Neuroprotective Effect of Bax-Inhibiting Peptide on Neonatal Brain Injury
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Background and Purpose—Mitochondria play a critical role in mediating cell death in both the adult and immature brain. The cyclophilin D mitochondrial membrane permeability transition pore is critical in adult ischemia, whereas in neonatal hypoxic–ischemic (HI) brain injury, mitochondrial permeabilization appears to be primarily Bax-dependent. The aim of this study was to evaluate the neuroprotective effect of a cell-penetrating Bax-inhibiting peptide (BIP) on neonatal mouse HI brain injury.

Methods—BIP (5 μL, 5 mg/mL) or a BIP-negative control (5 μL, 5 mg/mL) was injected intracerebroventricularly immediately before HI in postnatal day 9 mice. Mice were euthanized at different time points after HI for evaluation of brain injury, Bax activation, release of proapoptotic proteins, and caspase activation. The trace fear conditioning and cylinder tests were performed for evaluation of the functional recovery after BIP treatment.

Results—At 5 days after HI, there was a 41.2% reduction of brain injury in BIP-treated mice compared with BIP-negative control treated animals. Myelin basic protein and neurofilament quantification revealed that BIP reduced white matter injury. BIP treatment conferred improvement in both sensorimotor and memory functions at 7 weeks after HI. BIP protection was associated with a reduction of Bax activation, mitochondrial permeabilization, and downstream caspase activation.

Conclusions—Bax inhibition provides neuroprotection and functional improvement in a neonatal mouse model of HI. (Stroke. 2010;41:2050-2055.)

Key Words: apoptosis ■ Bax ■ neonatal ischemia ■ neuroprotection

Hypoxia–ischemia triggers a number injurious events: release of excitatory amino acids, increased intracellular calcium, accumulation of nitric oxide and other reactive oxygen species, loss of trophic factor support, and induction of c-jun kinases. At a certain threshold level, these upstream “stressors” will increase the pro- versus antiapoptotic Bcl-2 family protein balance, which will induce mitochondrial outer membrane permeabilization. Permeabilization of mitochondria will lead to release of cytochrome c (cyt c) and apoptosis-inducing factor (AIF), leading to activation of both caspase- and caspase-independent cell death. Indeed, combined inhibition of AIF and the caspases provides marked reduction of brain injury suggesting that blockage of mitochondrial permeabilization may offer a promising strategy for protection of the immature brain.

Cyclophilin D-dependent opening of the mitochondrial membrane permeability transition plays a marginal role in the setting of the immature brain response to injury. Instead, Bax-dependent mitochondrial outer membrane permeabilization appears to be the predominant mechanism of proapoptotic protein release, which agrees with earlier studies showing that Bax gene deletion reduces immature brain injury. Hence, our hypothesis is that pharmacological inhibition of Bax translocation/activation may be a useful strategy for protection of the immature brain. The purpose of the present study was to investigate the effect of a synthetic Bax-inhibiting peptide (BIP) on brain injury and functional impairment after neonatal hypoxia–ischemia.

Materials and Methods

Animals
Pregnant C57BL/6 mice were purchased from Charles River Laboratories (Sulzfeld, Germany) and gave birth in the animal facility at University of Gothenburg (Experimental Biomedicine, University of Gothenburg). Mice were housed with a 12-hour light/dark cycle. Free access to a standard laboratory chow diet (B&K, Solna, Sweden).
Sweden) and drinking water was provided. All animal experimentation was approved by the Animal Ethical Committee of Gothenburg (No. 193/09).

Hypoxia–Ischemia

At postnatal Day (PND) 9, mice of both genders were subjected to hypoxia–ischemia (HI) insult according to a method described previously. Briefly, mice were anesthetized with isoflurane (5.0% for induction and 3.0 to 1.5% for maintenance) in a mixture of nitrous oxide and oxygen (1:1). The left common carotid artery was ligated. Mice were returned to the cage and allowed to recover for 1 hour and were then placed in an incubator perfused with a humidified gas mixture (10.00% oxygen in nitrogen) at 36°C for 50 minutes. Normal control animals received no surgery, and sham animals were treated identically except for the carotid artery ligation.

Drug Administration

BIP-V5 (H-Val-Pro-Met-Leu-Lys-OH; 5 mg/mL, Cat. No. 196810; Calbiochem, Nottingham, UK) or BIP negative control (BIPN, 5 μL, 5 mg/mL, Cat. No. 196811; Calbiochem; H-Ile-Pro-Met-Ile-Lys-OH) dissolved in saline was injected intracerebroventricularly immediately before HI in PND 9 mice according to a method described previously. Pups were anesthetized with isoflurane (5.0% for induction and 3.0 to 1.5% for maintenance) and injected using a syringe attached to a microinjection pump (CMA, Stockholm, Sweden) at a speed of 1 μL/min and with a depth of 2.5 mm from the skin surface.

Assessment of Brain Damage

Five days post-HI, mice were deeply anesthetized and perfused intracardially with saline and 5% buffered formaldehyde (Histofix; Histolab, Göteborg, Sweden). The brains were paraffin-embedded and cut into 10-μm frontal sections. Sections were used for immunohistochemical staining for neurofilament (NF; SMI 312; Sternberger Monoclonal) and anti-phosphorylated neurofilament (NF; SMI 312; Sternberger Monoclonal) immunohistochemical staining (see subsequently).

Infarct volumes were identified as the MAP-2-negative staining volume in the ipsilateral hemisphere and was calculated according to the method described previously using the formula: $$V = \frac{SA \cdot \pi \cdot T}{p}$$, where V is the total volume, SA is the sum of the areas measured, p is the inverse of the sections sampling fraction, and T is the section thickness. The total tissue volume loss was calculated as the MAP-2-positive volume in the contralateral hemisphere minus the MAP-2-positive volume in the ipsilateral hemisphere. Neuropathological score was evaluated in MAP-2-stained sections using a semi-quantitative scoring system as described previously.

Subcortical white matter injury was analyzed by quantitative measurements of immunohistochemical positive staining for MBP and NF using Micro Image, Version 4.0 (Micro-Macro AB, Gothenburg, Sweden) as previously described. The proportion of immunoreactivity in the ipsilateral hemisphere was calculated as percentage of the staining in the contralateral hemisphere; therefore, the results were expressed as the percentage loss of MBP/NF for the ipsilateral hemisphere in relation to the contralateral hemisphere.

Caspase Activity Assays

Animals were euthanized by decapitation 1.5 hour, 3 hours, 6 hours, or 24 hours after HI (n=6 per time point). The brains were dissected out and ice-cold isolation buffer (15 mmol/L Tris-HCl, pH 7.6, 320 mmol/L sucrose, 1 mmol/L DTT, 1 mmol/L MgCl2, 3 mmol/L EDTA-K, 0.5% protease inhibitor cocktail: P8340; Sigma) was added. Brain tissue homogenization was performed by hand in a 2-mL glass/glass homogenizer (WVR International, Gothenburg, Sweden). The homogenates were centrifuged at 9200 x g for 15 minutes at 4°C, producing a cytosolic fraction for caspase-3 activity measurements. Cleavage of Ac-DEVD-AMC for caspase-3-like activity (Peptide Institute, Osaka, Japan) was measured using a SpectraMax Gemini microplate fluorometer (Molecular Devices, Sunnyvale, Calif) and expressed as pmol AMC released per milligram protein and minute.

Figure 1. Effect of Bax inhibition on HI brain injury on PND 9 mice. BIP reduced the total infarct volume (A), tissue loss (B), and the neuropathological score (C–D) at 5 days post-HI (PND 14). Representative photomicrographs of MAP-2-stained sections of PND 9 mouse brain at hippocampus level at 5 days (BIP n=18; BIP n=19; E) and 7 weeks (BIP n=17; BIP n=15; F) after HI in the BIP- (right panel) and BIPN- (left panel) treated groups. The injury was still obvious in hippocampus at 7 weeks after HI (F) and BIP injection reduced brain injury significantly as indicated by total pathological score (G) and the total tissue loss volume (H). *P<0.05, **P<0.01.
**Immunohistochemistry Staining**

At 1.5 hour, 3 hours, 6 hours, and 24 hours after HI, pups were deeply anesthetized and perfused intracardially with saline and 5% buffered formaldehyde (Histofix; Histolab). Brains were paraffin-embedded and cut into 6-μm frontal sections. Parallel sections were used to stain for the following primary antibodies: MAP-2, MBP, NF, AIF (D-20, Sc-9416; Santa Cruz Biotechnology, Inc), Bax6A7 (ab5714; Abcam plc, Cambridge, UK), cyt c (556433; BD Pharmingen), and active form of caspase-3 (557038; BD Bioscience Pharmingen). The positive cell counting for Bax, cyt c, and AIF were performed at 400× magnification (one visual field=0.196 mm²).

The positive cells in the CA1, CA2, CA3, and DG of hippocampus were counted. In the cortex, 3 visual fields within an area displaying loss of MAP-2 were counted. The sum number of positive cells from all regions mentioned from each section was calculated.

The colocalization of Bax6A7 and cyt c was investigated by double-labeling immunofluorescence staining. Antigen recovery was performed as described previously followed by incubation with mouse anti-Bax6A7 (1:50) for 2 hours in TBS (0.2% Triton PBS) at 20°C for 2 hours. After washing, the sections were incubated with secondary antibodies, Alexa Fluor 555 donkey antimouse IgG (H+L), at 20°C for 60 minutes. After washing, the sections were incubated with fluorescein isothiocyanate-labeled mouse anti-cyt c (1:100; BioLegend, San Diego, Calif) for 60 minutes. After washing, the sections were mounted using Vectashield mounting medium.

**Trace Fear Conditioning Test**

At 7 weeks after HI, trace fear conditioning was conducted in Automatic reflex conditioner 7531 (inside dimensions 390×95×165 [height] mm; Cat. No. 7530; Ugo Basile) according to a method described previously with some modification. Animals were timed for freezing individually within total of 2 minutes’ time period and recorded by digital video cameras. Freezing was defined as absence of movement except for respiration. The procedure was conducted over 2 days (see Figure 3A). On Day 1, mice received pairing of a tone (20 seconds, 80 dB, 670 KHz) and a shock (2 seconds, 0.5 mA). The time interval between the tone and the shock was 2 seconds. On Day 2, each mouse was placed into the chamber for 2 minutes to record the baseline freezing (pretone freezing). The tone was presented once for 30 seconds, 80 dB, 670 KHz. No shocks were administered. Freezing was scored for 2 minutes after the tone presentation (tone-elicited freezing, posttone freezing).

**Cylinder Rearing Test**

The cylinder test was performed according to the method described previously with some modifications. Briefly, at 7 weeks after HI, animals were individually placed in a glass transparent cylinder (95 mm ø×180 mm height) and observed/video-recorded the initial forepaw (left/right/both) preference during full rear for 3 minutes. Forepaw use of the first contact against the wall after rearing and during lateral exploration was recorded according to a criteria suggested by Kadam et al. The relative proportion (%) of left (nonimpaired) forepaw contacts was calculated as: (left−right)/(right+left+both)×100%.

**Statistics**

StatView software was used. The Student t test was used when comparing 2 groups, whereas analysis of variance followed by Fisher protected least significant difference post hoc test was used when comparing ≥3 groups. Mann–Whitney U test was used to compare the neuropathology score. *P<0.05 was considered statistically significant. All data are expressed as mean±SEM.

**Results**

**BIP Reduced Gray and White Matter Injury After HI**

At 5 days after HI, there was a 41.2% reduction of brain injury in BIP-treated mice compared with BIPN-treated controls. The infarct volume in the BIP group was 2.8±0.4 mm³ (n=19) versus 4.8±0.8 mm³ (n=18) in the BIPN group (P=0.041; Figure 1A). Tissue area loss was reduced at most levels of the brain except for the most posterior sections (Figure 1B). This was confirmed by the total neuropathology score, showing a significantly decreased neuropathology score in the BIP treatment group (7.8±0.8) if compared with the BIPN treatment group (10.7±0.9, P<0.03; Figure 1C). The reduced injury was more pronounced in the cortex and striatum (Figure 1D). The long-term effect was evaluated at 7 weeks after HI using total tissue loss (Figure 1H) and total pathological score (Figure 1G). The injury was mainly located in the hippocampus (Figure 1F) and BIP treatment significantly reduced brain injury (Figure 1G–H).

MBP and NF quantification in the subcortical white matter showed that BIP treatment reduced white matter injury. MBP loss (%) in the BIP treatment group was 23.2±3.8 versus 41.8±3.7 (P=0.001) in BIPN treatment group (Figures 2A and C). NF loss (%) was 25.4±2.5 in BIP-treated group versus 34.8±2.6 (P=0.02) in BIPN-treated group (Figures 2B and D).

**BIP Improved the Functional Recovery After HI**

To evaluate functional recovery in mice after BIP treatment, the cylinder test for sensorimotor function and trace fear
conditioning test for memory function were performed 7 weeks after HI. The trace fear conditioning test showed there are no significant differences between the normal control and sham-treated animals (Figure 3B). BIPN-treated animals had significantly less posttone freezing compared with sham-operated animals (39.1% ± 7.4% in BIPN versus 73.8% ± 7.4% in sham controls, P = 0.005), whereas there were no significant differences between BIP and the sham control animals (P = 0.17; Figure 3B). Freezing tended to normalize after BIP treatment, because BIP-treated animals had significantly more freezing than the BIPN-treated animals post–tone (Figure 3B; 60.7% ± 6.3% in BIP versus 39.1% ± 7.4% in BIPN, P = 0.024).

In the cylinder rearing test, control animals do not exhibit any left versus right preference in the cylinder rearing test, whereas injured mice use their left forepaw more often than the right due to the left-sided brain injury so both BIP- and BIPN-treated mice were significantly impaired compared with sham control animals 7 weeks after the insult (BIPN 53.3% ± 6.8% versus sham 11.0% ± 5.2%, P < 0.0001; BIP 25.8% ± 6.0% versus sham 11.0% ± 5.2%, P = 0.02; Figure 3C). The impairment was, however, markedly attenuated in the BIP versus the BIPN group (P = 0.0053; Figure 3C).

**BIP Reduced Bax Activation and Apoptosis After HI**

To detect Bax translocation and activation, we used an antibody (6A7) that primarily detects the Bax conformation associated with apoptosis. HI induced clear Bax 6A7 immunostaining as compared with controls, in which no positive cells were visible. There was a significant reduction in the number of Bax 6A7-positive cells after BIP treatment compared with BIPN at 1.5 hours and 3 hours after HI (Figures 4A and 5). The decrease in Bax 6A7 staining after BIP was accompanied by reduced cytosolic cyt c immunoreactivity at 6 hours after HI (Figures 4B and 5). AIF translocation at 3 hours (Figures 4C and 5) as well as subsequent caspase-3 activation (Figure 6A) and expression of the cleaved form of caspase-3 (Figure 6B). These data suggest...
that BIP reduces Bax activation, subsequent mitochondrial permeabilization, and downstream caspase activation.

**Discussion**

In the present study, we found that Bax inhibition provided by an inhibiting peptide derived from Ku70 administered immediately before HI reduced gray and white matter injury and improved the sensorimotor and memory function. The protection was associated with a reduction of Bax activation and decreased mitochondrial release of the proapoptotic proteins cyt c and AIF. These results together with the fact that Bax gene deficiency confers neuroprotection and our recent data showing an important role of Bax in mitochondrial permeabilization in vivo and vitro strongly indicate that Bax is a potential target for pharmacological neuroprotection of the neonatal brain.

Under normal conditions, Bax resides primarily in the cytoplasm in its monomeric form. In response to apoptotic stimuli, Bax undergoes conformational change involving exposure of lipophilic N and C terminal domains that allows insertion into the outer mitochondrial membrane, oligomerization with other Bax and/or Bak monomers, and finally pore formation. Direct evidence is lacking that Bax-dependent pore formation really occurs in neurons in the neonatal brain after HI, but Bax has been shown to translocate to mitochondria. Presently, we found an early progressive increase of 6A7 Bax immunostaining after HI. The 6A7 antibody recognizes the N-terminal epitope that is hidden under normal conditions but exposed when Bax is activated and required for Bax mitochondrial membrane integration. These data thus offer further support that Bax activation precedes the release of proapoptotic proteins and downstream activation of executioner caspases.

BIP derives from Ku70 protein that was primarily identified as a critical component of the Ku complex required for nuclear repair of DNA strand breaks. In addition, the cytosolic form of Ku70 binds and inhibits Bax. This interaction depends on a Bax-binding domain (corresponding to amino acids 578 to 583) of Ku70 and the N-terminal 53 amino acids of Bax. BIP amino acid sequence corresponds to that of the Bax-binding domain of Ku70 and has been shown to effectively inhibit Bax-dependent apoptosis and possesses excellent cell penetrating abilities. In the present study, we found that Bax inhibition by BIP administered immediately before HI reduces brain infarct volume by 41.2% in gray matter. We also found that Bax inhibition provides protection of the white matter after HI, which is important because the white matter is vulnerable in term and especially in preterm infants. To date, this is the first evidence that Bax inhibition can reduce not only gray matter, but also white matter injury in neonatal HI.

There is not always a close correlation between morphological and neurological outcome in various animal models of brain injury. Thus, it is important to perform assessments of neurological functions as a complement to morphological outcome measures. Indeed, we found that BIP treatment not only reduced the brain lesion, but also improved sensorimotor function after neonatal HI in rats and has now been adapted for use in mice.

**Figure 5.** Representative immunostainings showing Bax- (3 hours post-HI), cyt c- (6 hours post-HI), and AIF- (3 hours post-HI) positive cells before (Cont, left panel) and after HI in the hippocampus ipsilateral hemisphere in BIP- (right panel) and BIPN- (middle panel) treated mouse brains (A). The double labeling of Bax6A7 and cyt c indicated colocation at 3 hours (upper panel) and 6 hours (bottom panel) after HI (B). Bar=10 μm.

**Figure 6.** Effect of BIP on caspase-3 activation. A, Fluorimetry quantification of caspase-3-like activity in brain homogenates after HI at different time points. *P<0.05. B, Representative photomicrographs showing immunodetection of the active form of caspase-3 in the hippocampus (upper panel, bar=50 μm) and in the cortex (lower panel, bar=20 μm; n=6/group).
sensitive, and reproducible test that allows observation of behavior under unforced conditions. 
Fear conditioning to either a cue or a context is a test that represents a form of associative learning that has been well characterized in many species. 
However, to our knowledge, this is the first study using a fear condition test to evaluate the memory function recovery after neonatal mouse HI brain injury.

To conclude, BIP treatment provides significant brain protection in both the gray and the white matter and also improves functional recovery and may thus constitute a framework for further investigation to determine if a pharmacologically improved, or selectively delivered, BIP derivative might be a clinically applicable neuroprotective strategy.

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Disclosures
None.

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