Original Article

Bax inhibiting peptide reduces apoptosis in neonatal rat hypoxic-ischemic brain damage

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Abstract: Neonatal hypoxic ischemic encephalopathy (HIE) has been reported to induce apoptosis in neonates. We, therefore, analyzed the ability of Bax-inhibiting peptide (BIP) to provide neuroprotective effects during hypoxic-ischemic brain damage (HIBD). Seven-day-old wistar rat pups (n = 198) were randomly divided into a sham-operated group (Group S, n = 18), saline group (Group C, n = 90) and BIP group (Group B, n = 90). Pathological changes in the cerebral tissues of rat pups were analyzed using hematoxylin and eosin stain, TUNEL and Western blot. The expression of cytochrome c and caspase-3 was determined using western blot technique. Rat pups demonstrated neurobehavioral alteration in Groups C and B. TUNEL-positive cells in the left hippocampus were significantly increased in Group C and Group B after HIBD (P < 0.01) when compared with Group S. There was a marked reduction in TUNEL positive cells in subgroups B1 through B4 when compared with the respective subgroups C1 through C5. Compared with Group S, the expression of caspase-3 and cytochrome c was significantly increased in Groups C and B (P < 0.01). The difference in expression of caspase-3 and cytochrome c between subgroups B1 through B4 and C1 through C4 was significant (P < 0.01). In conclusions, the neuro-protective effect of BIP was due to a reduction of nerve cell apoptosis in our neonatal HIE rat model. We propose that BIP has potential as a neuro-protective drug in neonatal HIE cases.

Keywords: Neonatal hypoxic ischemic encephalopathy, hypoxic-ischemic brain damage, bax-inhibiting peptide, apoptosis, necrosis, bax-inhibiting peptide

Introduction

Hypoxic-ischemic encephalopathy (HIE) refers to the hypoxic-ischemic brain damage (HIBD) caused by asphyxia and anoxia in neonates. Perinatal HIE occurs in 1 to 3 per 1000 live full-term births [1]. Fifteen to twenty percent of affected newborns will die during the postnatal period, and an additional 25% will develop severe and permanent neuropsychological sequelae, including mental retardation, visual motor or perceptive dysfunction, increased hyperactivity, cerebral palsy and epilepsy [2-4]. Perinatal HIE is the most important cause of cerebral damage and long-term neurological sequelae in the perinatal period in both full term and preterm infants. The serious neurological sequelae caused by perinatal HIE affect the quality of life of affected infants and result in an increased burden on society. Unfortunately, currently there is no effective therapies available. Therefore, studies of the pathogenesis of HIE are urgently needed in order to find effective measures to decrease the morbidity and mortality caused by HIBD.

Previous studies have demonstrated that free radical production, inflammation, glutamate excitatory toxicity and endothelial cell dysfunction are involved in the pathological mechanisms of HIE [5]. Ultimately, these pathological conditions lead to neurocyte death by necrosis or apoptosis. Apoptosis plays a more important role in neurocyte deficiency and dysfunction [6]. Hill et al. demonstrated that neurocyte apoptosis occurs in HIBD by utilizing a seven-day old rat pup model of HIBD [7].

Apoptosis can be executed by two distinct molecular mechanisms: the intrinsic (caspase-
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9-dependent) and extrinsic (caspase-8-dependent) pathways. Both pathways lead to the activation of the final player, caspase-3 [8-11]. Activation of caspase-3 results in DNA fragmentation, loss of normal cell contacts, cell blebbing, cell condensation and subsequent phagocytosis [12, 13].

During caspase-mediated apoptosis, mitochondria integrate lethal and survival signals and thereby play a crucial role in deciding the fate of the cell. Integral mitochondrial membrane permeability (MMP) is key to this decision process [3, 14]. Bax, a member of the Bcl-2 family [15], translocates from the cytosol to the mitochondria in response to apoptotic stimuli and causes the release of apoptogenic factors, such as cytochrome c (Cyt C) [16-18]. Bax-mediated cell death is implicated as one of the major causes of pathology in damaged tissue. Neurodegenerative diseases in which Bax-mediated cell death plays important role include Alzheimer’s disease [6], Parkinson’s disease [7] and ischemia/reperfusion-induced organ damage [8].

Sawada et al. [19] used a yeast-based functional screening system to search for Bax inhibitors and demonstrated that cloned human Ku70 is a potential Bax suppressor protein. Ku70 is the 70K subunit of Kuantigen, a heterodimeric complex composed of Ku70 and Ku80. Pentapeptides based on Ku70 are known as Bax inhibiting peptides (BIPs) and suppress Bax-mediated cell death in human cancer cell lines [20]. Therefore, we hypothesized that BIP has the potential to be a novel therapeutic candidate that can protect against apoptotic damage in neonatal HIE.

Materials and methods

Animals

One-hundred and ninety-eight healthy Wistar rat pups of either sex (12 to 16 g, SCXK 20090007, 7 days old) supplied by the Experimental Animal Center of Qingdao City were used in experiments. Seven-day old pups were anesthetized with 10% chloral hydrate (3 ml per kg body weight by intraperitoneal injection). The right common carotid artery (CCA) was exposed, separated from nerves and veins and permanently ligated with 4-0 surgical silk. After the wound was sutured, the pups were allowed to recover from anesthesia and returned to the cage. The entire surgical procedure never exceeded eight min. Sham-operated pups underwent the same operative procedure except that the exposed carotid artery was not ligated. Two hrs after surgery, pups were placed in an airtight 3 L container and exposed to a humidified 8% mixture of nitrogen and oxygen. The container was partially submerged in a 37°C water bath to maintain a constant thermal environment. After being exposed to hypoxia for 150 min, pups were returned to the cage until they were sacrificed. During the whole process, animals were kept away from bright light and noise.

Rat pups were randomly divided into three groups. In Group S (sham group, n = 18) a midline vertical neck incision was performed and sutured under anesthesia, without common carotid artery (CCA) ligation and hypoxia, then 0.2 ml saline was given intraperitoneally. In Group B (BIP group, n = 90) BIP (Merck Germany) was injected in the left lateral ventricle 0, 6, 12, 24 and 72 hrs after the HIBD procedure (described above). In Group C (control group, n = 90) the left lateral ventricle was injected with saline and all other procedures were the same as Group B. Group B and C were randomly divided into five subgroups (n = 18 for each group, groups were numbered B1 to B5 and C1 to C5, respectively) according to the different drug injection time points. All experimental designs were approved by the hospital ethics committee.

Intracerebroventricular injection

Lateral ventricle injection was performed according to the previously described method [22]. Preliminary tests using methylene blue tracing and anatomic localization demonstrated that the method was feasible. Anesthetized pups were secured in a miniature stereotaxic apparatus in a prone position, the scalp incised and the lambdoid suture exposed. The location of each injection in relation to the lambda was 2.0 mm rostral and 1.5 mm lateral by compass and ruler and then the 10 µl microsyringe (10
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Table 1. Expression of TUNEL-positive neurons in the CA1 region of the hippocampus at different time points (X ± S)

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>1 d (number/area)</th>
<th>3 d (number/area)</th>
<th>7 d (number/area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>18</td>
<td>2.97 ± 1.10</td>
<td>2.93 ± 1.17</td>
<td>2.81 ± 1.05</td>
</tr>
<tr>
<td>C</td>
<td>18</td>
<td>56.99 ± 9.78*</td>
<td>45.46 ± 8.33*</td>
<td>28.28 ± 5.98*</td>
</tr>
<tr>
<td>B1</td>
<td>18</td>
<td>33.26 ± 5.59*▲▼★◆</td>
<td>22.05 ± 5.65*▲▼★◆</td>
<td>14.22 ± 4.19*▲▼★◆</td>
</tr>
<tr>
<td>B2</td>
<td>18</td>
<td>45.06 ± 6.03*▲</td>
<td>30.58 ± 6.03*▲</td>
<td>20.12 ± 4.31*▲</td>
</tr>
<tr>
<td>B3</td>
<td>18</td>
<td>47.04 ± 6.34*▲</td>
<td>34.74 ± 5.03*▲</td>
<td>20.59 ± 4.40*▲</td>
</tr>
<tr>
<td>B4</td>
<td>18</td>
<td>48.13 ± 9.67*▲</td>
<td>38.41 ± 8.31*▲</td>
<td>23.76 ± 5.87*▲</td>
</tr>
<tr>
<td>B5</td>
<td>18</td>
<td>56.90 ± 9.71*▲</td>
<td>45.41 ± 8.37*▲</td>
<td>28.24 ± 5.91*▲</td>
</tr>
</tbody>
</table>

PS: Compared with Group S *P < 0.01. Compared with Group C ▲P < 0.01. Compared with Group B2 ★P < 0.01. ★P < 0.01. Compared with Group B3 ☆P < 0.01. Compared with Group B4◆P < 0.01. Compared with Group C ΔP > 0.05.

Figure 1. Expression of TUNEL-positive neurons in the CA1 region of the hippocampus at different time points in brain sections of a rat pup hypoxia induced brain damage model. Group S is the SHAM operated group. Group C is the control group. Group B rats were treated with Bax inhibiting peptide after HIBD procedures.

Figure 1. Expression of TUNEL-positive neurons in the CA1 region of the hippocampus at different time points in brain sections of a rat pup hypoxia induced brain damage model. Group S is the SHAM operated group. Group C is the control group. Group B rats were treated with Bax inhibiting peptide after HIBD procedures.

Specimen collection

Six rat pups from each group were sacrificed on day one, three and seven after injection. Three pups were used for western blot analysis and three for histological tissue section procedures. Brains were excised by the method described below. Rats were anesthetized with an intra-peritoneal injection of chlo-ral hydrate. Rat pups were fixed on the operation panel. The sternum was opened and the heart was exposed. A 5 ml syringe was inserted from the left ventricle to the aorta, fixed by silk thread ligation and 4% paraformaldehyde was injected into the heart. At the same time, the right atrium was opened to allow free blood flow. The above technique was repeated until clear liquid flowed from the atrium and the extremities became pale and stiff. Next, the head and neck were severed and the skull was systematically stripped to expose cerebral tis-sue. Cortex without blood color demonstrated good perfusion. The entire brain was removed and immediately placed in 4% paraformalde-hyde solution.

Tissue section and HE staining

Brain specimens preserved in 4% paraformaldehyde were kept in the solution for 24 h. After 24 h 1 to 2 cm² wide and 0.2 to 0.3 cm thick tissue blocks were rinsed with running water for about 12 h. Brain tissue blocks were then dehydrated with alcohol of different concentrations and soaked in di-methyl benzene under 65°C before being embedded in paraffin. Continuous coronal slicing was performed at the parahippo-campal gyrus level (4 µm thick). The sections were placed on to polylysine treated glass slides and dried under 65°C for at least eight hours and then kept at room temperature.

Brain tissue sections were stained with hema-toxylin and eosin (HE). The slides were then mounted using neutral balsam.

Cell apoptosis assay

The in situ end-labeling primary Terminal Deoxynucleotidyl Transferase-Mediated Uridine 5’-Triphosphate-Biotin Nick End Labeling (TUN-EL) Kit (Roche Diagnostics (GmbH, Shanghai, China) was used to detect apoptosis in the brain tissue. Two neighboring dewaxed sec-tions were acquired from each brain tissue specimen. The sections were then incubated with 1:200 proteinase K for 10 min, followed by
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**Table 2.** Cyt C expression density in the CA1 region of the hippocampus at different time points (x ± s) (n = 18)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. (n)</th>
<th>Time after HIBD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 d</td>
</tr>
<tr>
<td>S</td>
<td>6</td>
<td>0.0368 ± 0.0109</td>
</tr>
<tr>
<td>C</td>
<td>18</td>
<td>0.4061 ± 0.0127</td>
</tr>
<tr>
<td>B1</td>
<td>18</td>
<td>0.2871 ± 0.0251</td>
</tr>
<tr>
<td>B2</td>
<td>18</td>
<td>0.3136 ± 0.0079</td>
</tr>
<tr>
<td>B3</td>
<td>18</td>
<td>0.3361 ± 0.0190</td>
</tr>
<tr>
<td>B4</td>
<td>18</td>
<td>0.3670 ± 0.0106</td>
</tr>
<tr>
<td>B5</td>
<td>18</td>
<td>0.3907 ± 0.0125</td>
</tr>
</tbody>
</table>

PS: Compared with Group S *P < 0.01. Compared with Group C ▲P < 0.01, △P > 0.05. Compared with Group B2 ☆P < 0.05. Compared with Group B3 ☆P < 0.01. Compared with Group B4 ◆P < 0.01.

Figure 2. Cyt C expression in the CA1 region of the hippocampus at different time points (x ± s) (n = 18) in brain sections from the rat pup hypoxia induced brain damage model. Group S is the SHAM operated group. Group C is the control group. Group B rats were treated with Bax inhibiting peptide after HIBD.

Western blot analysis

Brain tissues were stored in aliquots at -80°C until needed for further analysis. Western blotting was carried out to detect the levels of caspase-3 and Cyt C in the cortex. Brain tissue samples were extracted with lysis buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 2 mg/ml apronin and 1 mM PMSF) for 20 min at 4°C in the presence of protease inhibitors. Extracts were centrifuged at 10,000 × g for 15 to 20 min at 4°C. Supernatants containing total protein were then harvested. Proteins were separated in precast gels by protein electrophoresis system (BioRad Mini-PROTEAN Tetra II BioRad, Hercules, CA, USA) followed by transfer to a nitrocellulose membrane (NC) for about eight hrs at room temperature in blocking buffer (5% nonfat dry milk, 0.1% PBS or 5% bovine serum albumin (BSA)). The membrane was incubated with primary antibodies against caspase-3 and Cyt C Boaosen Biotechnology, Inc. (Beijing, China) overnight at 4°C or for one h at room temperature. After incubation, membranes were washed five times for three min. Proteins were visualized using anti-mouse or anti-rabbit IgG antibodies (at a dilution of 1:4000), conjugated to horseradish peroxidase (HRP) for 40 min at room temperature. Bound proteins were visualized using DBA and detected using the chemiluminescence imaging system (Fusion X7 Peqlab, Lutterworth, UK). The relative protein levels were calculated based on β-actin.

Statistical analysis

Statistical analysis was performed using the paired Student’s t-test of the SPSS 17.0 statisti-
Table 3. Expression of caspase-3 in the CA1 region of the hippocampus at different time points (x ± s) (n = 18)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. (n)</th>
<th>Time after HIBD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 d</td>
<td>3 d</td>
</tr>
<tr>
<td>S</td>
<td>6</td>
<td>0.042 ± 0.0127</td>
</tr>
<tr>
<td>C</td>
<td>18</td>
<td>1.108 ± 0.0032*</td>
</tr>
<tr>
<td>B1</td>
<td>18</td>
<td>0.380 ± 0.0307**</td>
</tr>
<tr>
<td>B2</td>
<td>18</td>
<td>0.607 ± 0.0072*</td>
</tr>
<tr>
<td>B3</td>
<td>18</td>
<td>0.785 ± 0.0149*</td>
</tr>
<tr>
<td>B4</td>
<td>18</td>
<td>0.878 ± 0.0102**</td>
</tr>
<tr>
<td>B5</td>
<td>18</td>
<td>1.097 ± 0.0074**</td>
</tr>
</tbody>
</table>

PS: Compared with Group S *P < 0.01. Compared with Group C ^P < 0.01, _P > 0.05. Compared with Group B2 *P < 0.01, _P < 0.05. Compared with Group B3 *P < 0.01. Compared with Group B4 *P < 0.01.

Results

Evaluation of neurobehavior with HIBD

In Group S, no clinical abnormalities were observed.

In Group C, pups showed dysphoria, cyanosis and tachypnea at 20 mins; hemiparesis, circling and convulsions at 30 mins; and, finally, somnolence and coma occurred within 1 hr.

In Group B, behavior similar to Group C was observed; however, the degree of improvement after treatment was better than that observed in Group C. Symptoms in Group B had almost disappeared after 24 h.

Macroscopic appearance

In Group S, the left and right cerebral hemispheres were symmetrical, without swelling or congestion. Overall, the morphology and texture of the brain tissue were normal.

In Group C, mild to moderate brain swelling was observed and became most obvious on the seventh day after HIBD. This was accompanied by ligation of the cerebral hemisphere, congestion and necrosis like tissue liquefaction.

In Group B, similar but less severe injury was seen, as compared with Group C.

Microscopic appearance after HE staining

In Group S, HE stained hippocampal brain tissue sections demonstrated normal structure and morphology, regular cell arrangement of the pyramidal neurons and complete cell outlines with intact, center-positioned nuclei and a clear entoblast. Cellular degeneration and necrosis could not be observed.

In Group C, regular cell arrangement of the pyramidal neurons was observable in the hippocampus one day after the procedure, along with cellular swelling. On the other hand, nuclear membranes were unclear, entoblasts disappeared and cellular degeneration and necrosis were detected. Three days after the HIBD procedure, decreased cell density was observed and cells appeared disorganized. Additionally, the gap between pyramidal neurons widened.
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Astrocytes around the blood capillary swelled and cellular degeneration and necrosis were more prominent. After seven days, cellular swelling was reduced and gliocyte hyperplasia along with partial nuclear pyknosis and fragmentation were observed.

In Group B, the pathological changes observed in the B1, B2 and B3 groups were significantly less than those observed in Group C. Abnormal cell arrangements and partial or complete neuronal degeneration were observed in the cortex and hippocampus three days after the HIBD procedure. The pathological changes observed in Group B4 and Group B5 had no apparent differences when compared with those in Group C. Cell density in Group B1 was greater, with a more regular cell arrangement and less cell apoptosis and necrosis when compared with Group B2 through Group B5.

Apoptosis in the hippocampal area

TUNEL-positive cells observed under the light microscope exhibited unstained cytoplasm with the brown-stained pyknotic nuclei that are indicative of apoptotic cells. Apoptosis occurred at different time points in each group (Table 1).

Group S showed fewer TUNEL-positive cells than either Group B or Group C. The expression of TUNEL-positive cells was markedly increased in both Groups B and C from day one to day three, reaching a peak during day one (P < 0.01), and remained significant until day seven (P < 0.01). Compared with Group S, the number of TUNEL-positive cells was significantly higher in Group B1 through Group B4 (P < 0.01). Further, Group B1 had statistically higher values when compared to Group B2 through B4 (P < 0.01). Group B5, however, did not differ significantly from Group C (P > 0.05) (Table 1 and Figure 1).

Expression of caspase-3 and Cyt C

The expression of Cyt C was markedly increased in both Group B and Group C. The expression of Cyt C in Group B1 through Group B4 was significantly different from the expression in Group S (P < 0.01), and the expression in Group B1 differed significantly from the expression in Group B2 through B4 (P < 0.01). Similar to the TUNEL assay results, Group B5 did not differ significantly from Group C (P > 0.05) (Table 2 and Figure 2).

Discussion

The Ku70 based peptide BIP-V5 (Bax-Inhibiting Peptide, V5) effectively protects cells from apoptosis. BIPs can protect normal cells from Bax cytotoxicity, and a BIP based treatment strategy may be an effective method of reducing organ damage during degenerative diseases. The development and study of improved BIPs may, therefore, provide tools and information for the design of new cytoprotective therapies that could be of great potential clinical use.

In the experiment reported here, rat pups were given BIP by lateral ventricle injection to limit the drug effects to the cortex and hippocampus. In this way, adverse side effects of BIP administration could be avoided. However, this method of drug administration requires a high level of precision or else brain tissue will be damaged. Therefore, prior to performing the experiments described here, we repeatedly practiced ventricle injection using a methylene blue trace until we had achieved an error rate that was under 10%. After administration of BIP by lateral ventricle injection to rats that had been subjected to the HIBD procedure, we found a reduction in pathological symptoms in the cortex and hippocampus.

We observed apoptosis on days one through seven in the neonatal rat model. In neonatal HIBD or HIE, failure to produce cellular energy, glutamate release, intracellular Ca²⁺ accumulation, lipid peroxidation and nitric oxide accumula-
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The induction of apoptosis cumulatively leads to neurotoxicity and ultimately leads to cell death, mainly through the induction of apoptosis [23, 24].

Because apoptosis is the major cell death pathway in neonates, we hypothesized that anti-apoptotic molecules, such as BAX inhibiting peptide, may have the potential to minimize the symptoms of the HIBD.

In accordance with our hypothesis, we found that rats with an induced model of HIBD that were subsequently treated with Bax-inhibiting peptide had decreased brain damage, improved neurological outcomes and decreased incidence of apoptosis. It has been recently demonstrated that the early events in ischemic brain injury in neonates are necrosis followed by apoptosis [2, 25]. Thus, we cannot rule out the possibility that BIP induced prevention of early necrosis might have occurred in the brain of rats after the HIBD procedure and BIP administration. However, the reduction in apoptotic cells was accompanied by changes in the expression of apoptosis related molecules, caspase-3 and Cyt C, supporting our hypothesis that BIP is neuroprotective via an anti-apoptotic mechanism.

Two pathways are considered important for the induced release of Cyt C from mitochondria, namely, a Ca^{2+}-dependent CsA-sensitive process and a CsA-insensitive Bax-VDAC (voltage dependent anion channel) process [15, 26]. It has been suggested that Bax, a pro-apoptotic protein, interacts with and increases the opening of the mitochondrial VDAC, which leads to a loss in membrane potential and the liberation of Cyt C from mitochondria [27]. The full details of this process are not yet known, but translocation of the proteins Bim and Bax from the cytosol to the mitochondrial membrane is commonly believed to be involved [28].

Rats treated with BIP in this study demonstrated a marked reduction in the expression of Cyt C and caspase-3. Thus, all the findings in our study strongly suggest that, in our model of neonatal HIE/HIBD, BIP decreases apoptosis in neurocytes and that, in turn, the reduction of apoptotic activity decreases the damage to brain tissue after hypoxia-ischemic brain injury.

BIP plays an important role in neurocyte protection by binding to the Bax protein [18]. In our experiment, we observed reduced injury to the cortex in rats treated with BIP immediately after severe hypoxia, especially in the hippocampal CA3.

Conclusion

Our findings confirm the hypothesis that Bax inhibiting peptide reduces the apoptotic response in HIE-induced damage of the cortex and hippocampus in neonates. Thus BIP is neuroprotective after brain injury and BIP is a promising therapeutic agent for the treatment of ischemic brain injury in neonatal HIE/HIBD.

Disclosure of conflict of interest

None.

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