Nitric Oxide Inhibition Aggravates Ischemic Damage of Hippocampal but Not of NADPH Neurons in Gerbils

Giuseppe Sancesario, MD; Michelangelo Iannone, MD; Maria Morello, MSc; Giuseppe Nisticò, MD; Giorgio Bernardi, MD

Background and Purpose Nitric oxide may influence pathophysiology of brain ischemia in a complex way depending on the sources of its production either from neurons or endothelial cells. We investigated whether inhibition of nitric oxide synthesis affects postischemic neuronal death in hippocampus. Moreover, we evaluated whether the presence of nitric oxide synthase activity in specific neurons protects these against ischemia in the hippocampus, striatum, and sensorimotor cortex.

Methods To inhibit nitric oxide synthase, several dosing regimens of N-o-nitro-L-arginine methyl ester (L-NAME) were used (5 or 50 mg/kg IP, twice a day for 4 days, or 30 mg/kg IV) in gerbils. Control animals received either the isomer Nω-nitro-D-arginine methyl ester or the vehicle. The gerbils underwent 10-minute occlusion of carotid arteries under ether anesthesia and controlled body temperature while physiological parameters were monitored. Neuronal damage was assessed 5 days after ischemia using Nissl-stained sections of hippocampus. Nitric oxide synthase neurons were histochemically stained for reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase activity.

Results L-NAME treatments, but not the chronic one at 5 mg/kg, induced elevation of blood pressure (30% to 80%) greater than the control level, P<.01), as observed shortly before and after bilateral carotid occlusion. Postischemic neuronal loss in the CA1 through CA4 sectors was worsened by chronic pretreatment with L-NAME at 50 mg/kg (eg, CA1 neuronal counts per 100-μm length: 3.2±2.74, mean±SD; n=19; P<.01). After the acute (30 mg/kg) or chronic pretreatment at lower dosage (5 mg/kg) with L-NAME, neuronal loss was comparable to that of animals treated with the D-isomer or the vehicle (CA1 counts in vehicle-treated animals: 7.65±6.51, mean±SD; n=14). None of the L-NAME treatments affected postischemic survival of NADPH diaphorase-positive neurons in hippocampus, striatum, and sensorimotor cortex.

Conclusions These observations demonstrate that inhibition of endothelial and neuronal nitric oxide synthase activity does not modify resistance of nitric oxide–producing neurons to transient ischemia. The severe inhibition of nitric oxide production aggravates postischemic neuronal death in the hippocampus, whereas the mild inhibition is ineffective. (Stroke. 1994;25:436-444.)

Key Words • cerebral ischemia • hippocampus • nitric oxide • gerbils

The high vulnerability of some brain regions to ischemia has been attributed to enhanced extracellular glutamate levels induced by ischemic insults. In this condition, stimulation of glutamate receptors could induce an abnormal influx of ions and water into energy-exhausted neurons. Recent studies suggest that glutamate neurotoxicity can be mediated also by a neuronal messenger, nitric oxide (NO), which is produced enzymatically by NO synthase (NOS) from l-arginine.

There is evidence that glutamatergic agonists rapidly stimulate NO synthesis via receptor-operated calcium influx in rat cerebellar neurons in vitro. Inhibitors of NO synthesis prevent N-methyl-D-aspartate (NMDA)–induced necrosis of cerebral cortical neurons in primary cultures. In an in vivo model of epilepsy, hippocampal neuronal death has been suggested to be due to abnormal formation of NO. In addition to its proposed role in inducing glutamate–dependent neurotoxicity, NO may play a protective role. In fact, NOS-containing neurons, which occur in discrete brain regions, are selectively resistant to ischemia, NMDA–mediated toxicity, and some neurodegenerative diseases such as Huntington’s chorea. Furthermore, NO production may play an important function in the control of cerebral blood flow in rats.

To solve the dilemma between the neurotoxic and the protective roles of NO in the central nervous system, Brett and Snyder have suggested that NOS neurons would behave like macrophages: if NOS neurons are in the presence of large levels of glutamate, they will release a large amount of NO, killing NO–receptive neurons. This hypothesis appeared to be supported by the fact that, in a focal model of brain ischemia in the mouse and rat, inhibitors of NO synthesis were able to significantly reduce the size of brain infarct. However, these findings are still controversial because other authors, in models of focal or global forebrain ischemia,
demonstrated either no protective action or a worsening of postischemic neuronal damage after NO synthesis inhibition.\(^{17,20}\) The different dosing regimens of NOS inhibitors make these works hardly comparable. Because NO is produced in the brain in several cell types, including specific neurons, endothelial cells, and activated microglia and astrocytes, the role of NO in the pathophysiology of brain ischemia may be dependent on the cell type producing it.\(^{3-7}\)

This study attempts to elucidate the interrelationship between different cellular sources of NO and the postischemic neuronal damage in a gerbil model of transient forebrain ischemia. The occurrence of a selective pattern of neuronal damage in the CA1 hippocampal sector and in the dorsolateral striatum makes this model suitable to test anti-ischemic activity of pharmacological agents.\(^{21}\) We evaluated whether \(N^\circ\)-nitro-L-arginine methyl ester (L-NAME), a powerful but unselective NOS inhibitor, was able to affect the pattern of postischemic neuronal death in the hippocampus.\(^{22,23}\) At the same time, we studied whether L-NAME decreased the intrinsic resistance to ischemia of NOS-containing neurons, which are few in the hippocampus but widely distributed in the cerebral cortex and in the striatum.\(^{10,11,24}\) Several dosing regimens of L-NAME were used to attain standardized inhibition of endothelial and cerebral NOS activity, before or after ischemia.\(^{13,22}\) Because neural NOS specifically colocalizes with a paraformaldehyde-resistant reduced nickelamidine adenine dinucleotide phosphate diaphorase (NADPH-d) activity, \(m_{NOS}\), \(mg\) of NOS neurons was made using the histochemical NADPH-d method.\(^{24,25}\)

Materials and Methods

Adult Mongolian gerbils (Meriones unguiculatus, M.I.L. Morini, Reggio E, Italy) of both sexes (\(n=150\)) weighing 50 to 70 g were used. They were maintained on a 12-hour light/12-hour dark cycle (light on 7 AM to 7 PM) and had free access to food and water. L-NAME and \(N^\circ\)-nitro-D-arginine methyl ester (D-NAME) were obtained from Sigma (Milan, Italy) and dissolved in saline before injection.

For the morphological study, the gerbils were randomly assigned to different groups and treated with several dosing regimens of L-NAME before or after transient bilateral carotid occlusion (BCO), as summarized in Table 1.\(^{13,22}\) Control animals (\(n=29\)) received either the vehicle or the isomer D-NAME to demonstrate that the effects of L-NAME are stereospecific (Table 1). In addition, other gerbils (7 for each group), treated according to the above dosing regimens, were studied to monitor systemic mean arterial blood pressure (MABP) before, during, and 5 minutes after BCO (Table 2). Blood gases were checked before and after BCO (Table 2). In the L-NAME posttreated animals, only MABP was recorded 1 hour after BCO with the animals under ether anesthesia.

Between 9 and 12 AM, all gerbils were anesthetized with ether. A polyethylene catheter was inserted into the femoral artery and vein before dissection of the carotids to allow recordings of physiological parameters (\(n=31\)), as well as intravenous injection of the drug in the acute pretreated group (\(n=19\)). The common carotid arteries were dissected free via a ventral neck incision and occluded with a microaneurysm clamp. Anesthesia was stopped immediately before the occlusion of the carotids. After 10 minutes of occlusion, the clips were removed and the skin incision was sutured. Two needle electrodes were then placed in the temporalis muscles to record the interhemispheric electroencephalographic (EEG) activity immediately after the ischemic period. A complete flattening of EEG activity, which was observed for about 15 minutes, was used as an indicator of the induced ischemia.\(^{26}\) The few animals (\(n=3\)), from different groups, that displayed no postischemic EEG flattening were discarded from the histological analysis. Rectal temperature was monitored and kept between 36°C to 37°C during surgical operation by means of a heating pad. Body temperature measurement also was taken at 2 hours after carotid occlusion.

After 5 days, the gerbils were reanesthetized with pentobarbital (2.5 mg IP) and transcardially perfusion-fixed with 4%
### Table 2. Experimental Parameters

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Parameters</th>
<th>Resting</th>
<th>Carotid Occlusion*</th>
<th>Reperfusion (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic pretreatment, twice a day for 4 days before carotid occlusion</td>
<td>MABP</td>
<td>71.4±3.8</td>
<td>119.3±11.7</td>
<td>74.0±11.0 (5)</td>
</tr>
<tr>
<td></td>
<td>PaO₂</td>
<td>126.8±56.5</td>
<td>149.0±20.0</td>
<td></td>
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<tr>
<td></td>
<td>PaCO₂</td>
<td>34.0±8.0</td>
<td>33.0±4.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>7.2±0.0</td>
<td>7.3±0.0</td>
<td></td>
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<tr>
<td>Saline (n=7)</td>
<td></td>
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<tr>
<td>L-NAME, 5 mg/kg (n=7)</td>
<td>MABP</td>
<td>70.7±6.1</td>
<td>111.4±19.5</td>
<td>83.2±11.5 (3)</td>
</tr>
<tr>
<td></td>
<td>PaO₂</td>
<td>121.0±21.1</td>
<td>113.6±25.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PaCO₂</td>
<td>24.3±5.7</td>
<td>27.5±7.3</td>
<td></td>
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<tr>
<td></td>
<td>pH</td>
<td>7.3±0.1</td>
<td>7.2±0.0</td>
<td></td>
</tr>
<tr>
<td>L-NAME, 50 mg (n=7)</td>
<td>MABP</td>
<td>94.3±7.9 t</td>
<td>112.9±38.6</td>
<td>95.0±5.0 t (3)</td>
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<td>PaO₂</td>
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<td>128.4±14.4</td>
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<tr>
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<td>31.1±6.1</td>
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<tr>
<td></td>
<td>pH</td>
<td>7.3±0.1</td>
<td>7.3±0.1</td>
<td></td>
</tr>
<tr>
<td>Acute pretreatment, IV bolus</td>
<td>Saline (n=3)</td>
<td>MABP</td>
<td>71.7±2.3</td>
<td>123.3±5.8</td>
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<tr>
<td></td>
<td></td>
<td>PaO₂</td>
<td>139.8±16.0</td>
<td>103.7±22.1</td>
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<tr>
<td></td>
<td></td>
<td>PaCO₂</td>
<td>32.1±5.6</td>
<td>38.0±9.8</td>
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<tr>
<td></td>
<td></td>
<td>pH</td>
<td>7.3±0.0</td>
<td>7.2±0.0</td>
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<tr>
<td>L-NAME, 30 mg/kg (n=7)</td>
<td>MABP</td>
<td>101.4±6.3 t</td>
<td>128.7±15.1</td>
<td>97.5±4.2 t (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PaO₂</td>
<td>131.0±41.8</td>
<td>116.0±41.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PaCO₂</td>
<td>35.2±4.3</td>
<td>34.3±7.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH</td>
<td>7.2±0.1</td>
<td>7.2±0.1</td>
</tr>
</tbody>
</table>

L-NAME indicates N⁶-nitro-L-arginine methyl ester; MABP, mean arterial blood pressure.

Data are mean±SD. Resting values were measured 5 minutes before carotid occlusion and postischemic values 5 minutes after reperfusion.

*In each group, some animals died 4 to 5 minutes after carotid occlusion. MABP values (mm Hg), sampled every 60 seconds during the ischemic period, were expressed as mean±SD of the peak response.

†P<.01 compared with saline-treated groups (Student’s t test for unpaired data).

‡P<.05.

Buffered formaldehyde pH 7.4 after a brief rinse with saline and heparin (0.1%) at room temperature. The brains were removed, kept in cold buffer for 2 hours, and stored in phosphate-buffered saline (PBS) overnight.

Histological stainings for nerve cells were performed to evaluate general brain morphology and tissue damage, while NOS neurons were histochemically stained for their high NADPH-d activity. Coronal slices of the caudate putamen and of the dorsal hippocampus 50 or 400 /μm thick were cut with a vibratome. The 400-μm-thick slices were dehydrated in graded alcohols and embedded in paraffin. Coronal sections 6 to 7 μm thick were stained with cresyl violet or with hematoxylin and eosin. Free-floating 50-μm-thick slices were directly stained for NADPH-d activity according to the method of Sherer-Singler et al, rinsed in PBS, and mounted onto polylysinated slides. The sections were coverslipped with Permount (Fisher) and examined using a light microscope (Leitz).

Severity of neuronal damage was evaluated by the number of surviving neurons, ie, conventionally intact cells when stained with cresyl violet, in the dorsal hippocampus. The mean number of surviving neurons per 100-μm length was separately calculated in the CA1, CA2, CA3, and CA4 hippocampal sectors (0.7 to 1.2 mm posterior to bregma, according to Del Thiessen and Yahr) to accurately estimate the extent of neuronal damage. Cell counting was performed in three serial sections in a blind fashion by two independent observers, using the light microscope equipped with a 25x objective.

NADPH-d reactive neurons were counted in three serial sections over the dorsal hippocampus, the sensorimotor cortex, and the rostral caudate putamen (3.5 to 3.0 mm anterior to bregma), using the light microscope equipped with a 10x objective. As no obvious differences were present among L-NAME-treated animals, the counts of NADPH-d positive neurons (expressed as mean number per square millimeter) were performed in sham-operated animals and in animals after ischemia that were chronically pretreated with saline or with the higher L-NAME dose. Neuronal counts of hippocampal neurons in sectors CA1 through CA4 and of NADPH-d neurons in the areas of interest, generated for the two hemispheres in each brain, were summed to give a single value for each animal.

The numbers of animals dying (mortality) during BCO and within 24 hours after ischemia were compared using the χ² test. Student’s t test for unpaired data was used to evaluate differences in rectal temperature and MABP between the control ischemic groups and each ischemic L-NAME-treated subgroup. ANOVA of the transformed data was used to compare differences in the loss of CA1-CA4 hippocampal neurons between the ischemic saline-treated group and each ischemic L-NAME-treated subgroup. ANOVA was used also to compare differences in survival of NADPH-d neurons in each area of interest between different groups after the ischemic insult. Data are presented as mean±SD; when P<.05, differences were considered significant.
**Results**

MABP values were markedly affected by L-NAME treatments and by BCO.\(^{30,31}\) In control animals, baseline MABP was 71.43±3.78 mm Hg, and it rose abruptly during BCO (mean peak response, 119.29±11.70 mm Hg), returning to baseline values 30 to 40 seconds after reperfusion (Table 2). The hypertensive response induced as a consequence of BCO reached similar mean peak values in groups of control and L-NAME–treated gerbils (Table 2). However, in the resting state as well as at 5 minutes after ischemia, animals pretreated acutely, intravenously, or chronically, intraperitoneally, with the higher dose showed an elevation of MABP values of 30% to 80% with respect to control levels (Table 2). Gerbils pretreated with the lower L-NAME dose (5 mg/kg) were normotensive, as observed 5 minutes before and after BCO. Animals posttreated in the reperfusion period with L-NAME (50 mg/kg IP) showed a marked rise of MABP values (110±5.4 mm Hg) at 1 hour after ischemia (P<.01).

During BCO, some saline-treated gerbils exhibited respiratory distress and a fall in MABP, and they died in a few minutes (4 of 21). In the reperfusion period, two animals (2 of 21) lay motionless in the cage and were found dead the following day. Mortality of saline-treated gerbils was 29% at 24 hours after ischemia (Table 1). After L-NAME chronic treatments, but not after L-NAME in intravenous bolus, the mortality at 24 hours after BCO amounted to more than 50% (Table 1). However, mortality showed significant differences only between the ischemic saline group and that receiving intraperitoneal L-NAME chronically at a higher dose (P=.03).

No significant intergroup differences were observed in the values of body temperature. Temperature was maintained between 36°C to 37°C during cerebral ischemia, but it rose to 38°C to 39°C during the postischemic period in all groups (Table 1). All groups whose physiological parameters were monitored showed systemic acidosis before and after ischemia, probably as a secondary effect of a longer anesthesia (20 to 30 minutes) (Table 2). Indeed, those animals in which histological brain damage was studied underwent just 4 to 5 minutes of anesthesia before BCO. Seizures either spontaneous or related to manipulation of the gerbils did not occur during the postischemic period in any group of animals.

**Hippocampal Damage**

In comparison to sham-operated gerbils, BCO for 10 minutes induced a severe loss of CA1 and CA2 neurons in 84.7% of the saline-treated gerbils (Fig 1B; Table 3). Occasionally, the neuronal damage slightly expanded into other hippocampal subsectors.

None of the L-NAME dosing regimens, given acutely or chronically before or after BCO, protected the CA1 and CA2 neurons against transient ischemia (Table 3). Actually, the L-NAME–pretreated group at higher dosage showed more severe neuronal loss in the CA1 and CA2 as well as in the more resistant CA3 and CA4 sectors (Table 3; Fig 1C). The chronic L-NAME posttreatment also induced a worsening of hippocampal neuronal loss, which approached statistical significance in the CA2 sector (P=.056) (Table 3). After D-NAME treatment, the severity of neuronal damage in the hippocampus was similar to that of saline-treated animals (Table 3).

**NADPH-d Neurons**

NADPH-d reactivity can be detected histochemically as a bright blue reaction product, which heavily and specifically stains neuronal bodies, dendritic trees, and axonal networks. In sham-operated gerbils, either pretreated with saline or with L-NAME (Fig 2, A and B), scattered NADPH-d neurons were observed in the sensorimotor cortex (neuronal count/mm²: 4.24±0.97, mean±SD; n=3) and in the striatum (12.73±5.13; n=3); very few of them were present in the different hippocampal sectors (0.14±0.14; n=3). The appearance of NADPH-d neurons changed after ischemia, mainly in the striatum. After ischemia, in the saline-treated...
TABLE 3. Neuronal Cell Counts per 100-μm Length in the CA1-CA4 Sectors of the Hippocampus

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>n</th>
<th>CA1</th>
<th>CA2</th>
<th>CA3</th>
<th>CA4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham operation</td>
<td>3</td>
<td>32.3±7.3</td>
<td>32.3±4.0</td>
<td>28.5±2.5</td>
<td>29.4±7.4</td>
</tr>
<tr>
<td>Carotid occlusion Saline</td>
<td>14</td>
<td>7.6±6.5</td>
<td>6.3±4.1</td>
<td>22.0±11.2</td>
<td>13.8±4.7</td>
</tr>
<tr>
<td>L-NAME 5 mg/kg IP twice a day for 4 days before</td>
<td>6</td>
<td>4.6±2.6</td>
<td>5.8±2.4</td>
<td>15.6±2.2</td>
<td>11.7±2.8</td>
</tr>
<tr>
<td>L-NAME 50 mg/kg twice a day for 4 days before</td>
<td>19</td>
<td>3.2±2.7*</td>
<td>3.8±2.0†</td>
<td>14.5±5.4*</td>
<td>9.6±3.6*</td>
</tr>
<tr>
<td>L-NAME 50 mg/kg twice a day for 4 days after</td>
<td>4</td>
<td>1.2±0.5</td>
<td>1.9±1.4†</td>
<td>22.6±2.8</td>
<td>14.7±0.8</td>
</tr>
<tr>
<td>L-NAME 30 mg/kg IV bolus immediately before</td>
<td>6</td>
<td>5.5±4.3</td>
<td>8.8±5.5</td>
<td>19.1±5.0</td>
<td>13.8±2.1</td>
</tr>
<tr>
<td>D-NAME 50 mg/kg twice a day for 4 days before</td>
<td>5</td>
<td>5.6±9.0</td>
<td>8.2±10.1</td>
<td>24.1±19.8</td>
<td>13.4±2.9</td>
</tr>
</tbody>
</table>

L-NAME indicates N⁰-nitro-L-arginine methyl ester; D-NAME, N⁰-nitro-D-arginine methyl ester. Data are mean±SD.

*P=.01 vs carotid occlusion+saline treatment by ANOVA.
†P≤.05.

In ischemic L-NAME-treated gerbils, the density and the distribution of NADPH-d positive neurons were not different from those of ischemic saline-treated animals (P>.45) even after chronic pretreatment at the higher dose (cell count/mm²: 2.63±1.28, mean±SD, in the striatum; 2.1±1.13 in the sensorimotor cortex; and 0.84±0.86 in the hippocampus; n=9). As in saline-treated animals, NADPH-d neurons survived in areas of the dorsolateral striatum, showing severe neuronal loss in Nissl-stained sections of five of nine L-NAME ischemic animals (data not shown).

Discussion

The present experiments in gerbils do not support the hypothesis that the ischemic neuronal damage is depen-
dent on an abnormal production of NO, nor that the resistance of NOS/NADPH neurons to ischemia is due to their constitutive NOS activity. Instead, the inhibition of endothelial and neuronal NO synthesis results in a more severe ischemic neuronal damage in the hippocampus.

Endothelial NOS activity can be rapidly inhibited in vivo. Intravenous bolus injection of L-NAME (30 mg/kg) almost immediately causes an increase in blood pressure, which lasts for 1 to 2 hours and is associated with a significant fall in cerebral blood flow in the neocortex as well as in cerebral deep structures. 13,31 Probably, a single intravenous injection of L-NAME (50 mg/kg) also induces a certain degree of neural NOS inhibition, as it is known that a single intraperitoneal injection of L-NAME (50 mg/kg) attenuates brain NOS activity by 50%. 22 Applied shortly before ischemia, the intravenous dosing regimen had significant consequences in neither the survival of the animals nor the severity of the histological damage.

A chronic pretreatment with nitroarginine derivative is required to markedly decrease neuronal NOS activity in intact animals. 22 L-N^6-Nitroarginine, 5 and 50 mg/kg IP injected in rats twice a day for 5 days, reduces the brain NOS activity to 50% or 95%, respectively, for more than 14 hours (the longest time point measured). 32 Such a severe brain NOS inhibition does not induce behavioral changes or evident EEG disturbances in unanesthetized freely moving gerbils. 26 Whether the slow rate of brain NOS inhibition in vivo depends on the presence of the blood-brain barrier or on the fact that endothelial and neuronal NOS are different isoforms is not known. 6,32

The effect of chronic L-NAME treatment on brain NOS seems to be of longer duration than on endothelial NOS. 22,31 Although L-NAME 5 mg/kg IP induced a rise of MABP for about 90 minutes (from about 70 to about 95 to 100 mm Hg, data not reported), the animals chronically pretreated with this lower dose were normotensive when they underwent BCO at 2 to 4 hours after the last injection of the drug, at a time when 50% of brain NO synthesis is known to be inhibited. 22 Again, such degree of NOS inhibition in the brain was not sufficient to affect postischemic neuronal damage.

Long-lasting inhibition of both endothelial and neuronal NOS is induced by a chronic regimen of L-NAME at higher dose. Indeed, L-NAME 50 mg/kg IP caused an elevation of MABP, still present at 4 hours after the last injection (the longest time point measured), at a time when 90% of brain NOS should be inhibited. 22 Such a concurrent inhibition of endothelial and neuronal NOS resulted in a significant increase in the mortality rate after ischemia and in more severe neuronal damage in the hippocampus. The D-NAME did not have the same effects. It is worth noting that posts ischemic hippocampal damage was more severe in animals in which an almost complete inhibition of NO synthesis was induced before rather than after the induction of ischemia. This could be explained by the fact that L-NAME pretreatment may result in greater ischemia during and immediately after BCO. Although mean peak values of blood pressure are similar during BCO in animals treated with saline or L-NAME, the increase in blood pressure during BCO is far less after L-NAME pretreatment with 30 mg/kg IV in bolus or with 50 mg/kg IP for 4 days. It is unknown whether L-NAME can change the carotid baroreflex and/or the hypertensive response of brain to ischemia. However, the smaller pressor response to BCO occurs simultaneously with other vascular effects of L-NAME, such as an increase in vascular resistance in the brain and changes in cerebrovascular reactivity. 33 Altogether, NOS inhibition results in constriction of cerebral vessels and in reduction of cerebral blood flow. These effects have been observed before and after carotid occlusion. 13,34 In light of this, L-NAME pretreatment may also result in greater ischemia during carotid occlusion. The cerebrovascular effects of L-NAME can be dependent on the inhibition of both endothelial and neuronal NOS. Because NO, produced in neurons and endothelial cells, regulates vasodilatation and is involved in the coupling of cerebral blood flow to neuronal activation, it is conceivable that the vasoconstrictory effect of L-NAME is increased, and cerebral blood flow is further decreased under experimental conditions in which endothelial and neuronal NO synthesis are simultaneously and markedly inhibited. 13,31,35,36

Whether hemodynamic disturbances induced by chronic L-NAME regimen (50 mg/kg) may fully account for the higher mortality and for the worsening of posts ischemic hippocampal neuronal damage is unknown. NO may also have a direct effect on the functional recovery of neurons during the posts ischemic period. In a previous study in gerbils that received chronic L-NAME regimen at higher doses (50 mg/kg), we observed a delayed recovery of spontaneous electro cortical activity, which is depressed by transient ischemia. 26 These results may be dependent on changes in cerebral blood flow induced by L-NAME treatment. Nevertheless, a more severe functional impairment of the cortex after L-NAME is also consistent with a recent article reporting that NO, the synthesis of which is stimulated by NMDA agonists, can in turn induce a blockade of NMDA receptors. 37 Moreover, NO could decrease the neuronal levels of cytosolic free calcium by a cyclic nucleotide-independent mechanism. 38 Altogether these data suggest that NO could represent a protective feedback mechanism after an ischemic insult both at the vascular level, inducing vasodilatation, and at the neuronal level, preventing overstimulation of NMDA receptors.

It is important to note that the basic topographical distribution of NOS activity in the brain does not match the pattern of regional neuronal damage. 21,24 The hippocampus, the region most vulnerable to transient global ischemia, contains only occasional NOS neurons and sparse nerve fibers in the CA1 through CA3 sectors as well as in the dentate gyrus. 10,12,24 This implies that NO would require additional regional factors on NO-receptive neurons, such as high density of NMDA receptors, to exhibit a possible toxic action. Alternatively, we can hypothesize that a baseline low level of constitutive NO in hippocampus makes this region easily vulnerable to ischemia.

Both actions of NO, the protective as well as the deleterious one, have recently been suggested to take place in different stages of focal ischemia. 19 The neuroprotective aspects mediated by NO might have a rapid onset and take place during and immediately after the ischemic insult, activating vasodilatation, preventing
were spared in the dorsolateral striatum of saline-treated animals after ischemia of the forebrain for 10 minutes. An almost absolute sparing of such neurons in the cortical regions or in the most vulnerable areas of the hippocampus and striatum, as demonstrated by comparative observations of those regions stained either with cresyl violet or for NADPH-d activity after nitroarginine application (1 mmol/L). Consequently, it has been suggested that NOS inhibition actually protects brain against focal ischemic insult or whether it merely prevents the acute swelling of the infarcted area.

**NADPH-d Neurons**

Our results demonstrate that inhibition of NOS activity in vivo is ineffective on NADPH-d histochemical staining. This is in accordance with preliminary in vitro experiments of Bredt et al. that reported no changes in NADPH-d staining after nitroarginine application (1 mmol/L). Subsequently, it has been suggested that NOS and NADPH-d activities are located on two independent portions of the same enzyme, but it might be also that nitroarginine analogues serve as substrate for some enzyme other than NOS. Present evidence shows that NOS activity cannot account for the resistance of NADPH-d neurons to ischemia. None of the L-NAME treatments affected the survival of the NADPH-d neurons in the cortical regions or in the most vulnerable areas of the hippocampus and striatum, as demonstrated by comparative observations of those regions stained either with cresyl violet or for NADPH-d activity. This suggests that the diaphorase activity of NOS may provide a neuroprotective mechanism to NOS/NADPH-d neurons, unrelated to NO synthesis. Indeed, enhanced NADPH:quinone reductase activity, a similar enzyme to NADPH-d, prevents in vitro glutamate-induced toxicity.

In this study, only some of the NADPH-d neurons were spared in the dorsolateral striatum of saline-treated animals after ischemia of the forebrain for 10 minutes. An almost absolute sparing of such neurons after ischemia has recently been reported. The discrepancy between this result and the present study may be explained by the interferences of stress occurring in chronically saline-treated animals before BCO. It is known that glucocorticoids inhibit the induction of NOS in macrophages and in several tissues. It would be interesting to investigate whether stress or corticosterone therapy may affect the NADPH-d activity and the susceptibility of NADPH-d neurons to ischemia.

In conclusion, resistance of NOS/NADPH-d neurons to ischemia is not related to NOS activity. Rather than being neurotoxic, NO produced by endothelial cells and neurons may participate in the homeostatic responses of the brain to global ischemia. The relative role of endothelial, neuronal, and glial NO production in the pathophysiology of neuronal death in different experimental models of brain ischemia could be better characterized when more selective inhibitors are available for the different isoforms of NOS in the various compartments.

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**References**


The article by Sancesario et al investigates the role of nitric oxide (NO) in cerebral ischemic damage using a gerbil model of transient global ischemia. Two major findings were reported. First, inhibition of NO synthase (NOS) by administration of N^o-Nitro-L-arginine methyl ester (L-NAME) fails to protect CA1 hippocampal neurons. Rather, chronic treatment at doses that reduce NOS activity by 90% worsens hippocampal damage. Second, inhibition of NO synthesis does not affect the survival of NOS/nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase–containing neurons, suggesting that NO production is not responsible for the purported resistance of these cells to neurotoxicity.

Evidence has accumulated that NO may have opposing roles in cerebral ischemic damage. On the one hand, NO is a potent vasodilator that may be beneficial in the acute phase of cerebral ischemia by increasing blood flow to the ischemic territory. In support of this hypothesis, early administration of the NO precursor L-arginine reduces focal cerebral ischemic damage. On the other hand, NO at higher concentrations inhibits mitochondrial enzymes and leads to production of peroxynitrite and, as such, is extremely toxic. Therefore, some studies have reported that inhibition of NO synthesis reduces glutamate-mediated neuronal damage in vitro and ameliorates focal cerebral ischemic damage in vivo. In addition, NO appears to interact directly with the N-methyl-D-aspartate (NMDA) receptor resulting in downregulation of the receptor activity and attenuation of neurotoxicity. The redox milieu of the tissue may determine whether NO, or related nitroso compounds, will cause downregulation of NMDA damage. Whether NO is “protective” or “destructive” will, therefore, depend on numerous factors including, for example, the cellular source of NO and its targets, the amount of NO being produced, the stage of evolution of the ischemic damage, the degree of glutamate receptor activation, and
Nitric oxide inhibition aggravates ischemic damage of hippocampal but not of NADPH neurons in gerbils.
G Sancesario, M Iannone, M Morello, G Nisticò and G Bernardi

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