

REVIEW

Mitochondrial mechanisms of neural cell apoptosis

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Abstract

The importance of calcium overload, mitochondrial dysfunction, and free radical generation to neuropathological processes has been recognized for many years. Only more recently has evidence accumulated that the programmed cell death process of apoptosis plays an integral role not only in the development of the nervous system, but in the loss of cells following acute neurological insults and chronic disease. In 1996 came the landmark discovery that cytochrome *c*, an evolutionary old and essential component of the respiratory chain, has a second and deadly function when it escapes the mitochondrion: triggering the cell death cascade. A flurry of activity has since ensued in an effort to understand the

mechanistic events associated with mitochondrial permeabilization during apoptosis and regulation by an enigmatic family of proteins characterized by homology to the proto-oncogene Bcl-2. This review discusses the evidence for various release mechanisms of apoptotic proteins (e.g. cytochrome *c*) from neural cell mitochondria, focusing particularly on roles for calcium, Bax, p53, and oxidative stress. The need for new drugs that act at the level of the mitochondrion to prevent apoptosis is also highlighted.

Keywords: Bax, Bcl-2, cytochrome *c*, membrane permeability transition, oxidative stress, p53.

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Death pathways in neurodevelopment and degeneration

The significance of cytochrome *c* to the apoptotic process was revealed by the finding that mitochondrially released cytochrome *c* combines with apoptosis protease activating factor-1 (Apaf-1), procaspase-9, and dATP in the cytosol, producing active caspase-9 (Zou *et al.* 1997; Li *et al.* 1998). The activation of this initiator caspase then leads to the proteolytic activation of caspase-3, the primary effector caspase of the cell (Fig. 1). This pathway is referred to as the mitochondrial pathway or ‘intrinsic pathway’ of caspase activation, and often involves the release of additional mitochondrial proteins such as second mitochondrial-derived activator of caspase/direct IAP-associated binding protein with low PI (Smac/DIABLO) and HtrA2/Omi, which antagonize inhibitor of apoptosis (IAP) proteins, and apoptosis inducing factor (AIF) and Endonuclease G (EndoG), which contribute to DNA fragmentation. A second, ‘extrinsic’ pathway of caspase activation exists where the binding of a death ligand such as Fas to its death receptor triggers auto-processing of caspase-8 to its active form, which then directly activates caspase-3 (Muzio *et al.* 1996). The

pro-apoptotic Bcl-2 family protein Bid bridges the two pathways by translocating to mitochondria and releasing cytochrome *c* after truncation by caspase-8 (Fig. 2) (Li *et al.* 1998; Luo *et al.* 1998). The intrinsic pathway of cell death appears to be the primary mode of cell elimination during synaptic development (Chang *et al.* 2002) while both pathways can be recruited in neurodestructive processes, e.g. ischemic brain injury.

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Abbreviations used: AIF, apoptosis-inducing factor; ANT, adenine nucleotide translocase; BIP, Bax inhibitory peptide; DIABLO, direct IAP-associated binding protein with low PI; IAP, inhibitor of apoptosis protein; MAC, mitochondria apoptosis-induced channel; NGF, nerve growth factor; NMDA, N-methyl-D-aspartate; PARP-1, Poly(ADP) ribose polymerase-1; PTP, permeability transition pore; ROS, reactive oxygen species; Smac, second mitochondria-derived activator of caspase; TUDCA, tauroursodeoxycholic acid; VDAC, voltage-dependent anion channel.

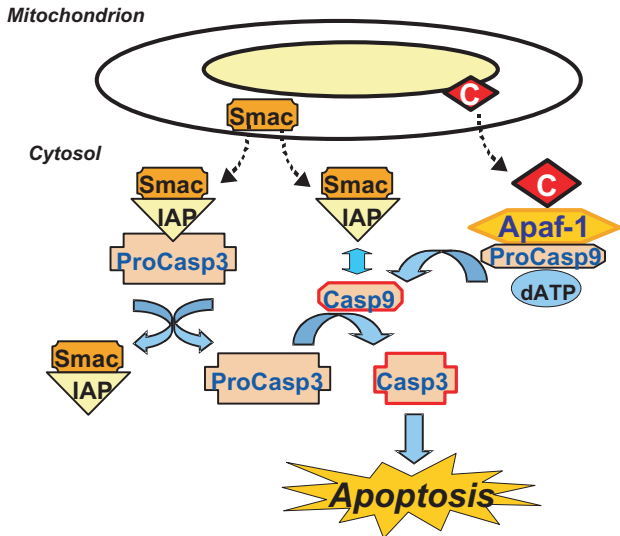


Fig. 1 Intrinsic pathway of caspase activation. Cytochrome *c* (C) released from mitochondria combines with apoptosis protease activating factor-1 (Apaf-1) and procaspase-9 (ProCasp9) in the cytosol to produce active caspase-9 (Casp9). Caspase-9 cleaves procaspase-3 to activate caspase-3 (Casp3) and initiates apoptosis. The release of Smac (Second Mitochondria-derived Activator of Caspase)/DIABLO (direct IAP-associated binding protein with Low PI) from mitochondria relieves caspase-inhibition by inhibitor of apoptosis proteins (IAP), allowing caspase activation by cytochrome *c*.

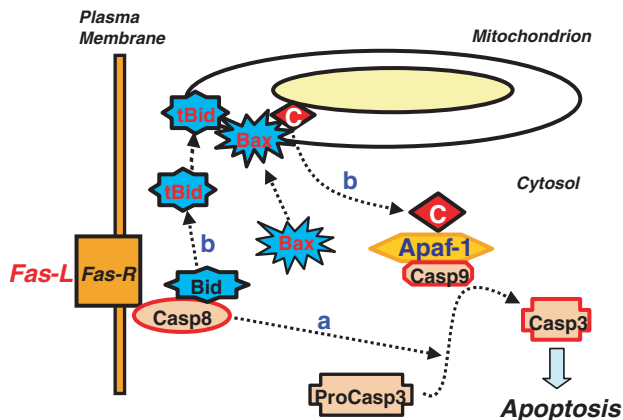


Fig. 2 Recruitment of the intrinsic mitochondrial apoptotic pathway following death receptor ligation by caspase-8 cleaved Bid. In the death receptor pathway (i.e. Fas), Fas ligand (Fas-L) binding to Fas receptor (Fas-R) activates caspase-8 (Casp8). In some cell types (Type I cells), caspase-8 activation is sufficient to cleave procaspase-3 (ProCasp3) to active caspase-3 (Casp3) and induce apoptosis (path a). However many cell types (Type II cells) require amplification of the caspase cascade by cytochrome *c* (C) release initiated by caspase-8 cleaved Bid (path b). Caspase-3 is proteolytically activated by the initiator caspase-9 (Casp9).

In addition to the well-characterized caspase-mediated intrinsic and extrinsic apoptotic pathways, mitochondrial AIF release mediates a caspase-independent cell death cascade that is also important in neurons. AIF translocation from mitochondria to the nucleus has been observed in photoreceptors following retinal detachment (Hisatomi *et al.* 2001), in rat cortex and hippocampus following traumatic brain injury (Zhang *et al.* 2002), in neonatal rat brain following hypoxia-ischemia (Zhu *et al.* 2003), in rat hippocampal CA1 neurons following transient global ischemia (Cao *et al.* 2003), in cortical neurons following N-methyl-D-aspartate (NMDA) treatment (Yu *et al.* 2002), peroxynitrite treatment (Zhang *et al.* 2002), or oxygen/glucose deprivation (Cao *et al.* 2003), and in primary neuronal and microglial cells following exposure to pneumococcus (Braun *et al.* 2001). Poly(ADP-ribose) polymerase-1 (PARP-1) inhibitors or genetic deletion of PARP-1 prevents AIF-mediated death of NMDA-treated cortical neurons, indicating that AIF release lies downstream of PARP-1 activation in this excitotoxicity model (Yu *et al.* 2002). Although caspase inhibitors had no effect on PARP-1 mediated cell death, AIF release in response to various apoptotic stimuli appears to be a separate event from cytochrome *c* release and requires caspase activation (Arnoult *et al.* 2002), suggesting that AIF release may be differentially regulated in caspase-independent versus caspase-dependent cell death. Interestingly, the recently described Harlequin mouse mutant has an 80% reduction in AIF expression yet displays progressive degeneration of terminally differentiated cerebellar and retinal neurons associated with oxidative stress (Klein *et al.* 2002). This finding is contrary to what would be expected if AIF functions solely as an apoptotic factor in the nervous system. Overexpression of AIF in cerebellar granule neurons was protective against peroxide-mediated cell death suggesting that, in addition to its pro-death function in the nucleus, AIF may also function as a free radical scavenger at the mitochondria (Klein *et al.* 2002).

Genetic lesion studies in mice and mouse embryos have provided strong evidence for the contribution of Bcl-2 family proteins to naturally occurring cell death during nervous system development. Pro-apoptotic Bax is required for the cell death program in many populations of neurons, including peripheral ganglia, motor neurons in the spinal cord, neurons in the trigeminal brainstem nuclear complex, and some neurons in the cerebellum, retina, and hippocampus (Deckwerth *et al.* 1996; White *et al.* 1998; Fan *et al.* 2001). Mouse embryos deficient in anti-apoptotic Bcl-X display massive cell death of immature neurons of the brain, spinal cord, and dorsal root ganglia that is largely rescued by codeletion of Bax, providing evidence that Bcl-X is an essential negative regulator of the Bax-mediated programmed cell death pathway *in vivo* (Motoyama *et al.* 1995; Shindler *et al.* 1997). *In vitro* experiments using wild type and Bax deficient cerebellar granule neurons

demonstrated involvement of the intrinsic pathway by showing that BH3-only protein-mediated Bax activation, cytochrome *c* release, and caspase activation was responsible for eliciting developmental-like apoptosis following withdrawal of depolarizing concentrations of potassium chloride (Harris & Johnson 2001).

Although brain mitochondria have been extensively investigated as a primary target of excitotoxic calcium loads and reactive oxygen species leading to acute energy failure, new evidence reveals that the BH3-only signal transduction cascade mediating programmed cell removal also contributes substantially to neural injury. The BH3-only protein Bid is cleaved during focal ischemia in a caspase-8 dependent fashion and Bid-deficient mice display a significant reduction in infarct volume that coincides with protection against cytochrome *c* release and impaired caspase-3 activation (Plesnila *et al.* 2001). Bad, another member of the BH3-only subgroup of Bcl-2 family proteins, regulates the threshold for mitochondrial cytochrome *c* release in neurons by inactivating anti-apoptotic Bcl-2 proteins (Datta *et al.* 2002). Although normally held in check by survival kinase-mediated phosphorylation, excitotoxic elevation of intraneuronal calcium can lead to Bad dephosphorylation and activation through the Ca²⁺-dependent phosphatase calcineurin (Wang *et al.* 1999). *In vivo* evidence suggests that Bad activation contributes to apoptotic death following traumatic spinal cord injury (Springer *et al.* 2000) and kainic acid-induced seizures (Henshall *et al.* 2002). Studies using double-knockout mice of Bax and its close relative Bak revealed that BH3-only proteins such as Bid require these multidomain Bcl-2 homologs for their cytochrome *c*-releasing and killing ability (Wei *et al.* 2001); however, the precise biochemical actions of these proteins has been a subject of considerable debate.

The mitochondrial permeability transition hypothesis

A phenomenon called the mitochondrial permeability transition was the initial mechanism proposed to mediate the release of mitochondrial intermembrane constituents. Identified as early as the 1960s, mitochondria undergo swelling following exposure to high calcium and oxidative stress. The swelling results from the opening of the mitochondrial permeability transition pore (PTP) in the inner membrane. Solute entry (≤ 1500 Da) precipitates water influx, leading to expansion of the matrix space within the highly convoluted inner membrane and dissipation of the electrochemical gradient. The comparatively rigid outer mitochondrial membrane eventually ruptures, releasing the contents of the intermembrane space including cytochrome *c*. Reconstitution of the inner membrane adenine nucleotide translocator (ANT) together with the matrix protein cyclophilin D and the outer membrane voltage-dependent anion channel (VDAC) in proteoliposomes mimics mitochondrial permeability transition pore activity observed in isolated

mitochondria (Crompton *et al.* 1998). Co-purification, co-immunoprecipitation, and yeast-two-hybrid screening have demonstrated an interaction of Bax with the ANT (Marzo *et al.* 1998b) and some investigators have found evidence supporting induction of the mitochondrial PTP as the mechanism for Bax-mediated apoptosis (Marzo *et al.* 1998a; Pastorino *et al.* 1998) although many find that Bax-induced cytochrome *c* release and apoptosis occurs in the absence of mitochondrial permeability transition (Eskes *et al.* 1998; Polster *et al.* 2003). New evidence suggests that Bax and Bak may contribute indirectly to mitochondrial permeability transition by controlling endoplasmic reticulum-to-mitochondria calcium signaling in some apoptotic paradigms (Scorrano *et al.* 2003; Zong *et al.* 2003).

PTP inhibitors, such as cyclosporine A and bongkrekic acid, have been used to investigate the involvement of the mitochondrial permeability transition in neural cell death. Although PTP inhibitors are protective in some models (Budd *et al.* 2000; Cao *et al.* 2001), in most cases inhibition of cytochrome *c* release was not demonstrated and non-PTP effects were not excluded, such as protective general inhibition of protein synthesis (Wigdal *et al.* 2002). Detailed analysis from our lab and others using both biochemical and imaging techniques have excluded a role for the PTP in the mechanism of Bax-induced cytochrome *c* release from isolated brain mitochondria (Brustovetsky *et al.* 2003; Polster *et al.* 2003). Nevertheless, the PTP may still play a role in neural cell death, perhaps by signaling Bax translocation to mitochondria (De Giorgi *et al.* 2002), participating in intracellular Ca²⁺ or ROS signaling, or regulating the release of larger mitochondrial apoptogenic proteins that occurs downstream of cytochrome *c* release. Recently it was shown that both Bax and Bak are dispensible for NMDA-induced death of cerebellar granule neurons (Lindsten *et al.* 2003), perhaps suggesting a comparatively greater role for calcium in forms of neuronal death that are not purely apoptotic. However, whether PTP is a cause or the result of irreversible excitotoxic neuronal injury is, at this juncture, unclear.

The outer membrane channel hypothesis

As an alternative to non-selective mitochondrial outer membrane permeabilization due to membrane breakage, it is possible that a discrete outer membrane protein-conducting channel forms to allow cytochrome *c* and its death-promoting cohorts to escape the intermembrane space during apoptosis. One proposal is that Bax combines with the outer membrane, non-selective metabolite channel VDAC to increase its permeability or form hybrid channels that are large enough to flux cytochrome *c*. Bax and VDAC but neither protein alone was capable of passing cytochrome *c* across planar lipid bilayers (Shimizu *et al.* 1999). Also, Bax was co-immunoprecipitated with VDAC, and antibodies that blocked ¹⁴C-sucrose uptake by VDAC-containing liposomes also blocked mammalian apoptosis (Shimizu *et al.* 1999;

Shimizu *et al.* 2001). However, although Tsujimoto and colleagues first proposed that Bax and Bak mediate cytochrome *c* release by modulating the conductance state of VDAC in 1999, independent studies have yet to reproduce their findings. Conflicting results, e.g. the findings that Bax expression in VDAC-deficient yeast induces large conductance mitochondrial channels and cytochrome *c* release (Pavlov *et al.* 2001), lead many to favor the hypothesis that Bax does not require native mitochondrial proteins for outer membrane permeabilization.

N-terminal epitope-specific Bax antibodies and trypsin digestion patterns were used to demonstrate that active Bid can induce a conformation change in Bax or Bak (Desagher *et al.* 1999; Wei *et al.* 2000) that precedes the formation of higher order complexes (Eskes *et al.* 2000). Associated with this conformational change was a shift in mitochondrially associated Bax from a loosely bound, alkali-extractable form to an alkali-resistant form that is characteristic of integral membrane proteins. The formation of oligomers is required for the ability of Bax to release entrapped molecules from liposomes in cell-free assays (Antonsson *et al.* 2000) and endogenous high molecular weight Bax oligomer/complexes have been found in mitochondrial membranes of apoptotic cells (Antonsson *et al.* 2001) supporting a physiological role for Bax multimerization. Inhibition of cytochrome *c* efflux by size-specific dextrans from protein-free liposomes treated with Bax estimated a Bax pore size of ~ 30 Angstroms that was consistent with a Bax tetramer (Saito *et al.* 2000). However, in contrast to larger Bax complexes that have been detected within cells (Antonsson *et al.* 2001; Nechushtan *et al.* 2001), proteinaceous channels formed by more than four Bax molecules could not be detected in this liposomal system. Furthermore, the predicated size of tetrameric Bax channels was insufficient to account for the release of large mitochondrial proteins observed during apoptosis, although there is also now evidence that the release of several mitochondrial intermembrane space proteins (e.g. Smac/DIABLO and AIF) occurs by more than one mechanism, or is at least differentially regulated by various pro-apoptotic proteins (Adrain *et al.* 2001; Arnoult *et al.* 2002; Kandasamy *et al.* 2003).

The lipid pore hypothesis

A substantial shift in the thinking in the field occurred when investigators began considering a role for mitochondrial lipids in the permeabilization process. The hypothesis of Bax-induced lipid pore formation was first proposed but largely ignored in 1999, when Basañez and co-workers found that Bax decreased planar phospholipid bilayer stability and diminished membrane linear tension, corresponding to a lowered energy requirement for the formation of a lipidic pore (Basanez *et al.* 1999). Newmeyer's group recently revisited this hypothesis by demonstrating that mitochondrial lipids, particularly cardiolipin, are essential for Bax-induced

permeabilization of vesicles and showing remarkably that 2000 kilodalton dextran molecules could be released from liposomes without visible changes in structure (Kuwana *et al.* 2002). However, they were not able to exclude the possibility that the liposome membrane resealed during the process of preparation for electron microscopy. Additional support for a lipid pore-mediated mechanism of Bax permeabilization came from observations that altering intrinsic membrane curvature in lipid vesicles can inhibit or promote Bax activity (Basanez *et al.* 2002) and that amphiphilic cations that interact strongly with mitochondrial lipids can prevent Bax-induced permeabilization of isolated mitochondria or liposomes without affecting the ability of Bax to integrate into membranes (Polster *et al.* 2003). Atomic force microscopy was used to directly visualize a toroidal-shaped pore formed by oligomeric Bax in synthetic lipid bilayers, demonstrating proof-of-concept in an artificial system (Epanand *et al.* 2002). Assessing the ability of dibucaine and propranolol to inhibit MAC (mitochondria apoptosis-induced channel), a novel high conductance channel that was electrophysiologically characterized on mitochondrial outer membranes of apoptotic cells (Pavlov *et al.* 2001), will aid in bridging the gap between events detected in isolated systems and intact cells. Intriguing new observations that Bax and Bak colocalize with proteins involved in mitochondrial fission and fusion at mitochondrial constriction sites in apoptotic cells suggest a role for regulation of lipid dynamics *in vivo* (Karbowski *et al.* 2002). Antagonism of the mitochondrial fission protein Drp1 inhibits Bax-mediated cytochrome *c* release and apoptosis in cells (Frank *et al.* 2001) by a process that is still unknown and may prove difficult to address in simplified systems, e.g. isolated mitochondria or liposomes.

Regulation by p53

Induction of the transcriptional activating factor p53 by oxidative stress, hypoxia, etc. results in apoptosis mediated by the intrinsic (mitochondrial) pathway (Miller *et al.* 2000). p53 stimulates the expression of several Bcl-2 family genes including Bax and multiple BH3-only proteins, e.g. Bid, Noxa, and PUMA (Sax and El Deiry 2003). In addition to elevating the levels of proteins that mediate release of cytochrome *c*, p53 stimulates apoptosis by a transcription-independent pathway (Caelles *et al.* 1994). The molecular mechanism by which p53 activates apoptosis independent of transcription apparently involves the direct binding of p53 to one or more anti-apoptotic mitochondrial proteins, e.g. Bcl-X_L, thereby inhibiting their ability to suppress Bax- or Bak-mediated pore formation and cytochrome *c* release (Fig. 3) (Chipuk *et al.* 2003; Mihara *et al.* 2003). The ability of other nuclear factors such as TR3/nur77 and histone H1.2 to directly stimulate cytochrome *c* release from mitochondria has also been described (Li *et al.* 2000; Konishi *et al.* 2003), indicating that nuclear-to-mitochondrial signaling may be a

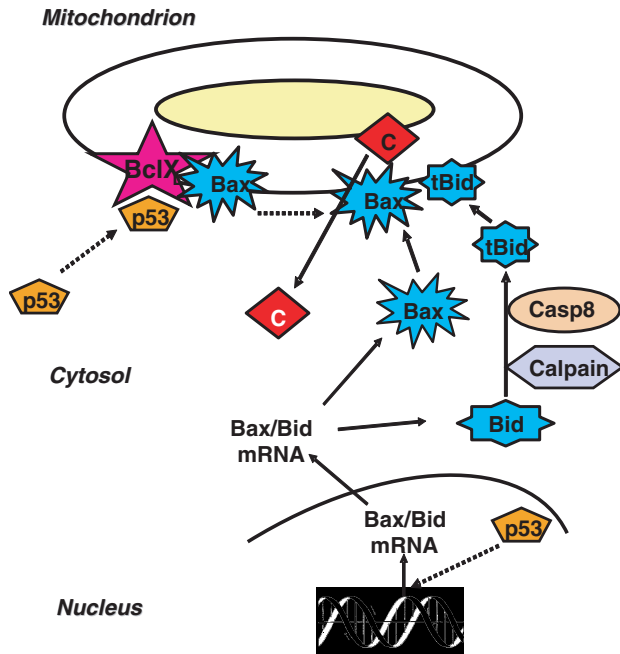


Fig. 3 Regulation of the intrinsic mitochondrial apoptotic pathway by p53. Various stressful stimuli associated with neurologic disorders, e.g. oxidative stress, stimulate the expression of p53. This gene product transcriptionally activates several pro-apoptotic genes, including Bax, Bid, Noxa, and PUMA. In addition, p53 stimulates apoptosis by a non-transcriptional mechanism involving direct binding to anti-apoptotic mitochondrial proteins, e.g. Bcl-X_L, inhibiting their ability to block outer membrane pore formation by Bax and Bak.

common theme in apoptotic death. However the importance of these nuclear instigators of the mitochondrial death pathway has yet to be investigated in neural cells.

Role of reactive oxygen species

A significant rise in reactive oxygen species (ROS) occurs in both apoptotic and necrotic neural cell death. Examination of Bax deficient and Bax heterozygous mouse sympathetic neurons revealed that the amount of ROS produced subsequent to nerve growth factor (NGF) withdrawal directly correlates with Bax gene dosage (Kirkland *et al.* 2002). In Bax ^{-/-} neurons, no elevation of ROS occurred and cytochrome *c* release in response to growth factor deprivation was completely suppressed. The complex I inhibitor rotenone abrogated the increase in ROS, indicating a mitochondrial source for the reactive oxygen species.

Cai and Jones initially proposed that intracellular redistribution of cytochrome *c* is responsible for the oxidative stress that often accompanies apoptosis (Cai and Jones 1998). We have found that Bax-induced release of cytochrome *c* from isolated brain mitochondria causes increased production of ROS (Starkov *et al.* 2002), likely via stimulation of superoxide production at Complex I of the electron transport chain (Kushnareva *et al.* 2002). This observation supports the

hypothesis that Bax-dependent mitochondrial ROS generation lies downstream of cytochrome *c* release. This concept is challenged by measurements of the timing of stimulated ROS production in mouse sympathetic neurons, which was found to precede the release of cytochrome *c*, as measured by immunofluorescence (Kirkland *et al.* 2002). However, evidence in rat sympathetic neurons suggests that partial cytochrome *c* release may be detectable by subcellular fractionation and immunoblotting earlier than when the change in immunostaining pattern becomes apparent (Martinou *et al.* 1999). Lipid peroxidation, particularly of cardiolipin, can cause dissociation of cytochrome *c* from its electrostatic interaction with the inner mitochondrial membrane (Ott *et al.* 2002). Thus, release of the intermembrane soluble pool of cytochrome *c* by Bax could potentially lead to ROS generation that would then enhance cytochrome *c* release, further enhancing the production of ROS. Activated caspase-3 was also reported to enhance mitochondrial ROS downstream of cytochrome *c* release by damaging complexes I and II (Ricci *et al.* 2003). Although the mechanism of mitochondrial ROS generation by Bax is not clear, a membrane-permeant form of reduced glutathione was able to limit the ROS burst and cytochrome *c* release in Bax^{+/+} neurons deprived of NGF (Kirkland *et al.* 2002), pointing to an important role of ROS and possibly lipid or protein oxidation in the mitochondrial permeabilization process.

Neuroprotective interventions

As the apoptotic pathway has become better defined and its contribution to neurodegenerative disorders irrefutable, the development of anti-apoptotic therapeutics has predictably become a focus of investigation. Suppression of apoptosis at the level of caspases via caspase inhibitors is one approach that has been actively explored with modest success (Bilsland and Harper 2002). A factor complicating this strategy, however, is that mitochondrial dysfunction often occurs even in the presence of caspase inhibition, leading to an impairment in ATP production and an increase in the generation of reactive oxygen species (Chang *et al.* 2002). Thus, caspase inhibitors may merely delay cell death. Alternatively, neurons surviving injury in the presence of caspase inhibitors may still be functionally impaired and contribute to poor neurologic outcome.

In contrast to the inhibition of caspases, genetic deletion of Bax and Bak confers long-term resistance to apoptosis in cultured cells and prevents upstream apoptotic changes in mitochondria, e.g. loss of apoptogenic factors and membrane potential (Wei *et al.* 2001). Thus, the challenge is to develop or identify pharmacologic agents capable of inhibiting apoptosis at steps upstream of Bax and Bak-induced mitochondrial changes (Fig. 4). In neurons, the relocalization of Bax from the cytosol to the mitochondria represents one of the first steps in programmed cell death (Putcha *et al.* 1999). Although Bax has an N-terminal mitochondrial targeting

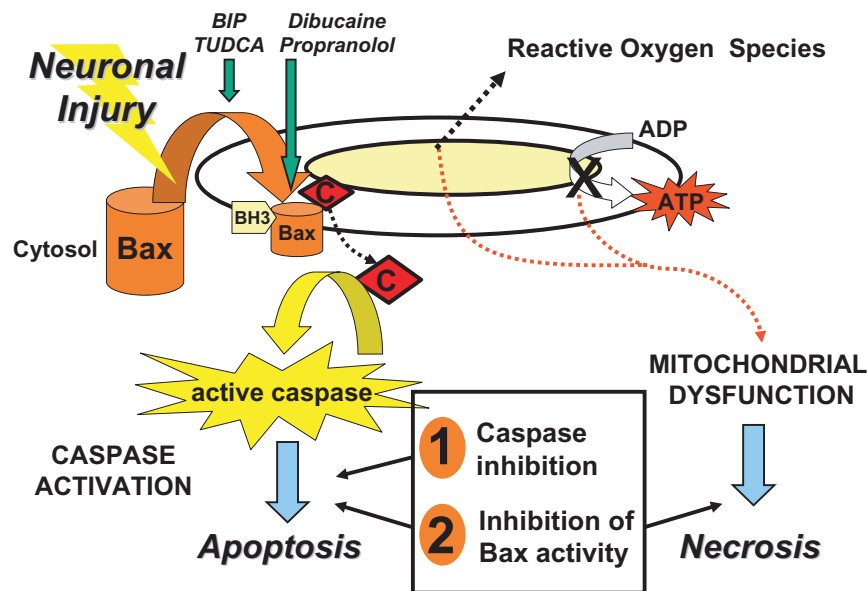


Fig. 4 Neuroprotection strategies targeting the intrinsic pathway of apoptosis. Until recently, the development of caspase inhibitors that cross the blood–brain barrier has been the major focus of neuroprotective studies that target apoptosis. However, because mitochondrial damage occurring upstream of caspase activation can lead to necrotic cell death due to oxidative stress and energy failure, inhibition of Bax

function at the mitochondria is potentially a more effective therapeutic strategy. Bax-inhibiting Peptide (BIP) and tauroursodeoxycholic acid (TUDCA) prevent Bax translocation and association with mitochondria, while dibucaine and propranolol inhibit Bax-induced cytochrome *c* release downstream of Bax membrane insertion.

signal (Cartron *et al.* 2003), in healthy cells it is largely retained in the cytosol either by binding to a 24 amino acid anti-apoptotic peptide called humanin (Guo *et al.* 2003), or through an interaction with the DNA repair protein Ku70 (Sawada *et al.* 2003b). A cell permeable five amino acid peptide BIP (Bax-inhibiting peptide) designed from the Bax binding domain of Ku70 protected cells against Bax-dependent apoptotic stimuli in multiple cell types (Sawada *et al.* 2003a), demonstrating the possibility of inhibiting Bax-induced death at the initial stage of activation. Tauroursodeoxycholic acid limits Bax association with mitochondria (Rodrigues *et al.* 2003) while dibucaine and propranolol inhibit Bax-induced cytochrome *c* release downstream of Bax mitochondrial insertion (Polster *et al.* 2003), showing promise for the development of drugs that interfere with the membrane permeabilizing activity of Bax.

In contrast to Bax, Bak is constitutively present at mitochondria in healthy cells, and although Bax and Bak are often considered to have redundant pro-death functions, recent experiments with isolated rat brain mitochondria implicate distinct mechanisms in the release of cytochrome *c* (Brustovetsky *et al.* 2003). VDAC2 was recently shown to inhibit mitochondrial outer membrane permeabilization by Bak but not Bax (Cheng *et al.* 2003), while humanin and Ku70 appear to be specific Bax antagonists (Guo *et al.* 2003; Sawada *et al.* 2003b), demonstrating distinct mechanisms of regulation as well. Calcium-induced permeability transition

is also likely to contribute to neuronal cell death under many circumstances and new permeability transition pore inhibitors are being developed (Walter *et al.* 2000; Cesura *et al.* 2003; Chinopoulos *et al.* 2003). Thus, therapeutics focused solely on suppressing Bax translocation or function may display incomplete efficacy due to the existence of alternate, parallel, or compensatory death pathways.

Bcl-2 and Bcl-X_L are two prominent native inhibitors of apoptosis and Bax and Bak function. Early investigations focused on the ability of Bcl-2 to regulate inner mitochondrial permeability transition, cellular redox state, and oxidative damage-induced cell death (Hockenbery *et al.* 1993; Susin *et al.* 1996; Kowaltowski *et al.* 2000). This approach was followed by research on binding interactions between Bcl-2 or Bcl-X_L and Bax/Bak or BH3-only proteins, and regulation of mitochondrial outer membrane permeability (Wei *et al.* 2001). The ability of Bcl-2 and Bcl-X_L to strengthen antioxidant defenses, to inhibit mitochondrial permeability transition, and to inhibit permeability transition pore-independent cytochrome *c* release makes these proteins attractive multitarget neuroprotective candidates. Early attempts to deliver the Bcl-X_L protein directly to the brain via fusion to a protein transduction-conferring TAT peptide have been fruitful, with several studies demonstrating protection in rodent ischemic injury models (Cao *et al.* 2002; Kilic *et al.* 2002). Because of the multifaceted nature of cell death pathways in the nervous system, it is likely that a cocktail of

therapeutics will ultimately prove most effective in treating both acute and chronic neurodegenerative disorders.

Conclusions

Although mitochondrial inner membrane permeability transition contributes to acute necrotic neural cell death, the selective permeabilization of the outer membrane caused by Bax and other Bcl-2 family proteins is likely to play a more causative role in apoptosis. Mitochondrial lipids are thought to play an important role in the outer membrane permeabilization process, although the nature of Bax- or Bak-induced protein or lipid pores in intact cells remains to be defined. Adding to the complexity, it is now apparent that mitochondrial apoptotic proteins are not always released in synchrony, for instance, Smac/DIABLO and AIF release are sometimes seen only after caspase activation has occurred downstream of cytochrome *c* redistribution. Thus, although considerable progress has been made toward unravelling the mechanism(s) of release of apoptotic proteins from mitochondria during programmed cell death, our understanding of the process is far from complete. Various mechanisms discussed in this review may be recruited by different stimuli or in different neural cell types, and undoubtedly additional players remain to be discovered. Further characterization of events that occur at mitochondrial membranes during apoptosis will provide novel targets for upstream therapeutic interventions in the apoptotic pathway that may prove effective in the treatment of both acute neurologic disorders and chronic neurodegenerative diseases.

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