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Mitochondria, calcium and pro-apoptotic proteins as mediators in cell death signaling

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Abstract

Cellular Ca^{2+} signals are crucial in the control of most physiological processes, cell injury and programmed cell death through the regulation of a number of Ca^{2+}-dependent enzymes such as phospholipases, proteases, and nucleases. Mitochondria along with the endoplasmic reticulum play pivotal roles in regulating intracellular Ca^{2+} content. Mitochondria are endowed with multiple Ca^{2+} transport mechanisms by which they take up and release Ca^{2+} across their inner membrane. During cellular Ca^{2+} overload, mitochondria take up cytosolic Ca^{2+}, which in turn induces opening of permeability transition pores and disrupts the mitochondrial membrane potential ($\Delta\psi_m$). The collapse of $\Delta\psi_m$ along with the release of cytochrome c from mitochondria is followed by the activation of caspases, nuclear fragmentation and cell death. Members of the Bcl-2 family are a group of proteins that play important roles in apoptosis regulation. Members of this family appear to differentially regulate intracellular Ca^{2+} level. Translocation of Bax, an apoptotic signaling protein, from the cytosol to the mitochondrial membrane is another step in this apoptosis signaling pathway.

Key words
- Ca^{2+}
- Mitochondrial Ca^{2+} uptake
- Mitochondrial Ca^{2+} efflux
- Permeability transition
- Apoptosis
- Bcl-2 family
- Bax and apoptosis

Cell death and apoptosis

Cell death is a primordial event in cell life and there are several mechanisms by which cells achieve death. Necrotic death and apoptotic death are characterized by biochemical and morphological differences. Necrosis is usually involved in some death stimuli and therefore is always pathological, accidental and not very well regulated. Conversely, apoptosis or programmed cell death is a regulated physiological process that is involved in cell maintenance, development, tumor regression, hormone-induced atrophy, and cell-mediated immunity. Apoptosis also occurs during certain pathological states, such as ischemia-reperfusion damage, infarction, neurodegenerative diseases and viral or chemical toxicity, and can be triggered by activation of certain death receptors on the plasma membrane or by cellular stress. The main interest in apoptotic mechanisms is the fact that apoptosis involves a cascade of biochemical events that is tightly regulated. Some of the elements in this cascade are the activation of specific cysteine proteases, called caspases, mitochondrial release of death factors, and finally the characteristic changes in

nuclear morphology and DNA fragmentation (1). Several lines of evidence suggest that a change in cellular Ca^{2+} handling and an increase in cytosolic Ca^{2+}, $[Ca^{2+}]_c$, might be associated with apoptotic signaling (2).

The mitochondrial membrane potential ($\Delta\psi_m$) has been identified as an important hallmark associated with apoptotic cell death. During apoptosis nuclear disintegration can be preceded by loss of $\Delta\psi_m$ (3), and disruption of $\Delta\psi_m$ associated with several factors such as an increase in reactive oxygen species production and elevation of $[Ca^{2+}]_c$ (4). Under certain circumstances, loss of $\Delta\psi_m$ during apoptosis is prevented by cyclosporine A, indicating that permeability transition may be involved in apoptosis (5). During apoptotic signaling, cytochrome c, apoptosis-inducing factor, caspases and other mitochondrial factors like smac/DIABLO, are released from mitochondria (6). Both apoptosis-inducing factor and cytochrome c, when released from mitochondria into the cytosol, induce activation of pro-caspases and caspases. Release of cytochrome c can be related to the full opening of the permeability transition pore (PTP) (7). However, as discussed below, the precise nature of the release of cytochrome c and other factors from mitochondria certainly requires further examination.

Mitochondria and cell death

A number of investigators have shown that during cell death mitochondria lose their $\Delta\psi_m$. This loss of $\Delta\psi_m$ was inhibited by cyclosporine A, a PTP inhibitor, suggesting that PTP may be involved in this process (4,5).

The PTP has been reported to be a megachannel on the mitochondrial membrane and the opening of this pore may cause the release of solutes up to 1.5 kDa, inducing the collapse of the proton gradient and thus impairing ATP synthesis and leading to mitochondrial dysfunction (8). Although the existence of such a mechanism has been established for over 20 years (9), the exact nature of the pore and its molecular structure are still unknown. It has been proposed that the PTP is composed of a mitochondrial cyclophylin D located in the matrix and is bound to the inner mitochondrial membrane (10). The binding of cyclophylin D to the adenine nucleotide translocase on the inner membrane causes a conformational change in adenine nucleotide translocase (11). In addition, other mitochondrial constituents, such as creatinine kinase, located in the inter-membrane space, mitochondrial porin known as voltage-dependent anion channel, present on the outer membrane, may all form part of the PTP complex (12).

It is known that the opening of the PTP in the high conductance mode is triggered by increases in Ca^{2+} concentration and changes in mitochondrial voltage, pH and redox state (13). Reactive oxygen species are also related to the opening of the pore (14). Because of the dramatic change in membrane permeability that occurs upon opening of the PTP and collapse of the $\Delta\psi_m$, ATP synthesis is uncoupled from oxidative phosphorylation and mitochondrial swelling occurs, leading to cellular pathology.

It has been shown and discussed more recently that opening of the PTP occurs in a low conductance state which can be related to the transient openings of the pore (15,16). Although this mechanism is still not well understood, it might be related to Ca^{2+} signaling (15,16), oxygen utilization, and to the reduction in $\Delta\psi_m$ reducing the formation of superoxide due to leakage of electrons from the mitochondrial electron transport chain (17). Further investigation of this subject is required.

Proteins of the Bcl-2 family and apoptosis. Mitochondrial modulation

Members of the Bcl-2 family form a group of proteins that play important roles in the

regulation of cell death under both physiological and pathological conditions. Members of this family promote either cell survival, as in the case of Bcl-2 and Bcl-X_L, or cell death, as in the case of Bax and Bid (2,18). In living cells, Bax and Bid are predominantly soluble proteins (19), Bcl-2 is associated with the membranes of various organelles including endoplasmic reticulum, mitochondria, and nuclei (20), and Bcl-X_L exists in both soluble and membrane-bound forms (19). During apoptosis, Bcl-2 remains bound to the membranes, but the cytosolic forms of Bax, Bid and Bcl-X_L have been found to redistribute from the cytosol into the membranes, in particular mitochondrial membranes (19,21). The mechanisms leading to Bax and Bcl-X_L redistribution into the mitochondria are still unknown. Bid is believed to be post-translationally cleaved by caspase-8 to enable its translocation into mitochondria (21).

X-ray crystallography and solution NMR analyses of the recombinant Bcl-X_L have indicated that this protein shares a high degree of structural similarity to the translocation domain of diphtheria toxin (22), which can insert into lipid bilayers (23). This suggests that Bcl-2, Bcl-X_L, and Bax may form ion channels (24). *In vitro* studies have shown that the insertion of Bax causes the release of cytochrome c from mitochondria (25). Cytochrome c has been proposed to interact with Apaf-1 (26). This complex, in the presence of dATP, can catalyze the activation of caspases to proteolyze cellular constituents (27). Figure 1 shows a schematic representation of the apoptotic cascade and the interaction of Bax and Bid with mitochondria.

Some reports have suggested that this release of cytochrome c is associated with Bax interaction with adenine nucleotide translocase (7) or voltage-dependent anion channels (28), both of which are putative components of the PTP. Thus, release of cytochrome c can be associated with PTP activation and the attending loss of $\Delta\psi_m$.

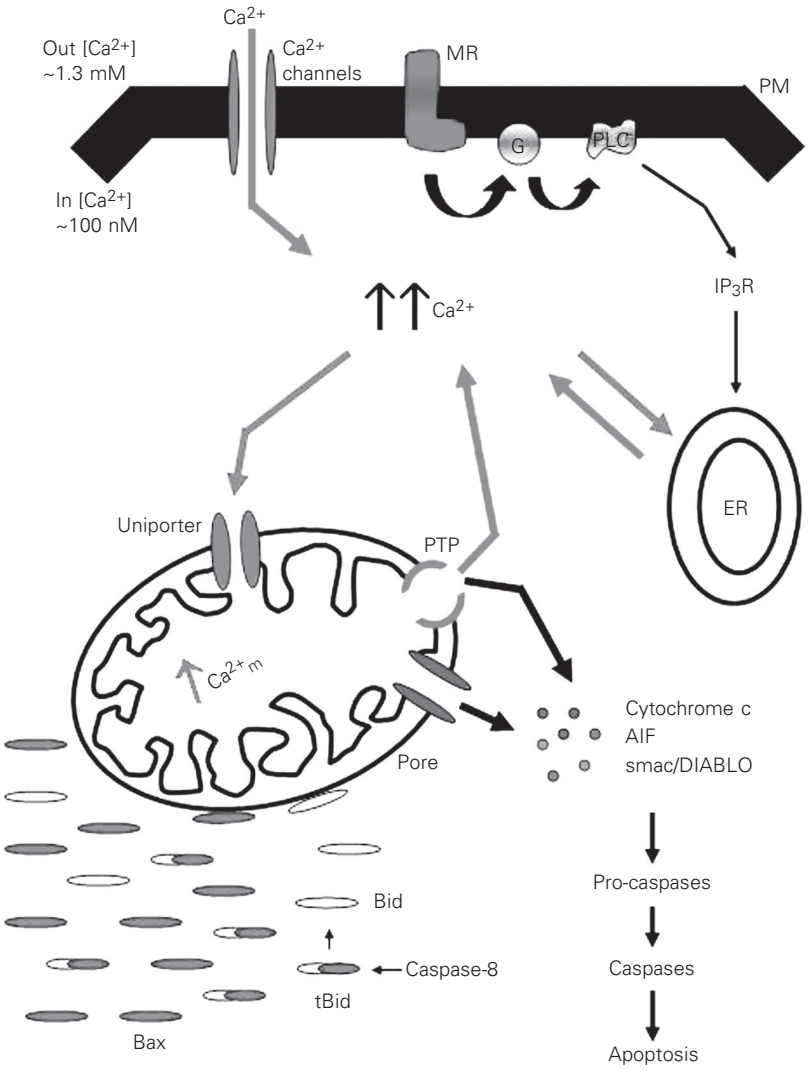

Figure 1. Ca^{2+} homeostasis and signaling under physiological and cell death conditions. Without stimuli, extracellular Ca^{2+} concentration is approximately 1.3 mM and cytosolic concentration is about 100 nM. When metabotropic receptors (MR) are stimulated cytosolic Ca^{2+} increases either by opening of membrane Ca^{2+} channels or release from intracellular stores. Transient increases in cytosolic Ca^{2+} stimulate mitochondrial uptake of Ca^{2+} which is found in close proximity to the sarcoendoplasmic reticulum. Mitochondrial Ca^{2+} (Ca^{2+}m) accumulation leads to opening of the permeability transition pore (PTP; either at a high or low conductance). In the presence of an apoptotic signal cells may overexpress pro-apoptotic proteins such as Bax and Bid. Bax can translocate from the cytosol to the membranes, especially mitochondria. When Bid is cleaved (truncated Bid, tBid) by caspases, Bid translocates into the mitochondria increasing Bax toxicity. Bax and Bid lead to a permeabilization of mitochondrial membranes via mechanisms that are still under investigation (e.g., pore formation, opening of PTP). This permeabilization leads to the release of cytochrome c and other factors such as apoptosis-inducing factor (AIF), second mitochondrial activator of caspases (smac/DIABLO) that activate pro-caspases and down caspases that execute the proteolytic cascade, DNA fragmentation and cell death. During apoptotic stimuli, Ca^{2+} may be released from the endoplasmic reticulum (ER) and be taken up by mitochondria. The Ca^{2+} content of the intracellular stores can accelerate apoptosis, but the exact relationship between Ca^{2+} signaling and apoptosis is still unknown. PM, plasma membrane; IP_3R, inositoltriphosphate receptor; G, G protein; PLC, phospholipase C.

However, cytochrome c release can occur in the absence of permeability transition and collapse of $\Delta\psi_m$ (29). In addition, under special circumstances permeability transition has been shown not to be involved in Bax-induced cytochrome c release (30). Since the nature of PTP and its molecular identity are unknown, the mechanism of PTP activation and its participation in cytochrome c release and loss of $\Delta\psi_m$ are not clearly understood and need further investigation.

Bax as apoptotic protein

Bax is a pro-apoptotic member of the Bcl-2 family. Members of this family can promote either cell survival, as is the case for Bcl-2 and Bcl-X_L, or cell death, as is the case for Bax and Bak. Bax was first identified as a Bcl-2 binding partner by immunoprecipitation (31). Subsequently it was shown that overexpression of Bax can accelerate cell death in response to various apoptosis stimuli (32). Physiologically, Bax plays an important role in neuronal development and spermatogenesis. Animals that are deficient in Bax have increased numbers of neurons and males are known to be sterile (33). Under pathological conditions such as cerebral and cardiac ischemia, upregulation of Bax has been reported in the afflicted area of the tissues, implicating the participation of this protein in the promotion of neuronal and cardiomyocytic cell death (34). In certain cases of human colorectal cancer, mutations were found in the gene encoding Bax, suggesting that inactivation of Bax promotes tumorigenesis by rendering the tumor cells less susceptible to cell death (35).

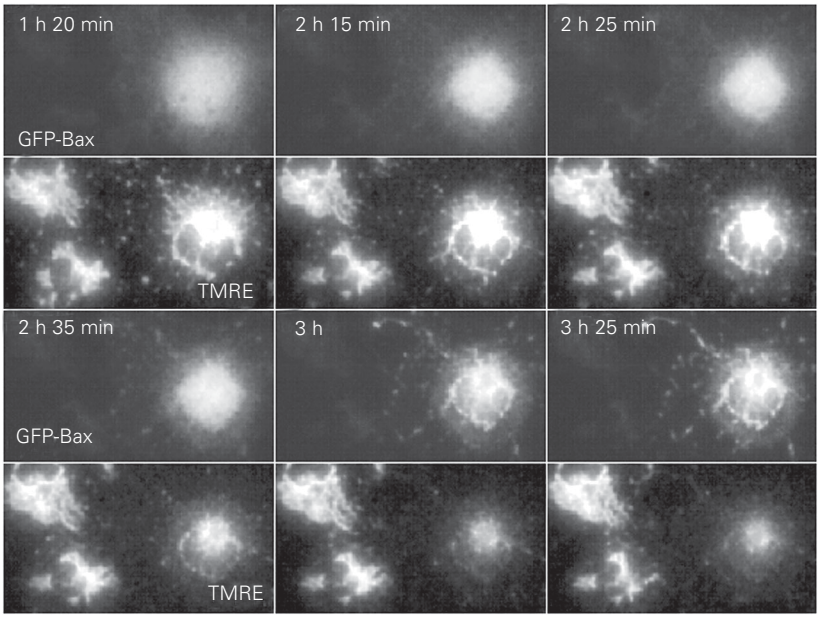

Figure 2. Loss of mitochondrial membrane potential ($\Delta\psi_m$) is associated with Bax translocation to mitochondria. Healthy Cos-7 cells expressing GFP-Bax (rows 1 and 3) were co-stained with tetramethylrhodamine ethyl ester (TMRE, 50 nM) for $\Delta\psi_m$ visualization (rows 2 and 4). Cells were then treated with the apoptotic inducer staurosporine (STS, 1 µM) and fluorescences of GFP-Bax and TMRE were simultaneously recorded. The cells on the right were transfected with GFP-Bax (rows 1 and 3) while two others on the left were not transfected with GFP-Bax but were stained with TMRE (rows 2 and 4). In the first panel at a specific time (1 h and 20 min) after STS treatment, Bax (row 1) was still soluble in the cytosol and the $\Delta\psi_m$ (row 2) was well maintained. Over time, GFP-Bax shifted from a diffuse cytosolic state to a punctuated mitochondrial-bound localization (row 3). The presence of GFP-Bax in the mitochondria promoted the collapse of $\Delta\psi_m$ as indicated by the decrease in TMRE fluorescence (row 4). This loss may occur concomitantly with GFP-Bax translocation to mitochondrial membranes. In the two untransfected cells the collapse of $\Delta\psi_m$ was not observed.

Bax shares with other members of the Bcl-2 family the feature of having three conserved regions named BH (Bcl-2 homology) domains 1-3 (36). Several lines of evidence show that these domains can be important for the regulatory functions of these Bcl-2 family proteins. In addition, Bax and a number of Bcl-2 family members also possess a hydrophobic segment at their C-terminal ends. For Bcl-2, this hydrophobic segment is required to anchor the protein to various organelles, including endoplasmic reticulum, mitochondria, and nuclear outer membranes (36). The 3-D structures of the Bax and its pro-survival antagonist Bcl-X_L have recently been deciphered (37). These two proteins appear to share a significant structural homology with the translocation domain of diphtheria toxin, especially in a helical loop domain formed by α-helices 5 and 6. This particular domain of diphtheria toxin has been shown to penetrate the lipid bilayer to form pores (22,23).

In healthy cells, Bax is predominantly a soluble monomeric protein (19) despite the fact that it possesses a C-terminal hydropho-

bic segment. This hydrophobic domain, unlike those of Bcl-2 and Bcl-X_L, is sequestered inside a hydrophobic cleft (37). Upon induction of apoptosis by a variety of agents, a significant fraction of Bax has been observed to translocate from the cytosol to the membrane fractions, in particular, the mitochondria (19,38). This translocation process appears to involve a conformational change in Bax leading to the exposure of its C-terminal hydrophobic domain (39). After translocation, Bax may form large oligomers once they reach the membrane. These oligomers are associated with cytotoxic activities and have not been seen in other proteins such as Bid and Bad (40). Deletion of the Bax C-terminal hydrophobic domain abrogated the ability of the mutant protein to translocate to mitochondria and greatly attenuated its ability to promote cell death. On the other hand, point mutations of Bax that target the expressed proteins to mitochondria greatly increased Bax toxicity (39,40). The translocation of Bax to mitochondria is associated with the release of cytochrome c and the loss of $\Delta\psi_m$ (25,29). These phenomena may be related to the recent observations that Bax can form ion channels or pores in mitochondrial membranes (41). Cytochrome c activates caspase-3 leading to the proteolysis of the cell while the loss of $\Delta\psi_m$ corresponds to a decrease in cellular energy production. The pro-apoptotic activity of Bax, however, can be counteracted by co-expression with pro-survival factors Bcl-2 and Bcl-X_L, which can block Bax translocation to mitochondria during apoptosis (42). In Figure 2 we show Cos-7 cells tranfected with a GFP-Bax construct. Using this approach, it is possible to visualize that Bax is soluble in the cytosol of healthy cells and translocates to membranes upon apoptotic stimuli like staurosporine. In our study, we observed that the collapse of $\Delta\psi_m$ occurred just before the complete translocation of Bax to mitochondrial membranes (38). In cells co-expressing Bax and Bcl-X_L, staurosporine was not able to induce either $\Delta\psi_m$ collapse or Bax translocation.

BH3 domain proteins and cell death

Bcl-2 family members are classified as having one or more Bcl-2 homology domains (BH domain 1-4). Some members of this family that possess the BH3 domain alone were named "BH3 domain only". The BH3 domain proteins such as Bid, Bad, and Bik are critical for heterodimerization with other family members. This interaction may be critical for death-regulatory activity. Caspase cleavage of truncated Bid (tBid) exposes the BH3 domain, causing its translocation to mitochondria and promoting cytochrome c release (43). In neuronal cells, it has been shown that the BH3 domain causes a permeabilization of the outer membrane leading to cytochrome c release without affecting inner membrane integrity or permeability transition. These effects may be dependent on the presence of Bax (44). The caspase-activated tBid is believed to trigger the homo-oligomerization of the pro-apoptotic family members such as Bak and Bax (45). This oligomerization can induce the release of cytochrome c (46). The precise mechanisms by which cytochrome c is released is not understood; however, to a certain extent, release can be rescued by exogenous cytochrome c, showing a reversible component of mitochondrial respiratory dysfunction during apoptosis (47).

Ca^{2+} as a sensor in cell death

Intracellular Ca^{2+} changes have been implicated in cell death and have been associated with a number of pathological states such as cardiac ischemia, muscular dystrophy, neuronal damage during cerebral ischemia, and hypoglycemia (48). In the central nervous system Ca^{2+} overload can give rise to excitotoxicity, leading to cellular damage and eventually cell death. Excitotoxic activation of glutamate

receptors causes a large influx of Ca^{2+} into the cell, resulting in mitochondrial Ca^{2+} overload and oxidative damage. It has been postulated that the PTP is associated with the accumulation of Ca^{2+} (49); however, others have suggested that the opening of the pore may be a consequence of reactive oxygen species production (50).

Mitochondria regulate Ca^{2+} levels by utilizing the energy derived from the chemiosmotic potential gradient ($\Delta\psi_m$) to transport Ca^{2+} into and out of their matrix. Mitochondria take up Ca^{2+} through uniporters in mitochondrial inner membranes, utilizing energy derived from $\Delta\psi_m$ (51). This process, however, can be inhibited by polycations such as ruthenium red. This uptake mechanism serves to support Ca^{2+}-sensitive mitochondrial enzymes and acts as a mechanism for removal of $[Ca^{2+}]_c$ from local areas of the cell.

Ca^{2+} efflux from mitochondria into the cytosol occurs via at least two different mechanisms, an Na^+-dependent transport process and an Na^+-independent one that exchange Ca^{2+} for Na^+ or H^+, respectively. The Na^+-dependent mechanism, also known as mitochondrial Na^+/Ca^{2+} exchanger, is mainly found in heart and brain mitochondria and is an electrogenic process (52). In contrast, the Na^+-independent efflux, is nonelectrogenic, and is present in liver and kidney mitochondria, and behaves as an active $Ca^{2+}/2H^+$ exchanger (51).

Cytosolic Ca^{2+} has been proposed to play an important role in the triggering of apoptotic signals in the regulation of cell death-specific enzymes such as endonucleases (53). Recent studies have shown that Ca^{2+} stores in the endoplasmic reticulum may play a role in apoptotic induction, since a reduction of Ca^{2+} release from the endoplasmic reticulum can prevent mitochondrial damage. Endoplasmic reticulum Ca^{2+} release can also sensitize mitochondria to release cytochrome c, and pro-apoptotic proteins such as Bax and Bak have been suggested to play a role in this process (54). Calcium signals have been identified as one of the major signals which converge on mitochondria to trigger the mitochondrial-dependent pathway of cardiac apoptotic cell death (55). Currently, it is not clear what roles Bcl-2 family members play in Ca^{2+} signaling during cell death. Some investigators have shown that they play a modulatory role, since overexpression of these proteins can change Ca^{2+} concentration in the cytosol or in the intracellular stores. Overexpression of Bcl-2 has been reported to prevent Ca^{2+} release from the endoplasmic reticulum in response to apoptotic inducers such as thapsigargin (56). Recently, it has been shown that overexpression of Bax in cultured cells caused a loss of endoplasmic reticulum content, and depletion of endoplasmic reticulum by rynodine receptors enhances Bax interaction with mitochondria (57). Also, caffeine and other xanthines that mobilize intracellular Ca^{2+} can act synergistically with p53 to induce cell death involving Bax translocation (58).

The role of different members of the Bcl-2 family and their relation with calcium signals is still unknown. Recent reports have associated tBid with Ca^{2+} signaling and propagation to mitochondria. It is possible that tBid induces a selective permeabilization of the outer mitochondrial membrane to inositoltriphosphate-induced mitochondrial Ca^{2+} signaling (59). This change in mitochondrial permeability might be related to the tBid-induced remodeling of mitochondrial structure that evokes a mobilization of the cytochrome c stores (60). The consequences of the effect of tBid and its interactions with Bax translocation and Ca^{2+} homeostasis still await elucidation.

Currently, little is known about the mechanism of apoptosis. The role of the Bcl-2 family, the PTP and Ca^{2+} signaling, and how these pathways interact to regulate this process are still unknown. Further work is needed to elucidate these mechanisms in order to understand their role in tissue homeostasis and disease progression.

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