Nitric Oxide Production During Focal Cerebral Ischemia in Rats

Abraham Kader, MD; Vincent I. Frazzini, MD; Robert A. Solomon, MD; Rosario R. Trifiletti, MD, PhD

Background and Purpose: Nitric oxide has been implicated as a mediator of glutamate excitotoxicity in primary neuronal cultures.

Methods: A number of indicators of brain nitric oxide production (nitrite and cyclic guanosine monophosphate [cGMP] concentrations and nitric oxide synthase activity) were examined after bilateral carotid ligation and right middle cerebral artery occlusion in adult rats.

Results: Brain nitrite was significantly increased in the right versus left cortex 5, 10, and 20 minutes after middle cerebral artery occlusion ($P<.05$), with a return to baseline at 60 minutes. There were no significant changes in cerebellar concentrations. Cortical levels of cGMP were increased at 10, 20, and 60 minutes after occlusion, with significant right-to-left differences ($P<.05$). Cerebellar concentrations of cGMP were also increased but without significant side-to-side differences. Nitric oxide synthase activity increased approximately 10-fold from baseline 10 minutes after occlusion in the right cortex but decreased markedly by 60 minutes from its peak at 10 minutes. The right-to-left difference in nitric oxide synthase activity was significant at 20 minutes ($P<.05$). Pretreatment of rats with N0-nitro-L-arginine, a nitric oxide synthase inhibitor, abolished the rise in nitrite and cGMP.

Conclusions: These results suggest that a sharp transient increase in the activity of nitric oxide synthase occurs during the first hour of cerebral ischemia, which leads to a burst in nitric oxide production and activation of guanylate cyclase. (Stroke. 1993;24:1709-1716.)

**KEY WORDS** • cerebral arteries • cerebral ischemia • nitric oxide

**Materials and Methods**

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Nitrite and nitrate are the major stable metabolites of NO under aerobic conditions, and NO has been shown to activate brain guanylate cyclase, leading to cGMP production.11,12 In the present study, we have examined brain nitrite, cGMP, and NOS activity ex vivo during the first hour of focal cerebral ischemia, using a rat model of bilateral carotid/unilateral middle cerebral artery (MCA) occlusion. We found changes in these quantities that are best explained by a transient increase in NOS activity and NO production in ischemic regions of the brain. Further measurements of nitrite and cGMP in animals pretreated with NO2-Arg suggest that the production of NO and cGMP is mediated by NOS.

Materials

All materials were of the highest purity available and were obtained from Sigma Chemical Co, St Louis, Mo, unless otherwise specified.

Animal Surgery and Animal Tissue Processing

Male Wistar rats weighing 250 to 350 g were anesthetized with an intraperitoneal injection of chloral hydrate (400 mg/kg). Both carotid arteries were isolated but not occluded with a 4.0 silk tie, with preservation of the vagus nerve. Middle cerebral artery occlusion (MCAO) was performed using a modification of techniques described by Tamura et al.13 With the animal in the left
lateral decubitus position, an oblique incision was made between the inferior margin of the orbit and the tragus. The exposed temporalis muscle was dissected from the cranium to reveal the inferotemporal fossa. After removal of the coronoid process of the mandible, a craniectomy was made using a saline-cooled electric drill (Motoool; Dremel, Denver, Colo.). The dura was opened and the MCA cauterized with a microbipolar unit (Malis bipolar coagulator; Codman, Randolph, Mass) for a distance of 4 mm. The MCA was then divided with microscissors just after the point where it crosses the olfactory nerve. Immediately after, both carotid arteries were occluded with the silk tie that had been previously used to isolate them; this was considered the time of onset of ischemia. The average length of time between the MCA and bilateral carotid artery occlusions was 15 seconds. Sham-operated animals had the MCA and both carotid arteries exposed but not occluded. The model of bilateral carotid and unilateral MCA occlusion was used for this study because we found in preliminary experiments that it reliably results in a very large stroke ipsilateral to the side of MCAO without histological evidence of infarction on the opposite side in Wistar rats.

Contralateral temporalis muscle temperature was monitored throughout the experiment in all rats (YSI Telethermometer 400; YSI, Yellow Springs, Ohio) and maintained at 36.5°C throughout the experiment. Rats were killed by decapitation at 0, 5, 10, 20, and 60 minutes after the onset of ischemia. The head was immersed in running cold water for 20 seconds and the brain removed. Using a rodent brain matrix (Activational Systems Inc, Warren, Mich), a 4-mm coronal section was made with the posterior cut 2 mm behind the optic chiasm. From this coronal section, the right and left cortices were separated with a scalpel and each was placed in 2.0 mL of ice-cold 100 mmol/L potassium phosphate (pH 7.5) containing 1 mmol/L NaCl, ethylenediaminetetraacetic acid (EDTA). Right and left cerebella were similarly treated. All tissues were rapidly sonicated in ice-cold buffer for 15 seconds in a thermally regulated sonicator (Artek Ultrasonic 2000; Dynatech Lab, Chantilly, Va). Sonicates were then processed as described below.

Drug Treatments of Animals

NOS-Nitro-L-arginine was dissolved in a vehicle of 1:2:2 (vol/vol/vol) 1N HCl/proplylene glycol/100 mmol/L phosphate-buffered saline (pH 7.5). Eighteen hours before being subjected to surgery as described above, animals were injected intraperitoneally with 0.1 mL of the NO2-Arg solution (at the appropriate concentration to provide 50 mg/kg) or vehicle. Control experiments (R.R.T., unpublished data, 1992) demonstrated a greater than 90% inhibition of NOS at these doses for 1 to 30 hours after NO2-Arg administration. All animals appeared to tolerate NO2-Arg well at this dose.

Nitrite Assay

Whole brain nitrite levels were measured by a fluorometric method. Briefly, 1.5 mL of brain sonicates prepared as described above were centrifuged at 14 000 rpm for 10 minutes at 4°C in an Eppendorf microcentrifuge. One milliliter of the supernatant was withdrawn and subjected to nitrite analysis; 0.5 mL of 0.04% (wt/vol) 4-hydroxyocoumarin in 1:1 (vol/vol) dimethylformamide/2N HCl was added, and the mixture was incubated for 5 minutes at 0°C. Then, 50 μL of 8% (wt/vol) sodium thiosulfate was added, followed by a 5-minute incubation at room temperature. Next, 0.5 mL of 1.5 mol/L NaOH was added, and fluorescence was determined at room temperature in a Farrand model A4 fluorometer (Farrand Optical Co, New York, NY) using 340-nm excitation and 470-nm emission filters. Relative fluorescence was measured with respect to a sample of 1.0 mL of sonication buffer and calibrated with nitrite internal and external standards. Nitrite values are expressed as picomoles per millgramm soluble protein.

Cyclic GMP Assay

Cyclic GMP was determined by a commercially available radioimmunoassay kit using tritium-labeled cGMP as a tracer (Amersham, Inc). Crude sonicate (250 μL) (prepared as described above) was placed in a boiling water bath for 10 minutes to activate phosphodiesterases. The heated sonicate was cooled and spun at 14 000 rpm for 10 minutes at 4°C in an Eppendorf microfuge. Supernate (50 μL) was incubated with 25 μL of tritium-labeled cGMP tracer and 25 μL of anti-cGMP antibody. After a 2-hour incubation at 4°C, 0.5 mL of ice-cold 60% (wt/vol) ammonium sulfate was added, and the mixture was incubated an additional 15 minutes at 4°C. Tubes were again spun at 14 000 rpm for 10 minutes at 4°C. The resulting pellet was dissolved in 1.0 mL of water and counted for tritium at 49% efficiency in a liquid scintillation spectrometer. cGMP values were computed using internal and external unlabeled cGMP standards and are expressed as femtomoles per milligram total protein.

Nitric Oxide Synthetase Assay

Nitric oxide synthase was assayed by a slight modification of the procedure of Bredt and Snyder. The assay mix (175 μL) was composed of 25 μL of analyte; 25 μL of 50 mmol/L N-(N-2-hydroxyethylpiperazine)-N2-ethane-sulfonic acid (HEPES) (pH 7.4) or 7.14 mmol/L l-Nmonomethyl-l-arginine (L-NMMA) in buffer (for “sample” and “blank” tubes, respectively); 100 μL of an “NOS mix” containing (final concentrations) 50 mmol/L HEPES (pH 7.4), 1 mmol/L NADPH, 1 mmol/L EDTA, 1.25 mmol/L CaCl2, and 10 mg/mL of pure calmodulin (Sigma). Reaction was started by the addition of 25 μL of 100 mmol/L (3H)L-arginine. After a 5-minute incubation at 37°C, 2.0 mL of “NOS stop” (20 mmol/L HEPES [pH 5.5]/2 mmol/L EDTA) was added with vortexing. The mixture was applied to a Dowex AG50WX-8 (Na+ form) column preequilibrated in water and the column eluted with 2.0 mL of water. The entire flow-through (4.0 mL) was counted by liquid scintillation spectrometry at 49% efficiency. The counts per minute of “sample” less “blank” is a measure of L-NMMA-sensitive conversion of (3H)L-arginine to (3H)L-citrulline by NOS.

Protein Assay

Protein was assayed by the method of Bradford, using bovine serum albumin as an external standard.

Statistical Analysis

Right and left brain regions were compared by two-tailed, paired, Student’s t test of log-transformed data.
Comparisons of brain regions at different times of ischemia were done by one-way analysis of variance (ANOVA) of log-transformed data. Linear curve fits were done by unweighted least squares. Nonlinear curve fits were done by best (third-degree) spline curve fitting using commercially available software (Delta Graph Professional, Delta Point).

Results

Brain Nitrite Levels After Middle Cerebral Artery Occlusion

Nitrite ion is the major breakdown product of NO under aerobic conditions. Nitrite is then slowly oxidized to nitrate, which is then excreted from the body. Control experiments (data not shown) indicated that nitrite is stable for more than 3 hours at 37°C when incubated with brain homogenates. We reasoned that, over the time course of these experiments (less than 1 hour), nitrite levels are more likely to change than nitrate levels. Using the fluorometric method of Ohta et al,14 we determined nitrite levels in ipsilateral and contralateral cerebral cortex or cerebellum after 0, 5, 10, 20, or 60 minutes of ischemia. Results are shown in Fig 1. Brain nitrite increased in ipsilateral versus contralateral cortex 5, 10, and 20 minutes after ischemia (P<.05). Disparity reached maximal levels at 5 to 10 minutes but normalized at 60 minutes. We observed an increase in absolute nitrite concentrations, which was significant (P<.025) at 20 minutes but approached significance at 5 and 10 minutes (P<.1). There was also a significant decrease in absolute nitrite concentration on the contralateral hemisphere at 20 and 60 minutes but not 5 and 10 minutes. However, if both right and left hemisphere data are pooled together at 0 minutes, this decrease in contralateral nitrite at 20 and 60 minutes loses its statistical significance. Finally, no significant right-to-left differences or differences from 0 minutes of ischemia were observed in the cerebellum (Fig 1B).

When a percent side-to-side disparity was calculated in each animal using the formula [(Value) ipsilateral−(Value) contralateral]/(Value) contralateral×100% and then averaged, we found a significant nitrite concentration disparity at 5, 10, and 20 minutes compared with 0 minutes (Fig 1C). No significant disparity in nitrite concentrations was observed in the cerebellum. There was no rise in nitrite concentrations in sham-treated animals (Table 1).

Brain Cyclic GMP Levels After Middle Cerebral Artery Occlusion

There was a substantial (approximately fivefold) increase in cGMP in both cortex and cerebellum, which was more markedly increased in the ipsilateral cortex at 10, 20, and 60 minutes (Fig 2). Unlike nitrite, which returned to baseline by 60 minutes, changes in cGMP were sustained. On the right cortex and cerebellum, cGMP concentrations at 10, 20, and 60 minutes were significantly increased when compared with 0 minutes of ischemia by single-factor ANOVA (P<.05). There were also statistically significant differences in right versus left cortical cGMP concentrations at 10, 20, and 60 minutes but not in the cerebellum. Similar conclusions are reached when the data are analyzed by percent disparity. There was no elevation in cGMP in sham-operated animals.

Percent ipsilateral/contralateral disparities in levels of nitrite and cGMP were, on a rat-by-rat basis, strongly positively correlated in cerebral cortex; representative data after 20 minutes of MCAO are shown in Fig 3. Significant positive correlation was also found at 10 minutes but not in the cerebellum at any time point examined (data not shown).

Effect of Pharmacologic Blockade of Nitric Oxide Synthase on Brain Nitrite and Cyclic GMP Levels

If the observed changes in nitrite and cGMP levels reflect consequences of NOS-catalyzed de novo synthesis of NO from l-arginine, then pharmacologic inhibition of NOS should block changes in both. We pretreated rats with either NO2-Arg or vehicle (see "Materials and Methods") and then subjected them to MCAO for 10 minutes. Brain nitrite and cGMP were determined (Tables 1 and 2, respectively). Pretreatment of animals with NO2-Arg abolished the increase of both nitrite and cGMP at 10 minutes, which suggests that these increases are mediated by NO production via NOS. Interestingly, the nitrite concentration in ipsilateral cortex was lower than that in contralateral cortex in drug-treated animals (P=.028) and lower than that in ipsilateral cortex in placebo-treated animals (P=.016) (Table 1). Furthermore, although nitrite was slightly higher on the contralateral side at 10 minutes in drug-versus placebo-treated animals, this was not statistically significant.

Vehicle injections in control animals did not block the observed increases in nitrite and cGMP. In addition, there were no significant differences between vehicle-treated animals and those with no treatment (Tables 1 and 2).

Brain Nitric Oxide Synthase Activity After Middle Cerebral Artery Occlusion

Taken together, the changes in brain nitrite and cGMP, their correlation, and the abolition of these changes by NOS inhibition are best explained by a transient differential activation of brain NOS after MCAO. If such activation involves an enduring change in NOS activity such as might be conferred by phosphorylation or dephosphorylation, it might be possible to detect changes in NOS activity ex vivo after MCAO. Accordingly, we measured NOS activity in ipsilateral and contralateral cerebral cortex and cerebellum 0, 10, 20, or 60 minutes after ischemia (Fig 4). We found that brain NOS activity was significantly increased in ipsilateral cortex at 10 and 20 minutes compared with 0 minutes of MCAO. There was a 10-fold increase in NOS activity at 10 minutes, which decreased dramatically by 60 minutes. We also found significant side-to-side differences at 20 minutes (P<.05, ANOVA) and a trend toward significance at 10 minutes (P<.1, ANOVA). No significant side-to-side changes were detected in the cerebellum.

Discussion

The major finding of the present study is that NO production is rapidly and transiently increased in ischemic cerebral cortex after focal cerebral ischemia. Several lines of evidence lead to this conclusion: (1) Ipsilateral cerebral cortical nitrite levels were increased (relative to contralateral cortex) after MCAO, peaking
Fig 1. Line graphs of nitrite levels in ipsilateral and contralateral cerebral cortex or cerebellum after 0, 5, 10, 20, or 60 minutes of ischemia. A, Nitrite levels in cerebral cortex after middle cerebral artery occlusion (MCAO). Nitrite values in ipsilateral cortex were significantly increased from those in contralateral cortex (two-tailed paired Student's t test on log-transformed data) at 5 minutes ($P=.028$, 5 df), 10 minutes ($P=.0043$, 13 df), and 20 minutes ($P=2.1e-7$, 16 df); values at 0 and 60 minutes were not significantly different. On the ipsilateral side, log-transformed values compared with 0 minutes of ischemia by single-factor analysis of variance (ANOVA): 5 minutes ($F=3.54$, $P=.082$, 14 df), 10 minutes ($F=3.15$, $P=.091$, 21 df), 20 minutes ($F=5.89$, $P=.023$, 24 df), and 60 minutes ($F=0.19$, $P=.67$, 17 df). B, Nitrite levels in cerebellum after MCAO. No significant ipsilateral vs contralateral differences ($P>.05$) were observed at 0, 5, 10, 20, or 60 minutes (two-tailed Student's t test on log-transformed data). On the ipsilateral side, log-transformed values compared with 0 minutes of ischemia by single-factor ANOVA: 5 minutes ($F=1.54$, $P=.2429$, 11 df), 10 minutes ($F=0.009$, $P=.93$, 19 df), 20 minutes ($F=2.99$, $P=.099$, 20 df), and 60 minutes ($F=0.44$, $P=.52$, 14 df). C, Ipsilateral vs contralateral disparities in nitrite levels in cortex and cerebellum after MCAO. Cerebral cortex values compared with 0 minutes of ischemia by single-factor ANOVA: 5 minutes ($F=5.10$, $P=.041$, 14 df), 10 minutes ($F=4.69$, $P=.043$, 21 df), 20 minutes ($F=28.3$, $P=.0021$, 22 df), and 60 minutes ($F=2.25$, $P=.15$, 17 df). There were no significant differences by similar analysis at any time point in cerebellum.
Table 1. Effect of N°-Nitro-L-Arginine Treatment on Nitrite Concentration 10 Minutes After Middle Cerebral Artery Occlusion

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ipsilateral Cortex (n)</th>
<th>Contralateral Cortex (n)</th>
<th>Ipsilateral Cerebellum (n)</th>
<th>Contralateral Cerebellum (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min MCAO</td>
<td>779±112 (9)</td>
<td>937±79 (9)</td>
<td>756±212 (7)</td>
<td>959±205 (7)</td>
</tr>
<tr>
<td>10 min MCAO</td>
<td>1406±323* (13)</td>
<td>791±200 (13)</td>
<td>753±168 (13)</td>
<td>834±168 (13)</td>
</tr>
<tr>
<td>10 min MCAO, vehicle pretreatment</td>
<td>1567±327 (12)</td>
<td>867±53 (12)</td>
<td>577±86 (9)</td>
<td>615±175 (9)</td>
</tr>
<tr>
<td>10 min MCAO, NO₂-Arg (50 mg/kg) pretreatment</td>
<td>536±108† (6)</td>
<td>1066±183 (6)</td>
<td>548±42 (9)</td>
<td>511±60 (9)</td>
</tr>
<tr>
<td>Sham operated</td>
<td>871±214 (6)</td>
<td>739±48 (6)</td>
<td>514±98 (3)</td>
<td>558±13 (3)</td>
</tr>
</tbody>
</table>

Values are mean±SEM picomoles per milligram protein. MCAO indicates middle cerebral artery occlusion; NO₂-Arg, N°-nitro-L-Arginine.
*Significantly higher than 10 min MCAO on contralateral side and 0 min MCAO on ipsilateral side (P<.05, analysis of variance).
†Significantly lower than contralateral side (P<.05).

at 5 to 10 minutes after arterial occlusion and returning to baseline by 60 minutes. No significant changes were observed in ipsilateral or contralateral cerebellum. (2) Ipsilateral cerebral cortical cGMP levels were increased (relative to contralateral cortex) after MCAO. Changes in cGMP reached maximal levels of ipsilateral/contralateral disparity at 5 minutes and endured for at least 60 minutes. (3) On a rat-by-rat basis, ipsilateral and contralateral changes in cortical nitrite and cGMP were strongly positively correlated at 10 and 20 minutes of ischemia. (4) Pharmacologic inhibition of NOS before ischemia abolished the increase in ipsilateral cortical nitrite and cGMP. (5) When measured ex vivo, brain NOS activity transiently increased in all brain regions examined, but much more dramatically in ipsilateral cerebral cortex.

Recent experiments by Dawson et al suggested that NO mediates glutamatergic neurotoxicity via NMDA receptors in primary cerebral cortical cultures. Although glutamate production has been found to be increased in some animal models of ischemia, changes in NO in ischemic cerebral tissue have not been examined. The burst of NO production that apparently occurs in the first few minutes of focal ischemia may be the result of increased glutamate release and may, in itself, be responsible for the neuronal damage attributed to excitatory neurotransmitters during ischemia. NO, in turn, has been proposed to cause damage to tissues by combining with the superoxide anion to form peroxynitrite anion, which can decompose to highly toxic hydroxyl and nitrogen dioxide radicals.

There is mounting evidence that NO may be responsible for regulating or at least modulating cerebral blood flow. NOS inhibitors have been shown to block neurally induced vasodilation and NO immunoreactivity has been found in adventitial fibers of many blood vessels. Recent studies suggest that NO may be responsible for maintaining cerebral blood flow during ischemia.
vasodilatation also as well as mediating cerebral blood flow changes that occur as a result of changes in arterial carbon dioxide concentrations. Our finding of increased NO production by ischemic brain might be viewed as part of a homeostatic mechanism designed to lead to vasodilation in the face of ischemia. This would agree with the recent findings in which infarct size was increased in rats pretreated with NOS inhibitors. On the other hand, a large and/or rapid burst of NO may be toxic, which would account for the neuroprotective effect of NOS inhibitors reported in focal stroke in mice and neonatal rats. Modulating this burst in NO production to levels in which its vasodilatory effects are still present may be the optimal neuroprotective strategy. However, we cannot determine from our data which cortical levels of nitrite are sufficient to mediate vasodilatation or neurotoxicity.

The production of NO and cGMP are closely interrelated. NO activates guanylate cyclase, and glutamate (via NMDA receptors) has been shown to increase cGMP levels via NO in the cerebellum, and hippocampal slices, and primary cortical cultures. Although changes in NO during cerebral ischemia have not been previously examined, changes in cGMP have been reported by Magal et al in a model of global intrauterine ischemia. As in our experiments, Magal et al found a large increase in cGMP within minutes after onset of ischemia. These authors also showed that the increase in cGMP was due to rapid activation of brain guanylate cyclase. In view of our present results, it would appear that the findings of Magal et al might most easily be explained by increased brain NO production during ischemia.

**TABLE 2. Effect of N⁶-Nitro-L-Arginine Treatment on Cyclic Guanosine Monophosphate Concentration 10 Minutes After Middle Cerebral Artery Occlusion**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ipsilateral Cortex (n)</th>
<th>Contralateral Cortex (n)</th>
<th>Ipsilateral Cerebellum (n)</th>
<th>Contralateral Cerebellum (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min MCAO</td>
<td>715±278 (6)</td>
<td>743±172 (6)</td>
<td>636±159 (4)</td>
<td>792±118 (4)</td>
</tr>
<tr>
<td>10 min MCAO</td>
<td>2125±378* (7)</td>
<td>1487±231 (7)</td>
<td>1911±388 (7)</td>
<td>2475±640 (7)</td>
</tr>
<tr>
<td>10 min MCAO, vehicle pretreatment</td>
<td>2010±428 (6)</td>
<td>1278±308 (6)</td>
<td>1973±370 (7)</td>
<td>2784±800 (7)</td>
</tr>
<tr>
<td>10 min MCAO, NO₂-Arg (50 mg/kg) pretreatment</td>
<td>624±212† (6)</td>
<td>686±228 (6)</td>
<td>749±366 (6)</td>
<td>444±94 (6)</td>
</tr>
<tr>
<td>Sham operated</td>
<td>784±198 (5)</td>
<td>775±193 (5)</td>
<td>829±44 (3)</td>
<td>660±84 (3)</td>
</tr>
</tbody>
</table>

Values are mean±SEM femtomoles per milligram protein. MCAO indicates middle cerebral artery occlusion; NO₂-Arg, N⁶-nitro-L-Arginine.

*Significantly different from 0 min (P<.05, analysis of variance).
†Not significantly different from 0 min (analysis of variance).
ischemia. However, other factors such as carbon monoxide might also mediate cGMP production. An increase in carbon monoxide may be responsible for the increase in contralateral cGMP production at 10 minutes without a concomitant increase in nitrite. It is possible that although there is no histological evidence of ischemia in the contralateral hemisphere, a decrease in cerebral blood flow may occur, which could account for the observed rise in cGMP. A correlation between cerebral blood flow in this model and nitrite and cGMP production remains to be determined.

The decrease in ipsilateral but not contralateral nitrite in NO2-Arg–treated animals may be explained by recent work by Stamler et al., who showed that NO circulates in plasma mostly as an S-nitroso adduct of proteins. These proteins, of which serum albumin is the most common, serve as a source and sink of NO, buffering the concentration of free NO. After MCAO, blood flow to the right cortex is markedly diminished, thereby decreasing the only available source of NO (S-nitroso proteins) in the right cortex of these animals. In the left cortex, sufficient blood flow may be present to explain the apparently unchanged levels of nitrite.

We found a striking activation followed by an equally striking inactivation of NOS after MCAO, and we propose that this accounts for the NO burst after ischemia. At least two mechanisms can be invoked for NOS activation after MCAO. Focal ischemia leads to increased synaptic glutamate. Increased synaptic glutamate might increase postsynaptic calcium influx via NMDA receptors, increased calcium/calmodulin binding, and thereby NOS activation. While this sequence of events could lead to increased NOS activity in vivo, we find it difficult to be the sole explanation ex vivo. Alternatively, it has been shown that brain NOS has two serine groups that can be phosphorylated by distinct protein kinases, leading to decreased activity of the enzyme. If the rapid depletion of adenosine triphosphate that attends cerebral ischemia is rapidly coupled to NOS dephosphorylation, it would lead to an activation of enzyme that might persist ex vivo. Recently, the calcium/calmodulin–regulated phosphatase calcineurin has been demonstrated to dephosphorylate NOS, thereby activating it. The immunomodulatory drug FK-506 (in concert with the immunophilin FK binding protein) inhibits calcineurin and potently inhibits glutamate toxicity in cell culture. This implies that NOS activity in vivo is at least in part regulated by phosphorylation/dephosphorylation.

Several possible mechanisms might also be invoked to explain the inactivation of NOS that follows its rapid activation. Brain NOS is an enzyme that produces a product that is highly oxidizing yet requires numerous highly reduced cofactors (NADPH, reduced tetrahydrobiopterin, reduced flavin adenine dinucleotide, reduced flavin mononucleotide); therefore, rapid NO production might activate NOS. NO could also conceivably nitrosylate sulfhydryl groups in NOS, as has been demonstrated for plasma proteins, thereby inactivating it. Another possible inactivation mechanism involves the NMDA receptor. Recent work demonstrated that NO oxidizes a critical sulfhydryl group in the NMDA receptor, which might decrease calcium influx and thereby reduce NOS activity. Finally, activation of peptidases in ischemic tissue cannot be ruled out as a cause of NOS deactivation.

In summary, a sharp, transient increase in the activity of NOS results in a burst of NO production and activation of guanylate cyclase in the first hour of focal cerebral ischemia. The precise biochemical mechanisms underlying the activation (and inactivation) of NOS, as well as the pathophysiological consequences of excess NO in ischemic tissue, remain to be determined.

Acknowledgments

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18. Beckman JS, Beckman TW, Chen J, Marshal PA, Freeman BA. Apparent hydroxyl radical production by peroxyxinitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci U S A. 1990;87:1620–1624.
The accompanying article by Kader et al provides evidence that significant amounts of nitric oxide (NO) are produced within brain after the onset of focal cerebral ischemia. Although NO itself was not measured, elevations in nitrite ion, a breakdown product, cyclic guanosine monophosphate, and of NO synthase activity were detected by this group beginning shortly (minutes) after the onset of occlusion. The findings, suggestive of increased NO production, are consistent with data recently reported by Malinski et al using an NO-sensitive porphyrinic microsensor and electrochemical detection to measure NO directly. Malinski et al noted dramatic increases in NO from less than 10 nmol/L (baseline detection limit) to 2.2 µmol/L occurring 3 to 24 minutes after transient middle cerebral artery occlusion. NO levels then decreased slowly over the ensuing hour with a decay half-time of approximately 30 minutes. We can infer from the positioning of the electrode that NO was probably synthesized (at least in small part) within or close to the penumbra. Hence, the data from two laboratories describing the time course and production of NO using both direct and indirect methods appear consistent. Of course, it is not possible presently to determine the source(s) (neuronal, vascular) for the generated NO.

Whether or not bursts of NO continue to develop within salvageable brain over time seems relevant to the consequences of increasing NO levels within ischemic tissue. Compelling arguments have been advanced for both NO-mediated cytotoxic effects (eg, via peroxynitrite production and excitotoxic mechanisms) and for NO-mediated tissue-sparing effects based on antiplatelet and vasodilating actions; both are important. The prevailing mechanism may well depend in part on the amount of NO generated, the tissue compartment generating NO, the time after the onset of ischemia, the redox form of NO, and the integrity of guanylate cyclase and its dependent reactions. These complex considerations notwithstanding, it would be interesting to learn the results of tissue outcome studies after complete NOS inhibition in the model of focal cerebral ischemia characterized by Kader et al.

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