Nitric Oxide Production in the CA1 Field of the Gerbil Hippocampus After Transient Forebrain Ischemia: Effects of 7-Nitroindazole and $N^G$-Nitro-L-Arginine Methyl Ester

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Background and Purpose—The present study was designed to examine the time course of nitric oxide (NO) production and the source of NO in the CA1 field of the gerbil hippocampus after transient forebrain ischemia.

Methods—The production of NO in the CA1 field of the hippocampus after transient ischemia was monitored consecutively by measuring total NO metabolites ($NO_2^-$, $NO_3^-$ plus $NO_2^-$) with the use of brain microdialysis. 7-Nitroindazole (7-NI) and $N^G$-nitro-L-arginine methyl ester were used to dissect the relative contributions of neuronal NO synthase and endothelial NO synthase to the NO production. The histological outcomes of 7-NI in 5- and 10-minute global ischemia were also evaluated.

Results—The production of NO in the CA1 field of the hippocampus after ischemia was dependent on the severity of ischemia. Ischemia for 2 or 5 minutes did not induce a significant increase in $NO_2^-$ levels in the CA1 field of the hippocampus after reperfusion, whereas the 10- and 15-minute ischemias produced significant and persistent increases in $NO_2^-$ levels. 7-NI did not inhibit the basal $NO_2^-$ levels and showed no effects on $NO_2^-$ levels after 5 minutes of ischemia. However, it completely inhibited the increased $NO_2^-$ levels after 10 or 15 minutes of ischemia. 7-NI provided minor neuroprotection in 5 minutes but not in 10 minutes of global ischemia.

Conclusions—The increased NO level in the CA1 field of the hippocampus after ischemia is produced mostly by neuronal NO synthase, whereas the basal NO level mainly originates from endothelial NO synthase. The observed neuroprotective effect of 7-NI in 5-minute global ischemia in gerbils may not be due to neuronal NO synthase inhibition by this drug. (Stroke. 1999;30:669-677.)

Key Words: cerebral ischemia • microdialysis • nitric oxide • gerbils

The role of nitric oxide (NO) in the pathogenesis of ischemic brain damage has been controversial.1–4 Evidence has accumulated that NO may play either a neurotoxic or a neuroprotective role after cerebral ischemia. As a result of the multiple and contrasting actions of NO as well as the different sources of NO synthase (NOS), modulations of NO in ischemic brain have been shown to produce a variety of outcomes.5,6 Both neuronal NOS (type I; nNOS) and inducible NOS (type II; iNOS) activity have been proposed to be detrimental to the ischemic brain, whereas endothelial NOS (type III; eNOS) activity might be protective.3,4 However, little information is available on the contributions of 3 isoforms of NOS to NO production in the brain after ischemia. Since the effects of NO on the ischemic brain are thought to be dependent on the stage of the ischemic process and the sources of its production,3 it is necessary to elucidate the time course of NO production associated with transient cerebral ischemia and the source of NO in the posts ischemic brain.

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Certain brain regions, especially the CA1 region of the hippocampus, are selectively vulnerable to brief periods of global ischemia.7–9 The mechanism underlying this vulnerability is unknown, and the involvement of NO in the process of delayed neuronal death observed in this region has not been clarified. Although several studies showed that 7-nitroindazole (7-NI), a selective nNOS inhibitor, protected against ischemia-induced hippocampal damage in the gerbil and rat,10–12 NO production was not measured in these studies. In the present study we measured the amount of total NO metabolites ($NO_2^-$, $NO_3^-$ plus $NO_2^-$) by brain microdialysis to monitor the production of NO in the CA1 field of the hippocampus in a gerbil model of cerebral ischemia, and we examined the relationship between NO production and the severity of ischemia. We also evaluated the effects of 7-NI and $N^G$-nitro-L-arginine methyl ester (L-NAME) on NO production to determine the relative contributions of nNOS and eNOS to NO production. Furthermore, the histological
outcomes of 7-NI were examined in 5- and 10-minute global ischemia.

Materials and Methods

Animals

Male Mongolian gerbils (weight, 60 to 80 g) (Seiwa Experimental Animals, Fukuoka, Japan) were housed in a room controlled at 23±2°C with controlled lighting conditions (12 hours light/12 hours dark). Food and water were provided ad libitum. All animal use procedures were approved by the Committee on Animal Experimentation at Ehime University School of Medicine, Ehime, Japan.

Experiment 1: Effects of Transient Forebrain Ischemia on NO Production

The time courses of NO production in the CA1 field of the hippocampus after various durations of ischemia were examined. Thirty-five gerbils were divided into 6 groups. Seven animals served as controls and underwent sham operations without carotid artery occlusion. In the other groups, ischemia was induced for 2, 5, 10, 15, or 20 minutes (n = 7, n = 6, n = 5, n = 5, and n = 5, respectively). The gerbils were anesthetized and maintained with a mixture of 3% halothane and nitrous oxide/oxygen (1:1) with the use of a face mask. A midline neck incision was made, and both common carotid arteries were exposed and separated carefully from adjacent nerves and tissues. Silk threads (4-0) were looped around these arteries. After the animal was placed in a stereotaxic apparatus (David Kopf Instruments), the skull was exposed through a midline scalp incision. Two small burr holes were drilled to insert probes for brain temperature measurement and for microdialysis. The dura was carefully incised. A thermocouple needle probe was inserted in the right brain through a burr hole. The tip of the thermocouple probe was positioned ~3 mm anterior and 2 mm lateral to the bregma and 2 mm below the brain surface. A guide cannula (A-I-8, Eicom) was perpendicularly implanted into the right hippocampus (2 mm posterior and 2 mm lateral to the bregma and 1.4 mm below the brain surface). The administration of nitrous oxide was then discontinued, and the anesthesia was maintained with 2% halothane delivered with oxygen at 2 L/min throughout the experiment. The microdialysis probe (A-I-8-01, 1-mm-long dialysis membrane, OD 0.22 mm; molecular weight cutoff at 50 000; Eicom) was inserted through the guide cannula into the CA1 field of the right hippocampus and perfused with Ringer’s solution at a constant rate of 2 μL/min with a microinjection pump. After a stabilization period of 90 minutes, dialysates were collected every 15 minutes into microtubes on ice, then stored at −80°C until analysis. The threads around both common carotid arteries were pulled by 8-g weights to occlude the circulation. After various durations of ischemia, the threads were cut and removed to restore the blood flow. Rectal and brain temperatures were maintained at 37.0°C to 37.5°C with heating lamps during the experimental period.

In a separate group of animals, mean arterial blood pressure (MAP) and arterial blood gases were checked during ischemia and reperfusion. The animals were anesthetized and maintained as described above. A polyethylene catheter (PE-10; Becton Dickinson) was inserted into the femoral artery for monitoring MAPB and collecting blood samples. The bilateral common carotid arteries were dissected free by a ventral neck incision and occluded with aneurysm clips. After various durations of ischemia (2, 5, 10, 15, and 20 minutes), the clips were removed. The rectal temperature was monitored and kept at 37.0°C to 37.5°C during the experimental period.

Experiment 2: Effects of 7-NI and L-NAME on NO Production

The effects of 7-NI and L-NAME on NO production in the CA1 field of the hippocampus after ischemia were evaluated. Eighty-three gerbils were divided into 16 groups: nonischemic saline or L-NAME group; ischemic (5, 10, and 15 minutes) saline or L-NAME groups. Each group contained 5 to 7 gerbils. The animals were prepared as described above, and an intraperitoneal catheter was inserted into the abdominal space for drug administration. 7-NI (80 mg/kg) or L-NAME (60 mg/kg) was injected by means of the catheter. In the 5-minute ischemic groups, ischemia was induced 30 minutes after the injection of 7-NI or L-NAME. In the 10- and 15-minute ischemic groups, 7-NI or L-NAME was injected intraperitoneally immediately after the onset of ischemia. In the ischemic control groups, the animal was given peanut oil or saline. 7-NI was suspended in peanut oil (40 mg/mL) with sonication. L-NAME was dissolved in saline (10 mg/mL).

Experiment 3: Histology

The histological outcome of the 7-NI administration was examined. After anesthesia, a catheter was inserted into the abdominal space for drug administration. Twenty-seven gerbils were divided into 5 groups: sham-operated group (n = 5); 5-minute ischemic oil or 7-NI group (n = 6 for each); and 10-minute ischemic oil or 7-NI group (n = 5 for each). In the 5-minute ischemic groups, ischemia was induced 30 minutes after the injection of oil or 7-NI (80 mg/kg). In the 10-minute ischemic groups, oil or 7-NI was injected immediately after the onset of ischemia. The rectal temperature was monitored and maintained at 37.0°C to 37.5°C throughout the experiment.

Determination of NO3− in Dialysate

The amount of NO3− (NO2− plus NO3−) in the dialysates was determined by the 2,3-diaminonaphthalene (DAN) method with slight modification.14,15 NO2− in the dialysate was first reduced to NO3− by nitrate reductase, and then the amount of NO3− was determined. Briefly, an aliquot of the dialysate was incubated with 25 μmol/L β-nicotinamide adenine dinucleotide phosphate (reduced form [NADPH]), 25 μmol/L flavin adenine dinucleotide, and 10 μU of nitrate reductase in a final volume of 50 μL of 20 mmol/L Tris-HCl buffer (pH 7.6) for 60 minutes at room temperature while protected from light. To terminate the reaction, 150 μL of distilled deionized water was added. Then 20 μL of DAN solution (31.6 μmol/L in 0.62 mol/L HCl, prepared from 31.6 mmol/L stock solution in N,N-dimethylformamide [DMF]) was added and mixed immediately. After a 10-minute incubation at room temperature in the dark, the reaction was terminated with 300 μL of 93.32 mmol/L NaOH. The fluorescence was measured at an excitation wavelength of 365 nm and an emission wavelength of 405 nm with a spectrophotometer (FP-777, JASCO). A standard curve prepared with solutions of known NaNO3 concentrations (from 0.25 to 2 μmol/L) was run in each assay. To determine the rate of NO2− to NO3− conversion by nitrate reductase, a series of solutions of known NaNO3 concentrations (from 0.25 to 2 μmol/L) was also assayed. The total NO3− concentration (representing NO2− plus NO3−) in the dialysate was calculated from the standard curve. The level of NO3− alone in the dialysate was undetectable, and the NO2− to NO3− conversion was >90%. The detection limit of this method was 0.1 μmol/L, equivalent to ~1 pmol of NO3− in 10 μL of dialysate.

The in vitro recovery of NO2− and NO3− across the 1-mm dialysis probe was determined by immersing the microdialysis probes in a Ringer’s solution containing 10 μmol/L NaNO2 or 10 μmol/L NaNO3 at 37°C. The probe was perfused with Ringer’s solution at a rate of 2 μL/min, and fractions were collected every 15 minutes for the analysis of NO2− and NO3− levels.

Histological Analysis

All animals in the second experiment and those subjected to 15- and 20-minute ischemia in the first experiment were not allowed to recover from anesthesia at the end of microdialysis experiment. The animals were perfused transcardially with 0.9% saline and then with 10% buffered formalin solution. The brains were removed, and the location of the microdialysis probe was microscopically determined. The other animals in the first experiment and those in the third experiment were allowed to recover from anesthesia. Seven days later, the animals were anesthetized with pentobarbital again and transcardially perfusion-fixed with the same procedure as...
The physiological parameters during ischemia and reperfusion are presented in the following table:

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameters</th>
<th>Preischemia</th>
<th>Ischemia*</th>
<th>Reperfusion for 30 min</th>
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<tr>
<td>Sham-operated</td>
<td>MABP</td>
<td>77.5±12.6</td>
<td>82.2±5.3</td>
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<td></td>
<td>pH</td>
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<td></td>
<td>PaCO₂</td>
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<td>40.2±4.6</td>
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<tr>
<td></td>
<td>PaO₂</td>
<td>444.5±35.1</td>
<td>444.7±69.4</td>
<td></td>
</tr>
<tr>
<td>2-min ischemia</td>
<td>MABP</td>
<td>76.5±12.8</td>
<td>111.2±6.2</td>
<td>75.7±11.5</td>
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<tr>
<td></td>
<td>pH</td>
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<td>7.382±0.020</td>
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<tr>
<td></td>
<td>PaCO₂</td>
<td>34.1±2.6</td>
<td>37.8±1.7</td>
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<td></td>
<td>PaO₂</td>
<td>375.6±71.0</td>
<td>477.2±20.7</td>
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<tr>
<td>5-min ischemia</td>
<td>MABP</td>
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<td>110.0±7.1</td>
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<tr>
<td></td>
<td>pH</td>
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<td>7.379±0.022</td>
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<tr>
<td></td>
<td>PaCO₂</td>
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<td>PaO₂</td>
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<tr>
<td></td>
<td>pH</td>
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<td>7.339±0.021</td>
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<td>PaCO₂</td>
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<td>PaO₂</td>
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<tr>
<td>15-min ischemia</td>
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<tr>
<td></td>
<td>PaO₂</td>
<td>444.7±22.5</td>
<td>375.7±69.3</td>
<td></td>
</tr>
</tbody>
</table>

Data are mean±SD; n=4 for each group. *MABP values (mm Hg), sampled every 60 seconds during the ischemic period, were expressed as mean±SD of the peak response. †P<0.01 vs the sham-operated group.

Results

The Table shows MABP and blood gas parameters during ischemia and reperfusion. There were no significant differences in MABP, pH, PaCO₂, or PaO₂ among any of the groups before ischemia and 30 minutes after reperfusion. MABP was markedly increased during ischemia in all ischemic groups, but there were no significant differences among the ischemic groups.

The basal level of NOₓ⁻ (NO₂⁻ plus NO₃⁻) in the dialysate in the hippocampus was 0.26±0.10 μmol/L (n=35). The in vitro recoveries of NO₂⁻ and NO₃⁻ across the 1-mm dialysis membrane were estimated to be 14.6±2.0% and 13.8±2.5%, respectively (n=3).

Figures 1 and 2 show the changes in NOₓ⁻ levels in the dialysate in the hippocampus after various durations of ischemia. In the control group, the levels of NOₓ⁻ in dialysates in the hippocampus were stable throughout the experimental period. During ischemia, the NOₓ⁻ levels were decreased in all ischemic groups. In the 2- and 5-minute ischemic groups, the levels of NOₓ⁻ returned to the basal level 15 minutes after reperfusion and were not significantly different from those in the control group throughout the reperfusion period (Figure 1). In the 10- and 15-minute ischemic groups, the levels of NOₓ⁻ were significantly increased 30 minutes after reperfusion, and the elevation continued for 180 minutes after reperfusion. The levels at 45 and 60 minutes (233% and 236% of the basal level, respectively) and at 150 minutes (261% of the basal level) of

Chemicals and Reagents

DAN, DMF, NaOH, HCl, NaNO₂, and NaNO₃ were obtained from Wako Pure Chemical Industries. 7-NI, L-NAME, peanut oil, nitrate reductase (EC 1.6.6.2, from Aspergillus species), NADPH, and flavin adenine dinucleotide were purchased from Sigma.

Statistical Analysis

The data from microdialysis are expressed as mean±SD of percentage of basal levels (the mean of 2 values before ischemia or drug administration). Statistical significance was determined by ANOVA, followed by Fisher’s protected least significant difference test or by Student’s t test. The statistical analysis of histological data was performed with ANOVA followed by Scheffe’s test. Comparisons among the 6 groups of MABP and blood gases were analyzed by ANOVA followed by Scheffe’s test. A probability value of <0.05 was regarded as significant.

Described above. Paraffin sections, 5 μm thick, were stained with hematoxylin and eosin. The number of intact CA1 neurons per 1-mm length of stratum pyramidale was counted in the same level of coronal sections (2.0 mm posterior to bregma).
reperfusion in the 15-minute ischemic group were markedly greater than those of the other ischemic groups at the corresponding time points (Figure 2). In the 20-minute ischemic group, the increases in NOx levels were observed only at 45 and 60 minutes of reperfusion.

As shown in Figure 3, in nonischemic gerbils, 7-NI (80 mg/kg) had no effect on the levels of NOx in the hippocampus. L-NAME (60 mg/kg) decreased the levels of NOx to \( \approx 50\% \) of the control level.

Figure 4 shows the effects of 7-NI (A) and L-NAME (B) on NOx levels after 5-minute ischemia. 7-NI had no effect on the NOx levels before ischemia. L-NAME decreased the levels of NOx 30 minutes after the intraperitoneal injection. During the ischemic period for 5 minutes, the levels of NOx were decreased in all ischemic groups. In the ischemic oil and saline groups, the levels of NOx were not significantly different from those in the corresponding sham control groups throughout the reperfusion period. 7-NI had no effect on the levels of NOx during reperfusion, whereas L-NAME decreased the NOx levels to \( \approx 50\% \) of the sham control level.

As shown in Figures 5A and 6A, the increased levels of NOx after 10- or 15-minute ischemia were completely inhibited by 7-NI. L-NAME abolished the increased levels of NOx after 10-minute ischemia and decreased the NOx levels after 15-minute ischemia (Figures 5B and 6B). The levels from 30 to 60 minutes after reperfusion in the 15-minute ischemic L-NAME group were higher than those in the saline-injected sham group but significantly lower than those in the ischemic saline group (Figure 6B).

Figure 7 shows the effects of 7-NI on the neuronal density in the CA1 region of the hippocampus 7 days after 5 and 10 minutes of ischemia. The number of intact CA1 neurons was significantly lower in the ischemic oil and 7-NI groups than in the sham-operated group. In the 5-minute ischemic 7-NI group, the number of intact CA1 neurons was significantly greater than that in the 5-minute ischemic oil group. There were no significant differences in the number of intact CA1 neurons between the 10-minute ischemic oil group and the 10-minute ischemic 7-NI group.

**Discussion**

NO is an unstable molecule that is easily degraded into NOx and NOy. The dynamic evaluation of NO production is
thus thought to be difficult. Several studies used microdialysis to detect NO\textsubscript{2} and NO\textsubscript{3} as a measure of NO production in the brain.\textsuperscript{17–21} It has been shown that the levels of NO\textsubscript{x} in the dialysate can serve as a reliable indicator of NO production in the in vivo brain. In the present study we detected NO\textsubscript{3} levels in dialysate collected during 5 minutes of bilateral carotid artery occlusion. \( *P < 0.01 \) vs the corresponding sham groups; \( +P < 0.05, ++P < 0.01 \) vs the ischemic 7-NI or L-NAME group; \( ##P < 0.01 \) vs the ischemic oil or saline group.

Figure 4. Effects of 7-NI (A) and L-NAME (B) on NO\textsubscript{x} in the gerbil hippocampus after 5-minute ischemia. Dialysates were collected at 15-minute intervals. Data are mean±SD (bars) percentage of the basal level (by averaging 2 consecutive dialysate samples). B indicates the preischemic basal level. 7-NI (80 mg/kg) or L-NAME (60 mg/kg) was administered intraperitoneally at 30 minutes before ischemia (arrow). Values at 0 minutes represent NO\textsubscript{x} levels in dialysate collected during 5 minutes of bilateral carotid artery occlusion. \( *P < 0.01 \) vs the corresponding sham groups; \( +P < 0.05, ++P < 0.01 \) vs the ischemic 7-NI or L-NAME group; \( ##P < 0.01 \) vs the ischemic oil or saline group.

Figure 5. Effects of 7-NI (A) and L-NAME (B) on NO\textsubscript{x} in the gerbil hippocampus after 10-minute ischemia. Dialysates were collected at 15-minute intervals. Data are mean±SD (bars) percentage of the basal level (by averaging 2 consecutive dialysate samples). B indicates the preischemic basal level. 7-NI (80 mg/kg) or L-NAME (60 mg/kg) was administered intraperitoneally immediately after the onset of ischemia (arrow). Values at 0 minutes represent NO\textsubscript{x} levels in dialysate collected during 10 minutes of bilateral carotid artery occlusion. \( *P < 0.05, **P < 0.01 \) vs the corresponding sham groups; \( +P < 0.05, ++P < 0.01 \) vs the ischemic 7-NI or L-NAME group; \( ##P < 0.01 \) vs the ischemic oil or saline group.

It is clear that cerebral ischemia/reperfusion results in an increased production of NO.\textsuperscript{20–23} NO production in the brain depends on substrate availability, NOS levels, and NOS activity. In the present study we found that there was a significant decrease in NO production in the CA1 field of the hippocampus during ischemia. This is consistent with the requirement of oxygen for the formation of NO and L-citrulline from L-arginine. Furthermore, a decrease in shear stress on the endothelial wall could mediate the decrease in NO levels during ischemia.\textsuperscript{24}

After reperfusion, the increase in NO production may be attributable to the resupply of oxygen and the activation of constitutive NOS by an intracellular calcium elevation during reperfusion.\textsuperscript{25} Reperfusion may also lead to an increase in shear stress and the restoration of eNOS activity.\textsuperscript{24} In addition, eNOS and nNOS levels have been reported to increase rapidly in the rat brain after focal cerebral ischemia.\textsuperscript{26,27} In the present study NO production was not increased after 2- and 5-minute ischemia. Significant increases were observed after...
10 and 15 minutes of ischemia. However, the extent of NO production after 20 minutes of ischemia was decreased. These findings suggest that the threshold for the increase in NO production in the CA1 region of the hippocampus may be higher than 5-minute ischemia and lower than 10-minute ischemia and that the capacity of the CA1 field to produce NO may be limited (a plateau effect).

In this study, 7-NI did not reduce the basal NO production and exerted no effect on NO production after 5 minutes of ischemia. L-NAME, in contrast, decreased the basal NO production and NO production after 5 minutes of ischemia. L-NAME is a nonspecific inhibitor, acting on both nNOS and eNOS, whereas 7-NI has a high specificity for nNOS in vivo. We therefore believe that the basal NO and the NO after 5 minutes of ischemia are mainly produced by the action of eNOS. It was recently demonstrated that long-term potentiation in the CA1 region, which is mediated by NO, still exists in mice that lack nNOS and that eNOS immunoreactivity is selectively concentrated in the CA1 region of the hippocampus, suggesting that eNOS is responsible for most of the basal NO level in the hippocampus. Other studies, by using an ex vivo assay of NOS, indicated that 7-NI inhibited NOS in the hippocampus. The divergent results may be due to the fact that the ex vivo assay of NOS is not necessarily a reliable index of actual NO production in vivo and has less site specificity than microdialysis. However, since intraperitoneal administration of 7-NI even at high doses usually inhibits ex vivo total NOS activity in the supernatant fraction (where nNOS is thought to be more heavily concentrated than eNOS) by only 50% to 60%, it is possible that 7-NI does not produce complete inhibition of nNOS. Thus, 7-NI may not be capable of inhibiting nNOS sufficiently to inhibit basal activity but only evoked increases in activity.

Since the increased NO production after 10- or 15-minute ischemia was completely inhibited by 7-NI in the present study, NO production may be due to the action of nNOS. In the hippocampus, nNOS exists mainly in the interneurons and astrocytes, although it was also found in the CA1 pyramidal neurons. However, the interneurons and astrocytes are resistant to ischemia and presumably not affected by 5 minutes of ischemia. Therefore, the sustained increase in NO production after 10- or 15-minute ischemia may be caused by the activation of nNOS in interneurons and astrocytes. In the present study, 7-NI abolished the increased NO level in these groups but did not decrease the NO amount to the level below the basal level, implying that eNOS still partly contributes to an increase in NO after 10- and 15-minute ischemia. However, L-NAME did not abolish the increased NO production after 15 minutes of ischemia. The lack of complete abolition of the increased NO production...
may be attributed to the limited inhibitory ability of L-NAME.

It is well known that 5-minute ischemia produces complete destruction of the CA1 pyramidal cells in the gerbil. Because we did not observe an increase in NO production in the present 5-minute ischemic group, it is unlikely that NO produced from nNOS is involved in the vulnerability of the CA1 cells of the hippocampus after transient ischemia. Several investigators reported that 7-NI protected against ischemia-induced delayed neuronal death of the CA1 pyramidal neurons. We also found in the present study that 7-NI was neuroprotective in 5-minute global ischemia. Because NO production was not increased after 5-minute ischemia and 7-NI did not exert an influence on NO production after 5 minutes of ischemia, the observed neuroprotective effect of 7-NI may be derived from other unknown mechanisms rather than nNOS inhibition. However, there is a possibility that a large but short burst of NO at the start of reperfusion may occur in the 5-minute ischemic gerbils, while we failed to detect an increase in NO because of the long sampling intervals of the microdialysis technique. In addition, in the present study NO production was measured only in the early stage of reperfusion. Since the hippocampal CA1 neurons after ischemia deteriorate very slowly and NOS catalytic activity increases 4 days after 5-minute ischemia in this model, we cannot exclude the possibility that NO produced from iNOS in the late stages of the damage is involved in the mechanism of brain damage after global ischemia.

In the present study 7-NI inhibited the increased NO production after 10 minutes of ischemia, but it had no neuroprotective effect. However, failure to demonstrate a neuroprotective effect with 7-NI in 10-minute global ischemia may be a result of inadequate absorption, because 7-NI was given after the onset of ischemia in the 10-minute ischemic group. Thus, toxic amounts of NO could be produced during ischemia or early reperfusion and would not be inhibited in the 10-minute ischemic protocol. Because 10-minute ischemia may be too severe to evaluate its protection against ischemic injury and the dosing regimens of our present study may be improper, the question of whether the increased NO level after 10 and 15 minutes of ischemia is beneficial or detrimental to the brain cannot be answered by the present study. Although damage to the hippocampal CA1 sector after 5 minutes of ischemia occurs extremely slowly, the progression of damage becomes faster when the ischemic period is prolonged. The increased NO production after 10 and 15 minutes of ischemia may be involved in the progression of damage, because high concentrations of NO are toxic and interact with superoxide to produce the highly toxic peroxynitrite anion (ONOO·). According to the in vitro recovery of NO3⁻ observed in the present study, the extracellular concentrations of NO in the hippocampus after 10 and 15 minutes of ischemia are estimated to be ≈3.0 to 4.5 μmol/L. These concentrations are believed to be sufficient to elicit peroxynitrite formation. Therefore, an excessive production of NO may exert acutely lethal influences on the CA1 neurons.

In conclusion, the data in the present study indicate that NO production in the CA1 field of the hippocampus after ischemia depends on the severity of ischemia. The data also indicate that mainly eNOS contributes to the basal NO production, whereas nNOS is mostly responsible for the increased NO production after ischemia. The observed neuroprotective effect of 7-NI in 5-minute global ischemia in gerbils may be due to other unknown mechanisms rather than nNOS inhibition by this drug.

References

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**Editorial Comment**

Excessive production of NO during ischemia and reperfusion is thought to contribute to neuronal injury. Studies with pharmacological inhibitors and knockout mice implicate a role of nNOS in injury from focal cerebral ischemia. An increase in brain tissue NO concentration is supported by direct measurements of NO with microsensors and electron paramagnetic resonance spin traps, and by indirect measurements with nitrite plus nitrate concentrations in jugular venous plasma. During global cerebral ischemia, neuroprotection by pharmacological inhibitors is less consistent, and efficacy may depend on the severity and duration of ischemia.

In the present study Lei et al systematically investigated the temporal dynamics of NO production in gerbil hippocampus as a function of ischemic duration. Nitrite plus nitrate concentration in microdialysates was used as a marker of NO production. Several interesting findings were observed.

First, in contrast to the increase in NO reported during focal ischemia, NO production appears to decrease during global ischemia. Tissue oxygenation may decrease to a greater extent in this model than in focal ischemia and may limit oxygen availability for NO formation. The large decrease in shear stress on the endothelial wall may also contribute to the decrease in NO during ischemia.

Second, NO production rapidly recovered during reperfusion after 2 or 5 minutes of ischemia. However, longer ischemic durations were required to produce an increase in NO production during reperfusion to levels above the baseline values. This excessive increase in NO was inhibited by the nNOS inhibitor 7-NI. These results are consistent with impaired recovery of calcium homeostasis after prolonged ischemia leading to stimulation of NOS in neurons.

Third, 7-NI provided a modest amount of neuroprotection in CA1 neurons when administered before 5 minutes of ischemia but not when administered after 10 minutes of ischemia. Because NO production was not elevated after 5 minutes of ischemia, the authors suggest that 7-NI may act through a nonspecific mechanism. However, it is possible that their microdialysis assay system is not adequately sensitive for detecting a short-lived burst of NO at reperfusion or that NO increases beyond the 3-hour observation period. As ischemic duration is shortened, CA1 neuronal degeneration becomes progressively delayed, and any involvement of NO might also be delayed. The lack of effect of posttreatment 7-NI after 10 minutes of ischemia could be related to the increased severity of the insult, the delayed administration of 7-NI in the 10-minute ischemia group, or persistence of
activity of eNOS based in pyramidal neurons. Thus, some caution needs to be taken in interpreting the role of NOS in delayed neurodegeneration in hippocampus.

Fourth, 7-NI failed to decrease basal nitrite/nitrate levels in hippocampus, whereas L-NAME, which inhibits both nNOS and eNOS, reduced basal levels. These results imply that basal NO production is derived primarily from eNOS. This finding may be specific for hippocampus, where the endothelial isoform has been described in neurons. In other areas, 7-NI can decrease the basal level of cerebral blood flow, thereby implying tonic NO production by nNOS. It is also possible that the 7-NI administered in the present study does not completely inhibit nNOS. Furthermore, there may also be some leakage of nitrite/nitrate from plasma into the dialysate long after the probe insertion. L-NAME could selectively reduce this leakage by inhibiting peripheral sources of nitrite/nitrate and decreasing the diffusion gradient from plasma into brain tissue.

In summary, the present study provides new information on NOS kinetics during global ischemia and reperfusion. However, some precautions need to be considered in interpreting differences between L-NAME and 7-NI effects when applied to this indirect marker of NO production.

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Nitric Oxide Production in the CA1 Field of the Gerbil Hippocampus After Transient Forebrain Ischemia: Effects of 7-Nitroindazole and NG-Nitro-L-Arginine Methyl Ester
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