Review

Bench-to-bedside review: Mitochondrial injury, oxidative stress and apoptosis – there is nothing more practical than a good theory

Hülya Bayır[1,2] and Valerian E Kagan[2]

[1]Safar Center for Resuscitation Research, Department of Critical Care Medicine, School of Medicine, University of Pittsburgh, PA, USA
[2]Center for Free Radical and Antioxidant Health, Department of Environmental and Occupational Health, School of Public Health, University of Pittsburgh, PA, USA

Corresponding authors: Hülya Bayır, bayihx@ccm.upmc.edu. Valerian E Kagan, vkagan@eoh.pitt.edu

Abstract

Apoptosis contributes to cell death in common intensive care unit disorders such as traumatic brain injury and sepsis. Recent evidence suggests that this form of cell death is both clinically relevant and a potential therapeutic target in critical illness. Mitochondrial reactive oxygen species (ROS) have become a target for drug discovery in recent years since their production is characteristic of early stages of apoptosis. Among many antioxidant agents, stable nitroxide radicals targeted to mitochondria have attracted attention due to their ability to combine electron and free radical scavenging action with recycling capacities. Specific mechanisms of enhanced ROS generation in mitochondria and their translation into apoptotic signals are not well understood. This review focuses on several contemporary aspects of oxidative stress-mediated mitochondrial injury, particularly as they relate to oxidation of lipids and their specific signaling roles in apoptosis and phagocytosis of apoptotic cells.

The recent realization that mitochondria are at the intersection of the life and death of a cell, particularly through the involvement of mitochondrial damage in a range of diseases, has made them a promising target for drug discovery and therapeutic interventions [1]. Several types of critical illnesses are associated with cell death, both necrotic and apoptotic. The significant contributions of apoptotic pathways in cell death and to the pathogenesis of common intensive care unit (ICU) disorders, such as traumatic brain injury, acute respiratory distress syndrome, and sepsis, are well appreciated [2-4]. Because apoptosis usually includes mitochondrial injury, production of reactive oxygen species (ROS) and oxidative stress, this review is focused on apoptotic mechanisms through which oxidative stress realizes its signaling functions. This review is not a comprehensive analysis of available literature on this subject but rather is focused on several contemporary aspects of oxidative stress-mediated mitochondrial injury, particularly as they relate to oxidation of lipids and their specific signaling roles in apoptosis.
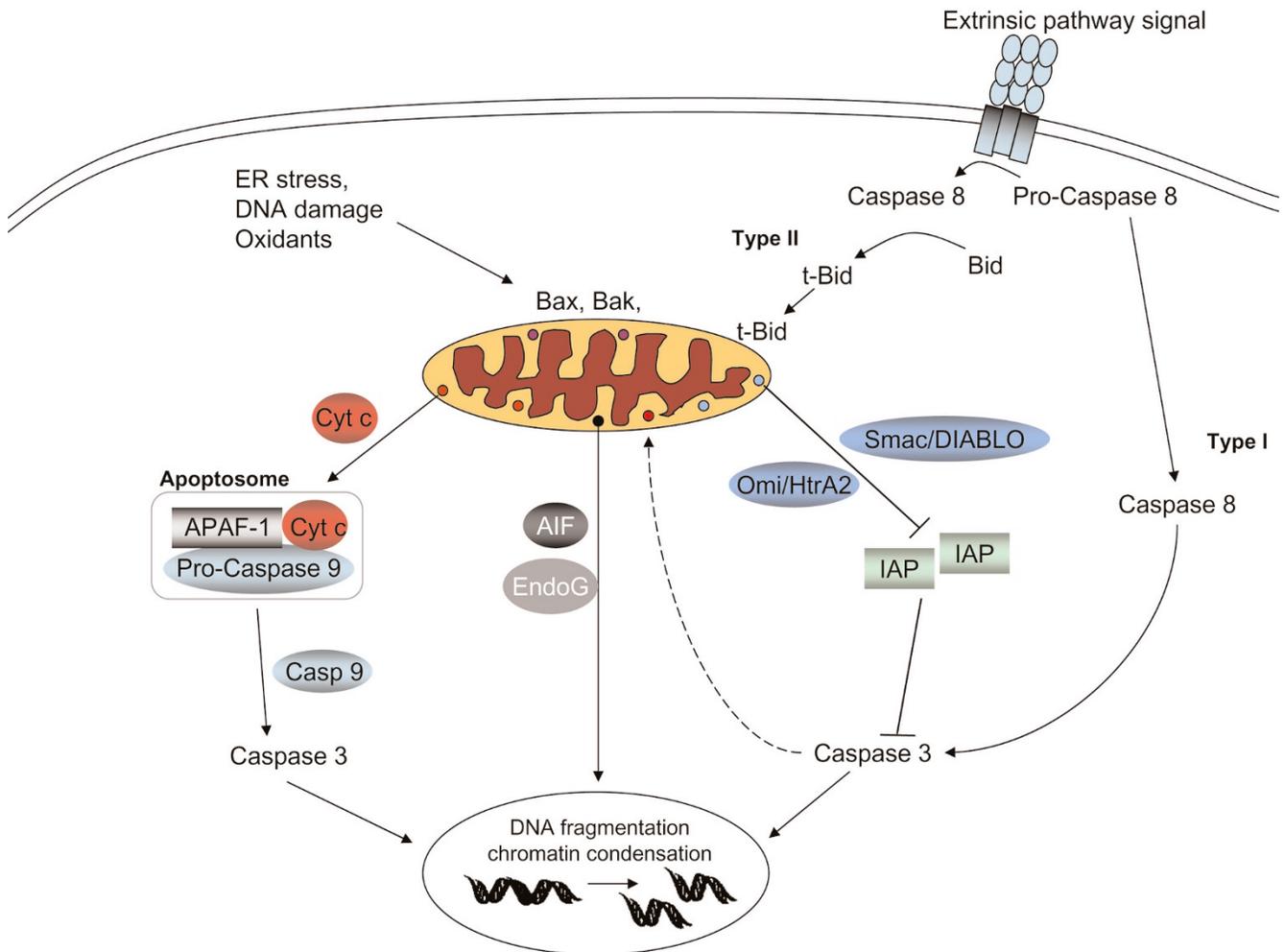
Apoptosis

Apoptosis, or programmed cell death, is a series of well coordinated and strictly controlled processes in which ligand binding to specific (death) receptors or cytotoxic insults result in the activation of several proteases and other hydrolytic enzymes, leading to proteolysis, DNA fragmentation and chromatin condensation [5]. Programmed cell death can be executed via extrinsic and intrinsic pathways depending on the stimulus leading to apoptosis (Figure 1). Extrinsic signals (such as cytokines, tumor necrosis factor (TNF)-α, Fas ligand, glucocorticoids) bind to their receptors and trigger intracellular signaling leading to caspase-8 activation [6]. The TNF and Fas ligand pathways can induce both apoptosis and cell survival depending on the intracellular signaling pathways that are induced by them, and the balance seems to be related to activation of nuclear factor-κB [6].

In the intrinsic apoptotic pathway, pro-apoptotic signals (such as the pro-papototic Bcl-2 family proteins Bax and Bak) translocate to mitochondria, resulting in mitochondrial membrane permeabilization. This in turn provides a route for release of intermembrane space proteins (such as cytochrome c, apoptosis inducing factor (AIF), Endo G and Smac/DIABLO (Second mitochondria-derived activator of caspase/direct IAP binding protein with a low pI)) into the cytosol. Once in the cytosol, cytochrome c promotes the formation of the 'apoptosome', a molecular platform for the activation of caspase-9. The apoptosome also includes the

Figure 1

Programmed cell death can be executed via extrinsic and intrinsic pathways depending on the stimulus leading to apoptosis. In the intrinsic apoptotic pathway, pro-apoptotic signals (such as pro-apoptotic Bcl-2 family proteins Bax and Bak) translocate to mitochondria, resulting in mitochondrial membrane permeabilization. This in turn provides a route for release of intermembrane space proteins (such as cytochrome c (Cyt c), apoptosis inducing factor (AIF), Endo G and Smac/DIABLO (Second mitochondria-derived activator of caspase/direct inhibitor of apoptosis protein binding protein with a low pI)) into the cytosol. Once in the cytosol, cytochrome c promotes the formation of the 'apoptosome', a molecular platform for the activation of caspase-9. In turn, active caspase-9 catalyzes the proteolytic activation of caspase-3. This leads to DNA fragmentation and chromatin condensation. Caspase 3 may also serve to amplify the initial death signal by helping to promote further cytochrome c release from mitochondria. Smac/DIABLO and Omi stress-regulated endoprotease/high temperature requirement protein A2 (Omi/HtrA2) promote apoptosis indirectly by binding to and antagonizing members of the inhibitor of apoptosis protein (IAP) family. AIF and Endo G, on the other hand, translocate from the cytosol to the nuclear compartment, leading to DNA fragmentation and chromatin condensation. Extrinsic signals bind to their receptors and trigger intracellular signaling, leading to caspase-8 activation. Activation of caspase-8 by extrinsic stimuli (such as tumor necrosis factor-α, Fas ligand) involves mitochondria-dependent signaling (in type II cells) and results in cleavage of the pro-apoptotic Bcl-2 family protein Bid to t-Bid. Translocation of t-Bid to the mitochondria is believed to be one of the signals for mitochondrial events during apoptosis. In type I cells, on the other hand, execution of apoptosis occurs without significant participation of mitochondria. APAF-1, apoptosis protease activating factor 1; ER, endoplasmic reticulum.

apoptosis protease activating factor 1 (APAF-1) and ATP/ dATP. In turn, active caspase-9 catalyzes the proteolytic activation of the effector caspases. This leads to the expression of two key apoptotic phenotypes, namely, exposure of phosphatidylserine (PS) on the outer surface of plasma membrane and DNA fragmentation and degradation. Recent studies show that caspase 3 may also serve to amplify the

initial death signal by helping to promote further cytochrome c release from mitochondria [7]. Smac/DIABLO promote apoptosis indirectly, by binding to and antagonizing members of the inhibitor of apoptosis protein (IAP) family. AIF and Endo G, on the other hand, translocate from the cytosol to the nuclear compartment, leading to DNA fragmentation and chromatin condensation [5,8].

The mechanisms by which the intermembrane space proteins are released from mitochondria remain controversial [9]. In general, two mechanisms have been described, the occurrence of either of which might depend on the nature of the apoptotic stimulus. In the first mechanism, a permeability transition pore opens in the inner membrane, allowing water and molecules up to 1.5 kDa to pass through [10]. Although alternative models have been proposed [11], most models imply that the adenine nucleotide transporter in the inner membrane and the voltage dependent anion channel in the outer membrane are components of this pore [12]. Opening of the permeability transition pore leads to equilibration of ions across the inner mitochondrial membrane with loss of mitochondrial transmembrane potential ($\Delta\psi$) and swelling of the matrix as water enters. The latter can result in sufficient swelling to break the outer membrane, resulting in the release of pro-apoptotic factors from the intermembrane space to the cytosol.

The second mechanism is mediated by Bcl-2 family members acting directly on the outer mitochondrial membrane. In this process, oligomerization of pro-apoptotic Bcl-2 family members Bax and Bak plays an essential role in the permeabilization of the outer mitochondrial membrane [13]. We and others have shown that Bax/Bak translocation to mitochondria causes ROS generation and cardiolipin (CL) oxidation in the mitochondria [14-15]. In Bax/Bak double knock out cells, ROS production and CL oxidation are decreased. These cells, but not cells lacking only one of these proteins, have been found to be resistant to a variety of apoptotic stimuli, including oxidants, that act through the mitochondrial pathway [16]. Irrespective of its mechanisms, permeabilization of the mitochondrial outer membrane is considered a point of no return for programmed cell death because of the release of caspase activators such as cytochrome c.

Mitochondria appear to be involved in both the extrinsic and intrinsic apoptotic pathways. While the intrinsic apoptotic pathway is mitochondria dependent, cells undergoing programmed death via the extrinsic pathway can be classified as type I and II depending on whether the mitochondria is involved or not. In type I cells, execution of apoptosis occurs without significant participation of mitochondria. This kind of cell death mechanism may be important for developmental tissue remodeling [18]. In type II cells on the other hand, mitochondria are involved in the execution of apoptosis as a second loop. In some membrane death receptor-mediated systems, activation of caspase-8 by extrinsic stimuli (such as TNF-α and Fas ligand) involves mitochondria-dependent signaling and results in cleavage of the pro-apotic Bcl-2 family protein Bid to t-Bid. Translocation of t-Bid to mitochondria is believed to be one of the signals triggering mitochondrial events during apoptosis [5].
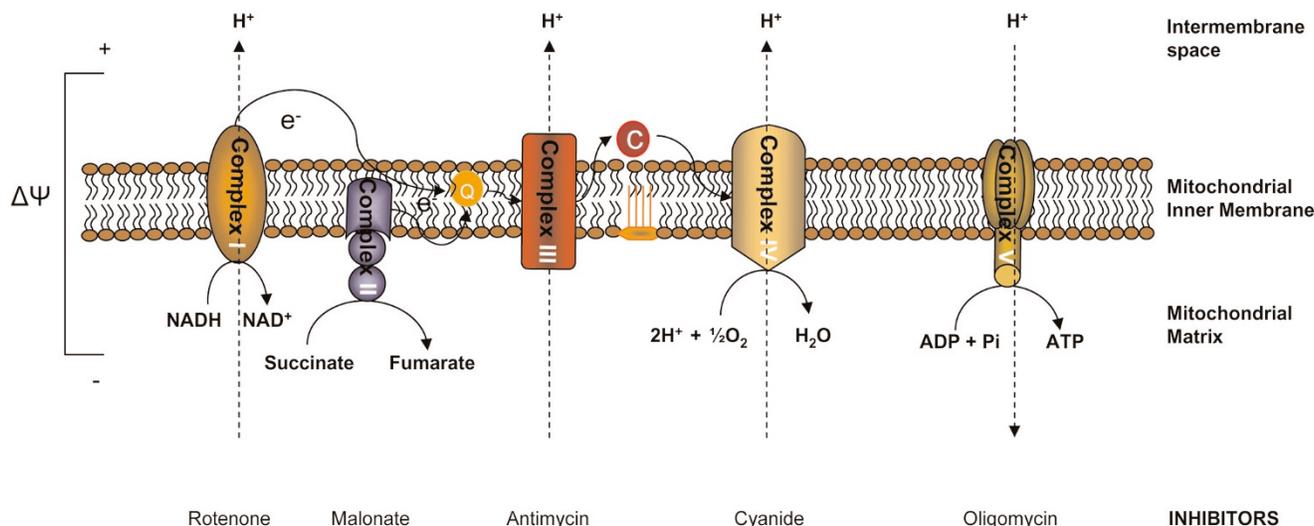
Mitochondrial energy production
Mitochondria play a critical role in cellular energy production via electron transport chain-dependent synthesis of ATP.

Derangements in mitochondrial function mostly affect cells that have a high energy demand, such as neurons and cardiac myocytes. The mitochondrial electron transport chain is composed of five multimeric complexes [19]. Electron transport between complexes I to IV is coupled to extrusion of protons from complexes I, III and IV into intermembrane space, creating an electrochemical gradient ($\Delta\psi$) across the inner mitochondrial membrane (Figure 2). This movement of electrons generates an alkaline matrix and an acidic intermembrane space. Protons then flow through complex V (ATP synthase), which utilizes the energy to synthesize ATP from ADP [20].

Since molecular oxygen is the terminal electron acceptor in the mitochondrial electron transport chain, assessments of mitochondrial function are often performed through the measurement of oxygen consumption in isolated mito-chondria. Intact mitochondria provided with inorganic phosphate and an oxidizable substrate (such as succinate) respire relatively slowly in the absence of ADP. Addition of ADP initiates a sudden burst of vigorous respiration and the ADP is rapidly phosphorylated to produce ATP. This is called coupled respiration: oxygen consumption is coupled to the production of ATP. When all the ADP is depleted, the respiration rate returns to its original level. The active rate in the presence of ADP is called 'state 3 respiration' and the slower rate when all the ADP has been phosphorylated is referred to as 'state 4 respiration'. State 4 respiration reflects the rate of leakage of proton back across the inner mitochondrial membrane into the matrix and is not coupled to complex V or ATP synthesis. The ratio of state 3 to state 4 is called the respiratory control index. This ratio varies with the substrate and the experimental details; in general, it is at least 5 or greater if the isolated mitochondria are in good condition. In state 3 respiration, control of respiration may involve several components, including the adenine nucleotide translocator and cytochrome c oxidase [20-24]. The extra-mitochondrial ATP/ADP ratio, extramitochondrial Pi concen-tration and the supply of hydrogen also play significant roles in controlling respiration [25]. Under disease conditions decreased state 3 respiration and increased state 4 respiration may be observed. Many compounds can inhibit mitochondrial respiration; the precise pattern of inhibition differs from one compound to the next. Important inhibitors are cyanide, antimycin, rotenone and oligomycin, all of which block different components of the electron transport chain (Figure 2). A small group of compounds called uncoupling agents cause unrestrained oxygen uptake in the absence of ADP. None of the energy released during this type of oxidation is captured; it is all dissipated as heat [26].

An important component of the electron transport chain, cytochrome c is located in the intermembrane space and shuttles electrons between mitochondrial complexes III and IV. In addition to this well known and essential function, cytochrome c plays a critical role in the activation of

Figure 2

Mitochondrial energy production. The mitochondrial electron transport chain is composed of five multimeric complexes. Electron transport between complexes I to IV is coupled to extrusion of protons from complexes I, III and IV into the intermembrane space, creating an electrochemical gradient ($\Delta\psi$) across the inner mitochondrial membrane. Protons then flow through complex V (ATP synthase), which utilizes the energy to synthesize ATP from ADP. Some common mitochondrial respiratory chain inhibitors are shown. C, cytochrome c; Q, ubiquinone.

caspases during apoptosis [27]. Release of cytochrome c from mitochondria is one of the early stages of the apoptotic program, designating a point of no return in a cell's disintegration. It is also believed that the detachment of cytochrome c from the inner membrane and its release into the cytosol is one of the rate limiting events in the execution of the apoptotic program [28].

Mitochondrial dysfunction in critical illness

Mitochondrial dysfunction has been reported during critical illness in the ICU. We will briefly mention studies on sepsis and severe traumatic brain injury. The reader is referred to excellent reviews specifically describing mitochondrial dysfunction in these disease states [29-31]. Long term laboratory models of sepsis (>12 hours) and sparse human data have shown decreases in mitochondrial activity or ATP concentrations [32-34]. In septic shock patients examined within 24 hours of ICU admission, the degree of skeletal muscle mitochondrial dysfunction was associated with the severity of the disease [32]. In this work, tissue ATP levels were significantly lower in non-survivors than in an orthopedic surgical control population, but they were maintained in those who survived sepsis. Complex I activity had a significant inverse correlation with norepinephrine requirements and nitrite/nitrate concentrations. The pathogenesis of mito-chondrial dysfunction during sepsis is complex and multi-factorial. Nitric oxide (NO), with its inhibitory effects on electron transport chain complexes, is believed to play an important role [29]. However, it has also been shown that low levels of NO stimulate mitochondrial proliferation, suggesting

that effects of NO on mitochondrial function during sepsis depend on its concentration and the timing of its release [35]. An alternative idea suggested by some investigators is that mitochondrial dysfunction during sepsis may be due to an adaptive cellular strategy to reduce cellular energy expenditure, similar to hibernation [29].

Studies in traumatic brain injury also suggest derangements in mitochondrial function in experimental models and humans [30]. Mitochondrial dysfunction has been documented after both experimental and clinical head injury [30,36-39]. Studies in rodents suggest that alterations in mitochondrial function begin early and may persist for days following injury. A recent work evaluated the time course of cortical mitochondrial dysfunction in adult mice after experimental traumatic brain injury [40]. The results showed impairment in mitochondrial bioenergetics concomitant with accumulation of an oxidative stress marker, 4-hydroxynonenal, as an index of global lipid peroxidation. In addition, CL hydroperoxides have been identified as one of the major contributors to overall lipid peroxidation and mitochondrial dysfunction early after injury [41].

Growing interest has been focused on developing new therapeutic strategies able to combat mitochondrial dys-function. Several pharmacological agents are currently under investigation, including novel antioxidants, uncoupling proteins and mitochondrial permeability transition pore inhibitors [30,42]. One of these agents, cyclosporine, a mitochondrial permeability transition pore inhibitor, has shown benefits in

Figure 3
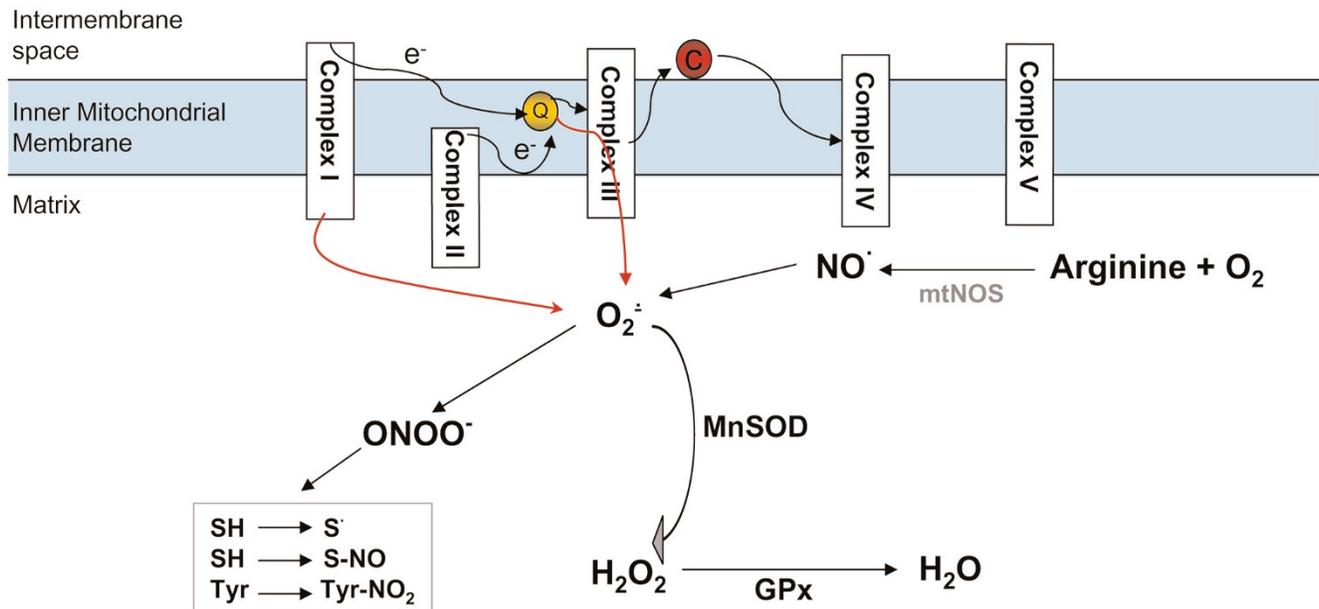
Mitochondrial oxidative stress. In the mitochondria, superoxide can be produced by respiratory complexes. Complex I in the brain and complex III in the heart and lung seem to be the primary sources of mitochondrial superoxide production. Superoxide is detoxified by manganese superoxide dismutase (MnSOD) to hydrogen peroxide (H_2O_2) in the mitochondria. Glutathione peroxidases (GPxs) convert hydrogen peroxide to water. Nitric oxide (NO) generated from (mitochondrial) nitric oxide synthase (mt)NOS can compete with MnSOD and form peroxynitrite (ONOO⁻). Peroxynitrite in turn initiates thiol oxidation or nitrosylation and tyrosine nitration. C, cytochrome c; O2⁻, superoxide; Q, ubiquinone.

experimental traumatic brain injury models with improvement of mitochondrial function, cerebral metabolism and tissue damage [43,44]. The effect of cyclosporin on immune function and outcome is currently under investigation in clinical traumatic brain injury [45].

Oxidative stress

While small fluctuations in the steady-state concentrations of some free radicals may actually play a role in intracellular signaling in normal physiology [46], uncontrolled increases in the generation of free radicals exceed the capacity of antioxidant mechanisms to defend against them, resulting in oxidative stress with damage to lipids, proteins and DNA. Among the major biologically relevant free radical species in cells and biofluids are a one-electron product of oxygen reduction, the superoxide anion radical, and its dismutation product, hydrogen peroxide (H_2O_2). The latter can be further reduced to a highly potent oxidant, the hydroxyl radical; alternatively, hydrogen peroxide can be activated by heme-peroxidases to reactive (oxoferryl) intermediates, which are also very potent oxidants [47]. Up to 1% of all oxygen consumption is estimated to form superoxide [5]. In the mitochondria, superoxide can be produced by respiratory complexes and individual enzymes on the outer mitochondrial membrane, on both sides of the inner mitochondrial membrane and in the matrix [48]. The relative contribution of each site to the overall superoxide production

is tissue and respiratory state dependent. Complex I in the brain [49] and complex III in the heart and lung [48,50] seem to be the primary sources of mitochondrial superoxide production under normal conditions. The rate of superoxide production increases with state 4 respiration (when the electron transport chain slows down and becomes more reduced) and in the presence of inhibitors [20]. When the electron transport chain is dysregulated or disrupted, particularly at the level of complexes I and/or III, the production and release of superoxide radicals increase (Figure 3) [51]. Outside of mitochondria, NADPH oxidases, particularly in polymorphonuclear neutrophils, macrophages and endothelial cells [52], xanthine oxidase [53] and cytochrome P450-dependent oxygenases [54] are the major sources of superoxide production.

A family of specialized enzymes, superoxide dismutases (SODs), convert superoxide to hydrogen peroxide, a relatively stable molecule [55]. Although the dismutation of superoxide to hydrogen peroxide can occur spontaneously, the role of SODs is to increase the rate of the reaction to that of a diffusion-controlled process. In the cytosol and the intermembrane space of mitochondria, superoxide is eliminated by Cu,Zn-SOD, whereas in the matrix it is eliminated by MnSOD [48]. Glutathione peroxidase in turn reduces hydrogen peroxide to water and molecular oxygen by using glutathione [56].

Another important free radical species produced by cells is NO, whose formation from arginine and oxygen is catalyzed by a family of NO synthases (NOSs). Although its presence, localization and physiological role are debated, a mitochondrial NOS isoform (mtNOS), possibly associated with the mitochondrial inner membrane, has recently been described [57,58]. NO can bind to heme groups of hemoproteins, including cytochrome oxidase. In the latter case, the important consequence is inhibition of respiration [59]. Chemically, NO is relatively inert. Similarly, superoxide itself is not particularly redox reactive. However, the toxic potential of both small radical species may be enhanced by their interaction, which leads to the production of a very potent oxidant, peroxynitrite (ONOO-) [60]. NO and peroxynitrite exert differential inhibitory effects on the mitochondrial electron transport chain. Reactions of peroxynitrite with respiratory complexes and cytochrome c lead to their inactivation via free radical mechanisms, including thiol oxidation or nitrosylation and tyrosine nitration [61].

Mitochondrial ROS production and oxidative signaling in apoptosis

Mitochondrial ROS production is one of the very early events preceding collapse of mitochondrial membrane potential, release of pro-apoptotic factors, and activation of caspases [8]. However, specific mechanisms of enhanced ROS generation in mitochondria and their translation into apoptotic signals are not well understood. Cytochrome c, the major player in post-mitochondrial caspase activation, was found to have yet another, earlier function during the mitochondrial stage of apoptosis. This role includes its interactions with a mitochondria-specific phospholipid, CL. The result of this interaction is a high affinity cytochrome c-CL complex which acts as a specific and potent oxidant. In the presence of hydrogen peroxide, this complex functions as a CL-specific oxygenase catalyzing the oxidation of CL, which occurs early in apoptosis [62]. This has been established in a variety of cell lines undergoing apoptosis induced by pro-oxidant and non-oxidant pro-apoptotic agents *in vitro*. Our studies in experimental traumatic brain injury and hemorrhagic shock confirmed that this mechanism is also functional *in vivo* [41,63]. We have shown that selective oxidation of CL takes place during apoptosis while other, more abundant phospholipids (such phosphatidylcholine and phosphatidylethanolamine) remain non-oxidized in spite of the fact that they contain sufficient amounts of polyunsaturated fatty acid residues susceptible to oxidation [41].

The regulation of cytochrome c-CL interactions and the peroxidase function of the complex are not fully understood. However, several important details concerning this function of cytochrome c are beginning to emerge. For example, binding to CL turns off cytochrome c's function as an electron carrier but turns on its peroxidase activity [64]. Notably, oxidized CL (CLox) has a markedly lower affinity for cytochrome c and abandons the complex [65,66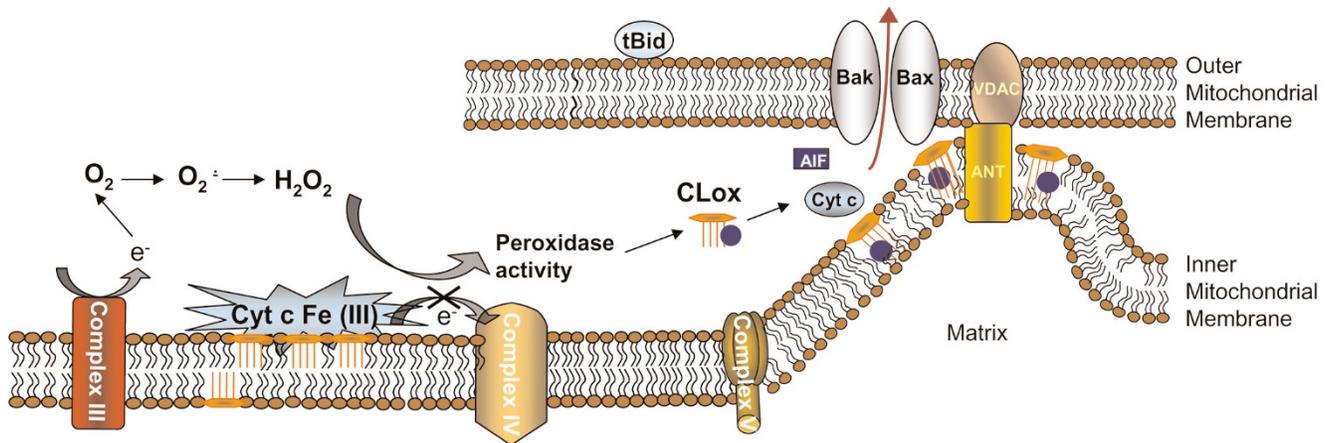]. CL oxidation products (CLox; mostly cardiolipin hydroperoxides) accumulate in the mitochondria, leading to the release of pro-apoptotic factors into the cytosol (Figure 4). The specific mechanisms through which peroxidized CL induces a permeability transition in mitochondria and the subsequent release of pro-apoptotic factors need further analysis; however, this important task of CLox has been documented both in cells during apoptosis as well as isolated mitochondria [67]. It is essential, therefore, to understand the metabolic mechanisms involved in controlling CLox content in mitochondria. Interestingly, CL hydroperoxides are endogenous substrates for phospholipid-hydroperoxide glutathione peroxidase (Gpx IV) [68], and transgenic mice overexpressing this enzyme are protected against oxidative stress-induced apoptosis [69].

Mitochondrial apoptosis as a target for drug discovery

Because apoptosis contributes to disease processes in many critical illnesses, such as sepsis, trauma and acute respiratory distress syndrome [2-4], it is a clinically relevant mechanism that may represent a potential therapeutic target [70]. Mitochondrial ROS have become a target for drug discovery in recent years because their production is characteristic of early stages of apoptosis. Among many antioxidant agents, stable nitroxide radicals have attracted attention due to their ability to combine radical scavenging action with recycling capacities [71]. By accepting one electron, nitroxide radicals are converted to their respective hydroxylamines. These hydroxylamines act as effective ROS scavengers to produce nitroxides, that is, they undergo recycling [72]. Furthermore, nitroxide radicals possess SOD mimetic activity [73,74]. Although nitroxides (such as 4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl (TEMPOL)) have been extensively investigated as successful cytoprotectors in a number of experimental models of oxidative stress [75-79], the required high concentrations (millimolar) have limited *in vivo* applications of nitroxides [80].

Several recent studies indicate that targeting of nitroxides to mitochondria enhances their anti-apoptotic activity [81-84]. Dessolin and colleagues [81] have reported that accumulation of TEMPOL in mitochondria was substantially increased by conjugating it with a Salen-Mn(III) complex of o-vanillin (EUK-134), and the targeted nitroxide delayed apoptosis after an exogenous oxidative insult. Dhanasekaran and colleagues [82] have also demonstrated that a mitochondria-targeted conjugate of nitroxide with a positively charged triphenylphosphonium (mitocarboxy peroxyl) inhibited peroxide-induced oxidative damage and apoptosis. We have previously reported a significant increase in the cytoprotective effects of 4-amino TEMPO (4-AT) by coupling it to a segment of a membrane-active compound, gramicidin S [84]. Furthermore, the activity of the mitochondrially targeted electron acceptor conjugates of 4-AT with gramicidin S prolonged survival in rats subjected to lethal hemorrhagic shock even in the absence of resuscitation with asanguinous fluids or blood [63].

Figure 4

Mitochondrial reactive oxygen species production and oxidative signaling in apoptosis. Interactions of cytochrome c (Cyt c) with the mitochondria specific phospholipid cardiolipin (CL) result in a high affinity cytochrome c-CL complex that acts as a specific and potent oxidant. In the presence of hydrogen peroxide, this complex functions as a CL-specific oxygenase catalyzing oxidation of CL. Binding with CL turns off cytochrome c's function as an electron carrier but turns on its peroxidase activity. Oxidized CL has a markedly lower affinity for cytochrome c and abandons the complex. CL oxidation products (CLox; mostly cardiolipin hydroperoxides) accumulate in the mitochondria, leading to the release of pro-apoptotic factors into the cytosol (Figure 4). AIF, apoptosis inducing factor; ANT, adenine nucleotide translocase; VDAC, voltage-dependent anion-selective channel.

Control of apoptosis and apoptotic clearance in critical illness

During critical illness, a number of cell types in different tissues undergo apoptosis [6,70]. Excessive cell death, including apoptotic cell death, contributes to the pathogenesis of traumatic brain injury and organ failure in sepsis [2,4]. Paradoxically, insufficiency of cell death may also be associated with the pathogenesis of critical illnesses. An important example is delayed apoptosis and ineffective clearance of neutrophils, which is associated with multiple organ failure [85,86]. Neutrophil apoptosis and their effective clearance are particularly important in modulation of the inflammatory response [87]. Prolongation of neutrophil life span may have important implications in delayed resolution of inflammation. Therefore, timely elimination of excessive neutrophils via apoptotic pathways is imperative. Neutrophils are produced at a rate of 1 to 2×10^{11} cells per day and they survive in the circulation for approximately 48 hours before undergoing apoptosis [88]. Neutrophils can undergo apoptosis via intrinsic and extrinsic pathways, the latter requiring mitochondrial amplification [89]. The role played by mitochondria in the regulation of cell life span is perhaps most crucial in neutrophils compared to other cell types in the body [90]. Mature neutrophils contain few mitochondria, although the amount of these apoptosis 'orchestrating' organelles is sufficient to trigger and execute common pathways of apoptosis and to express the signals essential for the effective clearance of them [89,91].

Macrophage recognition and uptake of apoptotic cells (also termed 'efferocytosis') is an important cell-cell interaction

regulating inflammation [92,93]. This interaction triggers not only effective clearance of apoptotic cells but also causes suppression of the inflammatory response, thus limiting the local tissue response and normally leading to quiet cell removal [94-96]. In contrast, inefficient apoptotic cell clearance is pro-inflammatory and immunogenic [97]. The recognition of different types of apoptotic cells, including neutrophils, by macrophages is largely dependent on the appearance on the cell surface of an anionic phospholipid, PS, which is normally confined to the cytosolic leaflet of the plasma membrane [98]. Thus, externalization of PS during apoptosis generates an 'eat-me' signal for macrophages [94,99]. Development and utilization of non-toxic molecules regulating apoptosis, and particularly phagocytosis of neutrophils, may provide a novel therapeutic strategy. Notably, asymmetric distribution of PS across the plasma membrane is mainly due to the activity of a specialized enzymatic mechanism, aminophospholipid translocase, APLT [98]. The enzyme contains redox-sensitive thiol groups and its inhibition by oxidative/nitrosative stress results in PS externalization (Figure 5) [100]. This suggests that nitrosylation of sensitive cysteine residues in APLT may be a promising strategy to research in the drug discovery programs aimed at regulating inflammatory responses. Indeed, our experiments with neutrophil-like HL-60 cells demonstrated that S-nitrosylation of critical cysteine residues in APLT using a cell-permeable trans-nitrosylating agent, S-nitroso-acetyl-cysteine, resulted in egression of PS, rendering these cells recognizable by macrophages [100]. This approach represents a new strategy in regulating the life-span of neutrophils and their clearance by macrophages. Further development of the strategy for

Figure 5

The recognition of apoptotic neutrophils by macrophages is largely dependent on the appearance on the cell surface of an anionic phospholipid, phosphatidylserine (PS), which is normally confined to the cytosolic leaflet of the plasma membrane. Asymmetric distribution of PS across the plasma membrane is mainly due to the activity of a specialized enzymatic mechanism, aminophospholipid translocase (APLT). S-nitrosylation of critical cysteine residues in APLT results in its inhibition, leading to PS externalization. This in turn triggers not only effective clearance of apoptotic cells but also causes suppression of inflammatory response. iNOS, inducible nitric oxide synthase; NO, nitric oxide.

expedited clearance of neutrophils *in vivo* may be useful in overcoming the consequences of insufficient and delayed elimination of neutrophils causing dysregulation and exacerbation of inflammatory responses. It is tempting to speculate that the delivery of reagents required for trans-nitrosylation of target cysteine residues in APLT may be achieved using novel nanoparticle-based vectors [101]. Lately, carbon nanotubes have demonstrated significant potential in the targeted delivery of their cargoes to accomplish temporally and spatially predetermined release of specific regulators and inhibitors [102,103]. While these strategies may look futuristic, recent exponential development of nanotechnologies promises that these plans are not overly optimistic.

Conclusion
In this review, we briefly considered several theoretical issues relevant to the role that mitochondria play in oxidative stress, oxidative signaling and the regulation of apoptosis. It is true that translation of these theoretical considerations into practical predictors of outcomes, biomarkers and new effective drugs in critical illnesses may take some time. However, we are encouraged by the previous experiences of many successful researchers in the field of drug discovery who proved that 'there is nothing more practical than a good theory'.

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Competing interests
The authors declare that they have no competing interests.

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