The Neuroprotective Effects of 6-cyano-7-nitroquinoxalin-2,3-dione (CNQX) Via Mediation of Nitric Oxide Synthase on Hypoxic-ischemic Brain Injury in Neonatal Rats

Ji Eun Jung, M.D., Kyung Hae Keum, M.D., Eun Jin Choi, M.D., Jin Kyung Kim, M.D., Hai Lee Chung, M.D. and Woo Taek Kim, M.D.
Department of Pediatrics, School of Medicine, Catholic University of Daegu, Daegu, Korea

Purpose: Current studies have demonstrated the neuroprotective effects of 6-cyano-7-nitroquinoxalin-2,3-dione (CNQX) 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 CNQX can protect the developing rat brain from HI injury via mediation of nitric oxide synthase.

Methods: In an in vivo model, left carotid artery ligation was done in 7-day-old Sprague-Dawley (SD) rat pups. The animals were divided into six groups; normoxia (N), hypoxia (H), hypoxia with sham-operation (HS), hypoxia with operation (HO), HO treated with vehicle (HV), and HO treated with CNQX at a dose of 10 mg/kg (HC). Hypoxia was made by exposure to a 2 hr period in the hypoxic chamber (92% N₂, 8% O₂). In an in vitro model, embryonic cortical neuronal cell culture of SD rats at 18-day gestation was done. The cultured cells were divided into three groups: normoxia (N), hypoxia (H), and hypoxia treated with CNQX (HC). 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 hr.

Results: In the in vitro and in vivo models, the expressions of iNOS and eNOS were reduced in the hypoxia group when compared to the normoxia group, whereas they were increased in the CNQX-treated group compared to the hypoxia group. In contrast, the expression of nNOS was showed reversely.

Conclusion: CNQX has neuroprotective property over perinatal HI brain injury via mediation of nitric oxide synthase.

Key Words: CNQX, Nitric oxide synthase, Cerebral ischemia, Neuroprotection

Introduction

Excitotoxicity is the pathological process by the overactivation of receptors for the excitatory neurotransmitter glutamate including increased extracellular amino acids (AA). First, immediate neuronal death with necrosis occurs following stimulation of AMPA and kainate receptor, Na⁺, Cl⁻, H₂O into cell, and osmocel lysis within a few min.

Second, delayed neuronal death with apoptosis occurs following stimulation of NMDA receptor (metabotropic receptor = ligand-gated Ca⁺⁺ channel or voltage dependent (gated) Ca⁺⁺ channel), Ca⁺⁺ into cell, destruction of cerebral lipid, protein, nucleic acid, and cell death within a few hours or days¹.

Excitatory extracellular AA act on the central nervous system (CNS) through various receptors. Glutamate
receptors have been functionally divided into two main groups: ionotrophic and metabotropic glutamate receptors. Ionotrophic receptors act on cationic specific channels and have been classified into three structurally and pharmacologically distinct subtypes: NMDA, AMPA, and KA receptors. Mammals possess six NMDA receptor subunits (NR1, NR2A-D, NR3A), four AMPA receptor subunits (Glul–4) and five KA receptor subunits (Glu5–7, KA1, KA2). AMPA receptor possesses several agonists (5-Fluorowillardiine, AMPA, Domoic acid and Quisqualic acid) and antagonists (CNQX, GYKI-52466, GYKI53655, Kynurenic acid, NBQX, Perampanel and Tezampanel). AMPA receptor antagonists have received considerable attention in recent years, because they have shown excellent neuroprotection in various models of cerebral ischemia and neuronal injury. However, poor physical properties have been a major limiting factor in developing these as viable drug candidates.

The glutamate extracellular concentration is controlled by metabolic and neuronal pathways via release and uptake mechanisms. Stimulation of glutamate receptors induces neuronal nitric oxide (NO) release. NO is a highly reactive signal molecule in the CNS. It is a unique messenger molecule and a multifunctional biological mediator. It is synthesized from the guanidino nitrogen of L-arginine by nitric oxide synthases (NOS). The agent acts on interneuronal communications, synaptic plasticity, memory formation, receptor function, intracellular signal transduction and mediator release. However, pathological conditions may occur when higher fluxes of these mediators are generated, such as during the process referred to as excitotoxicity. Excitotoxicity produced by glutamate is initiated by a sustained increase of intracellular Ca++. Influx of Ca++ serves as a signal for activation of Ca++-calmodulin dependent and protein kinase C-regulated NOS. Activation of NOS generates NO, which can produce oxidative damage. In addition, elevated cytosolic free Ca++ can activate phospholipase A2, leading to subsequent generation of arachidonic acid. Metabolism of arachidonic acid can than produce free reactive oxygen species (ROS) and lipid peroxidation. Because NO is a reactive free radical, it has many potential targets to initiate neurotoxic cascades. NO toxicity may be amplified by the presence of superoxide radical, the one-electron reduction product of oxygen, since these species react at a diffusion-limited rate to form peroxynitrite, a potent oxidant. Thus, oxidative stress plays a critical role in excitotoxicity.

A rapid and reproducible spinal motor neuron death occurs after sciatic nerve transection in neonatal rats. Glutamate and its receptors has been implicated as possible mechanism for motor neuron death. Systemic application of CNQX to transected sciatic motor axons had a striking effect on motor neuron survival after axotomy in neonatal period. Therefore, CNQX prevents spinal motor neuron death following sciatic nerve transection in newborn rats. AMPA agonism may occur upstream to nNOS upregulation in inhibitory interneurons, Therefore, AMPA antagonists such as CNQX might downregulate nNOS expression and reduce NO levels. The production of NO after receipt of intra-cerebral KA injections can be modulated by pretreatment with competitive glutamate receptor antagonist; namely, CNQX. The used glutamate antagonist CNQX provided sufficient neuroprotection in sense of decreasing nitrite levels, but with different mechanisms and time dynamics.

To evaluate the mechanisms based on molecular biology and pharmacologic treatments in a HI brain injury, we investigated the neuroprotective effects of CNQX via mechanisms of NOS mediation on HI brain injury in neonatal rats.

Materials and Methods

1. Materials

CNQX (6-cyano-7-nitroquinolin-2,3-dione) was purchased from Sigma (St. Louis, MO, USA). Primary antibodies included the following: inducible NOS (iNOS) (1:1,000; StressgenBioreagents Corporation, Ann Arbor, MI, USA), endothelial NOS (eNOS) (1:1,000; Stressgen Bioreagents Corporation, Ann Arbor, MI, USA), neuronal NOS (nNOS) (1:1,000; StressgenBioreagents Corporation, Ann
Arbor, MI, USA) and β-Actin (1:1,000; Santa Cruz Biotechnology, CA, USA). Secondary antibodies used were goat anti-mouse or rabbit IgG–HRP (1:2,000; Santa Cruz Biotechnology, 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 modified Levine’s method in postnatal day 7 rat pups was used to the HI model of the neonatal rats. 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 premature (32–34 weeks-old) 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 were anesthetized using ether. The neck midline incision was made, and the left common carotid artery (CCA) was exposed and double ligated with 5–0 surgical silk. The skin was closed with 5–0 surgical silk. Age-matched sham controls had the carotid artery exposed under the same anesthesia but were not ligated. Total time of surgery never exceeded 5 minutes in any animal. After a 1 hr recovery period, the rat pups with the ligated carotid artery were placed in a hypoxia chamber in which the temperature and humidity were maintained at 37℃ and 90%, respectively. The chamber was then filled with 8% oxygen and 92% nitrogen mixture. The rat pups were exposed to hypoxia for 2 hr, then allowed to recover in room air, and placed with their mother and littermates. CNQX was prepared in phosphate-buffered saline (PBS) and injected intraperitoneally at a dose of 10 mg/kg 30 minutes before a hypoxic insult (n=6–8 in each group). Pups from each litter were randomly assigned and marked to the N, H, HS, HO, HV and HC groups.

3. Embryonic cortical neuronal cell culture

Culture of cortical neuronal cells from rat embryos was performed using the Brewer method. The fetal pups were removed from SD rats pregnant for 18 days. The fetal pups were washed in 100% ethanol and Hanks’ balanced salt solution (HBSS) (GibcoBRL, NY, USA). The brains of the fetal pups 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).

Cells were counted with about 2×10⁶ cells/mm² in each dish, then they were inoculated in plating Neurobasal media (GibcoBRL, NY, USA) (100 mL neurobasal, 2 mL B27 supplement, 0.25 mL glutamax 1, 0.1 mL 25 mM glutamate, 0.1 mL 25 mM 2-mercaptoethanol). The cell cultures were incubated in a CO₂ chamber. A fifth of the culture solutions were changed every three days and were replaced with feeding Neurobasal media (GibcoBRL, NY, USA) (100 mL neurobasal, 2 mL B27 supplement, 0.25 mL glutamax 1).

The cultured cells were divided into three groups: N, normoxia; H, hypoxia; HC, hypoxia treated with CNQX. The N group was prepared in 5% CO₂ incubators while the other groups (before a hypoxia injury) were cultured in 1% O₂ incubators (94% N₂, 5% CO₂) for 16 hr.

4. Brain extraction and protein isolation

Rats were killed under ether anesthesia 7 days after hypoxia. Brain tissues were immediately removed and left
cerebral (injured) hemispheres of each animal were frozen in liquid nitrogen and stored at -70°C until use. Frozen tissues and cells were homogenized in 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. Total protein was measured with a Bio-Rad Bradford kit (Bio-rad Laboratories, CA, USA).

5. Western blotting

Tissue or cell lysates (50 μg) were separated by electrophoresis on 12% SDS-polyacrylamide gel after denaturing in 5× SDS sample buffer (60 mM Tris-HCl [pH 6.8], 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue) in boiling water for 10 minutes and transferred to a polyvinylidenedifluoride (PVDF) membrane (Millipore, MA, USA) at a constant voltage of 10 V for 30 minutes. After transfer, the membrane was washed twice with 1x tris-buffered saline (20 mM Tris-HCl [pH 7.5], 137 mM NaCl) plus 0.1% Tween-20 (TBST pH 7.5). The blot was blocked with TBST containing 5% skim milk at room temperature for 1 hr and then incubated with primary antibody solution at 4°C overnight. After washing with TBST, the membrane was incubated with secondary antibody for 1 hr at room temperature. Signals were detected with an enhanced chemiluminescence (ECL) Plus Western Blotting Detection System (Amersham Biosciences, NJ, USA) or SUPEX (Neuronex, Pohang, Korea), followed by exposure to X-ray films for different periods. Each sample was conducted four times.

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

Each tissue or cell was subjected to extraction of mRNA using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA). Briefly, a piece of tissue was 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 resuspended at 65°C in diethlylpyrocarbonate (DEPC)-treated distilled water. RNA concentration was determined by GeneQuant 1,300 spectrophotometer (GeneQuant™ proRNA/DNA calculator, GEHealthcare, USA). The RNA was then stored at -70°C before further processing. Total RNA (1 μg) from each cell or tissue extract was reverse-transcribed using a 0.5 ng Oligo (dT) 15 primer (Promega, Madison, WI) and 20 U RNase inhibitor (Promega, Madison, WI), 1 mM dNTP (Promega, Madison, WI), 1x RT buffer and 200 U M-MLV reverse transcriptase (Promega, Madison, WI) in a 20 μL reaction. The reaction mixture was reverse transcribed for 1 h at 37°C and 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 done using the Mini Opticon™ Real-Time PCR System (Bio-Rad Laboratories, CA, USA) with SYBR Green detection and Tm analysis. iQTM SYBR Green Supermix was purchased from Bio-rad (Bio-Rad Laboratories, CA, USA). Amplification is performed in a total volume of 20 μL containing 10 μL of kit-supplied iQ™ SYBR Green Supermix, 1 μL (2 pmol) of each primer, 7 μL RNase-free water, 1 μL

<p>| Table 1. Primer Pairs and Annealing Temperature for Real-time PCR |
|------------------------|------------------------|-------------------|</p>
<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequence (5'-3')</th>
<th>Annealing</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>F:AGGCTCTGGCTTGTAGCTAGT</td>
<td>55°C</td>
</tr>
<tr>
<td></td>
<td>R:TTCTCTGCTGACTCCTAGACT</td>
<td></td>
</tr>
<tr>
<td>eNOS</td>
<td>F:GGATTCTGGACAGACCGTAC</td>
<td>57°C</td>
</tr>
<tr>
<td></td>
<td>R:GGAGGACGTGGCACCAACTCT</td>
<td></td>
</tr>
<tr>
<td>nNOS</td>
<td>F:CCCTGGAGGGCTCTGTGAGCG</td>
<td>59°C</td>
</tr>
<tr>
<td></td>
<td>R:CTGACTGAGATCATCTAGGCG</td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>F:TTGCTCGACCCACTGCTGCTG</td>
<td>53°C</td>
</tr>
<tr>
<td></td>
<td>R:GACAGGATGCAGAAGGAGAT</td>
<td></td>
</tr>
</tbody>
</table>
cDNA. Cycling conditions used were as follows: denaturation at 95℃ for 1 minute, annealing shown in Table 1, extension at 72℃ for 30 seconds, 40 cycles. The relative quantification in gene expression was determined using the 2^-ΔΔCt method (Livak and Schmittgen, 2001). Each sample was conducted six times.

8. Statistics analysis

Data were analyzed using the SPSS version 12 statistical analysis package. Examined data were assessed using the t-test, GLM (general lineal 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. Gross morphologic changes in the perinatal hypoxic-ischemic brain injury

Gross morphologic changes in the perinatal hypoxic-ischemic brain injury (in vivo) showed that the percentage of left hemisphere area compared to right hemisphere area were 100.1% in the normoxia (A, N), 104.4% in the hypoxia without operation (B, H), 103.5% in the hypoxia with sham operation (C, HS), 81.6% in the hypoxia with operation (D, HO), 85.1% in the D treated with vehicle (E, HV) and 93.6% in the D treated with CNQX (F, HC) (Fig. 1). The percentage of hemisphere area was measured by using a densitometer (Multi Gauge Software: Fuji Photofilm, Tokyo, Japan).

2. The expressions of iNOS, eNOS, and nNOS antibodies by western blots in the perinatal HI brain injury

The expressions of the iNOS and eNOS antibodies were decreased in the HO and HV groups compared to the N, H and HS groups, whereas they were relatively increased in the HC group as compared to the HO and HV groups (P<0.05) (Fig. 2A and B). In contrast, the expression of the nNOS antibody was increased in the HO and HV groups compared to the N, H and HS groups, whereas it was decreased in the HC group compared to the HO and HV groups (P<0.05) (Fig. 2C).

Fig. 1. Gross morphologic changes revealed in the perinatal hypoxic-ischemic brain injury (in vivo). A, normoxia; B, hypoxia without operation; C, hypoxia with Sham operation; D, hypoxia with operation; E, D treated with vehicle; F, D treated with CNQX. Percentage of left hemisphere area compared to right hemisphere area are 100.1% (A), 104.4% (B), 103.5% (C), 81.6% (D), 85.1% (E), and 93.6% (F).
3. The expressions of iNOS, eNOS, and nNOS mRNAs by real-time PCRs in the perinatal HI brain injury

The expressions of the iNOS and eNOS mRNAs were decreased in the HO and HV groups compared to the N, H and HS groups, whereas they were relatively increased in the HC group as compared to the HO and HV groups ($P<0.05$) (Fig. 3A and B). In contrast, the expression of the nNOS mRNA was increased in the HO and HV groups compared to the N, H and HS groups, whereas it was decreased in the HC group compared to the HO and HV groups ($P<0.05$) (Fig. 3C).

4. Morphologic changes in the embryonic cortical neuronal cell culture of rat

Researchers observed the cortical neuronal cells using light microscopy under high magnification ($\times200$). The cells in the N group (Fig. 4A) appeared normal, while those in the H group (Fig. 4B) showed cellular swelling with indistinct nuclear shapes. Cells in the HC group (Fig. 4C) had a similar appearance to those in the N group.

5. The expressions of iNOS, eNOS, and nNOS by western blots (Fig. 5A) in the embryonic cortical neuronal cell culture

The expressions of the iNOS and eNOS antibodies were decreased in the H group compared to the N group, whereas they were relatively increased in the HC group as compared to the H group ($P<0.05$) (Fig. 5B and C). In contrast, the expression of the nNOS antibody was increased in the H group compared to the N group whereas it was decreased in the HC group compared to the H group ($P<0.05$) (Fig. 5D).

6. The expressions of iNOS, eNOS, and nNOS by real-time PCRs in the embryonic cortical neuronal cell culture

The expressions of the iNOS and eNOS mRNAs were decreased in the H group compared to the N group, whereas they were relatively increased in the HC group as compared to the H group ($P<0.05$) (Fig. 6A and B). In contrast, the expression of the nNOS mRNA was increased.
in the H group compared to the N group, whereas it was decreased in the HC group compared to the H group ($P < 0.05$) (Fig. 6C).

**Discussion**

Hypoxic–ischemic (HI) brain injury in the perinatal period remains a major cause of acute brain injury by activating a cascade of biochemical events. In other words, when oxygenation fails, ATP formation drops and excessive calcium influx occurs through voltage–sensitive calcium channels leading to glutamate release with activation of NMDA and AMPA glutamate receptors. At the core of the mechanism is a vicious cycle in which glutamate receptor stimulation causes activation of Na+ channels, leading to further glutamate release and NMDA and AMPA receptor overstimulation. The output of the cycle is an enduring production of NO and free radicals from neuronal and glial sources, and is responsible for delayed neuronal death. Perinatal hypoxic–ischemic encephalopathy (HIE) ultimately leads to permanent neurological dysfunction including mental retardation, cerebral palsy, learning disability, or
epilepsy).

Multiple pathogenic mechanisms have been implicated in the neuronal cell death that occurs within a few hours to a few days following the perinatal HI brain injury. These events include the following: energy failure, membrane depolarization, brain edema, an increase of neurotransmitter release, the inhibition of neurotransmitter uptake, an increase of receptor-mediated intracellular Ca++, the production of oxygen-free radicals, lipid peroxidation, a decrease of cerebral blood flow, immune/inflammatory
activation, and destructive process such as glutamate and NO neurotoxicity. Programmed cell death, or apoptosis, also plays a key role in delayed neuronal cell death after ischemic injury, particularly in the developing brain\textsuperscript{1,2,38).}

A variety of therapeutic strategies are employed to protect against the pathologic mechanisms of HI injury. These include induction of hypothermia\textsuperscript{19), antagonism of excitatory amino acid neurotransmitter receptors\textsuperscript{20), anti-inflammatory agents\textsuperscript{21), and neurotropic factors\textsuperscript{22), and inhibitors of the caspase pathway\textsuperscript{38) and of stress kinases\textsuperscript{23).}

Pharmacological agents which mediate one or more of these processes may provide neuroprotection for patients against this condition. Furthermore, study of neuroprotective agents may provide important information about treatment for perinatal HI brain injury. Most studies in seeking therapeutic targets in HI brain damage have focused on antagonists of glutamate receptors, inhibitors of NOS and free radicals and antiapoptotic pathways.

The neonatal rat HI model and the cortical cell culture model of rat embryos have been well characterized and used extensively to search for neuroprotective agents\textsuperscript{31). The Levine\textsuperscript{15) method has been widely used as an animal model of perinatal HI brain injury, and many investigators have reported the morphological, biophysical and biochemical changes following HI brain insult. This model of HI brain injury results in a reproducible pattern of hemispheric injury that is ipsilateral but not contralateral to the carotid ligation. The 7–day postnatal rat was originally chosen for study because at this stage of development the animal’s brain is histologically similar to that of a 32– to 34–week gestation human fetus or newborn infant. Therefore, HI brain injury in 7–day–old rats can be considered as analogous to perinatal asphyxia in premature infants\textsuperscript{1).

Our studies showed that as brain was exposed to hypoxia, the volume of affected brain was reduced compared to ones of contralateral unaffected brain. However, the affected brain treated with CNQX was restored to the similar volume of normal brain. The cortical neuronal cells cultured in the normal oxygen environment appeared normal, whereas those exposed in the hypoxic oxygen environment showed cellular damages. The affected cells treated with CNQX appeared similar to normal cells.

A physiological role for NO in the nervous system has been established by demonstrations that arginine derivatives, which are potent and selective inhibitors of NOS, block neuronally mediated relaxation of the intestine\textsuperscript{25–27) and stimulation of cGMP formation by glutamate in the cerebellum\textsuperscript{28). NO is formed from arginine in brain and endothelial cells by NOS, which has been purified to homogeneity and molecularly cloned\textsuperscript{28) from rat brain. Immunocytochemical studies have localized NOS to select neuronal populations in the brain, as well as to neurons in the retina, adrenal medulla, and intestine, and to nerve fibers in the posterior pituitary\textsuperscript{28). Pathological situations such as hypoxia and ischemia can lead to excess release of glutamate and its accumulation in the extracellular space, which initiates the pathway of neuronal death known as excitotoxicity\textsuperscript{29).}

Excitotoxicity results in the production of NO. NO is produced by NOS, of which there are three major isoforms: nNOS, eNOS, and iNOS\textsuperscript{30). The three NOS isoforms are expressed in the central nervous system, eNOS and nNOS are constitutive and second messenger–regulated enzymes that play physiological roles in crucial processes such as vasodilation and synaptic plasticity\textsuperscript{31). Generally, iNOS is absent in the resting cells and is triggered in response to stimuli such as bacteria or proinflammatory cytokines and lead to the production of greater amounts of NO\textsuperscript{31). iNOS activity has been demonstrated in a wide variety of stimulated cells like macrophages, neutrophils, vascular smooth cells, and glial cells in the CNS\textsuperscript{32).}

NO exerts both neurotoxic and neuroprotective effects in focal cerebral ischemia\textsuperscript{34). Under cerebral ischemia, high concentrations of NO generated by the calcium–dependent activation of the constitutive nNOS and by the activation of the iNOS in macrophages and other cell types intervene in inflammatory and cytotoxic actions that lead to neuronal death\textsuperscript{31). Studies using pharmacological inhibitors of NOS or isoenzyme–deficient mutant mice, suggest that increased NO production by nNOS or iNOS during ischemia is cyto-
toxic, although the precise molecular mechanisms of toxicity remain to be elucidated\(^5\)\(^6\). In contrast, NO generated by the activation of the constitutive eNOS have protective effects that decrease the ability of platelets to aggregate, prevent leukocyte endothelial adhesion, and increase vascular dilation and cerebral blood flow\(^7\)\(^8\). Enhanced NO production from eNOS may also promote angiogenesis in damaged tissue\(^8\)\(^9\). The mechanisms causing postischemic eNOS upregulation are currently unknown. But vascular eNOS protein expression is believed to increase very rapidly after focal ischemia. The increase in NO formation has been implicated in the pathogenesis of HI brain damage in neonatal rats\(^9\)\(^10\). NOS inhibitors and some agents that inhibit NO formation in vivo have been shown to be useful in the treatment of hypoxic and ischemic brain injury\(^10\)\(^11\).

In the present study, western blots and real-time PCRs of brain homogenates and cortical cell cultures to measure the expression of the different NOS isoforms in neonatal HI brain injury revealed that hypoxia induced a significant change in the expression of all isoforms of NOS. After hypoxia, the expressions of iNOS and eNOS were increased, but while that of nNOS was decreased. This result is different from that mentioned above, but this has been suggested that eNOS or iNOS is neuroprotective, whereas nNOS is neurotoxic. The pattern of change in expressions of NOS isoform mRNAs after hypoxia was the same as the western blot results of the NOS isoforms. In the CNQX-treated group before or after hypoxia, the expression patterns of all NOS isoforms were reversed. These data suggest that CNQX might exert some neuroprotective effect via a mechanism of NOS mediation.

한글요약

목적: 6-cyano-7-nitroquinoxalin-2,3-dione (CNQX)는 저산소성허혈성뇌병증, 외상성뇌손상, 흥분독성과 같은 뇌 손상 동물 모델에서 보호 효과가 있다고 발표되고 있지만 신생아기 에 그 효과에 대해서는 아직 제한적이다. 저자들은 CNQX를 이용하여 저산소성허혈성뇌손상 동반한 발달 과정에 있는 환자 뇌에 산화질소합성효소 중재를 통한 낮 보호 효과를 알아보기 하였다.

방법: 생체내 실험으로 생후 7일된 신생 환자의 좌측 총 경동맥을 결찰한 후 6개군(정상산소군, 수술없이 저산소군, sham 수술 후 저산소군, 수술 후 저산소군, vehicle 투여 후 저산소군, CNQX 투여 후 저산소군)으로 나누었고, 저산소 손상은 특별히 제작한 통속에서 2시간 동안 8% O\(_2\)에 노출시켰다. CNQX은 주향 30분에 체중 kg당 10 mg를 투여하였다. 생체외 실험으로 새끼간 18일된 테아 백서의 대뇌피질 세포를 배양하여 정상산소군은 5% CO\(_2\) 배양기(95% air, 5% CO\(_2\))에 두었고, 저산소군과 뇌손상 전 CNQX 투여군(10 \(\mu\)g/mL)은 1% O\(_2\) 배양기(94% N\(_2\), 5% CO\(_2\))에서 16시간동안 뇌세포손상을 유도하였다. 생체내 외 실험 모두 산화질소합성효소와 관련된 iNOS, eNOS, nNOS 항체와 primer를 이용하여 western blotting과 실시간 중합효소연쇄반응을 시행하였다.

결과: 산화질소합성효소와 관련된 생체외·내 실험에서 iNOS와 eNOS의 발현은 저산소군에서 정상산소군보다 감소하였으나 CNQX 투여군에서는 저산소군보다 증가하였다. nNOS의 발현은 반대로 표현되었다.

결론: 본 연구에서 CNQX는 산화질소합성효소 중재를 통해 주산기 저산소성 허혈성 뇌손상에서 신경보호 역할을 하는 것을 알 수 있었다.

References


