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Forum Review

Bax, Reactive Oxygen, and Cytochrome *c* Release in Neuronal Apoptosis

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ABSTRACT

Half of all neurons produced during embryogenesis undergo apoptotic death shortly before birth or soon thereafter. Two cell culture models have been used extensively to investigate the cellular and molecular mechanisms underlying apoptosis during neuronal development: (a) sympathetic neurons deprived of their required neurotrophic factor, nerve growth factor, and (b) cerebellar granule neurons deprived of serum in low-potassium medium. A dramatic increase in mitochondrial-derived reactive oxygen species (ROS) occurs during the apoptotic death of both of these cell types. These ROS lie downstream from the proapoptotic protein, Bax. Bax normally resides in the cytoplasm, but translocates to the outer mitochondrial membrane during apoptosis. Once associated with mitochondria, Bax causes release of apoptogenic factors from the mitochondria into the cytoplasm, thus inducing or augmenting the apoptotic cascade. Although there is much controversy about the exact mechanism by which Bax causes release of these factors, recent evidence suggests that the Bax-induced ROS are critical for this release to occur in both sympathetic and cerebellar granule neurons. Because Bax is critical for the apoptotic death of many other types of neurons, it is likely that increased ROS is important for the death of these cells as well. *Antioxid. Redox Signal.* 5, 589–596.

INTRODUCTION

DURING METAZOAN DEVELOPMENT AND TISSUE TURNOVER in mature multicellular organisms, a great many cells die by the cell suicide process known as apoptosis (33). This death is physiologically appropriate, serving distinct functions in different tissues. Apoptosis is particularly prominent in the developing nervous system. About half of all neurons produced during neurogenesis die during a period of development corresponding approximately to the time at which synaptic contact with target tissue is established. Evidence suggests that the purpose of this death is appropriate matching of innervation density with target size. The primary determinant of which neurons survive developmental apoptosis is attainment of a sufficient quantity of neurotrophic substance from their target or other tissues (51). Neurons that fail to acquire an adequate amount of neurotrophin undergo apoptotic death.

The best characterized models of developmental apoptotic neuronal death are cultures of sympathetic neurons deprived of nerve growth factor (NGF) and cultures of repolarized/serum-starved cerebellar granule neurons. These cells die with most of the features common to many types of cells undergoing apoptosis. These features include atrophy, sustained induction of several immediate early genes, condensation of nuclear chromatin, cleavage of DNA between nucleosomes, release of proapoptotic factors from mitochondria, and activation of caspase family proteases (8, 12, 13, 44, 46, 49). Cell death with these characteristics occurs not only in a physiologically appropriate manner, but also pathologically, for example, in some neurodegenerative conditions such as Alzheimer's disease (70) and in neurotrauma and stroke (5, 14, 62).

There is considerable evidence that reactive oxygen species (ROS) contribute to the apoptotic death of many types of cells, including neurons. Recent findings suggest that one means by

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which ROS affect neuronal apoptosis is via an influence of ROS on release of proapoptotic factors from mitochondria into the cytoplasm (34, 35, 62). Here we discuss evidence suggesting that mitochondrial-derived ROS either directly or indirectly induce release of the apoptogenic protein cytochrome *c* from the intermembrane space of mitochondria into the cytoplasm of neurons undergoing death caused by neurotrophin withdrawal.

THE ROLE OF MITOCHONDRIA AND CYTOCHROME *C* IN APOPTOSIS

Extensive evidence indicates a central role for mitochondria in the apoptotic death of many types of cells, including neurons (8, 12, 30, 49). The principal means by which mitochondria influence apoptosis is by releasing proapoptotic agents into the cytoplasm. Prominent among these agents is cytochrome *c*. The mature form of cytochrome *c* (holocytochrome *c*) is normally found in cells only between the inner and outer mitochondrial membranes where it functions in the electron transport chain to carry electrons from respiratory complex III to respiratory complex IV. Holocytochrome *c* induces apoptotic death when released from the mitochondria into the cytoplasmic compartment (12, 36, 42). Once in the cytoplasm, cytochrome *c* binds to apoptosis activating factor 1 (Apaf-1) and causes formation of a complex known as the apoptosome (1) that consists of multiple molecules of cytochrome *c*, dATP, Apaf-1, and caspase-9. Formation of the apoptosome activates caspase-9, which then cleaves and activates other caspases that degrade many protein substrates, leading to cell death (41, 71). Several other proapoptotic proteins sequestered in mitochondria also redistribute into the cytoplasm during apoptosis. These include endonuclease G, Smac/Diablo, apoptosis inducing factor, and, perhaps, some caspases (7, 15, 40, 63).

Bax in neuronal apoptosis

Members of the Bcl-2 family of proteins can have pro- or antiapoptotic effects (38). Despite much research effort, the mechanism(s) by which these proteins influence cell survival remains unclear. Antiapoptotic family members, such as Bcl-2 and Bcl-x_L, localize primarily to the outer mitochondrial membrane (OMM) (48). These proteins function, in part, to retain cytochrome *c* and other apoptogenic factors within the mitochondrial intermembrane space. Proapoptotic family members, like Bax, reside primarily in the cytoplasm of healthy cells and translocate to the OMM during apoptosis (53, 69). Once at the OMM, these proteins promote release of apoptogenic factors from the mitochondria into the cytoplasm. Bax-induced release of mitochondrial factors is essential for the programmed cell death of many types of neurons (67). Bax deficiency blocks the apoptotic death of NGF-deprived sympathetic neurons and repolarized/serum-starved cerebellar granule neurons in culture (9, 12, 47).

The mechanism(s) by which Bax induces the release of apoptogenic factors from mitochondria in these and other cells is undetermined (45). Bax, like other Bcl-2 family members, is a channel-forming protein (59). Bax can form channels in liposomes that are large enough to allow passage of cytochrome *c* (57). However, it is unclear whether such channels function as routes of exit for cytochrome *c in vivo*. When associated with the OMM, Bax induces a high-conductance channel that may be large enough to allow passage of cytochrome *c* and other proteins from the mitochondrial intermembrane space into the cytoplasm (52). However, the conductance of these channels is much higher than those produced by Bax alone in lipid bilayers, suggesting that Bax may regulate the opening of other OMM pores and that these pores serve as the main route of egress for proapoptotic factors. One OMM channel regulated by Bcl-2 family proteins and implicated in cytochrome *c* release is the voltage-dependent anion conductance (VDAC) (65). VDAC may open to form a channel in the OMM that is large enough to allow cytochrome *c* and other agents to exit from the intermembrane space (43). Recent findings show that Bax itself can cause transit of large molecules across membranes containing the lipid cardiolipin (39). The underlying mechanism is unknown, but may involve formation of lipidic pores (2). The bulk of published data suggests at least two routes of exit for apoptogenic factors from mitochondria, direct Bax-induced OMM permeabilization and Bax-induced VDAC opening.

Free radicals in neuronal apoptosis

A free radical is any molecular species that has one or more unpaired electrons (23). ROS, which include superoxide (O_2^-), singlet oxygen, hydroxyl radicals, and nitric oxide, are the principal biological free radicals. The primary source of O_2^- is the mitochondrial electron transport chain where ~1–6% of O_2 consumed is reduced to O_2^- by leakage of electrons from the respiratory complexes (66). The O_2^- produced rapidly converts to hydrogen peroxide (H_2O_2) by the dismutation reaction catalyzed by the enzyme superoxide dismutase: $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. The H_2O_2 subsequently converts to H_2O by oxidation of the small tripeptide glutathione (GSH) via a reaction catalyzed by the enzyme glutathione peroxidase: $H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$. Reduction of the oxidized glutathione (GSSG) in a reaction catalyzed by glutathione reductase creates more GSH that then converts more H_2O_2 to H_2O. In this way, GSH neutralizes the potentially damaging effects of these ROS. The enzyme catalase also detoxifies H_2O_2. However, in neurons, this is a minor H_2O_2 detoxification pathway.

Hockenbery *et al.* (28), Kane *et al.* (32), and Ellerby *et al.* (17) demonstrated that Bcl-2 has an antioxidant effect and presented evidence that this effect is part of the means by which Bcl-2 blocks apoptosis. Their work was challenged by Jacobson and Raff (31) and Shimizu *et al.* (61), who showed that Bcl-2 blocks apoptosis in what was assumed to be anaerobic conditions where free radical production should be greatly reduced. However, the Jacobson and Shimizu articles made two assumptions that now appear erroneous. First, they assumed that Bcl-2 has only one site of action. Later work demonstrated that antiapoptotic Bcl-2 family proteins block apoptosis at multiple sites (6, 37, 56). Therefore, even if Bcl-2 promotes cell survival via an antioxidant mechanism, there may be other Bcl-2-influenced antiapoptotic pathways that do not involve free radicals. Second, and more important, the assumption that anaerobosis blocks free radical formation may be incor-

rect. Esposti and McLennan (18) demonstrated that cells produce large amounts of free radicals even in supposedly anaerobic conditions. Therefore, there is not yet an adequate understanding of the role of the redox effects of Bcl-2 family proteins in apoptotic death. Recent findings have led to resurgence of interest in the role of ROS in apoptotic death (50).

Sympathetic neurons induced to undergo apoptotic death by NGF deprivation and cerebellar granule cells induced to undergo apoptosis by membrane potential repolarization/serum starvation both show significantly increased levels of cellular ROS before becoming committed to die. These ROS lie downstream from Bax (35). Antioxidants and antioxidant enzymes (Cu/Zn superoxide dismutase) have potent antiapoptotic effects on these cells, suggesting a role for the ROS in cell death (16, 20, 21, 34, 60). Little is known about how ROS promote neuronal apoptosis. ROS may act as signaling molecules that initiate apoptosis by activating downstream death effectors rather than by direct damage to cellular components (58). Madesh and Hajnóczky (43) reported that O_2^- induces VDAC-dependent permeabilization of the OMM in HepG2 cells and that the induced conductance is great enough for passage of large amounts of cytochrome *c*. Therefore, ROS may signal VDAC to open and release mitochondrial contents, causing apoptosis.

BAX GENE DOSAGE DETERMINES ROS LEVELS DURING NEURONAL APOPTOSIS

We discovered that application of antioxidants to cultures of NGF-deprived sympathetic neurons not only inhibits apoptosis and increased ROS in those cells, but also blocks release of cytochrome *c* from the mitochondria into the cytoplasm (34). Additionally, we found that application of H_2O_2 to cultures caused rapid cytochrome *c* redistribution. Because Bax deletion also prevents apoptosis by preventing cytochrome *c* release, we decided to investigate the relationship between ROS and Bax in NGF-deprived neurons. We first determined levels of Bax protein in cultures of sympathetic neurons from *bax* wild-type ($bax^{+/+}$), *bax* hemizygous ($bax^{+/-}$), and *bax* knockout ($bax^{-/-}$) mice. We found that *bax* gene dosage determines Bax protein concentration in these cells (35). Bax protein was absent in cells from $bax^{-/-}$ mice and was at about half the wild-type level in neurons from $bax^{+/-}$ mice.

We used the redox-sensitive dye 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluoresceindiacetate (CM-H_2DCFDA) and confocal microscopy to do a detailed temporal analysis of cellular ROS levels in NGF-deprived sympathetic neurons having each *bax* genotype. A comparison in rat and mouse sympathetic neurons of several widely used redox-sensitive dyes revealed that CM-H_2DCFDA was far superior to the other dyes for this type of analysis (34). Figure 1 shows that the fluorescence of this dye increased after NGF withdrawal, indicating increased ROS levels. *bax* genotype determined the degree of this increase (linearly related to Bax concentration). Bax deletion completely prevented the ROS burst. We found a similar effect of Bax dosage on the ROS burst occurring during the apoptotic death of cerebellar granule neurons (35).

Depleting the mitochondrial electron transport chain of cytochrome *c* can augment mitochondrial ROS production (4).

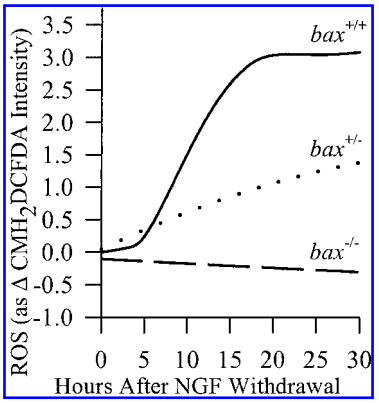

FIG. 1. Bax concentration determines ROS levels in NGF-deprived mouse sympathetic neurons. Withdrawal of NGF causes a greater increase of ROS in the somas of neurons taken from $bax^{+/+}$ animals than in neurons taken from $bax^{+/-}$ animals. ROS does not increase in NGF-deprived neurons from $bax^{-/-}$ mice. ROS levels are shown as fold increase in the intensity of the redox-sensitive dye, CM-H_2DCFDA, above baseline values (adapted from 34, 35).

Therefore, one possible explanation for the ROS increase after NGF deprivation was that Bax caused cytochrome *c* release and, secondarily, the ROS burst. However, a temporal analysis of cytochrome *c* redistribution indicated that increased ROS in both rat (34) and mouse (Fig. 2; 35) sympathetic neurons begins long before detectable cytochrome *c* redistribution and, therefore, ROS could not have been caused by extensive depletion of cytochrome *c* from the electron transport chain.

To determine whether mitochondria produced the ROS, we tested the effects of specific inhibitors of mitochondrial respiratory complexes on CM-H_2DCFDA intensity in NGF-deprived cells. The resulting data suggest that the primary source of ROS in both NGF-deprived rat and mouse sympathetic neurons is the mitochondrial electron transport chain. Specifically, the data suggest that electrons enter the chain at respiratory complex I and leak to O_2 to form O_2^- at either

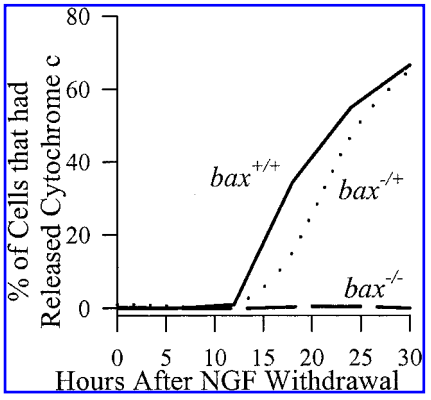

FIG. 2. Time course of release of cytochrome *c* from the mitochondria of NGF-deprived sympathetic neurons taken from $bax^{+/+}$ and $bax^{+/-}$ mice. Cells from $bax^{-/-}$ mice do not release cytochrome *c* (adapted from 34, 35).

complexes I, II, or III (34, 35). Therefore, the Bax-induced ROS derive from the electron transport chain, but do not appear to result from depleting the chain of cytochrome *c*.

ROS promote apoptosis of NGF-deprived neurons by causing cytochrome c *release*

Increasing cellular GSH levels blocks ROS, cytochrome *c* release, and apoptotic death after NGF deprivation (34, 35). A membrane-permeant form of GSH blocks death when applied to cultures up to the time of commitment to die, suggesting that ROS affect a late event in the apoptotic cascade. The main determining factor for death commitment in these cells is release of cytochrome *c* from mitochondria into the cytoplasm (12). Consistent with a role for ROS in cytochrome *c* release, we found that application of membrane-permeant GSH ethyl ester rapidly blocked cytochrome *c* redistribution in NGF-deprived cells (Fig. 3). We also determined whether an artificially induced prooxidant state could cause cytochrome *c* redistribution. Figure 4 shows that treatment of cultures with H_2O_2 induced rapid cytochrome *c* release. The cellular ROS measured in the H_2O_2 -treated cells were similar to those observed after NGF withdrawal (35). Surprisingly, we found that H_2O_2 was more effective at causing cytochrome *c* released in neurons from *bax*$^{-/-}$mice than in neurons from *bax*$^{+/+}$ mice. We have no ready explanation for this difference (GSH levels in *bax*$^{+/+}$ and *bax*$^{-/-}$ neurons were similar). However, the data clearly show that Bax is dispensable for release, that there is one or more OMM exit routes for cytochrome *c* that do not require Bax, and that the ROS levels after NGF deprivation are sufficient to cause cytochrome *c* redistribution.

To determine whether H_2O_2 might have nonspecifically damaged mitochondria, we tested mitochondrial integrity after H_2O_2 treatment. Electron microscopy showed no change in mitochondrial morphology after this treatment. One hundred

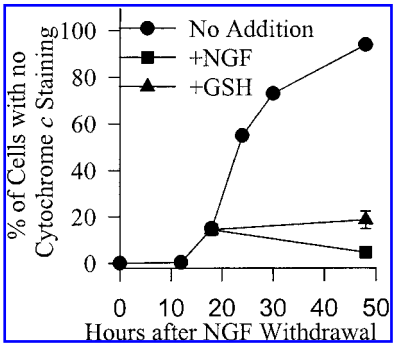

FIG. 3. Addition of NGF or GSH ethyl ester (10 m*M*) to NGF-deprived mouse sympathetic neurons immediately blocked cytochrome *c* release. Cultures were deprived of NGF and maintained in the caspase inhibitor, boc-aspartyl (OMe)-fluoromethylketone (BAF; 30 μ*M*), for 18 h to block death. At this time, medium containing BAF and either NGF or GSH ethyl ester was added to some cultures. Forty-eight hours after the initial withdrawal, cultures were immunostained and the percentage of cells with complete loss of cytochrome *c* determined by immunocytochemistry as described (34, 35). BAF treatment does not affect rate of cytochrome *c* release. $n = 101–159$ neurons. Error bars in this and the subsequent figures are SEM.

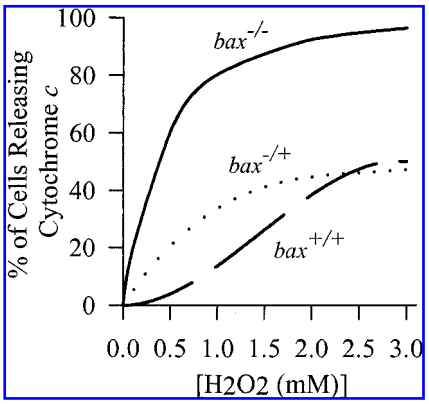

FIG. 4. A prooxidant state causes rapid release of cytochrome *c* from the mitochondria of mouse sympathetic neurons. Mitochondria in neurons having the indicated genotypes release all detectable cytochrome *c* within an hour of exposure to the indicated concentrations of H_2O_2. Surprisingly, H_2O_2 was more effective at causing release in *bax*$^{-/-}$ neurons than in *bax* wild-type or hemizygous neurons (adapted from 34, 35). There is, at present, no ready answer for this finding.

percent of cells had mitochondria that retained a membrane potential after H_2O_2 treatment. Washout of H_2O_2 followed by maintenance in NGF-containing medium rescued 100% of the cells and led to repletion of mitochondrial cytochrome *c* stores. These data indicate that the ROS did not cause gross, nonspecific damage to mitochondria that might lead to cytochrome *c* redistribution. Additionally, these, and other data that we published, suggest that the opening of a large inner mitochondrial membrane pore known as the mitochondrial permeability transition pore (mPTP) (22) does not occur during the apoptosis of either sympathetic neurons or cerebellar granule cells (34, 35, 68). The mPTP induces cytochrome *c* release by causing mitochondrial swelling and subsequent rupture of the OMM. Because mitochondrial swelling does not occur during the apoptotic death of both of these cell types, it is unlikely that mPTP is important for apoptogenic factor release in them.

Evidence that caspase activity has prooxidant effects during neuronal apoptosis

Tan *et al.* (64) found that a caspase inhibitor blocks increased ROS during the apoptotic death of a hippocampal cell line. To determine if a similar phenomenon occurs during apoptotic death of sympathetic neurons, we tested the effects of broad-spectrum caspase inhibitors on ROS levels in NGF-deprived cells. These inhibitors attenuated, but did not prevent, the ROS burst (Fig. 5). These data suggest that caspase activity contributes to the prooxidant state after NGF withdrawal. Ricci *et al.* (55) recently reported that caspases increase mitochondrial ROS production in several cell types. This effect is mediated by caspase attack on mitochondrial respiratory complexes or the proteins that regulate them. This finding may account for all of the attenuating effects of caspase inhibitors on cellular ROS levels although, at present, other prooxidant effects of caspases (*e.g.*, degradation of antioxidant proteins) cannot be excluded.

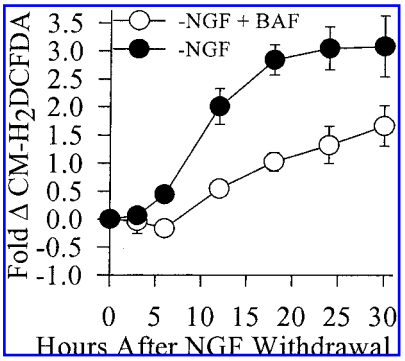

FIG. 5. Attenuation of increased ROS in NGF-deprived mouse sympathetic neurons by the broad-spectrum caspase inhibitor BAF. BAF (30 μ*M*) was added at the time of NGF deprivation. A similar effect of BAF was found in NGF-deprived rat sympathetic neurons (34). The broad-spectrum caspase inhibitor zVAD had a similar effect. $n = 38–152$ neurons.

The MLK/JNK signaling pathway in neuronal apoptosis

c-Jun N-terminal kinase (JNK) (11) is activated downstream of mixed-lineage kinases (MLK) (27) in NGF-deprived sympathetic neurons. Various forms of cellular stress, including ROS, activate JNK (3). However, Deckwerth *et al.* (10) reported that JNK is activated in NGF-deprived sympathetic neurons from *bax*$^{-/-}$ mice. We found no increase in ROS in NGF-deprived *bax*$^{-/-}$ neurons (35) and, like Deckwerth *et al.* (10), found that JNK was activated in these cells to a level equivalent to that observed in NGF-deprived *bax*$^{+/+}$cells. Therefore, JNK activation does not lie downstream from the Bax-induced ROS burst in these cells.

The MLK/JNK pathway contributes to the apoptotic death of NGF-deprived sympathetic neurons and other cell types in part by activating the transcription factor c-Jun (19, 24, 46). The MLK/JNK pathway causes up-regulation of BH3-only (death domain) proteins. These proteins, which are members of the Bcl-2 family of proteins, function to promote the translocation of Bax to the mitochondria (26, 29, 54). Ham *et al.* (25) reported that a dominant negative c-Jun inhibits release of cytochrome *c* from mitochondria during the apoptotic death of NGF-deprived sympathetic neurons. Based on our published data (34, 35), we propose that ROS promote apoptosis in neurons, at least in part, by acting as messenger molecules that cause release of apoptogenic factors from mitochondria. We suggest that the MLK/JNK pathway up-regulates BH3-only family proteins, causing Bax to associate with mitochondria and increase production of ROS. These ROS then induce or augment release of cytochrome *c* and other apoptogenic factors by inducing VDAC to open to a high-conductance state that allows their passage. The cytoplasmic cytochrome *c* then activates caspases that attack mitochondrial respiratory complexes, further increasing mitochondrial ROS production (Fig. 6).

CONCLUSIONS

A role for ROS in apoptotic death has been suspected for at least 10 years. Originally, it was suggested that ROS might contribute to death via direct damage to cellular components. Although it remains possible that some of the deleterious effects of ROS on apoptotic cells result from such damage, recent evidence suggests more subtle mechanisms. Specifically, ROS appear to act as a signaling molecule that can induce downstream apoptotic processes. In Bax-dependent neuronal apoptosis, a Bax-dependent ROS increase appears to be part of the mechanism by which Bax causes release of proapoptotic factors from mitochondria into the neuronal cytoplasm. The mechanism by which Bax causes increased ROS and how the ROS then cause release of mitochondrial apoptogenic fac-

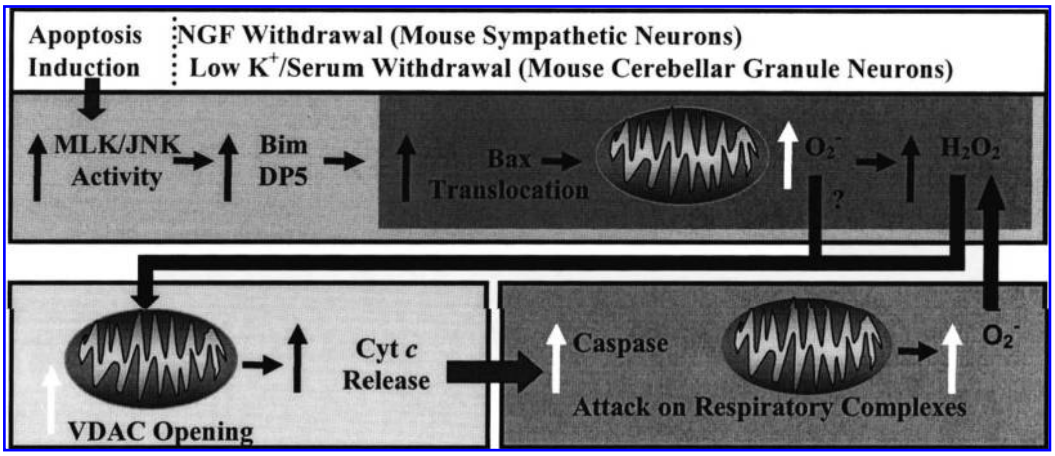

FIG. 6. Known and hypothesized events occurring in neuronal apoptosis. The white arrows indicate hypothesized events. We propose that apoptotic stimuli, such as NGF deprivation, cause activation of the MLK/JNK pathway in neurons. This pathway up-regulates BH3-only proteins, such as Bim and DP5, that cause Bax to translocate to the OMM and increase mitochondrial O_2^- production. Either the O_2^- or downstream ROS resulting from dismutation of O_2^- induces opening of VDAC, which acts as an exit route for release of cytochrome *c* from the mitochondrial intermembrane space. Cytoplasmic cytochrome *c* then activates caspases that attack mitochondrial respiratory complexes, which then produce more ROS in a positive feedback cycle.

tors is unknown, but may involve ROS-induced opening of VDAC (Fig. 6). Cytochrome *c* is released over a short period from all mitochondria in a cell. Such rapid release implies that a signal coordinates the redistribution from different mitochondria. We suggest that ROS may be the signal mediating this coordination.

ACKNOWLEDGMENTS

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ABBREVIATIONS

BAF, boc-aspartyl (OMe)-fluoromethylketone; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; JNK, c-Jun N-terminal kinase; MLK, mixed lineage kinase; mPTP, mitochondrial permeability transition pore; NGF, nerve growth factor; O_2^-, superoxide; OMM, outer mitochondrial membrane; ROS, reactive oxygen species; VDAC, voltage-dependent anion conductance.

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