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MOLECULAR MECHANISMS OF MITOCHONDRIAL APOPTOGENIC FACTOR RELEASE THROUGH PORES AND MEGACHANNELS

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ABSTRACT

Mitochondrial membrane permeabilization is a biochemically well-defined phenomenon that occurs in response to numerous physiological and pathological processes that regulate cell survival. In many situations, mitochondrial membrane permeabilization is triggered by an excess of reactive oxygen species (ROS), Ca^{2+} overload, and the interference of BH3-only proteins of the BCL-2 family, as well as by activated caspases that can act on components of the inner or outer membrane to cause the opening, assembly and/or activation of membrane mitochondrial permeability transition pores. These pores permit the release of apoptogenic factors such as cytochrome *c*, apoptosis-inducing factor, Smac/Diablo, HtrA2/Omi and endonuclease G from the intermembrane space to the cytosol where they mediate many of the biochemical and morphological features of apoptosis and necrosis. In this review, we discuss the pharmacological, genetic and biochemical evidence that proteins, protein complexes and membrane structures can form pores through which apoptogenic factors can be released from mitochondria.

Key words: Apoptosis, BCL-2, caspases, mitochondrion, permeability transition pore, reactive oxygen species, voltage-dependent anion channel

INTRODUCTION

The mitochondrion is a fundamental organelle that controls many energy-linked pathways involved in cell survival and death. The mammalian mitochondrial genome (~16 kb) is circular and encodes 13 proteins required for energy production, as well as 22 tRNAs and two rRNAs. Mitochondrial DNA (mtDNA) lacks protective histones and polyamines, and its repair system is less efficient than that of the nuclear system. As a result, the frequency of oxidative damage, substitutions and deletions in mtDNA is ~15-fold higher than in nuclear DNA, and there is evidence for a direct relationship between mutations in mitochondrial genes and the pathogenesis of many human conditions, such as Alzheimer´s disease, Parkinson´s disease, cancer and premature aging [29].

Most of the mitochondrial proteins (~1000) anchored in the inner and outer mitochondrial membranes, intermembrane space and matrix are encoded in the nucleus, and are vital for mitochondrial biogenesis and function. These proteins include components of the electron-transport chain, oxidative phosphorylation and Kreb´s cycle, as well as transporters and channels for the entry of ions and polypeptides into the organelle. Whereas the functions of these components are quite well known, we are only beginning to understand how the protein-transporting channels of the inner mitochondrial membrane translocase (Tim complex) and the outer mitochondrial membrane translocase (Tom complex) recognize and target proteins into mitochondria after synthesis in the cytosol [16].

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ABBREVIATIONS

ABC transporter, ATP binding cassette transporter; AIF, apoptosis-inducing factor; ANT, adenine nucleotide translocase; Apaf-1, apoptotic protease activating factor; BH, BCL-2 homology; BIR, baculoviral IAP repeat; BCL-2, B cell lymphoma; CAD, caspase-activated DNAse; CARD, caspase recruitment domain; CK, creatine kinase; DED, death effector domains; DISC, death-inducing signaling complex; DR, death receptor, Endo G, endonuclease G; FADD, Fas-associated protein with a death domain; GK, glycerol kinase; HK, hexokinase; IAP, inhibitor of apoptosis protein; MAC, mitochondrial apoptosis-induced channel; ROS, reactive oxygen species; SOD, superoxide dismutase; tBID, truncated BID; TIM, the inner mitochondrial membrane translocase; TOM, the outer mitochondrial membrane translocase; TNF, tumor necrosis factor; TRADD, TNF receptor associated protein, TRAF, TNF receptor-associated factor, TRAIL, TNF-related apoptosis-inducing ligand; VDAC, voltage-dependent anion channel.

Mitochondria produce and/or release several molecules with key roles in the initiation and execution of necrotic and apoptotic cell death. Reactive oxygen species (ROS), calcium (Ca^{2+}), ATP, inorganic phosphate (Pi), and the apoptogenic proteins cytochrome *c*, caspases, apoptosis-inducing factor (AIF), Smac/Diablo, HtrA2/Omi and endonuclease G (Endo G) are the most important examples [12,21,24]. The distinct concentrations and translocation kinetics of these proteins into the cytosol suggest that their activation and release from mitochondria occurs through well-regulated mechanisms and through poorly characterized pores or channels [1,30,40]. Mitochondrial bioenergetic metabolism and ATP levels have an important influence on this protein transport (Fig. 1).

Cytochrome *c* is a soluble, 14 kDa colored protein located in the intermembrane space where it acts as an electron carrier in the mitochondrial respiratory chain. The heme group in the porphyrin molecule of cytochrome *c* contains an iron atom that changes from Fe^{3+} to Fe^{2+} after accepting an electron [29]. Cytochrome *c* by itself is not cytotoxic, but can interact with Apaf-1 (apoptotic protease activating factor) to activate caspase-9, which then initiates the activation of executioner caspases -3, -6 and -7 [9]. The CARD (caspase recruitment domain)-CARD domain interaction between procaspase-9 and Apaf-1 occurs only in the presence of cytochrome *c* and ATP or dATP [5].

The release of cytochrome *c* is not fatal since cytoplasmic proteins known as inhibitors of apoptosis (IAPs) prevent the unintended activation of caspase-9 and of caspases 3 and 7 [5,42,44]. The functional unit in each IAP is the baculoviral IAP repeat (BIR), which contains about 80 amino acids folded around a central zinc atom [44]. XIAP, c-IAP-1 and c-IAP2 contain three BIR domains each. The third BIR domain (BIR3) interacts with caspase-9 to inhibit the activity of this enzyme. The linker region between BIR1 and BIR2 selectively targets caspases -3 and -7. The activity of IAPs is regulated by Smac/Diablo, a structural homolog of the *Drosophila* proteins Reaper, Hid, and Grim [44]. Smac/Diablo, a protein expressed from a nuclear gene, is located in the mitochondrial intermembrane space. When released from mitochondria, this protein competes with caspases for the same binding site on IAPs. Since IAPs inhibit the activity of caspases, interaction with SMAC/Diablo reduces the inhibition of these enzymes and facilitates their activation during apoptosis [5,42,44].

HtrA2/OMI belongs to the HtrA (high-temperature requirement) protein family and is also encoded by a nuclear gene. This protein translocates to the mitochondrial intermembrane space and, at normal temperature, acts as a chaperone. However, at elevated temperature, this protein becomes a serine protease that cleaves noxious proteins that accumulate in mitochondria. When released into the cytosol, HtrA2/OMI blocks IAPs through its N-terminal and degrades these caspase inhibitors [42,44].

Mitochondria release two other pro-apoptotic proteins to the cytosol after cytochrome *c*, namely, Endo G and AIF, a 57 kDa flavoprotein (12,41). These proteins are not normally soluble in the intermembrane space and their release may require either caspase activation or another proteolytic system [40-42]. The differences in their size, shape and kinetics of diffusion suggest that the pore or channel involved in their release is distinct from that involved in the release of cytochrome *c* [1,30,40-42].

AIF is a protein that translocates from mitochondria to the nucleus, where it binds to DNA to stimulate chromatin condensation and DNA degradation [19]. The resulting morphology of the condensed chromatin is, however, distinct from that caused by caspase-activated DNAse (CAD). Endonuclease G is also a DNAse that participates in nuclear DNA fragmentation [12,19].

The voltage-dependent anion channel (VDAC) is a potential candidate for the efflux of cytochrome *c* and other apoptogenic factors, but experimental evidence also supports the existence of (i) a pore consisting of BAX subfamily members, (ii) a pore formed by the BAX subfamily in conjunction with VDAC, (iii) a mitochondrial apoptosis-induced channel (MAC), and (iv) rupture of the outer mitochondrial membrane. The biochemical and morphological events associated with permeabilization of the inner and outer mitochondrial membrane and/or opening of these pores and channels have been extensively discussed elsewhere [12,19,21,24,32,48]. In this review, we provide an overview of some of the recent findings in this field and discuss questions that are currently the focus of considerable research.

Formation of reactive oxygen species and their effects on mitochondria

The biochemical and bioenergetic mechanisms that control ROS production are fairly well understood [29]. Under normal circumstances, the electron-transporting complexes I-IV and one H^+-transloating ATP synthetic complex (complex V, F_oF_1-ATP synthase), coenzyme Q and cytochrome *c* carry out oxidative phosphorylation. Complex II is entirely of nuclear origin, whereas complexes I, III and IV are encoded by nuclear and mtDNA genes. Oxidative phosphorylation by the respiratory enzyme complexes transfers electrons ($H^- \rightarrow H^+ + 2e$) from a reducing equivalent NADH or $FADH_2$ to O_2. The total proton-motive force across the inner mitochondrial membrane is the sum of a large force derived from the mitochondrial membrane potential ($\triangle\psi m$) and a smaller force derived from the H^+ concentration gradient ($\triangle pH$), and is used to drive protons (H^+) from the intermembrane space into the matrix via the ATP synthase, a transmembrane protein complex that uses the energy of H^+ flow to synthesize ATP from ADP and Pi. This electrochemical proton gradient is also required to import mitochondrial proteins and to regulate the transport of metabolites across the mitochondrial membrane [16,29].

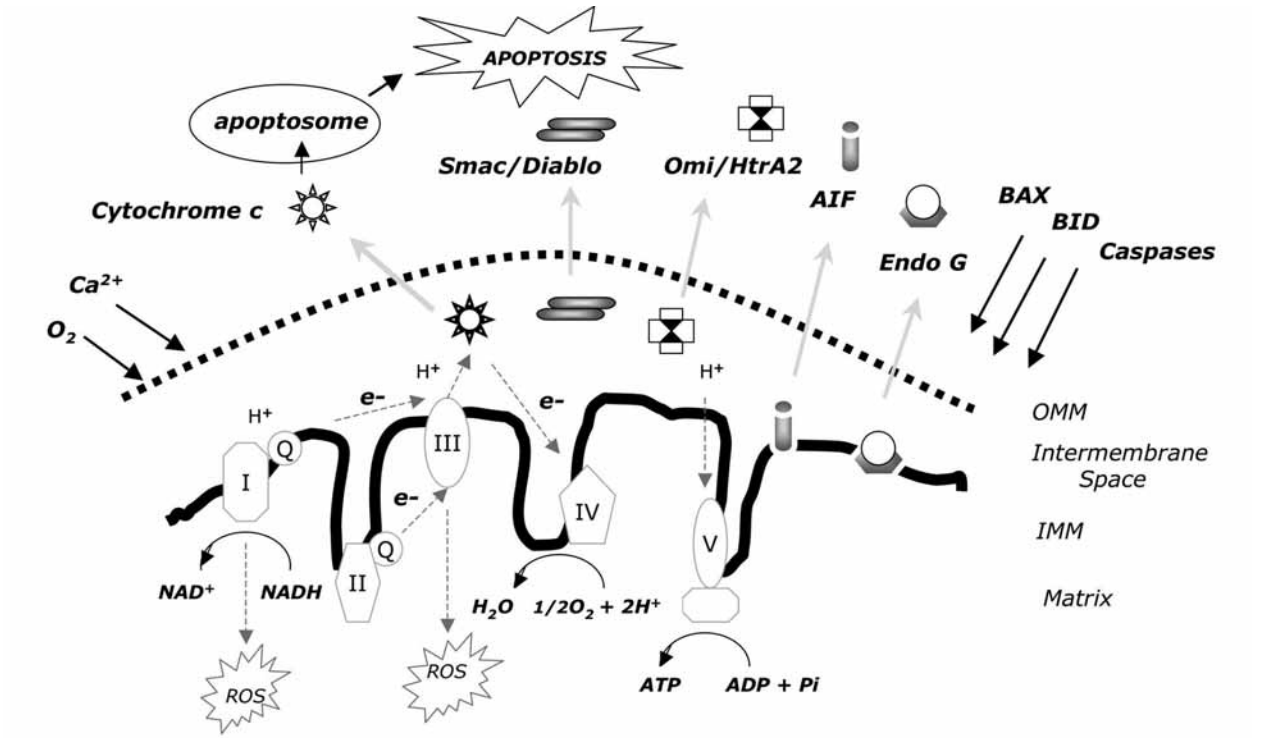

Figure 1. Bioenergetic pathways and molecular mechanisms involved in mitochondrial membrane permeabilization and the translocation of cytochrome *c*, AIF, Smac/Diablo, HtrA2/Omi and Endo G from the intermembrane space into the cytosol. In cells exposed to many apoptotic stimuli, a massive uptake of Ca^{2+} and O_2 will impair mitochondrial metabolism, leading to disruption of the mitochondrial electron transport chain and a deficiency in the pumping and removal of ATP, H^+ and NAD^+, as well as insufficient enzymes and substrates to convert excess O_2 to H_2O. This deficiency will increase the production of ROS, thereby enhancing the oxidation and/or reduction of inner and outer membrane lipids and proteins and increase the permeability of these membranes to metabolites and ions. Changes in the operation of pores formed by voltage-dependent anion channels (VDAC), adenosine nucleotide transporters (ANT) and regulatory proteins will subsequently cause swelling of the mitochondrion and rupture of the outer mitochondrial membrane. In an alternative scenario, the activation of caspases outside or inside the mitochondrion could also initiate the proteolytic cleavage of accessory and regulatory proteins, including BID and BAX, leading to the assembly and operation of pores formed by BAX multimeric complexes or the association of VDAC, ANT, BAX and other regulatory proteins. The concentrations and translocation kinetics of these proteins into the cytosol suggest that their release occurs through different pores and channels and depends on the pathways that contribute to cell death.

Disruption of the $\triangle\psi m$, $\triangle pH$ and inner membrane permeability prevents the importation of mitochondrial proteins and Ca^{2+} release, and uncouples oxidative phosphorylation, with cessation of ATP synthesis and the oxidation of $NAD(P)H_2$ and glutathione. All of these events can cause increased production of oxygen byproducts and cell death. Various mechanisms allow mitochondrial metabolism to adjust to changes in the levels of oxygen byproducts such as superoxide anions, hydrogen peroxide, hydroxyl radicals and ROS [29].

Oxygen (O_2) is converted to superoxide anions ($O_2^{\cdot-}$) by xanthine oxidase or respiratory complexes I (NADH-ubiquinone (Q) oxido-reductase) and III (ubiquinol-cytochrome *c* oxido-reductase). $O_2^{\cdot-}$ is converted to hydrogen peroxide (H_2O_2) by the superoxide dismutases SOD1 (cytosolic CuZn superoxide dismutase), SOD2 (intramitochondrial manganese superoxide dismutase), and SOD3 (extracellular CuZn peroxidase dismutase). These enzymes catalyze the reaction:

$$2H^+ + O_2^{\cdot-} + O_2^{\cdot-} \rightarrow H_2O_2 + O_2 \qquad (1)$$

H_2O_2 is converted to H_2O by catalase or glutathione peroxidase (GPX) that converts reduced glutathione (GSH) to oxidized glutathione (GSSG), according to the reaction:

$$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG \qquad (2)$$

GPX is located in mitochondria and in the cytosol such that H_2O_2 is removed from either compartment, depending on the availability of glutathione. Alternatively, $O_2^{\cdot-}$ reacts with proteins that contain iron-sulphur centers such as aconitase (4Fe-4S), succinate dehydrogenase and NADH-ubiquitone oxidoreductase to cause damage and the release of ferrous ions (Fe^{2+}). Fe^{2+} participates in the Fenton reaction, which forms hydroxyl radicals ($OH^{\cdot}$) from hydrogen peroxide:

$$H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^{\cdot} + Fe^{3+} \qquad (3)$$

Early experiments showed that ROS, mainly OH radicals, can oxidize the thiol groups (-SH) of outer and inner mitochondrial membrane proteins [18]. Physiologically, the targets of these molecules can be redox-activated ion channels and pores [reviewed in 27 and 46] that can be adversely affected by alterations in the levels of ROS; such alterations may result in these channels or pores acting as a "transition permeability pore".

Mitochondrial Ca^{2+} overload and cytochrome *c* release

Ca^{2+} is a key regulator of mitochondrial responses to the effects of many physiological and pathological factors [6,46]. Ca^{2+}-dependent stimulation of NAD(P)H and consequent ATP production, via activation of Ca^{2+}-sensitive dehydrogenases and metabolite carriers, serves to adapt the energy and substrate production necessary for cell growth and other cellular functions. Stimulation with ceramide, H_2O_2 and arachidonic acid causes cells to release Ca^{2+} stored in the endoplasmic reticulum and bound to cytoplasmic proteins such as parvalbulin, calretinin and calbindin. Mitochondrial Ca^{2+} overload results in adenine nucleotide depletion and elevated phosphate (Pi) levels [18] that can lead to oxidative stress. The combination of Ca^{2+} and ROS alters protein and lipid organization in the inner membrane by interacting with the anionic head of cardiolipin and thiol groups [18,27]. Mitochondrial swelling via the entry of water and solutes into the matrix space, and the osmotic pressure exerted by solutes, cause rupture of the outer membrane. However, it is still unclear whether the initial release of cytochrome *c* and other apoptogenic factors occurs through a membrane lesion or an organized molecular structure (pore or channel) in the outer mitochondrial membrane [12,19,21,24,25,48]. Sesso discusses the evidence for release by membrane rupture (see accompanying review in this issue [37]).

Caspases disrupt mitochondrial membrane barrier function

Caspases are cytosolic cysteine proteases that play a central role in several intracellular proteolytic pathways by structurally and functionally changing various proteins involved in apoptosis, inflammation, the cell cycle and differentiation [10,38]. Since zymogen forms of caspases -2, -3, -7, -8 and -9 occur in small amounts in the intermembrane space of mitochondria from liver, kidney, heart, brain and spleen, as well as several cell lines, these enzymes are considered to play a regulatory role in mitochondrial functions. There is considerable evidence to support the role of caspases [22,26], particularly caspase-2 [23], caspase-3 [22,31,32] and caspase-7 [22], in processes that influence permeabilization of the outer mitochondrial membrane and the formation of permeability transition pores that allow the release of cytochrome *c*.

Under certain conditions, caspase-3 can reach the intermembrane space and cleave the 75 kDa subunit of complex I (NDUFS1 protein). This is followed by the inhibition of oxygen consumption by complexes I and II [31]. However, this enzyme is unable to directly permeabilization the outer mitochondrial membrane [32]. During apoptosis, activated caspase-7 translocates from the cytosol to mitochondria and can associate with mitochondrial and microsomal fractions [7], although the mitochondrial targets of this caspase remain to be identified. Recombinant caspase-2 is an efficient promoter of outer membrane permeabilization and the release of cytochrome *c* and Smac/Diablo from isolated mitochondria [9,23]. Caspase-2 and Bax/Bak may have mutual effects in mitochondrial permeabilization and cytochrome *c* release since caspase-2 has a higher affinity for cardiolipin [9]. In a recent study, Lakhani *et al*. [22] showed that embryonic fibroblasts and thymocytes from mice with double-knockouts for caspase-3 and -7 were highly resistant to mitochondrial and death receptor-mediated apoptosis. Consistent with this, caspase $7^{-/-}$ and caspase $3^{-/-}$ cells showed a pronounced delay in cytochrome *c* release and Bax translocation, whereas the mitochondrial membrane potential was unaffected. These findings indicate that caspases -3 and -7 are critical mediators of these mitochondrial events.

One of the ways to induce the release of apoptogenic factors is by the nonselective permeabilization of mitochondrial membranes to ions and metabolites. Since there are several classes of mitochondrial transporters, channels and pores involved in the diffusion of ions, protons, metabolites and heavy metals through mitochondrial spaces, these proteins could contribute to membrane permeabilization and the nonselective passage of molecules, including proteins such as cytochrome *c*. This hypothesis was suggested by work in our laboratory which showed the presence of potential cleavage sites for caspases in proteins belonging to the Ca^{2+}, K^+ and Cl^- channel and ABC (ATP binding cassette) transporter families that operate exclusively in mitochondria [3]. Since some of these proteins are structurally and functionally linked to apoptosis via TRAF (TNF receptor-associated factor), FADD (Fas-associated protein with a death domain) and CARD (caspase recruitment domain) domains [11], we assume that they interact at a specific step during the extrinsic and intrinsic pathways of cell death. Hence, substrate cleavage could cause conformational changes and a switch to a nonselective channel/transporter function, or even the rupture and formation of megapores with different modes for releasing apoptogenic factors. This hypothesis is under investigation [3].

The voltage-dependent anion channel (VDAC) complex as a regulator of apoptogenic factor release

The most common pathway for the translocation of metabolites through the outer membrane is the voltage-dependent anion channel (VDAC), three isoforms of which are expressed in multicellular organisms [8,34,45]. The archetypal VDAC1 is a large diameter β barrel structure (~3 nm) composed of one α helix and 13 β strands, the channel of which adopts multiple conductance states with special selectivity for cations and anions [34]. The association of the VDAC with proteins such as adenine nucleotide translocase (ANT), hexokinase (HK), cyclophilin, creatine kinase (CK), glycerol kinase (GK), the peripheral benzodiazepine receptor and BCL-2 family members has been proposed to explain the role of this channel in apoptosis (see below) [8,34,43,45]. However, there is no direct evidence to support this mechanism.

The VDAC exists in an open configuration that allows the free exchange across the outer membrane of most metabolites up to 5 kDa. However, in this physiological state, the VDAC is not permeable to cytochrome *c*. ANT is located in the inner membrane and interacts directly with VDAC to form a complex that changes its transmembrane topology in response to functional mitochondrial states and physical interactions with CK, HK, GK and other cytosolic proteins [45]. At voltages <30 mV, the pore has a diameter of 4 nm and is in the anion-selective, high conductance state referred to as the open state. This open state allows the passage of ATP^{-4}, HPO_4^{-2}, $succinate^{-2}$ and other negatively charged small molecules. Above 30 mV, the diameter decreases to 2 nm, the conductance decreases to 2 nS and the selectivity changes to cationic. This VDAC state favors the flux of Ca^{2+} and Na^+. The permeability at high conductance (>100 mV) leads to dissociation of the VDAC-ANT complex. VDAC then adopts a non-physiological open state that could permit cytochrome *c* release from the intermembrane space [8,45], although this remains to be confirmed [33,34]. In contrast to this mechanism, various studies have suggested that massive cytochrome

c diffusion can occur as a consequence of VDAC closure, which ultimately results in osmotic swelling of the matrix and rupture of the outer membrane [33,34,43]. Direct participation of VDAC and ANT in cytochrome *c* release has been discarded because mitochondria devoid of cyclophilin D and ANT show a permeability transition in response to various apoptotic factors [2,34,43]. Further clarification of these important issues awaits a more complete molecular characterization of the putative permeability transition pore.

BID, BAX and BAK form pores in the mitochondrial outer membrane

Proteins of the BCL-2 family, which consists of pro-apoptotic and anti-apoptotic factors, share one or more BCL-2 homology (BH) domains [4,21,35]. The proteins BAX and BAK possess three BH domains whereas BID, BAD and BIM possess only a BH3 domain; the anti-apoptotic members BCL-2 and BCL-X_L have four BH domains (BH1-4). Certain pro- and anti-apoptotic members, such as BCL-2, BCL-X_L and BAK, reside predominantly in the outer mitochondrial membrane, whereas BAX, BID and BAD translocate from the cytosol to the outer mitochondrial membrane when cells undergo apoptosis [4,21,25,35].

A wild variety of cytotoxic agents that damage DNA or inhibit RNA and protein synthesis can induce the intrinsic pathway of apoptosis when the pro-apoptotic member BAX is specifically activated [25]. After activation, BAX undergoes a conformational change and inserts into the outer mitochondrial membrane as higher order homo-oligomers of multiple sizes to form a mega-channel that may involve the cooperation of BAK [4,21,25,35].

The pro-apoptotic member BID is specifically activated when cells enter the extrinsic pathway of apoptosis in response to the binding of CD95/Fas/APO-1, TNF, TRAIL and other members of this cytokine family to their specific cell death receptors [5,38]. Receptor activation results in the recruitment of procaspase-8 to a cell death receptor inducing signaling complex known as death-inducing signaling complex or DISC [5,38]. This large complex stimulates a conformational change in procaspase-8 and its proteolytic cleavage and auto-activation. Caspase-8 now acts on BID, releasing a truncated form of BID (tBID) that promotes permeabilization of the outer mitochondrial membrane, in a manner similar to its homolog BAX [4,35].

The production of cells and mice doubly deficient in BAX and BAK revealed that BH-3-only molecules such as BID, BIM, BAD and NOXA are unable to induce outer mitochondrial permeability in the absence of both BAX and BAK. tBID or BIM activates BAX and BAK, and oligomerized forms of BAX can insert into the outer mitochondrial membrane. BCL-2 anti-apoptotic proteins may act by sequestering activated tBID away from BAX and BAK proteins [4,21,35].

Monomeric BAX shows no channel-like conductance. However, a recent study has shown that oligomeric BAX and tBID can induce such conductance in planar membranes [33]. The most intriguing observation of this study was that the addition of tBID to a membrane containing many VDACs increased the membrane conductance, selectivity and voltage properties of these channels. This finding supports the idea that the insertion of tBID into a membrane may alter the local lipid environment and induce VDAC closure indirectly [34], although there is no evidence that these two proteins physically associate within the membrane [33].

In healthy, intact cells, BAX, BAK and BCL-2 are located in the endoplasmic reticulum and play an important role in regulating Ca^{2+} release [36]. This observation led to the suggestion of an additional mechanism that could account for their biological effects. BCL-2 family proteins apparently reduce the release of endoplasmic reticulum Ca^{2+} that normally occurs in response to death signals that activate the IP_3 pathway. Conversely, BAX and BAK promote Ca^{2+} release that in turn causes mitochondrial overload, matrix swelling and outer membrane rupture. Therefore, it is quite possible that the pro- and anti-apoptotic effects of these proteins that culminate in membrane permeabilization and opening of a mitochondrial permeability transition pore may be regulated by intraorganellar Ca^{2+} waves [36].

Evidence for novel mitochondrial apoptosis-induced channels

Recent reports have provided evidence for a new channel known as the mitochondrial apoptosis-induced channel (MAC), the pharmacological and electrophysiological properties of which can be reproduced in yeast and human cell models of apoptosis [15,28]. The MAC conductance correlates well with BAX oligomerization in the outer membrane and this activity can be prevented by overexpression of Bcl-2, but not by cyclosporin A [15,28]. More importantly, this novel, voltage-

dependent pore, which is ~3.0-3.5 nm in diameter and has a conductance of ~3 nS, is permeable to cytochrome *c in vitro* [13,15,17]. The molecular nature of this channel has not been determined, although preliminary experiments suggest that its ion conductance differs from that of VDACs and of channels formed by members of the inner (Tim) and outer (Tom) membrane protein complexes [13,17].

The inner (Tim) and outer (Tom) membrane complexes consist of several large and small proteins that form water-filled channels which mediate the translocation of nuclear proteins, including the apoptogenic factors cytochrome *c*, AIF, Endo G, Smac/Diablo and HtrA2/Omi, into mitochondria [16]. Disturbance of the activity of the Tim23 complex with synthetic peptides and antibodies causes matrix swelling, activation of a conductance channel and cytochrome *c* release [20]. Similarly, the genetic inactivation of Tim50, a subunit of the Tim23 complex, in a human cell line and in zebrafish embryos can cause mitochondrial dysfunction and cell death [14].

Tom22 forms a complex of approximately 400 kDa with Tom20, Tom40 and Tom70 that efficiently coordinates the recognition and translocation of preproteins into mitochondria [47]. We recently used CaSPredictor software [11] to examine the potential cleavage sites of these proteins by caspases and found suitable sites in Tom22, Tom 50 and Tom 70 [3]. The cleavage of these proteins could result in a pore large enough to allow the passage of cytochrome *c* or block the entry of pro-apoptogenic factors into mitochondria. This interesting possibility is under investigation [3].

Recently, a pore composed of the lipid ceramide (ceramide channel) has also been implicated in the permeabilization of the mitochondrial outer membrane [39]. Mitochondria contain a three-fold higher concentration of ceramide in the outer membrane than in the inner membrane [39]. Ceramides differ from other lipids in that they can form intermolecular hydrogen bonds to produce columns of ceramide residues that can form channels of variable sizes. The channel conductance (generally multiples of 4 nS) is proportional to the number of columns that form the ceramide channel. A channel consisting of 14 columns is theoretically capable of releasing a protein of up to 60 kDa [39]. Hence, these channels are good candidates for the release of mitochondrial apoptogenic factors.

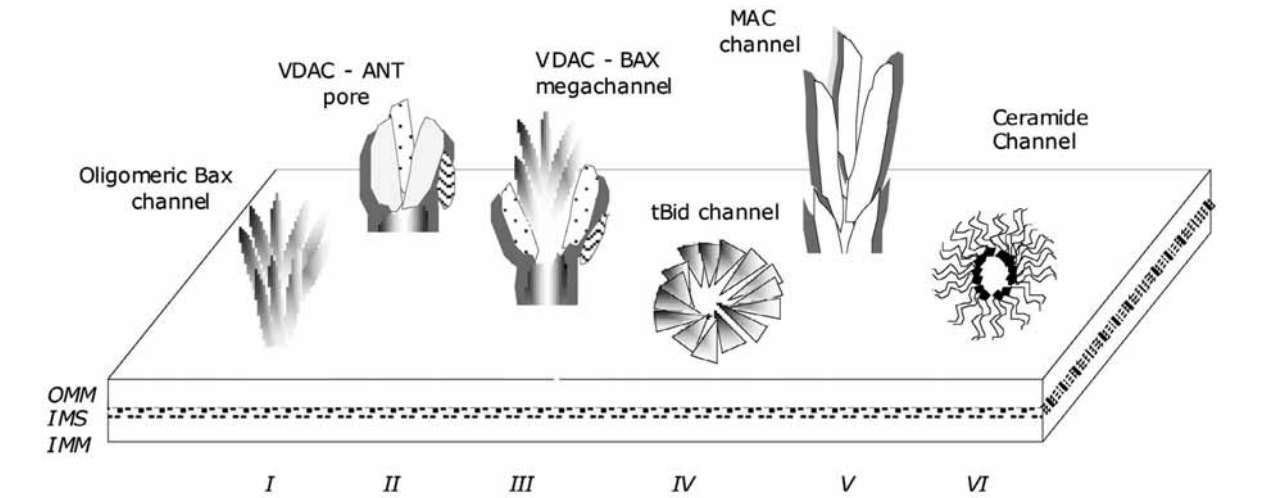

Figure 2. Structural model and molecular features of pores and channels that regulate mitochondrial membrane permeabilization and the release of mitochondrial apoptogenic factors. At high concentrations, the oligomeric forms of BAX (I) and tBID (IV) have channel-like properties [33]. The complex consisting of VDAC, ANT and oxidative phosphorylation enzymes can form channels (II) with different conductances and selectivities [8,43]. The possible insertion of BAX into VDAC channels (III) may lead to the formation a megachannel [21,32-35]. The MAC channel (V) is a new, larger pore whose activity is also affected by BCL-2 and BAX [13,15,17,28]. This pore has channel-like conductances that differ from those of other candidate channels [13,15,17,28]. Ceramide, a membrane sphingolipid, can also form large, protein-permeable channels in membranes (VI) that allow the release of proteins up to 60 kDa.

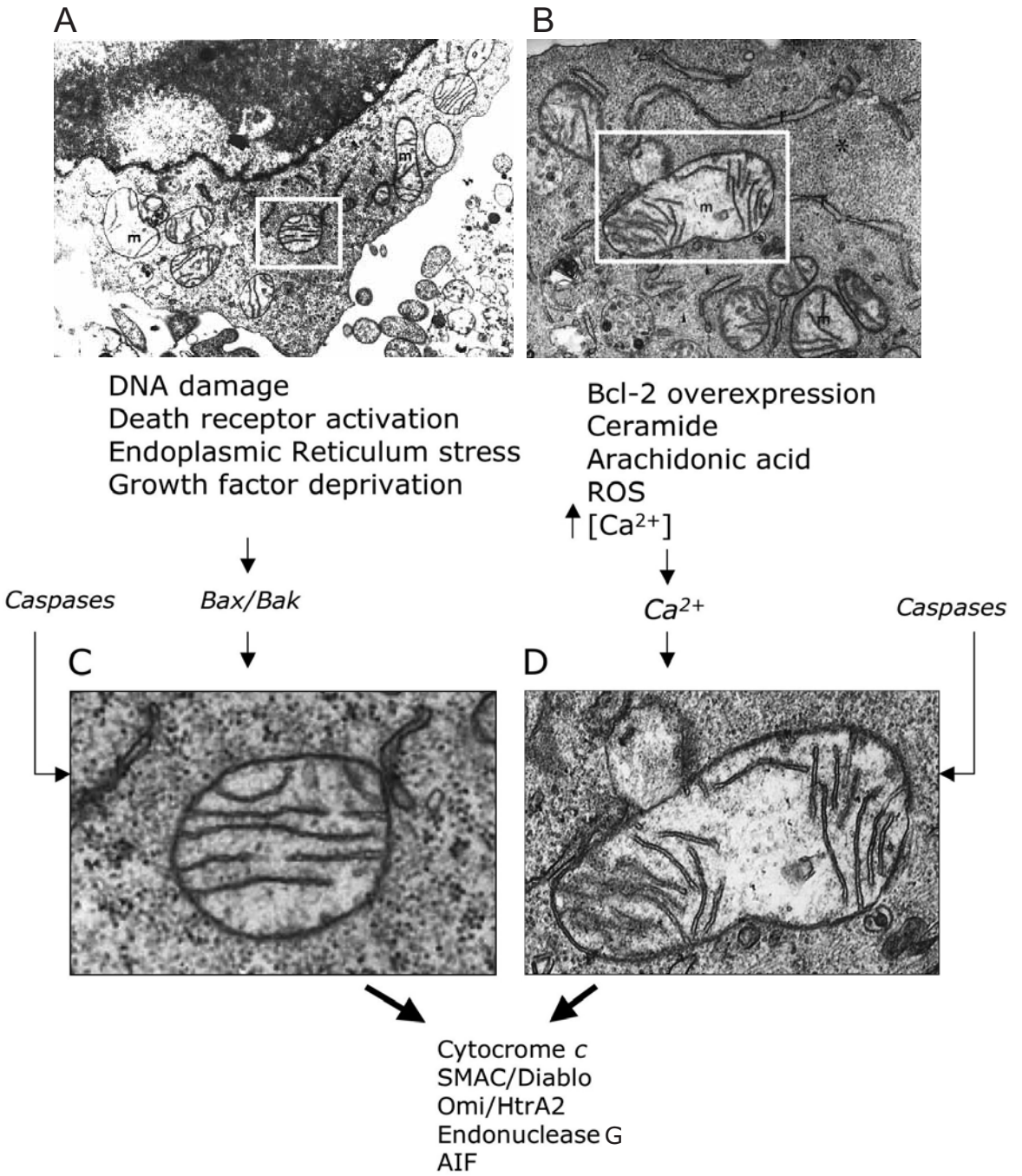

Figure 3. Electromicrographs of typical morphological changes in mitochondria in a WEHI-164 cell exposed to TNF-α for 20 h (100 ng/ml) (**A**) and for 4 h (10 ng/ml) (**B**). The images in **C** and **D** illustrate the classic ultrastructural changes in the matrix and outer and inner mitochondrial membranes in response to the agents indicated. The mitochondrion in **C** shows a mechanism in which the membrane permeabilization and release of apoptogenic factors do not cause ultrastructural changes. This pathway may require the participation of BAX and BAK. The mitochondria in **D** show a second mechanism in which a Ca^{2+} overload causes swelling of the matrix, permeabilization of the inner membrane and the passage of solutes, leading to expansion and rupture of the outer mitochondrial membrane. In either of these mechanisms, caspases 2, 3 and 7 [22,24] also contribute to amplification of the initial damage caused by low levels of ROS or Ca^{2+}. Cross-talk between these two mechanisms may be critical for the death of certain types of cells. (**A**) Bar = 0.5 µm; (**B**) Bar = 0.25 µm; (**C**) and (**D**) Bars = 0.1 µm.

CONCLUSIONS

Numerous studies have provided new insights into the molecular mechanisms by which several biomolecules, as well as biochemical and bioenergetic reactions inside and outside mitochondria can alter the permeability of the inner and outer mitochondrial membrane to allow the release of AIF, cytochrome *c*, Endo G, HtrA2/Omi and Smac/Diablo, all of which leave mitochondria with different transport mechanisms and kinetics. These proteins may reach the cytosol through one or more megapores or channels, the molecular nature remains to be fully determined. These "permeability transition pores" probably consist of inner and outer membrane proteins that include (i) VDAC and ANT, (ii) BAX proteins, (iii) tBID proteins and (iv) VDAC and BAX proteins (Fig. 2). There is also evidence that the release of apoptogenic factors occurs through mitochondrial apoptosis-inducing channels (MAC), large ceramide-based channels, and rupture of the outer mitochondrial membrane (Fig. 3). The regulatory co-factors and mechanism of operation of these multimolecular complexes have been extensively investigated, but are still not fully understood.

Pharmacological, genetic and biochemical data suggest that the formation and opening of these channels are controlled by classic mitochondrial permeability inducers (Ca^{2+} and ROS), as well as by some members of the caspase family (caspases -2, -3 and -7) and the pro-apoptotic proteins BAX, BAK and BID. Our understanding of the role of caspases, BAX and BID is based on experiments showing their direct effect on lipid membranes and isolated mitochondria. Caspase-2 and the cleaved forms of BAX and BID can directly permeabilize the mitochondrial outer membrane. Caspase-3 can enter mitochondria and cleave components of the electron transport chain such as the NDUF1 subunit of complex I. Several other mitochondrial channels and transporters for ions, metals and metabolites, as well as components of the Tim/Tom complexes, are also putative caspase substrates. Their cleavage may result in structural alterations that could make the channel/transporter nonselective or lead to their rupture and the formation of megapores with different mechanisms of releasing apoptogenic factors. The validity of this mechanistic model needs to be assessed experimentally. A better understanding of the operation of these transporters, channels and pores will contribute to the design of new drugs capable of amplifying or blocking the release of apoptogenic factors.

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