Inhibition of nitric oxide production during global ischemia ameliorates ischemic damage of pyramidal neurons in the hippocampus

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Abstract. We examined the relationship between nitric oxide (NO) production and delayed neuronal death (DND), in the rat hippocampus induced by 21 minutes of transient global ischemia produced by the occlusion of both of the common carotid arteries combined with systemic hypotension. NO production during ischemia and reperfusion was investigated by quantifying the nitrite (NO$_2^-$) levels of the in vivo microdialysis samples collected every 3 minutes from the hippocampus. To determine the origin of NO production, we studied the effects of the focal administration of N$^G$-nitro-L-arginine methyl ester (L-NAME), an inhibitor of the constitutive NO synthase (NOS). We also carried out systemic administration of a selective neuronal NOS inhibitor, 7-nitroindazole (7-NI). Rats were grouped as follows: group 1 (n = 22), vehicle; group 2 (n = 19), L-NAME; group 3 (n = 12), 7-NI; and group 4 (n = 12), a sham operation. The role of NO in the hippocampal DND was investigated histologically one week after ischemia. The level of NO production was significantly decreased in groups 2 and 3 as compared to group 1 in which NO production was significantly increased (p < 0.05). The density of remaining neurons in the CA1 area was significantly reduced only in group 1 (p < 0.01). Taken together, it can be concluded that NO production by neuronal NOS during ischemia and reperfusion resulted in DND in the CA1 region of the rat hippocampus. (Keio J Med 50 (3): 182–187, September 2001)

Key words: in vivo microdialysis, delayed neuronal death, N$^G$-nitro-L-arginine methyl ester, 7-nitroindazole, rat

Introduction

The hippocampus is known to be one of the brain areas “vulnerable” to an ischemic insult.¹ Some clinical cases of the global ischemia due to transient systemic hypotension, in which neurological deficits such as amnesia often develop as sequelae, have been reported to have pathological lesions restricted to hippocampal neurons.²,³ Some form of vascular dementia may be attributed to the vulnerability of the hippocampus to ischemia. The pathophysiological change corresponding to these cases in animal models is thought to be delayed neuronal death (DND), which is slowly progressive cell death originally found in the CA1 sector of the gerbil hippocampus after transient global ischemia.⁴ Although this selective vulnerability of the CA1 sector of the hippocampus to cerebral ischemia is well known, the mechanism underlying this vulnerability has not yet been elucidated.

The glutamate-calcium theory⁵,⁶ is one of the most well known theories that can explain neuronal death, including the mechanism of DND not directly mediated by energy failure. During ischemia, subsequent activation of NMDA-type receptors induces a massive influx of Ca$^{2+}$ into neurons. Secondary to the elevation of intracellular calcium, constitutive nitric oxide synthase (NOS) is activated by calmodulin and results in NO production.

The participation of NO in DND has not been demonstrated. Elucidating the mechanism of slowly progressive neuronal death, including DND, is important, since it concerns the treatment of not only global
ischemia but also the “penumbra” around the ischemic core. However, few studies have been done on the direct relation between the NO production and DND.7–9

The purpose of this study is to investigate the relationship between the NO production during cerebral ischemia and reperfusion, and the histological changes observed in the rat hippocampus one week after transient global ischemia.

**Materials and Methods**

Male Sprague-Dawley rats weighing 250–350 g were anesthetized by an intraperitoneal injection of pentobarbital sodium (40 mg/kg). The body temperature was continuously monitored and maintained at 37.0–37.5°C during ischemia and reperfusion with a thermostatically controlled heating pad connected to a rectal thermistor probe. The unilateral femoral vein and a tail artery were catheterized for induction of exsanguination and continuous monitoring of the blood pressure respectively. The blood pressure was recorded continuously using a multi-pen recorder via a pressure transducer. Both of the common carotid arteries were isolated and loops made of PE-10 polyethylene catheter were passed around the carotid arteries for subsequent occlusion. Animals were placed in a stereotaxic frame with the skull level. Following skull exposure, a small burr hole was drilled at 4.8 mm posterior and 5.3 mm lateral to the bregma, bilaterally, allowing insertion of a double-luminal type microdialysis probe (outer diameter 220 µm, length 3 mm, cellulose membrane) into the hippocampus so that the probe tip was positioned 4.8 mm posterior, 5.3 mm lateral, and 7.5 mm ventral to the bregma. The microdialysis probe was perfused with Ringer's solution (NaCl, 147 mM; KCl, 4 mM; and CaCl2, 2.5 mM) at a constant rate of 2 µl/min employing a microsyringe pump. After a 2-hour equilibration period, the dialysate was collected every 3 min in polyethylene tubes kept at 15°C.

Physiological parameters (blood pressure, PaO2, PaCO2, and the pH of arterial blood) were measured before the induction of ischemia.

Global ischemia was induced by occlusion of both common arteries using polyethylene loops with systemic hypotension. The mean arterial pressure was maintained below 50 mmHg by drawing blood from the femoral vein. The focal cerebral blood flow was measured by laser-Doppler flowmetry in several cases to ensure that ischemia had been induced. After 21 min of ischemia, cerebral reperfusion was achieved by releasing the polyethylene loops around the common carotid arteries and normalizing the blood pressure. The animals were decapitated 6 hours (n = 4), 24 hours (n = 4) and one week after ischemia (n = 4), and the brains were immediately immersed in 4% formaldehyde. This fixation method assured optimal visualization of the hippocampus, and did not affect the ischemic damage grading used in this analysis. The brains were excised 1 mm in front of the insertion sites of the microdialysis probes and cut into 3 mm coronal slices. Sections (10 µm) were cut from an area in close proximity to the probe insertion sites and stained with hematoxylin and eosin for ischemic grading and neuronal counting.

For the *in vivo* microdialysis study, the animals studied were divided into the following groups: Group 1 (n = 22): global ischemia and reperfusion were induced as previously described. Group 2 (n = 19): focal administration of 4 mM NOS nitro-L-arginine methyl ester (L-NAME), a constitutive NOS inhibitor, via a microdialysis probe was started 24 min before inducing ischemia in the same manner as in group 1. L-NAME was administered continuously for 60 min. Group 3 (n = 12): 7-nitroindazole (7-NI), a neuronal NOS inhibitor, was suspended in peanut oil by sonication. 7-NI (30 mg/kg) was so insoluble in water that it had to be administered intraperitoneally 30 min before inducing ischemia in the same manner as in group 1. Group 4 (n = 12): a sham operation was performed. The unilateral femoral vein and a tail artery were catheterized. Blood pressure was recorded continuously. Both common carotid arteries were isolated and loops made of PE-10 polyethylene catheter were passed around the carotid arteries, but carotid artery occlusion was not performed.

**NO2** levels in dialysate samples were measured by the Griess reaction.11 Briefly, samples were injected into a flow-through system and delivered by a carrier solution containing 1% NH4Cl at 10 µl/min, reaching a mixing tee where the samples react with the Griess agents (one part 1% sulfanilamide and one part 0.1% N-(naphthyl)-ethylenediamine) to yield an azo dye after incubation at 40°C. Subsequently, the samples underwent spectrophotometric analysis to determine **NO2** levels. We have already demonstrated the absorbance at 546 nm to be proportional to **NO2** levels in our system.11 In the statistical analyses, the data were expressed as a percentage of the baseline level, the mean value of the preceding three samples before ischemia and reperfusion.

The grading scale developed for this study took into account the severity of the ischemic damage. The nucleus of an ischemic neuron deteriorates in stages that can be readily assessed by microscopic observation at ×320 magnification in hematoxylin-eosin stained 10 µm plastic sections.

The following histological changes were graded into three categories as reported by Eke et al.12 histologically normal, mild ischemic changes (e.g., smeared nuclear-cytoplasmic border, mild nuclear shrinkage,
and mild cytoplasmic eosinophilia), and severe ischemic changes (e.g., a triangular shrunken neuron lacking nuclear-cytoplasmic detail, and strong eosinophilic changes in the cytoplasm). Neurons with recognizable nuclei were counted in each area of the hippocampus (e.g., CA1, CA2, and CA3) and classified into the ischemic categories described above. The degree of ischemic change in each area of the hippocampus was expressed as the density of surviving neurons and as the percentage of each category. The mean and standard deviations for the degree of ischemic change in all areas are depicted for each group. The sections were graded in a double-blinded manner by a neuropathologist who was blind to the identification of the groups.

The ANOVA multiple measurement and Mann-Whitney test were used for the microdialysis and histological studies, respectively.

The Experimental Committee of Keio University approved the experimental protocol as meeting the Experimental Animal Guidelines of Keio University School of Medicine.

### Results

There were no significant differences in physiological parameters among the four groups studied (Table 1). In group 1, global ischemia gave rise to a significant increase in NO$_2^-$ 15, 18 and 21 min after the onset of ischemia and 6, 9, 12 and 15 min after reperfusion, as compared to group 4. In group 2, administration of L-NNAME induced a significant decrease in NO$_2^-$ 18 min after onset of ischemia and 12 min after reperfusion compared to group 1 (\(p < 0.05\)). In group 3, 7-NI also induced a significant decrease 18 and 21 min after onset of ischemia and 9, 12 and 15 min after reperfusion compared to group 1 (\(p < 0.05\)). ANOVA multiple measurement. Values are mean and standard deviation.

![Fig. 1](image)

**Fig. 1** NO$_2^-$ levels in dialysate samples, collected by in vivo microdialysis technique, were measured by the Griess reaction. In group 1, global ischemia gave rise to a significant increase in NO$_2^-$ 15, 18 and 21 min after the onset of ischemia and 6, 9, 12 and 15 min after reperfusion compared to group 4 (\(\ast p < 0.05\), \(\ast\ast p < 0.01\)). In group 2 (\(\triangleright\)), administration of L-NNAME induced a significant decrease in NO$_2^-$ 18 min after onset of ischemia and 12 min after reperfusion compared to group 1 (\(p < 0.05\)). In group 3 (\(\bigcirc\)), 7-NI also induced a significant decrease 18 and 21 min after onset of ischemia and 9, 12 and 15 min after reperfusion compared to group 1 (\(p < 0.05\), \(\overline{\overline{p}} < 0.01\)). ANOVA multiple measurement. Values are mean and standard deviation.

Ischemia

<table>
<thead>
<tr>
<th>pH</th>
<th>$P_O_2$ (mmHg)</th>
<th>$P_CO_2$ (mmHg)</th>
</tr>
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<tbody>
<tr>
<td>Group 1 (n = 22)</td>
<td>7.42 ± 0.36</td>
<td>84.1 ± 6.4</td>
</tr>
<tr>
<td>Group 2 (n = 19)</td>
<td>7.43 ± 0.16</td>
<td>85.1 ± 3.7</td>
</tr>
<tr>
<td>Group 3 (n = 12)</td>
<td>7.41 ± 0.03</td>
<td>82.6 ± 8.5</td>
</tr>
<tr>
<td>Group 4 (n = 12)</td>
<td>7.44 ± 0.36</td>
<td>82.5 ± 7.4</td>
</tr>
</tbody>
</table>

### Discussion

Our present data suggest a causal relationship between NO production by neuronal NOS, during and shortly after ischemia, and neuronal death of pyramidal neurons in the CA1 region of the rat hippocampus one week after ischemia. Although Lei et al. reported a continuous temporal profile of NO production during global ischemia and reperfusion, and the protective
effect of 7-NI against DND in the gerbil hippocampus, no data from rats have as yet been reported. Differences among species are very important when subtle changes, such as measuring small quantities of nitrite and distinguishing between protective and harmful effects of NOS, are considered.

In the present study, 21 min global ischemia increased the level of NO production in the hippocampus although a previous study reported a decrease in that of NO production during ischemia. This increase in NO production was seen during both the latter half of the ischemic period and the reperfusion period. The lack of an NO production increase just after the onset of ischemia may be explained by the very short half-life (less than a few seconds) of NO. NO, an unstable molecule with unpaired electrons, can combine with several other molecules and is metabolized immediately after its production. The hypoxic state during ischemia is likely to cause some delay in $\text{NO}_2^-$ formation, since the rate of NO oxidation is critically dependent upon the oxygen concentration. Enhancement of NO production during ischemia must be transient, since the supply of oxygen or L-arginine, both of which are required for NO production, gradually decreases with the progression of ischemia. Besides, NOS itself may degenerate in response to an ischemic insult. The total amount of NO production during the ischemic period may decrease even if NO production increases transiently during the ischemic period. Thus, to evaluate the production of NO precisely during ischemia, NO should be monitored continuously with measurements taken at short intervals as in our study. The production of NO also increased during the reper-

Fig. 2 Temporal profile of neuronal death on hippocampus CA1 was confirmed. A, coronal brain sections (10 μm) of the CA1 sector of hippocampus of animals killed at indicated time points after global ischemia were stained with hematoxylin and cosin. Scale bar, 50 μm. B, The density of neurons in CA1 six hours and twenty-four hours after ischemic insult and also treated with 7-NI was not significantly different from that of control. However, the density of neurons in CA1 one week after ischemia was significantly decreased against other. *p < 0.05, Mann-Whitney’s U test. Values are mean ± SD.

Fig. 3 The density of neurons in hippocampus CA1 was estimated from hematoxylin/eosin-stained section one week after transient global ischemia. The density in group 2, group 3 and group 4 was significantly larger than that in group 1. *p < 0.01, Mann-Whitney’s U test. Values are mean ± SD.

Fig. 4 The histological changes, estimated from hematoxylin/eosin-stained section one week after transient global ischemia, were graded into three categories. The ratio of normal neurons in CA1 in group 3 was significantly higher than that in group 1. S: severe ischemic change, M: moderate ischemic change, N: normal. *p < 0.05, Mann-Whitney’s U test. Values are mean ± SD.
fusion period and the amount of NO was higher than that in the ischemic period in the present study. This may result from an increased supply of the NOS substrate, L-arginine, and oxygen due to reperfusion. Although we did not measure NO itself but rather its product, NO$_2^-$, the measurement of NO$_2^-$ alone is sufficient to evaluate the NOS activity in the brain. Nitrate (NO$_3^-$) measurement appears to be unnecessary, since (1) the nitrogen atoms in NO$_2^-$ and NO$_3^-$ are derived exclusively from one of the two nitrogens in arginine$^{14}$ and (2) NO$_2^-$ is not converted to NO$_3^-$ in the brain tissue during the early phase of ischemia.$^{16}$ The rationale for measuring NO$_2^-$ in this model has been discussed in detail elsewhere.$^{17}$

In the present study, administration of L-NAME during and shortly after ischemia exerted a neuroprotective effect in the hippocampal CA1 region one week after ischemia. The effect of systemic administration of L-NAME on ischemia is controversial. Sance-sario, et al.$^{18}$ reported that intraperitoneal administration of L-NAME (50 mg/kg) every other day that was initiated four days before 10-minute ischemia induced by common carotid artery occlusion (CCAO), resulted in a greater ischemic damage to CA1 neurons five days after ischemia.$^{18}$ On the other hand, Shapira et al. showed that intraperitoneal administration of the constitutive NOS inhibitor, N$^G$-nitro-L-arginine (L-NA)(5 mg/kg), 4 hours before 5-minute ischemia induced by CCAO, would suppress ischemic changes in CA1 neurons six days after ischemia.$^{19}$ Stronsznajder et al.$^{20}$ and Kohno et al.$^{8}$ also suggested that constitutive NOS inhibitors had a neuroprotective effect one-week after a 5-minute ischemic insult. These discrepancies may be due to the amount of the NOS inhibitor used, that is, a large amount of the NOS inhibitor (10–50 mg/kg) would exaggerate the ischemic damage to CA1 neurons. We speculate that small amounts of constitutive NOS inhibitors can suppress neuronal NOS, thereby apparently exerting neuroprotective effects. However, a large amount of a constitutive NOS inhibitor may also suppress endothelial NOS, possibly producing a neurotoxic effect. In our study, 4 mM L-NAME was focally administered via a microdialysis probe and a neuroprotective effect was observed. L-NAME is so lipid-soluble that it can readily penetrate neurons near the microdialysis probe to attenuate neuronal NOS activity. To confirm the above assumption, we further investigated the effect of 7-NI. 7-NI, a selective neuronal NOS inhibitor, was administered intraperitoneally in the same model, which demonstrated a neuroprotective effect on the outcome of ischemic neuronal damage at one week after transient ischemia. This result suggests that the neuronal NOS activity in the hippocampus is neurotoxic, that is, neuronal NOS may play a neurotoxic role in delayed neuronal death as described in other studies.$^9$ To investigate neuronal death resulting from ischemia, the influence of hypothermia should be considered, since hypothermia is known to be neuroprotective.$^{21}$ In the present study, however, hypothermia exerted no neuroprotective effects. The effect of inducible NOS should also be considered. However, the effects of focally administering L-NAME and 7-NI persisted for about one day$^{22}$ and inducible NOS activity peaked two days after transient ischemia. Therefore, it is probable that L-NAME or 7-NI itself had no direct influence on the activity of inducible NOS in our study. All three groups were equally influenced by inducible NOS or the one week process of maturation of neuronal death in our study. Thus, it can be concluded that suppression of neuronal NOS activity during ischemia and reperfusion exerted protective effects against DND.

NO production triggers several events during ischemia and early phase reperfusion, including depletion of DNA by peroxynitrite (ONOO$^-$),$^{23}$ inhibition of superoxide dismutase in mitochondria,$^{24}$ reversible suppression of mitochondrial oxidation via competition with oxygen,$^{25}$ and suppression of GAPDH activity resulting in exhaustion of ATP.$^{26}$ Fragmentation of DNA, which is due to the depletion of nuclear DNA, activates poly ADP-ribose polymerase (PARP) in the nucleus. As PARP is an enzyme which adds ADP-ribose from NAD to protein in the nucleus, DNA damage activates PARP to add a large amount of ADP-ribose to histone and PARP itself. Each addition of ADP-ribose consumes one molecule of NAD which is synthesized by four molecules of ATP. Damage to mitochondrial enzymes by NO also leads to exhaustion of NAD and ATP, resulting in energy failure and neuronal death.$^{27}$ The suppression of NO production during ischemia and the early phase of reperfusion might attenuate the pathological mechanisms described above and significantly diminish neuronal death during the first week after ischemia.

Our experiment demonstrated a causal relationship between NO production by neuronal NOS, during and shortly after ischemia, and neuronal death of pyramidal neurons in the CA1 region of the rat hippocampus one week after ischemia. These data suggest that NO synthesized by neuronal NOS promotes DND in rats.

References


4. Kirino T: Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res 1982; 239: 57–69


