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Postnatal Developmental Regulation of Bcl-2 Family Proteins in Brain Mitochondria

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Abstract

Although it has been long recognized that the relative balance of pro- and antiapoptotic Bcl-2 proteins is critical in determining the susceptibility to apoptotic death, only a few studies have examined the level of these proteins specifically at mitochondria during postnatal brain development. In this study, we examined the age-dependent regulation of Bcl-2 family proteins using rat brain mitochondria isolated at various postnatal ages and from the adult. The results indicate that a general down-regulation of most of the proapoptotic Bcl-2 proteins present in mitochondria occurs during postnatal brain development. The multidomain proapoptotic Bax, Bak, and Bok are all expressed at high levels in mitochondria early postnatally but decline in the adult. Multiple BH3-only proteins, including direct activators (Bid, Bim, and Puma) and the derepressor BH3-only protein Bad, are also present in immature brain mitochondria and are down-regulated in the adult brain. Antiapoptotic Bcl-2 family members are differentially regulated, with a shift from high Bcl-2 expression in immature mitochondria to predominant Bcl-x_L expression in the adult. These results support the concept that developmental differences in upstream regulators of the mitochondrial apoptotic pathway are responsible for the increased susceptibility of cells in the immature brain to apoptosis following injury.

Keywords

Bcl-2; BH3-only; brain; mitochondria; apoptosis; immature

Developmental differences in energy metabolism, glutamate excitotoxicity, response to oxidative stress, and susceptibility to apoptosis distinguish the immature brain response to injury from that of the adult (Vannucci and Hagberg, 2004; Blomgren and Hagberg, 2006; Robertson et al., 2006). Extensive experimental evidence indicates that the involvement of apoptosis in acute neurodegeneration is age dependent (Johnston et al., 2001, 2002; Vannucci and Hagberg, 2004; Northington et al., 2005). More prominent activation of caspase-3 and induction of apoptosis are reported for the immature relative to the adult brain following both traumatic brain injury (Bittigau et al., 1999; Pohl et al., 1999) and ischemia/reperfusion (Liu et al., 2004; Zhu et al., 2005). A potential mechanism is indicated by reports of increased level of expression of the apoptosome component Apaf-1 and of caspase-3 in the immature relative to adult brain (Yakovlev et al., 2001). Developmental regulation of the mitochondrial apoptotic pathway at other levels is also reported. The release of apoptogenic proteins cytochrome c (Cy

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C) and AIF from mitochondria following hypoxia/ischemia is also age dependent, being more pronounced in the immature than in the adult brain (Zhu et al., 2005). We have also shown previously that mitochondria isolated from immature but not adult brain are particularly sensitive to Bax-BH3-peptide-induced Cyt C release and demonstrated that one mechanism involved in this effect is the increased association of Bax with immature brain mitochondria (Polster et al., 2003). These studies, suggest that developmental changes that occur upstream in the apoptotic pathway, particularly expression of mitochondria-localized Bcl-2 family proteins, also play a critical role in regulating the susceptibility to apoptosis in the immature brain through modulating the release of apoptogenic proteins that activate both caspase-dependent (i.e., Cyt C) and caspase-independent death mechanisms (e.g., AIF; Polster et al., 2003; Zhu et al., 2005).

The Bcl-2 family consists of pro- and antiapoptotic proteins characterized by the presence of at least one of the four Bcl-2 homology (BH) domains. Proapoptotic proteins are subdivided into multidomain (BH1–3; Bax, Bak, and Bok) and BH3-only proteins (Bid, Bim, Bad, Bmf, Dp5/Hrk, Puma, Noxa, Bik, Bnip3) sharing only the amphipathic α -helical BH3 region (Adams and Cory, 1998; Gross et al., 1999; Huang and Strasser, 2000). Bcl-2 proteins regulate the permeability of the outer mitochondrial membrane (OMM) and the release of apoptogenic proteins (e.g., Cyt C, AIF, EndoG, Smac/Diablo) from the mitochondrial intermembrane space through mechanisms involving mitochondrial relocation of proapoptotic members (e.g., Bax, BH3-only proteins) and a number of protein–protein interactions at the OMM. The OMM permeabilization is triggered by Bax/Bak in cooperation with BH3-only proteins and is inhibited by antiapoptotic members (e.g., Bcl-2, Bcl-x_L, Mcl-1, Bcl-w). Two models are proposed to explain the interactions among Bcl-2 family proteins that lead to Bax/Bak oligomerization and OMM permeabilization. The hierarchical activation model based on the model proposed by Letai et al. (2002) indicates that binding of derepressor BH3-only proteins (e.g., Bad, Noxa) to antiapoptotic Bcl-2 proteins results in displacement of direct activator BH3-only (Bid, Bim, and Puma), the only proteins that can directly interact with Bax/Bak and induce their oligomerization in the OMM (Letai et al., 2002; Kuwana et al., 2005; Kim et al., 2006). A second model holds that any BH3-only protein or combination that is sufficient to bind and neutralize all antiapoptotic Bcl-2-like proteins present in mitochondria will displace a “primed” or preactivated Bax/Bak protein from antiapoptotic members and will induce OMM permeabilization (Chen et al., 2005). In this model, the strong apoptotic activity of Bid, Bim, and Puma correlates with their ability to bind all antiapoptotic Bcl-2 family members and not direct interaction with Bax/Bak.

Although it has been long recognized that the relative balance of pro- vs. antiapoptotic Bcl-2 proteins at the mitochondria is critical for OMM permeabilization and commitment to apoptosis, only a few studies have examined the relative levels of Bcl-2 proteins present in mitochondria during postnatal brain development. Using mitochondria isolated at various postnatal ages and from the adult brain, we examined in this study the developmental regulation of mitochondrial Bcl-2 family proteins. Our results support the concept that developmental differences in the level of mitochondrial Bcl-2 proteins regulate the susceptibility of brain cells to initiate apoptosis following injury.

MATERIALS AND METHODS

Antibodies and Reagents

Reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated. The following antibodies were used in this study: mouse monoclonal anti-Bcl-2 (sc-7382, clone B2; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-Bcl-x_{L/S} (No. 14-9701-63; eBioscience, San Diego, CA), rabbit polyclonal anti-Bcl-x_L (sc-7195, H-62; Santa Cruz), rabbit polyclonal anti-Mcl-1 (sc-718; Santa Cruz Biotechnology), rabbit polyclonal anti-

Bax (No. 06-499; Upstate, Chicago, IL), rabbit polyclonal anti-Bak (No. 06-536; Upstate), rabbit polyclonal anti-Bok (No. 4521; Cell Signaling Technology, Danvers, MA), rabbit polyclonal anti-Bid (sc-11423, FL-195; Santa Cruz Biotechnology), anti-Bim (No. 4582 from Cell Signaling; No. 14-6265 from eBioscience), mouse monoclonal anti-Bad (No. 336420; BD Transduction Laboratories, San Jose, CA), mouse monoclonal anti-VDAC (No. 529534; Calbiochem, La Jolla, CA), goat polyclonal anti-Puma (sc-19187, N-19; Santa Cruz Biotechnology), goat polyclonal anti-Noxa (sc-11718, P-19; Santa Cruz Biotechnology), anti-AIF (sc-9416, D-20; Santa Cruz Biotechnology), and anticytochrome C (No. 556433; Pharmingen, San Diego, CA). The OXPHOS Western blotting kit containing a mixture of monoclonal antibodies (Mitosciences, Eugene, OR) was used to examine the relative levels of several subunits of the mitochondrial oxidative phosphorylation complexes I–V.

Isolation of Mitochondria

Mitochondria were isolated at different postnatal ages [postnatal day (P) 3, P7, P14, P21, and P31 \pm 1 day] and from adult (P60–P90) Sprague-Dawley rat forebrains, according to the procedure described in (Polster et al., 2003), yielding a mixture of synaptosomal and nonsynaptosomal mitochondria. All animal protocols have been approved by the University of Maryland Institutional Animal Care and Use Committee. Mitochondria were isolated separately from three to six animals for each age, and the level of mitochondrial proteins was examined by immunoblotting. After the initial homogenization step of the rat forebrains, an aliquot of total brain homogenate was collected and stored at -70°C for subsequent analysis of total brain levels of various proteins. The protein concentration of isolated mitochondria and brain homogenates was determined by using the bicinchoninic acid assay method (Pierce, Rockford, IL).

Western Blot Analysis

For analysis of protein expression, the isolated mitochondria and brain homogenates were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (30 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM DTT, 2 mM MgCl_2 , 1 mM NaVO_4) containing a mixture of protease inhibitors (Protease Inhibitor Cocktail Set I; Calbiochem). Equal amounts of mitochondria (25–50 μg) or total brain lysate (75 μg) from each sample were separated by SDS-PAGE on 4–12% Novex Bis-Tris gels (Invitrogen, Carlsbad, CA) and then transferred to nitrocellulose membranes. The membranes were blocked overnight in Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) and 0.1% Tween-20, then incubated with the primary antibodies (1 hr at room temperature or overnight at 4°C), followed by washing and incubation with the corresponding HRP-conjugated secondary antibodies (Pierce, Rockford, IL). For subsequent detection of voltage-dependent anion channel (VDAC) and other proteins, the membranes were stripped with the Restore Western Blot Stripping Buffer (Pierce). Detection of the immunoreactive bands was performed with an enhanced chemiluminescence system (GE Healthcare Bio-Sciences, Piscataway, NJ). Densitometric analysis of band intensities was performed with Image J (1.37) software gel analysis tools (<http://rsb.info.nih.gov/ij/index.html>). The relative level of each Bcl-2 family protein was normalized to the mitochondrial protein VDAC detected on the same membrane, and the relative band intensity were expressed as percentage of band intensities at P3.

Statistical Analysis

The results of Western blot quantification are expressed as mean \pm SEM. The statistical significance of the results was assessed either by Student's *t*-test when comparing two groups or, as indicated, by one-way analysis of variance (ANOVA) followed by Tukey post hoc test for multiple group comparisons. Data expressed as percentage were square root transformed prior to analysis, because this tended to result in a more normal distribution. The SigmaStat

3.0 software (Systat Software, Inc., Point Richmond, CA) was used, with $P < 0.05$ considered as significant.

RESULTS

Since mitochondrial developmental changes are likely to affect the expression of multiple proteins, a preliminary analysis was performed to identify a candidate for normalization of protein levels in isolated mitochondria. Several non-Bcl-2 family proteins including subunits of the oxidative phosphorylation complexes I–V and the outer membrane protein VDAC were examined for developmental changes by immunoblotting of equal amounts of mitochondria isolated from the immature (P3, P7, P14, P21, P31) or adult rat forebrain. As shown in Figure 1A,C, the levels of the COX II subunit of the OXPHOS complex IV increases postnatally, and a similar trend is noted for the core 2 subunit of complex III. In contrast, the levels of two other proteins, the Ip 30-kDa subunit of the OXPHOS complex II and the F1 α subunit of the F1F0 ATP-ase (complex V), are only marginally increased during the early postnatal period (P3–P14). Complex I was not detected with standard exposure time. The expression of VDAC examined on the same blots did not significantly change during post-natal development (Fig. 1A,C). VDAC and F1 α were subsequently used for normalizing the expression of individual Bcl-2 family proteins. We also examined the expression of AIF, another non-Bcl-2 family apoptotic protein. AIF is also expressed in mitochondria at relatively constant levels during postnatal development (Fig. 1B,C), and no significant changes were detected when its levels were expressed as ratio to VDAC (not shown).

Antiapoptotic Bcl-2 and Bcl-x_L Are Differentially Regulated in Mitochondria During Postnatal Brain Development

The postnatal developmental regulation of several antiapoptotic Bcl-2 family proteins was examined at the same ages in isolated brain mitochondria. The rat Bcl-2 protein was detected as a single band migrating at ~26 kDa. The rat Bcl-x_L was detected as a doublet of approximately 29–30 kDa. As shown in Figure 2A, the antiapoptotic Bcl-2 protein is expressed at high levels in brain mitochondria early postnatally (P3–P7). A decreasing trend in the mitochondrial Bcl-2 protein level is observed with increasing age and a statistically significant change is noted after the first 2 postnatal weeks. Bcl-2 is still detected in adult brain mitochondria at 20% \pm 5.9% of the P3 level (Fig. 2A). Mitochondrial Bcl-x_L was easily detectable at all ages during postnatal life to adulthood (Fig. 2B). Bcl-x_L protein level in mitochondria remains relatively constant during postnatal development. A decreasing trend ($P = 0.075$) is noted in the adult to 63.4% \pm 23.7% of the P3 level. The same antibody (anti-Bcl-x_{L/S}) also detected a lower molecular weight band corresponding to the proapoptotic Bcl-x_S isoform that was up-regulated with increasing age (Fig. 2B). By using another antibody, an additional Bcl-x-immunoreactive band whose levels increase during postnatal brain development was detectable at ~27 kDa and likely represents the Bcl-x β isoform (Gonzalez-Garcia et al., 1994; not shown). Mcl-1, another antiapoptotic Bcl-2 family member, was also reported to be expressed in the mouse brain (Mori et al., 2004); however, we were unable to identify Mcl-1 clearly in isolated rat brain mitochondria (not shown).

Adult Brain Mitochondria Are Deficient in All Multidomain Proapoptotic Bcl-2 Proteins

Multidomain proapoptotic Bcl-2 proteins include Bax, Bak, and the less well characterized protein Bok. Whereas Bax is considered to be localized predominantly in the cytosol in healthy cells, it can also loosely associate with mitochondria (Polster et al., 2003). Bak is a constitutive membrane protein localized at mitochondria and other intracellular membranes (e.g., endoplasmic reticulum), and Bok is also thought to localize primarily at mitochondria.

The expression of Bax, Bak, and Bok was examined in isolated rat brain mitochondria as described above. Confirming previous results, high levels of Bax (~21 kDa) were associated with brain mitochondria early postnatally (P3 and P7). A statistically significant decrease in the level of mitochondria-associated Bax is detected by P14, and the protein level declines gradually with increasing age to $7.2\% \pm 2\%$ from its P3 level in the adult, where the protein is poorly detectable (Fig. 3A). Proapoptotic Bak, migrating as a single band at ~26 kDa, is expressed at high levels in brain mitochondria, and no statistically significant changes are detected during postnatal brain development up to P31. Similar to Bax, however, mitochondrial Bak expression declines sharply in adult mitochondria to approximately $16.8\% \pm 5.2\%$ from its P3 level (Fig. 3B). We found that, in addition to Bax and Bak, immature brain mitochondria also express the multidomain protein Bok. Although two different isoforms of *Bok* (*Bok-L* and *Bok-S*) were reported previously (Hsu and Hsueh, 1998), the antibody used here detected a single band migrating at ~18 kDa, corresponding to the Bok-L isoform. Although Bok is detected at high levels during the first postnatal week (P3 and P7), it is rapidly down-regulated by P14 and becomes almost undetectable in adult mitochondria (Fig. 3C). These results indicate that, unlike mitochondria isolated from the immature brain, adult brain mitochondria are relatively deficient in all multidomain proapoptotic Bcl-2 proteins.

Proapoptotic BH3-Only Proteins Are Present in Mitochondria Early Postnatally and Are Down-Regulated in the Adult Rat Brain Mitochondria

Early models indicate that, in healthy cells, some BH3-only proteins are sequestered away from mitochondria and translocate to the OMM following induction of apoptosis. More recent studies indicate, however, that similar to Bax, BH3-only proteins (e.g., Bim) can be constitutively present in mitochondria prior to an apoptotic stimulus (Zhu et al., 2004; Gomez-Bougie et al., 2005). Insofar as BH3-only proteins have distinct binding affinities for various antiapoptotic Bcl-2 proteins and some of them (direct activators) can also bind and activate multidomain Bax/Bak, the levels and the type of BH3-only proteins that are present in mitochondria can be critical in determining the sensitivity to OMM permeabilization and apoptosis. We therefore analyzed the mitochondrial expression of several “activator” and “derepressor” BH3-only proteins during postnatal development.

As shown in Figure 4A, the derepressor BH3-only protein Bad was prominently expressed in immature rat brain mitochondria. Although a statistically significant decline in mitochondria-associated Bad is observed during postnatal period by P14 and at later ages, adult brain mitochondria still contain significant amounts of Bad (~25% from the P3 level). We examined next the three activator BH3-only proteins Bim, Bid, and Puma. With the antibody used in this study, Bim_{EL} was the only isoform clearly detectable among the three Bim isoforms reported for other tissues (O'Reilly et al., 2000). Mitochondrial Bim_{EL} declines significantly from P7 to adult-hood, when it is still detectable at 18.3% of the P3 level (Fig. 4B). Bid is a unique BH3-only protein that conveys proteolytic signals to mitochondria. Cleavage of Bid by multiple proteases results in generation of a truncated Bid fragment (tBid) that translocates to mitochondria and induces Bax/Bak activation. Although the antibody we used can also recognize the truncated Bid fragment (not shown), we were unable to detect the presence of tBid clearly in isolated mitochondria. Full-length Bid was, however, readily detectable. Mitochondria-associated full-length Bid decreases gradually after P7 and is almost undetectable in adult brain (Fig. 4C). The p53-induced activator BH3-only protein Puma was expressed in mitochondria almost exclusively during the first post-natal week (P3 and P7) and was undetectable at later ages (Fig. 4D). Another p53-induced BH3-only protein, Noxa, could not be detected with the antibody used here (data not shown).

Mitochondrial Localization of Proapoptotic BH3-Only Bad and Bim Is Modulated With Age

To test whether the age-dependent expression of proapoptotic multidomain and BH3-only proteins in mitochondria correlates with their total cellular level, we compared the relative levels of these proteins in mitochondria and total brain lysates from immature (P7) and adult brain. Similar to the results obtained in isolated mitochondria, the total cellular levels of multidomain Bax and Bak as well as those of several BH3-only proteins also decreased with increasing age (Fig. 5A). As shown in Figure 5B, this is reflected by a similar ratio of the mitochondrial to brain homogenate levels in both P7 and adult samples for Bax, Bak, and Bid (Fig. 5B). As expected, the mitochondria to brain homogenate ratio for Bak, a constitutive membrane protein located primarily in mitochondria, was higher than for the other proapoptotic proteins. A significant increase in the fraction of Bim localized to mitochondria was observed at P7, suggesting that selective translocation of this protein to mitochondria occurs in the immature developing brain. A similar trend was also detected for Bad, although it did not reach statistical significance.

DISCUSSION

Although the developmental regulation of several Bcl-2 family proteins has been previously reported (Merry and Korsmeyer, 1997; Shimohama et al., 1998; Krajewska et al., 2002; Polster et al., 2003), the expression of many potent proapoptotic Bcl-2 family proteins, including BH3-only proteins, has not been examined specifically in mitochondria in the postnatal brain. In this study, we expanded our previous analysis of the expression of Bcl-2 proteins in mitochondria during postnatal brain development to include all multidomain proapoptotic Bcl-2 proteins, some of the most potent BH3-only proteins (i.e. activator BH3-only), and antiapoptotic Bcl-2 proteins. The results are summarized in Table I.

Levels of mitochondrial Bcl-2 and Bcl-x_L are differentially regulated. In contrast to previous results (Robertson et al., 2006), by using another antibody that is more specific for Bcl-x_L, we found that Bcl-x_L is expressed at constant levels during postnatal brain development. Persistence of Bcl-x_L at relatively high levels in the adult mitochondria reflects its antiapoptotic role in the adult brain and could also be related to Bcl-x_L involvement in regulating synaptic plasticity (Jonas et al., 2003; Jonas, 2006). Stable postnatal expression of Bcl-x_L contrasts with the dramatic reduction (~80%) in mitochondrial Bcl-2 levels present in brain of adult compared with P3 animals. Other antiapoptotic Bcl-2 proteins are also expressed in the adult brain, including Mcl-1 and Bcl-w, and may play protective roles against seizure induced neuronal death and ischemic brain injury, respectively (Hamner et al., 1999; Sun et al., 2003; Mori et al., 2004). Mcl-1 could not be clearly identified in isolated brain mitochondria, possibly because of its low expression in the brain and preferential localization to specific brain regions such as hippocampus. Although further studies will be needed to clarify the expression of this protein, Bcl-2 and Bcl-x_L appear to be the dominant antiapoptotic proteins present in rat brain mitochondria during postnatal development and in the adult.

The multidomain proapoptotic proteins Bax and Bak are critically involved in activation of the mitochondrial apoptotic death pathway, as cells deficient in both these proteins display long-term protection against multiple apoptotic stimuli (Wei et al., 2001). Bax/Bak deficiency results in defects in apoptotic death of neural progenitor cells (for review see Lindsten et al., 2005). Although Bok was recently implicated as a mediator of p53-induced death in neuroblastoma cells (Yakovlev et al., 2004), almost nothing is known about the developmental regulation and potential role of Bok in apoptosis in the brain. We found that, similar to Bax and Bak, Bok is also expressed at significant levels in immature but not adult brain mitochondria. This is consistent with a previous study reporting the expression of Bok mRNA in the brain (Suominen et al., 2001). The increased expression of Bok in mitochondria in the first postnatal week

suggests that Bok could be involved in developmental death occurring during this period in the rat brain, potentially complementing Bax/Bak activity.

Early models indicate that, in healthy cells, many BH3-only proteins (e.g., Bid, Bim, Bad) are sequestered away from mitochondria through different mechanisms (for review see Huang and Strasser, 2000). Induction of apoptosis triggers translocation to the OMM, where, depending on their relative affinities, BH3-only proteins bind and inactivate a distinct subset of antiapoptotic Bcl-2 proteins (Chen et al., 2005). More recent studies indicate, however, that similar to Bax, BH3-only proteins, including Bax/Bak activators (e.g., Bim), can also constitutively reside in mitochondria prior to an apoptotic stimulus (Zhu et al., 2004; Gomez-Bougie et al., 2005; Becker and Bonni, 2006). Displacement of mitochondria-localized BH3-only proteins from the antiapoptotic Bcl-2 proteins could trigger Bax/Bak oligomerization and OMM permeabilization (Letai et al., 2002; Kim et al., 2006; Del et al., 2007). We found that multiple BH3-only proteins are present in mitochondria in the immature brain, and their relative levels decrease progressively with increasing age. By comparing the relative mitochondrial vs. total brain levels, we found that the fraction of Bim localized to mitochondria is increased in the immature (P7) brain compared with the adult. A similar trend was also noted for Bad. These results suggest that an active translocation of Bad and Bim to mitochondria occurs at this age in the immature brain and likely reflects their involvement in developmental neuronal death at P7. Consistent with an involvement of these proteins in apoptosis in the immature brain, a recent study performed in immature mice (P7) deficient in Bid, Bim, and Bad demonstrated the involvement of Bim and Bad but not Bid in ischemic brain injury in the immature brain (Ness et al., 2006). We did not observe the presence of tBid in mitochondria, but full-length Bid was easily detected. Recent studies showed that, in several cell types, including neurons, full-length Bid also translocates to mitochondria and induces cell death in the absence of cleavage (Sarig et al., 2003; Konig et al., 2007). Puma, another direct activator BH3-only protein, was also detected in mitochondria during the first post-natal week. The pattern of Puma expression in brain mitochondria correlates with the previously reported expression of p53 in the postnatal rat brain, which peaks at P5 when increased apoptosis occurs, decreases by P7 (Poulaki et al., 1999), and is almost undetectable in the adult brain (Chung et al., 2000). The expression of Bok, which is also induced by p53 (Yakovlev et al., 2004), is similarly expressed primarily in the first postnatal week.

The high expression of multiple proapoptotic proteins in the early postnatal brain reflects to some extent changes in cells dying through apoptosis as part of the naturally occurring developmental programmed death. In the rat basal forebrain, caspase-3-positive apoptotic cells (mostly neurons) peak at P1 and P5 and are still detected during the first 2 weeks of postnatal life (Sophou et al., 2006). It is unlikely, however, that such changes are exclusively present in dying apoptotic cells. Studies *in vitro* in neural cells (Polster et al., 2003) and oligodendrocytes (Soane et al., 1999; Itoh et al., 2003) as well as in nonneuronal systems indicate that profound changes in regulation of Bcl-2 family proteins occur in a differentiation-dependent manner and suggest a coordinated regulation of susceptibility to apoptosis with differentiation stage. Another aspect of postnatal brain development that could conceivably affect expression of proteins present in isolated mitochondria is the relative increase in the number of astrocytes compared with neurons and the increase in synapses that occur during this period; however, there are currently no comparisons available of apoptotic proteins present in mitochondria from these different locations.

To our knowledge, the present study is the first to report that, in contrast to the antiapoptotic Bcl-2 proteins that are differentially regulated, all multidomain proapoptotic Bcl-2 proteins (Bax, Bak, and Bok) are relatively deficient in the adult relative to immature brain mitochondria. Insofar as multidomain Bax/Bak-type proteins appear to be absolutely required for OMM permeabilization and induction of apoptosis by BH3-only proteins, the results

suggest that adult brain mitochondria will also display increased resistance to most other BH3-only proteins or BH3 peptides. Our results could explain findings that release of apoptogenic factors Cyt C and AIF from mitochondria is reduced in the adult compared with the immature brain following hypoxia/ischemia (Zhu et al., 2005) and suggest a potential mechanism involved in the relative shift from apoptotic to necrotic death in the adult brain following injury. In addition to the results from our study on mitochondrial apoptotic regulators, studies have found that other mitochondrial factors regulating susceptibility to neural cell death, i.e., sensitivity to Ca²⁺-induced mitochondrial permeability transition, are also developmentally regulated during the postnatal period (Robertson et al., 2004; Eliseev et al., 2006). Further understanding of the role these factors play in neurologic outcome following insults, e.g., cerebral ischemia and trauma, may lead to development of neuroprotective interventions that are optimized according to age.

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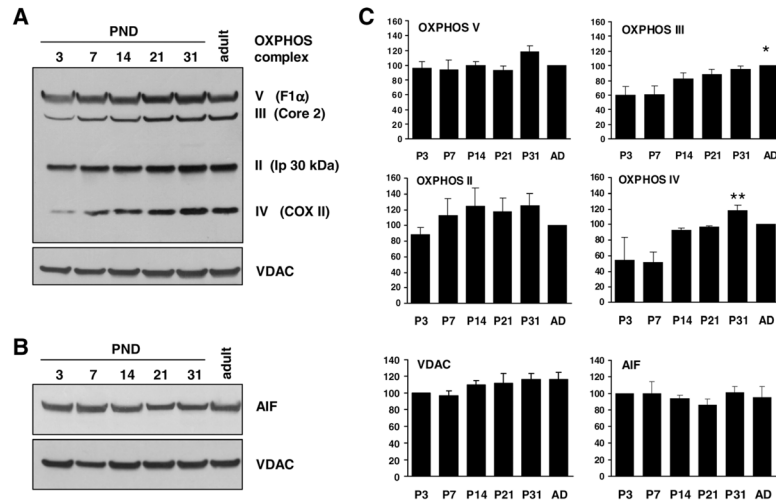
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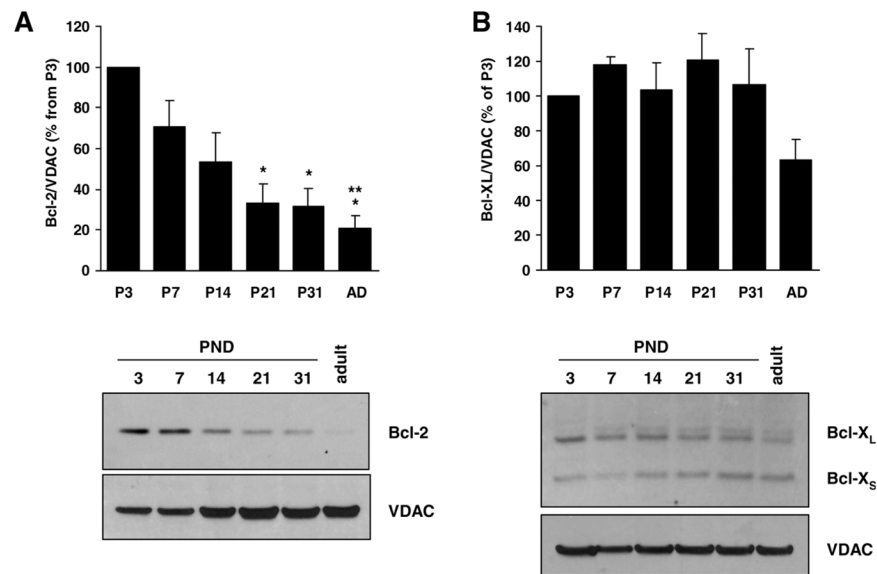
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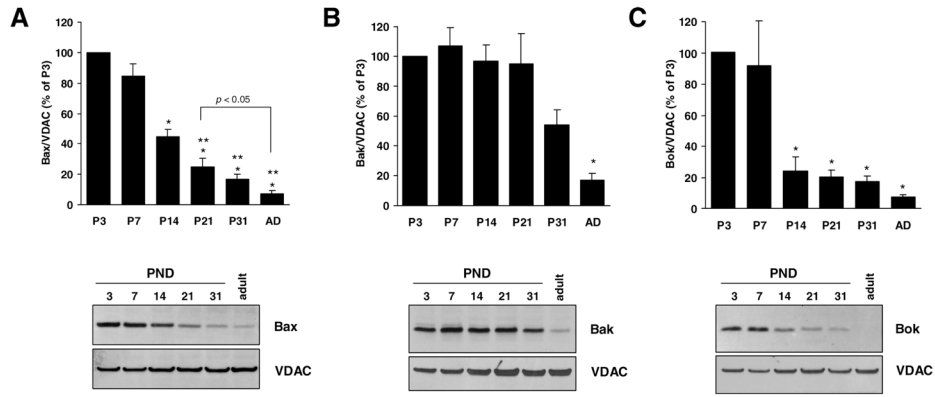
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**Fig. 1.**

Expression of mitochondrial OXPHOS complexes and AIF during postnatal brain development. **A:** Expression of several subunits of the mitochondrial OXPHOS complexes I–V was examined in isolated rat brain mitochondria at the indicated postnatal day (PND) and in adult brain mitochondria using the OXPHOS Western blotting kit (Mitosciences). The expression of VDAC was examined on the same membranes to control for equal loading of mitochondrial protein. A representative of three different immunoblots is shown. **B:** Developmental regulation of AIF was examined as described above at the indicated ages in isolated mitochondria. A representative of four different immunoblots is shown. **C:** Densitometric analysis of the level of subunits of OXPHOS complexes II–V ($n = 3$), VDAC ($n = 6$), and AIF ($n = 4$). Data are expressed as mean \pm SEM. * $P < 0.05$ vs. P3, ** $P < 0.05$ vs. P7.

**Fig. 2.**

Expression of antiapoptotic Bcl-2 and Bcl-x_L in mitochondria during postnatal brain development. **A:** Level of antiapoptotic Bcl-2 in rat brain mitochondria was examined by immunoblotting using equal amounts of mitochondria (50 µg/lane) isolated from rat brain at various ages postnatal (P3–P31) and from the adult rat brain. VDAC immunoblotting was performed on the same membranes. The relative levels of Bcl-2 were normalized to VDAC, and the results are expressed as percentage of the P3 level. Data are expressed as mean ± SEM (n = 5–6). **P* < 0.05 vs. P3. ***P* < 0.05 vs. P7. **B:** Expression of Bcl-x_L in mitochondria was examined similarly by immunoblotting, using VDAC as control (n = 4). The relative Bcl-x_L level after normalization to VDAC was expressed as percentage of the P3 level.

**Fig. 3.**

Developmental regulation of multidomain proapoptotic Bcl-2 proteins in brain mitochondria. **A:** Expression of proapoptotic Bax was examined by immunoblotting at the indicated ages in isolated brain mitochondria. The relative levels of Bax are expressed as percentage of the P3 level. Data are expressed as mean \pm SEM ($n = 6-7$). $*P \leq 0.002$ vs. P3 and P7, $**P < 0.05$ vs. P14. **B:** Immunoblot analysis of Bak expression in mitochondria during postnatal development and scan densitometric analysis of the relative Bak/VDAC levels; $n = 4-5$. $*P < 0.05$ vs. P3, P7, P14, P21, and P31. **C:** Expression of the multidomain proapoptotic protein Bok in mitochondria during postnatal development. A representative immunoblot is shown, and the relative Bok/VDAC levels during postnatal brain development are expressed as percentage of the P3 level ($n = 4$). $*P < 0.005$ vs. P3, $P < 0.05$ vs. P7.

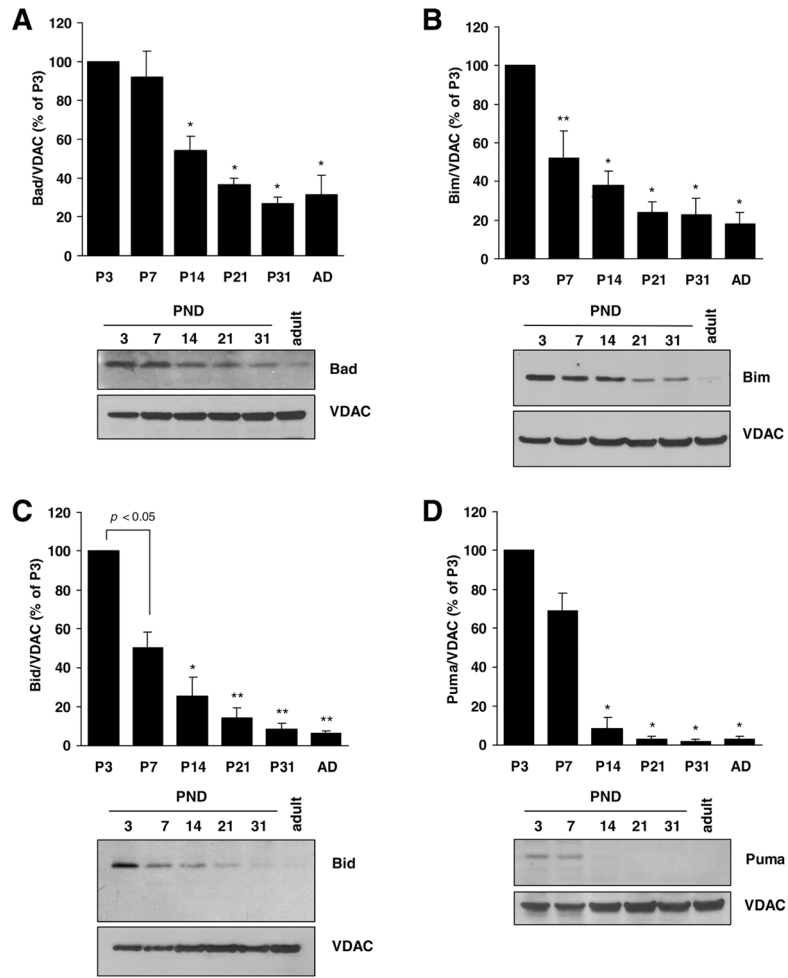
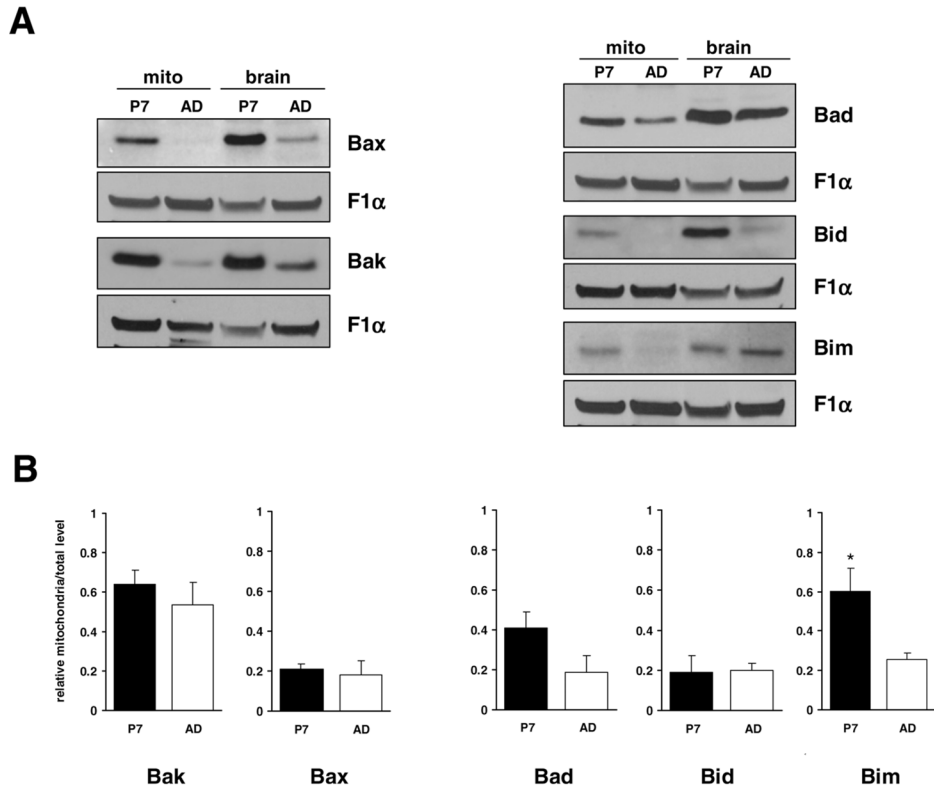


Fig. 4. Developmental expression of mitochondrial BH3-only proteins during postnatal brain development. The presence of several BH3-only proapoptotic proteins in isolated brain mitochondria was analyzed at the indicated age by immunoblotting using VDAC as control. Representative immunoblots demonstrating that significant levels of Bad, Bim, Bid, and Puma are present in the immature brain mitochondria. The relative level of the proteins is also expressed as percentage of the P3 value. Data are expressed as mean \pm SEM (n = 4). **A:** * $P < 0.01$ vs. P3, $P < 0.05$ vs. P7. **B:** * $P < 0.001$ vs. P3, ** $P < 0.01$ vs. P3. **C:** * $P < 0.001$ vs. P3, ** $P < 0.001$ vs. P3, $P < 0.005$ vs. P7. **D:** * $P < 0.001$ vs. P3 and P7.

**Fig. 5.**

Relative mitochondrial vs. total cellular level of proapoptotic Bcl-2 family proteins during postnatal brain development. **A:** Protein fraction present in mitochondria (mito) from the total cellular level (brain) was examined in immature (P7) and adult rat brain mitochondria for several proapoptotic Bcl-2 family proteins by loading lysates of isolated mitochondria and total brain homogenates on the same gels and immunoblotting with the indicated antibodies for multidomain (Bax and Bak) and BH3-only proteins (Bad, Bid, Bim). The F1 α subunit of the F1Fo ATPase localized predominantly in mitochondria was used to verify protein loading and to normalize the relative levels of proapoptotic proteins. **B:** Results of quantification analysis following normalization to F1 α were expressed as ratio of mitochondria to total protein levels present in brain homogenates for each of the proteins in A at P7 and in the adult. Data are expressed as mean \pm SEM (n = 4). * P < 0.05 vs. adult.

TABLE I

Summary of Bcl-2 Family Protein Expression in Mitochondria During Postnatal Brain Development

Subfamily	Protein	Expression during postnatal development	Expression in adult relative to P3 (%)
Antiapoptotic	Bcl-2	Decrease	20.80
	Bcl-x _L	Constant	63.40
Proapoptotic multidomain	Bax	Decrease	7.20
	Bak	Decrease	16.60
	Bok	Decrease	7.10
Proapoptotic BH3-only	Bad	Decrease	31.30
	Bim	Decrease	18.30
	Bid	Decrease	6.06
	Puma	Decrease	2.60