Effects of nitric oxide synthase inhibition on changes in cerebral oxygenation and cerebral blood flow during kainic acid-induced seizures in newborn rabbits

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ABSTRACT

Changes in cerebral oxygenation and cerebral blood flow during kainic acid (KA)-induced seizures were studied in newborn rabbits. A nitric oxide (NO) synthase inhibitor, N-ω-nitro-L-arginine methyl ester (L-NAME), was also used to investigate effects of endogenous NO on the cerebral hemodynamics during KA-induced seizures. We measured concentrations of oxyhemoglobin (HbO₂), deoxyhemoglobin (HbR), and total hemoglobin (tHb) in brain tissue being monitored by near-infrared spectroscopy (NIRS), and cerebral blood flow (CBF) by laser Doppler flowmetry. Changes in the mean arterial blood pressure (MABP), and electroencephalography (EEG) were also monitored continuously. The KA administration caused abnormal epileptic discharges and metabolic acidosis in all the rabbits without significant differences in PaO₂, PaCO₂, hemoglobin, blood glucose, and rectal and brain temperatures. Our study showed that KA-induced seizures in newborn rabbits caused an increase in CBF and a maintenance of cerebral oxygenation, and that L-NAME administration induced significant decreases in HbO₂, tHb and CBF, and a significant increase in HbR in animals with KA-induced seizures, compared with those without seizures. The inhibition of these changes by an nitric oxide synthase (NOS) inhibitor indicates that the cerebral vasodilation during KA-induced seizures is mediated by the endogenous NO in the neonatal brain.

Abbreviations

NO: nitric oxide
L-NAME: N-ω-nitro-L-arginine methyl ester
KA: kainic acid
CBF: cerebral blood flow
NOS: nitric oxide synthase
NIRS: near-infrared spectroscopy
HbO₂: oxyhemoglobin
HbR: deoxyhemoglobin
tHb: total hemoglobin
MABP: mean arterial blood pressure
EEG: electroencephalography
INTRODUCTION

Although neonatal seizures are one of the most important features of neurological diseases in the neonatal period, regulation of the cerebral oxygenation during neonatal seizures has never been clearly understood. It is important to elucidate changes in not only cerebral blood flow (CBF) but also cerebral oxygenation during seizures, because the cerebral oxygenation changes, including the factor of the cerebral metabolic rate of oxygen, have major effects on brain damage. Near-infrared spectroscopy (NIRS) is a modern technique for the non-invasive monitoring of tissue oxygenation, and is based on the measurement of the characteristic absorption of near-infrared light by the chromophores, oxyhemoglobin and deoxyhemoglobin, within cerebral tissue\(^1\). The NIRS allows us to measure continuous changes in concentrations of oxyhemoglobin (HbO\(_2\)) and deoxyhemoglobin (HbR), and the total hemoglobin concentration in brain tissue (tHb)\(^2\).

Furthermore, mechanisms underlying the regulation of cerebral hemodynamics during seizures has never been clarified in the neonatal brain. One of the candidates modulating the cerebral vascular tone during seizures is nitric oxide (NO). The NO, a diffusible and highly reactive molecule, is synthesized from L-arginine and oxygen by a calcium-calmodulin-dependent NO synthase (NOS) in a variety of cells, including endothelial cells, neurons, and platelets\(^3\). NO derived from the endothelium has been suggested to increase cyclic GMP in vascular smooth muscle thereby causing vasodilation\(^4\). It has been found that inhibition of NOS prevents the increase in CBF during drug-induced seizures in adult animals\(^5\)\(^\text{--}\)\(^8\), suggesting that NO mediates the vasodilation during seizures. However, the role of NO in cerebral circulation during seizures has never been clearly understood in the neonatal brain.

In this study we measured changes in cerebral tissue oxygenation using the noninvasive technique of NIRS during kainic acid (KA)-induced seizures. We used a model of seizures induced by KA, a non-N-methyl-D-aspartate (non-NMDA) receptor agonist, known to be a neurotoxin and potent convulsant. Systemic administration of KA induces typical limbic seizures, the behavioral and electroencephalographic characteristics of which have been extensively described\(^9\). The aim of this study was to investigate the relationship between the changes in cerebral oxygenation and CBF in the newborn brain. To assess the role of endogenous NO during seizures in the neonatal brain, we also tested whether NO synthase inhibition causes deterioration of cerebral hemodynamics.

MATERIALS AND METHODS

Animal model. Twelve female rabbits aged two weeks and with a body weight of 185--280 g (median 235 g) were examined. Anesthesia was induced with diethyl ether inhalation, and then tracheostomy and intubation were performed. After pancuronium bromide (0.1 mg/kg) had been injected through an ear vein, mechanical ventilation was carried out with a volume-limited ventilator (Harvard Rodent Ventilator Model 683; Harvard Apparatus Co., Natick, MA) with air at a respiratory rate of 30 breaths per min (bpm), with administration of intravenous pentobarbital sodium (3 mg/kg), as necessary. The mean arterial blood pressure (MABP) and heart rate (HR) were continuously monitored through a femoral artery with a strain gauge transducer system. Arterial blood was taken from the femoral artery for measurement of arterial blood gas and hemoglobin concentrations (Ciba-Corning 288; Ciba-Geigy, Summit, NJ), and the blood glucose level (Glucostix, Miles Sanko Co., Japan). Bipolar needle electrodes were placed in the frontal and parietal regions of the left hemisphere through small holes, and a referential electrode was placed in the ear for electroencephalography (EEG) (Rectigraph-8K, San-ei, Japan). The rectal temperature was continuously monitored and maintained at 37°C automatically by means of a temperature controller (ATC-101B, Unique Medical Co., Tokyo, Japan) throughout the experiment. The brain temperature was also monitored with a fine needle temperature probe (PTN-800, Unique Medical Co.) placed 5 mm lateral and 5 mm proximal to the sagittal and coronal sutures, respectively, at a depth of 5 mm from the cortical sur-
Measurement of CBF. The CBF was measured continuously in the right parietal cortex by a laser Doppler flowmeter (LDF) (ALF 2100, Advance Co., Tokyo, Japan). A 1–2 mm diameter hole was drilled at a site 5 mm lateral and 5 mm proximal to the sagittal and coronal sutures, respectively. The dura was left intact and an LDF probe (tip diameter 0.8 mm) was positioned 0.5 mm above the dural surface.

Measurement of cerebral oxygenation. Intracranial oxygenation was monitored during experiments by NIRS (NIR 1000; Hamamatsu Photonics, Hamamatsu, Japan). Six wavelengths of near-infrared light (780, 808, 830, 847, 867 and 911 nm), were transmitted to a rabbit’s head through a fiber optic bundle, the end of which (the optode) was applied to the temporal region. Light emerging from the head was collected by another optode attached to the contralateral temporal area and transmitted to the photomultiplier tube of the spectrometer by another fiber optic bundle. The distance between the two optodes was always 3.0 cm. To prevent interference by background illumination, the animal’s head was wrapped in a light-excluding bandage. Changes in the intracerebral concentrations of HbO2 and HbR were calculated, using the modified Lambert-Beer law, by the least squares curve-fitting technique, using previously established extinction coefficients10. The NIRS optodes were attached tightly to the rabbit scalp with adhesive tape.

All experimental procedures were carried out in strict accordance with the guidelines of the National Institute of Neuroscience, National Center of Neurology and Psychiatry, and were approved by the Ethical Committee of the Institute.

Experimental protocol. A period of stabilization of at least 30 min was allowed after each preparation. After baseline data collection for at least 5 min to obtain reference values, 12 mg/kg KA (Sigma Chemical Co., St. Louis, MO, USA), distilled in 1 ml of 0.9% saline was administered (n = 6). All variables were monitored throughout the experiment for at least 60 min. At more than 60 min after KA injection, when EEG recordings showed apparently regular or irregular spikes, l-NAME (Sigma Chemical Co.) at 20 mg/kg in 1 ml saline was given (seizure group). In order to determine the effect of l-NAME on the rabbits without seizures, the other rabbits (n = 6) were given 1 ml saline only, and then 20 mg/kg l-NAME in 1 ml saline was administered (non-seizure group).

In all experiments NIRS, CBF, MABP, EEG, and the brain and body temperatures were monitored and recorded every 5 seconds, and EEG was recorded continuously.

Data analysis and statistical methods. The values for periods of at least 1 min during baseline data collection were averaged to obtain reference values. To assess the changes in NIRS values, relative values (differences from reference values) were obtained. All values were averaged for 1 min every 5 min to reveal the changes in NIRS, MABP and CBF due to KA, l-NAME and saline injection. The changes in CBF were calculated as percentages of the baseline value.

Comparisons between the baseline values (before KA injection) and those at different times after KA injection were performed by means of one-way repeated measured analysis of variance (ANOVA) followed by the Fisher’s Protected Least Significant Difference (PLSD) test. The data for the seizure and non-seizure groups were analyzed by means of the unpaired Student’s t-test. A value of p < 0.05 was considered statistically significant. The results were expressed as means ± SD.

RESULTS

Physiologic variables. The values for PaO2, PaCO2, pH, arterial base excess (BE), hemoglobin concentration, rectal temperature, and brain temperature before and after KA injection are shown in Table 1. The KA induced significant decreases in pH and BE, but no change in PaO2, PaCO2, hemoglobin, and rectal or brain temperature. There were no significant differences between before and after l-NAME injection in any physiologic variable in either the seizure or non-seizure group. Blood glucose ranged from 70 to 110 mg/dl for all samples in all animals.

EEG. Figure 1 shows typical examples of experimental EEG records before (a) and after (b, c, d) KA injection. A decrease in amplitude and an
Table 1 Physiologic variables. *, p < 0.05, compared with the baseline value by means of one-way repeated measured ANOVA followed by the Fisher’s PLSD test. mean (SD).

<table>
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<th>KA-induced seizure group</th>
<th>Non-seizure group</th>
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<tr>
<td></td>
<td>Baseline Before l-NAME After l-NAME</td>
<td>Baseline Before l-NAME After l-NAME</td>
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<tr>
<td>PaO₂ (mmHg)</td>
<td>108.3(11.5) 109.4(28.4) 112.8(28.9)</td>
<td>106.7(19.6) 116.6(18.2) 105.7(14.7)</td>
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<tr>
<td>PaCO₂ (mmHg)</td>
<td>39.7(2.7) 41.1(5.4) 42.3(5.6)</td>
<td>42.4(4.0) 39.9(5.8) 39.2(5.6)</td>
</tr>
<tr>
<td>pH</td>
<td>7.40(0.04) 7.18(0.11)* 7.14(0.09)*</td>
<td>7.38(0.04) 7.42(0.06) 7.46(0.07)</td>
</tr>
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<td>BE (mmol/L)</td>
<td>0.8(4.1) -12.8(2.7)* -11.8(4.5)*</td>
<td>0.2(4.2) 1.7(3.6) 2.5(3.3)</td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>11.6(2.1) 10.8(2.8) 9.9(1.4)</td>
<td>11.7(2.0) 11.9(1.6) 11.6(2.5)</td>
</tr>
<tr>
<td>Rectal temperature (°C)</td>
<td>36.9(0.4) 36.9(0.3) 37.0(0.1)</td>
<td>37.1(0.2) 36.9(0.2) 37.0(0.1)</td>
</tr>
<tr>
<td>Brain temperature (°C)</td>
<td>36.2(0.6) 36.1(2.4) 36.0(1.3)</td>
<td>36.5(0.5) 36.2(1.1) 36.5(0.8)</td>
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Increase in frequency occurred soon after KA injection (Figure 1b). High amplitude sharp waves were first detected at 463 ± 98 (mean ± SD) sec after systemic KA administration. The epileptiform activity then showed a cyclic pattern, i.e. recurrent waxing and waning in amplitude, in all rabbits (Figure 1c). The cyclic pattern of epileptic activity then appeared more frequently at shorter intervals in all rabbits, eventually evolving into status epilepticus, identified by uninterrupted epileptiform activity (Figure 1d). All rabbits in our study showed similar EEG patterns induced by systemic KA administration, and they continued to the end of the experiment. There was no apparent EEG change after l-NAME administration in either seizure or non-seizure group.

Effects of KA-induced seizures. Typical experimental records, showing changes in HbO₂, HbR, tHb, MABP and CBF are shown in Figure 2. The KA administration induced decreases in HbO₂ and tHb, followed by increases in HbR, CBF and MABP soon after KA injection by 15 to 20 min. Afterwards, the decrease in HbO₂ and tHb, and the increase in HbR and MABP gradually returned to the values before KA administration. l-NAME administration induced significant decreases in HbO₂, tHb and CBF, and increases in HbR and MABP. The mean and SD changes in HbO₂, HbR, tHb, MABP and %CBF are shown in Figure 3. The HbO₂ significantly decreased 10 to 20 min after KA administration compared with the value at 0 min (baseline), and then recovered to basal value by 30 min, but no significant changes were seen at 25 to 60 min (Figure 3A). The HbR increased by 15 min after KA injection, and then returned to baseline values (Figure 3B). Although there were significant decreases in tHb 10 to 15 min after KA administration, they tended to increase subsequently, with significant increase 45 to 55 min (Figure 3C). MABP significantly increased 5 to 20 min after KA injection, and then returned to baseline values by 35 min (Figure 3D). The %CBF showed significant increases 5 to 60 min after KA injection (Figure 3E).

Effects of l-NAME administration. The differences between before and after l-NAME injection in HbO₂, HbR, tHb, MABP and %CBF in the KA-induced seizure or non-seizure group are shown in Figure 4. The HbO₂, tHb and %CBF significantly decreased, and HbR increased after l-NAME in the seizure group, compared with the non-seizure group. There were significant increases in MABP after l-NAME injection in both groups, but no significant difference between the two.

DISCUSSION

Our study demonstrated that KA administration induced transient attenuation of cerebral oxygenation, i.e. decreases in HbO₂, and an increase in HbR soon after KA administration, and that these changes were followed by a tendency to return toward the baseline levels during KA-
induced seizures, with no significant differences from 25 min after KA administration. On the other hand, CBF showed significant increases after KA injection throughout our experiment. There was also significant increase 45 to 55 min after KA administration in tHb, reflecting an increase in cerebral blood volume. The reason why KA caused the transient deterioration in cerebral oxygenation in spite of the increase in CBF remains unclear. However, it is possible that the cerebral metabolic rate of oxygen increased more than the increase in CBF during the early period of KA-induced seizures, which resulted in the attenuation of cerebral oxygenation observed in our study. The cerebral oxygenation 30 to 60 min after KA administration in this study was the same as the baseline value, although the CBF was significantly higher than the baseline. This could also be caused by a greater increase in the cerebral metabolic rate than in CBF during that period. Our data showed that measurement of cerebral oxygenation by means of non-invasive technique, NIRS, during seizures in the neonatal brain provided important information in addition to CBF.
Our study also demonstrated that an NOS inhibitor induced significant decreases in HbO$_2$, tHb and CBF, and a significant increase in HbR in the animals with KA-induced seizures, compared with in those without seizures. It is not likely that these changes were caused by changes in MABP, blood gas or other physiologic variables, because there were no significant differences in these physiologic valuables between before and after L-NAME injection in the two groups. These findings appear to indicate that the maintenance of cerebral oxygenation and the increased CBF during KA-induced seizures are mediated by endogenous NO. This assumption is further supported by recent findings that topical application of both glutamate and kainate in vivo increased the diameter of cerebral arterioles, which is attenuated by NOS inhibition. The assumption is also supported by other findings that seizures induced by either systemic administration of kainate or topical application of bicuculline in adult rats caused increased local cerebral blood flow, which is inhibited by NOS inhibitors.

The role of NO in the brain during drug-induced seizures remains controversial. Some investigators have demonstrated that NOS inhibitors have anticonvulsant and neuroprotective effects after seizures induced by KA, NMDA, bicuculline, pilocarpine, bupiva-
caine\textsuperscript{(6)}, pentylentetrazole (PTZ)\textsuperscript{(17)}, tetracaine\textsuperscript{(18)}, and l-cysteine\textsuperscript{(19)}. In contrast, NOS inhibitors have been shown to be neurodestructive during seizures induced by KA\textsuperscript{7, 8, 20, 21}, NMDA\textsuperscript{22}, quinolinic acid\textsuperscript{23}, bicuculline\textsuperscript{24}, pilocarpine\textsuperscript{25}, soman\textsuperscript{26}, and PTZ\textsuperscript{27}. Although our study demonstrated that the production of endogenous NO mediated the maintenance of cerebral oxygenation and the increase in CBF, it is still unclear whether NO during seizures is neuroprotective or neurodegenerative, because NO, a oxygen radical, has been shown to be neurotoxic\textsuperscript{29}.

The l-NAME, which is widely used as an inhibitor of NO synthesis in studies on the modulation of vascular tone by NO, allows prolonged inhibition of NOS\textsuperscript{5, 4, 29}. There are at least three kinds of NOS isozyme in the brain; neuronal NOS, inducible NOS, and endothelial NOS. It is not known which NOS isozyme was inhibited by l-NAME during the seizures in our study. However, it is unlikely that a possible candidate is inducible NOS, because NO was considered to be generated at an early stage of the KA-induced seizures in our experiment. Further studies are required to examine which isofrom of NOS is activated and contributes to the alteration of cerebral circulation during seizures in the neonate.

Both histological and physiological observations revealed that newborn rabbits are developmentally similar in maturity to human neonates, because the 11- to 12-day-old rabbit brain possesses a thin subependimal germinal layer with sparse myelination in the white matter of the cerebrum, and, physiologically, immature electrical activity remains until 10 to 15 days after birth\textsuperscript{30}. Therefore, the two-week-old young rabbits used in our study are a suitable model for investigating cerebral hemodynamics and oxygenation in neonates.

In conclusion, our study showed that KA-induced seizures in newborn rabbits caused an increase in CBF and a maintenance of cerebral oxygenation. The inhibition of these changes by an NOS inhibitor indicates that the cerebral vasodilation during KA-induced seizures is mediated by the endogenous NO in the neonatal brain.

Acknowledgement. This study was supported by a grant from the Tokyo Medical University and the Ministry of Health and Welfare, and Education, Japan. The authors thank Dr Satio Takashima for valuable advice and discussions, and the staff of the department of Mental Retardation and Birth Defect Research, National Institute of Neuroscience of NCNP for their cooperation.

REFERENCES


10) Wray S, Cope M, Delpy DT, Wyatt JS, Reynolds E : Characterization of the near infrared absorption spectra of cytochrome aa\textsubscript{3} and haemoglobin for the non-invasive monitoring of cerebral oxy-
新生仔家児カイニン酸誘発けいれんモデルにおける
一酸化窒素合成酵素阻害剤の脳酸素化状態と
脳血流量の変化におよぼす影響

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要旨：新生仔家児カイニン酸 (KA) 誘発けいれんモデルにおける脳酸素化状態と脳血流量の変化について検討した。同時に一酸化窒素合成酵素（NOS）阻害剤である N-ω-nitro-l-arginine methyl ester (L-NAME) を用い、カイニン酸誘発けいれん時の脳循環動態に及ぼす内因性一酸化窒素 (NO) の役割について検討した。近赤外線分光測定法 (NIRS) により脳組織の酸化型ヘモグロビン (HbO₂)，還元型ヘモグロビン (HbR) と総ヘモグロビン (tHb)，レーザードプラール流量計による脳血流量 (CBF)，平均血圧 (MABP)，および脳波 (EEG) を連続的に測定した。KA投与により全例に異常でんかん波が出現し，血液ガス上代謝性アシドーシスをきたしたが，PaO₂，PaCO₂，血液ヘモグロビン，血糖値，直腸温および脳温に変化はなかった。新生仔家児 KA 誘発けいれん時，脳血流は増加し，脳酸素化状態は維持されることが観察された。また，KA 誘発けいれん時の L-NAME 投与は，けいれんのない群と比べ，CBF と脳酸素化状態の有意な低下を示した。以上より，KA 誘発けいれん時，脳血管拡張によりと思われる脳循環動態の変化が確認され，それらが NOS 阻害剤で抑制されることにより新生児脳における KA 誘発けいれん時の脳血管拡張に NO が関与していることを示した。

キーワード：一酸化窒素，けいれん，L-NAME，脳血流，近赤外線分光測定法