Mitochondrial Oxidative Stress: Implications for Cell Death

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Annu. Rev. Pharmacol. Toxicol. 2007. 47:143–83

First published online as a Review in Advance on October 9, 2006

The *Annual Review of Pharmacology and Toxicology* is online at http://pharmtox.annualreviews.org

This article's doi: 10.1146/annurev.pharmtox.47.120505.105122

0362-1642/07/0210-0143$20.00

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Key Words

apoptosis, cardiolipin, mitochondria, necrosis, reactive oxygen species

Abstract

In addition to the established role of the mitochondria in energy metabolism, regulation of cell death has emerged as a second major function of these organelles. This seems to be intimately linked to their generation of reactive oxygen species (ROS), which have been implicated in mtDNA mutations, aging, and cell death. Mitochondrial regulation of apoptosis occurs by mechanisms, which have been conserved through evolution. Thus, many lethal agents target the mitochondria and cause release of cytochrome *c* and other pro-apoptotic proteins into the cytoplasm. Cytochrome *c* release is initiated by the dissociation of the hemoprotein from its binding to the inner mitochondrial membrane. Oxidation of cardiolipin reduces cytochrome *c* binding and increases the level of soluble cytochrome *c* in the intermembrane space. Subsequent release of the hemoprotein occurs by pore formation mediated by pro-apoptotic Bcl-2 family proteins, or by Ca^{2+} and ROS-triggered mitochondrial permeability transition, although the latter pathway might be more closely associated with necrosis. Taken together, these findings have placed the mitochondria in the focus of current cell death research.

INTRODUCTION

The mitochondria's role in the regulation of cell death is now well established. The generation of reactive oxygen species (ROS) and the release of proteins from the intermembrane space of mitochondria lead to the activation of different modes of cell death (1). Interestingly, the observations that alterations of mitochondrial function and release of cytochrome *c* may be of importance for cell death have a long history (2). Prior to the "epoch of apoptosis" cell death was studied quite intensively in radiation biology, and investigation of radiation-induced alterations in the cellular bioenergetic machinery was one of the most actively developing areas in the 1950s and 1960s. During the search for mechanisms of the early massive cell death observed in radiosensitive tissues, suppression of oxidative phosphorylation in mitochondria from thymus and spleen was described after whole-body X-irradiation of rats (3). This phenomenon was not seen in the mitochondria from radioresistant tissues (liver and heart) (4). Further, the suppression of oxidative phosphorylation in radiosensitive tissues was associated with the formation of pyknotic nuclei (5). Using selective dyes, it was found that there was a retardation of electron transport between cytochromes *b* and *c* in thymus mitochondria, which also exhibited lower levels of cytochrome *c* after irradiation (6). Thus, the radiation lesion was believed to be due to the looser binding of cytochrome *c* to the inner mitochondrial membrane (IMM) after X-irradiation.

This observation was in accordance with the finding that addition of exogenous cytochrome *c* could stimulate oxidative phosphorylation in mitochondria isolated from radiosensitive, but not from radioresistant, tissues of X-irradiated rats (7). Loss of cytochrome *c* was not a result of its simple escape from the mitochondria because additional washing of the mitochondrial fraction with isotonic buffer did not increase the "cytochrome *c* effect" (8). In vitro irradiation of isolated mitochondria also did not cause increased release of the hemoprotein. Thus, it was suggested that the perturbation of mitochondrial electron transfer in radiosensitive tissues was due to a controlled release of cytochrome *c* from the mitochondria into the cytosol.

Interestingly, only a modest decrease in ATP levels was found in thymus, spleen, and ascites tumor cells following irradiation (9). This observation suggested that the loss of functional cytochrome *c* from the mitochondria was not large enough to markedly depress ATP production by the mitochondria, and that this loss could be compensated for by glycolytic phosphorylation systems. Moreover, it was shown that the retardation of mitochondrial electron transport and subsequent impairment of ATP production and promotion of the generation of ROS are important steps in the induction of cell death. Developments since 1996 have revealed additional mechanisms of importance for mitochondrial initiation of cell death, i.e., caspase activation by the released cytochrome *c*, as well as the release of additional proteins to activate caspase-independent events that are now believed to be critical for the cell death process (for a review, see 10). At present, it seems that a combination of protein release from the mitochondria and maintenance of a sizable intracellular ATP pool is required for the execution of the suicide program, and that mitochondrial protein release is associated with enhanced ROS production by these organelles. In this review

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we discuss the current view on the role of mitochondria in cell death regulation and how this process is affected by their redox state.

OXIDATIVE STRESS

Mitochondria: The Main Source of ROS in Cells

ROS are implicated in a wide variety of pathologies, including malignant diseases, type II diabetes, atherosclerosis, chronic inflammatory processes, ischemia/reperfusion injury, and several neurodegenerative diseases (11). In addition, ROS play a regulatory role in cellular metabolic processes by activation of various enzymatic cascades as well as several transcriptional factors.

Oxidative stress is generally defined as an imbalance that favors the production of ROS over antioxidant defenses; however, the precise mechanisms by which ROS produce cellular injury remain elusive. The majority of ROS are products of mitochondrial respiration. Approximately 1%–2% of the molecular oxygen consumed during normal physiological respiration is converted into superoxide radicals. The one-electron reduction of molecular oxygen produces a relatively stable intermediate, the superoxide anion ($O_2^{\cdot-}$), which can be regarded as the precursor of most ROS. The one-electron reduction of oxygen is thermodynamically favorable for most mitochondrial oxidoreductases (12). The mitochondrial electron transport chain contains several redox centers that may leak electrons to molecular oxygen, serving as the primary source of superoxide production in most tissues. Superoxide-producing sites and enzymes were recently analyzed in detail in a comprehensive review (13).

There is growing evidence that most of the $O_2^{\cdot-}$ generated by intact mammalian mitochondria in vitro is produced by Complex I; the contribution by other complexes and sites seems to be relatively low (**Figure 1**). This $O_2^{\cdot-}$ production occurs primarily on the matrix side of the IMM (14). $O_2^{\cdot-}$ production by Complex I was also found to be markedly stimulated in the presence of succinate, the substrate of Complex II, indicating that a reverse electron flow is involved (15). However, measurement of $O_2^{\cdot-}$ production in intact isolated mitochondria is more complicated than the assessment of $O_2^{\cdot-}$ generation by isolated respiratory chain complexes, or submitochondrial

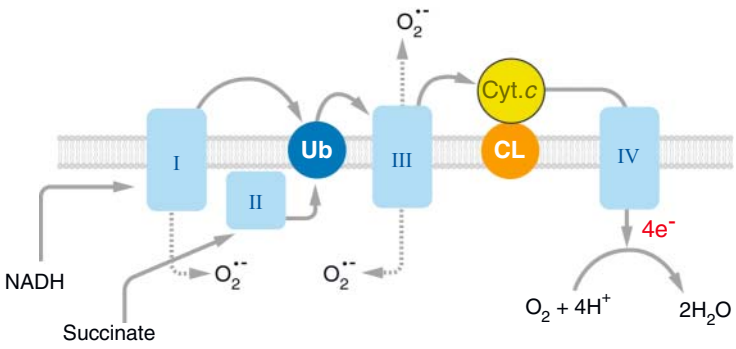

Figure 1

Formation of reactive oxygen species by the mitochondrial respiratory chain. CL: cardiolipin; Cyt. *c*: cytochrome *c*; Ub: ubiquinone.

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particles, because of its inability to cross the IMM and its rapid conversion to hydrogen peroxide by the Mn-dependent superoxide dismutase (MnSOD) present in the mitochondrial matrix (16). Although Complex I accounts for the bulk of $O_2^{\cdot-}$ production under in vitro conditions (submitochondrial particles), this is not necessarily true under in vivo physiological conditions, in particular, at high NADH/NAD^+ ratio (17).

In addition to Complex I, Complex III is regarded as an important site of $O_2^{\cdot-}$ production (18, 19), especially when mitochondrial respiration is suppressed by antimycin, a specific inhibitor of center "i" of Complex III (**Figure 1**). $O_2^{\cdot-}$ produced at this site appears at both sides of the inner membrane (20). Ubiquinone, a component of the mitochondrial respiratory chain connecting Complex I with III and Complex II with III, is regarded as a major participant in formation of $O_2^{\cdot-}$ by Complex III (21, 22). The oxidation of ubiquinone proceeds in a set of reactions known as the Q-cycle, and unstable semiquinone is responsible for $O_2^{\cdot-}$ formation. Specific Complex III inhibitors, such as antimycin and myxothiazol, are important tools in deducing both the site and the source of $O_2^{\cdot-}$ production. Myxothiazol (an inhibitor of center "*o*" of Complex III) decreases $O_2^{\cdot-}$ production, suggesting that center "*o*" is responsible for $O_2^{\cdot-}$ generation within Complex III.

Despite the presence of various antioxidants and detoxifying enzymes, the mitochondria appear to be the most powerful intracellular source of ROS; according to one estimation, the steady-state concentration of $O_2^{\cdot-}$ in the mitochondrial matrix is approximately five- to tenfold higher than that in the cytosol or nuclear space (23). The dismutation of superoxide anions by superoxide dismutase results in H_2O_2 production. Subsequent interaction of H_2O_2 and $O_2^{\cdot-}$ in a Haber-Weiss reaction, or Fe^{2+}- (or Cu^{2+})-driven cleavage of H_2O_2 in a Fenton reaction, can generate the highly reactive and toxic hydroxyl radical. In addition to the respiratory chain, monoamine oxidase (MAO), a flavoprotein localized on the outer mitochondrial membrane, is another important mitochondrial source of ROS, in particular of H_2O_2. MAO catalyzes the oxidative deamination of primary aromatic amines along with long-chain diamines and tertiary cyclic amines and is a quantitatively important source of H_2O_2. Because H_2O_2 easily passes through mitochondrial membranes, MAO can contribute to an increase in the steady state concentrations of ROS within the mitochondrial matrix and cytosol.

Stimulation of $O_2^{\cdot-}$ and H_2O_2 production by mitochondria was demonstrated in the presence of nitric oxide (24). Mitochondrial NO production was first reported in liver, but has since been found in mitochondria isolated from different tissues. It now appears that mitochondrial nitric oxide synthase (mtNOS) is an integral protein of the IMM and is involved in the regulation of mitochondrial functional activity. Nitric oxide synthases (NOSs) constitute a family of enzymes that catalyze the NADPH-dependent oxidation of L-arginine to yield L-citrulline and NO. mtNOS behaves biochemically as a constitutive NOS isoform considering the Ca^{2+} requirement for enzymatic activity and its constitutive expression. NO might affect respiratory chain activity in different ways. For example, reversible inhibition of mitochondrial respiration can result from the interaction of NO with cytochrome oxidase, i.e., by S-nitrosation of key cysteine residues of Complex IV. In fact, NO was

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recently identified as a physiological regulator of electron transfer and ATP synthesis by modulating cytochrome oxidase activity. Further, NO and peroxinitrite, $ONOO^-$, which is formed during simultaneous production of NO and O_2^-, can also cause inactivation of Complex I of the mitochondrial respiratory chain, while Complex II and Complex III remain unaffected. Because reduced thiols or light prevented the inhibition of Complex I, it was proposed that this process involves S-nitrosation or Fe-nitrosylation (25). Inhibition of Complex I by NO and $ONOO^-$ mimics the action of rotenone, a classical inhibitor of this complex, and also increases O_2^- formation by the complex. Hence, S-nitrosation of Complex I can reversibly increase H_2O_2 production by mitochondria, which is of potential importance in cell signaling and/or pathology (26). Inactivation of Complex I by NO or $ONOO^-$ is observed in cells or tissues expressing inducible NOS, and may be relevant to inflammatory pathologies, such as septic shock, and to Parkinson's disease (27).

As described below, the mitochondria possess multiple antioxidant defense systems, including superoxide dismutase, glutathione peroxidase, thioredoxin and thioredoxin reductase, peroxiredoxin and glutaredoxin, as well as water- and lipid-soluble antioxidants (e.g., vitamins C and E). Under some circumstances, the mitochondrial respiratory chain can also contribute to mitochondrial antioxidant defense, e.g., by oxidation of succinate. Owing to certain kinetic advantages, succinate can monopolize the mitochondrial respiratory chain, causing reduction of pyridine nucleotides, due to so-called reversed electron flow, and excessive reduction of ubiquinone (Coenzyme Q). Although ubiquinone acts as a prooxidant in its semiquinone form, when fully reduced it acts as an antioxidant. Therefore, preferential succinate oxidation can provide a tool against excessive accumulation of ROS by increasing the proportion of fully reduced ubiquinone.

Targets of ROS

ROS generated by mitochondria, or elsewhere in the cell, can cause damage to cellular macromolecules, including nucleic acids, phospholipids, and proteins.

Lipid modification. Polyunsaturated fatty acid residues in phospholipids are extremely sensitive to oxidation (28). The hydroxyl radical (generated via the Fenton reaction) is one of the most potent inducers of lipid peroxidation, although its reactivity limits its targets to macromolecules in the immediate vicinity of its site of formation. Fe^{2+} is a powerful catalyst of lipid peroxidation and several experimental models of iron overload in vivo have demonstrated increased polyunsaturated fatty acid (PUFA) oxidation in mitochondria, as well as lysosomal fragility. Maximal rates of lipid peroxidation are observed when the ratio of Fe^{2+}/Fe^{3+} is 1:1 (29).

DNA damage. Oxidative damage to DNA causes modification of the purine and pyrimidine bases, the deoxyribose backbone, single and double strand–breaks, as well as cross-links to other molecules. Many of these DNA modifications are mutagenic, contributing to cancer, aging, and neurodegenerative diseases (30).

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Damage to protein. Although protein oxidation is less well characterized, several types of damage have been demonstrated, including oxidation of sulfhydryl groups, oxidative adducts on amino acid residues close to metal-binding sites, reactions with aldehydes, protein-protein cross-linking, and protein fragmentation (31, 32).

The direct oxidation of amino acids, in particular lysine, arginine, proline, and threonine residues, promotes the formation of protein carbonyls (33, 34). Formation of a protein carbonyl can dramatically alter the tertiary structure of a protein, resulting in its partial or complete unfolding. The unfolding increases protein hydrophobicity, which confers a strong propensity for the protein to form potentially deleterious protein-protein interactions. Hence, oxidation of proteins leads to loss of their normal functions, e.g., enzymatic activity, channel forming properties, etc., and to enhanced susceptibility to proteolytic degradation.

Mitochondrial Targets of ROS

Mitochondria are not only a major source of ROS generation in aerobic cells, but they are also a sensitive target for the damaging effects of oxygen radicals. One of these targets is mitochondrial DNA (mtDNA), which encodes 13 polypeptides, 22 transfer RNAs (tRNAs), and 2 ribosomal RNAs (rRNAs), all of which are essential for electron transport and ATP generation by oxidative phosphorylation (35). This requires the assembly of the protein products of both the mitochondrial and nuclear genomes into functional respiratory complexes. mtDNA, therefore, represents a critical cellular target for oxidative damage that could lead to lethal cell injury through the loss of electron transport, mitochondrial membrane potential, and ATP generation. mtDNA is especially susceptible to attack by ROS owing to its close proximity to the electron transport chain, the major locus for free-radical production, and the lack of protective histones. For example, mitochondrially generated ROS can trigger the formation of 8-hydroxydeoxyguanosine, a lesion arising as a result of oxidative DNA damage; the level of oxidatively modified bases in mtDNA is 10- to 20-fold higher than that in nuclear DNA. Oxidative damage induced by ROS is probably a major source of mitochondrial genomic instability leading to respiratory dysfunction.

An important mechanism of $O_2^{\cdot-}$ toxicity is the direct oxidation and inactivation of iron-sulfur (Fe-S) proteins, such as aconitases, and the associated release of iron (16). The inactivation of mitochondrial aconitase may have at least two major consequences. First, the formation of an inactive $[3Fe\text{-}4S]^+$ cluster results in the simultaneous release of Fe^{2+} and H_2O_2. In fact, $O_2^{\cdot-}$-mediated inactivation of Fe-S-containing enzymes may pose a significant oxidative burden because it provides equimolar amounts of H_2O_2 per mole of $O_2^{\cdot-}$ (36). The release of Fe^{2+} and H_2O_2, ingredients of the Haber-Weiss and Fenton reactions, can also result in generation of the potent hydroxyl radical, which can oxidize mitochondrial proteins, DNA, and lipids, thereby amplifying $O_2^{\cdot-}$-initiated oxidative damage. Whether aconitase inactivation results in free radical formation and toxicity in vivo remains unknown.

Mitochondrial aconitase plays a key role in the Krebs cycle, catalyzing the conversion of citrate to isocitrate. Inhibition of mitochondrial aconitase, even partial, could result in Krebs cycle dysfunction and have an impact on energy production

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and cell viability. A recent study further documents that the mitochondrial aconitase is associated with protein-mtDNA complexes called nucleoids. In this novel context, aconitase functions to stabilize mtDNA, perhaps by reversibly remodeling nucleoids to directly influence mitochondrial gene expression in response to changing cellular metabolism (37). In addition, $O_2^{\cdot-}$ can inactivate several other iron-sulfur proteins, such as Complex I NADH dehydrogenase. $O_2^{\cdot-}$ revealed a mild efficiency toward succinate dehydrogenase (23, 38). Oxidized proteins are recognized by proteases and degraded; new replacement protein molecules are then synthesized de novo.

ROS formation and stimulation of lipid peroxidation in mitochondria can lead to extensive suppression of mitochondrial metabolism. Lipid peroxides alter vital mitochondrial functions, such as respiration and oxidative phosphorylation, inner membrane barrier properties, maintenance of mitochondrial membrane potential ($\Delta\psi$), and mitochondrial Ca^{2+} buffering capacity (39–42). Mitochondrial lipid peroxidation products can impair the barrier function of membranes by either directly interacting with the protein and/or indirectly interacting with the lipid moieties in the membrane (43).

A potentially deleterious effect of ROS production in mitochondria is facilitation of Ca^{2+}-dependent mitochondrial permeability transition (MPT), which plays a key role in certain modes of cell death. In addition to ATP production in aerobic cells, the mitochondria play a crucial role in the regulation of intracellular Ca^{2+} homeostasis. Mitochondria can take up and retain Ca^{2+}; however, this ability is limited. Accumulated Ca^{2+} is subsequently released from the mitochondria along with other matrix solutes, especially when Ca^{2+} sequestration is accompanied by oxidative stress and depletion of adenine nucleotides. This phenomenon was described some 30 years ago by Haworth & Hunter in a series of seminal papers (44–46) in which they showed that Ca^{2+} uptake stimulates drastic changes in mitochondrial morphology and functional activity owing to the opening of a nonspecific pore in the mitochondrial inner membrane, commonly known as the MPT pore.

Oxidative stress markedly sensitizes mitochondria toward MPT induction; according to some experimental data, mitochondrially generated ROS are involved in MPT induction (47). The basic unit of the MPT pore is the VDAC-ANT-CyP-D (voltage-dependent anion channel-adenine nucleotide translocase-cyclophilin D) complex located at contact sites between the mitochondrial inner and outer membranes. The VDAC-ANT complex is known to attract other proteins, in particular kinases (e.g., hexokinase, glycerol kinase). Recent studies identified the ANT as one important target for ROS induced by anticancer drugs, such as doxorubicin and arsenic trioxide. In particular, it was demonstrated that doxorubicin-induced cardiac toxicity correlates with oxidation of SH-groups in ANT and a decrease in ANT protein concentration, with inhibition of mitochondrial respiration and increased probability of MPT pore formation. The role of VDAC as a target for ROS is less clear, although it has been demonstrated recently that $O_2^{\cdot-}$ modulates the opening of VDAC reconstituted into liposomes causing release of entrapped FITC–cytochrome *c* (48).

Hence, oxidative stress and impaired Ca^{2+} homeostasis both contribute to mitochondrially mediated cellular damage. MPT is a mechanism causing mitochondrial failure, which can lead to necrosis owing to ATP depletion or to caspase-dependent

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apoptosis if MPT induction occurs in a subpopulation of mitochondria and remaining organelles are still able to maintain the mitochondrial membrane potential and produce ATP.

Regulation of Mitochondrial ROS Production (Antioxidants)

As discussed above, the mitochondrial respiratory chain has long been recognized as an effective source of ROS, originating from the disproportionation of superoxide anions formed mainly at the levels of Complex I and III of the respiratory chain. Within the mitochondrial matrix, MnSOD converts superoxide to hydrogen peroxide, which can be metabolized by glutathione peroxidase or peroxiredoxin (with the exception of cardiomyocytes, mitochondria in mammalian cells do not contain catalase) or diffuse from the mitochondria into the cytosol. Mitochondria contribute 20%–30% of the cytosolic steady-state concentration of H_2O_2 (49), and $O_2^{\cdot-}$ cannot cross biological membranes except in the protonated form, which constitutes only a very small fraction of the superoxide pool at physiological pH (50). However, part of the $O_2^{\cdot-}$ generated during mitochondrial respiration can also be vectorially released into the intermembrane space (**Figure 1**). The mechanism underlying this release considers the formation of ubisemiquinone at the Q_0 site of the ubiquinone pool in the vicinity of the intermembrane space (51). At least in some cell types, CuZnSOD is present in the mitochondrial intermembrane space (52), where it could convert superoxide to hydrogen peroxide for further diffusion into the cytosol. Alternatively, when present in the intermembrane space, $O_2^{\cdot-}$ might be scavenged by cytochrome *c* or diffuse into the cytosol through pores in the OMM, notably the voltage-dependent anion channel, VDAC (49). Finally, under conditions of stimulated NO production, $O_2^{\cdot-}$ might react with nitric oxide to form highly reactive peroxynitrite, $ONOO^-$.

Hence, the mitochondria are a major source of intracellular ROS formation and therefore in need of constant protection from the toxic action of these species. Such protection is provided by various low-molecular-weight antioxidants, as well as by multiple enzymatic defense systems (**Figure 2**). Among the antioxidants, ubiquinone and vitamin E have been found to be particularly important. Selective delivery of synthetic antioxidants to mitochondria has been made possible by attachment of a lipophilic triphenylphosphonium cation to α-tocopherol (MitoVit E) or to ubiquinone (MitoQ) (53). The positive charge of these antioxidant derivatives enables them to accumulate in mitochondria because of the negative membrane potential inside these organelles and to provide significant protection from toxic oxidative stress. Further, recent studies suggest that dietary vitamin E supplementation can enrich hepatic mitochondria with protective levels of α-tocopherol in a dose-dependent manner, and that this may prove beneficial in the treatment of oxidative stress–mediated liver disease, particularly when this is of mitochondrial origin (54).

Most important for mitochondrial antioxidant protection is the tripeptide glutathione, GSH (L-γ-glutamyl-L-cysteinylglycine), and multiple GSH-linked enzymatic defense systems (**Figure 2**). Although there is no evidence for glutathione biosynthesis in mitochondria, these organelles have long been known to have their distinct glutathione pool, which was early found to be critical for cell survival. In fact,

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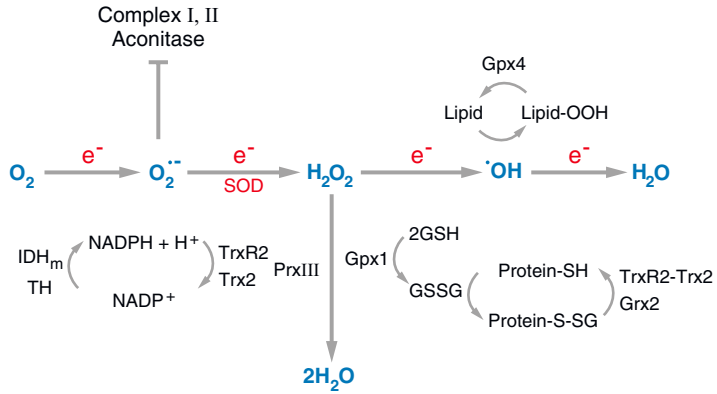

Figure 2

Formation, effects, and inactivation of reactive oxygen species in mitochondria. GSH: reduced glutathione, GSSG: glutathione disulfide; Gpx: glutathione peroxidase, Grx: glutaredoxin; IDH_m: mitochondrial isocitrate dehydrogenase; NADP: nicotinamide adenine dinucleotide phosphate; Prx: peroxiredoxin; SOD: superoxide dismutase; TH: transhydrogenase; Trx: thioredoxin, TrxR: thioredoxin reductase.

it was suggested that toxic cell death was often better correlated to depletion of the mitochondrial GSH pool than to overall intracellular GSH depletion. Further support for the importance of this pool is provided by the recent finding that mitochondrial GSH was more resistant to depletion upon inhibition of glutathione synthesis than other intracellular GSH pools (55). Among GSH-linked enzymes involved in mitochondrial antioxidant defense are glutathione peroxidases (Gpx) 1 and 4. Glutathione peroxidases catalyze the reduction of H_2O_2 and various hydroperoxides, with GSH as the electron donor. Gpx1 is the major isoform and is localized predominantly in the cytosol, but a small fraction is also present within the mitochondrial matrix. In contrast, Gpx4 (also known as phospholipid hydroperoxide glutathione peroxidase) is membrane-associated, with a fraction localized to the intermembrane space of the mitochondria, possibly at the contact sites of the two membranes (56). Gpx4 reduces hydroperoxide groups on phospholipids, lipoproteins, and cholesteryl esters. Because of its small size and large hydrophobic surface it can interact with, and detoxify, membrane lipid hydroperoxides much more efficiently than the alternative pathway, phospholipase A_2-Gpx1; the affinity of PLA_2 for lipid hydroperoxides is much lower than that of Gpx4 (57). Therefore, Gpx4 is considered to be the primary enzymatic defense system against oxidative damage to cellular membranes. Finally, a newly discovered member of the family of GSH-linked mitochondrial redox enzymes is glutaredoxin 2 (Grx2), which was recently cloned and found to be present as both mitochondrial and nuclear isoforms (58). Glutaredoxins catalyze glutathione-dependent dithiol reaction mechanisms, reducing protein disulfides, and monothiol reactions, reducing mixed disulfides between proteins and GSH (de/glutathionylation).

The mitochondrial thioredoxin system, which includes thioredoxin 2 (Trx2) and thioredoxin reductase 2 (TrxR2), is another potential source of disulfide reductase activity required for maintaining mitochondrial proteins in their reduced state

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(**Figure 2**). Thioredoxins catalyze reduction of protein disulfides at much higher rates than Grx (59). The physiological significance of this system is emphasized by the finding that disruption of the *Trx2* gene in the mouse results in embryonic lethality (60). The thioredoxin system can also interact with the peroxiredoxins (Prx), which constitute a novel family of thiol-specific peroxidases that rely on Trx as the hydrogen donor for the reduction of hydrogen peroxide and lipid hydroperoxides (61). One Prx isoform, Prx III, is exclusively detected in mitochondria (62). Upon reaction with H_2O_2, the redox-sensitive Cys residue of each subunit of the Prx III homodimer is oxidized to Cys-SOH, which then reacts with neighboring Cys-SH of the other subunit to form an intermolecular disulfide (63). The disulfide is reduced specifically by Trx2, which is subsequently regenerated by TrxR2 at the expense of NADPH.

The uncoupling proteins (UCPs) are inner membrane carriers, which have also been implicated in the control of ROS homeostasis (64). The first UCP isoform identified, UCP1, is involved in thermogenesis and expressed specifically in brown adipose tissue mitochondria, in which it confers a regulated proton leak across the inner membrane (65). The physiological functions of UCP2 and UCP3, which are expressed in other tissues, are unknown. However, knockout mice display signs of increased ROS production and oxidative tissue damage. There are also reports of O_2^--mediated activation of UCPs, although this has not been confirmed in other studies (66). Therefore, the physiological importance of the UCPs in the control of mitochondrial ROS generation remains unclear and should be subject to further studies.

ROS IN CELL PHYSIOLOGY AND DEATH

Introduction to Cell Death Mechanisms

All mammalian cells contain an intrinsic program necessary to carry out cell suicide. Cell death is an evolutionarily conserved and genetically regulated process that is important for morphogenesis, embryonic development, and for the maintenance of homeostasis in adult tissues. Different modes of cell death are defined by morphological criteria, without a clear reference to precise biochemical mechanisms. Based on these criteria several modes of cell death are now known, e.g., apoptosis, necrosis, autophagy, mitotic catastrophe, anoikis, excitotoxicity, wallerian degeneration, cornification, etc. However, the molecular mechanisms involved in the first three modes have been best characterized. A detailed classification of cell death was recently reported (67).

The term apoptosis was coined by Kerr and colleagues in 1971 (68), describing a particular mode of cell death that is characterized by rounding-up of the cell, reduction of cellular volume (pyknosis), condensation of chromatin, fragmentation of the nucleus (karyorhexis), plasma membrane blebbing, and maintenance of an intact plasma membrane until the very late stage of the process. Approximately 20 years later, a new family of proteases were discovered, the caspases, and found to be intimately related to most forms of apoptotic cell death. Hence, caspase activation was suggested as an important criterion of this mode of cell death (67, 69). Caspases

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constitute a family of proteases that are synthesized as proenzymes, which have very low intrinsic activity and require activation, either by proteolytic maturation or by interaction with an allosteric activator (for review see 70). Proteolytic automaturation might be the result of the activation of a so-called high proximity mechanism. Based on the size of the prodomain, caspases can be divided into long and short prodomain-containing enzymes. Long prodomain caspases, i.e., caspase-2, -8, -9, and -10, belong to the group of initiator caspases, whereas short prodomain caspases, i.e., caspase-3, -6, and -7, belong to the group of effector enzymes. Activation of initiator caspases can be achieved by a series of poly-protein complexes and it is mediated by various apoptotic signaling pathways. In the extrinsic, receptor-mediated pathway, the ligation of receptors is followed by formation of the death-inducing signaling complex (DISC), which results in the activation of procaspase-8 (71, 72) (**Figure 3**). Caspase-8 can directly activate procaspase-3, which cleaves target proteins, leading to

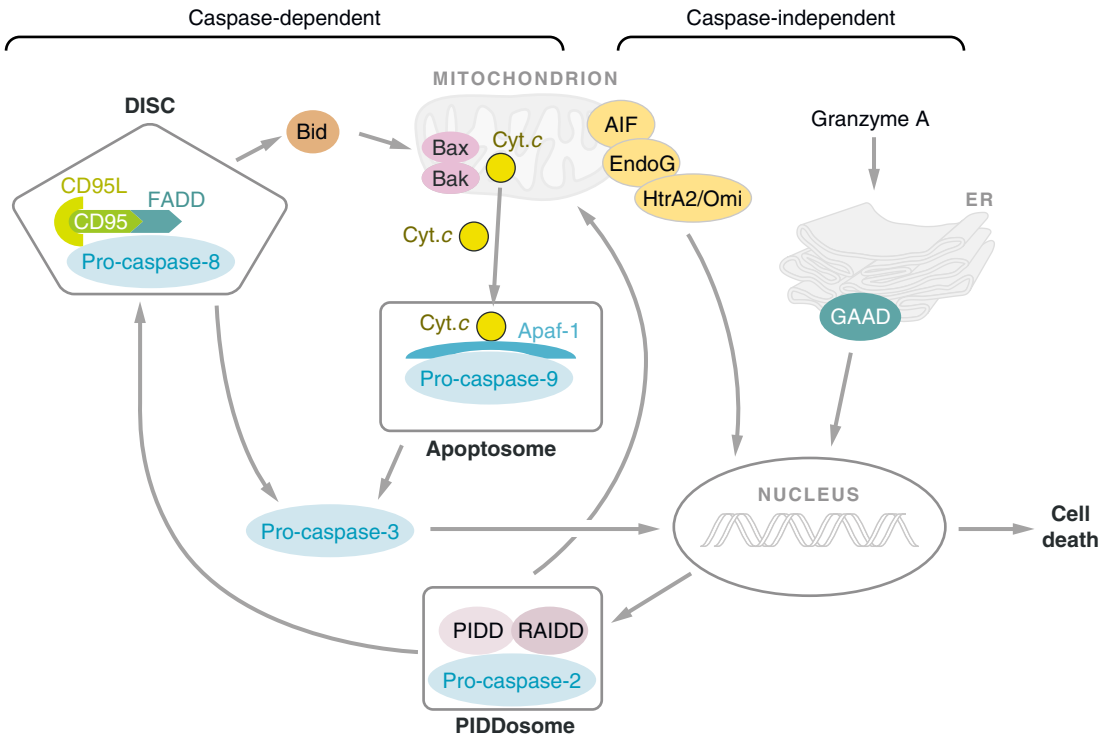

Figure 3

General apoptotic pathways. AIF: apoptosis inducing factor; Apaf-1: apoptosis activating factor 1; Bak: Bcl-2 homologous antagonist/killer; Bax: Bcl-2-associated X protein; Bid: BH3 interacting domain death agonist; Cyt. *c*: cytochrome *c*; DISC: death-inducible signaling complex; Endo G: endonuclease G; ER: endoplasmic reticulum; FADD: Fas-associated death domain; FLICE: Fas-like ICE; GAAD: granzyme A-activated DNase; HtrA2/Omi: High–temperature requirement protein A2; PIDD: p53-induced protein with a death domain; RAIDD: RIP-associated ICH-1 homologous protein with a death domain.

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apoptosis. However, in most cell types, caspase-8 first cleaves Bid, the Bcl-2 family protein, which, in turn, induces the translocation, oligomerization, and insertion of the other family members, Bax and/or Bak, into the outer mitochondrial membrane (OMM) (73). This is followed by permeabilization of the OMM and the release of several proteins from the mitochondrial intermembrane space, including cytochrome *c*, which forms a cytosolic apoptosome complex with apoptosis activating factor-1 (Apaf-1) and procaspase-9 in the presence of dATP. This results in the activation of procaspase-9, which leads to the activation of procaspase-3 and other effector caspases (74).

In the intrinsic pathway, death signals act directly or indirectly on the mitochondria, resulting in the release of cytochrome *c* and formation of the apoptosome complex (**Figure 3**). This cell death pathway is controlled by Bcl-2 family proteins (regulation of cytochrome *c* release), inhibitor of apoptosis proteins (IAPs) (inhibition of caspases) (75), second mitochondrial activator of caspases (Smac) (76), and HtrA2/Omi (negative regulator of IAPs) (77). Apoptosome function is also regulated by the oncoprotein prothymosin-α (Pro-T) and the tumor suppressor putative HLA-DR-associated protein (PHAP), although this regulation occurs at different levels (78).

When DNA damage is the trigger of the apoptotic response, the initially activated caspase is procaspase-2 (**Figure 3**). This initiator caspase is activated within the PIDDosome complex, which includes procaspase-2, PIDD (p53-inducible protein with a death domain), and RAIDD (RIP-associated Ich-1/Ced-3-homologous protein with a death domain) (79, 80). Activation of caspase-2 leads to the release of cytochrome *c* and subsequent formation of the apoptosome complex (81). This, in turn, is followed by the caspase cascade and the cleavage of cellular proteins leading to the biochemical and morphological alterations typical of apoptosis and, finally, cell death.

Another protein family that plays an important role in the apoptotic process is the Bcl-2 family (82). Proteins from this family can be divided into two groups: pro- and antiapoptotic members. Importantly, a majority of these proteins fulfill their function at the level of mitochondria (83). Thus, on one hand, the presence of Bcl-2 or Bcl-X_L prevents permeabilization of the OMM and release of proteins from the intermembrane space, thereby rescuing cells from death. On the other hand, transcriptional or posttranscriptional regulation of proapoptotic multidomain members of this family, such as Bax and/or Bak, leads to their activation by the BH3-only proteins from the same family, e.g., Bid, Puma, or Noxa. Activation of Bax/Bak is characterized by their oligomerization and insertion into the OMM, followed by permeabilization of these membranes (see below), release of proapoptotic proteins from the mitochondria, and cell death. Depending on which proteins that are released from the intermembrane space of the mitochondria, cells might die via activation of either caspase-dependent or caspase-independent pathways (**Figure 3**). The latter involves the release from mitochondria and translocation into the nucleus of at least three proteins: apoptosis inducing factor (AIF) (84), endonuclease G (EndoG) (85), and HtrA1/Omi (77). Nuclear localization of AIF is linked to chromatin condensation and appearance of high-molecular-weight chromatin fragments. Importantly, an obligatory cofactor for AIF-mediated chromatin condensation is cyclophilin A; however, the precise

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mechanism of the chromatin condensation catalyzed by these two proteins is still unclear. Although translocation of EndoG into the nucleus is essential for the formation of the degradosome complex in the nematode *Caenorhabditis elegans* (86), the role of this enzyme in the death of mammalian cells remains elusive. The third mitochondrial protein in this group, HtrA2/Omi, is a serine protease that can also mediate caspase-independent cell death (87). Upon release from mitochondria, HtrA2/Omi accumulates in the cytosol. One of the substrates of HtrA2/Omi is the antiapoptotic protein HAX-1 (bearing Bcl-2-homology BH1 and BH2 domains), which resides in the OMM and can be cleaved by HtrA2/Omi when present in the mitochondria. Two other substrates of HtrA2/Omi are Ped/Pea-15 (as inhibitor of the DISC and of stress kinase) and the IAP. However, the precise mechanism of activation of caspase-independent death by HtrA2/Omi is unknown.

Recently, a new caspase-independent, granzyme A (GrA)-mediated pathway was described (88). After being delivered into the target cell cytoplasm via Ca^{2+}-dependent, perforin-mediated pores, GrA triggers the activation of a caspase-independent pathway, which is characterized by the formation of single-stranded DNA nicks and the appearance of apoptotic morphology. The endonuclease involved in formation of DNA strand-breaks in this system was identified as GAAD (GrA-activated DNase), also known as NM23-H1. GAAD activity is inhibited by its specific inhibitor IGAAD, also known as the SET complex, which is located in the endoplasmic reticulum (ER) (**Figure 3**). This complex contains an inhibitor of protein phosphatase 2A (pp32), the nucleosome assembly protein SET, HMG2, and Ape1 [apurinic endonuclease-1, which is also known as redox factor-1 (Ref-1)]. In this pathway, GrA cleaves SET, HMG2, and Ape1, but not pp32, to release and activate GAAD. Active GAAD translocates into the nucleus to induce DNA strand breaks.

Currently, it is widely accepted that mitochondria play a key role in the regulation of cell death (1). In addition to the release of lethal proteins from the mitochondria via specific pores or other means of OMM permeabilization, a key initiative step in the cell death process, mitochondria can generate and release highly toxic ROS, which can also contribute to cell death. These events are accompanied by changes of mitochondrial structure, which also seem to be important for the mitochondrial regulation of cell death.

Mitochondrial Structural Reorganization and Its Role in Cell Death

Mitochondria are dynamic organelles that exist as networks in the cellular cytoplasm. During development, cell division, and under stress conditions, the mitochondria within these networks can change in number and morphology. These mitochondrial dynamics are dictated by the equilibrium between fusion and fission of the organelles (for review, see 89). Fusion is controlled by a complex regulatory system of multiple proteins that fuse both the OMM and IMM in a coordinated manner, which maintains the integrity of both membranes as well as the intermembrane space and matrix compartments. Similarly, fission proceeds in a controlled way that prevents the leakage of soluble compounds from both the matrix and intermembrane space.

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During the past several years, considerable progress has been made in our understanding of the molecular mechanisms of mitochondrial fusion. Under the electron microscope, the early stages of mitochondrial fusion resemble an onion slice and are termed Nebenkern. Upregulation of *fzo1* (*Drosophila melanogaster* fuzzy onions gene) occurs just prior to Nebenkern formation and disappears when mitochondrial fusion is completed (90). *fzo1* encodes a large transmembrane GTPase that does not share detectable homology with the dynamin family of GTPases. Mutations in the *fzo* gene inhibit fusion. This gene is very conservative and homologs were found in various organisms. Interestingly, disruption of the individual mammalian homologs of Fzo1, mitofusins 1 and 2 (Mfn1 and 2), results in the formation of fragmented mitochondria, a phenotype consistent with inhibition of mitochondrial fusion (91). Although knockout of either Mfn1 or 2 resulted in mitochondrial fragmentation, the function of each of these proteins is slightly different. Thus, knockout of Mfn1 causes the formation of small round mitochondria, whereas knockout of Mfn 2 results in the formation of short punctuate-form mitochondria and the appearance of much larger oval organelles. These proteins are also responsible for the regulation of mitochondrial mobility, and Mfn1 and 2 mutant cells are characterized by an increased number of mitochondria with significantly lower $\Delta\Psi m$. This effect might be a consequence of inhibition of a $\Delta\Psi m$ complementation mechanism that in wild-type cells could result from the frequent fusion and fission of mitochondria (91). Mfns span the OMM twice, having a short loop between the transmembrane domains located in the mitochondrial intermembrane space (92, 93). Interaction between OMM and IMM mediated by these proteins is essential for the proper, synchronized fusion of the two mitochondrial membranes.

Another protein, Ugo1, is also essential for mitochondrial fusion in yeast (94). It also localizes to the OMM, with its N-terminal domain facing the cytosol and the C terminus localized in the intermembrane space. However, it is unclear whether Ugo1 acts simultaneously with, or independently of, Fzo1. An additional protein, Mgm1, the large dynamin-like GTPase, was also shown to play a role in yeast mitochondrial fusion and remodeling of the IMM. This protein localizes to the external side of the IMM, and its loss results in the fragmentation of the mitochondrial reticulum. A human homolog of this protein, OPA1 (detected in patients with dominant optic atrophy), is attached to the outer leaflet of the IMM, close to the cristae (95). Expression of the full-length OPA1 induced the formation of a filamentous network of mitochondria, whereas reduction of OPA1 levels by RNAi results in globular and fragmented mitochondria as a consequence of reduced fusion. A genetic analysis of the determinants of OPA1 function reveals a requirement for Mfn1, but not Mfn2. In fact, OPA1 impinges on Mfn1-dependent fusion to regulate morphology of the mitochondrial reticulum. In spite of accumulating data, it is still unclear if all proteins described above control the same stage of mitochondrial fusion and operate simultaneously, or whether several independent stages of mitochondrial fusion exist.

Multiple proteins are involved in the regulation of mitochondrial fission and in the control of mitochondrial morphology in mammalian cells. Thus, dynamin-related protein, Drp1, which belongs to the dynamin family of GTPases, localizes to punctuate foci on the OMM and regulates mitochondrial morphology (96). Overexpression

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of a dominant-negative Drp1 resulted in the formation of interconnected, fused mitochondria, a phenotype consistent with inhibition of Drp1-mediated mitochondrial fission. Drp1-containing structures also colocalize with ER structures and peroxisomes, mediating fission of the latter and suggesting a role for Drp1 in the fission of multiple membrane targets (97). Microscopy revealed that, at the mitochondrial fission sites, Drp1 is present in multiple copies and can assemble into multimeric ring-like structures essential to execute mitochondrial fission. The presence of a coiled coil region, a typical mediator of protein-protein interactions, suggests the presence of other proteins in the fission foci. Indeed, hFis1, the ortholog of the yeast Fis1p protein, which is essential for yeast mitochondrial division, was recently identified (98). Overexpression of this protein, which, similarly to Drp1, is localized to the OMM, resulted in mitochondrial fission. Interestingly, this was inhibited by a dominant-negative Drp1. Fragmentation of the mitochondrial network induced by hFis1 was followed by the release of several proteins from the intermembrane space of mitochondria.

Rab32, a small GTPase in the Rab protein family involved in the regulation of membrane trafficking, is localized predominantly to mitochondria (99). Expression of Rab32 is involved in the regulation of mitochondrial morphology, and point mutations in the GTP-binding site induced formation of abnormally fused mitochondria. Rab32 sequesters protein kinase A to mitochondria, although it is unclear whether the interaction of Rab32 with PKA is important for the regulation of mitochondrial morphology.

More than 60 years ago it was shown that mitochondrial fragmentation occurs within injured cells (100). However, only recently has accumulating evidence suggested that mitochondrial fission might be involved in the regulation of cell death (101). Hence, it has been shown that in some experimental systems in which the mitochondrially mediated apoptotic pathway is activated, mitochondrial fragmentation occurs and is blocked by the expression of dominant-negative Drp1. In addition, during apoptosis, Mfn1-dependent mitochondrial fusion was significantly inhibited, and overexpression of Mfn1 and 2 protected cells from etoposide-induced death (102). Likewise, as mentioned above, expression of hFis1 results in mitochondrial fission, release of cytochrome *c*, and, finally, apoptosis. Interestingly, downregulation of hFis1 with siRNA prevents apoptosis to a much greater extent than Drp1 silencing (103).

As mentioned above, both pro- and antiapoptotic Bcl-2 proteins exert their function at the level of mitochondria. The proapoptotic Bcl-2 family proteins, Bax and Bak, are able to make specific pores in the OMM through which cytochrome *c* and other dangerous proteins might be released to activate both caspase-dependent and -independent apoptotic pathways. Recently, it was shown that Bax and Bak colocalize with Drp1 and Mfn2 to the large foci on the surface of the OMM (104). This interesting observation implies a role for some Bcl-2-family proteins in the mitochondrial fission machinery and raises the question if Bax/Bak-mediated release of mitochondrial proteins precedes fission of mitochondria or vice versa, or if both processes occur simultaneously but independently of each other. Elimination of Drp1 activity by siRNA, or by overexpression of dominant-negative Drp1, inhibits mitochondrial fission and the release of cytochrome *c*; however, Bax can still translocate

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to mitochondria and coalesce into foci, suggesting an interaction between these processes.

It seems that mitochondrial fragmentation has an evolutionarily conserved role in the regulation of cell death. Indeed, using life microscopy it was shown that programmed cell death in *C. elegans* embryos is associated with mitochondrial fragmentation, and that this process is caspase-independent because it occurs also in animals with mutant Ced-4 or Ced-3 (105). Importantly, fragmentation was not seen in animals lacking Egl-1, the protein acting upstream of Ced-4/Ced-3. Like in mammalian cells, expression of wild-type Drp1 was able to increase fragmentation, whereas expression of a dominant-negative form prevented this process. However, surprisingly, both gain-of-function and loss-of-function mutations in the nematode's Bcl-2 homolog protein, Ced-9, also blocked fragmentation. These data question the precise role of Ced-9 in mitochondrial fission and regulation of cell death.

Further evidence for the similarities in cell death regulation between nematodes and mammals comes from the observation that Ced-9 expression in mammalian cells promotes mitochondrial fusion (106). Ced-9 was found to immunoprecipitate with Mfn2, but not with other effectors of the mitochondrial fusion/fission machinery, such as Mfn1, Drp1, or OPA1. Like Ced-9, Bcl-X_L also promoted fusion and was able to bind to Mfn2. In addition, the expression of Ced-9 efficiently regulated Ced-4-dependent Ced-3 activation also in mammalian cells, but failed to affect Bax-induced cytochrome *c* release and apoptosis induced by a variety of stimuli. Remarkably, cells overexpressing both Ced-9 and Egl-1 contained extensively fragmented mitochondria but failed to release cytochrome *c* or undergo apoptosis. Altogether, these results suggest that mitochondrial fragmentation may not be a prerequisite for apoptosis, at least in some situations. Because mitochondrial fission occurs in both living and dying cells, and prevention of this event in *C. elegans* was able to rescue approximately 20% of the cells, it is also unclear how the cell distinguishes between fission occurring in viable cells and fission during cell death. Further experiments are required to address these questions.

Another morphological change in mitochondria, namely, reorganization of the cristae, has also been implicated in cell death (107). It was shown that the BH3-only protein, tBid, induced a striking remodeling of mitochondrial structure with mobilization of the cytochrome *c* stores in the cristae. This reorganization does not require tBid's BH3-domain and is independent of Bak; however, it was inhibited by cyclosporine A. During this process, individual cristae become fused and the junctions between the cristae and the intermembrane space open. In addition to tBid, another BH3-only protein, Bik, was also shown to be involved in cristal rearrangements (108). However, Bik is located in the ER and was found to initiate an early release of ER Ca^{2+} in response to apoptotic stimuli, which was followed by the activation of a Ca^{2+}-regulated target, Drp1, and resulted in remodeling and opening of the cristae. Hence, in addition to regulating mitochondrial fission, Drp1 might be involved in the control of cristal organization, suggesting that both mitochondrial fission and changes in cristal morphology might play a role in cell death via regulation of cytochrome *c* release. Currently, the possible role of ROS in these processes has not yet been studied.

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ROS and Different Mechanisms of Cytochrome *c* Release

Induction of mitochondrial permeability transition. There are currently several mechanisms that might explain the OMM permeabilization during cell death. One such mechanism, which may be engaged during necrotic and apoptotic cell death, involves the induction of MPT and is due to the opening of nonspecific pores in the IMM followed by osmotic swelling of the mitochondrial matrix, rupture of the OMM, and the release of intermembrane space proteins, including cytochrome *c* (109, 110) (**Figure 4**).

For a long time, MPT was regarded as the prime mechanism responsible for the permeabilization of the OMM. Opening of pores in the IMM and subsequent uncoupling of mitochondria would lead to active hydrolysis of cytosolic ATP (uncoupling-stimulated ATPase activity). As a result, the ATP content would drop, causing a perturbation of cytosolic Ca^{2+} homeostasis and activation of various catabolic enzymes (proteases, phospholipases, etc). Hence, this model of OMM permeabilization

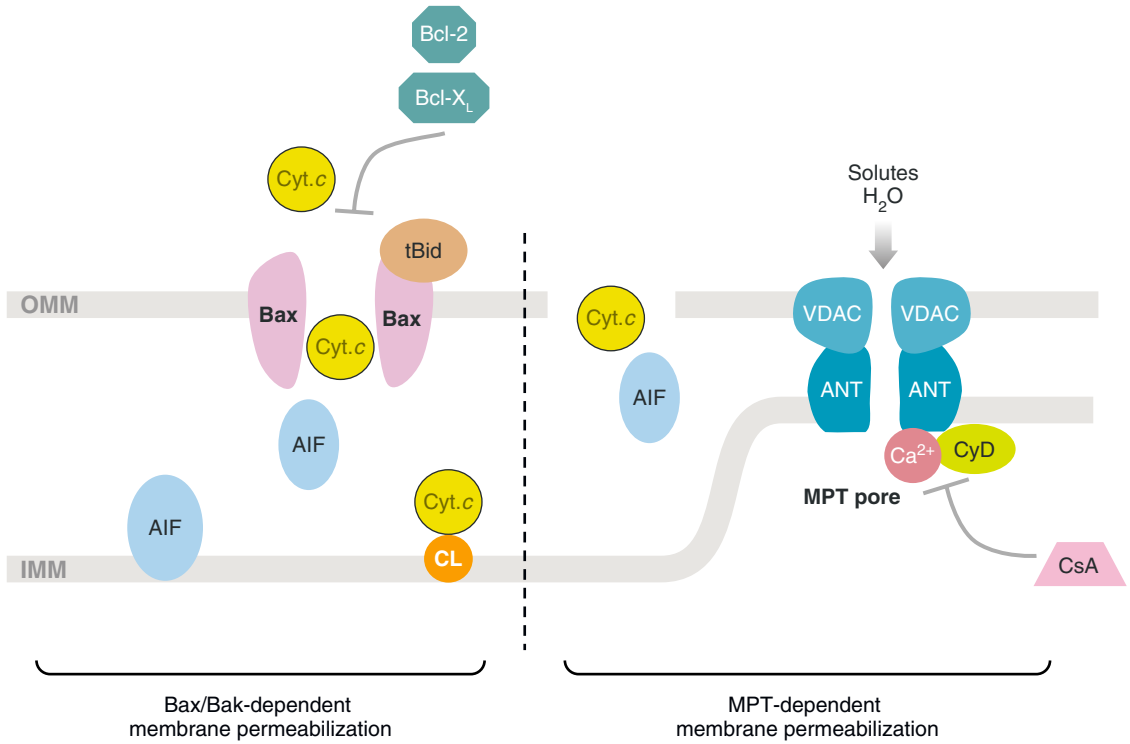

Figure 4

Mechanisms of mitochondrial outer membrane permeabilization during cell death. AIF: apoptosis inducing factor; ANT: adenine nucleotide translocase; CL: cardiolipin; Cyt *c*: cytochrome *c*; CyD: cyclophilin D; CsA: cyclosporin A; IMM: inner mitochondrial membrane; MPT: mitochondrial permeability transition; OMM: outer mitochondrial membrane; VDAC: voltage-dependent anion channel.

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may be most relevant under conditions associated with localized mitochondrial Ca^{2+} overload (111). However, transient pore opening might also occur whereby a small fraction of mitochondria would have open pores at a given time (112). In this case, mitochondrial protein release would occur without observable large-amplitude swelling, or drop in membrane potential, of the entire organelle population. This process can be observed also under normal physiological conditions, especially in mitochondria located in close proximity to calcium hot spots, microdomains in which the local concentration of ionized calcium far exceeds the average concentration measured throughout the cytosol (113). This local Ca^{2+} concentration might be high enough to induce Ca^{2+} overload and subsequent pore opening. Therefore, under the influence of apoptotic stimuli, the frequency of such spontaneous pore opening and closure might increase, contributing to translocation of intermembrane space proteins into the cytosol.

Recent observations have questioned the importance of MPT for the release of cytochrome *c* from the mitochondria under apoptotic conditions. Thus, overexpression of cyclophilin-D, a component of the MPT pore complex, had opposite effects on apoptosis and necrosis; whereas NO-induced necrosis was promoted, NO- and staurosporine-induced apoptosis was inhibited. These findings suggest that MPT leads to cell necrosis, but they argue against its involvement in apoptosis (114). Similarly, cyclophilin-D-deficient cells died normally in response to various apoptotic stimuli, but were resistant to necrotic cell death induced by ROS and Ca^{2+} overload. In addition, cyclophilin-D-deficient mice showed resistance to ischemia/reperfusion-induced cardiac injury. These results suggest that the cyclophilin-D-dependent MPT regulates some forms of necrotic, but not apoptotic, cell death (115, 116).

Bcl-2 family proteins and mitochondrial outer membrane permeabilization. Another mechanism of outer membrane permeabilization involves members of the Bcl-2 family of proteins. The Bcl-2 family consists of more than 30 proteins, which can be divided into three subgroups: Bcl-2-like survival factors, Bax-like death factors, and BH3-only death factors. Residues from BH1, 2, and 3 form a hydrophobic groove, with which BH3-only death factors interact through their BH3-domain, whereas the N-terminal BH4-domain stabilizes this pocket (117).

Early indications of the importance of these proteins for the release of cytochrome *c* were obtained in 1997, when two groups independently showed that overexpression of Bcl-2 prevented the efflux of cytochrome *c* from the mitochondria in apoptotic cells as well as the initiation of apoptosis (118, 119). It was concluded that one possible mechanism by which Bcl-2 can prevent apoptosis is to block cytochrome *c* release from mitochondria. The same year, the ability of Bax to stimulate cytochrome *c* release was demonstrated in yeast overexpressing Bax (120). It was found that Bax-induced growth arrest of yeast cells was caused by two defects in the respiratory chain: (*a*) a decrease in the amount of cytochrome *c* oxidase, the terminal enzyme of the respiratory chain, and (*b*) a dramatic release of cytochrome *c* into the cytosol. Other components of the mitochondrial inner membrane (the bc1 complex and F0F1-ATPase) were unaffected. Coexpression of Bcl-X_L almost fully prevented the effect of Bax.

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The importance of Bax interaction with mammalian mitochondria for the apoptotic process was shown by Jurgensmeier and colleagues (121). Addition of submicromolar amounts of recombinant Bax protein to isolated mitochondria induced cytochrome *c* release, whereas a peptide representing the Bax BH3 domain was inactive. When added to purified cytosol, neither mitochondria nor Bax alone induced proteolytic processing and activation of caspases. In contrast, addition of a combination of Bax and mitochondria triggered release of cytochrome *c* from the mitochondria and induced caspase activation in the cytosol. Supernatants from Bax-treated mitochondria also triggered caspase processing and activation, whereas recombinant Bcl-X_L protein abrogated Bax-induced release of cytochrome *c* from isolated mitochondria and prevented caspase activation.

Several assumptions were made to explain the ability of Bax to release cytochrome *c* from mitochondria (**Figure 4**). In experiments with lipid membranes, it was shown that Bax forms pores in the lipid bilayer and triggers the release of liposome-encapsulated carboxyfluorescein, which can be blocked by Bcl-2 (122). However, using isolated mitochondria, Narita et al. (123) reported that proapoptotic Bcl-2 family proteins, Bax or Bak, can release cytochrome *c* by interacting with MPT pore components, in particular, the voltage-dependent anion channel (VDAC). In addition to cytochrome *c* release, Bax and Bak caused mitochondrial alterations typical of MPT, such as loss of $\Delta\psi$ and swelling of the organelles. All of these changes were Ca^{2+}-dependent and were prevented by MPT inhibitors, cyclosporin A (CsA) and bongkrekic acid (123). Furthermore, antibodies that inhibited VDAC activity prevented Bax-induced cytochrome *c* release and loss of mitochondrial membrane potential (124). Contrary to this data, it was demonstrated that an inhibitor of pore opening, Mg^{2+}, also stimulated cytochrome *c* release and that other MPT inhibitors could not block the Mg^{2+}-induced efflux (125). These results strongly suggest the existence of two distinct mechanisms leading to cytochrome *c* release, one of which is stimulated by calcium and inhibited by CsA, whereas the other is Bax-dependent and Mg^{2+}-sensitive, but CsA-insensitive.

Bax, as well as Bak, were further shown to release cytochrome *c* in an MPT-independent manner via interaction with VDAC. VDAC is known to be responsible for most of the metabolite flux across the mitochondrial outer membrane (126). However, even in the open state it is not large enough (3 nm) to allow penetration of cytochrome *c* (14 kDa). On the other hand, Bax and Bak stimulated the opening of VDAC incorporated into liposomes and allowed encapsulated cytochrome *c* to pass, and the passage was prevented by Bcl-X_L (127). In contrast to this report, Rostovtseva and colleagues (128) found no electrophysiologically detectable interaction between VDAC channels isolated from mammalian mitochondria and either monomeric or oligomeric forms of Bax. In contrast, another proapoptotic protein, tBid, proteolytically cleaved by caspase-8, affected the voltage gating of VDAC by inducing channel closure. The latter finding is in accordance with recent work by Colombini and colleagues, which also indicates that apoptosis is associated with VDAC closure rather than opening, and that the resultant decrease in metabolite fluxes over the mitochondrial membranes leads to formation of ceramide channels mediating cytochrome *c* release and caspase activation (129). Hence, it appears that the

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role of VDAC in the permeabilization of the OMM during apoptosis requires further study.

Permeabilization of the OMM was shown to be a prerogative of the oligomeric form of Bax, whereas monomeric Bax was ineffective (130). Oligomerization of Bax is a result of binding to the truncated form of the BH3-domain-only proapoptotic protein Bid (131) (**Figure 4**). Hence, it was shown that tBid triggers the homooligomerization of Bax (or Bak) (132), resulting in the release of cytochrome *c* from mitochondria. Cells lacking both Bax and Bak, but not cells lacking only one of these proteins, have been found to be resistant to tBid-induced cytochrome *c* release and apoptosis (133). Moreover, Bax- and Bak-deficient cells were also resistant to a variety of apoptotic stimuli that act through the mitochondrial pathway, such as staurosporine, UV radiation, growth factor deprivation, etoposide, and the ER stress stimulus thapsigargin. Thus, activation of a multidomain proapoptotic Bcl-2 family member Bax or Bak appears to be a predominant gateway to mitochondrial release of proteins required for cell death in response to diverse stimuli.

As mentioned above, different mechanisms of tBid-induced release of cytochrome *c* were proposed. tBid added to mouse liver mitochondria stimulated cytochrome *c* release via mechanisms that did not require the BH3-domain of tBid or the presence of Bak (Bax), but were sensitive to CsA (107). According to the authors, tBid induces structural rearrangement of mitochondria; individual cristae become fused and the junctions between the cristae and the intermembrane space become open. The ability of CsA to block tBid-induced release of cytochrome *c* suggested that the MPT pore complex was involved in this remodeling process. However, the physiological importance of this pathway is doubtful because the amount of tBid required for cytochrome *c* release via remodeling of cristae was almost two orders of magnitude higher than that required for activation of the Bax/Bak-mediated pathway (134).

The permeability of the OMM is regulated by the concerted operation of pro- and antiapoptotic proteins. Antiapoptotic proteins, such as Bcl-2, Bcl-X_L, Mcl-1, and Bcl-w, interact with the proapoptotic proteins Bax and Bak, thereby preventing their oligomerization. For instance, Mcl-1 suppresses the pore-forming activity of Bak by the formation of complexes with this proapoptotic protein (135). This type of regulation is quite specific; for example, Bak can be bound by both Mcl-1 and Bcl-X_L but not by Bcl-2 or Bcl-w (136). Suppression of the proapoptotic activity of Bax was also shown to occur via formation of complexes with the DNA repair factor Ku70. After apoptosis induction, Ku70 becomes acetylated at particular lysine residues, causing dissociation of Bax (137).

Disturbance of the balance between anti- and proapoptotic Bcl-2 family members in favor of the latter can proceed by mechanisms involving BH3-only proteins that bind to and occupy the antiapoptotic proteins, thereby liberating Bax and Bak. For example, the BH3-only proteins PUMA and NOXA, which are expressed in a p53-dependent manner upon DNA damage, were shown to cause OMM permeabilization (138, 139). Coimmunoprecipitation studies showed that NOXA binds to Bcl-2 and Bcl-X_L, depending on a functional BH3 motif of NOXA, but not to Bax. Interestingly, cytosolic p53 can not only induce PUMA and NOXA but can also directly activate Bax

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and thereby cause the OMM permeabilization (140), although the precise mechanism of this activation is still obscure.

Multiple mechanisms of cytochrome *c* release can coexist within one model of cell death. Thus, arsenic-induced cytochrome *c* release triggered by low (up to 20 μM) doses of As_2O_3 was found to be Bax/Bak-dependent and, hence, completely blocked in Bax/Bak double-knockout mouse embryonic fibroblasts (141). However, at higher arsenic concentrations, cytochrome *c* release was caused by a direct effect of the toxicant on mitochondria resulting in MPT induction, presumably via increased ROS production at the level of Complex I of the respiratory chain and oxidative modification of SH-groups in ANT, which occurred to a similar extent in both wild-type cells and cells lacking Bax and Bak (141).

Bcl-2 family proteins not only regulate the permeabilization of the OMM via formation of pores but also can modulate MPT induction. Thus, the presence of a higher level of Bcl-2 protein in mitochondria of Zajdela hepatoma was found to be the cause of a delay in MPT induction when compared with liver mitochondria (142). Conversely, it has been demonstrated that cell death resulting from Bax overexpression can occur via induction of MPT because it was prevented by inhibition of the MPT with CsA in combination with the phospholipase A_2 inhibitor aristolochic acid (143). Inclusion of recombinant oligomeric Bax in the incubation buffer markedly stimulated Ca^{2+}-triggered MPT induction (144).

In contrast to MPT-induced cytochrome *c* release, the release mediated by Bcl-2 family proteins occurs without apparent alterations of ultrastructure and main mitochondrial functions, even when the loss of cytochrome *c* was almost complete (145). Apparently, under these circumstances mitochondrial $\Delta\psi$ could be maintained via hydrolysis of glycolytic ATP because the severe loss of cytochrome *c* should have blocked mitochondrial respiration.

Oxidation of Cardiolipin Modulates Cytochrome *c* Release

Cytochrome *c* is normally bound to the inner mitochondrial membrane by an association with the anionic phospholipid cardiolipin (**Figure 4**). Cardiolipin is present only in mitochondria and is found primarily in the IMM. Because of its unique structure among phospholipids, cardiolipin confers fluidity and stability to the mitochondrial membrane. The molecular interaction between cardiolipin and cytochrome *c* involves electrostatic interactions at the A-site of cytochrome *c*, whereas hydrophobic interactions and hydrogen bonding take place at its C-site. In fact, one of the acyl chains of cardiolipin is inserted into a hydrophobic channel in cytochrome *c*, whereas the other extends into the phospholipid bilayer (146).

Unlike other phospholipids, the fatty acyl groups on cardiolipin are essentially restricted to C18 chains. The dominant C18 chain in mammals is linoleoyl (18:2), with oleoyl (18:1) and linolenoyl (18:3) also present. The unsaturated nature of the acyl chains confers functional specialization to cardiolipin, which appears to be required for optimal function of many of the proteins involved in mitochondrial energy metabolism. The degree of unsaturation of the acyl chains is also important for the

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synthesis of cardiolipin. Palmitate, a saturated (16:0) free fatty acid found in plasma at high concentrations during ischemia, is known to trigger apoptosis in many cell types. Importantly, palmitate-induced apoptosis in rat neonatal cardiomyocytes has been attributed to a decrease in cardiolipin content, which correlates almost stoichiometrically with the extent of cytochrome *c* release into the cytosol (147). These findings are consistent with the observation that palmitate (16:0) increased apoptosis (with concomitant cytochrome *c* release), whereas oleate (18:1) supported proliferation of breast cancer cells (148). Hence, it appears that palmitate stimulates cardiolipin turnover and decreases the concentration of unsaturated fatty acid precursors needed for optimal cardiolipin synthesis, thereby reducing overall amounts of this phospholipid.

An additional view on the role of cardiolipin in cytochrome *c* release from mitochondria that has surfaced recently is the hypothesis that cardiolipin is also required for permeabilization of the OMM. Although, as mentioned above, cardiolipin is present almost exclusively in the IMM, it also can be found at low levels in the OMM, depending on the source of the mitochondria. In contrast to outer membrane vesicles from rat liver mitochondria, which are virtually free of cardiolipin, outer membrane vesicles from yeast mitochondria, as well as those from *Neurospora crassa*, contain more cardiolipin than can be accounted for by contamination with IMM fragments (149). Thus, although cardiolipin is primarily an IMM phospholipid, it has some access to the outer membrane (150).

Experiments with liposomes composed of lipids mimicking the OMM, or the contact sites between the OMM and IMM, revealed that tBid binds poorly to liposomes resembling the OMM, but it binds effectively to liposomes resembling the contact sites. Analysis of the role of individual phospholipids demonstrated that the most efficient binding was observed in the presence of 20% cardiolipin. Although the cardiolipin content used in the liposome experiments far exceeds its physiological content in the OMM, the authors concluded that cardiolipin seems to be required for the recruitment of tBid to mitochondria, in particular, at contact sites where it would be enriched (151).

The requirement of cardiolipin for cytochrome *c* release was supported by a study showing that cardiolipin is obligatory for Bax-mediated pore formation in liposomes (152). Specifically, the authors used reconstituted membrane and/or synthetic liposomes encapsulating fluorescently labeled dextran molecules to demonstrate that Bax-mediated dextran release required the presence of cardiolipin in the liposomes. Bid, or its BH3-domain peptide, activated monomeric Bax to produce membrane openings that allowed the passage of very large (2 megadalton) dextran molecules, mimicking the translocation of mitochondrial proteins during apoptosis. This process required cardiolipin and was inhibited by antiapoptotic Bcl-X_L. Thus, they concluded that mitochondrial protein release in apoptosis can be mediated by supramolecular openings in the OMM, promoted by BH3/Bax/lipid interaction and directly inhibited by Bcl-X_L. However, it is uncertain how protein-lipid interaction might lead to formation of the huge pores in the OMM. Besides, as highlighted in Reference 153, it is difficult to understand why such pores would not also be formed in the IMM in which the content of cardiolipin is incomparably higher than in the OMM.

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To study the role of cardiolipin in OMM permeabilization, we used a model of cardiolipin-deficient and wild-type yeast mitochondria (154). It was found that neither the mitochondrial association of exogenous recombinant Bax nor the resulting cytochrome *c* release was dependent on the cardiolipin content of the yeast mitochondrial membranes. In our experiments, Bax associated equally with both wild-type and cardiolipin-deficient mitochondrial membranes under conditions that led to the release of cytochrome *c* from both strains. However, we found that cytochrome *c* was bound more loosely to the cardiolipin-deficient IMM compared with the wild-type control.

Considering binding of cytochrome *c* to the IMM, it appears that the electrostatic and hydrophobic interactions between cardiolipin and cytochrome *c* must be breached for cytochrome *c* to leave the mitochondria. Apparently, simple permeabilization of the OMM by oligomeric Bax in a low-ionic-strength medium is insufficient for cytochrome *c* release and activation of the caspase cascade. It was early found that cardiolipin oxidation decreases its binding affinity for cytochrome *c* and, more recently, that oxidative modification of cardiolipin facilitates cytochrome *c* mobilization from the IMM (155). Based on these results, we hypothesized that cytochrome *c* release during apoptosis occurs by a two-step process involving first the detachment of the hemoprotein from the IMM, followed by permeabilization of the OMM and the release of cytochrome *c* into the extramitochondrial milieu. These findings indicate that cardiolipin plays an important role not only in mitochondrial energy metabolism but also in the retention of cytochrome *c* within the intermembrane space.

Hence, accumulating data suggest that ROS facilitate the detachment of cytochrome *c* from cardiolipin before its release into the cytoplasm via pores in the OMM formed by proapoptotic proteins. Indeed, early studies utilizing bovine heart submitochondrial particles showed that mitochondrially generated ROS decreased the content of cardiolipin in the membrane, and that this was concomitant with a decrease in the activity of cytochrome *c* oxidase (156). The cardiolipin content of the particles could be restored with exogenously added cardiolipin, but not with peroxidized cardiolipin. Studies of myocardial ischemia in the perfused rabbit heart have also revealed that increased ROS production leads to cardiolipin oxidation and depletion, combined with inhibition of Complex IV activity in subsarcolemmal mitochondria. It is of interest to note that phospholipase A_2-mediated generation of lysocardiolipin was a late event, and that cardiolipin oxidation was primarily responsible for the depletion of cardiolipin seen in this model. In addition, Vogelstein's group reported that oxidative degradation of mitochondrial cardiolipin occurred during p53-mediated apoptosis (157). Furthermore, a model of glutamate toxicity in neurons demonstrated that cytochrome *c* is released from mitochondria in a ROS-dependent fashion (158), and a burst of ROS in growth factor–deprived neurons was found to damage mitochondria by causing a profound loss of cardiolipin (159). A host of more recent studies have also shown a correlation between preserved cardiolipin content and resistance to apoptosis upon manipulation of various mitochondrial antioxidant enzymes, such as peroxiredoxin, glutaredoxin 2, and Gpx4 (see discussion below).

Selective peroxidation of cardiolipin was recently demonstrated by Kagan and colleagues to precede cytochrome *c* release during apoptosis (160). Looking for the

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mechanism of cardiolipin oxidation, the authors found that cytochrome *c*, in complex with cardiolipin, catalyzes H_2O_2-dependent cardiolipin peroxidation, which, in turn, facilitates the detachment of cytochrome *c* from the outer surface of the inner mitochondrial membrane and its subsequent release into the cytoplasm through pores in the outer membrane. Quantitative characterization of the peroxidase activity of cytochrome *c* revealed that at low ionic strength and high cardiolipin-cytochrome *c* ratio, the peroxidase activity of the cardiolipin/cytochrome *c* complex was increased more than 50-fold. This catalytic activity correlates with partial unfolding of cytochrome *c*, and an increase in the peroxidase activity preceded the loss of protein tertiary structure. It seems that electrostatic cardiolipin-cytochrome *c* interactions are central to the initiation of the peroxidase activity, whereas hydrophobic interactions are involved when the tertiary structure of cytochrome *c* is lost (161). The finding that cytochrome *c* might change its structure during apoptosis was unexpected because several groups have shown that even small changes in the structure of cytochrome *c* resulted in abrogation of its proapoptotic function (162). In the presence of cardiolipin in vitro, cytochrome *c* peroxidase activity was activated at lower hydrogen peroxide concentrations than seen with cytochrome *c* in the absence of phospholipid (161). Altogether, this suggests that redistribution of cardiolipin in the mitochondrial membranes combined with increased production of hydrogen peroxide can switch on the peroxidase activity of cytochrome *c* and cardiolipin oxidation in mitochondria, and that this might be a prerequisite step in the execution of apoptosis. The peroxidase function of the cardiolipin–cytochrome *c* complex is compatible with the proposed two-step hypothesis of cytochrome *c* release and also provides an explanation for the protective effects reported for multiple mitochondrial antioxidant enzymes (155). It also emphasizes the importance of mitochondrial ROS production as an integrated component of the apoptotic program.

One of the possible sources of ROS in mitochondria of apoptotic cells is the p66Shs protein, a redox enzyme that utilizes reducing equivalents derived from the mitochondrial electron transfer chain through the oxidation of cytochrome *c* to produce H_2O_2 in the intermembrane space (163). Redox-defective mutants of p66Shc are unable to induce mitochondrial ROS generation and swelling in vitro, or to mediate mitochondrial apoptosis in vivo. Interestingly, a fraction of this cytosolic enzyme localizes within mitochondria, where it forms a complex with mitochondrial Hsp70. Upon induction of apoptosis, dissociation of this complex is followed by the release of monomeric p66Shc and its interaction with cytochrome *c* to generate hydrogen peroxide.

Recently it has been shown that NO suppresses the peroxidase activity of cytochrome *c* in the cardiolipin–cytochrome *c* complex, suggesting a role for NO as a regulator of cell death via inhibition of cardiolipin oxidation (164). This is in agreement with earlier observations of the role of mitochondrially generated NO as an antioxidant, causing inhibition of oxidation of mitochondrial lipids and proteins and protecting membranes from oxidative damage (165). However, additional work is required to clarify the precise mechanism of the antiapoptotic function(s) of NO.

The two-step concept of cytochrome *c* release from mitochondria during apoptosis has been confirmed in several subsequent studies. For example, recent observations

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demonstrated that in the absence of Complex I inhibitors, recombinant oligomeric Bax protein elicited only minimal cytochrome *c* release (~18%) from brain mitochondria. However, when the mitochondria were incubated with both recombinant Bax and Complex I inhibitors, which were shown to stimulate ROS production and, hence, cardiolipin oxidation, up to 65% of the mitochondrial cytochrome *c* was released. Thus, in accordance with the two-step concept, neither ROS production via Complex I inhibition nor permeabilization of the OMM with Bax alone triggered overt release of cytochrome *c*, whereas their combination resulted in a marked release of this proapoptotic molecule (166). In another study, suppression of Complex I activity stimulated intramitochondrial oxidative stress, which, in turn, increased the releasable soluble pool of cytochrome *c* within the mitochondrial intermembrane space (167). Upon mitochondrial permeabilization by Bax, more cytochrome *c* was released into the cytosol from brain mitochondria with impaired Complex I activity. Based on these results, the authors proposed a model in which defects of Complex I lower the threshold for activation of mitochondrially dependent apoptosis by Bax, thereby rendering compromised neurons more prone to degenerate.

It is known that cells from Bax, Bak double-knockout mice are resistant to most inducers of apoptosis and that the presence of these proteins is normally required for OMM permeabilization. However, their exact role in cardiolipin oxidation-dependent cytochrome *c* release is still unclear. It was demonstrated, however, that mitochondrial production of ROS increased in the presence of Bax plus a BH3-domain peptide (168), apparently due to stimulated $O_2^{\cdot-}$ production by the respiratory chain following cytochrome *c* release (169). Increased ROS production may further contribute to cytochrome *c* release by activating lipid peroxidation (170) and thereby facilitating cytochrome *c* dissociation from cardiolipin (171). Clearly, the precise mechanism(s) of interaction of these proteins with oxidized cardiolipin remain to be investigated, as well as the nature of the promoting effect of cardiolipin hydroperoxides on OMM permeabilization. The interaction of tBid—the C-terminal cleavage fragment of the proapoptotic Bcl-2 protein, Bid—with the cardiolipin-cytochrome *c* complex also needs to be characterized.

As mentioned above, VDAC, a constituent of the MPT pore complex, represents a binding site for different proapoptotic proteins, and oxidation of cardiolipin at mitochondrial contact sites can be expected to modify the interaction of VDAC with these proteins, in particular tBid. Hence, Madesh & Hajnocky (48) showed that both $O_2^{\cdot-}$ and H_2O_2 facilitate Ca^{2+}-induced MPT pore opening, but release of cytochrome *c* was triggered only by $O_2^{\cdot-}$. The effect of $O_2^{\cdot-}$ was dose-dependent and also occurred in the absence of Ca^{2+} and was insensitive to MPT inhibitors. The IMM was not damaged, as $\Delta\psi$ was sustained in the presence of extramitochondrial ATP. It is worth mentioning that pretreatment of the cells with drugs or antibodies to block VDAC prevented superoxide-induced cytochrome *c* release. Similarly, VDAC-reconstituted liposomes released cytochrome *c* after exposure to $O_2^{\cdot-}$, and this release was prevented by VDAC blockers. The authors conclude that $O_2^{\cdot-}$-induced release of cytochrome *c* is independent of the inner membrane components of the pore and is a consequence of VDAC-dependent selective permeabilization of the OMM. It is, however, unclear why no release of cytochrome *c* was observed upon induction of MPT by Ca^{2+} and

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H_2O_2. These experiments were performed in a high-ionic-strength buffer, facilitating detachment of cytochrome *c* from the IMM, and permeabilization of the OMM upon MPT induction should have caused its release.

Thus, although our understanding of the detailed mechanisms that regulate the release of proapoptotic proteins from the mitochondria during the early phase of apoptosis is still not complete, the demonstration of a peroxidase function of the cardiolipin–cytochrome *c* complex represents an important step forward in this respect. Further work will have to characterize this phospholipid-protein interaction in detail and investigate the general implications of this mechanism in apoptosis.

Mitochondrial Antioxidant Systems in Protection from Cell Death

As discussed above, mitochondrial ROS production seems to be an integrated part of apoptotic and necrotic cell death. Accordingly, protection from oxidative stress by administration of antioxidants, such as vitamin E and ubiquinone, has been found to increase cell viability in multiple experimental model systems (54). In addition, under normal physiological conditions, cell viability and function are critically dependent on the maintenance of a balance between mitochondrial ROS generation and inactivation. Perturbation of this balance can result in cell death as well as the death of the organism.

The importance of a tight regulation of the mitochondrial redox balance is further emphasized by the broad spectrum of antioxidant enzymes present in the mitochondria, and the fact that deletion of several of these enzymes is incompatible with cell viability and also causes embryonic lethality. For example, disruption of the *MnSOD* gene results in early postnatal lethality (172), whereas *CuZnSOD*$^{-/-}$ mice show no apparent phenotypic alterations. This difference seems to be directly related to the different subcellular localization of the SODs because *MnSOD* deletion can be compensated for by the targeting of CuZnSOD into the mitochondrial matrix. Further, disruption of the mitochondrial thioredoxin system (*Trx2*$^{-/-}$) also confers embryonic lethality (60), whereas *Gpx1*$^{-/-}$ mice are healthy except under conditions of extreme oxidative stress. The latter finding may be due to the fact that peroxiredoxin (Prx III) is more important for mitochondrial H_2O_2 catabolism than Gpx1. In contrast, disruption of the *Gpx4* gene causes embryonic lethality, and cell lines from *Gpx4*$^{+/-}$ mice are hypersensitive to H_2O_2 and other agents that cause oxidative stress (173) (**Figure 2**).

A large number of studies have been devoted to the effects of modulation of mitochondrial antioxidant enzymes on cell viability and susceptibility to apoptotic cell death. Heterozygous *MnSOD* knockout mice exhibit numerous alterations in mitochondrial function, including inhibition of aconitase and Complex I and II of the respiratory chain, enhanced susceptibility to induction of permeability transition, and increased lipid peroxidation (174). In contrast, overexpression of *MnSOD* attenuates mitochondrial ROS generation, protects respiratory function, and blocks apoptosis in some experimental models. Although $O_2^{\cdot-}$ in the mitochondrial matrix has been proposed to activate uncoupling proteins (UCPs), thereby providing a feedback mechanism that would limit further $O_2^{\cdot-}$ production by the respiratory chain, there was no evidence for altered UCP activities in the studies of the *MnSOD* transgenic mice (66).

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A cytoprotective effect of UCP2 linked to attenuated ROS production and caspase activation has been observed in mice subjected to stroke and brain trauma (175).

As mentioned above, disruption of the *Trx2* gene results in a lethal embryonic phenotype that is associated with massive apoptosis during early embryogenesis (60). Trx2-deficient chicken cells were also reported to undergo apoptosis in the absence of exogenous triggers and to be hypersensitive to treatment with agents that induce oxidative stress (176). Similar results have been obtained in experiments with glutaredoxin 2, in which *Grx2* overexpressing HeLa cells were found to be less susceptible to mitochondrially mediated apoptosis (177), whereas glutaredoxin 2–deficient cells were hypersensitive to triggers of oxidative cell damage (178). The glutaredoxin system has also recently been suggested to be involved in the antiapoptotic effect of 17-β-estradiol in H_2O_2-treated cardiac H9c2 cells (179).

Mitochondrial Prx III is also dependent on the mitochondrial thioredoxin system for activity and is probably the most important H_2O_2-metabolizing system in these organelles. A recent study in HeLa cells has shown that knockdown of Prx III using RNA interference technology resulted in increased intracellular levels of H_2O_2 and sensitized the cells to induction of mitochondrially mediated apoptosis by staurosporine or TNF (63). These effects were reversed by ectopic expression of *Prx III* or mitochondrially targeted catalase. Prx III was found to be more abundant than Gpx1, and the study supports the hypothesis that Prx III is the most important H_2O_2-scavenging enzyme in the mitochondria (**Figure 2**).

Gpx4, also known as phospholipid hydroperoxide glutathione peroxidase, represents the most extensively studied enzyme involved in mitochondrial antioxidant defense and protection from apoptosis. *Gpx4*-null mice die in utero by mid-gestation, and $Gpx^{+/-}$ mice show reduced survival upon γ-irradiation (173). Cell lines from these animals are extremely vulnerable to oxidative injury, whereas transgenic mice overexpressing *Gpx4* were found to be less susceptible to diquat-induced liver injury as well as apoptosis induced by oxidative stress (180). Protection from apoptosis was early reported to be specific for mitochondrially expressed *Gpx4* and to be related to suppression of the mitochondrial pathway of apoptosis signaling (181). More recent findings have linked the inhibitory effect of Gpx4 on apoptosis to a protection of the adenine nucleotide translocase from the loss of activity that is otherwise associated with apoptosis, possibly by inhibition of cardiolipin oxidation (182). It is of interest to note that the antiapoptotic effects of peroxiredoxin (63), glutaredoxin 2 (177), and Gpx4 (182) have all been related to a possible protection of cardiolipin from peroxidation during the early stage of apoptosis (**Figure 2**).

Glutathione-linked antioxidant defense systems depend on the availability of GSH for activity, and there is ample evidence for a critical importance of the mitochondrial GSH pool for cell survival. Hence, glutathione disulfide formed in these reactions must be efficiently reduced back to GSH by mitochondrial glutathione reductase. This, in turn, relies on the availability of mitochondrial NADPH, which is also providing reducing equivalents for TrxR2 and is, therefore, of critical importance for the function of the thioredoxin and peroxiredoxin systems. NADPH is regenerated by mitochondrial $NADP^+$-dependent isocitrate dehydrogenase (IDPm) and by the proton-translocating nicotinamide nucleotide transhydrogenase located in the IMM,

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which catalyzes the reversible reduction of $NADP^+$ by NADH in a reaction linked to proton translocation across the membrane (183). Not unexpectedly, modulation of the activity of both enzymes has been reported to be inversely related to cellular susceptibility to apoptosis (184, 185).

Finally, novel work on p53 has further emphasized the importance of the mitochondrial redox state in the regulation of cell death. It has long been known that $p53^{-/-}$ cells demonstrate increased ROS production when compared to their wild-type counterparts, and a recent study has shown that p53 switch-down by siRNA technology causes oxidation of DNA, which could be inhibited by N-acetyl cysteine (186). Hence, it appears that loss of p53 function is associated with impaired cellular antioxidant defense owing to lack of activation of several p53 target genes, e.g., MnSOD and Gpx1. Another recently discovered target gene is TIGAR, which seems to halt glycolysis while stimulating NADPH generation via the pentose phosphate shunt (187), resulting in increased GSH levels that are essential to scavenge ROS. To compensate for the loss of energy production by inhibited glycolysis, the p53 protein also induces production of a copper transporter, SCO_2, which participates in the assembly of cytochrome *c* oxidase in the mitochondria and thereby facilitates ATP production by oxidative phosphorylation (188).

Thus, it appears that the mitochondrial antioxidant defense systems are of critical importance for the regulation of apoptosis. Although further work is certainly required to characterize the mechanism(s) of protection, inhibition of cardiolipin oxidation during the early apoptotic phase might contribute to protection. It is also remarkable that there seems to be so little functional overlap between the various systems, and that modulation of the activity of one particular defense system is directly reflected on cellular susceptibility to undergo apoptosis. For example, it is difficult to understand why a modest decrease in glutaredoxin 2 activity is not compensated for by the mitochondrial thioredoxin system, and why manipulation of peroxiredoxin activity exerts such pronounced effects on apoptosis susceptibility, when there are so many other cellular scavengers of the easily diffusible H_2O_2.

CONCLUDING REMARKS

During the past decade, it has become apparent that the mitochondria play a critical role in the regulation of both apoptotic and necrotic cell death, and that mitochondrial outer membrane permeabilization and release of intermembrane space proteins are important features of both processes. Of the mechanisms involved, mitochondrial permeability transition appears to be associated mainly with necrosis, whereas the release of proapoptotic proteins during early apoptosis is regulated primarily by the Bcl-2 family of proteins. However, there is abundant evidence for interaction and cooperation between these two mechanisms, although the detailed role of the VDAC in mitochondrial permeabilization requires further studies. The importance of the potential interaction of the proapoptotic Bcl-2 family members, tBid/Bax/Bak, with other proteins in the outer mitochondrial membrane, e.g., components of the protein import (TOM) complex, for pore formation also requires more detailed investigation. The same is true for the role of ROS and lipid peroxidation in triggering the release of

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proapoptotic proteins from the mitochondria. The hypothesis that the dissociation of cytochrome *c* from its binding to the outer surface of the inner membrane is facilitated by cardiolipin oxidation has received convincing support from several experimental studies, whereas the role of oxidized cardiolipin in outer membrane permeabilization and the release of proapoptotic proteins that are not bound to the inner membrane (e.g., Smac/DIABLO) is less clear. Mechanisms by which enhanced ROS formation is triggered during the early apoptosis phase also need further study, as does the relative contribution of the various mitochondrial antioxidant defense systems to the overall protection from apoptotic cell death. Thus, much work remains to be done before we can claim that we know the details of the mitochondrial regulation of cell death.

ACKNOWLEDGMENTS

Work in the authors' laboratory was supported by grants from the Swedish (3829-B04-09XAC) and Stockholm (041502) Cancer Societies, the Swedish Research Council (K2004-31X-02471-37A), the European Commission (QLK3-CT-2002-01956), and Gosta Fraenckel's Foundation for Medical Research. We apologize to authors whose primary references could not be cited due to space limitations.

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