 6-OHDA-induced caspase activity (Figure 2(c)). In addition, none of the antagonists had any effect on 6-OHDA-induced DEVDase activity in the absence of anandamide. Cannabinoids, particularly those possessing a phenolic ring, are known to exert receptor-independent effects on cells and the neuroprotective effects have been suggested to be related to the potent antioxidant properties of cannabinoids [28]. Since 6-OHDA-induced cell death can be mediated by oxidative stress [17, 20, 29], the effect of anandamide on H_2O_2-induced cell death was examined. Anandamide did not prevent cell death due to H_2O_2 as determined by MTT survival assay (Figure 3). This suggests that anandamide-mediated protection against 6-OHDA is not a consequence of its antioxidant ability.

3.3. Involvement of PI3K and ERK1/2 Signalling Pathways in the Prosurvival Action of Anandamide. Cannabinoid-dependent effects have been associated with several signal transduction pathways including activation of phosphatidylinositol 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) signalling pathways, both of which are linked to prosurvival signalling [30–32]. To gain insight into the molecular mechanism leading to cell survival, we studied the role of these signalling pathways in anandamide protection. Inhibition of PI3K with 40 μM LY294002 was found to exacerbate 6-OHDA toxicity (Figure 4(a)). Pre-treatment with LY294002 reversed anandamide-mediated protection, although not to the level in the absence of anandamide, probably due to the effect of LY294002 in enhancing 6-OHDA-induced toxicity.

To assess a possible role of MEK/ERK signalling in anandamide-mediated protection, we used Western blotting to examine the effect of anandamide on the phosphorylation status of ERK1/2. Treatment of PC12 cells with 25 μM anandamide resulted in a transient increase in ERK1/2 phosphorylation at 6 hours that returned to basal levels by 24 hours (Figure 4(b)). Pharmacological inhibition of ERK activation by 10 μM U0126 partially reversed the effect of anandamide on DEVDase activity, but without statistical significance (Figure 4(c)). This suggests that the increase in ERK1/2 phosphorylation does not make a major contribution to anandamide-mediated protection.

3.4. Anandamide Inhibits JNK Activation Induced by 6-OHDA. The stress kinase JNK has been reported to mediate 6-OHDA-induced cell death [20, 33]. Therefore, we examined the effect of anandamide on JNK activation by examining the phosphorylation status of JNK1 and JNK2. Treatment of PC12 cells with 100 μM 6-OHDA evoked a time-dependent increase in JNK1 phosphorylation with maximal activation after 6 hours (Figure 5(a)). There was a smaller increase in JNK2 phosphorylation that followed the same time course. Prior treatment with anandamide resulted in a marked inhibition of JNK1 phosphorylation

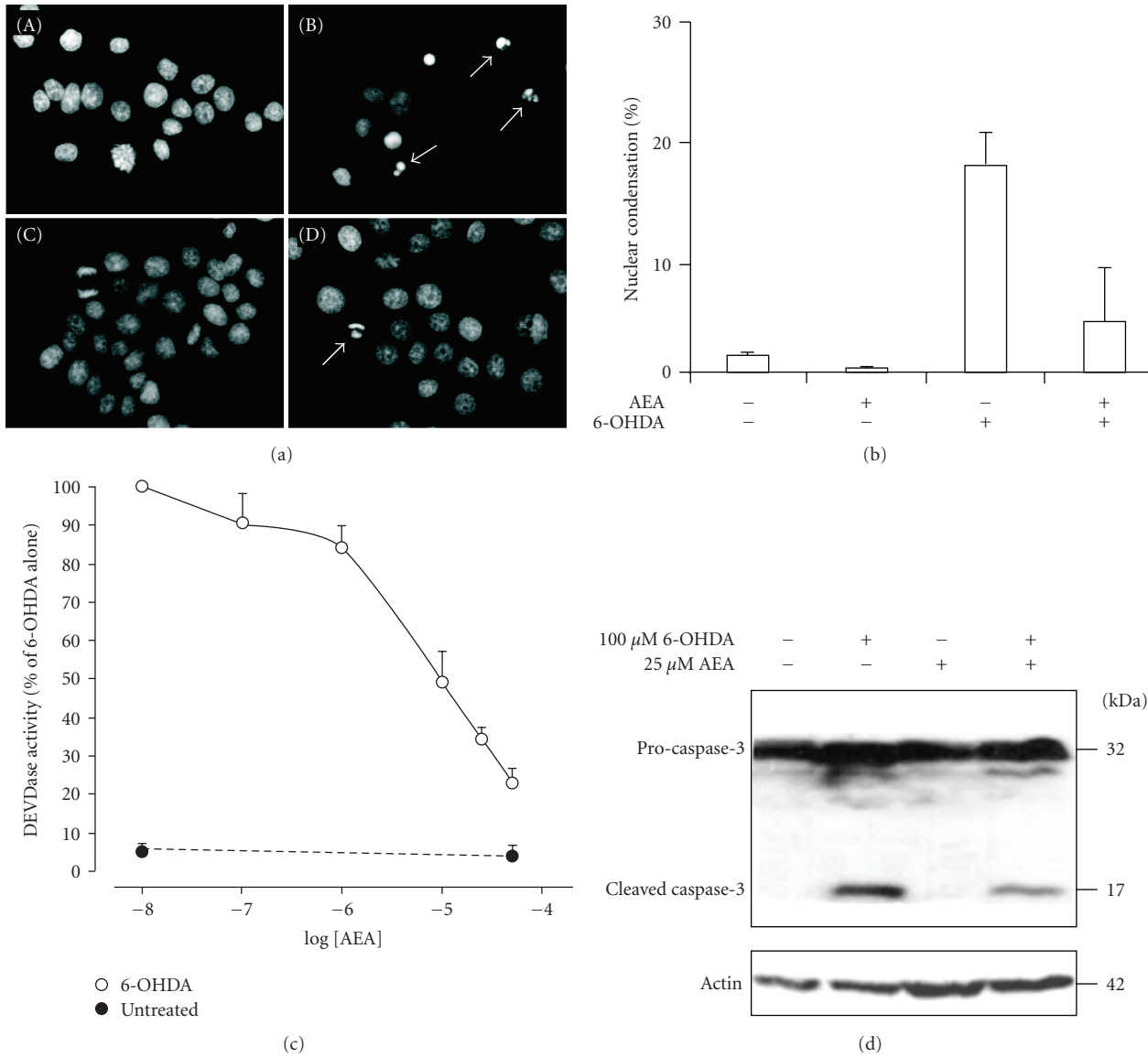

Figure 1: Inhibition of 6-OHDA-induced apoptosis by anandamide. (a) PC12 cells were treated with 25 μM anandamide for 24 hours and then exposed to 100 μM 6-OHDA for a further 24 hours. Cytocentrifuge preparations of control and treated cells were stained with DAPI to visualise nuclear changes. The apoptotic cells with condensed and fragmented nuclei are indicated by arrows. Cells were (a) untreated, (b) exposed to 6-OHDA, (c) treated with AEA only or (d) treated with AEA and then exposed to 6-OHDA. (b) The level of cell death due to 6-OHDA with and without prior anandamide treatment was quantified and expressed as a percentage of the total number of cells. The results shown are the average of two separate experiments ± range. (c) PC12 cells were treated with a range of concentrations of anandamide (0–50 μM) for 24 hours prior to exposure to 100 μM 6-OHDA for a further 24 hours. DEVDase activity was measured in whole cell extracts. Values represent the mean ± SEM of four independent determinations. (d) PC12 cells were exposed to 25 μM anandamide for 24 hours followed by treatment with 100 μM 6-OHDA for further 24 hours. Pro-caspase-3 processing was visualized by Western blotting. Actin was used as a loading control. The data are representative of two independent experiments.

(Figure 5(a)). These findings suggest that suppression of JNK activation may play an important role in anandamide-dependent protection against 6-OHDA and are in agreement with other studies showing that inhibition of JNK activity protects PC12 cells against 6-OHDA-induced apoptosis [20, 33–37].

Many JNK targets are implicated in cell death, including c-Jun and the BH3-only protein Bim [38]. Therefore, the effect of anandamide on these JNK targets was examined. 6-OHDA caused an increase in c-Jun phosphorylation which was reduced by anandamide pre-treatment (Figure 5(b)). Bim_{EL}, a pro-apoptotic member of the Bcl-2 family, is also a target of JNK. Treatment of cells with 6-OHDA caused a marked increase in Bim_{EL} expression by 12 hours (Figure 5(b)). Three Bim_{EL} bands were observed with the higher molecular weight forms probably reflecting

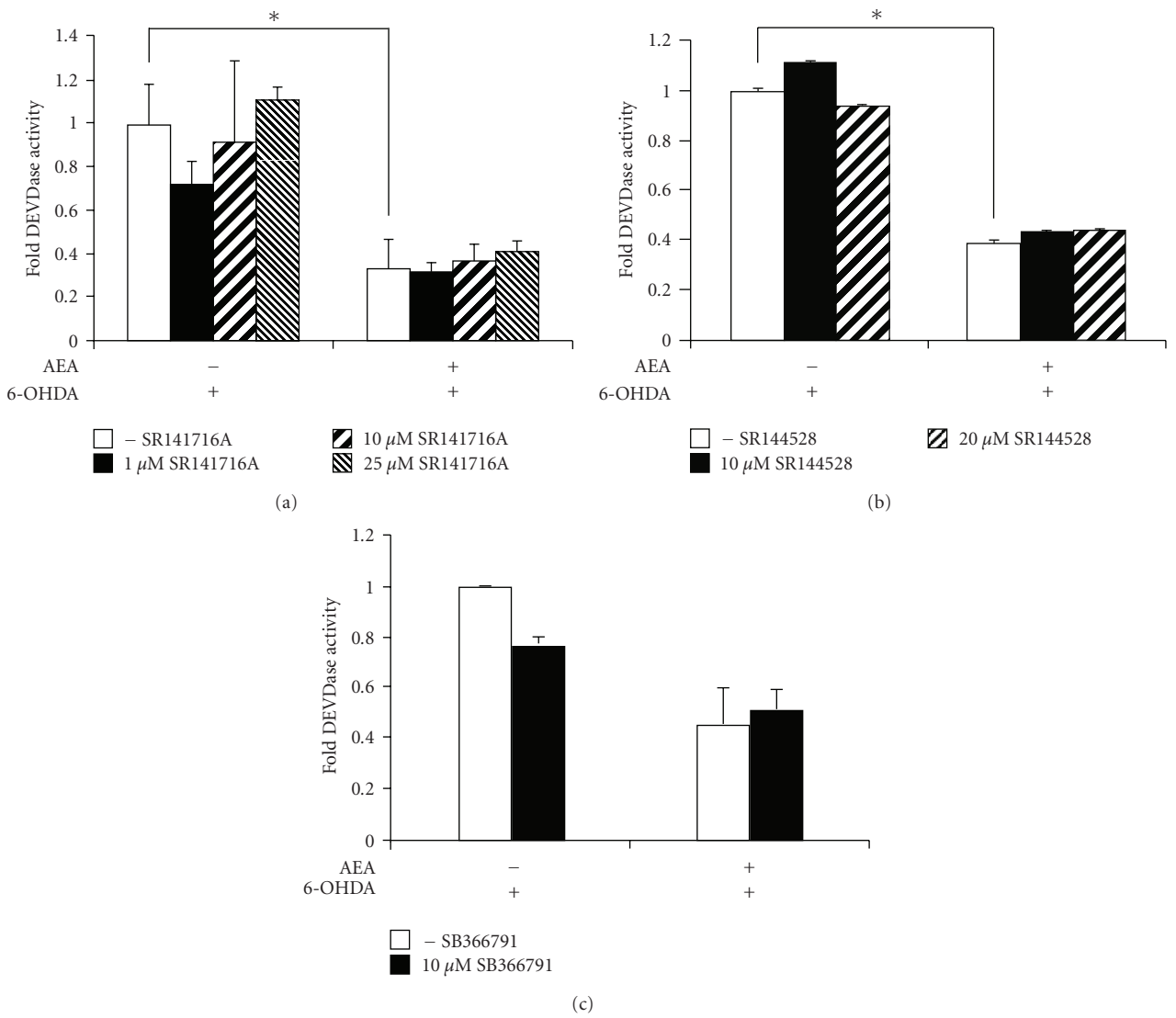

Figure 2: Anandamide-mediated prosurvival effect is cannabinoid and vanilloid receptor-independent. PC12 cells were treated with 25 μM anandamide for 24 hours, with or without selective receptor antagonists, and then exposed to 100 μM 6-OHDA for a further 24 hours. Effector caspase activity was measured by DEVDase assay in whole cell extracts. (a) Indicated concentrations of CB_1 receptor antagonist SR141716A were added 1 hours prior to incubation with anandamide. Values represent the mean ± SEM of 3 independent determinations, $^*P < .001$. (b) Indicated concentrations of CB_2 receptor antagonist SR144528 were added 1 hour prior to incubation with anandamide. Values represent the mean ± SEM of 3 independent determinations, $^*P < .001$. (c) The TRPV1 receptor antagonist SB366791 at 10 μM was added 1 hour prior to incubation with anandamide. The data shown are average of two separate experiments ± range.

phosphorylated species, since this protein is known to be regulated by phosphorylation [39, 40]. In fact, we have previously shown that these upper bands represent phosphorylated Bim_{EL}, since they disappear upon treatment of whole cell lysates with phosphatase [32]. Pre-treatment of the cells with anandamide did not appear to affect the time course or level of induction of Bim_{EL} (Figure 5(b)). However, there was a reduction in the higher molecular weight band for Bim_{EL} and an increase in the intensity of the lower molecular weight band at 24 hours of 6-OHDA with AEA treatment (Figure 5(b)). These data suggest that there is an anandamide-induced reduction in phosphorylation of Bim_{EL}, possibly due to JNK inhibition.

In order to confirm a role for JNK activation in 6-OHDA toxicity, cells were pre-treated with the JNK inhibitor SP600125 (4 μM). This inhibitor reduced phosphorylation of c-Jun by 6-OHDA and also caused a delay and a reduction in cleavage of caspase-3 to its active p17 fragment (Figure 5(c)). In the same experiment, 25 μM anandamide had caused complete inhibition of caspase-3 cleavage while inhibiting c-Jun phosphorylation, but to a lesser extent than SP600125.

4. Discussion

Here we provide the first evidence for direct protective effects of anandamide against 6-OHDA toxicity. Anandamide

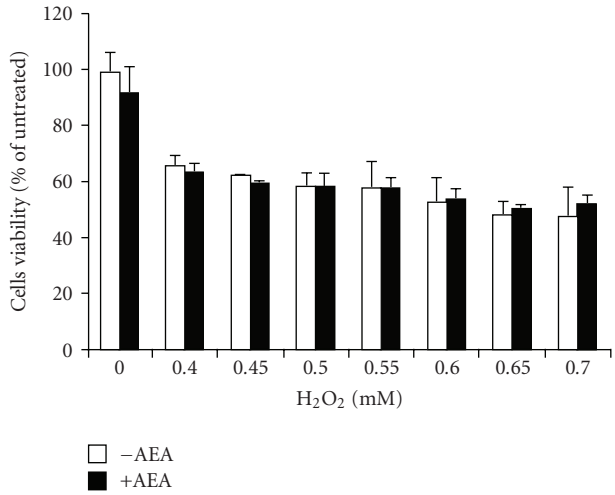

FIGURE 3: Effect of anandamide on H_2O_2 induced oxidative stress in PC12 cells. Cells were treated with 25 μM anandamide for 24 hours, followed by treatment with the indicated concentrations of H_2O_2 for another 24 hours. Cell viability was assessed by the MTT assay. The data shown are average of two separate experiments ± range.

blocks 6-OHDA-induced apoptosis in a concentration-dependent manner. This protection was upstream of caspase-3 activation since there was inhibition of DEVDase activity and a reduction in processing of pro-caspase-3. We were unable to demonstrate a role for CB_1, CB_2 or TRPV1 in anandamide protection. Nor did it involve ERK1/2 prosurvival signalling. However, a partial role for PI3K activation and suppression of JNK signalling were demonstrated. Cannabinoids have been shown to both induce and inhibit induction of cell death, (reviewed by [41]). Anandamide itself has been reported to be toxic [26] or nontoxic [42] to PC12 cells. In our study, anandamide, at the concentrations used, was not toxic even up to 48 hours of incubation (data not shown). Cannabinoid drugs have been shown by others to be protective in a variety of models of neurodegeneration [9, 41, 43], including neuronal cell death in models of Parkinson's disease [28]. For example, the non-selective synthetic cannabinoid receptor agonist HU210 was found to protect cerebellar granule neurons against 6-hydroxydopamine toxicity [28]. Furthermore, an in vivo study demonstrated that the plant-derived cannabinoids Δ^9-THC and cannabidiol protect the rat nigrostriatal dopaminergic pathway from a medial forebrain bundle injection of the catecholamine neurotoxin 6-hydroxydopamine [28]. Although that in vivo study did not include direct assessment of neuroprotection, the ability of Δ^9-THC or cannabidiol to ameliorate the effects of the 6-OHDA lesion on striatal dopamine concentration and tyrosine hydroxylase activity were shown. Our current data with anandamide support these earlier findings.

A possible role of metabolites of anandamide in the regulation of PC12 cell fate cannot be ruled out. Anandamide is enzymatically hydrolysed to ethanolamine and arachidonic acid [44]. In fact, this degradation has been shown to be necessary for its prosurvival effects in murine neuroblastoma cells where anandamide protective activity against serum deprivation was not mediated via CB_1/CB_2 or TRPV1 receptors and required a breakdown of anandamide to ethanolamine [9].

It has been reported that induction of oxidative stress is involved in 6-OHDA-induced toxicity and antioxidants can protect against hydrogen peroxide and superoxide anion-mediated cell death [20, 33–37, 45–47]. To our knowledge, anandamide has not been reported to possess antioxidant activity. We did not observe anandamide-mediated protection of PC12 cells against H_2O_2-induced death, suggesting that anandamide does not inhibit oxidative stress due to 6-OHDA. However, H_2O_2 is not the only product of 6-OHDA auto-oxidation. In addition, superoxide anions and *p*-quinone, may contribute to reactive oxygen species (ROS) production [48–50] and may be inhibited by anandamide, thus blocking JNK activation and phosphorylation of its downstream targets.

Cannabinoids have been shown to activate PI3K/Akt and MEK/ERK signalling pathways [51, 52]. While our results obtained with pharmacological inhibitors did not support a role for MEK/ERK signalling in anandamide protection against 6-hydroxydopamine, they did support an involvement of PI3K/Akt prosurvival signalling, since the PI3K selective inhibitor LY294002 reversed anandamide protection against 6-OHDA. In addition, the present data also support a possible role for inhibition of JNK activation in anandamide protection, since anandamide reduced 6-OHDA-induced JNK phosphorylation and JNK-dependent pro-apoptotic signalling, namely phosphorylation of c-Jun and possibly of Bim_{EL}.

JNKs are members of the mitogen-activated protein kinase (MAPK) pathway that is activated in response to many extracellular stimuli and different forms of environmental stress [53]. Our findings are in general agreement with recent reports showing that 6-OHDA induces JNK phosphorylation [36, 54] and that JNK activation mediates apoptosis induced by 6-OHDA in PC12 cells [45, 47, 54]. Furthermore, a number of studies showing protection by various agents against 6-OHDA neurotoxicity also show a concomitant suppression of JNK activation [20, 33–37]. We observed a large 6-OHDA-induced increase in phosphorylation of JNK1, and a minor increase in levels of phosphorylated JNK2 at 3–6 hours, which are accompanied by a concomitant increase in phospho-c-Jun and a delayed increase in the levels of Bim_{EL}. Furthermore, the JNK inhibitor SP600125 caused a partial reduction in caspase-3 cleavage due to 6-OHDA. This was in contrast to complete inhibition by anandamide. These findings are in agreement with a previous report showing that SP600125 partially protects against 6-OHDA toxicity in PC12 cells [47]. Taken together, these data suggest a partial contribution of JNK signalling in the toxicity of 6-OHDA, and that anandamide may mediate some of its protective effect through suppression of this pathway.

JNK promotes apoptosis in a number of ways. Through phosphorylation and activation of c-Jun it stimulates the transcription of c-Jun target genes, including *Bim* [38]. In addition, JNK phosphorylates certain members of the Bcl-2 protein family, associated with the mitochondrial

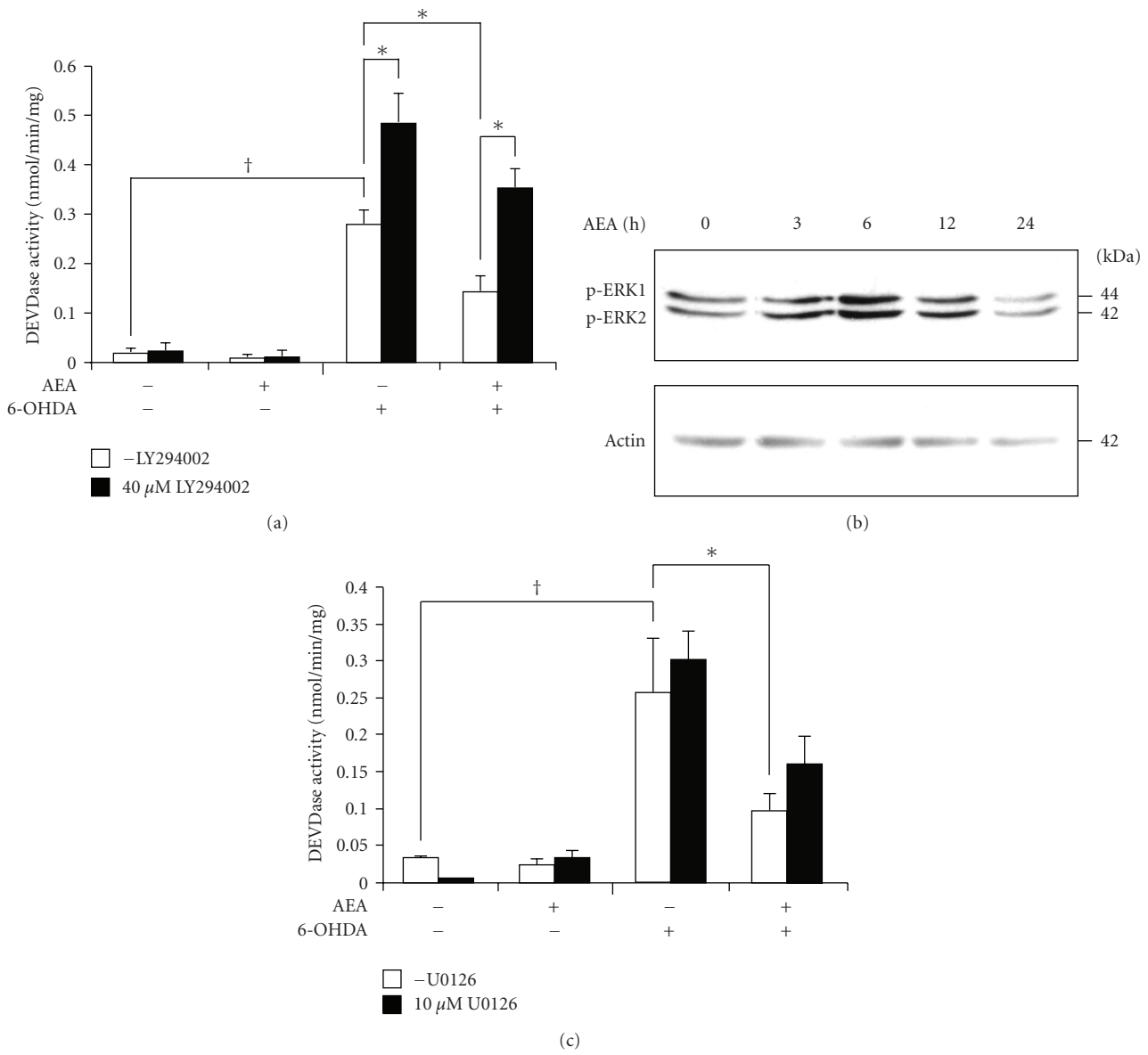

Figure 4: Role of PI3K and MAPK signalling in anandamide-mediated protection against 6-OHDA toxicity. (a) PC12 cells were pre-treated with 40 μM LY294002 for 1 hours prior to addition of 25 μM anandamide for 24 hours followed by treatment with 100 μM 6-OHDA for a further 24 hours. Values represent the mean ± SEM of 4 independent experiments. $^{*}P < .005, P^{\dagger} < .001$. (b) PC12 cells were treated with 25 μM anandamide for 0–24 hours. The phosphorylation state of ERK1/2 was analysed by Western blotting. Actin protein levels were also analysed as a loading control. The results are representative of two separate experiments. (c) 10 μM U0126, the MAPK pathway inhibitor was added 1 hour prior to incubation of PC12 cells with 25 μM anandamide for 24 hours and with 100 μM 6-OHDA for a further 24 hours. Values xrepresent mean ± SEM of three independent determinations. $P^{\dagger} < .001$, $^{*}P < .01$.

apoptotic pathway, including Bim_{EL}, Bmf, and Bcl-2 [38, 53]. For example, Bim_{EL} levels and function are regulated by phosphorylation by ERK, JNK and possibly Akt [40, 55]. Phosphorylation of Bim_{EL} at Ser65 by active JNK potentiates its pro-apoptotic activity [56]. These data support an attractive hypothesis that anandamide protects against 6-OHDA at the level of, or upstream of, JNK activation. This inhibition of JNK by anandamide thus prevents the activation of downstream apoptotic pathways, including activation of c-Jun and possibly phosphorylation of Bim_{EL}. However, since the level of cytoprotection by anandamide was far greater than that due to SP600125, despite similar JNK inhibition, it strongly suggests that anandamide utilises additional mechanisms to inhibit 6-OHDA toxicity.

It should be considered that in contrast to the data supporting endocannabinoid-mediated JNK inhibition, there are data reported by others [57–59] indicating that cannabinoids induce JNK activation, an effect that precedes apoptotic events including caspase-3 activation and DNA fragmentation. Thus, the neuroprotective and neurotoxic effects

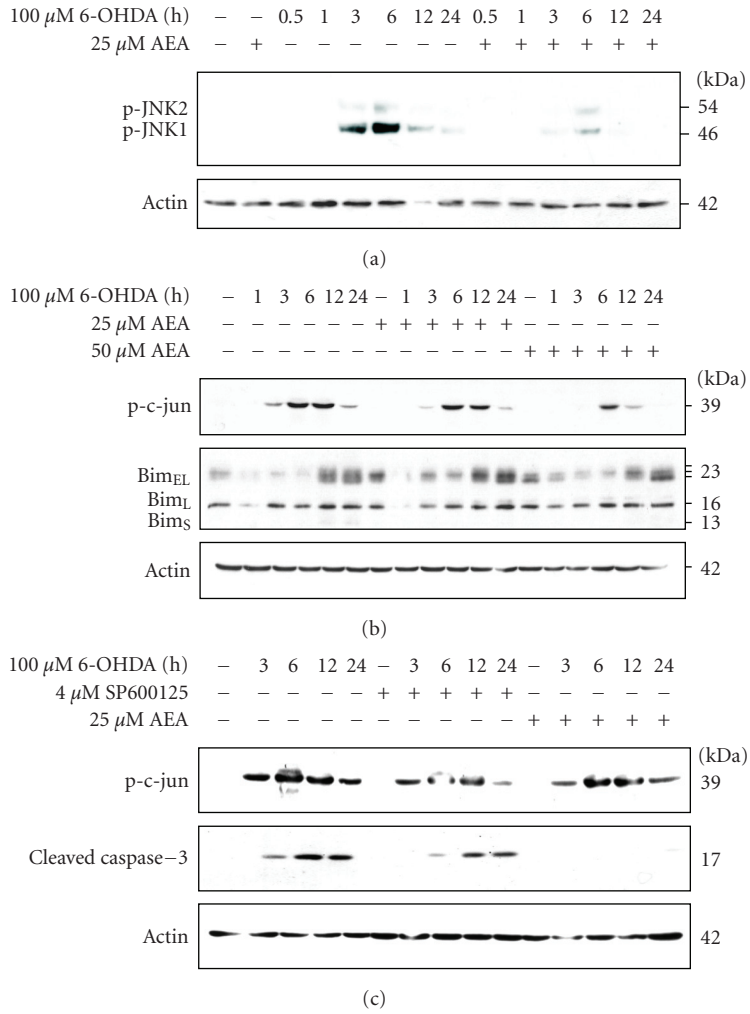

Figure 5: Anandamide partially protects against 6-OHDA-mediated apoptosis through inactivation of JNK signalling pathway. PC12 cells were treated with 25 μM or 50 μM anandamide for 24 hours and then exposed to 100 μM 6-OHDA for indicated periods of time. (a) The phosphorylation state of JNK1/2 was analysed by Western blotting. Actin protein levels were also analysed as a loading control. (b) The phosphorylation state of c-Jun and Bim isoforms were analysed by Western blotting. Actin protein levels were also analysed as a loading control. Blots shown are representative of three independent experiments. (c) Where indicated, cells were exposed to 4 μM SP600125 for 1 hour or 25 μM AEA for 24 hour prior to treatment with 100 μM 6-OHDA for further 24 hour. Phosphorylation of c-Jun and the processing of caspase-3 to the active 17 kDa fragment subunit were analysed by Western blotting. The levels of actin expression were also determined for loading control. The data shown are representative of two separate experiments.

of cannabinoids are likely to depend on a variety of factors, including toxic insults, doses and nature of cannabinoids (e.g., endocannabinoids versus phytocannabinoids), times of exposure and cell type [60, 61].

Here, for the first time we demonstrate that anandamide protects PC12 cells against 6-OHDA-induced apoptosis. These data and those of other studies may suggest potential therapeutic benefits of elevating anandamide and possibly other endocannabinoids in preventing the degeneration of nigrostriatal dopaminergic neurons in Parkinson's disease.

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Research Article

The Small Heat Shock Protein HSP25/27 (HspB1) Is Abundant in Cultured Astrocytes and Associated with Astrocytic Pathology in Progressive Supranuclear Palsy and Corticobasal Degeneration

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Filamentous tau-positive protein inclusions in neurons and glia are prominent features of a number of neurodegenerative disorders termed tauopathies. These inclusions are further characterized by the presence of heat shock proteins (HSPs). The group of small HSPs, namely, HSP27 and αB-crystallin, interact with the cytoskeleton, bind to nonnative proteins, and prevent their aggregation after stress. To further investigate their contribution to neurodegenerative diseases, we have analyzed the association of HSP27 with pathological lesions of tauopathies. Microarray and immunoblot analysis revealed that HSP27 is enhanced at the mRNA and protein levels in affected brains, and that it is associated with astrocytic pathology. The upregulation of HSP27 in tauopathies with gial pathology implies distinct mechanisms for glial and neuronal cells. This was sustained by cell culture studies, demonstrating that the small HSPs are specifically and prominently expressed in unstressed astrocytes and not in neurons and in neurons remained at a rather low level even after stress situations.

1. Introduction

Filamentous protein inclusions are observed in a number of neurodegenerative diseases. They contain a cellular protein, such as α-synuclein or tau, and often a variety of heat shock proteins (HSPs) and ubiquitin in addition to cytoskeletal proteins [1–4]. HSPs acting as molecular chaperones refold damaged proteins and target irreversibly damaged proteins for degradation to the ubiquitin-proteasome system to prevent proteotoxic damage. Their presence in cellular inclusions indicates that they were unsuccessfully upregulated in an attempt to prevent protein denaturation and aggregation. Furthermore it indicates that stress situations, such as oxidative stress, heat stress, or stress induced by proteasomal impairment, contribute to the pathogenesis of the disorders [3].

Pathological inclusions containing the microtubule-associated protein tau in its hyperphosphorylated form are a characteristic hallmark of a class of both sporadic and familial disorders, termed tauopathies [1, 2, 4]. While in Alzheimer's disease (AD) tau aggregates preferentially are formed in neurons, in frontotemporal dementias, such as Pick's disease, progressive supranuclear palsy (PSP), and corticobasal degeneration (CBD), tau-positive inclusions are consistent features not only in neurons but also in glia [5–8]. Neuronal and glial filamentous lesions in PSP and CBD are composed of hyperphosphorylated tau with four microtubule binding repeats, that is, 4R-tau [9]. Tau is a microtubule-binding protein that is abundantly expressed in the central nervous system. It is important for microtubule assembly and stability, and in normal brain is mainly located in axons and not expressed in healthy astrocytes [10]. Data from our laboratory have demonstrated that it is also an important constitutent of the oligodendroglial cytoskeleton [7, 11]. Based on their cellular origin, in PSP and CBD tau-positive glial inclusions are classified as tau-positive astrocytes and oligodendroglial coiled bodies [8, 9, 12]. Astrocytic inclusions vary among the diseases; they do not form solid inclusion bodies but rather exhibit diffuse or fibrillary staining patterns. Tufted

astrocytes and astrocytic plaques are typical for PSP and CBD, respectively, while tau-positive coiled bodies originating in oligodendrocytes can be found in both disorders [4, 12, 13].

The group of small HSPs with molecular weights in the range of 12–43 kDa is closely related [14, 15]. The best known representatives are αB-crystallin and HSP27, or its rat analogue HSP25. They are involved in a variety of cellular processes, particularly interact with cytoskeletal elements, are stress, inducible, display chaperone function and effectively bind to nonnative proteins, preventing the aggregation of proteins after stress [16, 17]. Cultured rat brain astrocytes constitutively express a high level of HSP25, which exerts protective effects against a variety of stress situations, while in oligodendrocytes αB-crystallin seems to be more important [18, 19]. In AD small HSPs have been found to be associated with astrocytes in senile plaques and not with neurons in neurofibrillary tangles [20]. αB-Crystallin immunoreactivity seemed to be specific to diseases with glial pathology [5, 21].

To further investigate the contribution of small HSPs to neurodegenerative diseases, we have analyzed the association of HSP27 with pathological lesions of PSP and CBD brains. Furthermore, since distinct pathogenic mechanisms and stress protein induction in neurons and astrocytes seem to contribute to tau pathology, we have compared HSP expression and upregulation in cultured neurons and astrocytes derived from rat brain. Immunoblot analysis demonstrates that HSP27 is upregulated in the brains of patients with PSP and CBD, and found in cell inclusions with astrocytic morphology. Furthermore, it is more prominently expressed in cultured astrocytes under normal and stress-induced conditions than in neurons.

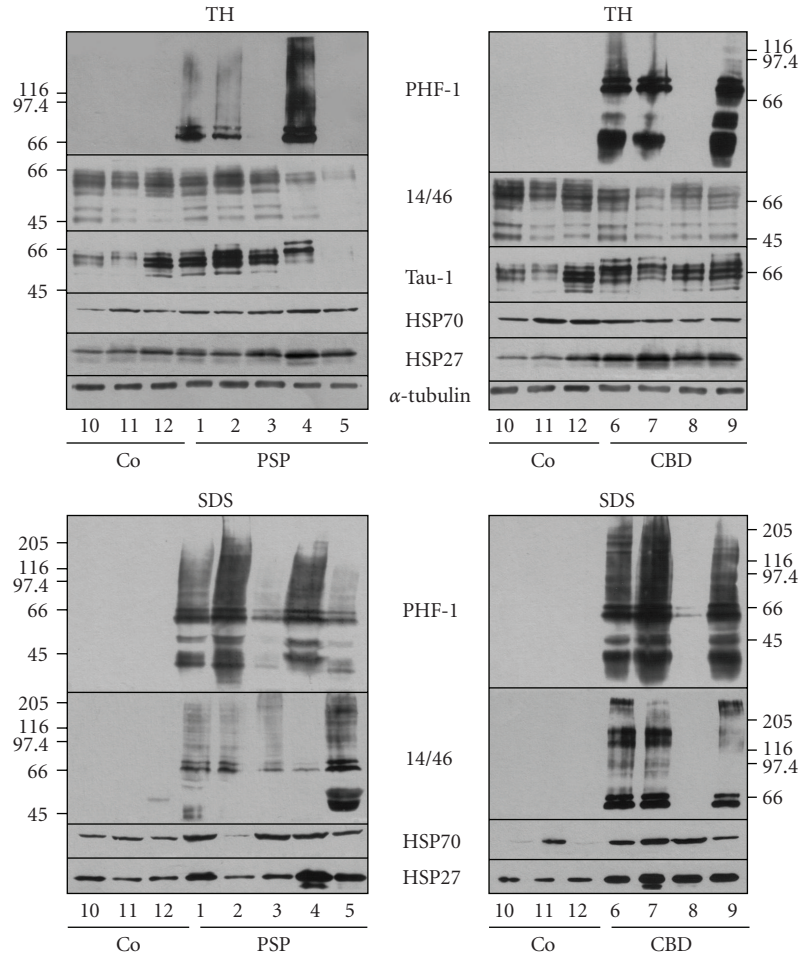
2. Materials and Methods

2.1. Materials and Antibodies. Cell culture media were from Invitrogen (Grand Island, NY).

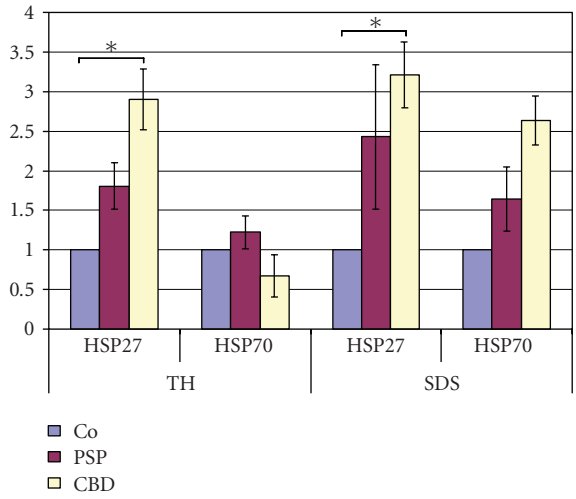
For Western Blot analysis or immunofluorescence the following antibodies were used: mAb anti-HSP70 (SPA-810), mAb anti-HSP/HSC70 (SPA-820), mAb anti-HSP60 (SPA-806), polyclonal anti-HSP40 (SPA-400), polyclonal anti-HSP32 (SPA-895), polyclonal anti-HSP25 (SPA-801), polyclonal anti-HSP27 (Spa-803), and mAb anti-αB-crystallin (SPA-222) from StressGen (Victoria, BC, Canada). Polyclonal anti-α-tubulin, mAb anti-α-tubulin, and mAb anti-GFAP were from Sigma (Taufkirchen, Germany). A mixture of mAbs anti-tau-14 and anti-tau-46 (both diluted 1 : 500) recognizing all Tau isoforms independently of phosphorylation, mAb PHF-1 (1 : 500) recognizing Tau phosphorylated at serine residues 396 and 404 and mAb Tau-1 specific for nonphosphorylated tau epitope located in amino acid residues 189–209 were used. The working dilution for Western Blot analysis was 1 : 1000 if not mentioned in parenthesis.

2.2. Sequential Biochemical Fractionation of Brain Tissue. Brain tissue was obtained from the Center for Neurodegenerative Disease Research and the AD Center Core at the University of Philadelphia, School of Medicine. Brain tissue of frozen globus pallidus from PSP ($n = 5$), CBD ($n = 4$) and normal control ($n = 3$), was homogenized in 8 mL/g high salt buffer (HS) (weight/vol) (50 mmol/L Tris, pH 7.5, 750 mmol/L NaCl, 5 mmol/L EDTA; all buffers were supplemented with protease inhibitor cocktail (cOmplete, Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors PhosSTOP (Roche Diagnostics)) and centrifuged at 50,000 × g for 30 minutes at 4°C. Aliquots of the total homogenate (TH) were taken for immunoblot analysis. Pellets were extracted with 3 mL/g of HS buffer containing 1% Triton X-100 (HS-T). To remove myelin, pellets were homogenized in 500 μL HS containing 1 mol/L sucrose. Floating myelin was discarded after centrifugation. The resulting pellets were homogenized in 2 mL/g of radioimmunoprecipitation assay (RIPA) buffer (50 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)). Afterwards, pellets were extracted in 2 mL/g SDS buffer (62.5 mmol/L Tris, pH 6.8, 1 mmol/L EDTA, 0.1% β-mercaptoethanol, 2% SDS, 10% glycerol). The SDS-insoluble pellet was further extracted by formic acid, but did not reveal enough material for analysis. Fivefold concentrated sample buffer (312.5 mmol/L Tris, pH 6.8, 5 mmol/L EDTA, 0.5% β-mercaptoethanol, 10% SDS, 50% glycerol, 0.05% bromphenol blue) was added to TH, HS, HS-T, and RIPA, and all samples were heated to 100°C for 5 minutes. Equal volumes of samples from each fraction were loaded on 7.5 and 10% polyacrylamide gels. All fractions were analyzed by immunoblot procedure; see below. In the result section only the TH and SDS-fractions are shown (Figure 1). α-Tubulin of TH was used to normalize data.

2.3. Immunoblot Analysis. Cellular monolayers of control and treated cells were washed with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 8.4 mM Na_2HPO_4; pH 7.4) once, scraped off in sample buffer (125 mM Tris, pH 6.7, 1 mM EDTA, 1% β-mercaptoethanol, 10% glycerol), containing 2% SDS, and boiled for 10 minutes. Protein contents in the samples were determined according to Neuhoff et al. [22]. For immunoblotting, total cellular extracts (10–20 μg protein per lane) were separated by one-dimensional SDS-PAGE using 7.5% or 10% polyacrylamide gels, and transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). The blots were saturated with TBS-T (20 mM Tris, pH 7.5, 136.8 mM NaCl, 0.1% v/v Tween 20) containing 5% dry milk and incubated with the individual antibodies overnight at 4°C. After washing, incubation with HRP-conjugated anti-mouse (Amersham Biosciences, Hercules, CA, USA; 1 : 3000) or anti-rabbit IgG (Biorad, Munich, Germany; 1 : 3000) was carried out for 1 hour, and blots were visualized by the enhanced chemiluminescence (ECL) procedure as described by the manufacturer (Amersham, Braunschweig, Germany). Quantitative evaluation of the immunoblots was carried out by densitometric scanning and ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).



(a)



(b)

Figure 1: Biochemical analysis of tauopathy and unaffected control brains. (a) Representative images of total homogenate (TH) and SDS-fractions of globus pallidus from PSP (left) and CBD (right) brains are shown. Brain tissue (1 g/8 ml high salt buffer) was homogenized and subjected to sequential extraction (see materials and methods). Equal amounts of samples were subjected to SDS-PAGE and immunoblot procedure was carried out with antibodies against tau, HSP27, HSP70 and α-tubulin as indicated on the right. Co represent brain samples of unaffected controls. In (b) quantitative evaluation of the HSP27 and HSP70 blots shown in (a) and (b) was carried out using ImageQuant software. α-Tubulin from TH was used to normalize the data. Asterisk: $P \geq .05$ according to t-test.

2.4. RNA Extraction and Microarray Analysis. To study the gene expression profiles total RNA was extracted of control and PSP globus pallidus brain tissue by Miltenyi Biotec (Cologne, Germany). Six human specific PIQOR microarrays (Miltenyi Biotec, Cologne, Germany) consisting of 1208 selected gene probes were used to generate an expression profile of mRNA in PSP brains versus control. For cDNA synthesis, RNA samples of two control brains and three PSP brains were pooled. cDNA synthesis and purification was carried out using the TSA-Labeling and Detection Kit (Perkin-Elmer, USA) according to the manufacturers instructions (see Perkin-Elmer, USA, Micromax TSA Labeling and Detection Kit for kits MPS521, MPS522 for details).

Hybridization and posthybridization were carried out using PIQOR Microarray Kit (Miltenyi Biotec, Cologne, Germany) according to the manufacturers instructions. Slides were scanned on a microarray scanner (GenePix 4000B, Axon Instruments, Foster City, USA). Acuity 4.0 (Molecular Devises, Califonia, USA) was used for signal quantification. Signals ≤ 0.5 refer to underexpressed genes, while signals ≥ 2 show overexpressed genes.

2.5. Immunohistochemistry. Tissue obtained at the time of autopsy was fixed in 10% formalin, paraffin-embedded and cut into 6 μm thick sections. Following antigen retrieval using Vector unmasking solution formalin-fixed brain sections were analyzed by immunohistochemistry as described previously [23], using the avidin-biotin complex (ABC) method (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). For double immunolabeling, sections were blocked in 5% ChemiBLOCKER (Chemicon, Millipore, Billerica, MA, USA) and 2% donkey serum in 0.1 M Tris, pH 7.6 supplemented with 0.1% Triton X-100 (TrisT) overnight at room temperature after antigen retrieval. First antibodies were diluted 1 : 100 in TrisT and incubated at room temperature overnight. After 6 washes for 30 minutes in 0.1 M Tris, pH 7.6, sections were incubated with secondary antibodies (1 : 100 in TrisT) (Jackson ImmunoResearch, West Grove, PA, USA) overnight at room temperature. Autofluorescence was blocked as described using Sudan Black B [24] and sections were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) containing 4′, 6-diamidino-2-phenylindole (DAPI). Digital images of immunohistochemical preparations were obtained using an Olympus BX 51 (Tokyo, Japan) microscope equipped with bright-field light sources with a digital camera, the DP71 (Olympus, Orangeburg, New York), and DP manager (Olympus). Fluorescent labelling was analysed using a Zeiss epifluorescence microscope (Oberkochen, Germany) equipped with a digital camera using a plan-neofluar objective (100× or 40× for overview images) or a Leica TCS SL confocal laser scanning microscope (Wetzlar, Germany).

2.6. Primary Neuronal Cell Culture. Single cell suspensions were prepared from cerebral hemispheres of 17-day-old rat embryos as previously described [25]. The tissue was homogenized in Eagles basal medium (BME), containing 0.5% heat-inactivated fetal calf serum (FCS). Cells were kept in serum-free medium supplemented with N2-Supplement (1 : 100; Invitrogen, Carlsbad, CA), L-glutamine (1 : 100; Invitrogen, Carlsbad, CA), 50 U/mL penicillin, and 50 μg/mL streptomycin. Experiments were carried out with 7-day-old cultures or as indicated. Twice per week, one-half of the culture medium was exchanged.

2.7. Astrocyte Culture. Primary cultures of glial cells were prepared from the brains of 1-2-day-old Wistar rats and astrocytes were prepared from the flasks after 6–8 days as described previously [26]. Briefly, astrocytes were trypsinated from culture flasks, after removal of microglia and oligodendrocyte precursor cells, seeded on 10 cm culture dishes, and kept in DMEM supplemented with 10% fetal calf serum (FCS). 3 days before the experiment medium was changed to DMEM containing 0.5% FCS.

2.8. Heat Shock Treatment. Culture dishes were sealed with parafilm and immersed for 30 minutes in a water bath at 44°C, as described before [27]. For the indicated recovery, cells were put into the incubator. Control cells were sealed for 30 minutes but remained in the incubator.

2.9. Oxidative Stress. Cells were treated with hydrogen peroxide (100 μM) for 30 minutes, the medium was replaced, and cells were put into the incubator for recovery as indicated.

2.10. Indirect Immunofluorescence. Cells were cultured on poly-L-lysine-coated glass coverslips (2.0×10^5 cells per 35 mm dish) and subjected to heat shock or oxidative stress as indicated. After washing with PBS, cells were fixed with ice-cold methanol for 7 minutes. The coverslips were washed three times and incubated for 1 hour with anti-GFAP and anti-α-tubulin antibodies, followed by the appropriate secondary antibodies (1 : 100) (Jackson ImmunoResearch, West Grove, PA, USA), washed with PBS and mounted. Nuclei were stained by DAPI (1.5 μg/mL) included in the mounting medium (Vectashield; Vector Laboratories, Burlingame, CA, USA). Fluorescent labeling was studied using a Zeiss epifluorescence microscope (Oberkochen, Germany) equipped with a digital camera using a plan-neofluar objective (100x).

3. Results

3.1. Increased Expression of Tau and HSP27 in Affected Brains of PSP and CBD Patients. Frozen tissue samples from the globus pallidus were obtained from 5 PSP and 4 CBD patients and from 3 control cases without neurological disease. The expression and solubility of tau was determined by sequentially extracting the brain tissue using buffers with increasing solubilization capabilities (see Methods section). The individual fractions were analyzed by immunoblot procedure using PHF-1 antibodies recognizing hyperphosphorylated tau, and tau-1 antibodies directed against nonphosphorylated tau, and a cocktail of t14 and

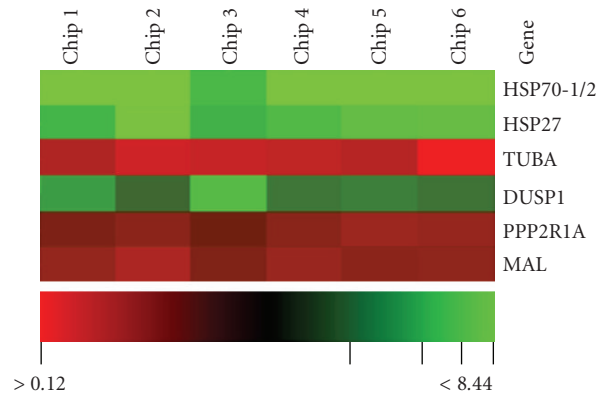


Figure 2: PIQOR microarray analysis of mRNA abundance in PSP versus normal control brains. Six chips were generated and statistically analyzed. Signals ≤ 0.5 (red) refer to underexpressed genes, while signals ≥ 2 (green) show overexpressed genes. The figure shows some genes out of 64 genes which are differentially regulated in PSP.: TUBA: α-tubulin; DUSP1: dual specifity protein phosphatase 1; PPP2R1A: serine/threonine protein phosphatase 2A; MAL: myelin and lymphocyte protein.

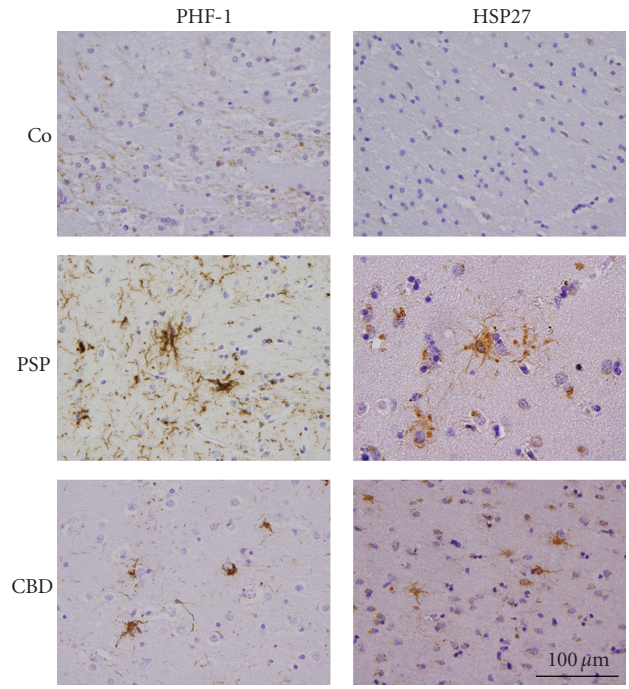


Figure 3: Expression of PHF-1 tau and HSP27 in globus pallidus of patients with PSP and CBD in comparison to unaffected control (Co). Adjacent sections were immunostained with PHF-1 (left) or HSP27 (right). PHF-1 staining demonstrates robust tau pathology in the affected brain regions. No pathology was detected in the brains of unaffected controls, while PSP and CBD brains show characteristic glial pathology and PHF-1 positive threads.

t46 antibodies. Figure 1 shows the immunoblot of the total brain homgenates (TH) and the least soluble SDS-fraction. The majority of hyperphosphorylated tau was detected in the SDS fraction and was present in all PSP and CBD brain samples, although at different levels, thus confirming the pathology (Figure 1). Brain tissue was homogenized as weight per volume, and equal volumes of the subsequent individual fractions were separated by SDS-PAGE. Since α-tubulin was at approximately the same level in the TH of all brain samples, it was used to normalize data (Figure 1(b)).

To study the expression profile of genes in normal and affected brains, RNA was isolated from PSP brain tissue and normal control tissue. RNA from 3 PSP and 2 normal control tissue samples was suitable and used for microarray analysis. Human specific PIQOR microarrays were used to analyze 1208 genes. Quantitative analysis revealed that 38 genes were repressed and 26 genes induced, including HSP27 and HSP70 (Figure 2). The induction of HSP27 mRNA was confirmed by RT-PCR analysis using the same RNA preparations (not shown).

This result prompted us to analyze the tissue extracts for the presence of HSP27 and HSP70 proteins (Figure 1(a)). Immunoblot analysis revealed that HSP27 was detectable in all fractions of the control and affected brain samples, and in comparison to the normal controls more abundant in the affected brains. In PSP brain extracts, HSP27 was elevated in the TH and most prominent in the least soluble SDS-fraction, and in CBD brains HSP27 was enriched in the TH and SDS-fraction. Also HSP70, as compared to control nonaffected brain tissues, was observable at an elevated level in the SDS-fraction of PSP and CBD brains (Figure 1(a)).

3.2. Increased HSP27 Immunoreactivity in Affected Brains. Adjacent sections of paraffin-embedded globus pallidus tissue were prepared and immunostained with PHF-1 antibodies, to confirm tau-pathology, and antibodies against HSP27. As compared to the normal controls, a marked immunostaining of PHF-1 and HSP27 was observed in the affected brains (Figure 3). HSP27 staining was mainly associated with cells with astrocytic morphologies (Figure 3). Although PHF-1 positive coiled bodies originating in oligodendrocytes were present in the brain sections of PSP and CBD, we did not detect coiled bodies positively stained by HSP27 in the analyzed sections. This indicates that HSP27 pathology is mainly associated with astrocytes. This was further confirmed by double immunolabelling of brain sections using antibodies against HSP27 and PHF-1 (Figure 4). HSP27 immunoreactivity was present in numerous cells with astrocytic morphologies. Furthermore, differences in astrocytic pathology between PSP and CBD brain sections were observed. In PSP, PHF-1 positive cells resembling tufted astrocytes were often also positive for HSP27 (Figure 4, PSP patient 3). In CBD brain sections, PHF-1 positive astrocytic plaques were seen, which in the center contained HSP27 immunoreactivity (Figure 4, CBD patient 6). Furthermore, large astrocytes which strongly were labelled by antibodies against HSP27 containing PHF-1 immunoreactivity in the cellular extensions were typically observable in CBD brains (Figure 4, CBD patient 6). The colocalization of PHF-1 and HSP27 was further confirmed by confocal microscopy. The overlay images demonstrate that while HSP27 is mainly

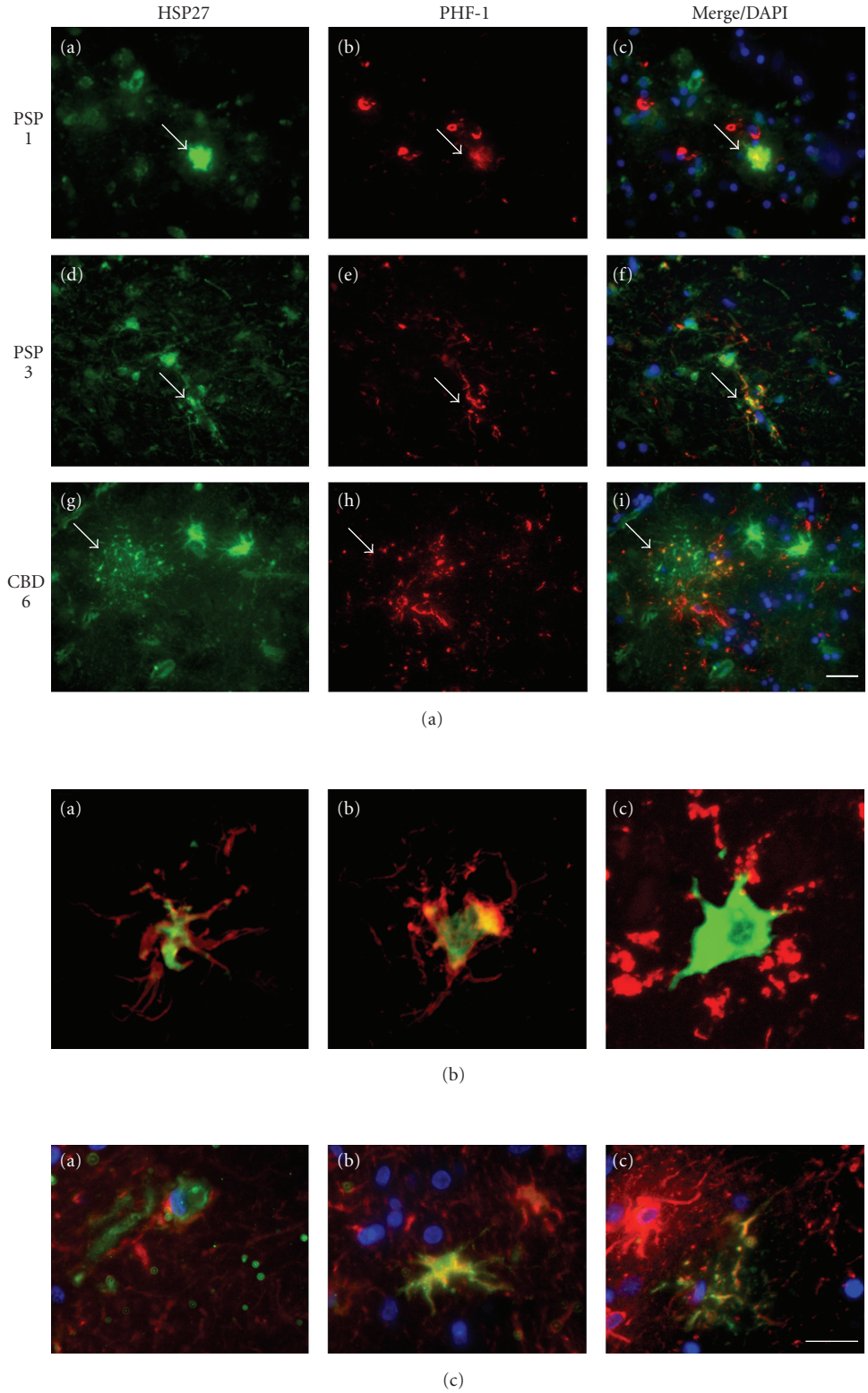


Figure 4: Colocalization of HSP27 with tau-immunoreactive inclusions. (b) Adjacent sections from affected brains, as indicated on the left, were double labelled for HSP27 (green) and PHF-1 tau (red). HSP27 colocalizes with a subset of astrocytic pathology. In PSP (a)–(f) astrocytic tufts positive for HSP27 and PHF-1 are seen (arrows) and in CBD (g)–(i), PHF-1 positive astrocytic plaques with HSP27 in the center are prominent (arrows). Scale bar, 25 μm. Nuclei are stained with DAPI in the overlay images. (c) Confocal overlay images of representative cells from brain sections as depicted in (a) are shown (a,b, PSP patient 3; c, CBD patient 6). (d) Brain sections were double labelled for HSP27 (green) and GFAP (red). All cells positively stained for HSP27 were GFAP positive, demonstrating their astrocytic origin. Overlay images are shown for PSP patient 1 (a) and CBD patient 6 (b) and (c). Nuclei are stained with DAPI. Scale bar, 20 μm.

seen in the center of the cell throughout the cytoplasm, PHF-1 immunoreactivity is prominent in the periphery and the cellular extensions (Figure 4(b)). The data further show that not all HSP27-positive cells or plaques are also positive for PHF-1 but were colocalized in a subset of tau-immunoreactive inclusions (Figure 4). Also, even in brain samples with relatively little PHF-1 protein expression as demonstrated in the immunoblots (Figure 1, PSP 3 and CBD 8), HSP27 was abundantly expressed and in a subset of cells was found in colocalization with PHF-1. The astrocytic identity of the cells was corroborated by double immunofluorecent staining tissue sections using antibodies against GFAP (glial fibrillary acidic protein) and HSP27. Figure 4(c) demonstrates that cells expressing HSP27 are also positive for GFAP and thus represent astrocytes.

3.3. Constitutive Expression and Differential Upregulation of HSPs in Cultured Rat Brain Neurons and Astrocytes. Previous data from our laboratory indicated that astrocytes in comparison to oligodendrocytes derived from the brains of newborn rats express a higher constitutive level of the small HSPs and thus might be protected from stress situations [18, 19]. Since tau-pathology is also prominent in neurons, and neuronal filamentous lesions and neuropil threads are found in PSP and CBD, we prepared cultures of rat cerebral neurons and astrocytes to compare the pattern of constitutively expressed HSPs. Cell extracts were subjected to immunoblot procedure using a panel of antibodies against several HSPs. Figure 5 demonstrates that astrocytes and neurons specifically differ in their abundance of the small HSPs, that is, HSP25 and αB-crystallin, while the higher molecular weight HSPs are similarly expressed in both cell types.

Next we tested if neurons and astrocytes differentially react to heat and oxidative stress. Cells were subjected either to a heat shock (HS; 30 minutes, 44°C, 18 hours recovery) or to hydrogen peroxide (OS; 100 μM, 30 minutes, 18 hours recovery). Immunoblot analysis reveals that HSP25 is upregulated in neurons after HS and OS, but under no condition is as abundantly expressed as in astrocytes (Figure 6). In neurons, αB-crystallin is inducible by HS but not detectable after OS, while in astrocytes it is enhanced after both stress conditions. In both cell types, HSP32 is induced after oxidative stress and HSP70 is induced only after heat stress, while HSP60 remains at the same level (Figure 6). The presence and upregulation of HSP25 in astrocytes after HS and OS was further confirmed by indirect immunofluorescence using antibodies against HSP25 and GFAP (glial fibrillary acidic protein). Figure 7 demonstrates that HSP25 is upregulated after HS and OS and distributed in the cell body and processes and partly is associated with glia filaments. Hence, these data show that HSPs are differentially expressed in neurons and glia, and that cells react to different stress situations by upregulation of different stress proteins. These data underline the functional significance of small HSPs in glia and point to the conclusion that cell type specific responses accompany pathological processes.

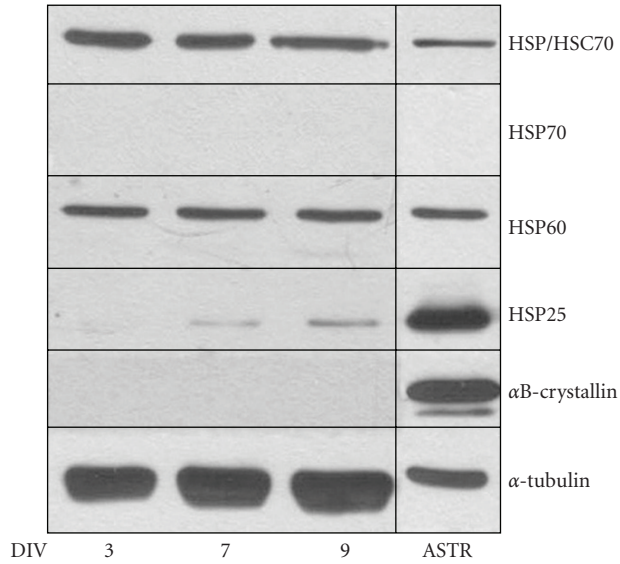


Figure 5: Cultured neurons and astrocytes constitutively express heat shock proteins at different levels. Cell lysates from astrocytes (ASTR) and neurons at the indicated days in vitro (DIV, 3, 7, 9) were prepared and equal amounts of proteins were subjected to immunoblot analysis using antibodies against various HSPs, as indicated on the right. Note that in comparison to neurons astrocytes constitutively express high amounts of HSP25 and αB-crystallin, while HSP60 and HSC 70 are similarly prominent in both cell types.

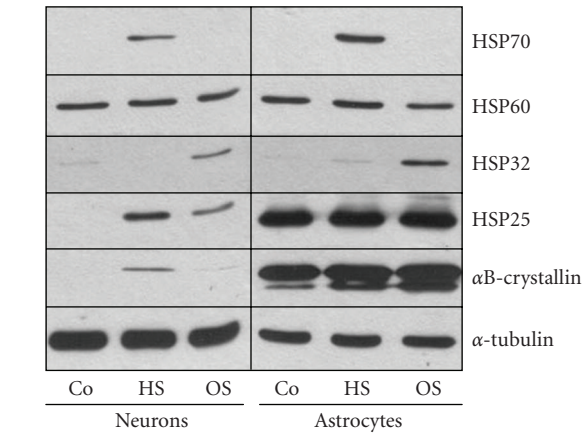


Figure 6: Differential stress responses of neurons and astrocytes. Neurons (7 div) and astrocytes were subjected to heat shock (HS; 44°C, 30 minutes) or oxidative stress (OS; 100 μM H_2O_2) and cell lysates were prepared after a recovery period of 24 hours and equal amounts of proteins were subjected to immunoblot analysis, using antibodies against a variety of heat shock proteins, as indicated on the right. Note that in both neurons and astrocytes, HSP70 is heat-inducible, while HSP32 is only induced by oxidative stress. Generally, the induction of HSPs after stress is more pronounced in astrocytes than in neurons.

4. Discussion

In tauopathies such as AD, CBD, PSP, and Pick's disease, tau is the major constituent of intracellular inclusions, and the fact that these can occur not only in neurons but also

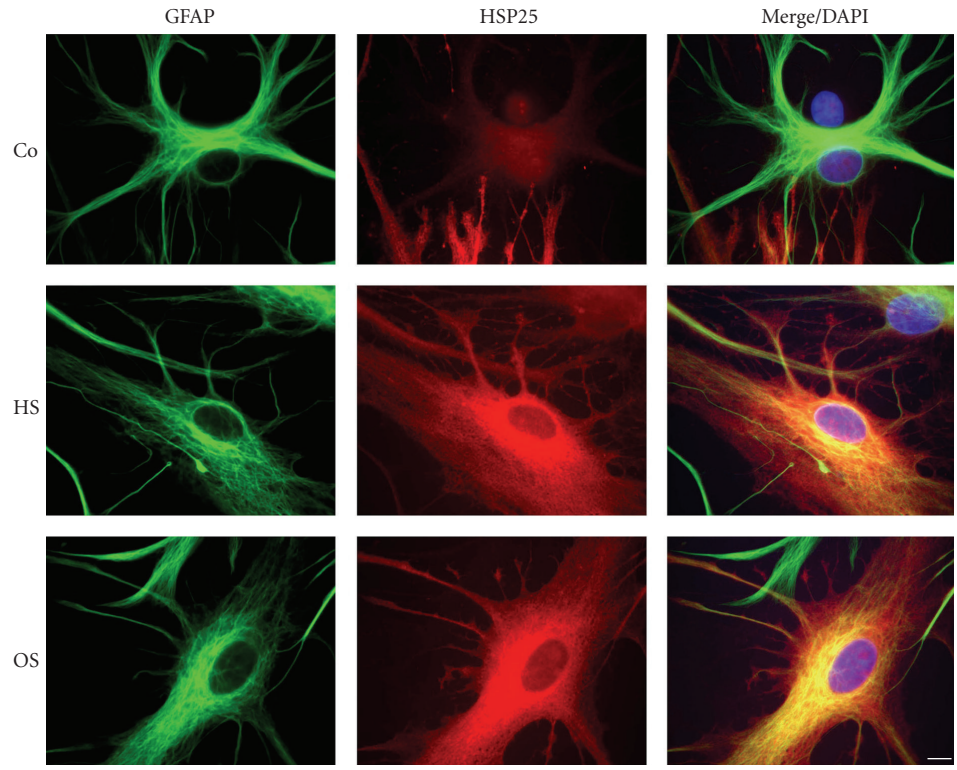


Figure 7: Cellular distribution of HSP25 in astrocytes. Astrocytes after heat shock (HS; 44°C, 30 minutes, 24 hours recovery) or oxidative stress (OS; H_2O_2 100 μM, 30 minutes, 24 hours recovery) were subjected to indirect immunofluorescence staining. Co: untreated control. Double labelling was carried out using monoclonal anti-GFAP and polyclonal anti-HSP25 antibodies. Nuclei were stained with DAPI. Scale bar = 20 μm.

in astrocytes and oligodendrocytes points out that glial cells contribute to the pathogenesis of neurodegenerative diseases [8]. Misfolded proteins assemble and accumulate before the onset of neurodegeneration, and it is likely that this is a mechanism aimed at removing denatured proteins to rescue the cells. When aggregates grow and enlarge, they may confer death signals and play a critical role in the pathogenesis of the diseases. It has been suggested that various types of inclusions may arise through common mechanisms. They contain components of the ubiquitin proteasome system and a variety of molecular chaperones [28]. Molecular chaperones provide a first line of defence against misfolded proteins and closely cooperate with the proteasomal machinery [29, 30]. HSP25/27 can confer resistance against apoptotic stimuli and might protect the cytoskeleton during stress [31, 32]. It undergoes dynamic assembly into large aggregates and oligomerization has been suggested to be required for chaperone function and substrate binding. The direct proof of a chaperone function of HSP27 in vivo is still missing, but evidence has accumulated that it contributes to an increased chaperone activity of the cells and binds unfolded proteins, which are then kept in a competent state to be refolded by HSP70 [33].

Here we examined the presence of HSP27 in PSP and CBD, representing tauopathies with abundant glial pathology [8, 12]. The data show that in affected brain tissue HSP27 is enhanced in comparison to the normal control, and a large proportion of HSP27 is only extractable with detergents disrupting the cytoskeleton, that is, 2% SDS, similarly to hyperphosphorylated tau. Also HSP70 is abundant in this fraction, and microarray analysis demonstrates that mRNA levels encoding HSP27 and HSP70 are enhanced in PSP brain samples. Immunohistochemical data further point to the fact that HSP27 is associated with cells displaying astrocytic morphologies, both in PSP and CBD brains, but we could not detect coiled bodies positively stained for HSP27. Double immunofluorescence labelling demonstrated that not all HSP27-positive cells displayed PHF-1 immunoreactivity, but astrocytic tufts positive for PHF-1 and HSP27 were prominent in PSP brain sections and PHF-1-positive astrocytic plaques with HSP27 immunoreactivity in the center were typically seen in CBD brain sections. Astrocytic tufts are prominent features of PSP brains and astrocytic plaques are characteristic in CBD [12]. Our data demonstrate that in both diseases a number of cells prominently express HSP27 without displaying tau pathology. This is also the case in brains of patients with a rather low level of PHF-1 protein expression. This might indicate that the stress response in astrocytes involving HSP27 upregulation precedes the formation of tau deposits and is attempted at protecting the cells against the protein aggregation process.

Our data support a previous study, demonstrating that immunostaining for αB-crystallin and also HSP27, although to a much lesser extent, was increased in CBD, PSP,

and FTDP-17 [21]. αB-Crystallin staining was specifically prominent in structures resembling coiled bodies in CBD and FTDP-17. In the three PSP cases analyzed, which displayed only mild tau pathology, also only mild αB-crystallin immunoreactivity was observable [21]. HSP70 immunostaining was not increased in both affected and unaffected brain regions, and HSP27 immunostaining was demonstrated predominantly in glial cells in the neocortex of all three pathologies, but this was not further persued biochemically in this previous study [21].

Thus, the upregulation of the small HSPs seems to be specific for glial pathology, and implies distinct pathogenic mechanisms for glial and neuronal cells. This is supported by our data, demonstrating that cultured neurons and astrocytes have a different set or amount of constitutively expressed HSPs and that the small HSPs, αB-crystallin and HSP25, are specifically and prominently expressed in unstressed astrocytes. Even after stress situations the levels of small HSPs in neurons remain low. Similarly, oligodendrocytes have a low constitutive level of both small HSPs, but respond to oxidative and proteasomal stress by prominent induction of αB-crystallin, while HSP25 is mainly induced after heat stress [18, 27]. Astrocytes are specifically resistant against a variety of stress situations and our recent data demonstrate that they are protected by their high constitutive level of HSP25 [19, 32]. Downregulation of HSP25 by siRNA approach caused actin filament breakdown in control cells in the absence of stress stimuli and sensitized the cells against oxidative and proteasomal stress [19]. Hence cell-type and stress-specific regulation is observable in cell culture and likely involved in disease progression.

The question remains, why small HSPs are associated with the insoluble protein fractions of stressed cells and affected brains. Indeed, although small HSPs may prevent protein aggregation by forming soluble complexes with aggregation prone substrate proteins, they are often found in insoluble detergent-resistant fractions [19, 21, 34]. Cell culture studies have revealed that at an early state of pathogenesis small aggregates and aggresomes are formed, these are aimed at shielding the remaining part of the cell body from unwanted interactions with misfolded proteins [32, 35, 36]. In glial cells, aggresomes are formed around the MTOC and small HSPs and ubiquitin are recruited to the growing inclusions as a major constituent [19, 37, 38]. When cells are overloaded with nonnative substrates, small HSPs coaggregate with nonnative proteins and are tightly associated with and might surround inclusion bodies. This situation is specifically promoted in stress situations, such as oxidative stress or stress exerted by proteasomal inhibition.

To summarize, small HSPs seem to be particular important in neurological diseases with glial pathology. They are prominent constitutents in inclusion bodies originating in astrocytes and oligodendrocytes. These are characteristic features of CNS diseases with cytoskeletal abnormalities. Although small HSPs can specifically interact with cytoskeletal components and might preserve cell shape and integrity, chronic overexpression, instead of protecting the cells, might further contribute to cell damage and disease progression.

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