Role of nitric oxide in traumatic brain injury in the rat

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Object. Although nitric oxide (NO) has been shown to play an important role in the pathophysiological process of cerebral ischemia, its contribution to the pathogenesis of traumatic brain injury (TBI) remains to be clarified. The authors investigated alterations in constitutive nitric oxide synthase (NOS) activity after TBI and the histopathological response to pharmacological manipulations of NO.

Methods. Male Sprague–Dawley rats underwent moderate (1.7–2.2 atm) parasagittal fluid-percussion brain injury. Constitutive NOS activity significantly increased within the ipsilateral parietal cerebral cortex, which is the site of histopathological vulnerability. 5 minutes after TBI occurred (234.5 ± 60.2% of contralateral value [mean ± standard error of the mean (SEM)], p < 0.05), returned to control values by 30 minutes (114.1 ± 17.4%), and was reduced at 1 day after TBI (50.5 ± 13.1%, p < 0.01). The reduction in constitutive NOS activity remained for up to 7 days after TBI (31.8 ± 6.0% at 3 days, p < 0.05; 20.1 ± 12.7% at 7 days, p < 0.01). Pretreatment with 3-bromo-7-nitroindazole (7-NI) (25 mg/kg), a relatively specific inhibitor of neuronal NOS, significantly decreased contusion volume (1.27 ± 0.17 mm³ [mean ± SEM], p < 0.05) compared with that of control (2.52 ± 0.35 mm³). However, posttreatment with 7-NI or pre- or posttreatment with nitro-l-arginine-methyl ester (l-NAME) (15 mg/kg), a nonspecific inhibitor of NOS, did not affect the contusion volume compared with that of control animals (1.87 ± 0.46 mm³, 2.13 ± 0.43 mm³, and 2.18 ± 0.53 mm³, respectively). Posttreatment with l-arginine (1.1 ± 0.3 mm³, p < 0.05), but not 3-morpholino-sydnonimine (SIN-1) (2.48 ± 0.37 mm³), significantly reduced the contusion volume compared with that of control animals.

Conclusions. These data indicate that constitutive NOS activity is affected after moderate parasagittal fluid percussion brain injury in a time-dependent manner. Inhibition of activated neuronal NOS and/or enhanced endothelial NOS activation may represent a potential therapeutic strategy for the treatment of TBI.

KEY WORDS • nitric oxide • nitric oxide synthase • fluid-percussion brain injury • l-arginine • nitric oxide inhibitor • rat

RECENTLY, divergent roles for the newly identified neuronal messenger molecule and oxygen radical, nitric oxide (NO), have been identified in various models of cerebral ischemia. Nitric oxide is synthesized from the semiessential amino acid l-arginine by a group of isozymes known as NO synthases (NOSs). Three isozymes of NOS have been identified: two constitutive NOSs (cNOS), neuronal NOS (nNOS; type I) and endothelial NOS (eNOS; type III), and one inducible isozyme (iNOS; type II). All three isozymes can be expressed in the central nervous system. Both eNOS and nNOS synthesize NO only when the [Ca++], concentration is elevated and calmodulin is bound to the enzyme. However, iNOS produces NO continuously and independently of [Ca++].

Neuronal NOS is primarily expressed in a small population of neurons throughout the central nervous system and perivascular nerves. Nitric oxide produced by nNOS is thought to mediate synaptic plasticity and neuronal signaling and may play an important role in neuronal injury under pathological conditions. Among the mechanisms of neuronal injury in traumatic brain injury (TBI), excitotoxicity and oxygen free radicals are believed to play important roles. Indeed, in TBI models, excitotoxicity has been implicated in studies demonstrating the massive release of excitatory amino acids at the trauma site and by the cytoprotective action of N-methyl-D-aspartate (NMDA) receptor antagonists. A role for oxygen radicals in the acute pathophysiological process of TBI has been suggested by studies showing elevations in free radicals. In addition, free radical scavengers, such as superoxide dismutase and lipid antioxidants, including methylprednisolone and tirilazad mesylate, have been reported to be neuroprotective in animals subjected to experimental TBI. Several studies have suggested that NO participates in NMDA-mediated neurotoxicity and hypoxic ischemic damage. Furthermore, NO itself may cause neuronal injury by reacting with superoxide (O₂⁻) to form peroxynitrite (ONOO⁻), a highly toxic free radical. Therefore, NO produced by nNOS may participate in the development of neuronal damage by excitotoxicity and/or oxygen free radicals following TBI.

Endothelial NOS is expressed in the endothelial layers.
of large vessels and in the choroid plexus of the normal rat brain. Small populations of neurons are also immunopositive for eNOS, including pyramidal cells of the CA1 through CA3 regions of the hippocampus and granule cells of the dentate gyrus. Nitric oxide produced by eNOS is believed to maintain a resting cerebral blood flow (CBF) and is an inhibitor of platelet aggregation and leukocyte adhesion to the blood vessel wall. Focal ischemia studies, in which the precursor of NO, L-arginine, as well as the NO donor, 3-morpholino-sydnonimine (SIN-1) have been administered to animals, have improved CBF levels and decreased infarction volume. These data support the hypothesis that early activation of eNOS might reduce the severity of neuronal injury. Because a potential mechanism of secondary brain damage after TBI is cerebral ischemia, methods that improve local CBF after brain injury would be expected to improve outcome.

Although elevated levels of NO have been documented immediately after TBI by using a microdialysis technique, the role of NO in the pathogenesis of TBI remains poorly understood. Therefore, we investigated the following: 1) the change in eNOS activity after TBI; 2) whether inhibition of NO by two types of NO inhibitors, Nω-nitro-L-arginine methyl ester HCl (L-NAME) (which inhibits all NOS isoforms) and 3-bromo-7-nitrodiazole (7-NI) (which inhibits nNOS in a relatively selective fashion), protects the brain from tissue injuries; and 3) whether NO generated from two drugs, L-arginine (a precursor of NO) and SIN-1 (which is a NO donor), protects the brain from tissue injury.

**Materials and Methods**

### Animal Groups

The NOS enzymatic activity experiments were performed in 67 male Sprague–Dawley rats weighing between 300 and 400 g. The animals were randomly divided into the following groups. Group 1, the control group, consisted of eight rats that underwent TBI 15 minutes after all monitoring preparations (four animals) or 1 hour after intraperitoneal injection of peanut oil or a 2-minute intravenous infusion of saline (four animals). Group 2, the pre-L-NAME–treated group, consisted of eight rats that received an intraperitoneal injection of 7-NI (25 mg/kg dissolved in saline) 1 hour before TBI. This concentration of L-NAME reduces parietal cortex NOS activity by approximately 60% 1 hour after administration. Group 3, the pre-7-NI–treated group, consisted of eight rats that received an intraperitoneal injection of 7-NI (25 mg/kg dissolved in saline) 1 hour before TBI. This concentration of 7-NI reduces parietal cortex NOS activity by approximately 60% 1 hour after administration. Group 4, the post-L-NAME–treated group, was composed of eight rats that were given a 2-minute intravenous infusion of L-NAME (15 mg/kg dissolved in saline) 1 hour before TBI. This concentration of L-NAME used in the pre-L-NAME–treated group. Group 5, the post-7-NI–treated group, consisted of eight rats that underwent TBI and were immediately given the same dose of L-NAME used in the pre-L-NAME–treated group. Group 6, the post-L-arginine–treated group, consisted of eight rats that underwent TBI and were immediately given a 2-minute intravenous infusion of L-arginine (300 mg/kg dissolved in distilled saline). Group 7, the post-SIN-1–treated group, was composed of eight rats that underwent TBI and were immediately given a 60-minute infusion of SIN-1 (3 mg/kg dissolved in distilled saline) via the right common carotid artery. Phenytoin HCl was infused concurrently via the right femoral vein with SIN-1 to counteract the hypotension produced by the NO donor. Dosages that maintained a stable blood pressure throughout the infusion period were determined in a preliminary series of experiments.

### Surgical Preparation

All animal use procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The basic surgical preparation for fluid-perfusion brain injury was performed according to methods previously described. Briefly, 1 day before injury, the rats were anesthetized with Equithesin (a mixture of nembutal, propylene glycol, ethanol, MgSO₄, and chloral hydrate). The animals were then placed in a stereotactic frame and a 4.8-mm craniectomy was made overlying the right parietal cortex (3.8 mm posterior to the bregma and 2.5 mm lateral to the midline). A plastic injury tube was placed over the exposed dura and was bound by adhesive; dental acrylic was then poured around the injury tube. After the acrylic had hardened, the scalp was closed and the animal was returned to its cage and allowed to recover overnight before undergoing TBI.

The next day, a fluid-perfusion device was used to produce experimental TBI. The rats were reanesthetized with 3% halothane in a gas mixture of 70% NO/30% O₂, and maintained at 0.5% halothane. An endotracheal tube was inserted orally and the animals were mechanically ventilated. During ventilation, the animals were paralyzed with an intravenous injection of pancuronium bromide (1 mg/kg/hour). Moderate head injury, ranging from 1.7 to 2.2 atm, was then produced. The fluid-perfusion device consists of a saline-filled plexiglass cylinder that is fitted with a transducer housing and injury screw adapted for the rat skull. The metal screw is firmly connected to the plastic injury tube of the anesthetized rat, and the injury is induced by the descent of a pendulum that strikes the piston. Temporal muscle temperature was monitored as an indirect measure of brain temperature throughout the experiment and maintained at a normothermic (37°C) level by using a feedback heating lamp located above the animal’s head. Rectal temperature, obtained 6.5 cm from the anus, was also monitored and maintained at normothermic level throughout the study by using a feedback heating lamp located above the animal’s body. Arterial blood pressure was monitored via the right femoral artery and recorded for as long as 60 minutes after TBI. Blood gas levels were obtained from arterial samples taken from the right femoral artery just before TBI and 60 minutes later. Blood glucose levels were also monitored. The animals in the sham operation group were treated for NOS activity measurements, were subjected to all of the same surgical procedures except for the actual insult, including preparatory anesthesia and placement of a plastic injury tube.

### Measurement of cNOS Activity

The rats were killed 5 minutes, 30 minutes, 1 day, 3 days, or 7 days after TBI or sham operation, and their brains were rapidly removed. A 4-mm-thick coronal brain slice was first cut at a level between 12 and 16 mm caudal from the rostral tip of the cerebrum, and was incubated in a total volume of 50 μl reaction buffer containing 50
mM Tris-HCl, pH 7.4, 6 μM tetrahydrobiopterin, 2 μM flavin adenine dinucleotide, 2 μM flavin adenine mononucleotide, 1 μCi/μl of [3H]L-arginine, and nicotinamide adenine dinucleotide phosphate (10 mM) freshly prepared in Tris-HCl (pH 7.4) and CaCl2 (6 mM) for 45 minutes at 37°C. For Ca2+-independent activity measurements, CaCl2 was omitted from the incubation buffer. The reaction was terminated by addition of 400 μl of buffer (stopping buffer) containing 50 mM of N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), pH 5.5, and 5 mM EDTA. The [3H]L-arginine was separated from the product [3H]L-citrulline by column chromatography using 0.1 ml Dowex GA50WX-8 (Na form). The reaction sample was transferred to a spin cup and was placed into a scintillation reaction sample was transferred to a spin cup and was placed into a scintillation solvent and counted in a liquid scintillation counter. The column was further washed with 200 μl of stopping buffer and the radioactivity of the eluate was quantitated as described earlier and added to the initial counts. Soluble protein concentration in brain supernatant was determined by means of the Bradford reaction12 in which bovine serum albumin was used as a standard. Constitutive NOS activity was calculated by subtracting Ca2+-independent NOS activity from total NOS activity and expressed as picomoles of citrulline formed per milligram of protein per minute. The results represent the percentage increase or decrease in enzymatic activity on the contralateral side compared with that of sham-operated control animals.

Pathological Assessment

Three days after TBI, a subset of rats were deeply anesthetized with halothane and were perfused transcardially with physiological saline for 1 minute followed by a mixture of 40% formaldehyde, glacial acetic acid, and methanol (1:1:8 v/v/v) for 20 minutes at a pressure of 100 mm Hg. The rat brains were extracted and stored in chilled fixative overnight. The next day, the brains were embedded in paraffin. Semiserial 10-μm sections were obtained through the neocortex and stained with hematoxylin and eosin. Quantitative assessment of the histopathological injury and determination of the contusion area at several coronal levels 0.8, 1.8, 3.3, 4.3, 5.8, 6.8, and 7.3 mm posterior to the bregma14 was accomplished by an investigator (K.W.) who was blinded to the experimental groups. For this purpose the area of tissue contusion was traced using a camera lucida microscopic attachment. The contusion volume was then calculated by the numeric integration of sequential areas.

Statistical Analysis

Data were expressed as the mean values ± SEM. Data were compared by performing the Kruskal–Wallis one-way analysis of variance by ranks. Additional group comparisons were evaluated using the Mann–Whitney U-test.

Sources of Supplies and Equipment

Coronal brain slices were cut using rodent brain matrices obtained from Harvard Apparatus, Inc., South Natick, MA. The assay for NOS was performed using the Alexis NOSdetect assay kit purchased from Stratagene, San Diego, CA. The Dowex GA50WX-8 (Na form) was included in this assay kit. The [3H]L-arginine was obtained from Amersham, Arlington Heights, IL.

Results

General Physiological Parameters

Physiological variables obtained before and 1 hour after TBI or sham operation in the rats used for the cNOS activity measurements are presented in Table 1. No significant difference was observed between groups.

Physiological variables obtained before and 1 hour after TBI in the rats used for the histopathological experiments are presented in Table 2. Most physiological variables were within normal limits. Alterations in mean arterial blood pressure (MABP) during the experiments are summarized in Fig. 1. A significant rise in MABP was ob-

**TABLE 1**

Physiological variables in 67 rats used for cNOS activity measurements

<table>
<thead>
<tr>
<th>Group (no. of rats) &amp; Timing</th>
<th>MABP (mm Hg)</th>
<th>pH</th>
<th>PaO2 (mm Hg)</th>
<th>PaCO2 (mm Hg)</th>
<th>Glucose (mg/dl)</th>
<th>TMT (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min post-TBI (7)</td>
<td>1 hr pre-TBI</td>
<td>113.0 ± 2.0</td>
<td>7.444 ± 0.007</td>
<td>115.8 ± 3.3</td>
<td>39.4 ± 0.5</td>
<td>145.9 ± 6.9</td>
</tr>
<tr>
<td>5 min post sham op (8)</td>
<td>1 hr preop</td>
<td>114.5 ± 2.6</td>
<td>7.451 ± 0.012</td>
<td>122.5 ± 3.1</td>
<td>39.5 ± 0.5</td>
<td>142.2 ± 11.1</td>
</tr>
<tr>
<td>30 min post-TBI (6)</td>
<td>1 hr pre-TBI</td>
<td>115.0 ± 5.5</td>
<td>7.450 ± 0.006</td>
<td>119.7 ± 4.1</td>
<td>38.3 ± 0.7</td>
<td>132.8 ± 8.6</td>
</tr>
<tr>
<td>30 min post sham op (6)</td>
<td>1 hr preop</td>
<td>117.3 ± 3.5</td>
<td>7.469 ± 0.021</td>
<td>120.8 ± 4.7</td>
<td>38.5 ± 0.4</td>
<td>131.0 ± 13.9</td>
</tr>
<tr>
<td>1 hr post-TBI (7)</td>
<td>1 hr pre-TBI</td>
<td>108.9 ± 2.7</td>
<td>7.495 ± 0.013</td>
<td>115.2 ± 3.5</td>
<td>36.7 ± 0.6</td>
<td>156.7 ± 18.6</td>
</tr>
<tr>
<td>1 hr post-TBI (7)</td>
<td>1 hr post-TBI</td>
<td>108.6 ± 2.8</td>
<td>7.465 ± 0.022</td>
<td>107.5 ± 2.1</td>
<td>41.4 ± 1.5</td>
<td>141.6 ± 13.2</td>
</tr>
<tr>
<td>1 day post sham op (8)</td>
<td>1 hr preop</td>
<td>114.3 ± 3.6</td>
<td>7.447 ± 0.006</td>
<td>121.2 ± 4.4</td>
<td>39.1 ± 0.7</td>
<td>132.4 ± 8.8</td>
</tr>
<tr>
<td>1 hr postop</td>
<td></td>
<td>112.6 ± 4.1</td>
<td>7.441 ± 0.011</td>
<td>118.9 ± 2.2</td>
<td>40.2 ± 0.9</td>
<td>133.7 ± 5.2</td>
</tr>
<tr>
<td>3 days post-TBI (6)</td>
<td>1 hr pre-TBI</td>
<td>114.9 ± 2.3</td>
<td>7.463 ± 0.015</td>
<td>121.6 ± 4.2</td>
<td>36.5 ± 0.6</td>
<td>140.3 ± 15.0</td>
</tr>
<tr>
<td>1 hr post-TBI (7)</td>
<td>1 hr post-TBI</td>
<td>101.4 ± 3.9</td>
<td>7.446 ± 0.013</td>
<td>112.9 ± 3.9</td>
<td>40.2 ± 1.1</td>
<td>128.6 ± 11.6</td>
</tr>
<tr>
<td>3 days post sham op (7)</td>
<td>1 hr preop</td>
<td>106.4 ± 3.7</td>
<td>7.448 ± 0.011</td>
<td>118.8 ± 4.6</td>
<td>39.2 ± 0.9</td>
<td>138.0 ± 8.4</td>
</tr>
<tr>
<td>1 hr postop</td>
<td></td>
<td>112.0 ± 4.2</td>
<td>7.452 ± 0.004</td>
<td>114.9 ± 4.3</td>
<td>39.2 ± 0.4</td>
<td>127.7 ± 12.6</td>
</tr>
<tr>
<td>7 days post-TBI (6)</td>
<td>1 hr pre-TBI</td>
<td>110.3 ± 0.3</td>
<td>7.440 ± 0.015</td>
<td>116.9 ± 3.3</td>
<td>40.1 ± 1.0</td>
<td>134.1 ± 8.0</td>
</tr>
<tr>
<td>1 hr post-TBI (7)</td>
<td>1 hr post-TBI</td>
<td>106.9 ± 2.8</td>
<td>7.434 ± 0.008</td>
<td>108.5 ± 3.3</td>
<td>41.2 ± 0.6</td>
<td>136.9 ± 5.5</td>
</tr>
<tr>
<td>7 days post sham op (6)</td>
<td>1 hr preop</td>
<td>111.3 ± 3.3</td>
<td>7.462 ± 0.010</td>
<td>122.5 ± 3.8</td>
<td>37.6 ± 0.8</td>
<td>126.8 ± 6.1</td>
</tr>
<tr>
<td>1 hr postop</td>
<td></td>
<td>113.0 ± 3.7</td>
<td>7.450 ± 0.006</td>
<td>110.8 ± 3.1</td>
<td>39.1 ± 0.4</td>
<td>132.7 ± 10.0</td>
</tr>
</tbody>
</table>

* Values are expressed as the mean ± standard error of the mean. Abbreviation: TMT = temporal muscle temperature.
served before and 1 hour after TBI in rats pretreated with l-NAME (Fig. 1 left) and at 1 hour after TBI in rats posttreated with l-NAME (p < 0.05, Fig. 1 right).

Constitutive NOS Activity After TBI

The time course of cNOS activity in the injured cortex is illustrated in Fig. 2. Constitutive NOS activity was significantly increased within the ipsilateral cerebral cortex 5 minutes after TBI (mean ± SEM: 234.5 ± 60.2%, compared with that of sham-operated rats, which was 77.3 ± 19.3%; p < 0.05). By 30 minutes, cNOS activity did not significantly differ from that of control animals (114.1 ± 17.4% compared with that of sham-operated rats, which was 81.6 ± 6.1%). However, by 1 day, cNOS activity was significantly reduced compared with that found in control animals (50.5 ± 13.1% compared with that of sham-operated rats, which was 119 ± 17.9%; p < 0.01). This reduction remained for up to 7 days after TBI (at 3 days, 31.8 ± 6% compared with that of sham-operated rats, which was 97.7 ± 21.5%, p < 0.05; at 7 days, 20.1 ± 12.7% compared with that of sham-operated rats, which was 137.6 ± 29.6%; p < 0.01). No further significant reduction was observed at 14 days after TBI (75.9 ± 17.1% compared with that of sham-operated rats, which was 101 ± 7.3%). In sham-operated rats, cNOS activity did not significantly differ during the study period.

Histopathological Outcome

In nontreated animals, a well-demarcated intracerebral contusion overlying the right external capsule was seen in all animals (Fig. 3A and B). In animals pretreated with 7-NI (Fig. 3C and D) or posttreated with L-arginine (Fig. 3E and F), the contusion area appeared smaller than that found in nontreated rats that had TBI.

Figure 4 upper shows contusion volumes in rats after the various drug treatments. In the pretreatment groups, 7-NI, but not l-NAME, produced significant reductions in total contusion volume (mean ± SEM: pre-7-NI–treated group 1.27 ± 0.17 mm³, p < 0.05; pre-l-NAME–treated group 2.13 ± 0.43 mm³) compared with the nontreated TBI group (2.52 ± 0.35 mm³). In contrast, in posttreatment groups, L-arginine significantly reduced the total contusion volume (1.1 ± 0.3 mm³, p < 0.05; Fig. 4 upper). However, there was no obvious protection given by SIN-1 treatment (2.48 ± 0.37 mm³). Furthermore, posttreatment with either NOS inhibitor did not provide significant protection (post-7-NI–treated group 1.87 ± 0.46 mm³; post-l-NAME–treated group 2.18 ± 0.53 mm³).

Figure 4 lower illustrates the rostrocaudal distribution of total contusion areas in each group. Contusion areas were significantly smaller in the pre-7-NI–treated group than in the nontreated TBI group at coronal levels three (bregma −3.3 mm, p < 0.01), four (bregma −4.3 mm, p < 0.01), and five (bregma −5.8 mm, p < 0.05). Post-L-arginine treatment also reduced contusion areas significantly at coronal levels three (bregma −3.3 mm, p < 0.05) and four (bregma −4.3 mm, p < 0.01).

Discussion

Nitric oxide synthase catalytic activity results demonstrate that in this fluid-percussion model of brain injury, Ca²⁺-dependent NOS activity was increased immediately after TBI and returned to baseline by 30 minutes. At 1, 3, and 7 days after TBI, NOS activity was significantly reduced. In cerebral ischemia models, cNOS activity was reported to be significantly increased at 10 and 20 minutes after middle cerebral artery (MCA) occlusion, but dramatically decreased by 1 hour, 10 1 day, and 7 days.55 Saka-
moto, et al., have reported that NO end products are increased markedly at 10 and 20 minutes after cortical contusion in rats. Our NOS activity data after TBI therefore demonstrate patterns of altered NOS activity that are similar to those documented after cerebral ischemia.

Several factors may be considered to increase NOS activity after brain injury. In TBI, excitotoxicity has been implicated in the pathophysiological process by studies in which increased extracellular levels of excitatory amino acids are demonstrated at the trauma site. These factors may include synaptic transmission, neurotransmitter release, and metabolic changes.

FIG. 1. Left: Graph showing alterations in MABP (mean ± SEM) before and after TBI in nontreated (precontrol), pre-L-NAME–treated, and pre-7-NI–treated rats. Right: Graph showing alterations in MABP (mean ± SEM) in nontreated (postcontrol), post-L-NAME–treated, post-7-NI–treated, post-L-arginine–treated, and post-SIN-1–treated rats. Significant differences compared with control rats: *p < 0.05 and **p < 0.01.

FIG. 2. Bar graph depicting the time course of cNOS activity in the injured cortex of animals subjected to TBI or sham operation. Within the ipsilateral cerebral cortex, a significant increase in cNOS activity was observed at 5 minutes; this returned to control value by 30 minutes and was reduced at 1 day after TBI. The reduction remained for up to 7 days. Significant differences compared with sham-operated rats: *p < 0.05 and **p < 0.01.
glutamate elevations would be expected to increase Ca\(^{++}\) influx via NMDA receptor activation.\(^{23,119}\) Such a consequence might lead to cNOS activation because cNOS synthesizes NO only when the [Ca\(^{++}\)] concentration is elevated and calmodulin is bound to the enzyme.\(^{11,13,63}\) However, because an excess concentration of Ca\(^{++}\) was added during the present ex vivo NOS catalytic assays, it is unlikely that the change of Ca\(^{++}\) concentration itself activates NOS activity. Alternatively, Dawson and colleagues\(^{19}\) have proposed that the phosphorylation state regulates NOS catalytic activity. Thus, the increase in [Ca\(^{++}\)] after TBI might activate calcineurin, which may dephosphorylate the inactive phosphorylated cNOS and increase the amount of active dephosphorylated cNOS.

Several possible mechanisms might also be considered to explain the inactivation of cNOS that follows its rapid activation. The increase in extracellular glutamate, which may be considered an initiating factor in the activation of cNOS activity, has been observed to be maximally increased within 10 minutes after TBI. However, glutamate levels rapidly decrease following trauma.\(^{35,41}\) Excess NO has also been reported to inhibit NOS activity, possibly

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**Fig. 3.** Photomicrographs showing paraffin-embedded sections of rat brain stained with H & E 3 days after fluid-percussion brain injury in nontreated (A and B), pre-7-NI–treated (C and D), and post-L-arginine–treated (E and F) rats.  
A, C, and E: Focal contusion (asterisks) present at the gray–white matter interface lateral to the hippocampus. Original magnification × 25.  
B, D, and F: Higher magnification of subcortical contusion (asterisks). Original magnification × 250. The focal contusions appear smaller in the pre-7-NI–treated (C and D) and post-L-arginine–treated (E and F) rats.
Role of NO in traumatic brain injury
by binding of NO to the heme iron of NO synthase.\textsuperscript{56,90,101} Therefore, an increased extracellular glutamate level, \([\text{Ca}^{2+}]\) elevation, eNOS activation, as well as rapid NO production might inactivate NOS. In conditions in which the \([\text{Ca}^{2+}]\) concentration is high, NOS may be phosphorylated by protein kinase C, Ca\(^{2+}\)/calmodulin-dependent protein kinase,\textsuperscript{9} and be inactivated.

Because samples were obtained from structurally damaged sites, damage to neuronal cell bodies could have caused decreased nNOS activity. Brain trauma is also known to lead to early cerebrovascular abnormalities, including increased blood-brain barrier permeability, endothelial lesions, autoregulatory abnormalities, and microvascular obstruction.\textsuperscript{20,47,50,68,92,108,110} Thus, vascular endothelial damage may also have participated in a decrease of eNOS activity.

Recently, L-arginine is required for the formation of NO. Low O\(_2\) tensions have been reported to inhibit the synthesis of NO.\textsuperscript{82,97} Under physiological conditions, tissue PO\(_2\) levels can be significantly lower than those of venous blood.\textsuperscript{109} Most O\(_2\)-dependent enzymes require a higher Michaelis–Menton constant than occurs in most tissues.\textsuperscript{109} Thus, under pathological conditions, O\(_2\)-dependent enzymes may be faced with critical levels of O\(_2\) substrate. Hemodynamic data indicate that local CBF is reduced to 42% of control 30 minutes after moderate parasagittal fluid-percussion brain injury.\textsuperscript{28} Therefore, the reduction of CBF in the traumatic region may reduce the tissue O\(_2\) tension, leading to inactivation of cNOS.

In primary cortical neuronal cultures, glutamate and NMDA neurotoxicity are mediated largely by excess formation of NO.\textsuperscript{22,23} Coexposure of primary cortical neurons to NMDA and NOS inhibitors, flavoprotein inhibitors, calmodulin antagonists or agents that bind calmodulin, and calcineurin inhibitors, all of which decrease NOS catalytic activity, result in neuroprotection against NMDA neurotoxicity.\textsuperscript{19,20,22,23} These data indicate that under pathological conditions nNOS neurons are the key source of neurotoxic NO. The 7-NI, which is a selective nNOS inhibitor, has been shown to be neuroprotective in focal ischemia.\textsuperscript{8,14} The present data indicate that inhibition of nNOS by 7-NI before TBI could account for the decreased contusion volume in our TBI model. However, pretreatment with L-NAME failed to produce significant protection in TBI. Because L-NAME is a nonspecific NOS inhibitor, eNOS inhibition, which would lead to vascular constriction, may delete the neuroprotective effect of nNOS inhibition. Studies in which nNOS knockout mice are used support our speculation. In one study, the resistance of nNOS knockout mice to cerebral ischemia was reversed with the pharmacological inhibition of eNOS.\textsuperscript{52} On the other hand, posttreatment with 7-NI after TBI failed to provide protection. Furthermore, the measurement of NOS activity showed that activation of eNOS occurred within 30 minutes after TBI. Therefore, the post-traumatic period during which the inhibition of nNOS results in histopathological protection may be relatively short.

It has been demonstrated that L-arginine significantly reduces contusion volume. Vascular endothelial cells synthesize NO from L-arginine.\textsuperscript{90} Nitric oxide influences guanyl cyclase activity and increases cyclic guanosine monophosphate levels in smooth muscle, leading to relaxation.\textsuperscript{56,69} In a number of vascular beds L-arginine is a potent vasodilator whereas eNOS is responsible for the conversion of L-arginine into NO. This is based on the observation that L-arginine–induced pial dilation can be blocked by the topical application of L-NAME\textsuperscript{57} and that L-arginine does not readily pass through the blood-brain barrier.\textsuperscript{77} Therefore, intravenous administration of L-arginine may affect the NO production by eNOS but not that by nNOS. Indeed, L-arginine treatment has been reported to increase local CBF and to decrease infarction volume in experimental cerebral ischemia in rats.\textsuperscript{46,76,77} In the midline fluid-percussion model, DeWitt, et al.,\textsuperscript{25} reported that L-arginine treatment prevented posttraumatic hypoperfusion. In a cortical contusion model, Lee and associates\textsuperscript{46} demonstrated that L-arginine treatment prevented the injury-induced reduction of CBF and protected the brain from tissue injury. These results support the hypothesis that L-arginine may have improved histopathological outcome in the present study by improving posttraumatic CBF by means of NO formation through eNOS.

Findings in the L-arginine study could indicate that the early activation of eNOS might reduce the severity of neuronal injury after TBI. This hypothesis is again consistent with data obtained in NOS-deficient mice. Mice deficient in eNOS have larger infarctions compared with those with wild-type NO after MCA.\textsuperscript{51} Thus it is unclear why adding additional substrate to a saturated enzyme system would increase the production of NO.\textsuperscript{73} In this regard, Morikawa and colleagues\textsuperscript{26} have reported that intravenous infusion of 3 mg/kg of L-arginine did not increase blood flow distal to MCA occlusion, but infusion of 30 mg/kg or more of L-arginine did. This fact supports the hypothesis that either NOS remains unsaturated with substrate in pathological states or that L-arginine is compartmentalized in certain cells within the neuraxis.\textsuperscript{76} An alternative possibility is that other potentially vasoactive products of L-arginine may contribute to the increase in CBF after TBI.\textsuperscript{80}

Our data showed a time-dependent decrease in NOS activity 30 minutes after TBI. Therefore, in this model, the therapeutic window for L-arginine treatment may also be limited. Based on a closed head injury model in mice, Mesenge and colleagues\textsuperscript{3} have reported that L-arginine (300 mg/kg) treatment immediately after TBI did not alter the neurological deficit. In models of fluid-percussion brain injury, it is known that TBI produces early reductions in local CBF.\textsuperscript{24,27,122,113} Furthermore, parasagittal fluid-percussion brain injury produces a relatively small contusion.\textsuperscript{26,27,29,30} In contrast, more pronounced structural abnormalities have been described in models of cerebral

\begin{figure}[h]
  \centering
  \includegraphics[width=\textwidth]{figure4.png}
  \caption{Upper: Bar graph showing contusion areas (mean ± SEM) in nontreated (control), pre-L-NAME–treated, pre-7-NI–treated, post-L-NAME–treated, post-7-NI–treated, post-L-arginine–treated, and post-SIN-1–treated rats. Significant difference compared with nontreated rats: * p < 0.05. Lower: Bar graph showing contusion areas (mean ± SEM) in nontreated (TBI only), pre-L-NAME–treated, pre-7-NI–treated, post-L-NAME–treated, post-7-NI–treated, post-L-arginine–treated, and post-SIN-1–treated rats at seven coronal levels. Significant differences compared with nontreated rats: *p < 0.05 and **p < 0.01.}
  \label{fig:figure4}
\end{figure}
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Therefore, the protection afforded by l-arginine may be model specific,44,107 a point that may have important implications in the potential treatment of clinical TBI.

In this study, SIN-1, an NO donor that has been reported to reduce infarction volume by means of increasing CBF in an MCA occlusion model,106 failed to protect the brain from tissue damage after TBI. The synthesis of NO from l-arginine is dependent on NOS activity.60 Our data showed that NOS was activated within damaged tissue compared with the uninjured hemisphere 30 minutes after TBI. Therefore, if NO production from eNOS is substrate dependent77 and if more NO is produced by exogenous l-arginine treatment in histopathologically vulnerable TBI regions, it may lead to selective increases in local CBF in injured brain regions. In contrast, the formation of NO from SIN-1 is independent of NOS activity.237 Thus, SIN-1 administration would be expected to generate NO in both injured and uninjured brain regions and global vasodilation would potentially result in a “steal phenomenon” involving the contusion area. Finally at lower l-arginine concentrations, NOS can produce superoxide.50,111 Nitric oxide synthase generates NO and superoxide simultaneously, leading to peroxynitrite formation that can mediate cellular injury.111 Therefore, exogenous l-arginine treatment may prevent superoxide formation from NOS.

Conclusions

These results indicate that NO plays an important role in the pathophysiological process of moderate parasagittal fluid-percussion brain injury. We demonstrated a temporal response of cNOS activity in histopathologically vulnerable brain regions. In addition, treatment with a selective nNOS inhibitor or NO precursor decreased tissue injury. The NO pathway appears to represent an important target for treatment of TBI.

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