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Effects of Dizocilpine (MK-801) via Up-modulation of N-methyl-D-aspartate (NMDA) Receptors on Hypoxic-Ischemic Brain Injury in Neonatal Rats

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Objective : Several studies have demonstrated the neuroprotective effects of (+)-MK-801 hydrogen maleate (dizocilpine), in various animal models of hypoxic-ischemic (HI) brain injury. However limited data are available on the neonatal model of HI brain injury. The aim of the present study was to investigate the effects of dizocilpine and its mechanisms associated with NMDARs expression in neonatal rat model of HI brain injury.

Methods : In *in vivo* model, 7d-old rat pups underwent permanent unilateral carotid ligation. The animals were divided into six groups: 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. Dizocilpine (10 mg/kg) was administered intracerebrally to the rats 30 min before HI brain injury. Rat pups were exposed to hypoxia by placing them for 2 hours in hypoxic incubator (92% N_2, 8% O_2). In *in vitro* model, embryonic cortical neuronal cell cultures (from SD rats of embryonic days of 18) were done. The normoxia (N) group was prepared in 5% CO_2 incubators. The hypoxia (H), and hypoxia treated with dizocilpine (HD) groups were placed in 1% O_2 incubators (94% N_2, 5% CO_2) for 16 hours. In order to estimation of cell viability and growth, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was done. The degree of neuronal death was evaluated by morphometric method and the protein expression of each NMDARs was quantified by Real Time-PCR and Western blot.

Results : Both in the *in vitro* and *in vivo* models, the expressions of NMDAR subunits were lower in the hypoxia group than in the normoxia group, whereas they increased in the hypoxia treated with dizocilpine group compared to the hypoxia group. In *vitro* model, however, the expressions of NR1, NR2A mRNAs decreased in the H group when compared to the N group, whereas they increased a little in the HD group when compared to the H group.

Conclusion : Dizocilpine was modulated the degeneration of neuronal cell death in neonatal rat model of HI by preservation of NR expression.

Key Words : Dizocilpine, NMDAR, Glutamate receptor, Hypoxic-ischemic brain injury, Modulation

Abnormal glutamate receptor activation (excitotoxicity) may cause neuronal injury. Calcium influx through ionic channels of activated glutamate receptors is the predominant mediator of this excitotoxicity. Hypoxia and ischemia result in the over-accumulation of glutamate, which is an important neurotransmitter of the brain. It is responsible for many physiologic functions, including cognition, memory, movement, and sensation. The physiological and pathological

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effects of glutamate in the central nervous system (CNS) are mediated through its interaction with specific cell membrane receptors, of which the N–methyl–D–aspartate (NMDA), kainate, and α–Amino–3–hydroxy–5–methyl–4–isoxazolepropionic acid (AMPA) subtypes are the best characterized.[1] The function of the receptor may be modified by intracellular mechanisms such as phosphorylation/dephosphorylation, nitration, and pathways of free radical generation.

Glutamate has been implicated in the neuronal death because it can kill neurons by an NMDA receptor (NMDAR)–mediated mechanism. Paradoxically, the glutamate neurotoxicity can be prevented by blockade of glutamate receptors in experimental animals.[2] We have shown that recombinant human erythropoietin (rHuEPO),[3] and transforming growth factor–β1 (TGF–β1)[4] has neuroprotective effects via NMDAR modulation on perinatal hypoxic–ischemic (HI) brain injury.

Dizocilpine [10,11–dihydro–5S–methyl–5H–dibenzo[a,d]cyclohepten–5,10–imine,(2Z)–2–butenedioate ((+)–MK–801 hydrogen maleate) is an NMDAR antagonist, which prevents signal transmission by means of the blockade of NMDAR ion channel. The NMDAR ion–channel is an effective mechanism of Ca^{2+} entry into neurons. Studies have demonstrated that brain tissue hypoxia modifies the NMDAR ion–channel recognition and modulatory sites.[5] Hypoxia–induced increase in affinity of the recognition site and ion–channel MK–801 binding site has suggested that NMDA receptor–mediated calcium fluxes are increased during hypoxia. NMDARs are key factors in the progression of excitotoxicity and the effects of dizocilpine on NMDARs are clear and significant. Thus, NMDAR antagonists have been extensively studied for use in treatment of diseases with excitotoxic components (stroke, traumatic brain injury) and neurodegenerative diseases (Huntington's, Alzheimer's, and Amyotrophic Lateral Sclerosis). Several studies have demonstrated that treatment with dizocilpine after HI reduces subsequent morphologic brain damage in adult rat[6] and this drug also decreases the size of areas with an increased cerebral metabolic rate of glucose when given 1 to 5 h after HI.[7] In a previous study, we have demonstrated that the administration of dizocilpine could significantly protect against perinatal HI brain injury via some parts of anti–apoptotic mechanisms[8] and nitric oxide (NO)–mediated.[9] Thus, we hypothesized that dizocilpine may be protected against perinatal HI brain injury via modulation of NMDA receptors. Dizocilpine up regulates NMDAR (NR)2A protein levels and induces functional recovery of the ipsilateral hemi diaphragm following acute C2 hemi section in adult rats.[10] Dizocilpine also prevents the increased expression of NMDA–NR1 and NR2B subunits mRNA, observed in the hippocampus of rats tolerant to diazepam.[11]

In this study, we determined the effects of dizocilpine via up–modulation of NMDARs (NR1, NR2A, NR2B, NR2C, and NR2D) on hypoxic–ischemic (HI) brain injury in neonatal rats by using a neonatal rat model of HI brain injury (*in vivo*) and an embryonic cortical neuronal cell culture of embryonic rats (*in vitro*). Real–time PCRs and western blots evaluated the effects.

Materials and Methods

1. Materials

Dizocilpine was purchased from Sigma (St. Louis, MO, USA). The NR2A and NR2B antibodies were kindly provided by Dr. Il Soo Moon (1:1000, Department of Anatomy, College of Medicine, Dongguk University, Gyeongju, Korea). Mouse monoclonal β–Actin

and secondary goat anti-mouse or rabbit IgG-HRP antibodies were purchased from Santa Cruz Biotechnology (1:2000, CA, USA).

2. Animal model and drug administration

This study was performed in accordance with the approved animal use guidelines of the Catholic University of Daegu. The animal experiments were approved by the Animal Care and Use Committee at the Catholic University Medical Center of Daegu. We chose 7-day-old Sprague-Dawley (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.[12]

A total of 54 litters were used in this the experiment study. Six rats died during the HI experiment. Rat pups were randomly divided into six groups (n=8-10/group). No surgical procedure was not exposed to hypoxia (normoxia: N) (n=8) or was exposed to hypoxia (hypoxia: H) (n=8). Hypoxia with sham-operated pups underwent the same surgical procedure without ligation (hypoxia sham-operated: HS) (n=8). The pups were subjected to hypoxia with operation (hypoxia operation: HO) (n=8). Another pups received an intraperitoneal injection with PBS at the same volume with dizocilpine (hypoxia vehicle: HV) (n=7) or with dizocilpine at a dose of 10 mg/kg (hypoxia dezocilpine: HD) (n=9). Dizocilpine was prepared in phosphate-buffered saline (PBS) and injected intraperitoneally at a dose of 10 mg/kg 30 minutes before the hypoxic exposure. A modification of Levine preparation was used as a model for perinatal hypoxic-ischemic brain injury as previously described.[13] 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 min. 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 hours period of hypoxia (92% N_2, 8% O_2) by placing them in airtight containers partially submerged in a 37℃ 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℃ until use.

3. Embryonic cortical neuronal cell culture

Culture of cortical neuronal cells from rat embryos was performed using the Brewer method.[14] Dissociated cultures from Sprague Dawley rat embryonic (E18, both sexes) cerebral cortical neurons were prepared as follows: the isolated cortices free of meninges were dissected at 37℃ 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 and incubated at 37℃ water bath for 1 minute. After washing five times with 10 mL HBSS, the cells were moved in 1 mL HBSS, and dispersed by pipetting 6-7 times with a small-bore Pasteur pipette. The cell suspension was centrifuged at 1,000 rpm at 25℃ for 5 minutes and pellets were washed with HBSS (without phenol red). The pellet was resuspended in Neurobasal media supplemented with 2% B27 and 0.5 mM glutamax I. Cells were plated at a density of 2×10^6 cells/mm^2 in each dish precoated with 50 μg/mL poly-D-lysine. Cultures were maintained in Neurobasal media at 37℃ in a humidified atmosphere containing 5%

CO_2. Half of the medium was changed every 3 days.

The cultured cells were divided into three groups: a normoxia group (N), a hypoxia group (H), a hypoxia treated with dizocilpine (HD). The N group was prepared in 5% CO_2 incubators and the other groups (before a hypoxia injury) were placed in 1% O_2 incubators (94% N_2, 5% CO_2) for 16 hours.

4. 3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyl–tetrazolium bromide (MTT) assay

The MTT assay was used for estimation of cell viability and growth as originally described by Mosmann.[15] MTT was dissolved at a concentration of 5 mg/mL. Ten μL of the 5 mg/mL MTT stock solution was added to 96 well plate. After 4 hour of incubation at 37℃, media was removed and added 100 μL of the lysing buffer [Dimethyl sulfoxide (DMSO): 95% ethanol=1:1]. Absorbance of the samples was read at 492 nm using a microtiter plate enzyme–linked immunosorbent assay (ELISA) reader. The amount of formazan produced is proportional to the number of live and metabolically active cells.

5. 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℃ for 30 minutes and the supernatant was transferred to a new tube. Proteins were quantified using Bio–Rad Bradford kit (Bio–rad Laboratories, CA, USA) and taking spectrophotometric readings at 590 nm. Concentrations were estimated against a standard curve generated using BSA. Equal amounts of proteins (50 μg) were subjected to 12% SDS–PAGE after denaturing in 5 x SDS gel–loading buffer (60 mM Tris–HCl pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2–mercaptoehanol and 0.1% bromophenol blue) in boiling water for 10 minutes. After electrophoresis, proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) at a constant voltage of 10 V for 30 minutes. After transfer, the membrane was washed twice with 1 x Tris–buffered saline (TBS) plus 0.1% Tween–20 (TBST, pH 7.4) and preincubated with a blocking buffer (5% nonfat dry milk in TBST) at room temperature for 1 hour. Proteins were reacted with primary antibodies for overnight at 4℃ and then incubated with secondary antibodies for 1 hour at room temperature. Signals were detected using an enhanced chemiluminoescence (ECL) Plus Western Blotting Detection System (Amersham Biosciences, 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). Western blot was repeated four times in the same sample and the mean value was recorded.

6. Semiquantitation of the western blots

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

7. RNA extraction and real–time PCR

Total RNA was extracted from tissue with TRIzol reagent (Invitrogen Corporation, Calsbad, CA, USA). Briefly, samples were homogenized in 1 mL of TRIzol

reagent. Total RNA was separated from DNA and proteins by adding chloroform and was precipitated using isopropanol. The precipitate was washed twice in 100 % ethanol, air-dried, and re-diluted in diethylpyrocarbonate (DEPC)-treated distilled water. The amount and purity of extracted RNA was quantitated by spectrophotometry (GeneQuantTM pro RNA/DNA calculator, GE Healthcare, USA), and the RNA was stored at -70℃ pending further processing. For reverse transcription, total RNA (1 μg) was reverse transcribed for 1 hour at 37℃ in a reaction mixture containing 20 U RNase inhibitor (Promega, Madison, WI, USA), 1 mM dNTP (Promega), 0.5 ng oligo-(dT) 15 primer (Promega), 1 x RT buffer and 200 U M-MLV reverse transcriptase (Promega). The reaction mixture was then incubated at 95℃ for 5 minutes to stop the reaction. The cDNA was stored at -20℃ until further processing.

Real-time PCR was performed in 48-well PCR plates (Mini Opticon™ Real-Time PCR System, Bio-rad, USA) using the iQ™ SYBR Green Supermix (Bio-rad Laboratories, CA, USA). Amplification conditions are shown in Table 1. It was the same for all apoptotic and oxidant mRNA assayed: 94℃ for 5 minutes, followed by 40 cycles of 94℃ for 30 seconds, annealing temperature for 30 seconds, and 72℃ for 30 seconds. Real-time PCR data were analysed with LightCycler software (BIORad Lab, Hercules, CA, USA). All experiments were performed at least in six times.

Table 1. Primer Pairs and Annealing Temperature for Real-time PCR

Name	Primer Sequence (5'-3')	Annealing
NR1	F: AAGCCCAACGCCATACAGAT	53℃
	R: AGGCGGGTGGCTAACTAGGA	
NR2A	F: GCTATGGGCAGGCAGAGAAG	58℃
	R: GTGGTTGTCATCTGGCTCGC	
NR2B	F: GCTACAACACCCACGAGAAGAG	58℃
	R: GAGAGGGTCCACGCTTTCC	
NR2C	F: AACCACACCTTCAGCAGCG	56℃
	R: GGTTTCTTGCCCTTGGTGAG	
NR2D	F: CGATGGCGTCTGGAATGG	54℃
	R: AGATGAAAACTGTGACGGCG	

8. Statistics analysis

Data were analyzed using the SPSS version 12.0 statistical analysis package. Examined data were assessed using the *t*-test, and ANOVA. In each test, the data were expressed as the mean±tandard deviation, and *P*-value <0.05 was accepted as statistically significant.

Results

1. The expressions of NR2A and NR2B antibodies by western blots in the neonatal HI brain injury (*in vivo*)

The expressions of NR2A and NR2B antibodies were reduced in the HO and HV groups when compared to the N, H, and HS groups, whereas they were increased in the HD group compared to the HO and HV groups ($P<0.05$) (Fig. 1A and 1B).

2. The expressions of NR1, NR2A, NR2B, NR2C and NR2D mRNAs by real-time PCRs in the neonatal HI brain injury (*in vivo*)

The expressions of NR1, NR2A, NR2B, NR2C, and NR2D mRNAs were reduced in the HO and HV groups when compared to the N, H, and HS groups, whereas they were increased in the HD group compared to the HO and HV groups ($P<0.05$) (Fig. 2A, 2B, 2C, 2D, and 2E).

3. Morphologic changes in the embryonic cortical neuronal cell culture of rats (*in vitro*)

Researchers observed the cortical neuronal cells using light microscopy under high magnification (× 200). The cells in the N group (Fig. 3A) were well preserved, whereas the cells in the H group (Fig. 3B)

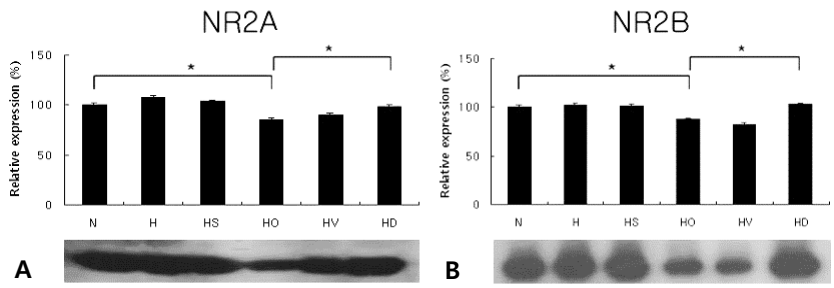

Fig. 1. Western blots of NR2A (A; N, 100±2.1; H, 107.5±2.1; HS, 103.2±2.1; HO, 85.1±1.7; HV, 90.5±1.8; HD, 98.5±1.9) and NR2B (B; N, 100±2.0; H, 101.6±2.0; HS, 101.2±2.0; HO, 87.9±1.8; HV, 82.3±1.6; HD, 102.6± 2.1) in the neonatal hypoxic-ischemic brain injury (*in vivo*) (n=4). Dizocilpine was administered at 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.

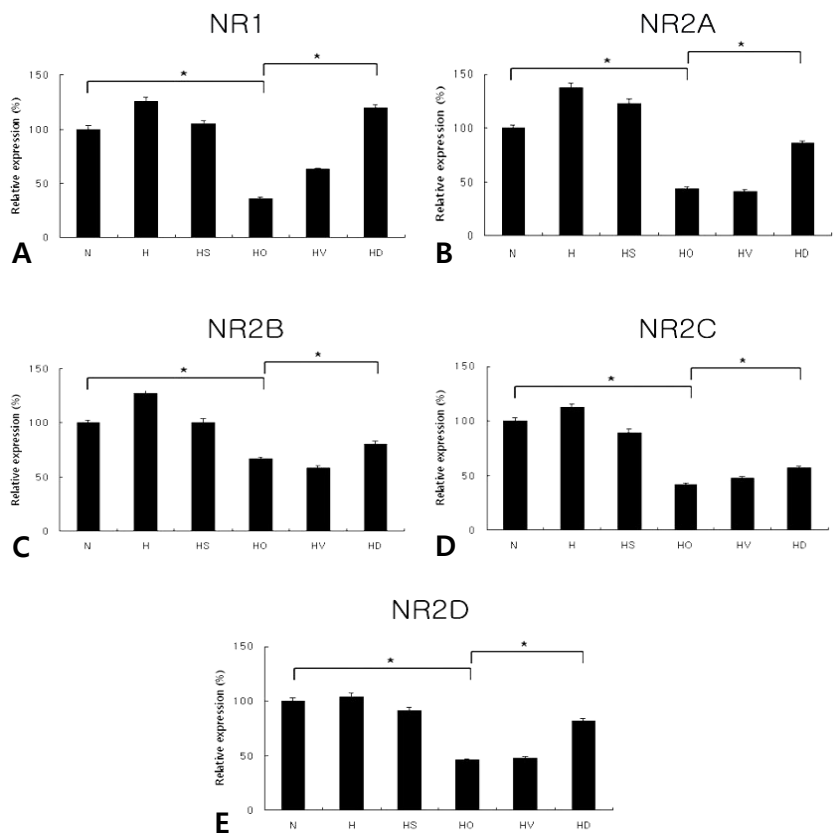

Fig. 2. Real-time PCRs of NR1 (A; N, 100±3.0; H, 125.7±3.8; HS, 104.9±3.1; HO, 36.1±1.1; HV, 62.9±1.8; HD, 119.7±3.4), NR2A (B; N, 100±3.1; H, 137.6±4.1; HS, 123.1±3.6; HO, 43.8±1.3; HV, 41.8±1.3; HD, 85.9±2.6), NR2B (C; N, 100±3.3; H, 127.5±3.8; HS, 100.7±3.0; HO, 66.9±2.0; HV, 58.6±1.8; HD, 80.7±2.4), NR2C (D; N, 100±3.1; H, 112.5±3.4; HS, 89.5±2.6; HO, 42.3±1.3; HV, 47.9±1.4; HD, 56.6±1.7), and NR2D (E; N, 100±3.1; H, 104.2±3.1; HS, 91.4±2.7; HO, 46.0±1.3; HV, 47.9±1.4; HD, 81.8±2.4) mRNAs in the neonatal hypoxic-ischemic brain injury (in vivo) (n=6). Dizocilpine was administered at 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.

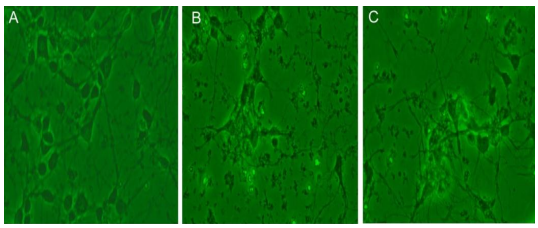

Fig. 3. Morphologic changes in the embryonic cortical neuronal cell culture of rat (*in vitro*). A, normoxia; B, hypoxia; C, hypoxia treated with dizocilpine.

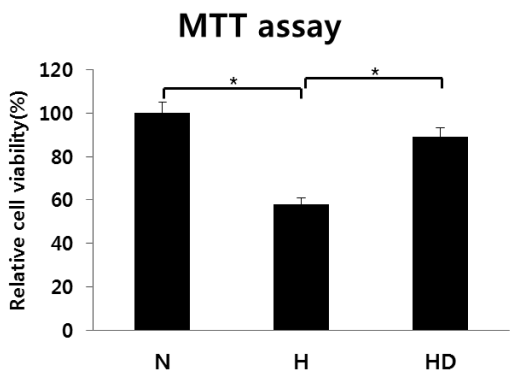

Fig. 4. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Dizocilpine was administered at 10 μg/mL. N, normoxia; H, hypoxia; HD, hypoxia treated with dizocilpine; *P<0.05, statistically significant vs. H.

showed cellular damages. The cellular patterns of the HD (Fig. 3C) appeared similar to those in the N group.

4. Cell viability of the *in vitro* hypoxic-ischemic brain injury, determined by the MTT assay

Cell viability was determined by the MTT assay. Cell viability in the H (58%) group was decreased when compared to the N (100%) group. Cell viability in the HD (89%) was increased more when compared to the H group (Fig. 4).

5. The expressions of NR2A and NR2B antibodies by western blots (Fig. 5A) in the embryonic cortical neuronal cell cultures (*in vitro*)

The expressions of NR2A and NR2B antibodies decreased in the H group when compared to the N group, whereas they increased in the HD group compared to the H group (P<0.05) (Fig. 5B and 5C).

6. The expressions of NR1, NR2A, NR2B, NR2C, and NR2D mRNAs by real-time PCRs in the embryonic cortical neuronal cell cultures (*in vitro*)

The expressions of NR2B, NR2C, and NR2D mRNAs

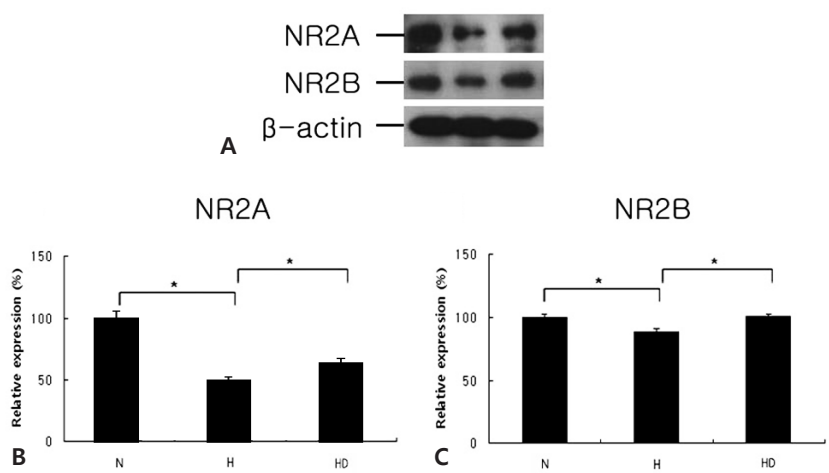

Fig. 5. Western blots (A) of NR2A (B; N, 100±2.1; H, 52.5±1.5; HD, 67.1±1.6) and NR2B (C; N, 100±2.1; H, 89.4±1.8; HD, 100.5±2.0) in the embryonic cortical neuronal cell culture (in vitro) (n=4). Dizocilpine was administered at 10 μg/mL. N, normoxia; H, hypoxia; HD, hypoxia treated with dizocilpine; *P<0.05, statistically significant vs. H.

decreased in the H group when compared to the N group, whereas they increased in the HD group compared to the H group (P<0.05) (Fig. 6C, 6D, and 6E). However, the expressions of NR1, NR2A mRNAs decreased in the H group when compared to the N group, whereas they increased a little in the HD group when compared to the H group (Fig. 6A and 6B).

Discussion

HI–induced cell death is not a single process, but rather multifactorial sequence that includes increased activation of excitatory amino acid neurotransmitter receptors, production of oxygen free radicals, increased intracellular Ca^{2+}, changes in cellular ion fluxes, and changes in expression of apoptotic and anti–apoptotic genes that contribute to cerebral dysfunction and cell death.[16]

The rat pup HI model and cell culture model of neurons prepared from cortex of rat embryos have been well characterized and used extensively to search for neuro–protective agents.[17] The most accepted rat pup

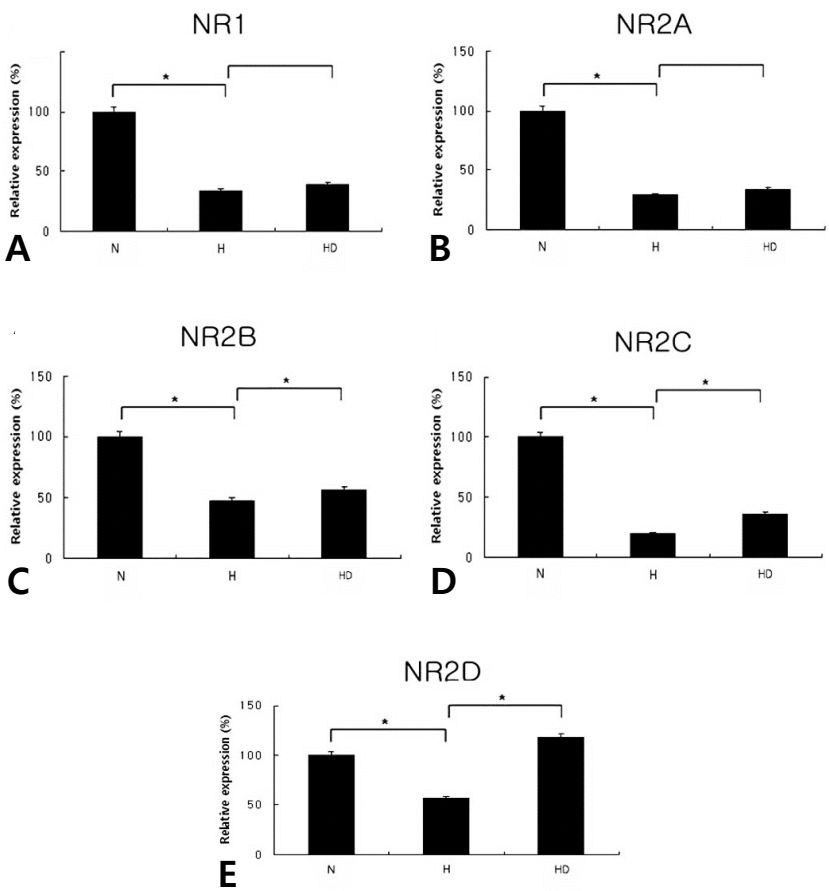

Fig. 6. Real-time PCRs of NR1 (A; N, 100±4.1; H, 34.0±1.4; HD, 39.5±1.6), NR2A (B; N, 100±4.0; H, 28.7±1.1; HD, 34.2±1.4), NR2B (C; N, 100±4.0; H, 47.5±1.9; HD, 56.1±2.2), NR2C (D; N, 100±4.2; H, 19.8±0.8; HD, 36.1±1.4), and NR2D (E; N, 100±4.1; H, 56.3±2.3; HD, 117.7±4.7) in the embryonic cortical neuronal cell culture (in vitro) (n=6). Dizocilpine was administered at 10 μg/mL. N, normoxia; H, hypoxia; HD, hypoxia treated with dizocilpine; *P<0.05, statistically significant vs. H.

HI model is a modification of the Levine preparation described by Rice et al.,[18] which includes a combination of ischemia, obtained by unilateral occlusion of the carotid artery, followed by exposure to hypoxia in 7-day-old rats. The neurodevelopmental stage of 7-day-old rats corresponds to that of newborn infants.[19] This animal model represents a useful tool for studying potential neuro-protective strategies capable of preventing or limiting perinatal HI injury in humans.

We have shown here that the cortical neuronal cells cultured in the normal oxygen environment appeared normal. In contrast, in neuronal cells exposed to hypoxia, those showed cellular damages. In a previous study, we have shown that cell viability of cortical neuronal cells was reduced after the hypoxic insult.[20] The affected cells treated with dizocilpine appeared similar to normal cells. Classically, cell death secondary to overactivation of glutamate receptors HI-induced changes in the NR2A and NR2B expression varies depending on the developmental stages. Pathological hyperexcitability of glutamate receptors can continue for long time following HI and treatment with the specific, noncompetitive NMDA antagonist dizocilpine blocks this activity and improves neuronal injury.[21,22]

The glutamate receptor family is made up of two main groups of receptors: the ionotropic glutamate receptors, which are ligand-gated ion channels sensitive to NMDARs, and AMPA/kainate receptors and the metabotropic glutamate receptors, which act through second messenger systems. The NMDARs are heterotetrameric channels composed of different subunits: NR1, NR2. The NR1 subunit is encoded by a single gene but exists as several variants generated by alternative splicing.[23] This NR subunit is expressed in most neurons, whereas the NR2 subunits (NR2A, NR2B, NR2C, and NR2D) are expressed in a cell-type specific manner, contributing to the functional and spatiotemporal diversities of the receptor complex.[24]

However, the demonstration of such specific age-related differences in the response to H/I suggests a basis for the changing sensitivity of the developing brain to excitotoxicity at the time of the insult, and could have longer lasting effects of synaptic events involved in recovery at different ages.

HI induced changes in NR2A and NR2B are specific to the developmental stage of the brain. NR2A levels in the right hemisphere of P7 pups were markedly reduced after 24 h of recovery. In contrast, NR2B, but not NR2A, was reduced after HI at P21.[25] Decreased expression and functionality of both NR2A and NR2B were also observed in adult rat hippocampus after transient cerebral ischemia.[26] In our study, the expression of NR1, NR2B were significantly reduced at 7 days after HI, compared to normoxia group, but were unaffected at 24 h, consistent with previous observations of HI in neonatal rats.[25] Band[27] reported that NR2D mRNA expressions are significantly decreased due to acute hypoxic stress of 6% O_2 for 5 h in rats. Under hypoxia receptors that include NR2D subunits have a greatly decreased channel open time compared to receptors without NR2D subunits. Therefore, less calcium goes into the cells through NR2 containing N2D subunits. The resulting lower internal calcium concentration could prevent calcium-mediated cell injury, and is thought to be an important mechanism of hypoxia resistance in neonatal mammals.[28]

The NMDA-type glutamate receptor is a predominant mediator of excitotoxicity in the immature brain due to abnormal receptor activation in the developing immature CNS.[29] The glutamate neurotoxicity (excessive glutamate), acting on the same glutamate receptors, over activate NMDARs, causing high levels of calcium ions (Ca^{2+}) to influx into the postsynaptic cells.[30] High Ca^{2+} concentrations activate a cascade of

cell degradation processes involving lipases, proteases, nitric oxide synthase, and a number of enzymes that damage cell structures often to the point of cell death.[31] The NMDAR in the CNS has several well–characterized regulatory and functional binding sites, including the glycin site, the glutamate site, and a site within the receptor–associated ion channel that binds phencyclidine and the noncompetitive antagonist dizocilpine maleate.[32]

Antagonism of the NMDAR following brain injury resulted in a decrease of neuronal damage.[33] Systemically administered ketamine exerts a partial neuroprotective action in the immature rat brain against hypoxic–ischemic damage.[34] Specifically, an improvement in functional recovery and cell survival occurs after administration of dizocilpine.[10, 35] According to previous reports, dizocilpine at NMDAR can protect neurons from HI.[36] However, there are not enough reports about the expressions of NR1, NR2A, NR2B, NR2C, and NR2D after administration of dizocilpine in neonatal HI brain injury. For a better understanding of modulation of NMDAR, the present study showed that the expressions of NR2A and NR2B antibodies lower than those in the normoxia group, whereas they higher in the dizocilpine–treated group compared to the hypoxia group. The expressions of NR1, NR2A, NR2B, NR2C, and NR2D mRNAs lower than those in the normoxia group, whereas they higher in the dizocilpine–treated group compared to the hypoxia group. These data suggest that dizocilpine might effectively modulate a mechanism of NMDAR in HI model.

In conclusion, our experiments (*in vivo*, *in vitro*) demonstrate that dizocilpine has effects for neuronal cells exposed to hypoxic condition. In addition, dizocilpine's effects on HI brain injury in neonatal rats may be via up–modulation of NMDAR. These results may be useful for the further development of clinical therapies for perinatal HIE induced by cerebral hypoxia.

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= 국 문 초 록 =

신생 백서의 저산소성 허혈성 뇌손상에서 NMDA 수용체 조절 기전을 통한 dizocilpine (MK-801)의 뇌신경 보호 효과

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목적 : 최근 N-methyl-D-aspartate (NMDA) 수용체 길항제인 dizocilpine (MK-801)는 저산소성 허혈성 뇌손상에서 신경 보호 효과가 있다고 발표되고 있지만 신생아기 저산소성 허혈성 뇌병증의 치료제로서 그 기전이 명확하게 밝혀지지 않았다. 저자들은 dizocilpine을 이용하여 주산기 저산소성 허혈성 뇌병증의 치료제로서 NMDA 수용체 조절을 통한 기전을 알아보고자 하였다.

방법 : 생체내 실험으로 저산소성 허혈성 뇌병증의 동물 모델에서는 생후 7일된 신생 백서의 좌측 총 경동맥을 결찰한 후 6개 군(정상 산소군, 수술 없이 저산소군, sham 수술 후 저산소군, 수술 후 저산소군, vehicle 투여 후 저산소군, dizocilpine 투여 후 저산소군)으로 나누었고, dizocilpine은 뇌손상 전 30분에 체중 kg당 10 mg를 투여하였으며, 저산소 손상은 특별히 제작한 통속에서 2시간 동안 8% O_2에 노출시켰다. 생체 외 실험으로 재태 기간 18일된 태아 백서의 대뇌피질 세포를 배양하여 정상 산소군은 5% CO_2 배양기(95% air, 5% CO_2)에 두었고, 저산소군과 뇌손상 전 dizocilpine 투여군(10 μg/mL)은 1% O_2 배양기(94% N2, 5% CO_2)에서 16시간 동안 뇌세포손상을 유도하였다. 세포의 viability를 확인하기 위하여 MTT assay를 실시하였다. 생체 내·외 실험 모두 NMDA 수용체의 아단위인 NR1, NR2A, NR2B, NR2C, NR2D primer를 이용하여 실시간 중합효소연쇄반응을 실시하였고, 또한 NMDA 수용체의 아단위 항체(NR2A, NR2B)를 이용하여 western blotting을 시행하였다.

결과 : 생체 내·외 실험에서 NMDA 수용체 아단위의 발현은 정상 산소군보다 저산소군에서 감소하였지만 dizocilpine 투여군에서는 저산소군보다 모두 증가하였다. 하지만 생체 외 실험에서 NR1, NR2A mRNA에서는 정상 산소군 보다 저산소군에서는 감소하였지만 dizocilpine에서는 저산소군보다 아주 적게 증가하였다.

결론 : 본 연구에서 dizocilpine은 저산소성 허혈성 뇌손상 모델에서 NMDA 수용체를 조절하는 효과가 있음을 알 수 있었다.

중심 단어 : Dizocilpine, NMDAR, Glutamate receptor, Hypoxic-ischemic brain injury, Modulation