

**TITLE**

**Mitochondrial Mechanisms of Neural Cell Death in Cerebral Ischemia**

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## **A. Cell death following cerebral ischemia and reperfusion**

Millions die annually from ischemic brain damage caused by stroke, subarachnoid hemorrhage, head trauma, shock, and post-ischemic injury following resuscitation from cardiac arrest. Many thousands of others either die or suffer permanent neurologic impairment following surgical procedures that are at risk for inducing cerebral hypoxia/ischemia. Neurologic morbidity and mortality are primarily the consequences of necrotic, apoptotic, and other forms of neuronal and non-neuronal cell death. The most effective neuroprotective interventions must therefore target mechanisms that contribute to each of these pathways.

Traditionally, brain cell death following cerebral ischemia has been considered to be primarily necrotic in nature. More recent research revealed, however, that the pattern of cell death in the post-ischemic brain is much more complex. Based on morphology alone, both necrotic and apoptotic death mechanisms, and according to recent studies autophagy, seem to contribute to some extent to the demise of injured cells. The extent of contribution of each of these mechanisms depends on the intensity and type of injury, whether it is due to focal or transient global cerebral ischemia, the brain region(s) affected, time post-injury, etc. While necrosis predominates in the center of a focal ischemic lesion (ischemic core) and can occur early (within minutes), cells with apoptotic morphology are generally reported in the penumbral region and peak at later stages (days, even weeks). In contrast, initiation of apoptotic molecular pathways has been observed within 30 min of reperfusion after global cerebral ischemia caused by cardiac arrest, even though most neurons that are dead or dying 24 hr later appear necrotic. A similar course of events has been described for the core region of ischemic strokes. It has been noted, however, that in the post-ischemic brain, dying neurons often assume morphologies that do not reflect purely apoptotic or necrotic characteristic features, but incomplete (apoptotic- or necrotic-like), or mixed phenotypes. Accordingly, some studies have proposed the existence of a necrosis-apoptosis continuum. Multiple cell death mechanisms can be activated in the injured cells, and in some models even within the same cells. The phenotype acquired by the dying cells reflects thus the degree of completion of these death pathways (i.e. availability of ATP is required for completion of apoptosis). It has been suggested that a process of secondary necrosis results from

rapid failure to fully develop the apoptotic pathways due to rapid depletion of energy stores. Such shifts from apoptosis to necrosis have also been demonstrated in *in vitro* models, and many studies begin to reveal the existence of extensive cross-communication between various cell death pathways. While necrosis has been considered for long time just as an accidental, unregulated form of cell death, there is now substantial evidence indicating that similar to other death pathways (i.e. apoptosis), execution of at least certain forms of necrosis is also highly regulated. Several specific mediators of necrotic death have been characterized, including RIP1 kinase induced by DR ligands, PARP-1 overactivation and multiple mitochondrial alterations (i.e., MPT, ROS production; see below) (Fig. 1). The term “necroptosis” has been proposed to distinguish the regulated necrotic death from accidental necrosis. A central role in coordinating many of the cell death pathways implicated in ischemia/reperfusion is held by mitochondria.

## **B. Mitochondria mediate both necrotic and apoptotic cell death**

Mitochondria have long been considered as subcellular targets of ischemic brain injury. The significance of ischemic mitochondrial injury was initially thought to be limited to the effects on maintaining sufficient cellular ATP levels to avoid necrotic cell death. Mitochondria were subsequently characterized as a major source of toxic reactive oxygen species (ROS), contributing to acute excitotoxic neuronal death in response to elevated intracellular  $\text{Ca}^{2+}$ . Mitochondria are also responsible for executing early steps in apoptosis, through their release of proteins, e.g., cytochrome c and apoptosis initiating factor (AIF), that acquire toxic functions in the cytosol or nucleus (Fig. 1). In general, relatively mild mitochondrial injury results primarily in apoptotic cell death, whereas more extensive injury leads to necrosis as a result of metabolic failure.

Excessive neuronal accumulation of  $\text{Ca}^{2+}$  that occurs during excitotoxic stimulation and during ischemia/reperfusion is not the only mediator of mitochondrial injury. Mitochondria are highly sensitive targets of reactive oxygen species (ROS) generated by several systems, including the mitochondrial electron transport chain (ETC), cyclooxygenases,  $\text{Fe}^{2+}$ -catalyzed formation of hydroxyl radicals from hydrogen peroxide, NAD(P)H oxidases, and peroxynitrite formed from the reaction of nitric oxide with

superoxide. These species and the influence that their metabolism has on mitochondrial redox state result in oxidative modification of proteins, DNA, RNA, and membrane lipids. Any of these modifications can affect the rate or energy coupling efficiency of oxidative phosphorylation. Oxidation of protein amino acids, including that due to tyrosine nitration and cysteine S-nitrosylation, is particularly prevalent during ischemia/reperfusion and appears responsible for inhibition of energy transduction both at the level of matrix enzymes, e.g., pyruvate dehydrogenase, and at the level of the electron transport chain.

### **C. Mitochondrial permeability transition activated by $\text{Ca}^{2+}$ and oxidative stress**

The combined exposure of mitochondria to high  $\text{Ca}^{2+}$  plus oxidative stress is far more toxic than either stress alone. The primary target for this pathologic synergism is the inner membrane permeability transition pore (PTP), which is responsible for initiating the mitochondrial permeability transition (MPT). The MPT is defined as a relatively non-specific increase in inner membrane permeability to solutes  $\leq 1500$  Da that results in osmotic swelling of the mitochondrial matrix volume and collapse of the mitochondrial membrane potential. In addition to uncoupling oxidative phosphorylation, PTP opening allows for release of critical small molecules from the matrix, including NAD(P)H and glutathione. Moreover, high amplitude swelling results in osmotic lysis of the relatively inflexible outer membrane, releasing cytochrome c, a peripheral membrane protein component of the respiratory chain, into the cytosol. Mitochondria that undergo these extreme consequences of PTP opening are irreversibly damaged and only contribute to ATP hydrolysis, rather than ATP production. Depending on the tissue or cell type, PTP opening is inhibited at least to some degree by the presence of the immunosuppressive agent cyclosporin A (CsA). The molecular identification of the PTP is still controversial and may consist of proteins associated with the adenine nucleotide translocase and (or) the phosphate/hydroxyl ion exchanger. There is a general consensus, however, that cyclophilin D is necessary for full PTP activation and is the target of inhibition by CsA. The MPT is an attractive hypothesis as a primary mechanism underlying acute neural injury due to its activation by factors known to be associated with cerebral ischemia and because of its sensitivity to inhibition by CsA and other drugs demonstrated to be neuroprotective in some ischemic brain injury paradigms. While CsA

and other drugs that either interfere with, or compensate for mitochondrial energy failure may represent strategically sound attempts at inhibiting necrotic cell death caused by ischemia and reperfusion, recognition that apoptotic molecular pathways also contribute to ischemic neurodegeneration has led to alternative mitochondria-targeted anti-apoptotic interventions.

#### **D. Mitochondrial mechanisms of apoptotic death after cerebral ischemia**

##### *1. Mitochondrial apoptotic pathways*

Progress in the study of programmed cell death and apoptosis over the last two decades led to the unexpected discovery that in addition to their bioenergetic and metabolic roles, mitochondria are also the central regulators of the intrinsic (mitochondrial) apoptotic pathway. In some cells (classified as Type II cells), including neurons, mitochondria also play a major role in the extrinsic (death receptor (DR)) apoptotic pathway that is induced by DR ligands such as TNF- $\alpha$  or FasL.

The current understanding of mitochondrial involvement in apoptosis indicates that the key event in this process is the permeabilization of the outer mitochondrial membrane (OMM) and release of apoptogenic proteins from the mitochondrial intermembrane space into the cytosol, leading to cell demise in a caspase-dependent or -independent manner. The best characterized apoptogenic proteins include cytochrome C (Cyt C), apoptosis-inducing factor (AIF), Smac/DIABLO, endonuclease G (EndoG) and HtrA2/Omi. The strategic positioning of mitochondria at the intersection of cell death and survival pathways is highlighted by the dual role (with respect to cell death and survival), of many apoptogenic proteins. In addition to the classic example of Cyt C, other apoptogenic proteins initially identified as death inducers when released into the cytosol (i.e., AIF and HtrA2/Omi) were also found to promote neuronal survival and resistance to oxidative stress at their mitochondrial location.

The release of apoptogenic proteins from mitochondria can occur through two distinct mechanisms involving either selective OMM permeabilization by protein/proteo-lipidic pores, or non-specific (mechanical) rupture of the OMM. The most important regulators of the intrinsic pathway of apoptosis are the Bcl-2 family proteins that regulate primarily OMM permeability, although other extra-

mitochondrial mechanisms (i.e. ER-related) are also described. Selective permeabilization of the OMM is triggered by the BH3-only subgroup of Bcl-2 proteins (e.g. Bid, Bim, Bad, Noxa, Puma etc.). Specific stimuli or non-specific cell stress can induce activation of constitutively expressed BH3-only proteins (BOP) (e.g. Bid, Bim, Bad) and/or trigger the expression of several additional BH3 only-proteins (i.e., Noxa, Puma) all of which translocate to mitochondria. The activity of BH3-only proteins at mitochondria results in oligomerization of multidomain pro-apoptotic Bcl-2 proteins (e.g. Bax/Bak) and permeabilization of the OMM through pore formation. This selective OMM permeabilization occurs without loss of the inner mitochondrial membrane (IMM) integrity and is antagonized by Bcl-2-like anti-apoptotic members (Bcl-2, Bcl-x<sub>L</sub>, Bcl-w, and Mcl-1).

Another mechanism of release of apoptogenic proteins involves non-selective rupture of the OMM. Most commonly this occurs following PTP opening, osmotic swelling of mitochondria and physical rupture of the OMM. Although hallmarks of MPT, i.e. loss of IMM potential ( $\Delta\Psi$ ), swelling of mitochondria, and protection by CsA, are observed in some cases in dying cells that display classic apoptotic morphology, the role of MPT in apoptosis has been controversial. MPT was proposed initially as a universal mechanism of mitochondrial apoptotic death accounting for the release of apoptogenic proteins. Recent studies using cells from cyclophilin D deficient mice demonstrate that MPT is not required for apoptosis. In contrast, cyclophilin D deficiency protects cells against oxidative stress-induced necrotic death. Regardless of its requirement for apoptosis, the importance of the MPT mechanism in pathologic neuronal death is highlighted by recent findings that animals deficient in cyclophilin D display a marked resistance to ischemic brain injury.

The involvement of mitochondria-dependent death pathways in brain ischemia has been demonstrated in both focal and global ischemia using small and large animal models, and evidence for their activation has also been found in humans. While both pathways appear to play critical roles in ischemia/reperfusion-induced neuronal death, their relative contribution varies greatly depending on the model (focal/global), brain region (cortex vs. hippocampus), age (immature vs. mature) and gender. Such results highlight the need for a better understanding at a molecular level and improved recognition in a clinical context of potentially distinct phenotypes of ischemic injury that are age or gender-

dependent. Similarly, the data support further development of targeted therapies for ischemic injury (i.e. specific for the immature vs. adult brain and gender-specific). Critical regulators of the mitochondrial-apoptotic pathway in ischemic brain injury are discussed below.

## *2. Bcl-2 family proteins*

Bcl-2 family proteins regulate cell death pathways by acting at the mitochondrial level where they control the release of death-inducing proteins from the mitochondrial intermembrane space (IMS) to the cytosol through regulation of the OMM permeability. Recent studies indicate that Bcl-2 family proteins also affect this process indirectly through their actions at other intracellular locations, i.e. at the endoplasmic reticulum level by regulating  $Ca^{2+}$  fluxes. Numerous studies document a role of for both pro- and anti-apoptotic Bcl-2 family proteins in neuronal injury and survival in pathologic conditions such as ischemia.

The Bcl-2-like proteins protect neurons and other cell types against a wide variety of apoptotic insults. Although labeled as “anti-apoptotic”, these proteins (e.g., Bcl-2, Bcl-X<sub>L</sub>) can also protect against necrotic cell death. At least for Bcl-2 a role in the regulation of cell death associated with autophagy (also termed autophagic cell death), through its interaction with the autophagy regulator protein beclin-1 is also documented. This type of cell death, characterized morphologically by the lack of chromatin condensation and presence of massive autophagic vacuolization of the cytoplasm has been also shown to contribute to cell death following ischemic brain injury. Early studies indicated that overexpression of Bcl-2 exerts a powerful neuroprotective activity in cerebral ischemia as well as in other models of acute or chronic brain injury. Subsequent studies reported similar neuroprotective properties against ischemic injury for several other anti-apoptotic Bcl-2 proteins, including Bcl-x<sub>L</sub>, Mcl-1 and Bcl-w (**Table 1**). The strong neuroprotective effect of Bcl-2 likely results from to its multiple pro-survival activities and is especially important in brain ischemia where multiple neuronal death types (apoptotic, necrotic and autophagic) are frequently observed.

Although not completely understood, the antiapoptotic activity of Bcl-2 and related proteins is in part due to their ability to heterodimerize with Bax/Bak-type (multidomain) proapoptotic proteins and/or to sequester BH3-only proteins. According to the “rheostat” model proposed by the late Stanley Korsmeyer, Bcl-2-like anti-apoptotic proteins bind and neutralize pro-apoptotic Bcl-2 proteins, and their relative balance determines cell death or survival. BH3-only proteins display distinct binding affinities for individual Bcl-2-like proteins and their killing efficiency correlates with their ability to target and inactivate multiple pro-survival proteins.

In addition to the Bax/Bak neutralizing activity, the anti-apoptotic and anti-necrotic activity of Bcl-2-like proteins involves regulation of other mitochondrial processes, including mitochondrial  $\text{Ca}^{2+}$  uptake capacity, MPT, and redox state. Studies on Bcl-2, Bcl-X<sub>L</sub> and Mcl-1, indicate that their cytoprotective activities are in part due to increased protection against oxidative stress via elevating the expression of enzymes actively involved in the defense against ROS. This effect may involve modulation of transcription factor activity and subsequent expression of antioxidant enzymes. It now appears that upregulation of the antioxidant defense systems by Bcl-2 is a “preconditioning” response to the elevation of mitochondrial ROS production by increased Bcl-2 expression. This response may explain Bcl-2 protection against death caused by oxidative stress throughout the cell, including at the mitochondrial level where Bcl-2 protects against  $\text{Ca}^{2+}$  and peroxide-induced MPT activation. This “antioxidant” component of the anti-death Bcl-2 proteins may be particularly important in acute ischemic injury where excitotoxicity and oxidative stress play major roles in neuronal cell death.

The role of multidomain pro-apoptotic protein Bax has been extensively studied and there is now strong evidence for involvement of Bax in neuronal death following cerebral ischemia both in the adult and in the immature brain. The mechanism of Bax activation has been the subject of intense studies in recent years. Overall, Bax appears as a “molecule on the edge” that can be potentially activated by a wide array of stimuli, either through a specific/instructive pathway involving distinct molecular interactions at multiple levels, or through other less specific pathways involving various physico-chemical changes of Bax. Both pathways involve changes in the inactive monomeric Bax conformation (similar to an “unfolding” process) and generation of active Bax molecules (aBax; i.e. with an exposed

N- or C-terminal domain). Once generated, aBax molecules are either bound and blocked by Bcl-2-like proteins (in surviving cells) or, if accumulated in excess of anti-apoptotic binding partners, lead to assembly of Bax oligomers (oBax) and pore formation in the OMM (**Fig 3**).

The multi-step process of Bax-dependent pore formation can be modulated at several levels by numerous factors, including proteins that “sequester” Bax in the cytosol (i.e. Humanin, Ku70, 14-3-3), the lipidic composition of the OMM, etc. The specific/instructive pathway of Bax activation is highly regulated at multiple levels. Its induction most commonly involves activation of the BH3-only subgroup of Bcl-2 proteins, but can also occur in response to other non-Bcl-2 family proteins (e.g. p53). These proteins can be viewed as “inverse chaperones” assisting the unfolding (rather than folding) and acquiring of activated Bax conformations. In addition, non-specific pathways of Bax activation have also been described, resulting from structural modifications of mBax induced by heat, pH changes or oxidative stress. This process can also be easily replicated *in vitro*, (e.g. by detergents). It is not known whether all of these mechanisms, particularly the non-specific ones, are involved in Bax activation following ischemic brain injury. The efficacy of hypothermia in reducing ischemic brain injury could suggest a role for such non-specific mechanisms, not only for Bax, but also for activation of many other mediators of neuronal dysfunction and death, including the MPT. If this is the case, therapeutic interventions focusing exclusively on the specific molecular pathways of activation are not likely to provide full (or persistent) protection against cell death. While both non-specific and specific pathways of Bax activation could lead to a “common” effector molecule/conformation (i.e., oligomeric oBax), the intermediate steps could be quite distinct. Therefore, it is not clear whether a single small molecular inhibitor such as those developed recently will efficiently block Bax activation through multiple pathways. Combinatorial therapies of ischemic brain injury should therefore continue to investigate concomitant targeting of both non-specific and specific molecular alterations.

The BH3-only proteins act upstream of multidomain proteins as sensors/transducers of apoptotic stimuli and trigger the activation of the multidomain Bax/Bak proteins. Despite employing similar mechanisms of action, the contribution of individual BH3-only proteins to apoptosis in different models of brain injury is at least partially selective. For instance, Bid deficiency protects neurons against

ischemic injury in the adult brain, Dp5/Hrk deficiency protects against axotomy-induced neuronal death, and Bim and Bad contribute to seizure induced neuronal death. Multiple BH3-only proteins, including Bad, Bid, Bim, Puma, Noxa, Dp5/Hrk and Bnip3 are up-regulated, activated, and subsequently translocate to mitochondria following cerebral ischemia. Experiments using either knockout mice (i.e. Bid, Bad, Bim) or antisense down regulation (i.e. Noxa) indicate that while some BH3-only proteins are required for initiation of neuronal death following cerebral ischemia, activation of other BH3-only proteins, e.g., Puma, is dispensable (**Table 1**). Concomitant up-regulation and activation of multiple BH3-only proteins in response to cerebral ischemia reflects the functional redundancy among these proteins, thereby weakening their usefulness as individual targets for pharmacologic intervention. This redundancy is also evident during brain development where no major defects in apoptotic death within the brain are observed in mice lacking the expression of individual BH3-only proteins (i.e. Bid, Bad, Bim).

Recent studies revealed that the involvement of many Bcl-2 family proteins in brain cell death and survival is multifaceted and much more subtle than previously appreciated. Some of these proteins are involved in part of the extensive cross-communication between cell death/survival pathways previously considered distinct (e.g. regulation of apoptotic, autophagic and necrotic death by Bcl-2; **Fig 1**). Similarly, several Bcl-2 family proteins, initially identified and studied mostly as cell death/survival mediators, are now known to function as regulators of basic physiologic processes in healthy cells (e.g. Bad involvement in glucose homeostasis and Bcl-x<sub>L</sub> in mitochondrial morphology and synaptic neurotransmission). Successful targeting of Bcl-2 proteins for therapeutic purpose is also complicated by the fact that many Bcl-2 family members are expressed as multiple isoforms, often with opposed functions. In some cases, isoforms are species-specific. For instance, two isoforms of Mcl-1 are expressed in humans and only one in other mammals. Species selective isoform diversity is one of several factors that limit rapid translation of experimental findings from animal models to human pathology.

Despite the complexity of pathogenic mechanisms of ischemic brain injury, remarkable progress in basic science and understanding of cell death mechanisms over the last two decades led to

development of several small molecule drugs and therapeutic peptides/proteins (**Table 2**), some of which are already in clinical trials for various neurodegenerative diseases or cancers. Among these, caspase inhibitors protect neurons against cell death in animal models of brain ischemia. The potential switch from apoptosis to necrosis in the presence of caspase inhibition, as well as recognition that caspase-independent pathways are also activated in the post-ischemic neurons (i.e., AIF release from mitochondria) suggest, however, that interfering with upstream steps in the death cascade should provide increased protection.

The discovery of the essential role played by the multidomain pro-apoptotic proteins (Bax and Bak) in activation of the mitochondrial apoptotic pathway, and of several endogenous regulators of Bax activation led to the development of several new cytoprotective agents. Among these are a cell-permeable Bax-inhibiting peptide (BIP) derived from the Bax-binding region of Ku70, and a small Bax-sequestering peptide humanin which, although endogenous, can also be artificially delivered inside cells by attaching it to small cell penetrating peptides (i.e. the HIV-1 TAT-PTD, protein transduction domain). Tauroursodeoxycholic acid (TUDCA) inhibits Bax translocation to mitochondria and has been shown to be neuroprotective in a rat stroke model. Two small molecule inhibitors of Bax channel activity (Bci1 and Bci2) that inhibit Cyt C release from mitochondria have been recently discovered. Inhibition of Bax channel activity by Bci1 and 2 protects against apoptosis and is neuroprotective in an animal model of global ischemia. In addition to targeting core apoptotic regulators such as Bax, pharmacologic inhibition of p53 using Pifithrin- $\alpha$ , PARP-1 inhibitors (i.e., 3-AB) and RIP1-kinase inhibitors (i.e., necrostatin) also confer protection against ischemic brain injury and have therapeutic potential.

The recent development of protein transduction technology has provided a new approach for neuroprotection by facilitating the delivery of proteins and peptides into cells and tissues including the brain. Although the mechanism by which small cell-penetrating peptides (protein transduction domains) mediate intracellular delivery of various attached cargoes (i.e., peptides or proteins) remains debated, the neuroprotective potential of this strategy has been demonstrated by several studies in cells and models of brain injury in mice. Delivery of the antiapoptotic Bcl-X<sub>L</sub>, FNK (a modified Bcl-X<sub>L</sub>) or GDNF as

a fusion protein with the HIV-1 TAT (transactivator of transcription) protein transduction domain (PTD) has been reported to reduce the severity of ischemic injury in mouse models.

### *3. Caspase-dependent apoptosis*

The release of Cyt C into the cytosol initiates a molecular cascade leading to caspase-dependent cell death. In the presence of deoxy-ATP (dATP), Cyt C binds to Apaf-1 and triggers activation of the initiator pro-caspase-9 within the apoptosome. Active caspase-9 then activates effector caspase-3 and -7 that in turn cleave a large number of substrates responsible for DNA fragmentation and cell disassembly.

Numerous studies document the contribution of the activation of this pathway to neuronal death in both focal and global cerebral ischemia models. In models of focal cerebral ischemia, the release and relocation of Cyt C from mitochondria to cytosol and the presence of activated caspase-3 is detected in the ischemic penumbra. In addition, caspase-3 deficiency has been shown to render mice partially resistant to ischemic injury. While most studies indicate that caspase activation occurs in a delayed manner (days or even weeks after the initial injury), activation of both pro-apoptotic Bcl-2 family proteins (e.g. Bid) and caspase cleavage (caspase-3, -8, -10, -14), possibly mediated by calpains, can also occur as early as 30 min during reperfusion following global cerebral ischemia. Some studies indicate that neurons containing activated caspase-3 are additionally present in the necrotic core following focal cerebral ischemia. While information in humans is much more limited, reports indicate an increase in pro-caspase-3 within hours following ischemic stroke. Similarly, activated caspase-3 and cleavage of PARP have been reported in some neurons several days after cardiac arrest and reperfusion.

Cleavage of PARP-1, a widely used marker of caspase-3 activation, illustrates some of the cross-communication occurring between different cell death pathways (**Fig.1**). While sustained overactivation of PARP-1 can lead to a necrotic-like cell death, cleavage of PARP-1 by activated caspase-3 can shift the cell death outcome toward apoptosis. Activation of caspase-3 and -9 is endogenously controlled by the inhibitor of apoptosis (IAP) family proteins. Their important role is highlighted by studies indicating that overexpression of the IAP protein XIAP promotes neuronal survival after cerebral ischemia.

#### *4. Caspase-independent apoptosis*

Classically, apoptosis execution was thought to require activation of the caspase family of cysteine proteases. However, cell death with morphological features of apoptosis, or so-called “caspase-independent apoptosis” is now known to contribute significantly to ischemic brain injury. While like other forms of cell death, caspase-independent apoptosis cannot be defined by a single linear biochemical cascade, key players have emerged. Not surprisingly, attention has once again focused on mitochondria, as well as a second family of destructive cysteine proteases, the calcium-activated calpains.

The first evidence for the existence of a caspase-independent mitochondrial apoptosis factor came from the treatment of purified nuclear extracts with a protein mixture derived from calcium-treated mitochondria (**Fig. 4**). The mitochondrial protein responsible for nuclear fragmentation, dubbed AIF for “apoptosis-inducing factor”, is now known to translocate from the mitochondrial intermembrane space to the nucleus following mitochondrial outer membrane permeabilization. AIF nuclear localization and its ability to fragment DNA require association with cyclophilin A, a cytosolic peptidyl prolyl cis-trans isomerase distinct from cyclophilin D that co-translocates to the nucleus. Mice with an attenuation in either AIF or cyclophilin A expression exhibit neuronal sparing following the induction of experimental ischemic brain injury. The engineering of an AIF mutant containing a nuclear export sequence elegantly confirmed the importance of nuclear translocation in AIF-mediated apoptosis.

Nuclear poly (ADP-ribose) polymerase-1 (PARP-1) was identified as an upstream mediator of AIF dependent cell death. This enzyme catalyzes the formation of poly (ADP) ribose and nicotinamide from NAD<sup>+</sup> and functions in DNA repair. However, when DNA damage becomes excessive, e.g. oxidative damage following ischemia/reperfusion brain injury, overactivation of PARP and resulting NAD<sup>+</sup> depletion can occur. The brains of PARP-knockout male mice are remarkably spared from ischemic injury induced by transient middle cerebral artery occlusion. The delivery of neutralizing AIF antibodies attenuates PARP-dependent neuronal cell death induced by either activation of calcium permeable NMDA-type glutamate receptors or by the DNA alkylating PARP activator N-methyl-N'-nitro-N-

nitrosoguanidine (MNNG). The mechanisms by which PARP initiates a caspase-independent program of cell execution remain to be fully elucidated but likely involve both metabolic dysfunction resulting from cytosolic and perhaps mitochondrial NAD<sup>+</sup> catabolism as well as the production of toxic poly-ADP ribose (PAR) polymers. Interestingly, in contrast to PARP-1 knockout males, PARP-1 knockout female mice exhibit increased rather than decreased infarct volume after focal ischemia/reperfusion. Gender-based differences in biochemical death pathways, although poorly studied, are now receiving increasing attention. Preliminary data indicate that sex-based differences in the sensitivity of cells and animals to injury are far more widespread than previously suspected. While some of the differences are due to the action of circulating sex hormones (e.g. estrogen and progesterone), intrinsic genetic-based differences in cultures of XX vs. XY cells are also observed.

#### *5. Calpains in ischemic neural cell death*

Until recently, the steps leading to the nuclear translocation of AIF in caspase-independent neural apoptosis were not understood. It is now known that AIF release from mitochondria requires multiples steps. Outer membrane permeabilization is an initial requirement, that occurs downstream of Bid-induced Bax insertion into the outer membrane or following swelling-induced membrane rupture associated with opening of the Ca<sup>2+</sup>-activated PTP. A second requirement is proteolysis of the membrane-embedded N-terminus of AIF that mediates its detachment from the inner membrane. This processing is performed by calpain-1 endogenous to the mitochondrial intermembrane space as well as by extramitochondrial calpain-1, which accumulates at the mitochondria following oxygen/glucose deprivation. Calpain-cleaved Bid releases AIF from isolated brain mitochondria, but only in the presence of active exogenous calpain-1 that permeates the outer membrane and cleaves AIF. While the consequences of outer membrane permeabilization have normally been associated with the release of apoptogenic proteins, this experiment established that the passage of large proteins across the outer membrane following Bid/Bax-mediated permeabilization is bidirectional. The additional finding that calpain-cleaved but not full-length Bid releases AIF at similar concentrations demonstrates that calpain also augments Bid-induced outer membrane permeabilization.

MNNG treatment of mouse embryonic fibroblasts derived from knockout mice is a convenient way to genetically dissect additional biochemical pathways involved in AIF and PARP-dependent cell death. Calpain knockout fibroblasts are resistant to MNNG-induced apoptosis. However, caspase-3, caspase-9, cathepsin B, or cathepsin L ablation have no effect on this form of injury. These findings demonstrate that PARP-dependent cell death is calpain-dependent but caspase-independent and does not require the cysteine class of lysosomal proteases. Mitochondrial AIF release does not occur in Bax knockout or calpain knockout cells in the MNNG model, confirming the requirement for both outer membrane permeabilization and proteolysis for AIF efflux. In keeping with these genetic findings, pharmacologic inhibition of Bid or overexpression of the calpain inhibitor calpastatin blocks the release of AIF following focal or global ischemia, respectively, affording significant neuroprotection *in vivo*. Knockdown of AIF via the naturally occurring harlequin (Hq) mutation or siRNA delivery confirms that the AIF-mediated death pathway contributes to the size of the infarct following transient focal or global ischemia. Significantly, viral delivery of wild type AIF but not a calpain-resistant mutant restores the sensitivity of AIF-depleted hippocampal CA1 neurons to injury after transient global ischemia. Collectively, these experiments confirm that calpain processing of AIF plays a crucial role in caspase-independent cell death *in vivo*.

AIF is by no means the only target of calcium-dependent calpain proteases. Overexpression of the calpain inhibitor calpastatin confers additional protection to AIF-depleted neurons subjected to oxygen/glucose deprivation, demonstrating that the detrimental effects of increased calpain activity are clearly mediated by the processing of multiple targets. Several proteins that participate in classical, caspase-dependent apoptosis are also calpain substrates, including caspases-3, -7, -8, -9, and -12 as well as Bcl-2 family members Bid, Bax, and Bcl-X<sub>L</sub>. Cleavage of Bcl-2 family members by calpain favors both caspase-dependent and caspase-independent apoptosis by releasing mitochondrial cytochrome *c*, SMAC/DIABLO, and AIF. However, proteolytic inactivation of caspases following calpain overactivation favors caspase-independent apoptotic or necrotic cell death. Intriguingly, the processing of Bcl-X<sub>L</sub> by calpain (or caspase) serves the dual purpose of inactivating a potent anti-apoptotic molecule and generating a pro-death C-terminal fragment that has been detected in the postischemic

hippocampus in association with increased mitochondrial membrane conductance. Because most Bcl-2 family death regulators are expressed at higher levels in the developing brain compared to the adult, the immature brain may be especially vulnerable to the induction of apoptotic pathways associated with cleavage-dependent Bcl-2 family regulation following ischemic injury.

The extent of energy impairment, intracellular calcium deregulation, and oxidative stress all regulate whether cells die by caspase-dependent apoptosis, caspase-independent apoptosis, or acute necrosis. Caspase-dependent apoptosis requires sufficient ATP levels for apoptosome activation. Metabolic impairment resulting in the failure of ATP-dependent ion pumps leads to intracellular calcium rises that, if sustained, become irreversible. Cell swelling and a necrotic death characterized by loss of plasma membrane integrity is the frequent result. Caspase-independent apoptosis likely resides in the center of the apoptosis-necrosis spectrum. Large intracellular calcium rises that result from the opening of calcium permeable glutamate receptors and other cation channels favor calpain protease activation. In addition to potentiating the death-inducing activity of Bcl-2 family proteins and triggering AIF release, calpain-directed proteolysis of caspases inhibits classical apoptosis, leading to caspase-independent cell death. Caspase-independent apoptosis shares many of the morphological features of classical caspase-dependent apoptosis and likely serves as a secondary mechanism of limiting the inflammation response when intracellular ATP depletion impairs the initial programmed cell death response. The roles for calpain proteases in apoptosis following ischemic injury are summarized in **Fig. 5**.

## **E. Summary**

Before the seminal observation that cytochrome c is both released from mitochondria and stimulates caspase activation during many apoptotic cell death paradigms, it was believed that metabolic failure was the primary role of mitochondria in neural cell death following cerebral ischemia and reperfusion. Despite the dramatic increase in our knowledge of pathologic apoptosis, mitochondrial bioenergetic dysfunction is still considered a major cause of neuronal death following cerebral ischemia and must be ameliorated for clinical outcome to be improved. While still controversial, opening of the

inner membrane permeability transition pore in response to abnormal mitochondrial  $\text{Ca}^{2+}$  accumulation and oxidative stress is widely considered to be at least one important mechanism of metabolic failure during acute brain injury. Moreover, pharmacologic inhibitors of the mitochondrial permeability transition are currently being tested in clinical trials for acute brain injury. Other mechanisms include direct inactivation by reactive  $\text{O}_2$  and nitrogen species of critical mitochondrial metabolic enzymes in the tricarboxylic acid cycle and the electron transport chain. Catabolism of both cytosolic and mitochondrial NAD(H) by PARP-1 in response to its activation by oxidative stress is another important cause of metabolic dysfunction. Drugs or other interventions that reduce oxidative stress and inhibit PARP-1 activity also show promise as neuroprotectants.

Prior to the point of irreversible cellular metabolic failure, when both the plasma membrane and subcellular membranes lose their ability to retain both small and large molecules, apoptotic molecular pathways are typically activated in parallel with the macromolecular degradation that potentially leads to necrosis. These apoptotic pathways can be categorized as either not requiring mitochondrial involvement (extrinsic pathway) or dependent upon mitochondria (intrinsic pathway). The intrinsic pathway is either caspase-dependent or independent, based on whether or not cytochrome c-dependent formation of the apoptosome occurs. The intrinsic pathway is activated within 30 min in some models of cerebral ischemia and consists of post-translational modification of many proteins and complex protein-protein interactions between both pro- and anti-apoptotic members. Pharmacologic inhibitors of Bcl-2, the most powerful of the anti-apoptotic proteins, are being tested clinically for promoting the death of cancer cells, so it is likely that drugs which inhibit mitochondrial outer membrane pore formation, e.g., Bax inhibitors, will eventually be tested for cytoprotection, including for that following cerebral ischemia. Calpains, which catalyze  $\text{Ca}^{2+}$ -dependent proteolysis of both apoptotic and non-apoptotic proteins, are another important potential target of intervention for ischemic brain injury as they contribute to both apoptotic and necrotic cell death. Hopefully, in time, the clinical outcome following stroke, global cerebral ischemia, and other forms of acute brain injury will improve as effective combined therapies are developed that both preserve cerebral energy metabolism and inhibit the key molecular events that occur at mitochondria, where different apoptotic pathways converge.

## Figure Legends

**Fig. 1. Cell death pathways contributing to ischemic brain injury.** Anti-apoptotic (Bcl-2-like) and pro-apoptotic Bcl-2 family proteins including *multidomain* proteins (Bax) and the *BH3-only* (BOP) protein subgroup (divided into activators and de-repressors), are central regulators of the intrinsic mitochondrial apoptotic pathway that control the release of apoptogenic proteins from the mitochondrial intermembrane space into the cytosol. Apoptogenic mitochondrial proteins trigger cell death in a caspase-dependent (i.e. Cyt C) or -independent manner (i.e., AIF). Excitotoxic mechanisms resulting in massive  $[Ca^{2+}]_{ic}$  influx and calpain activation, PARP-1 overactivation leading to depletion of NADH pools affecting mitochondrial bioenergetic and metabolic functions, and RIP1 activation by DR ligands (i.e. TNF- $\alpha$ ), result in necrotic (or necrotic-like) cell death. Cross-communications exist between cell death pathways (red lines). Bcl-2-like proteins can interfere with both autophagic death, through binding to the autophagy regulator beclin-1, and necrotic death, through regulation of various mitochondrial processes, including mitochondrial  $Ca^{2+}$  uptake capacity, MPT, and redox state. Further cross-communication occurs at later stages through the activities of calpains and caspases.

**Fig. 2. Schematic representation of the mitochondrial intrinsic apoptotic pathway.** See text for details.

**Fig. 3. Potential mechanisms of Bax activation.** The multidomain pro-apoptotic Bcl-2 family member Bax can be activated through multiple mechanisms that involve either nonspecific physico-chemical modifications of the inactive monomeric Bax molecule (mBax), or specific interactions, in a BH3-domain-dependent or -independent manner, with one or more proteins. The best characterized mechanism of Bax activation involves the activity of BH3-only proteins (BOP). *Direct Activator* BOPs (i.e. Bim, Bid) bind Bax directly and promote its conformational change. *Derepressor/sensitizer* BOPs (i.e., Bad, Noxa) promote activation of Bax indirectly through binding to Bcl-2-like anti-apoptotic proteins and release of “pre-activated” Bax molecules sequestered by these proteins. While some BOP can act both as *activators* and *derepressors* (i.e. Bim, Bid and potentially Puma), others are thought to act

exclusively as derepressors (i.e., Bad, Noxa). Similar to BOP, some non Bcl-2 family proteins can also bind Bax directly (direct activation mechanism) or to Bcl-2 like proteins (derepressor mechanism). Non-specific mechanisms of Bax activation include heat, changes in intracellular pH and oxidative changes of Bax molecule induced by increased ROS generation. Activated Bax (aBax) translocates to mitochondria where it is either bound and neutralized by anti-apoptotic Bcl-2 like proteins or, if present in excess, it can assemble into Bax oligomers (oBax) and form pores into the OMM leading to its permeabilization (MOMP) and release of apoptogenic proteins.

**Fig. 4. Identification of a caspase-independent mitochondrial apoptosis-inducing factor using an *in vitro* reconstitution assay with subcellular fractions.** Depicted is a cell displaying mitochondrial AIF immunostaining and Hoescht nuclear counterstaining. AIF was first identified by 1) treating purified mitochondria with calcium, 2) isolating proteins released into the solution, 3) adding the protein mixture to extracted nuclei to induce nuclear fragmentation, and 4) subfractionating the protein mixture to identify the active component.

**Fig. 5. Calpain pathways contributing to ischemic brain injury.** Calpain-1 activation following ischemic injury is linked to  $\text{Ca}^{2+}$  influx through NMDA receptors. Proteolysis of Bcl-2 family members and apoptosis-inducing factor (AIF) contribute to the pathological release of cytochrome c (C) and truncated AIF, respiratory inhibition, and elevated reactive oxygen species (ROS) production. Mitochondrial sequestration of  $\text{Ca}^{2+}$  may also lead to matrix calpain activation and degradation of electron transport chain subunits, although this remains an active area of investigation.  $\text{Ca}^{2+}$  entry through non-NMDA receptor channels, mitochondrial calcium release, and failed  $\text{Ca}^{2+}$  extrusion ultimately lead to catastrophic “delayed  $\text{Ca}^{2+}$  deregulation” (DCD), calpain-2 activation, and disintegration of the cell.

## **Suggested reading**

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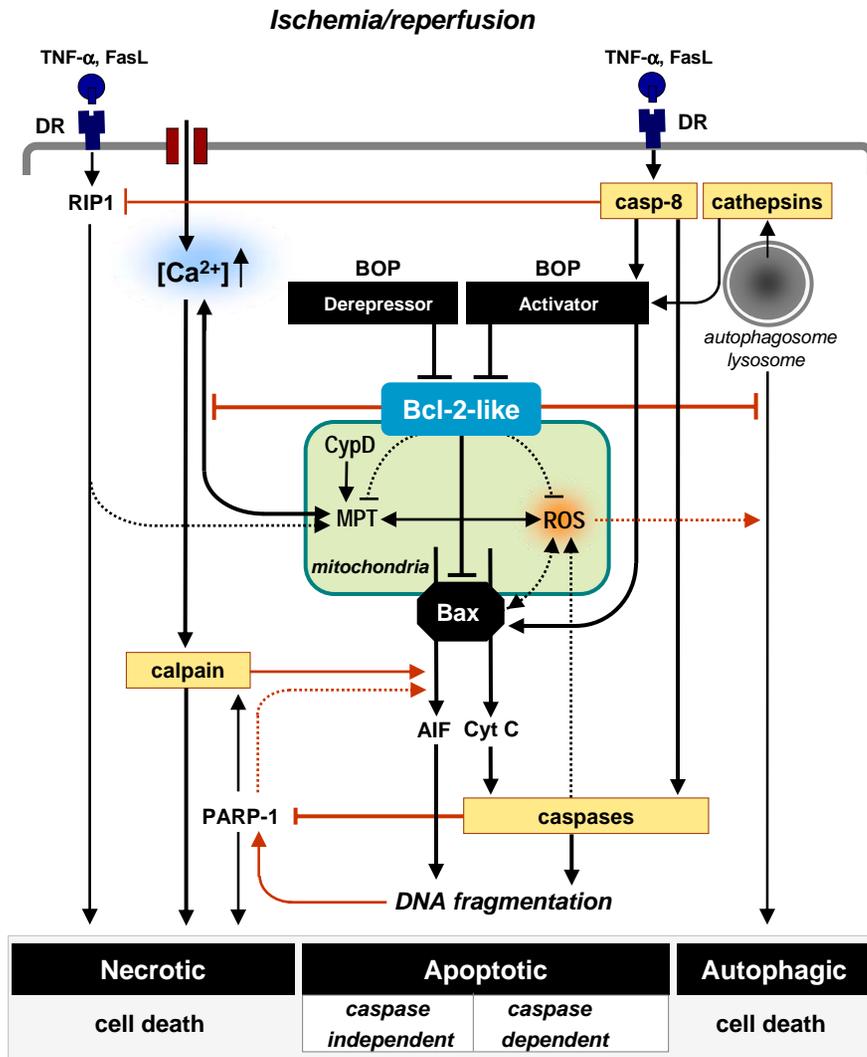
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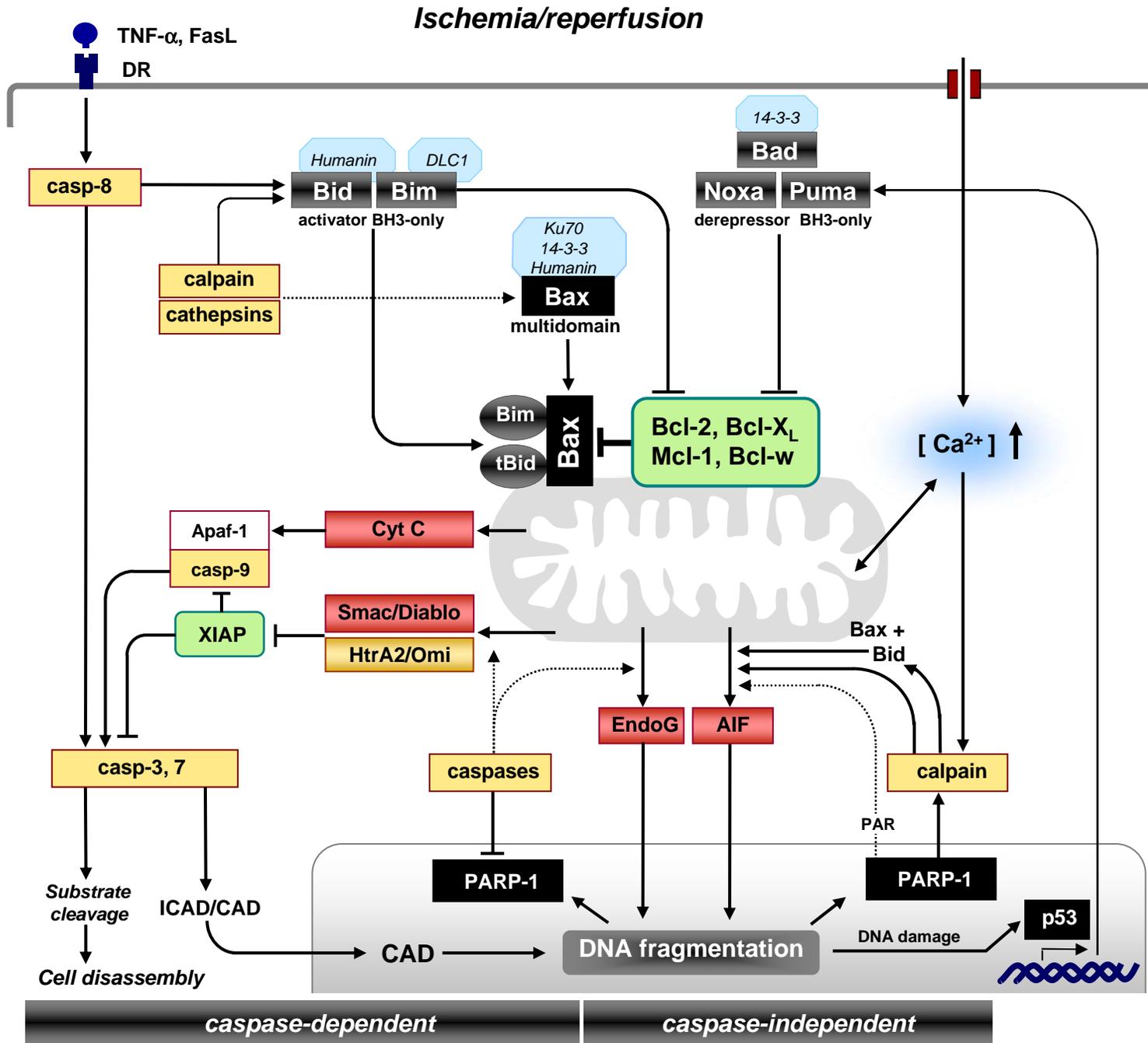
## **Acknowledgments**

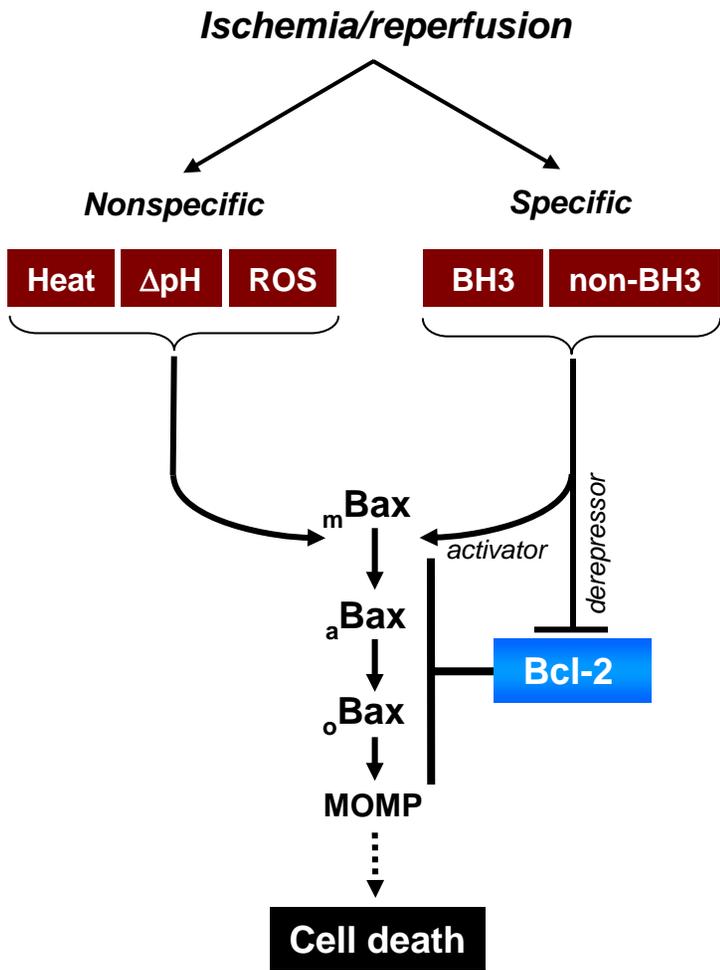
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**Fig. 1**

Fig. 2





*Fig. 3*

