Neuroprotective Effect of Dizocilpine (MK-801) via Anti-apoptosis on Hypoxic-ischemic Brain Injury in Neonatal Rats

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Purpose: Current studies have demonstrated the neuroprotective effects of dizocilpine (MK-801) in many animal models of brain injury, including hypoxic-ischemic (HI) encephalopathy, trauma and excitotoxicity, but limited data are available for those during the neonatal periods. Here we investigated whether dizocilpine can protect the developing rat brain from HI injury via anti-apoptosis.

Methods: In an in vitro model, embryonic cortical neuronal cell culture of Sprague-Dawley (SD) rats at 18-day gestation was done. The cultured cells were divided into three groups: normoxia (N), hypoxia (H), and hypoxia treated with dizocilpine (HD). The N group was prepared in 5% CO2 incubators and the other groups were placed in 1% O2 incubators (94% N2, 5% CO2) for 16 hours. In an in vivo model, left carotid artery ligation was done in 7-day-old SD rat pups. The animals were divided into six groups; hypoxia (N), hypoxia (H), hypoxia with sham-operation (HS), hypoxia with operation (HO), HO treated with vehicle (HV), and HO treated with dizocilpine (HD). Hypoxia was made by exposure to a 2 hour period of hypoxic incubator (92% N2, 8% O2).

Results: In the in vitro and in vivo models, the expressions of Bcl-2 in the hypoxia groups were reduced compared to the normoxia group. whereas those in the dizocilpine-treated group were increased compared to the hypoxia group. However, the expressions of Bax and caspase-3 and the ratio of Bax/Bcl-2 were revealed reversely.

Conclusion: Dizocilpine has neuroprotective property over perinatal HI brain injury via anti-apoptosis.

Key Words: Anti-apoptosis, Dizocilpine, Hypoxic-ischemic brain injury, Neuroprotection

Introduction

Neurons are highly vulnerable to exposure of hypoxia and ischemia and related insults that other cell types are able to withstand. The mechanisms for this are poorly understood but studies on hypoxic-ischemic (HI) brain injury have identified at least one important participant such as overstimulation of excitatory amino acid receptors, including glutamate and aspartate.

Glutamate has been implicated in the neuronal death after HI brain injury because the antagonists of glutamate receptors have been found to be neuroprotective. Glutamate receptor activation is associated with the raised intracellular Ca2+ and the enzymatic production of NO from the amino acid L-arginine. Glutamate has long been known to kill neurons by an N-methyl-d-aspartate (NMDA) receptor-mediated mechanism. Paradoxically, subtoxic concentrations of NMDA protect neurons against glutamate-mediated excitotoxicity. Because NMDA protects neurons in physiologic concentrations of glucose and oxygen.
However, dizocilpine (MK-801), a non-competitive NMDA receptor antagonist, prevents signal transmission by means of the blockade of NMDA receptor ion channels. Dizocilpine acts by binding to a site located within the NMDA associated ion channel and thus prevents Ca\textsuperscript{2+} flux\textsuperscript{5).}

NMDA receptors are key in the progression of excitotoxicity. Thus NMDA receptor antagonists including dizocilpine have been evaluated for use in treatment of diseases associated with excitotoxicity, such as stroke, HI brain injury, and neurodegenerative diseases such as Huntington’s, Alzheimer’s, and amyotrophic lateral sclerosis. Dizocilpine has shown effectiveness in protecting neurons in cell culture and animal models of excitotoxic neurodegeneration\textsuperscript{6, 7).} In addition, dizocilpine was assessed by evaluating hippocampal behavioral and histologic outcomes in an experimental rat model of neonatal hypoxia ischemia\textsuperscript{8).}

Dizocilpine reduces neuronal damage and preserves learning and memory in a rat model of traumatic brain injury. The neuronal caspase-3 expression, neuronal nitric oxide synthase (nNOS)-positive neurons and OX-42-positive microglia were all increased in transient brain injury animals. After dizocilpine treatment neuronal caspase-3 expression and nNOS-positive neurons were all significantly decreased. Dizocilpine could significantly inhibit the degeneration and apoptosis of neurons in damaged brain areas\textsuperscript{9).}

The mechanisms of protective effect of NMDA receptor stimulation on apoptosis of neurons at their early stage of development are poorly understood. Anti-apoptotic effects of NMDA is connected with inhibition of fragmentation of DNA via caspase-3-independent mechanism\textsuperscript{10).}

In addition, the efficacy of dizocilpine was evaluated in a rat model of retinal ischemia\textsuperscript{11).} NMDA receptors may have an important role in ischemia–reperfusion insult as well as in mediating ischemia-induced apoptosis of retinal neurons. After intravitreal NMDA injection, ultrastructural features consistent with classic apoptotic changes were noted in degenerating cells in the retinal ganglion cell layer and the inner nuclear layer. NMDA plus dizocilpine did not show these changes\textsuperscript{12).}

Dizocilpine can prevent excitatory neuronal death, but at higher concentrations, it can also induce neuronal death in the limbic system. This dizocilpine–induced selective neurotoxicity has been proposed as an animal model for dementia and psychosis. The results suggest that the dizocilpine–induced neuronal death was apoptotic\textsuperscript{13), whereas at high doses or after continuous administration it induces neuronal degeneration or necrotic–like irreversible neuronal death.

In this study, we determined the protective abilities of dizocilpine via mechanisms of anti-apoptosis on a HI brain injury by using an rat model of a HI brain injury (in vivo) and an embryonic cortical neuronal cell culture of rats (in vitro). Dizocilpine effects were evaluated by using western blots and real-time PCRs.

**Materials and Methods**

1. **Materials**

(+)-MK-801 hydrogen maleate (dizocilpine) was purchased from Sigma (St. Louis, Saint Louis, MO, USA). Primary antibodies included the following: Bcl-2 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bax (1:1,000; Cell Signaling technology, Beverly, MA, USA), caspase-3 (1:1,000; Cell Signaling technology), and β-Actin (1:1,000, Santa Cruz Biotechnology). Secondary antibodies used were goat anti-mouse or rabbit IgG–HRP (1:2,000, Santa Cruz Biotechnology).

2. **Embryonic cortical neuronal cell culture**

The cortical neuronal cell culture of rat embryos were prepared as previously described\textsuperscript{14).} Sprague–Dawley (SD) rats pregnant for 19 days were anesthetized with ether for 5 minutes at room temperature and the uterus was removed. The fetal pups were washed in 100% ethanol and Hanks’ balanced salt solution (HBSS) (GibcoBRL, Grand Island, NY, USA). The brains of the fetal pups were dissected at 37°C HBSS containing 1 mM sodium pyruvate and 10 mM HEPES (pH 7.4). The dissected brain cortical tissues were then placed in 2 mL trypsin. Trypsinisation of cells was performed...
under gentle agitation for 1 minute at 37°C water bath and reaction was stopped by washing the tissues five times with 10 mL HBSS. The cells were moved in 1 mL HBSS, passed 6-7 times via pasteur pipette with tiny holes and dispersed. The cell suspension was centrifuged at 1,000 rpm at 25°C for 5 minutes and pellets were washed with HBSS (without phenol red). Following final centrifugation cells were resuspended in plating neurobasal medium (GibcoBRL) (100 mL neurobasal, 2 mL B27 supplement, 0.25 mL glutamax II, 0.1 mL 25 mM glutamate, 0.1 mL 25 mM 2-mercaptoethanol) and counted using a Neubauer hemicytometer.

After cell counting, the cells resuspended in plating neurobasal medium (GibcoBRL) and plated at approximately 2×10^6 cells/mm² in each dish. Cells were cultured in a CO₂ chamber with 1/5 of the culture solution changed every three days with feeding neurobasal medium (GibcoBRL) (100 mL neurobasal, 2 mL B27 supplement, 0.25 mL glutamax I).

The cultured cells were divided into three groups: a normoxia group (N), a hypoxia group (H), and a hypoxia treated with dizocilpine (HD). The N group was prepared in 5% CO₂ incubators and the other groups (before a hypoxia injury) were placed in 1% O₂ incubators (94% N₂, 5% CO₂) for 16 hours. The experiments were repeated four times (n=4) in the western blots and six times (n=6) in the real time PCRs.

3. Animal model and drug administration

This study was performed in accordance with the approved animal use guidelines of the Catholic University of Daegu. A modification of Levine preparation was used as a model for perinatal hypoxic-ischemic brain injury as previously described. We chose 7-day-old SD rat pups weighing between 12 and 16 g because HI brain injury in 7-day-old rats can be considered similar to perinatal asphyxia in the full-term infants. The sexes were not differentiated since there are no differences in terms of neonatal HI brain injury between male and female rats. The rat pups underwent permanent unilateral carotid ligation. The midline of the neck was incised at the longitudinal plane under ether anesthesia. The left common carotid artery was permanently ligated with 5-0 surgical silk. Total time of surgery never exceeded 5 minutes. Animals were excluded from the study if there was bleeding during ligation or respiratory arrest resulting from anesthesia. Following a 1 hour period of recovery, the animals were exposed to a 2 hour period of hypoxia (92% N₂, 8% O₂) by placing them in airtight containers partially submerged in a 37°C water bath to maintain a constant thermal environment. After this hypoxic exposure, the pups were returned to their dams for the indicated time. Pups were killed at 7 days after the hypoxic insult. Left cerebral hemispheres from rat brains were immediately removed, frozen in liquid nitrogen and stored at −70°C until use.

The pups were divided into six groups. No surgical procedure was not exposed to hypoxia (N) or was exposed to hypoxia (H). Hypoxia with sham-operated pups underwent the same surgical procedure without ligation (HS). The pups were subjected to hypoxia with operation (HO). Another pups received an intraperitoneal injection with phosphate–buffered saline (PBS) at the same volume with dizocilpine (HV) or with dizocilpine at a dose of 10 mg/kg (HD). Dizocilpine was prepared in PBS and injected intraperitoneally at a dose of 10 mg/kg 30 minutes before the hypoxic exposure. The experiments were repeated four times (n=4) in the western blots and six times (n=6) in the real time PCRs.

4. Protein isolation and western blotting

Samples of brain tissue and cell were homogenized and total protein was extracted using a protein lysis buffer containing complete protease inhibitor cocktail tablets (Roche Applied Science, Mannheim, Germany), 1 M Tris–HCl (pH 8.0), 5 M NaCl, 10% Nonidet P-40 and 1 M 1,4-dithio-DL-threitol (DTT). After incubation for 20 minutes on ice, the samples were centrifuged at 12,000 rpm at 4°C for 30 minutes and the supernatant was transferred to a new tube. Proteins were quantified using Bio–Rad Bradford kit (Bio–rad Laboratories, Hercules, CA, USA) and taking spectrophotometric readings at 590 nm. Concentrations were estimated against a standard curve generated using BSA. Total protein (50 μg) was subjected to electrophoreses in
12% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) after denaturing in 5× sodium dodecyl sulfate gel-loading buffer (60 mM Tris–HCl [pH 6.8], 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol and 0.1% bromophenol blue) in boiling water for 10 minutes. Proteins were then transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) using a semi-dry transfer apparatus at a constant voltage of 10 V for 30 minutes. Membranes were blocked in TBS, 0.1% Tween-20 containing 5% nonfat powdered milk. Proteins were reacted with primary antibodies for overnight at 4°C, and then incubated with secondary antibodies for 1 hr at room temperature. Signals were detected using an enhanced chemiluminescence (ECL) Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ, USA) or SUPEX (Neuronex, Pohang, Korea), and expose to film and develop image. Then analyzed using Kodak X-Omat film or an image analyzer LAS1000 (Fuji Photo Film, Tokyo, Japan). Each sample was conducted four times.

5. Semiquantitation of the western blots

The intensity of the corresponding western blot band was measured by using a densitometer (Multi Gauge Software: Fuji Photofilm) and was calculated as the ratio of the signal intensity in the ischemic hemisphere compared to the contralateral hemisphere.

6. RNA extraction and real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA). Incubate the homogenized samples for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Add 0.2 mL of chloroform per 1 mL of TRIzol reagent. After centrifugation, the aqueous phase was transferred to a fresh tube and the RNA from the aqueous phase was precipitated by mixing with isopropyl alcohol. The precipitate was washed twice in 100% ethanol. At the end of the procedure, briefly the RNA pellet was dried and RNA was dissolved in diethylpyrocarbonate (DEPC)-treated distilled water. From each sample was taken for GeneQuant 1,300 spectrophotometer (Gene Quant™ proRNA/DNA calculator, GE Healthcare, Amersham, Buckinghamshire, UK) measurement of RNA concentration. The RNA was then stored at −70°C before further processing. Total RNA was used in a reverse transcriptase reaction. For real-time PCR (For reverse transcription), total RNA (1 μg) was reverse transcribed for 1 hour at 37°C in a reaction mixture containing 20 U RNase inhibitor (Promega, Madison, WI, USA), 1 mM dNTP (Promega, Madison, WI), 0.5 ng Oligo (dT) 15 primer (Promega), 1x RT buffer and 200 U M-MLV reverse transcriptase (Promega). The reaction mixture was then incubated at 95°C for 5 minutes to stop the reaction. The cDNA was then stored at −20°C before further processing.

Real-time PCR was performed in 48 well PCR plates (Mini Opticon™ Real-Time PCR System, Bio-rad Laboratories). Each reaction mixture contained 10 μL iQ™ SYBR Green Supermix (Bio-rad Laboratories), 1 μL template, and 2 pmol each primer were added and adjusted with sterile water to a final volume of 20 μL. The initial denaturation was performed at 94°C for 5 minutes, followed by 40 cycles at 94°C for 30 seconds (denaturation), 53–59°C (Table 1) for 30 seconds (annealing), and 72°C for 30 seconds (extension), and a final incubation at 72°C for 7 minutes to ensure complete strand extension. Real-time PCR data were analysed with LightCycler software (Bio-rad Laboratories). Relative expression levels of genes of interest were calculated as the difference between the specific ratios (NMDA receptor/Beta-actin) and were further adjusted on the basis of their differences in CT values (number of cycles to reach threshold). Each sample was conducted six times.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence (5’-3’)</th>
<th>Annealing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>F:TTGAGGTCTTCCAGCAATGGG&lt;br&gt;R:GGTGGAACTCTTTGAGGA</td>
<td>57°C</td>
</tr>
<tr>
<td>Bax</td>
<td>F:GCTGATGGCAACTTCAACT&lt;br&gt;R:ATGATGGTTCTGATCAGCTCG</td>
<td>55°C</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>F:AATTCAAGGGGAGGCTATG&lt;br&gt;R:GCTTGTGCGCGTACAGTTTC</td>
<td>56°C</td>
</tr>
<tr>
<td>β-Actin</td>
<td>F:TTGCTGATCCACATCTGCTG&lt;br&gt;R:GACAGGATGCAGAAGGAGAT</td>
<td>53°C</td>
</tr>
</tbody>
</table>
7. Statistics analysis

Data were analyzed using the SPSS version 12 statistical analysis package. Examined data were assessed using the t-test, GLM (general linear model), and ANOVA. In each test, the data were expressed as the mean±SD, and P<0.05 was accepted as statistically significant.

Results

1. Morphologic changes in the embryonic cortical neuronal cell culture of rat (in vitro)

The cortical neuronal cells were observed using light microscopy under high magnification (×200). The cells in the N group were well preserved (Fig. 1A), whereas the cells in the H group showed cellular damages (Fig. 1B). The cellular patterns of the HD (Fig. 1C) appeared similar to those in the N group.

2. The expressions of Bcl-2, Bax and caspase-3 antibodies by western blots (Fig. 2A) in the embryonic cortical neuronal cell culture (in vitro)

The expression of Bcl-2 was reduced in the H group compared to the N group, whereas it showed a greater increase in the HD group compared to the H group (P<0.05) (Fig. 2B). Conversely, the expressions of Bax and caspase-3 and the ratio of Bax/Bcl-2 were greater in the H group than in the N group, whereas they were reduced in the HD group compared to the H group (P<0.05) (Fig. 2C-E).

3. The expressions of Bcl-2, Bax and caspase-3 mRNAs by real-time PCRs in the embryonic cortical neuronal cell culture (in vitro)

The expression of Bcl-2 was reduced in the H group compared to the N group, whereas it showed a greater increase in the HD group compared to the H group (P<0.05) (Fig. 3A). Conversely, the expressions of Bax and caspase-3 and the ratio of Bax/Bcl-2 were greater in the H group than in the N group, whereas they were reduced in the HD group compared to the H group (P<0.05) (Fig. 3B-D).

4. Gross morphologic changes in the neonatal hypoxic-ischemic brain injury (in vivo)

Gross morphologic changes in the neonatal hypoxic-ischemic brain injury (in vivo) showed that the percentage of left hemisphere area compared to right hemisphere area are 107.3% in the normoxia (A; N), 101% in the hypoxia without operation (B; H), 105% in the hypoxia with Sham operation (C; HS), 86% in the hypoxia with operation (D; HO), 82.5% in the D treated with vehicle (E; HV) and 98% in the D treated with dizocilpine (F; HD) (Fig. 4).

5. The expressions of Bcl-2, Bax and caspase-3 antibodies by western blots in the neonatal hypoxic-ischemic brain injury (in vivo)

The expression of Bcl-2 was reduced in the HO and HV groups compared to the N, H and HS groups, whereas it showed a greater increase in the HD group compared to the HO and HV groups (P<0.05) (Fig. 5A). Conversely, the expressions of Bax and caspase-3 and the ratio of Bax/Bcl-2 were greater in the HO and HV groups than in the N, H and HS groups, whereas they were reduced in the HD group.
Fig. 2. Western blots (A) of Bcl-2 (B: N, 100±2.0; H, 92.4±1.8; HD, 101.8±2.0), Bax (C: N, 100±2.5; H, 116.3±2.9; HD, 107.3±2.7) and caspase-3 (D: N, 100±3.1; H, 116.4±3.5; HD, 92.0±2.8) in the embryonic cortical neuronal cell culture (in vitro) and the ratio of Bax/Bcl-2 expression (E) were revealed (n=4). The dizocilpine was administered at a dose of 10 μg/mL. N, normoxia; H, hypoxia; HD, hypoxia treated with dizocilpine; *P<0.05, statistically significant vs. H.

Fig. 3. Real time PCRs of Bcl-2 (A: N, 100±6.1; H, 43.5±2.6; HD, 75.3±4.5), Bax (B: N, 100±6.4; H, 148.5±8.9; HD, 35.4±2.1) and caspase-3 (C: N, 100±5.2; H, 131.9±6.6; HD, 91.4±4.5) mRNAs in the embryonic cortical neuronal cell culture (in vitro) and the ratio of Bax/Bcl-2 expression (D) were revealed (n=6). The dizocilpine was administered at a dose of 10 μg/mL. N, normoxia; H, hypoxia; HD, hypoxia treated with dizocilpine; *P<0.05, statistically significant vs. H.
Fig. 4. Gross morphologic changes in the neonatal hypoxic-ischemic brain injury (in vivo) were revealed. (A) normoxia; (B) hypoxia without operation; (C) hypoxia with Sham operation; (D) hypoxia with operation; (E) D treated with vehicle; (F) D treated with dizocilpine. Percentage of left hemisphere area compared to right hemisphere area are 107% (A), 101% (B), 105% (C), 86.0% (D), 82.5% (E) and 98.0% (F).

Fig. 5. Western blots of Bcl-2 (A: N, 100±5.6; H, 105.4±7.5; HS, 119.5±5.9; HO, 54.3±2.7; HV, 70.0±3.5; HD, 118.2±5.9), Bax (B: N, 100±5.0; H, 97.4±4.8; HS, 1,190±15.7; HV, 300.8±15.0; HD, 106.2±5.3) and caspase-3 (C: N, 100±5.2; H, 91.9±4.6; HS, 102.8±5.1; HO, 172.5±8.6; HV, 221.1±11.1; HD, 99.8±5.0) in the neonatal hypoxic-ischemic brain injury (in vivo) and the ratio of Bax/Bcl-2 expression (D) were revealed (n=4). The dizocilpine was administrated at a dose of 10 mg/kg. N, normoxia; H, hypoxia without operation; HS, hypoxia with Sham operation; HO, hypoxia with operation; HV, HO treated with vehicle; HD, HO treated with dizocilpine; *P<0.05, statistically significant vs. HO.
compared to the HO and HV groups (P<0.05) (Fig. 5B-D).

6. The expressions of Bcl-2, Bax and caspase-3 mRNAs by real-time PCRs in the neonatal hypoxic–ischemic brain injury (in vivo)

The expression of Bcl-2 was reduced in the HO and HV groups compared to the N, H and HS groups, whereas it showed a greater increase in the HD group compared to the HO and HV groups (P<0.05) (Fig. 6A). Conversely, the expressions of Bax and caspase-3 and the ratio of Bax/Bcl-2 were greater in the HO and HV groups than in the N, H and HS groups, whereas they were reduced in the HD group compared to the HO and HV groups (P<0.05) (Fig. 6B-D).

Discussion

Hypoxic–ischemic encephalopathy (HIE) during the perinatal period, a single most important cause of acute mortality and chronic disability in newborns, usually occurs as a result of intrauterine hypoxia or asphyxia during birth\(^7\). It gives rise to neurological disability and even neonatal death. The incidence of asphyxia at birth is around 0.2–0.4% in full-term newborn infants and approaches 60% in preterm infants\(^8\). Between 20 and 50% of asphyxiated babies who exhibit HIE, die during the newborn period\(^9\). Of the survivors, up to 25% have permanent neuropsychological handicaps in the form of cerebral palsy, with or without associated mental retardation, learning disabilities or epilepsy\(^10\). These can include attention deficit disorders and minimal brain disorder syndromes, and may form the basis for psychiatric and neurodegenerative diseases later in life\(^11\).

Neuronal injury may be caused by overstimulation of excitatory amino acid receptors, including glutamate and aspartate. This excitotoxicity is predominantly mediated by calcium influx through ionic channels of activated glutamate receptors. Hypoxia and ischemia result in overaccumulation of the excitatory amino acid, glutamate. Glutamate, the principal neurotransmitter of the brain, is responsible for many physiologic functions, including cognition, memory, movement, and sensation. Pathophysiologically, excessive glutamate activates NMDA, α-amino-3-hydroxy-5-methyl-
4-isoxazolepropionate, and kainate glutamate receptors. Glutamate along with coagonist glycine stimulates NMDA receptors to increase intracellular calcium, which triggers a cascade of intracellular reactions, activating phospholipases, proteases, protein kinases, phosphatases, and nitric oxide synthase (NOS). The NOS causes increased NO production, which may damage DNA by base deamination to result in DNA strand breaks. Damaged DNA activates poly (adenosine 5′-diphosphoribose) polymerase to add deoxyadenosinetriphosphate to the ends of nicked DNA, resulting in depletion of energy sources from the cell. These processes ultimately lead to cell death, which can be necrotic or apoptotic in nature.

The precise events which initiate the cascade leading to cell death after HI are still incompletely understood, but are undoubtedly multifactorial. It is likely partly related to excessive entry of calcium into cells both during and after HI, loss of trophic support from growth factors, induction of free radicals during hypoxia and early reperfusion, and leakage of enzymes and proteins causing extensive inflammatory reactions. In addition, necrotic cells appear in patches. In contrast, apoptotic cells are characteristically scattered throughout the tissue and initially show condensation of chromatin at the nuclear periphery and reduction of nuclear size and cell volume.

The Bcl-2 protein blocks a distal step in an evolutionarily conserved pathway for programmed cell death and apoptosis. Bcl-2 is the founding member of the Bcl-2 family of apoptosis regulator proteins. These proteins govern mitochondrial outer membrane permeabilization (MOMP) and can be either pro-apoptotic (Bax, BAD, Bak, and Bok among others) or anti-apoptotic (including Bcl-2 proper, Bcl-xL, and Bcl-w, among an assortment of others). Some Bcl-2 family proteins can induce (pro-apoptotic members) or inhibit (anti-apoptotic members) the release of cytochrome c into the cytosol which, once there, activates caspase-9 and caspase-3, leading to apoptosis. The majority of Bax is found in the cytosol, but upon initiation of apoptotic signaling, Bax undergoes a conformation shift, and inserts into organelle membranes, primarily the outer mitochondria membrane.

The family of caspases are two types of apoptotic caspases: initiator (apical) caspases and effector (executor) caspases. Initiator caspases (e.g. caspase-8, -9) cleave inactive pro-forms of effector caspases, thereby activating them. Effector caspases (e.g. caspase-3, -6, -7) in turn cleave other protein substrates within the cell, to trigger
the apoptotic process. The initiation of this cascade reaction is regulated by caspase inhibitors. Caspases are crucial mediators of programmed cell death (apoptosis). Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins. However, the specific requirements of this (or any other) caspase in apoptosis have remained largely unknown until now. Pathways to caspase-3 activation have been identified that are either dependent on or independent of mitochondrial cytochrome c release and caspase-9 function. Caspase-3 is essential for normal brain development and is important or essential in other apoptotic scenarios in a remarkable tissue-, cell type- or death stimulus-specific manner.

Stimulation of NMDA receptors induces apoptosis in rat brain. NMDA-induced cell death was completely inhibited by the NMDA receptor antagonist dizocilpine. This result suggests that apoptotic mechanisms are involved in excitotoxin-induced cell death.

We used the expressions of Bcl-2 antibodies and mRNAs, as ant apoptosis, and the expressions of Bax and caspase-3, as pro-apoptosis, using western blots and real-time PCR to detect apoptotic properties in perinatal HI brain injury. In the present study, the expression of Bcl-2 was reduced in the hypoxia group when compared to the normoxia group, whereas it was increased in the dizocilpine-treated group compared to the hypoxia group. In contrast, the expressions of Bax and caspase-3 and the ratio of Bax/Bcl-2 were showed reversely.

In conclusion, our experiments demonstrate that dizocilpine is able to prevent the degeneration of neonatal cerebral neuronal cells caused by a hypoxic insult. In addition, dizocilpine neuroprotective effects on HI brain injury in neonatal rats may be via anti-apoptosis. The present study may be useful for the further development of clinical therapies for perinatal HI encephalopathy induced by cerebral hypoxia.

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