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Postnatal brain development and neural cell differentiation modulate mitochondrial Bax and BH3 peptide-induced cytochrome *c* release

BM Polster[1,3], CL Robertson[1,2], CJ Bucci[1], M Suzuki[4] and G Fiskum*[,1]

[1] Department of Anesthesiology, University of Maryland School of Medicine, 685 W. Baltimore St., MSTF 5.34, Baltimore, MA 21201, USA
[2] Pediatrics, University of Maryland School of Medicine, 685 W. Baltimore St., MSTF 5.34, Baltimore, MA 21201, USA
[3] Neuroscience Program, in partial fulfillment of a Ph.D.
[4] Biochemistry Section, Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, 10 Center Drive, Room 5D-37, Bethesda, MD 20892, USA
* Corresponding author: G Fiskum, Department of Anesthesiology, University of Maryland, Baltimore, MSTF 5.34, 685 W. Baltimore St., Baltimore, MD 21201, USA. Tel: +1 410 706 4711; Fax: +1 410 706 2550; E-mail: gfisk001@umaryland.edu

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Abstract

Bax mediates cytochrome *c* release and apoptosis during neurodevelopment. Brain mitochondria that were isolated from 8-day, 17-day, and adult rats displayed decreasing levels of mitochondrial Bax. The amount of cytochrome *c* released from brain mitochondria by a peptide containing the BH3 cell death domain decreased with increasing age. However, approximately 60% of cytochrome *c* in adult brain mitochondria could be released by the BH3 peptide in the presence of exogenous human recombinant Bax. Mitochondrial Bax was downregulated in PC12S neural cells differentiated with nerve growth factor, and mitochondria isolated from these cells demonstrated decreased sensitivity to BH3-peptide-induced cytochrome *c* release. These results demonstrate that immature brain mitochondria and mitochondria from undifferentiated neural cells are particularly sensitive to cytochrome *c* release mediated by endogenous Bax and a BH3 death domain peptide. Postnatal developmental changes in mitochondrial Bax levels may contribute to the increased susceptibility of neurons to pathological apoptosis in immature animals.

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Abbreviations: NGF, nerve growth factor; BH, Bcl-2 homology

Introduction

Apoptosis plays a prominent role during the development of the nervous system by eliminating excess cells and ensuring the establishment of appropriate synaptic connectivity.[1,2] Bax knockout mice have been used to demonstrate that Bax is required for the cell death program in many populations of neurons.[3–7] The redistribution of Bax from the cytosol to the mitochondria triggers efflux of mitochondrial cytochrome *c* that initiates apoptosis in sympathetic neurons deprived of nerve growth factor (NGF), cerebellar granule neurons cultured in low potassium, and human SH-SY5Y neuroblastoma cells exposed to staurosporine.[8–10] Culturing of cerebellar granule neurons in low potassium or withdrawal of NGF from cultured sympathetic neurons induces the expression of the Bcl-2 homology (BH) 3 death-domain-containing Bcl-2 family proteins Bim and DP5 that kills cells by a mechanism dependent on Bax.[10–13]

In the cerebral cortex and cerebellum, Bax levels are high at birth and downregulated during development.[14] A total of 72 h NGF differentiation of PC12 neural cells was reported to decrease total cellular Bax.[14] Although a decrease of Bax during brain maturation has been described, the amount of Bax associated with the mitochondria during brain development has yet to be explored. Identification of changes in Bax expression and localization during postnatal brain maturation may help to explain the increased susceptibility of immature animals to apoptotic cell death following brain injury compared to mature animals.

We previously demonstrated that a BH3 death-domain peptide mimics the ability of endogenous BH3 domain-containing proteins to trigger Bax membrane insertion and the release of cytochrome *c* from mitochondria.[15] Forebrain mitochondria from mature rats did not release cytochrome *c* in response to BH3 peptide although release occurred from mitochondria within permeabilized cerebellar granule neurons and cortical astrocytes that were cultured from immature animals. In this study, we tested the hypotheses that: (1) unlike adult rat forebrain mitochondria, mitochondria from the brains of immature animals possess endogenous Bax and release cytochrome *c* in response to BH3 peptide and (2) NGF differentiation of neural cells decreases mitochondrially associated Bax protein and reduces the sensitivity of mitochondria to BH3-peptide-mediated cytochrome *c* efflux.

Results

Bax localizes to the mitochondria in the brains of immature but not mature animals

Previous experiments were unable to demonstrate detectable levels of Bax associated with mitochondria isolated from the

brains of mature rats.[15] These mitochondria also did not release cytochrome *c* following exposure to a 34 amino-acid peptide containing the BH3 cell death domain of Bcl-2 family proteins.[15] In contrast to mitochondria from adult rats, mitochondria isolated from 8- and 17-day animals possess significant immunoreactive 21 kDa Bax that comigrated with recombinant Bax protein, as shown by the immunoblots provided in Figure 1a. The level of Bax associated with the mitochondria decreased with increasing age of the animal, and was barely detectable in adults. Similar changes were observed in whole forebrain homogenates taken from the same animals. Although Bax was scarcely measurable in adult brain mitochondria, it was readily apparent in adult brain tissue homogenates (Figure 1a). A Bax immunoreactive band at approximately 50 kDa was detected in mitochondria that increased with age; however, because this band did not comigrate with the Bax dimer at 42 kDa present in the standard and was not detected by another Bax antibody (data not shown), it most likely represents nonspecific immunoreactivity.

The inner membrane protein cytochrome oxidase subunit I (COX I) and the outer membrane protein voltage-dependent anion channel (VDAC) were detected on the same immunoblot in an attempt to normalize the amount of Bax in the mitochondria. The levels of these proteins increased during development (Figure 1a). Thus, both the absolute and relative levels of brain mitochondrial Bax declined greatly during the first two months of postnatal development. The mitochondrial level of the Bax homologue Bak also decreased during development (Figure 1b), however, to a lesser extent than that of Bax.

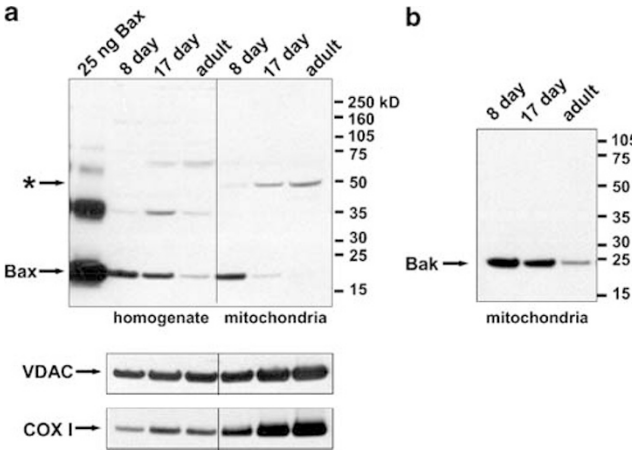

Figure 1 Rat forebrain mitochondrial Bax and Bak levels during postnatal development. **(a)** Mitochondrial suspensions isolated from 8-day old, 17-day-old, and adult rat forebrains (30 μg protein) and from unfractionated forebrain homogenates (100 μg protein) were subjected to gel electrophoresis on a single gel and levels of Bax were determined by immunoblot. VDAC and COX I levels were also assessed by immunodetection following stripping and reprobing of the same membrane. A Bax immunoreactive band in the mitochondria that likely represents non-specific immunoreactivity is designated by *. **(b)** Isolated brain mitochondria (30 μg protein) were assessed for Bak by immunoblot. Data are representative of two independent experiments

Mitochondria isolated from the brains of immature rats release cytochrome c in response to BH3 peptide

Mitochondria isolated from 8- and 17-day-old and adult rats were treated with BH3 peptide (60 μM) and mitochondrial suspensions were analyzed for cytochrome *c* release by enzyme-linked immunosorbant assay (ELISA). Results are expressed as the mean percentage ($\pm$ S.E.) of cytochrome *c* that was present in the supernatant compared to the total in the supernatant plus pellet. A significant increase in cytochrome *c* release following a 16-min exposure to BH3 peptide compared to timed control incubations was detected (Figure 2, two-way ANOVA, $P<0.05$). The use of Tukey's multiple comparison's test to examine differences among age groups revealed a significant interaction between treatment and age. When background cytochrome *c* release was subtracted from release observed in the presence of the BH3 peptide, the average total release observed for 8-day-old, 17-day-old, and 2-month-old rats was 14, 10, and 4%, respectively.

Reconstitution experiments using mitochondria from adult rats were performed to provide support for the hypothesis that the developmental reduction in mitochondrial Bax is responsible for the associated diminution of cytochrome *c* release evoked by the BH3 peptide. Isolated adult brain mitochondria were exposed to human full-length recombinant Bax (100 nM) alone or BH3 peptide (60 μM) plus exogenous Bax. ELISA measurements of cytochrome *c* release provided no evidence that either BH3 peptide (see Figure 2) or exogenous Bax alone released substantial amounts of cytochrome *c* (Figure 3). However, in the presence of BH3 peptide, robust cytochrome *c* release was observed in the presence of full-length recombinant Bax.

NGF differentiation of PC12S cells decreases mitochondrial Bax levels and sensitivity to BH3 peptide-induced cytochrome c release

An *in vitro* cell differentiation paradigm was used to further test the hypothesis that sensitivity to cytochrome *c* release by BH3 peptide is dependent on the presence of endogenous Bax. Undifferentiated PC12S cells were treated for 72 h with NGF to produce a sympathetic neuron-like phenotype that was confirmed by morphology.[14,16,17] Mitochondria from NGF-differentiated PC12S cells contained less endogenous Bax than mitochondria from undifferentiated cells (Figure 4). The level of the antiapoptotic Bcl-2 family protein Bcl-X_L also decreased; however, the level of the Bax homologue Bak was unchanged. Mitochondria from NGF-differentiated PC12S cells displayed a decreased sensitivity to cytochrome *c* release in response to BH3 peptide compared to mitochondria from the undifferentiated cells, despite the finding that they contain less antiapoptotic Bcl-X_L protein (Figure 5a). ELISA measurements indicated a near complete loss of cytochrome *c* release from undifferentiated PC12S cells following treatment with the BH3 peptide at 0.5 μM, whereas release of cytochrome *c* from differentiated cells was incomplete at a 10-fold higher peptide concentration (Figure 5b). Thus, the sensitivity of mitochondria to BH3 peptide-induced release of cytochrome *c* correlated with the amount of Bax that was associated with the isolated mitochondria.

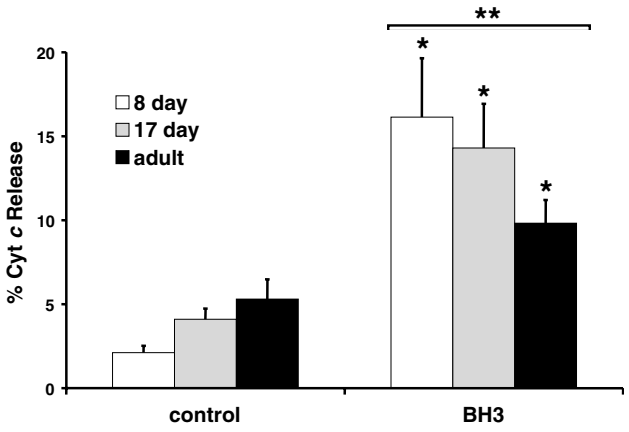

Figure 2 Release of cytochrome *c* from brain mitochondria isolated from 8-day old, 17-day-old, and adult rats released in response to BH3 peptide. Brain mitochondria (0.25 mg/ml) isolated from 8-day-old (n=3), 17-day-old (n=3), or adult (>6 weeks, n=6) rats were incubated at 30°C in KCl media supplemented with 5 mM succinate, 2 μM rotenone, 4 mM $MgCl_2$, 3 mM ATP, and 250 μM EGTA. After 2 min of incubation, BH3 peptide (60 μM) or vehicle control was added. After 16 min of additional incubation, the suspension was centrifuged at 13 400 × *g* for 5 min and cytochrome *c* (Cyt *c*) was detected in the supernatant (sup) and pellet (pel) fractions by ELISA. Cytochrome *c* release is expressed as the mean percentage (± S.E.) of total Cyt *c* that was present in the supernatant compared to the total in the supernatant plus pellet. * Significant difference between control and BH3 peptide treatment across groups. ** Significant effect of age on this difference (two-way ANOVA, $P<0.05$)

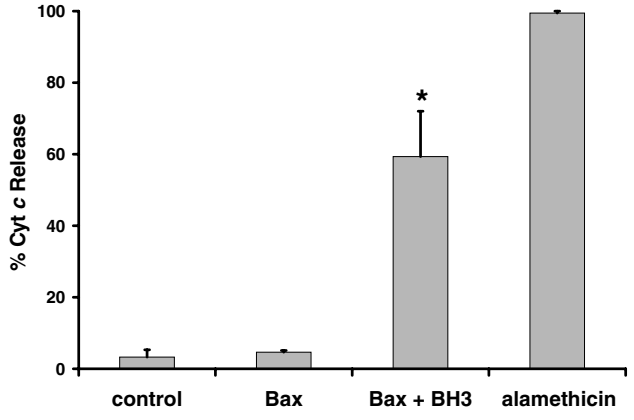

Figure 3 Release of cytochrome *c* from brain mitochondria isolated from mature rats in the presence of BH3 peptide and exogenous full-length Bax. Mitochondria (0.25 mg/ml) from adult (2 to 3-month-old) rat forebrains were incubated under the conditions described in Figure 2. Full-length recombinant Bax (100 nM) was present from the start of the incubation period when indicated; otherwise, vehicle control was present. BH3 peptide (60 μM) or vehicle control was added at 2 min as indicated and cytochrome *c* release was assessed after 16 min of additional incubation as described for Figure 2. *Significant difference between control and Bax+BH3 peptide (one-way ANOVA with Tukey's *post hoc* test, n=4, $P<0.05$). No significant difference was found between control treatment and Bax alone

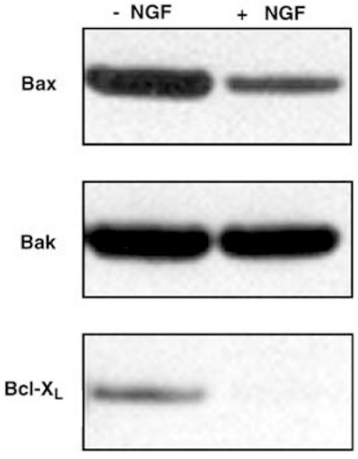

Figure 4 Mitochondrial Bax, Bcl-X_L, and Bak levels for undifferentiated and NGF-differentiated PC12S cells. Mitochondria were isolated from undifferentiated or NGF-differentiated PC12S cells and mitochondrial suspensions (30 μg protein) were subjected to gel electrophoresis and immunodetection for Bax. Bak and Bcl-X_L were detected on the same immunoblot following stripping and reprobing. Data are representative of two independent experiments

Discussion

This study is the first to demonstrate that brain mitochondria isolated from normal, immature animals (8- and 17-day old) possess endogenous Bax while Bax was barely detectable in mitochondria from adult rats (2 to 3-months old) (Figure 1). Since more total Bax protein was present in brain homogenates from immature *versus* mature rats, the detection of Bax at the mitochondria in immature rat brains could simply be the result of the normal proportional distribution of Bax within the cell. It was found in Cos-7 cells transfected with Bax-GFP that approximately 20% of the Bax fluorescence colocalized with mitochondrial staining in nonapoptotic cells.[18] Alternatively, a mechanism may exist for the increased targeting of Bax to the mitochondria in the brains of immature animals.

Previous evidence suggested that mitochondrial Bax is required for the release of cytochrome *c* by a BH3 death domain peptide that models the physiological action of proapoptotic BH3-only Bcl-2 family proteins, e.g., tBid and Bim.[15] Consistent with this interpretation, immature brain mitochondria released substantially more cytochrome *c* in response to BH3 peptide treatment than did mature brain mitochondria (Figure 2). Moreover, 60% release of cytochrome *c* was induced from adult brain mitochondria by BH3 peptide when they were also exposed to 100 nM full-length recombinant Bax, demonstrating the ability of exogenous Bax to reconstitute the phenotype of BH3 peptide-mediated cytochrome *c* release that was lost with the reduction in endogenous Bax (Figure 3). Isolated rat forebrain mitochondria consist of both astrocytes and neurons, and it is not known whether the relative contribution of mitochondria from these cell types to the mitochondrial preparation changes with age of the animal. It is therefore possible that the changes in mitochondrial Bax levels and sensitivity to BH3 peptide during postnatal development are a result of a shift in the heterogeneity of the mitochondrial preparation. However, in a previous study, we confirmed that the BH3 peptide was capable of releasing cytochrome *c* from both cultured astrocytes and neurons,[15] suggesting that brain maturation rather than changes in the heterogeneity of the preparation of forebrain mitochondria was more likely to account for the alteration in BH3 peptide sensitivity. The proportion of undifferentiated *versus* mature cells that are represented in

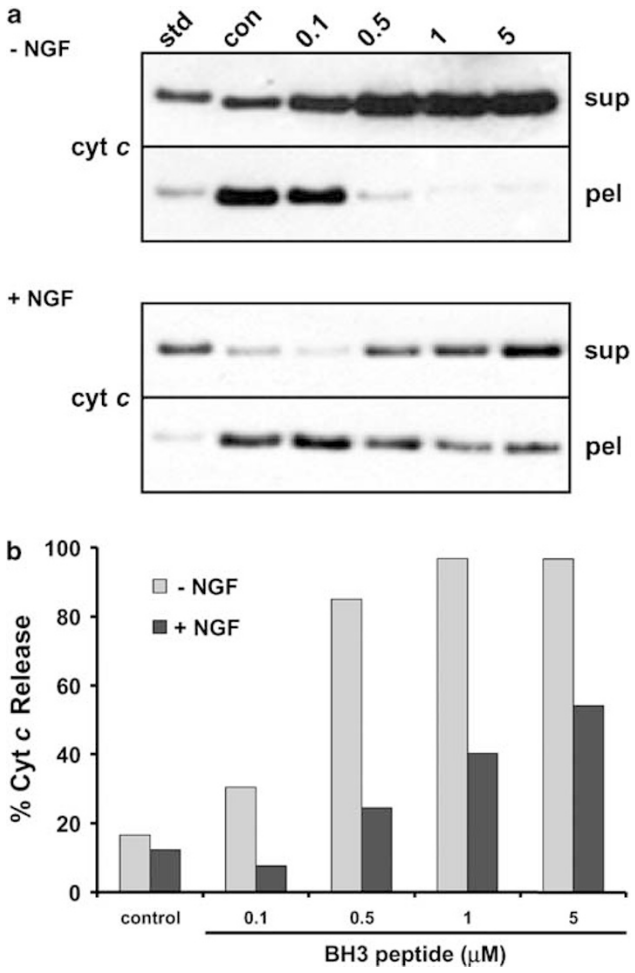

Figure 5 BH3 peptide-induced cytochrome *c* release by mitochondria from undifferentiated and NGF-differentiated PC12S cells. Mitochondria from undifferentiated (−NGF) or NGF-differentiated (+NGF) PC12S cells (0.5 mg/ml) were incubated under the conditions described in Figure 2 for immature brain mitochondria except that the incubation time was shortened from 16 to 8 min to minimize spontaneous release of cytochrome *c*. Release was detected by immunoblot **(a)** and also quantified by ELISA **(b)** as described under Materials and Methods. **(a)** cytochrome *c* standards (std) (4 ng) are shown to approximately equal exposure levels for – NGF *versus* +NGF treated PC12S cells for direct comparison. *Con*, control. Data are representative of two independent experiments

the forebrain mitochondrial population may determine the amount of mitochondrial Bax present and, thus, the associated amount of cytochrome *c* release that can be attained upon BH3 peptide treatment.

To further address this possibility, we examined the effect of differentiation on mitochondrial Bax levels and BH3 peptide-induced cytochrome *c* release in a homogeneous cell population. Upon differentiation of PC12S cells into a sympathetic neuron-like phenotype, a decrease in the level of mitochondrial Bax was observed (Figure 4) and mitochondria became less sensitive to the release of cytochrome *c* by BH3 peptide (Figure 5). Although Bak is also known to mediate cytochrome *c* release in mitochondria from some cell types,[6,19,20] the level of mitochondrial Bak was unaltered by NGF differentiation (Figure 4), suggesting that Bax is the required target for BH3 peptide-induced cytochrome *c* release in PC12 cell mitochondria. This finding is in agreement with the requirement for Bax, but not Bak, in sympathetic neuron cell death induced by NGF withdrawal.[21] However, we cannot exclude a potential role for Bak or related molecules in the brains of immature animals in response to different death stimuli *in vivo*.

The induction of neuronal apoptosis is a multistep process that includes the redistribution of Bax from the cytosol to the mitochondria, and the expression and/or translocation of proapoptotic BH3-only molecules.[21] The finding that mitochondria from brains of immature rats contain associated Bax and release cytochrome *c* in response to BH3 peptide may be important for the development of neuroprotective treatment strategies for pediatric brain injury. The elevated amount of Bax in these animals and the localization of Bax to the mitochondria suggest that immature animals may be more susceptible or 'primed' to undergo apoptotic cell death during brain injury. In support of this hypothesis, numerous studies using animals models have demonstrated prolonged, apoptotic neuronal death in the developing brain after hypoxic–ischemic injury,[22–25] with evidence for a particularly important role for caspase-3 in neuronal death in immature rats.[26] The contribution of apoptosis to cell death in mature rats using similar animal models is less clear; however, apoptosis appears to play a more pronounced role in immature when compared directly to mature rats.[24,27] Apoptosis may also play a dominant role in neural cell death following head trauma in immature animals.[28,29] Future investigations will focus on testing the hypothesis that cells with endogenous Bax localized to the mitochondria display increased susceptibility to apoptotic stimuli by circumventing the requirement for Bax translocation. Although it is difficult to isolate mitochondria from individual brain regions, it is also of interest to determine whether brain areas that have different sensitivities to pathological apoptosis exhibit similar changes in mitochondrial Bax levels and cytochrome *c* release.

In summary, elevated levels of proapoptotic mitochondrial Bax and increased susceptibility of immature brain mitochondria to cytochrome *c* release by BH3 death domain peptide compared to adult brain mitochondria may contribute to the apparent increased susceptibility of the developing brain to pathological apoptotic cell death following ischemic or traumatic injury. Although evidence supports the role of Bax and BH3 domain-only proteins, e.g., Bid, in acute brain injury of both adult and immature animals, the specific importance of different levels of endogenous mitochondrial Bax in promoting apoptosis requires verification. As an approach to this question, efforts are underway to determine if the lipid or protein composition of the outer membrane of brain mitochondria from immature animals or undifferentiated cells is altered in ways that promote the association of Bax and therefore Bax-mediated cytochrome *c* release and apoptosis.

Materials and Methods

Materials

Rat forebrain mitochondria were isolated from 8-day, 17-day, or adult (2 to 3-month-old) Sprague–Dawley rats according to a modification of the procedure of Rosenthal *et al.*,[30] yielding a combination of both

nonsynaptosomal and synaptosomal mitochondria. The nonspecific protease mixture nagarse was found to degrade mitochondrial Bax and was therefore excluded from the procedure. PC12S mitochondria were isolated according to the method of Moreadith and Fiskum[31] with slight modifications. The BH3 peptide spanned amino acids 53–86 of Bax (^{53}DASTKKLSECLKRIGDELDSNMELQRMIAAVDTD$_{86}$) and was synthesized by the Wadsworth Center Biochemistry and Peptide Synthesis Core using an Applied Biosystem 431A automated peptide synthesizer as previously described.[32] The peptide was prepared as dilute (15 μM–15 mM) stock solutions in distilled, deionized water just free to use. Full-length recombinant Bax was isolated as described.[33] Bax was stored as a 0.1 μg/ml stock in 100 mM NaCl, 20 mM Tris-HCl, pH 8.0 at 4°C. Other chemicals were obtained from Sigma Chemical Company, and all reagents were of the highest grade available.

Cell culture

PC12S cells, a morphological variant of rat pheochromocytoma PC12 cells that retain the ability to grow in tissue culture without poly-L-lysine treatment, were maintained as described.[17] Differentiation of PC12S cells was achieved by adding nerve growth factor (50 ng/ml) to the cell culture medium and treating the cells for 72 h as described.[14] The culture medium was replaced with fresh medium containing NGF every 24 h.

Determination of cytochrome c release

Isolated PC12S mitochondria (0.5 mg/ml) or rat brain mitochondria (0.25 mg/ml) were incubated in 0.25 ml of KCl assay medium (125 mM KCl, 2 mM P_i, 20 mM HEPES, pH 7.0) supplemented with 4 mM $MgCl_2$, 3 mM ATP, 0.8 mM ADP, 250 μM EGTA, 5 mM succinate, and 2 μM rotenone. For adult brain mitochondria, Bax (1–2 μg/ml) or vehicle control (100 mM NaCl, 20 mM Tris-HCl, pH 8.0) was also included where indicated. BH3 peptide or vehicle control (water) was added after 2 min of incubation. At 8 min (PC12S mitochondria) or 16 min (brain mitochondria) following the addition of BH3 peptide or vehicle control, mitochondria were pelleted by centrifugation at 13 400 $\times$ *g* for 5 min, and the supernatant and pellet were assayed for the presence of cytochrome *c* by immunoblot using enhanced chemiluminescence as described.[34] A longer incubation time for brain mitochondria was required because the kinetics of cytochrome *c* release from adult brain mitochondria in the presence of exogenous Bax were slower, likely because of a requirement for Bax association prior to activation by BH3 peptide. For quantitative comparisons, cytochrome *c* release was also determined using an ELISA kit (R&D Systems) according to the instructions of the manufacturer.

Detection of proteins in mitochondria and brain homogenates by immunoblot

A 1 ml aliquot of tissue homogenate from 8-day-old, 17-day-old, and adult rat forebrain was taken from the initial step of the mitochondrial isolation procedure and stored at -20°C. Protein concentrations of homogenates and mitochondria were determined by the Biuret assay and 100 μg of homogenate and 30 μg of mitochondria from the same animal were loaded on a single gel for immunodetection of Bax, Bak, cytochrome oxidase subunit I (COX I), and VDAC by enhanced chemiluminescence. Mitochondria from undifferentiated and NGF-differentiated PC12S cells (30 μg) were also compared on a single gel for Bax, Bak, and Bcl-X_L. Dilutions of antibodies was as follows: 1 : 500 anti-Bax (Upstate), 1 : 500 anti-Bak (Upstate), 1 : 500 anti-Bcl-XL (PharMingen), 1 : 2000 anti-Porin (Calbiochem), 1 : 2000 anti-COX I (Molecular Probes). A 15 min incubation in Restore Western Blot Stripping Buffer (Pierce) at 37°C with agitation was used for the stripping and reprobing of blots.

Statistical analysis

A two-way analysis of variance (ANOVA) with Tukey's *post hoc* test was utilized to determine statistical differences in cytochrome *c* release between control and BH3 peptide treatment among the different age groups (8-day, 17-day-old, and adult). A significant interaction between the two factors (treatment and age) was detected. A one-way ANOVA with Tukey's *post hoc* test was applied to examine statistical differences in cytochrome *c* release among control, Bax, and Bax+BH3 peptide treatments in adult brain mitochondria. In all cases, $P<0.05$ was considered significant.

Acknowledgments

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