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Forum Review

Cytochrome *c* Release from CNS Mitochondria and Potential for Clinical Intervention in Apoptosis-Mediated CNS Diseases

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ABSTRACT

Apoptosis is critical for normal development and tissue homeostasis. However, its abnormal occurrence has been implicated in a number of disorders, including neurodegenerative diseases and stroke. Translocation of cytochrome *c* (Cyt *c*) from mitochondria to the cytoplasm is a key step in the initiation and/or amplification of apoptosis. Here we discuss Cyt *c* release in apoptosis with its impact on the CNS and review our studies of Cyt *c* release from isolated rat brain mitochondria in response to several insults. Calcium-induced Cyt *c* release, as occurs in neurons during stroke and ischemia, involves rupture of the mitochondrial outer membrane (MOM) and can be blocked by inhibitors of the mitochondrial permeability transition (mPT). Thus, inhibitors of the mPT have shown efficacy in animal models of ischemia. In contrast, proapoptotic proteins, such as BID, BAX, and BAK, induce Cyt *c* release independently of the mPT without lysing the MOM. Several inhibitors of BAX-induced Cyt *c* release have shown promise in models of CNS apoptosis. Because of their distinct mechanisms for Cyt *c* release, both the mPT and proapoptotic proteins should be targeted for effective clinical intervention in CNS disorders involving apoptosis. *Antioxid. Redox Signal.* 7, 1158–1172.

INTRODUCTION

CYTOCHROME *C* (Cyt *c*) is a ubiquitous, heme-containing protein that normally resides in the space between the inner and outer mitochondrial membranes (74). The polypeptide is synthesized as an apoprotein in the cytoplasm and is then transported into the mitochondrion. In the intermembrane space, the heme is covalently attached to Cyt *c* by a heme lyase, and folding of the polypeptide into its native conformation occurs (70). In live cells, native Cyt *c* is found in the intermembrane space, often in association with the mitochondrial inner membrane (MIM) through interactions with phospholipids, including cardiolipin (46). The classical function of Cyt *c* in the intermembrane space is as a component of the electron transport chain where it accepts an electron from complex III (the *bc*1 complex) and transfers the electron to complex IV (cytochrome oxidase), leading to the reduction of oxygen to water (74). In 1996, another role for Cyt *c* as an apoptogenic agent was reported (62). Key to this function was the translocation of native Cyt *c* from mitochondria to the cytoplasm. Since the publication of that seminal study, Cyt *c* and the mechanism(s) for its release from mitochondria that allow it to act as a signaling molecule have been at the forefront of apoptosis research (26, 48). As apoptosis has been implicated in a number of CNS disorders, including neurodegenerative diseases, *e.g.,* Alzheimer's, Parkinson's (PD), and Huntington's (HD) diseases, amyotrophic lateral sclerosis (ALS), and damage as a consequence of stroke, pharmacological prevention of Cyt *c* release from mitochondria is being intensely investigated as an approach with potential to reduce significantly the clinical manifestations of apoptosis in the CNS (38, 68, 123).

PATHWAYS FOR APOPTOSIS LEADING TO THE RELEASE OF CYT *C* FROM MITOCHONDRIA

Two major pathways for apoptosis have been recognized in mammals, the extrinsic and intrinsic pathways (110), both

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leading to the translocation of Cyt *c* from mitochondria (Fig. 1). Overall, the apoptotic program in CNS cells in regard to these two pathways is similar to that in non-CNS cells, although there appear to be some distinctions, such as differential expression of certain proapoptotic proteins (88). However, in CNS disorders such as ischemia and stroke, a third pathway involving calcium has been identified (8, 68). This pathway also appears to be activated downstream of the two major apoptotic pathways in other cell types by Cyt *c* association with inositol 1,4,5-trisphosphate ($InsP_3$) receptors in the endoplasmic reticulum (ER), leading to increased cytoplasmic calcium and synchronized release of Cyt *c* from the bulk of mitochondria in a cell (13).

In the extrinsic pathway, signaling through cell-surface death receptors (some members of the tumor necrosis factor family, *e.g.,* Fas) in response to cell-surface ligation (*e.g.,* by FasL) initiates apoptosis (Fig. 1). Recruitment of adaptor proteins to the cytoplasmic domains of the death receptors leads to activation of caspase-8 through dimerization or "induced proximity" (98). In turn, caspase-8 cleaves BID, and then the truncated form of BID (tBID) acts on the proapoptotic factors BAK and BAX, inducing their oligomerization (54). BAK is a resident of the mitochondrial outer membrane (MOM). BID interacts with BAK, exposing the N-terminus of BAK and inducing its oligomerization (96). BID-induced BAX oligomerization also involves exposing the N-terminus of BAX (29). BAX is largely cytoplasmic, but appears to have a weak affiliation with the MOM. Targeting of BID to mitochondria, where it can interact with BAK and BAX, is facilitated by myristoylation (124). Oligomerized BAK and BAX are hypothesized either to form new channels in the MOM or to modify an existing channel, perhaps the voltage-dependent anion channel (VDAC), allowing for the release of Cyt *c* (67, 103). In the cytoplasm, Cyt *c* binds apoptotic protease activating factor-1 (Apaf-1), which, in conjunction with the binding of ATP or dATP, undergoes a conformational change leading to oligomerization (128). This enables binding of procaspase-9, inducing its cleavage and activation. The complex of Cyt *c*, Apaf-1, and caspase-9 is known as the apoptosome (59).

In the extrinsic pathway, caspase-8 activation alone would appear to be sufficient to induce apoptosis because it can activate the executioner caspases (caspase-3,-6, and -7) (113). There are certain transformed cells that undergo apoptosis through the extrinsic pathway without mitochondrial involvement initiated by BID activation. These are referred to as type I cells (99). However, in many cell types (referred to as type II), Cyt *c* release may be a requisite amplification step. For example, in mice treated with antibodies specific for Fas that induce signaling through the death receptor, Cyt *c* release from mitochondria was found to be necessary for hepatocellular apoptosis (122). Thus, the same treatment in mice deficient in BID (BID-gene knockout mice) did not cause death in liver cells. Similarly, in irradiated mice reconstituted with BAK/BAX double-deficient hematopoietic cells, activated T cells were not depleted following antigen-driven expansion (91). Normally, Fas up-regulation in activated T cells and its subsequent ligation are important mechanisms for clearing lymphocytes after an infection. The requirement for BAK or BAX in lymphocyte clearance implicates mitochondrial release of Cyt *c* as a critical component of the extrinsic apoptotic pathway in type II cells (91).

The intrinsic pathway can be activated independently of ligation of cell-surface death receptors and impacts mitochondria more directly. Ultraviolet irradiation, serum withdrawal, oxidants, *e.g.,* hydrogen peroxide, and staurosporine (a kinase inhibitor) all induce apoptosis by the intrinsic pathway (58). Such stress-induced insults appear to involve translocation of BAX to the MOM. Normally, cytoplasmic BAX is tightly associated

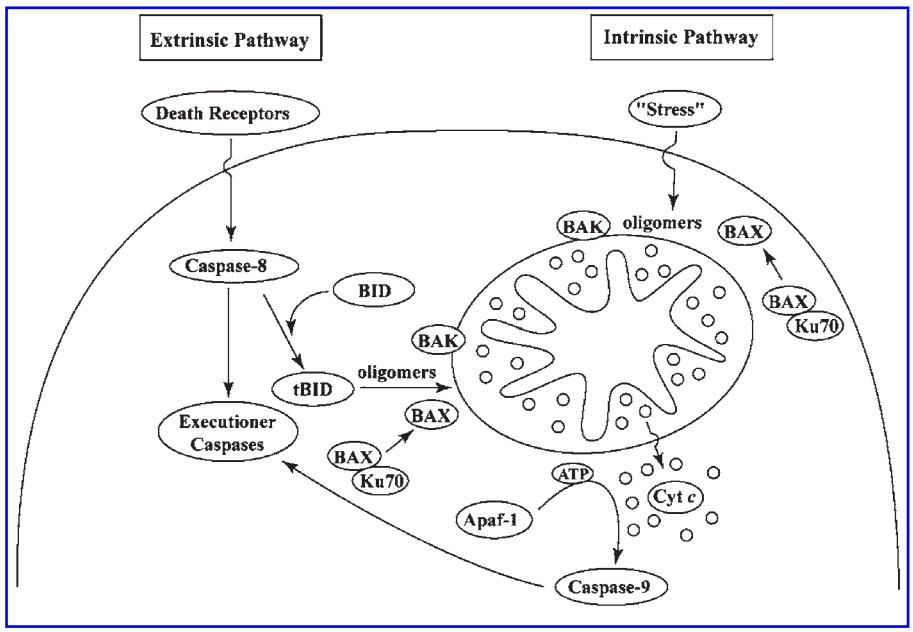

FIG. 1. The extrinsic and intrinsic pathways of apoptosis in mammalian cells. In type I cells, apoptosis through death receptor ligation involves the extrinsic pathway only, without recruitment of mitochondria. In type II cells, both the extrinsic and intrinsic pathways are involved in death receptor-induced apoptosis. The intrinsic pathway can also be activated under conditions of stress independently of the extrinsic pathway. BAK is a resident of the MOM, and BAX is considered to be primarily cytoplasmic.

with the DNA repair factor Ku70. Under stress conditions, and in response to cell damage, Ku70 becomes acetylated at particular lysine residues, causing dissociation of Ku70 and BAX (25). The liberated BAX is then more amenable to oligomerization. BAX oligomerization is not uniquely dependent on BID activation and can be activated by alternate mechanisms (103). For example, BAX-deficient mouse embryos have some resistance to apoptosis and, consequently, developmental abnormalities in the CNS do occur. However, BID-deficient embryos are normal in neural development (55). Thus, BAX would appear to be activated in a BID-independent manner in embryonic development of the CNS.

BAX appears to be the dominant proapoptotic protein involved in Cyt *c* release in the CNS (119). In some types of neurons, its counterpart, BAK, does not appear to play a role in Cyt *c* release during apoptosis. Indeed, BAK is not expressed in superior cervical ganglion neurons (88, 111). It has been reported that in neurons BAK is replaced by an alternatively spliced variant, N-BAK, which can be antiapoptotic in neurons (111).

BCL-2 FAMILY MEMBERS REGULATE CYT *C* RELEASE FROM MITOCHONDRIA

BAX and BAK can also be oligomerized in response to stress-induced insults that do not recruit BID. There are at least 10 BCL-2 homology 3(BH3) domain-only, BCL-2 family members, including BID, that induce Cyt *c* release from mitochondria (44). Several of these are shown in Fig. 2. These proapoptotic proteins are regulated by various mechanisms, *e.g.,* posttranslational modification, transcriptional activation, and sequestration (90). The dephosphorylated form of BAD and phosphorylated form of BIK bind to the antiapoptotic BCL-2 family members and antagonize their ability to inhibit Cyt *c* release (41, 115). BAD and BIK may also displace other BH3 domain-only proteins, such as BID, from being sequestered by the antiapoptotic BCL-2 family members (BCL-2, BCL-x_L, and BCL-w), thus allowing them to oligomerize BAK and BAX (56) (not shown in Fig. 2).

BIM, PUMA, and NOXA are regulated, at least in part, by gene transcription (55). Nerve growth factor withdrawal induces expression of BIM and HRK (45, 87). Also, BIM associates with microtubules by binding the dynein light chain, but this complex dissociates from microtubules early in apoptosis and translocates to mitochondria (89). Thus, BIM is regulated in part by sequestration. Peptides corresponding to segments of the BIM BH3 domain can oligomerize both BAX and BAK (56). However, it has been reported that intact BIM does not display this activity (44). BIM also binds the antiapoptotic BCL-2 family members (90). NOXA and PUMA are regulated at the transcriptional level by p53 in response to DNA damage (73, 77). These BH3 domain-only proteins bind antiapoptotic BCL-2 family members, but appear to mediate their effect somehow through BAK and BAX oligomerization. For example, BAK and BAX double-deficient mice do not respond to NOXA expression (21). Similarly, BAX-

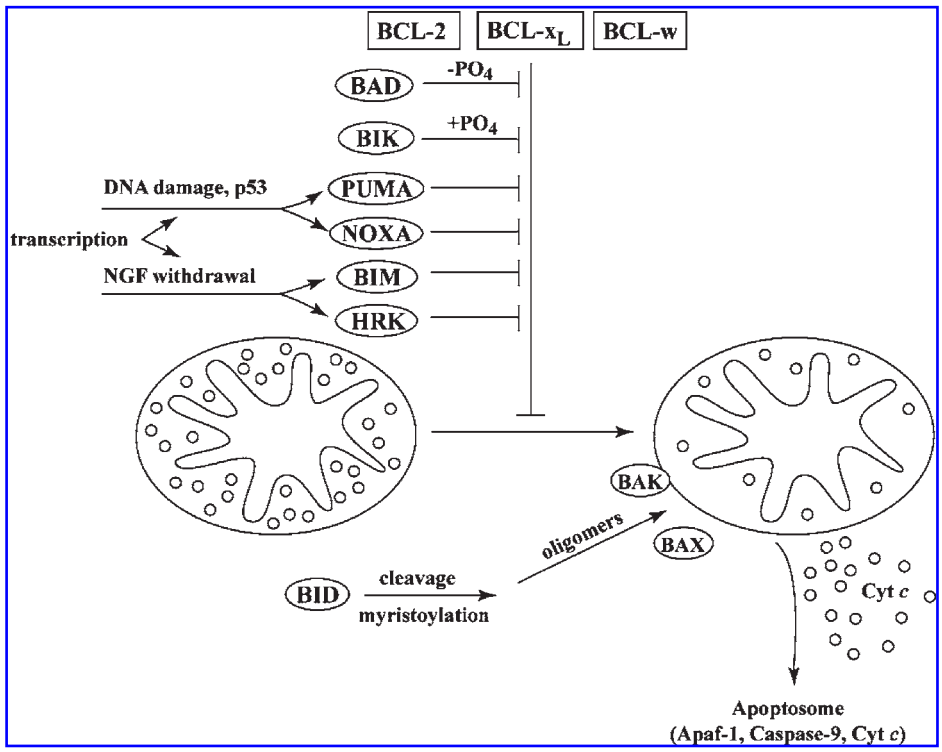

FIG. 2. Key regulators of Cyt *c* translocation from mitochondria in apoptosis. BCL-2 family members located in the MOM that prevent Cyt *c* translocation are boxed, and BCL-2 family members that induce Cyt *c* translocation are circled. Not all of the proapoptotic family members are depicted.

deficient neurons do not die in response to ectopic expression of BIM or HRK (42). The mechanism(s) responsible for the proapoptotic activity of these BH3-only molecules remain(s) unresolved.

In cancer cells, overexpression of BCL-2 and some closely related BCL-2 family members prevents the proapoptotic proteins BAK and BAX from releasing Cyt *c* from mitochondria and prolongs cell survival (2, 39). This is due either to the sequestration of apoptogenic BH3 domain-only BCL-2 family members or to inhibition by BCL-2 of activated BAK and BAX (103). Overexpression of BCL-2 also allows for survival of neurons in response to a variety of apoptotic insults (66).

CALCIUM-INDUCED RELEASE OF CYT *C* FROM MITOCHONDRIA

As mentioned, there is yet another pathway for Cyt *c* release from mitochondria that is not depicted in Fig. 1. Calcium can mediate a proapoptotic protein-independent pathway leading to mitochondrial involvement in apoptosis. Increased cytoplasmic calcium induces Cyt *c* release through lysis of the MOM and may not involve channel formation by apoptogenic proteins (discussed under *Proposed Mechanisms for Cyt* c *Translocation Through the MOM*). This pathway for Cyt *c* release is likely to be physiologically relevant in the CNS, particularly in stroke and ischemia (10, 11). In these disorders, calcium influx into neurons occurs in response to excess glutamate and aspartate release at synapses and overstimulation of glutamate receptors (60) (Fig. 3). Damaged neurons and astrocytes are additional sources for the excitatory amino acids, glutamate and aspartate. A major glutamate receptor involved in calcium (and sodium) influx is the *N*-methyl-D-aspartate (NMDA) type receptor, which is itself an ion channel in the cell membrane. Influx of calcium through NMDA-type receptors also mobilizes calcium from internal stores (60). The excess calcium is taken up by mitochondria through the calcium uniporter leading to the opening of mitochondrial permeability transition (mPT) pores. The redistribution of small molecules and water causes swelling of the mitochondrial matrix with subsequent lysis of the MOM and the release of Cyt *c*.

The calcium-induced pathway may also be engaged in other types of cells undergoing apoptosis as a consequence of Cyt *c* translocating to the ER and binding $InsP_3$ receptors (13). $InsP_3$ receptors modulate the levels of calcium released from the ER. Low levels of cytoplasmic calcium induce $InsP_3$ receptors to release calcium, whereas high levels of cytoplasmic calcium (>5 μ*M*) inhibit $InsP_3$ receptors from releasing calcium. Binding of Cyt *c* to $InsP_3$ receptors blocks the inhibitory effect of high calcium levels, allowing for sustained release of calcium from the ER. Calcium is then taken up by mitochondria, and subsequent opening of the mPT leads to swelling of the mitochondrial matrix, loss of MOM integrity, and release of Cyt *c* (83). Thus, whereas apoptogenic signals, *e.g.,* activated BID by oligomerizing BAK and BAX, may cause initial release of Cyt *c* from a few mitochondria in a cell, sustained calcium efflux from the ER due to Cyt *c* translocation to that organelle compromises the MOM and induces Cyt *c* release from the bulk of the mitochondria in a cell. Consequently, calcium that is released from the ER "synchronizes" the translocation of Cyt *c* from mitochondria within a cell (13, 37).

POSTTRANSLOCATION MECHANISMS PROTECTING AGAINST CYT *C*-INDUCED APOPTOSIS

Multiple mechanisms protect against the potentially lethal effect of the release of small amounts of Cyt *c* into the cytoplasm (Fig. 4). Several IAPs (inhibitors of apoptotic proteases) in the cytoplasm inhibit caspase activity (31). X-linked IAP (XIAP) blocks caspase-9, among other caspases, and thus functions as the most immediate IAP downstream of Cyt *c*

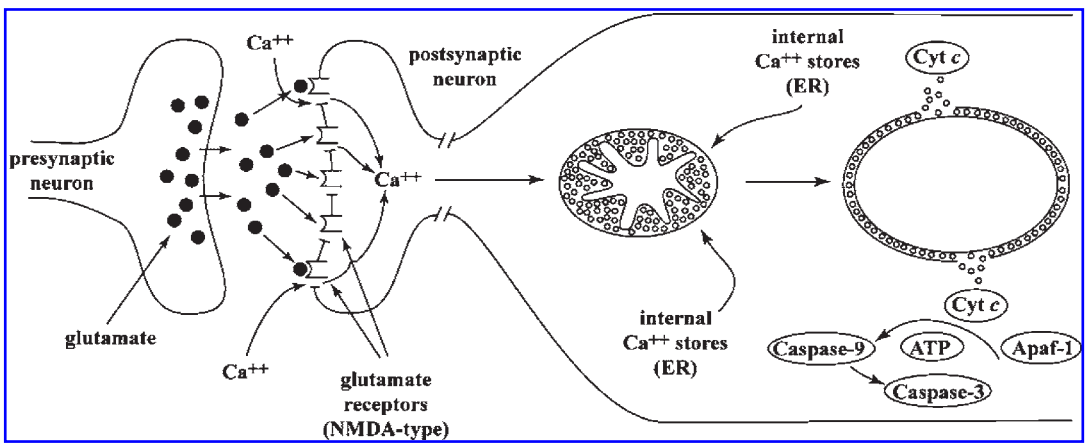

FIG. 3. Calcium-induced release of Cyt *c* from mitochondria in neurons in response to excitogenic signals (glutamate and aspartate). Binding of the excitatory amino acids to receptors, such as the NMDA ligand-gated ion channel, induces the influx of calcium. Subsequent uptake of calcium by mitochondria opens the mPT, allowing import of small molecules and water, leading to swelling of the matrix, lysis of the MOM, and release of molecules including Cyt *c* from the intermembrane space.

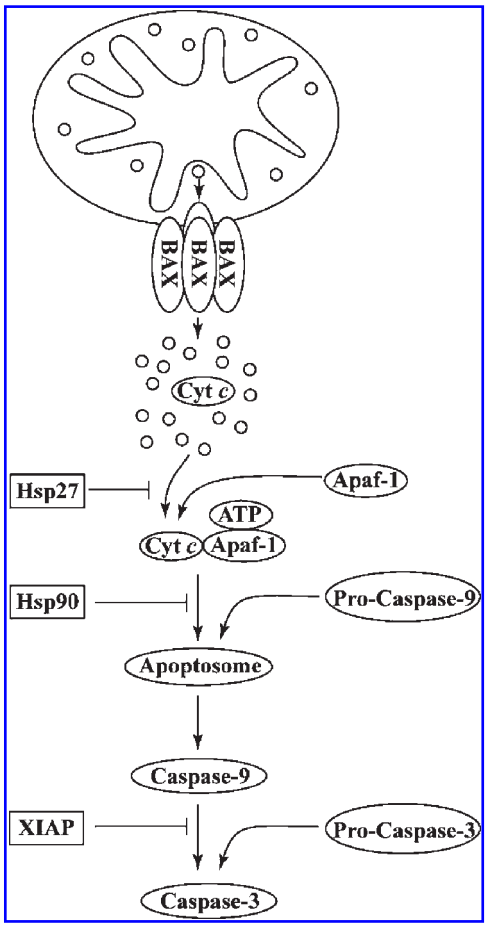

FIG. 4. Apoptosis regulators immediately downstream of Cyt *c* release (boxes). Apoptosome formation is inhibited by Hsp27 and Hsp90, whereas XIAP blocks caspase-9 activity.

release (43). XIAP can be blocked by Smac/DIABLO or HtrA2/Omi, which are also released from mitochondria (34, 104, 114). Their release may not occur concomitantly with the release of Cyt *c* but later in response to caspase activity (3). Injection of Cyt *c* into cultured neurons does not itself induce apoptosis because, in the absence of inhibitors of IAPs such as Smac/DIABLO, caspase activation remains blocked (30).

Cyt *c* has been shown to bind the heat shock protein, Hsp27 (15). This binding, in effect, sequesters cytoplasmic Cyt *c*, preventing its association with Apaf-1. Beyond its inhibitory effect on Apaf-1 activation, Hsp27 is a modulator of actin polymerization and stabilizes actin microfilaments (81). Actin depolymerizing agents, *e.g.,* cytochalasin D and cofilin, have been implicated as apoptogenic factors acting upstream of Cyt *c* release by unknown mechanisms (24, 81). High-level expression of Hsp27 that may occur in response to stress has also been shown to negatively regulate the translocation of Cyt *c* from mitochondria, perhaps by its effect on actin stabilization (81).

Another heat shock protein, Hsp90, can protect against Cyt *c*-induced apoptosis. Hsp90 interacts with Apaf-1 or Apaf-1/Cyt *c* complexes and blocks the formation of the apoptosome (80).

MULTIPLE ROLES FOR CYT *C* IN APOPTOSIS

In the last few years, it has become apparent that Cyt *c* plays more than one role in apoptosis. Beyond facilitating caspase-9 activation and allowing sustained calcium release from the ER (13, 59, 128), Cyt *c* has been reported to translocate to the nucleus, causing efflux of acetylated histone 2A into the cytoplasm and resulting in chromatin condensation (3). Addition of purified Cyt *c* to isolated nuclei or to chromatin mimicked these effects. It is not known how Cyt *c* induces the release of acetylated histone H2A, although a direct displacement reaction has been proposed, possibly involving interaction between Cyt *c* and DNA (76).

These are not the only "moonlighting" functions proposed for Cyt *c* in apoptosis resulting from its binding to different molecules. A summary of ligands known or hypothesized to bind Cyt *c* in apoptosis is given in Table 1. Cyt *c* has been shown to adopt weak peroxidase activity when associated with phospholipids such as cardiolipin and phosphatidylserine (for review, see 49). It has been proposed that this activity enables Cyt *c* to act as an executioner in reactive oxygen species (ROS)-induced cell death (49). For example, Cyt *c* could play a role in the oxidation and externalization of phosphatidylserine to the outer leaflet of the plasma membrane (49).

TABLE 1. CYT *C* LIGANDS IN APOPTOSIS

Subcellular location	*Ligand*	*Apoptotic effect*	*Reference*
Cytoplasm	Apaf-1	Activation of caspase-9	128
Cytoplasm	Hsp27	Sequestration of cytoplasmic Cyt *c* from Apaf-1; caspase-9 activation blocked	15
Cytoplasm	Phosphatidylserine*	Peroxidase activity; oxidation and externalization of phosphatidylserine*	49
MIM	Cardiolipin	Peroxidase activity; oxidation of cardiolipin and release from the MIM*	49
ER	$InsP_3$ receptors	Enhancement of ER calcium release	13
Nucleus	?	Translocation of acetylated histone 2A to the cytosol; chromatin condensation	76
Extracellular	?	Enhancement of apoptosis at high levels	4

*Hypothesized.

In addition to its translocation to subcellular compartments, Cyt *c* has also been shown to translocate to the extracellular space of apoptotic cells soon after its release from mitochondria (47, 93). It is unclear whether Cyt *c* binds to some protein that may function extracellularly to affect apoptosis in neighboring cells, although enhancement of staurosporine-induced apoptosis in primary neuronal cultures by extracellular Cyt *c* has been reported (4).

PROPOSED MECHANISMS FOR CYT *C* TRANSLOCATION THROUGH THE MOM

The pathway through which Cyt *c* exits mitochondria remains controversial. Several putative channel-forming polypeptides and binding partners have been implicated. There is actually more than one pathway, depending on the insult. Calcium causes lysis of the MOM and nonspecific release of Cyt *c*, whereas proapoptotic proteins do not appear to induce MOM lysis, at least initially, and release Cyt *c* more specifically. Clearly, the proapoptotic proteins BAK and BAX are required for Cyt *c* release from mitochondria in apoptosis (118). Thus, cells in which the genes encoding these proteins (or the protein BID, which induces their oligomerization) (122) have been deleted are protected from death induced by a variety of apoptotic insults. Although activated BID oligomerizes BAK and BAX, which have been shown to form channels in synthetic liposomes allowing for the release of Cyt *c* (54, 106), it has not yet been demonstrated that these are the actual channels through which Cyt *c* translocates in mitochondria. By using electrophysiological techniques, such as patch-clamping, it has been shown that the MOM pores formed by BAX are sufficiently large to accommodate Cyt *c* (82). They are also similar in size to the pores formed by BAX in liposomes and can be blocked by overexpression of BCL-2. Furthermore, Cyt *c* was found to decrease the conductance of these pores, implying that Cyt *c* can partition into them (40).

Consistent with the idea that homooligomers of BAX may form natural translocation pores for Cyt *c*, BAX formed tetramers allowing Cyt *c* translocation in synthetic liposomes (97). BAX oligomers have been isolated from mitochondria of apoptotic cells that either are slightly larger in size than tetramers or are even higher molecular weight complexes (9). Neither the VDAC nor the adenine nucleotide translocator (ANT), putative components of the mPT pore, was found to be associated with BAX in these complexes (9). In contrast, perhaps due to differing potencies and concentrations of the detergents used for MOM solubilization, in immunoprecipitation studies BAX and BAK were found to be associated with VDAC when these molecules were reconstituted into synthetic liposomes (106). In addition, VDAC was reported to associate with BAX in apoptotic, but not in healthy, PC12 cells and that anti-VDAC antibodies blocked BAX-induced Cyt *c* release from isolated liver mitochondria (106, 107). Similarly, glutathione *S*-transferase pull-down experiments identified both VDAC and ANT among the mitochondrial proteins that associate with BAX (27). However, the authors cautioned that their data did not indicate that the BAX complex with the mPT pore components was the actual Cyt *c* translocation channel. Rather, by binding components of the mPT pore, BAX may alter the properties of the pore (27).

Fluorescence resonance energy transfer approaches did not identify a stable BAX–VDAC complex in cells in which the mPT was gated using a lipophilic cation (28). In these experiments, BAX formed multimers and clustered in the MOM, leading the authors to conclude that the mPT pore is not part of the Cyt *c* releasing channel, but can signal BAX translocation to the MOM and BAX multimerization (28). In a different study, the VDAC2 isomer was found to be associated with BAK in live cells, but this association appeared to prevent BAK oligomerization rather than to facilitate it (22). Furthermore, the electrophysiologic properties of VDAC channels reconstituted in planar phospholipid membranes were reported to be unaffected by BAX, although tBID induced channel closure (95).

At present, due to the conflicting reports, the constitution of the mitochondrial pores formed by BAX and BAK in apoptotic cells remains undefined. From the accumulated data, the simplest hypothesis for the requisite roles of BAK and BAX is to form channels for Cyt *c* translocation either as homogeneous oligomers or as heterogeneous complexes in association with some other MOM molecule (67).

Prior to Cyt *c* translocation through the MOM, chemical and physical changes occur in mitochondria that appear to be required for Cyt *c* release. The formation of ROS leads to lipid peroxidation and dissociation of Cyt *c* from phospholipids, such as cardiolipin in the MIM (46, 53, 105, 108). This dissociation may be facilitated by phospholipase A_2, which is also activated by ROS (65). Phospholipase A_2 activity has been localized at the contact sites between the MOM and MIM (57). Interestingly, both BAX and tBID appear to interact with the contact sites (20, 33, 63). tBID may also induce remodeling of mitochondrial cristae, allowing Cyt *c* that is sequestered in enveloped cristae access to the inner leaflet of the MOM for translocation (103). Thus, downstream of the action of BH3-domain only BCL-2 family members and upstream of actual Cyt *c* release, two key events have been proposed: dissociation of Cyt *c* from MIM phospholipids and remodeling of the cristae. Both events would mobilize Cyt *c* for translocation across the MOM.

CYT *C* RELEASE FROM ISOLATED CNS MITOCHONDRIA

Once Cyt *c* begins to accumulate in the cytoplasm in response to apoptogenic stimuli allowing for the activation of caspase-9, downstream caspases and cytoplasmic accumulation of calcium may act on mitochondria causing further damage, such as MOM lysis (13, 64, 94). This may explain, in part, discrepancies in the literature regarding the requirement for MOM lysis and loss of membrane potential for Cyt *c* release in response to apoptotic stimuli. To avoid complications potentially presented by cytoplasmic factors, such as caspases and calcium, we have examined the mechanisms for Cyt *c* release using isolated mitochondria, focusing on CNS mitochondria because of the importance of Cyt *c* release and apoptosis in neurodegenerative diseases and stroke (38, 68, 123). The impact of apoptogenic agents on isolated mito-

chondria should directly reflect the initial insult leading to release of Cyt *c* in apoptosis. Here we summarize our studies of Cyt *c* release from rat brain mitochondria in response to several insults, including calcium, tBID plus endogenous BAK, and tBID plus exogenous BAX (16–18). We prepared mitochondria without the use of digitonin, which has been used in some studies to liberate synaptosomal mitochondria, because we have found that the detergent alters the pathway for Cyt *c* translocation (17) (see below).

To study the effect of calcium on Cyt *c* release, the incubation buffer was osmotically matched to the isolated mitochondria (16). In this case, Cyt *c* release occurred concomitantly with loss of membrane potential, swelling of the matrix (see Fig. 5), and lysis of the MOM shown by electron microscopy. This mechanism did involve the mPT because it could be blocked by the inhibitors cyclosporin A and ADP. However, it did not appear to involve VDAC, a putative component of the mPT pore. Dextran T70, an inhibitor of VDAC, did not decrease the amount of Cyt *c* release induced by calcium. Koenig's polyanion, another VDAC inhibitor, actually caused a significant increase rather than a decrease in the amount of Cyt *c* released. Thus, the mechanism for calcium-induced Cyt *c* release from isolated rat brain mitochondria involves mPT-dependent lysis of the MOM without participation of VDAC. This is consistent with findings from studies of calcium-induced Cyt *c* release from liver mitochondria (7, 50, 83, 100, 120).

It has been reported that calcium can induce Cyt *c* release from rat brain mitochondria by an mPT-independent mechanism (7, 101). This mechanism may not be physiologically relevant because we found that in one case, at least, it was due to the use of digitonin in preparing mitochondria (7). Thus, addition of a similar amount of digitonin to mitochondria isolated by Dounce homogenization in our experiments caused calcium to induce Cyt *c* release by an mPT-independent pathway (17).

Overstimulation of glutamate receptors in stroke and ischemia causes neuronal uptake of extracellular calcium, which is likely to be a key contributing factor in cell death (Fig. 3) (8, 60). Therefore, stimulation of glutamate receptors in cultures of CNS cells was also demonstrated to induce increased cytoplasmic calcium and lead to Cyt *c* release and to apoptosis (16). The extent of Cyt *c* released into the cytosol was similar to the extent of Cyt *c* released from isolated mitochondria following calcium treatment. In cultured neurons, Cyt *c* release was again mPT-dependent. Thus, the mechanism for Cyt *c* release from mitochondria in CNS cells stimulated with glutamate mimicked the mechanism for calcium-induced Cyt *c* release from isolated CNS mitochondria. In the cytosol of CNS cells, caspase-3 activity followed glutamate stimulation, indicating induction of apoptosis. These findings suggest that the mechanism for Cyt *c* release immediately as a consequence of stroke and ischemia in CNS cells, neurons in particular, may involve the mPT via effects of calcium on mitochondrial physiology (16).

In contrast to the effect of calcium, the proapoptotic proteins BAK and BAX induce Cyt *c* release from CNS mitochondria by mechanisms that do not involve the mPT and do not lead to matrix swelling and rupture of the MOM (Table 2, Fig. 5) (18). tBID acts on BAK present in the MOM to release Cyt *c*. This was shown using a polyclonal antibody specific for BAK to block Cyt *c* release that was induced by tBID (117). The amount of Cyt *c* released by tBID and its effect on BAK is small, but comparable to the amount of Cyt *c* released in response to calcium (Fig. 6). The low level of Cyt *c* released via BAK relative to BAX (see below) could reflect expression of BAK in only a subpopulation of cells from which mitochondria were obtained, *e.g.,* glial cells or certain types of neurons (88, 111). BAK-mediated Cyt *c* release may involve VDAC because it is attenuated by the VDAC inhibitor, Koenig's polyanion. If the VDAC2 isoform and BAK do associate in the MOM of live CNS cells as has been reported for a transformed cell line (22), it is possible that the VDAC inhibitor in our studies prevented the dissociation of these polypeptides in response to apoptogenic signals maintaining BAK in a monomeric form in the presence of tBID. However, this possibility has not been examined.

tBID plus BAX (full-length polypeptide) induced maximal Cyt *c* release from CNS mitochondria, *i.e.,* comparable to the amount of Cyt *c* released in response to alamethicin (Fig. 6). In some experiments, we used a truncated form of BAX lacking the hydrophobic C-terminal segment (BAXΔC) and found that it was only as effective as BAK in releasing Cyt *c* from CNS mitochondria. (Note that in Fig. 6 the effect of tBID plus BAXΔC is due to the effect of tBID on both endogenous BAK and BAXΔC.) Thus, the C-terminal segment of BAX seems to be required for maximal release of Cyt *c*. The significantly reduced amount of Cyt *c* released from

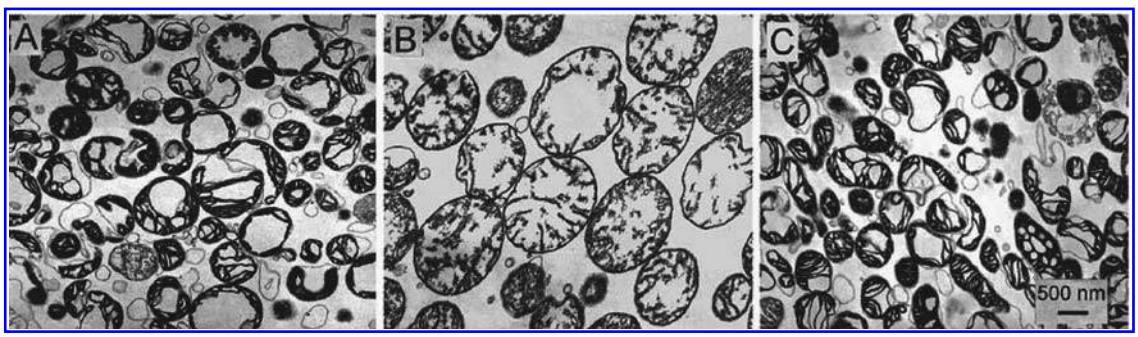

FIG. 5. Electron micrographs of rat brain mitochondria showing matrix swelling in response to calcium, but not to oligomerized BAX. (**A**) Untreated mitochondria. (**B**) Mitochondria incubated with calcium. (**C**) Mitochondria incubated with tBID plus full-length BAX. At higher magnification, rupture of the MOM in B can be discerned.

TABLE 2. COMPARISON OF MECHANISMS FOR CYT *C* RELEASE FROM ISOLATED CNS MITOCHONDRIA

Characteristic	*Calcium*	*tBID plus BAK*	*tBID plus BAXΔC*	*tBID plus full-length BAX*
Matrix swelling and MOM lysis	+	—	—	—
mPT involvement	+	—	—	—
Inhibition by VDAC blocker (Koenig's polyanion)	—	+	—	—
Maximal Cyt *c* release (comparable to alamethicin)	—	—	—	+

mitochondria due to oligomerized BAK compared with oligomerized BAX in our experiments may be due to the expression of the proapoptotic isoform of BAK in only a subset of cells in the brain, as mentioned (111). In addition, it has been reported that BAK oligomerization in rat proximal tubule cells is dependent on BAX (71). If the same is true for the CNS, then BAK oligomerization would not have been efficient in our experiments without the addition of exogenous BAX. Finally, BAX-mediated, but not BAK-mediated, Cyt *c* release is particularly relevant to apoptosis in neurons as demonstrated by the lack of naturally occurring death of neurons in BAX-deficient mice (119).

In isolated mitochondria, tBID plus BAX-induced Cyt *c* release did not involve the mPT (Table 2), did not lead to matrix swelling and MOM rupture (Fig. 5), and did not require VDAC (Table 2). Thus, there may actually be three distinct mechanisms for the release of Cyt *c* from isolated mitochondria in response to calcium and proapoptotic proteins, differentially defined in our studies by the involvement of the mPT in the response to calcium and by the possible involvement of VDAC in tBID plus BAK-mediated Cyt *c* release, but not in tBID plus BAX-mediated Cyt *c* release (18).

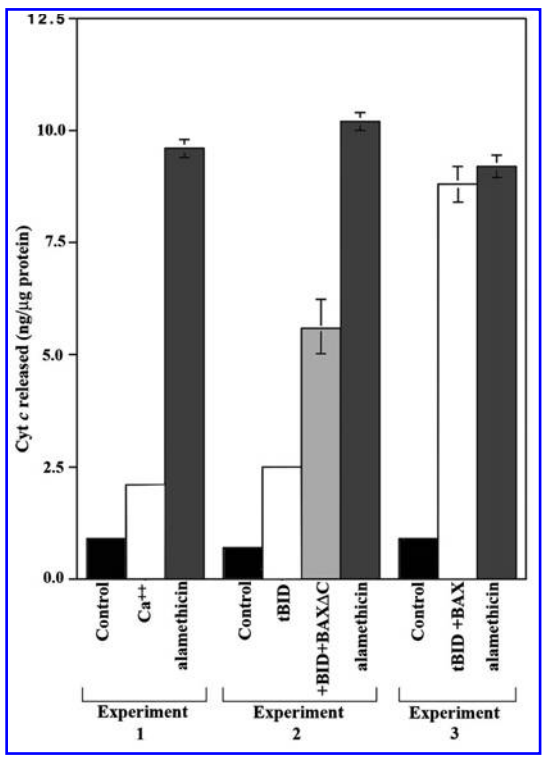

FIG. 6. Quantification of Cyt *c* release from isolated rat brain mitochondria in response to various insults. Cyt *c* in the supernates of rat brain mitochondrial suspensions was quantified by using an ELISA. The data were compiled from three separate experiments. Error bars represent SEM. Controls represent mitochondria in buffer only.

The relatively small amount of Cyt *c* released from mitochondria as a consequence of increased calcium despite matrix swelling and MOM rupture (Fig. 6) is consistent with evidence that MOM lysis is insufficient to mobilize the bulk of Cyt *c* in mitochondria. Full release likely requires the dissociation of Cyt *c* from binding phospholipids in the MIM, *e.g.,* by ROS production, antioxidant depletion, or phospholipase activity (46, 53, 65, 105, 108). Although there is evidence for remodeling of cristae as a consequence of tBID activity at the contact sites between the MOM and the MIM (103), neither tBID nor swelling of the matrix in response to calcium in our experiments mobilized the bulk of mitochondrial Cyt *c*, indicating the requirement for other mechanisms induced by the proapoptotic protein BAX for complete mobilization of Cyt *c* (Fig. 6). In our experiments examining BID- and BAX-induced Cyt *c* release, no obvious remodeling of cristae was observed (18).

TOWARD A UNIFYING HYPOTHESIS FOR APOPTOSIS-INDUCED CYT *C* RELEASE FROM MITOCHONDRIA

Proapoptotic proteins may mediate the specific release of Cyt *c* from mitochondria either by modifying an existing channel (perhaps VDAC for oligomerized BAK) or by forming a new channel (perhaps for oligomerized BAX) (67). In an individual cell, only a few of the mitochondria at or near the site of "stress" may be affected in this initial step (69). The actual translocation of Cyt *c* may involve additional membrane changes mediated by enzymes, *e.g.,* phospholipase A_2, and ROS that release Cyt *c* from its association with the MIM (46, 53, 65, 105, 108). In addition, in response to tBID, the cristae may open, allowing free access of Cyt *c* to the inner leaflet of the MOM. Initially, the potential apoptogenic activity of the low amount of cytoplasmic Cyt *c* may be attenuated by its binding to Hsp27, thus inhibiting formation of the apoptosome (15). As the quantity of cytoplasmic Cyt *c* increases due to the apoptogenic signaling to mitochondria in the area surrounding the site of stress, Hsp27 becomes limiting and the apoptosome begins to form. However, failure to release other proapoptotoic mitochondrial proteins, *e.g.,*

Smac/DIABLO, during the initial translocation of Cyt *c* prevents the apoptotic cascade from ensuing (64, 94). As active caspase-9 increases in response to apoptosome formation, downstream caspases eventually become activated, which, in conjunction with increased calcium release from the ER (see below), leads to lysis of the MOM, allowing release of other apoptogenic proteins, including Smac/DIABLO, from the intermembrane space (34). As Smac/DIABLO increases, IAP activity in the cytoplasm is limiting and amplification of the caspase cascade now occurs. Concomitantly, translocation of some cytoplasmic Cyt *c* to the ER and its binding to $InsP_3$ receptors increase cytoplasmic calcium (13). Uptake of calcium by mitochondria leads to mPT-dependent Cyt *c* release from the bulk of the mitochondria, *i.e.,* those mitochondria not initially affected by the stress, as well as the release of other apoptogenic proteins (13). The net effect is a wave-like release of Cyt *c*, from mitochondria along with a wave-like decrease in membrane potential. Thus, the loss of mitochondrial membrane potential observed *in situ* in response to apoptotic insults may not be coincident with the initial release of Cyt *c* but rather with the delayed, amplified release due to the effect of calcium and caspases (64).

POTENTIAL FOR PHARMACOLOGICAL REGULATION OF CYT *C* RELEASE FROM MITOCHONDRIA

There are multiple control points in apoptosis, and each of these, theoretically, has potential for regulation using pharmacological agents. As apoptotic pathways regardless of the insult eventually merge at caspase activation, there has been considerable effort identifying inhibitors of the caspases (35, 79). Caspase-3 and caspase-9 are the predominant caspases involved in neural development (123). These and other caspases are involved in ischemic brain injury and appear to play a role in neurodegenerative diseases (72). However, inhibition of caspase activity would likely not block mitochondrial damage resulting from the combined effects of apoptogenic proteins, calcium, and ROS. As Cyt *c* release from mitochondria is central to apoptosis initiation and/or amplification, approaches to regulate this step could have a significant impact preventing or controlling apoptosis-related maladies in the CNS, particularly in conjunction with other approaches, such as caspase inhibition.

In a CNS cell undergoing apoptosis, the initial release of Cyt *c* in response to apoptogenic proteins appears to be mPT-independent (18), consistent with some findings from the study of non-CNS cells (52, 116, 117, 125). Downstream events of proapoptotic protein-induced Cyt *c* translocation, such as release of calcium from the ER, may evoke mPT participation, although mPT opening would not necessarily be a component of the initiating events. However, in the case of ischemia and stroke, mPT inhibitors could be useful to block apoptosis in neurons because the mPT appears to be involved in the initial release of Cyt *c* in that case. For example, following a short ischemic episode in gerbils, reperfusion with immediate cyclosporin A treatment had a significant effect on survival of neurons (32). In other *in vivo* models of ischemia, proapoptotic proteins appear to play a role in neuronal death. Neurons from BID-deficient mice were largely, but not completely, resistant to ischemic death with a coincidental partial decrease in Cyt *c* release (85). It would seem likely that some of the neurons in this model succumbed to the effect of calcium on the mPT. BAX has been shown to translocate to mitochondria after cerebral ischemia (78), and overexpression of BCL-2 protects neurons in mice from experimental ischemia (66). As both opening of the mPT in response to calcium and proapoptotic proteins have been implicated in Cyt *c* release in ischemia, a combination of inhibitors of the mPT and of proapoptotic Cyt *c* releasing-proteins may be required to protect against death due to ischemia.

Hypothermia treatment may also be useful as it has been shown to reduce ischemia-induced cell death in animal models. In newborn rats subjected to focal ischemia by ligation of the left common carotid artery and hypoxia, reduction in body temperature for several hours markedly reduced Cyt *c* release and caspase-3 activity (127). Similarly, in focal cerebral ischemia experiments in adult rats, lowering body temperature by a few degrees dramatically decreased Cyt *c* release for several hours after the ischemic episode (121). Interestingly, BAX translocation to mitochondria was not significant within this time frame, indicating that the insult did not lead to immediate apoptogenic activity, but rather Cyt *c* release may have occurred through the calcium-induced pathway. Both BAX translocation and Cyt *c* release were observed at 24 h, suggesting that delayed damage was likely due to proapoptotic protein-induced Cyt *c* release.

In Table 3, a number of compounds are listed that have been shown to interfere with Cyt *c* release from mitochondria in either isolated mitochondria, cultured cells, or animal models. Some of these may affect the mPT, *e.g.,* cyclosporin A, minocycline, and rasagilene (5, 32, 112). Minocycline has been used to block apoptosis in spinal cord injury, experimental ALS, and HD (112). Rasagiline, which is currently used in clinical trials for PD, blocks mPT-dependent apoptosis induced by the neurotoxin, *N*-methyl(*R*)salsolinol (5). Upstream of the mPT in the calcium-induced Cyt *c* release pathway (see Fig. 3), the NMDA receptors can be inhibited by using antagonists such as ketamine (126). There are compounds that block the release of Cyt *c* from mitochondria by other mechanisms in models of CNS disorders. Among these are several that appear to protect against ischemia, including the ANT inhibitor, bongkrekic acid (19), the hydroxyl and peroxyl radical scavenger, cilostazol (23), and the activator of ATP-sensitive K^+ channels, diazoxide (61).

Other compounds listed in Table 3 are known to inhibit Cyt *c* release that is induced by proapoptotic proteins. These include propranolol and dibucaine, which act downstream of BAX insertion in the MOM (86). Their mechanism of action is unknown. These bicyclic aromatic, amphiphilic cations are effective *in vitro* at relatively high concentrations and, thus, will likely not be useful as therapeutic agents. However, it may be possible to prepare derivatives that are active at lower concentrations. In this regard, a number of 3,6-dibromocarbazole derivatives of 2-propanol have been shown to be effective in blocking Cyt *c* release from isolated HeLa cell mitochondria in response to BAX activation at concentrations approximately one order of magnitude more effectively than

TABLE 3. EXAMPLES OF COMPOUNDS THAT INHIBIT CYT *C* RELEASE FROM CNS MITOCHONDRIA

Compound	*Type of injury*	*Disease model*	*Mechanism*	*Reference*
Bongkrekic acid	Rat brain middle cerebral artery occlusion	Focal ischemia	ANT inhibition	52
Cilostazol	Rat left middle cerebral artery occlusion	Focal ischemia	Hydroxyl/peroxyl radical scavenger	116
Cyclosporin A	Gerbil carotid artery occlusion	Global ischemia	mPT inhibition	35
Diazoxide	Mouse middle cerebral artery occlusion	Focal ischemia	Activates ATP-sensitive K^+ channels	125
Dibucaine	Isolated rat brain mitochondria treated with BH3 peptide	Apoptosis	Inhibits BAX insertion	32
Flavopiridol	Neuron cultures treated with camptothecin to induce DNA damage	Chemotherapy-induced apoptosis	Cyclin-dependent kinase inhibition	85
Ketamine	Rat hippocampus/prevents Ca^{2+} influx	Global ischemia	NMDA receptor antagonist	78
Lithium carbonate	Rabbit brain neurotoxin		Blocks Al^{3+}-maltolate	127
Melatonin	Rat middle cerebral artery occlusion	Focal ischemia	Inhibits mPT	121
Minocycline	Rat spinal cord injury	Spinal paralysis	Inhibits mPT	19
Propranolol	Isolated rat brain mitochondria treated with BH3 peptide	Apoptosis	Inhibits BAX insertion	32
Rasagiline	Apoptosis in isolated mitochondria induced by *N*-methyl(*R*)salsolinol	PD	Inhibits mPT	23
Trifluoperazine	Gerbil carotid artery occlusion (enhanced the effect of cyclosporin A)	Global ischemia	Inhibits phospholipase A_2	61
Tauroursodeoxycholate	Rat neuronal cells: 3-nitropropionic acid-induced; R6/2 transgenic mouse model	HD	Inhibits BAX translocation; antioxidant	86

propranolol and dibucaine (14). Tauroursodeoxycholic acid prevents BAX insertion into the MOM and has been used in a transgenic animal model of HD (R6/2 model with a CAG expansion of *Htt* exon 1) to prevent striatal apoptosis (51). Such antiapoptogenic reagents, if effective through clinical trials, hold promise in ameliorating the conditions in patients with a variety of apoptosis-mediated CNS diseases.

CYT *C* AS A POTENTIAL CLINICAL MARKER FOR ABERRANT APOPTOSIS *IN VIVO*

Soon after the induction of apoptosis, Cyt *c* has been shown to appear in the extracellular fluid in *in vitro* studies (47, 93). Extracellular Cyt *c* appears to be intact, monomeric, and native in conformation (47). Whereas many proteins are cross-linked by transglutamination in apoptotic cells (84), Cyt *c* is spared despite its high content of lysine residues, a target of the transglutaminase reaction.

In several clinical trials, it has been shown that Cyt *c* appears in the blood of certain individuals, *e.g.,* patients with hematopoietic cancers who are undergoing chemotherapy (93) and individuals with aberrant apoptosis (see below). As Cyt *c* is released from apoptotic cells in its native state, the presence of Cyt *c* in serum can be quantified by enzyme-linked immunosorbent assay (ELISA) using antibodies specific for the native protein (47). Thus, Cyt *c* was found to be significantly increased in sera of patients with liver disease where apoptosis was involved, including viral hepatitis, autoimmune hepatitis, hepatocellular carcinoma, and primary biliary cirrhosis, among others (12). Cyt *c* was not elevated in patients with liver damage without apoptotic implications, *e.g.,* sarcoidosis,

Wilson's disease, and Budd–Chiari syndrome (12). In another study using ELISA, serum Cyt *c* was shown to correlate with systemic inflammatory response syndrome (SIRS) and was suggested to be useful in assessing organ dysfunction in patients with SIRS, including sepsis (1). More recently, increased serum Cyt *c* was detected in influenza virus patients presenting with encephalopathy. Infected individuals without encephalopathy had much lower serum levels of Cyt *c* (75). By western blot analysis, serum Cyt *c* levels were increased in patients with cancers of hematopoietic origin, and the levels of Cyt *c* increased further in response to chemotherapy, consistent with apoptosis in the malignant cells (93).

Taken together, these clinical trials strongly suggest that serum Cyt *c* may be an indicator of aberrant apoptosis *in vivo*. Importantly, Cyt *c* was not released from cells undergoing necrosis except in response to high temperature (47, 93), and so it may also appear in the serum of burn victims. Although serum Cyt *c* has not been reported in patients with CNS disorders other than influenza virus-induced encephalopathy (75), as apoptosis appears to be involved in a number of these diseases, it seems likely that elevated serum Cyt *c* would be observed in those patients, particularly when acute CNS damage is indicated.

CONCLUDING REMARKS

The importance of proapoptotic proteins, in particular BAX, in neuronal apoptosis implicates the release of Cyt *c* from mitochondria as a critical step. Pharmacological approaches to inhibit Cyt *c* release induced by proapoptotic proteins or calcium influx have shown promise in preventing neuronal damage in experimental stroke and in several models of neurodegenerative diseases. From studies of isolated CNS mitochondria and cultured neurons, it is clear that several mechanisms are involved in the translocation of Cyt *c* from mitochondria. During ischemia and stroke, calcium influx into neurons leads to lysis of the MOM through a pathway that involves the mPT. Thus, inhibitors of the mPT, such as cyclosporin A, have shown some efficacy in blocking Cyt *c* release effected by calcium in models of ischemia. In response to other apoptogenic insults, such as BAX oligomerization, the MOM is not lysed, at least during the initial release of Cyt *c*. Promising results with inhibitors that interfere with BAX insertion into the MOM, *e.g.,* tauroursodeoxycholate, or that act at a later step in the BAX-mediated pathway, *e.g.,* propranolol and dibucaine, may lead to the synthesis of analogues effective at physiologically relevant concentrations. In some disorders, it may be necessary to inhibit both the mPT and proapoptotic proteins where the initial insult and delayed effect induce different pathways for Cyt *c* release. Finally, quantification of Cyt *c* in serum may prove useful as a prognostic indicator of aberrant apoptosis in the CNS, as recently shown for influenza virus-induced encephalopathy.

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ABBREVIATIONS

ALS, amyotrophic lateral sclerosis; ANT, adenine nucleotide translocator; Apaf-1, apoptotic protease activating factor-1; BAXΔC, BAX lacking the hydrophobic C-terminal segment; BH3, BCL-2 homology 3; Cyt *c*, cytochrome *c*; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; HD, Huntington's disease; Hsp, heat shock protein; IAP, inhibitor of apoptotic proteases; $InsP_3$, inositol 1,4,5-trisphosphate; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; mPT, mitochondrial permeability transition; NMDA, *N*-methyl-D-aspartate; PD, Parkinson's disease; ROS, reactive oxygen species; SIRS, systemic inflammatory response syndrome; tBID, truncated BID; VDAC, voltage-dependent anion channel; XIAP, X-linked IAP.

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