Core and Penumbral Nitric Oxide Synthase Activity During Cerebral Ischemia and Reperfusion

Stephen Ashwal, MD; Beatriz Tone, BA; Hui Rou Tian, MD; Daniel J. Cole, MD; William J. Pearce, PhD

Background and Purpose—The present studies examined the hypothesis that the distribution of cerebral injury after a focal ischemic insult is associated with the regional distribution of nitric oxide synthase (NOS) activity.

Methods—Based on previous studies that certain anatomically well-defined areas are prone to become either core or penumbra after middle cerebral artery occlusion (MCAO), we measured NOS activity in these areas from the right and left hemispheres in a spontaneously hypertensive rat filament model. Four groups were studied: (1) controls (immediate decapitation); (2) 1.5 hours of MCAO with no reperfusion (R0); (3) 1.5 hours of MCAO with 0.5 hour of reperfusion (R0.5); and (4) 1.5 hours of MCAO with 24 hours of reperfusion (R24). Three groups of corresponding isoflurane sham controls were also included: 1.5 (S1.5) or 2 (S2.0) hours of anesthesia and 1.5 hours of anesthesia+24 hours of observation (S24).

Results—Control core NOS activity for combined right and left hemispheres was 129% greater than penumbral NOS activity \( (P < 0.05) \). Combined core NOS activity was also greater \( (P < 0.05) \) in the three sham groups: 208%, 122%, and 161%, respectively. In the three MCAO groups, ischemic and nonischemic core NOS remained higher than penumbral regions \( (P < 0.05) \). However, NOS activity was lower in the ischemic than in the nonischemic core in all three groups: R0 (29% lower), R0.5 (48%), and R24 (86%) \( (P < 0.05) \). Addition of cofactors (10 mmol/L tetrahydrobiopterin, 3 mmol/L flavin adenine dinucleotide, and 3 mmol/L flavin mononucleotide) increased NOS activity in all groups and lessened the decrease in ischemic core and penumbral NOS.

Conclusions—Greater NOS activity in core regions could explain in part the increased vulnerability of that region to ischemia and could theoretically contribute to the progression of the infarct over time. The data also suggest that NOS activity during ischemia and reperfusion could be influenced by the availability of cofactors. (Stroke. 1998;29:1037-1047.)

Key Words: cerebral ischemia, focal cerebrovascular disorders ischemia nitric oxide nitric oxide synthase rats

The neurotoxic effects of NO during focal cerebral ischemia are well recognized.\(^1\)\(^-\)\(^4\) Inhibition of neuronal NOS or use of neuronal NOS knockout models during MCAO reduces penumbral infarct volume with little if any reduction of the infarct core. Regional differences in NOS histochemical staining with the use of NADPH-diaphorase have also shown correlation with the regional vulnerability to ischemic injury.\(^5\) More recently, regional differences in cerebral NOS activity have also been demonstrated, with greatest activity in the cortex, striatum, hippocampus, hypothalamus, amygdala, and substantia nigra.\(^6\)\(^-\)\(^8\) These observations raise the question of whether there are differences in regional NOS activity, specifically in core and penumbral regions, and if so whether this may in part be responsible for the differences in the degree of ischemic vulnerability and subsequent brain injury. Although reduced CBF in core regions during ischemia is the major factor responsible for necrotic injury, other factors, including metabolic rate, capillary density, excitatory amino acid receptor concentration, and possible local regional differences in NOS activity, may also contribute to either the severity or progression of injury.

To investigate this possibility, we have chosen areas of known vulnerability to ischemia (ie, core and penumbra) and measured NOS activity in these regions under control and ischemic conditions. We used a well-established rodent filament model of unilateral proximal MCAO.\(^9\) In this model, the ischemic core first appears in the lateral striatum after approximately 30 minutes (Figure 1) and, as the duration of ischemia increases, spreads to the overlying cortex.\(^9\)\(^,\)\(^10\) The penumbra involves the adjacent ventrolateral neocortex. Core CBF in this model is reduced to near constant values at 20, 60, and 120 minutes of occlusion, averaging approximately...
Selected Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>BH4</td>
<td>tetrahydrobipterin</td>
</tr>
<tr>
<td>CBF</td>
<td>cerebral blood flow</td>
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<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>L-NAME</td>
<td>N\textsuperscript{G}-nitro-l-arginine methyl ester</td>
</tr>
<tr>
<td>MCA</td>
<td>middle cerebral artery</td>
</tr>
<tr>
<td>MCAO</td>
<td>middle cerebral artery occlusion</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>SHR</td>
<td>spontaneously hypertensive rats</td>
</tr>
<tr>
<td>TTC</td>
<td>2,3,5-triphenyltetrazolium chloride</td>
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10% of normal with penumbral flow approximately 15% to 20% of normal.\textsuperscript{8,11} In SHR models of permanent or temporary MCAO, reduction in infarct volume in the range of 31% to 72% has been reported in response to hypothermia, hemodilution, L-NAME, l-arginine, 3-morpholino-sydnonimine, ibuprofen, and tumor necrosis factor-\(\alpha\).\textsuperscript{12–16} Almost all recovery has been observed in (and thus pharmacologically defines) the ischemic penumbra and appears similar in magnitude to that observed in other rodent species.\textsuperscript{17}

Initially we determined NOS activity from regions designated as “core” and “penumbra” in control SHR who were immediately decapitated. Since our earlier studies suggested that the major neurotoxic effects of NOS-derived NO occur early during reperfusion rather than ischemia\textsuperscript{16} and because others have also shown fluctuations in whole brain NOS activity during ischemia and reperfusion,\textsuperscript{18,19} we determined NOS activity in three additional groups: (2) at the end of 1.5 hours of MCAO; (3) after 1.5 hours of MCAO and 0.5 hour of reperfusion; and (4) after 1.5 hours of MCAO and 24 hours of reperfusion. Our purpose was to determine whether differences in NOS activity were present in the ischemic core and penumbra compared with contralateral homologous regions and to determine to what degree this activity changed during ischemia and early or late reperfusion.

Because changes in NOS activity during ischemia or reperfusion could be influenced by NOS cofactor availability,\textsuperscript{20,21} we repeated the NOS assays in separate homogenate samples for core and penumbral regions from both hemispheres after adding the cofactors BH4, FAD, and FMN in concentrations shown to generate maximum activity.\textsuperscript{21} If cofactor addition restored NOS activity to preischemic or near-preischemic levels, then alterations in cofactor concentrations during ischemia or reperfusion may be important in the postischemic regulation of NOS activity.

Materials and Methods

NOS activity was determined in adult SHR with a modified version of the method reported by Breit and Snyder\textsuperscript{22} in core and penumbral regions in four separate series of experiments.\textsuperscript{23} In the first group (n=12), animals were anesthetized and immediately decapitated, and NOS activity was measured from right and left hemisphere brain regions that corresponded to the core (lateral striatum and overlying cortex) and penumbra (adjacent ventrolateral cortex), as described in detail below. The three other groups consisted of SHR undergoing 90 minutes of MCAO and either no reperfusion (R0), 0.5 hour of reperfusion (R0.5), or 24 hours of reperfusion (R24). Additional sham controls were done for the total time period of MCAO and reperfusion for these three groups (S1.5, S2.0, S24), and NOS activity was measured in designated core and penumbral regions to control for anesthetic effects on NOS activity. These studies served as controls for the effects of stress and surgery and also to determine whether inhalation anesthetics such as isoflurane, which have been shown to alter NOS activity, had similar effects in the current experimental protocols.\textsuperscript{22}

Establishment of Reversible Focal Cerebral Ischemia

Male SHR (weight, 325 to 400 g; n=78) were anesthetized with 2.5% isoflurane (Forane) and a 60%/40% air/oxygen mixture. SHR were intubated and mechanically ventilated with a Harvard rodent respirator at 60 breaths per minute and a tidal volume of 10 mL/kg body wt, as previously reported.\textsuperscript{23} End-tidal isoflurane concentration (2.0% to 2.5%) was determined with a Puritan-Bennett Datex Capnomac infrared anesthetic gas analyzer. Catheters were inserted into the femoral artery to monitor mean arterial pressure and for blood sampling and into the femoral vein for fluid administration. Arterial blood gas determinations, blood glucose concentration, and hematocrit were measured throughout the experiments. Pericranial temperature was maintained at 37°C throughout all experimental procedures.

Focal ischemia was accomplished with the use of a filament model of temporary unilateral proximal MCAO, as previously reported by our laboratory.\textsuperscript{11,23} which was based on the earlier studies of Tamura and coinvestigators.\textsuperscript{24} A 3- to 4-cm incision was made in the left cervical region, and the common carotid artery was exposed and covered for 5 to 10 minutes with 0.375% bupivacaine. The external carotid artery and several of its branches were ligated, and a length of suture was loosely passed under the internal carotid artery distal to the bifurcation to control vessel backflow. A bulldog vascular clamp was also placed on the common carotid artery approximately 1 cm proximal to the bifurcation. A nylon filament (No. 4) with a silicone-beaded tip (0.26 mm) was inserted into the external carotid artery and advanced approximately 19 mm or until resistance was felt. The filament was secured with 5–0 silk, the clamp and suture were removed, the area was irrigated with lactated Ringer’s solution, and the skin was closed. Previous studies from our laboratory in the adult SHR after 1.5 hours of occlusion and in the pup SHR after 4 hours have shown a reproducible infarct involving the striatum and overlying cortex.\textsuperscript{15,25}

Depending on the experimental protocol, the rats were killed as follows: (1) anesthetized and immediately decapitated; (2) after 1.5 hours of MCAO (R0); (3) after 1.5 hours of MCAO and 0.5 hour of reperfusion (R0.5); and (4) after 1.5 hours of MCAO, filament removal, and 24 hours of recovery (R24). In the three sham groups (S1.5, S2.0, and S24) that corresponded to the three occlusion/reperfusion experimental groups, all procedures and time periods...
were identical except for lack of filament insertion into the external carotid artery.

**Brain Sectioning for Measurement of NOS Activity and TTC Staining**

In each animal the brain was sectioned into three slices beginning 3 mm from the anterior tip of the frontal lobe (Figure 1). Section 2 (4 mm thick) was used for measurement of NOS activity, and sections 1 and 3 (3 mm thick) were used for estimating the area of mitochondrial ischemic injury.

Regions from the right and left hemispheres of section 2 that corresponded to the ischemic core and penumbra were dissected. We initially identified the midline between the hemispheres by making a longitudinal cut (from top to bottom) approximately 2 mm from the midline through each hemisphere. This was done to avoid mesial hemispheric structures, which are supplied primarily by the anterior cerebral artery. We then made a transverse diagonal cut at approximately the “2 o’clock” position (as shown in Figure 1) to separate the core (ie, striatum and overlying cortex) from the penumbra (adjacent cortex). As previously discussed, designation of these core and penumbral regions was based on pharmacological and histopathological studies by other investigators that defined the core to include subcortical structures, primarily the lateral caudoputamen and overlying cortex, whereas the adjacent ventrolateral cortex was designated as penumbra.2-11,13-17 We made minor modifications of these estimates of core and penumbra based on our earlier investigations in which L-NAMe reduced infarct volume in the SHR.26 In these studies, control infarct volume, determined at 24 hours with TTC staining, measured 29±1% of the left hemisphere and comprised striatal and overlying and anterior cortical regions, whereas in the L-NAMe group, infarct volume was 13±2% and the ischemic core primarily involved the striatum and some overlying cortex.

We used the histochemical stain TTC to verify that an ischemic insult had occurred and used only those animals with definite evidence of ischemia for measurement of NOS activity.16-25,27,28 SHR from the three MCAO groups that did not have evidence of ischemic injury were not included because sufficient control and sham groups were already being studied and we did not want to include animals who may have had incomplete ischemia from insufficient filament occlusion or an atypical cerebrovascular collateral circulation. We included only animals that had a defined area of ischemic injury that measured at least 10% of the hemispheric area. TTC, a mitochondrial stain, may delineate an area of ischemic mitochondrial injury as early as 15 to 20 minutes after occlusion but usually is considered to more accurately reflect ischemic injury at a minimum of 4, but preferably 24, hours of occlusion.24 Within the first 4 to 6 hours after injury, the TTC method more accurately correlates with other histopathological methods that measure infarct area when the stain is perfused.25,26 In the present series only 10% of the remaining [14 C]-arginine with <7% citrulline capture. The eluted volume, containing [14 C]citrulline, was measured by liquid scintillation counting. A separate standard curve was run with each assay to correct for interassay variations in quench and counting efficiency. Samples and standards were run in duplicate, and NOS activity was calculated as picomoles per milligram protein per minute.

**Effect of Cofactors on Core and Penumbral NOS Activity**

Because cofactor levels are generally not saturating, modest changes in cofactor availability may influence postischemic NOS activity. To further evaluate this possibility, we repeated the NOS assays for all regions after adding cofactors to separate homogenate samples. Our previous studies using SHR whole brain homogenates found a 255% increase in NOS activity with the addition of 10 μmol/L BH4, 3 μmol/L FAD, 3 μmol/L FMN, and 264 μmol/L calmodulin.21 In the present series only 10 μmol/L BH4, 3 μmol/L FAD, and 3 μmol/L FMN were added because our previous studies showed a relatively small effect of calmodulin on NOS activity. For each cofactor assay, the sample contained 25 μL of supernatant from a cerebral homogenate, 25 μL (45 pmol) of [14 C]-arginine, and 100 μL of reaction buffer (50 mmol/L HEPES, 1 mmol/L EDTA, 1 mmol/L CaCl2, and 1 mmol/L β-NADPH at pH 7.4). After a 10-minute incubation at 37°C, the reaction was terminated by addition of 2.0 mL of an ice-cold stop solution containing 20 mmol/L HEPES and 2 mmol/L EDTA at pH 5.5. The combined volume was then applied to Poly-Prep chromatography columns preloaded with 1.0 mL AG 50W-X8 resin (NaOH form; BIO-RAD) and rinsed with 2.0 mL distilled water. This preparation trapped ~99.8% of the remaining [14 C]-arginine with ~7% citrulline capture. The eluted volume, containing [14 C]citrulline, was measured by liquid scintillation counting. A separate standard curve was run with each assay to correct for interassay variations in quench and counting efficiency. Samples and standards were run in duplicate, and NOS activity was calculated as picomoles per milligram protein per minute.

**Data Analysis**

NOS activity data for all four experimental groups as well as the additional three sham groups were compared by means of a two-way ANOVA with region and duration of reperfusion as factors. In a separate analysis, we also examined the effect of cofactors on NOS activity for all regions and groups. To normalize for intrinsic differences in maximal NOS activity, cofactor effects were calculated as ratios of NOS activity observed in the absence of cofactors.
Core and Penumbral NOS During Ischemia/Reperfusion

Physiological Variables for Sham and MCAO/Reperfusion Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight, g</th>
<th>MAP, mm Hg</th>
<th>Heart Rate, bpm</th>
<th>pH</th>
<th>Paco₂, mm Hg</th>
<th>PaO₂, mm Hg</th>
<th>Hct</th>
<th>Glucose, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1.5</td>
<td>Baseline</td>
<td>420 (22)</td>
<td>121 (13)</td>
<td>304 (35)</td>
<td>7.42 (0.02)</td>
<td>39 (2)</td>
<td>145 (25)</td>
<td>52 (15)</td>
</tr>
<tr>
<td></td>
<td>Occlusion</td>
<td>116 (8)</td>
<td>330 (17)</td>
<td>7.40 (0.02)</td>
<td>39 (2)</td>
<td>149 (21)</td>
<td>47 (2)</td>
<td>138 (31)</td>
</tr>
<tr>
<td>S2.0</td>
<td>Baseline</td>
<td>405 (15)</td>
<td>143 (64)</td>
<td>314 (40)</td>
<td>7.40 (0.02)</td>
<td>39 (2)</td>
<td>143 (19)</td>
<td>48 (1)</td>
</tr>
<tr>
<td></td>
<td>Occlusion</td>
<td>117 (9)</td>
<td>340 (18)</td>
<td>7.40 (0.02)</td>
<td>38 (1)</td>
<td>135 (23)</td>
<td>49 (1)</td>
<td>132 (12)</td>
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<tr>
<td></td>
<td>Reperfusion</td>
<td>119 (10)</td>
<td>341 (18)</td>
<td>7.39 (0.02)</td>
<td>39 (1)</td>
<td>131 (27)</td>
<td>48 (1)</td>
<td>130 (17)</td>
</tr>
<tr>
<td>S24</td>
<td>Baseline</td>
<td>391 (10)</td>
<td>129 (10)</td>
<td>321 (29)</td>
<td>7.41 (0.03)</td>
<td>39 (3)</td>
<td>160 (18)</td>
<td>46 (1)</td>
</tr>
<tr>
<td></td>
<td>Occlusion</td>
<td>119 (9)</td>
<td>330 (21)</td>
<td>7.39 (0.02)</td>
<td>40 (2)</td>
<td>150 (17)</td>
<td>47 (1)</td>
<td>140 (24)</td>
</tr>
<tr>
<td>R0</td>
<td>Baseline</td>
<td>404 (21)</td>
<td>125 (10)</td>
<td>317 (16)</td>
<td>7.41 (0.02)</td>
<td>39 (2)</td>
<td>156 (19)</td>
<td>47 (1)</td>
</tr>
<tr>
<td></td>
<td>Occlusion</td>
<td>120 (5)</td>
<td>347 (20)</td>
<td>7.39 (0.02)</td>
<td>39 (2)</td>
<td>147 (7)</td>
<td>48 (1)</td>
<td>144 (21)</td>
</tr>
<tr>
<td>R0.5</td>
<td>Baseline</td>
<td>362 (30)</td>
<td>120 (16)</td>
<td>316 (31)</td>
<td>7.41 (0.04)</td>
<td>39.1 (3)</td>
<td>140 (27)</td>
<td>47 (2)</td>
</tr>
<tr>
<td></td>
<td>Occlusion</td>
<td>116 (10)</td>
<td>364 (18)</td>
<td>7.41 (0.02)</td>
<td>38 (1)</td>
<td>138 (21)</td>
<td>53 (14)</td>
<td>143 (24)</td>
</tr>
<tr>
<td></td>
<td>Reperfusion</td>
<td>111 (14)</td>
<td>345 (17)</td>
<td>7.40 (0.01)</td>
<td>38 (2)</td>
<td>147 (23)</td>
<td>44 (15)</td>
<td>192 (187)</td>
</tr>
<tr>
<td>R24</td>
<td>Baseline</td>
<td>395 (34)</td>
<td>120 (10)</td>
<td>308 (42)</td>
<td>7.41 (0.02)</td>
<td>37 (1)</td>
<td>158 (11)</td>
<td>46 (1)</td>
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<td>49 (10)</td>
<td>133 (17)</td>
</tr>
</tbody>
</table>

MAP indicates mean arterial pressure; bpm, beats per minute; and Hct, hematocrit.
Data are mean values for each variable for the indicated time period. Numbers in parentheses are ±1 SD. Scalp temperature was maintained at 37°C in all groups when the animals were anesthetized. The three sham groups (S1.5, S2.0, S24) were subjected to 1.5, 2.0, and 1.5 hours of isoflurane anesthesia. The S24 group was allowed to recover for 24 hours. The three MCAO groups (R0, R0.5, R24) were subjected to similar time periods of isoflurane.

Results
Physiological data (mean±1 SD) for all experimental groups are summarized in the Table. Data were obtained during baseline and occlusion in all groups and at the end of reperfusion in the R0 and R0.5 groups. For the two 24-hour groups (S24 and R24), physiological data at the end of reperfusion were not acquired because these animals no longer had catheters in place. There were no significant differences in any of the variables when any of the three sham groups or the three MCAO groups were compared.

Core and Penumbral NOS Activity in Control SHR
NOS activity was greater in the left (120%) and right (139%) core regions than in corresponding penumbral regions in the control group (n=12; P<0.05; Figure 2, top panel). Measurements of NOS activity (picomoles per milligram per minute) were as follows: left core, 1392±142; left penumbra, 634±85; right core, 1450±198; and right penumbra, 606±72. Significant differences were not found when we compared left versus right core or left versus right penumbra.

Combined (right+left) core NOS (1421±120) was 129% greater than combined penumbral NOS (620±54) (P<0.05).

Core and Penumbral NOS Activity in Isoflurane Sham Groups
Right and left hemisphere core NOS activity in each of the three sham groups was greater than the corresponding penumbral NOS activity (P<0.05; Figure 2, top panel). Differences were not observed when we compared left versus right core or left versus right penumbra in any of the three sham groups. Combined core activity in each of the three sham groups was significantly higher than combined penumbral activity: S1.5 (208%; n=8), S2.0 (122%; n=10), and S24 (161%; n=10) (P<0.05). Thus, as in the control groups, there were no side-to-side differences in NOS activity for either the core or penumbral regions in any of the sham groups, and NOS activity was consistently greater in the core than in the penumbral regions.

Compared with controls, core and penumbral NOS activity was little affected by administration of 2.5% isoflurane for 1.5 hours (S1.5) or 2 hours (S2.0). However, core and penumbral NOS activity was significantly increased in the group in which isoflurane was administered for 1.5 hours and the animals maintained for 24 hours (S24) compared with the control and other two sham groups (P<0.05). For example, NOS activity in the S24 group was higher in each brain region than in the S2.0 group as follows: left core (76%), left right hemisphere core NOS activity in each of the three sham groups was greater than the corresponding penumbral NOS activity (P<0.05; Figure 2, top panel). Differences were not observed when we compared left versus right core or left versus right penumbra in any of the three sham groups. Combined core activity in each of the three sham groups was significantly higher than combined penumbral activity: S1.5 (208%; n=8), S2.0 (122%; n=10), and S24 (161%; n=10) (P<0.05). Thus, as in the control groups, there were no side-to-side differences in NOS activity for either the core or penumbral regions in any of the sham groups, and NOS activity was consistently greater in the core than in the penumbral regions.

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Percent Area of Ischemic Injury

The percent area of ischemic injury measured $24\pm 2\%$ after 1.5 hours of occlusion (R0 group; n = 13), increased to $28\pm 3\%$ after 0.5 hour of reperfusion (R0.5 group; n = 12) and by 24 hours increased to $40\pm 2\%$ (R24 group; n = 13; $P<0.05$ compared with the other two groups). At the end of occlusion (Figure 3), this area of TTC pallor was located in the lateral caudoputamen and cortex and by 0.5 hour of reperfusion had extended more cortically and was more easily differentiated from nonischemic brain. By 24 hours the ischemic hemisphere was edematous, and the area of injury had continued to extend to adjacent regions, as shown.

Core and Penumbral NOS Activity During MCAO/Reperfusion

Core Versus Penumbral NOS

Both ischemic (left) and nonischemic (right) core NOS activity remained higher than corresponding penumbral regions at all times (Figure 4, top panel; $P<0.05$). These differences, expressed as the percentage that core NOS activity was greater than penumbra $[(\text{Core}−\text{Penumbra})/\text{Penumbra} \times 100]$, averaged 138%, 82%, and 156% in the ischemic hemisphere and 131%, 203%, and 92% in the nonischemic hemisphere at the end of occlusion (R0; n = 13), 30 minutes of reperfusion (R0.5; n = 12), and at 24 hours (R24; n = 13), respectively. As shown in Figure 4, even though the overall trend was for NOS activity to decrease in the ischemic hemisphere and to increase in the nonischemic hemisphere, the relation between core and penumbral NOS activity remained relatively constant.

Ischemic (Left) Versus Nonischemic (Right) Hemisphere

Core and penumbral NOS activity was consistently lower in the ischemic hemisphere than the nonischemic hemisphere at all times (Figure 4, top panel; $P<0.05$). In core regions, NOS activity was 29%, 48%, and 86% lower in the ischemic than the nonischemic hemisphere in the R0, R0.5, and R24 groups. Similar differences in penumbral NOS activity for the same groups were also observed, ie, 31%, 13%, and 90% lower in the ischemic versus nonischemic hemisphere.

Effects of Cofactor Addition on NOS Activity

Controls

Addition of cofactors to separate homogenate samples from the control group increased NOS activity in all brain regions (Figure 2, bottom panel). The percent increase in NOS activity between the “without” cofactors and “with” cofactors
groups were as follows: left core, 258%; left penumbra, 210%; right core, 285%; and right penumbra, 229% (P<0.05). For all regions combined, the average was 256%.

There were no significant differences in the percent increase in NOS activity between core and penumbra or between ipsilateral and contralateral homologous regions. NOS activity after cofactor addition remained significantly higher in core than in penumbral regions in both hemispheres (P<0.05).

To normalize for differences in NOS concentration, we used the NOS activities observed in the absence of added cofactors and compared them with the NOS activities present with saturating levels of cofactors (without/with ratio). This approach eliminated differences between samples due to differences in NOS concentration and expressed fractional activation as a function of endogenous cofactor concentrations. As shown in the top panel of Figure 5, the fractional activation of NOS supported by endogenous levels of cofactors did not vary significantly with region or side at any time in the sham groups. This finding suggests that the increase in total activity observed at 24 hours in the sham animals relative to controls (Figure 2, bottom panel) cannot be attributed to differences in cofactor availability and must instead be due to differences in NOS concentration and/or enzyme specific activity induced by isoflurane.

**Ischemic Groups**

In all groups and at all time points (Figure 4, bottom panel), addition of cofactors increased NOS activity significantly (P<0.05). For the four brain regions combined, the increase in NOS activity in the three MCAO groups averaged 434% (R0), 431% (R0.5), and 695% (R24). The effect of cofactor addition in these groups was significantly greater than the overall increase seen in the control group (256%) (P<0.05).

However, as shown in the bottom panel of Figure 5, the fractional activation of NOS supported by endogenous levels of cofactors did not vary significantly with region or side after 0 or 30 minutes of reperfusion but did vary significantly after 24 hours of reperfusion. In particular, after 24 hours of reperfusion values for fractional activation of NOS were not significantly different than control values on the noninfarcted right side but were dramatically depressed in both the core and penumbral regions of the infarcted left side. These findings indicate that differences in cofactor availability probably do not contribute to differences in total NOS activity at the end of occlusion or early during reperfusion but may be particularly important in infarcted regions after 24 hours of reperfusion.

**Discussion**

The concept of the ischemic penumbra was originally introduced to define brain regions in which blood flow was insufficient to sustain cellular electrical activity but above that required to maintain ionic gradients.
tion that progressively increases cell injury.\textsuperscript{30–33} As yet, events leading to infarct maturation remain incompletely understood.\textsuperscript{34}

The role of NO as it relates to the evolution of core and penumbral injury is complex, and an integrated model that takes into account its neuroprotective and neurotoxic roles has yet to be fully delineated.\textsuperscript{1–4} Protective effects due to enhancement of endothelial NOS synthesis and NO production at the vascular level resulting in increased CBF have been demonstrated.\textsuperscript{1,2,35,36} Likewise, neurotoxic effects due to neuronal NOS upregulation and increased neuronal NO release have also been shown.\textsuperscript{1,2,37}

**Core and Penumbral NOS Activity**

The principal finding of the present study, that NOS activity is greater in anatomically defined core than in penumbral regions, may explain in part why the ischemic core is more vulnerable to ischemic injury than the penumbra. Core NOS activity was 129\% higher in controls and 208\%, 122\%, and 161\% higher in the three sham groups. The higher NOS activity observed in core compared with penumbral regions was thus not due to ischemic effects because it was consistently observed in the control and sham groups. Likewise, core NOS activity during ischemia and at various stages of reperfusion remained higher than penumbra in both the ischemic and nonischemic hemispheres. While it is clear that the principal reason for core necrotic injury during MCAO relates to the severity and duration of reduced CBF, other factors appear to contribute to the cascade of injury that occurs during ischemia and reperfusion.\textsuperscript{29,30} Our earlier studies in a rat model of transient MCAO suggested that the principal neurotoxic effects of NO occur early during reperfusion, whereas during ischemia vascular NO might act to ameliorate ischemic injury by maintaining CBF.\textsuperscript{16} Thus, the observations of the present study make the important suggestion that higher core NOS activity may be an additional critical factor early during reperfusion that accounts for the preferential vulnerability of “core” regions compared with “penumbral” regions. Recent data in a rat model of MCAO, showing that L-NAME, an inhibitor of NOS, reduced necrotic cell injury in the ischemic core but not apoptotic cell death in the penumbra, support these observations.\textsuperscript{38} However, it is also important to recognize that the present study examined NOS activity ex vivo and that this only defines the potential for NO production and is not necessarily a reliable index of actual NO output. Additional studies either directly measuring core and penumbral NO production during ischemia in vivo (eg, microdialysis) or using cultured cell or brain slice techniques in conjunction with NOS immunocytochemical studies to determine the number of NOS neurons, such as in the study of Zhang et al,\textsuperscript{37} would be of importance.

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**Figure 4.** Core and penumbral NOS activity in control (n=12), R0 (n=13), R0.5 (n=12), and R24 (n=13) groups (see “Materials and Methods”). Values for both ischemic (left [L-PEN]) and nonischemic (right [R-PEN]) penumbral NOS activity were lower than those for corresponding core regions at all times (\(P<0.05\)). NOS activity was lower in the ischemic core (left [L-CORE]) than in the nonischemic core (right [R-CORE]) in all three MCAO groups (\(P<0.05\)). NOS activity in the ischemic core increased 18\% from control (CTL) by 1.5 hours of MCAO and then decreased significantly by 0.5 hour (31\%) and 24 hours (78\%) of reperfusion (\(P<0.05\)). Corresponding NOS activity in the nonischemic core increased 60\%, 27\%, and 51\% at these times compared with controls. Left penumbral and right penumbral NOS activity decreased by 0.5 hour of reperfusion, but by 24 hours left penumbral NOS continued to decrease, whereas right penumbral NOS increased above control values (+). Addition of cofactors increased NOS activity in all regions (\(P<0.05\)) and at all times after ischemia (\(P<0.05\)).
Greater NOS activity in the ischemic core may also explain why the ischemic core expands over time in the absence of progressively worsening CBF. In the rat filament model of temporary MCAO, core CBF (lateral caudoputamen and overlying cortex) during ischemia decreases to approximately 10% of normal and penumbra flow (adjacent neocortex) to 15% to 20% of normal.9,10 By most criteria, such penumbral flow values should not cause infarction unless associated with some other metabolic event.17,29–34 Also, in models of temporary MCAO, core and penumbral CBF are rapidly restored early during recirculation.39,40 Earlier studies have also shown that NOS-containing neurons are relatively resistant to hypoxic or ischemic injury and that the neurotoxic effects of NO are believed to be due to diffusion of NO to adjacent susceptible neurons.2–5 Thus, higher core NOS concentrations could generate an NO gradient particularly near the core-penumbra interface, where greater amounts of neuronally derived NO could be released, diffuse to adjacent vulnerable penumbral neurons, and over time bring about extension of the infarct. Additional evidence supporting this possibility can be found in recent MCAO studies that used diffusion-weighted MRI in wild-type versus neuronal NOS–deficient mice.21 The neuronal NOS–deficient mice had smaller infarct volumes and smaller peri-infarct (ie, penumbral) zones, which were believed to be due to reduced NO production.

Several methodological issues need to be considered. The first concerns the nature of the NOS isoform that we were measuring. It is likely that we were primarily measuring the neuronal isoform.8,21 Earlier studies have shown that inducible NOS is calcium independent and that the time course of inducible NOS upregulation peaks 2 days after MCAO.42 In the present study all NOS activity that we measured was calcium dependent, and we only examined NOS levels within the first 24 hours after ischemia. It is also unlikely that endothelial NOS activity was significant because our studies used only cleared homogenate supernatants that have been reported to contain little endothelial NOS activity.43,44

Another issue relates to the extent of regional cerebral differences in NOS activity and whether there is any rationale for the marked differences that we observed between core and penumbra. Temporal profiles of neuronal NOS mRNA after permanent MCAO have shown upregulation as early as 15 minutes after occlusion.37 More importantly, the pattern of increased immunoreactivity corresponded to the ischemic core. These findings are supported by direct and indirect measurements of NOS activity. Indirect assays for NO synthesis in rat brain have shown very high activity in the cerebellum as well as robust striatal (caudoputamen) and cortical activity.45,46 Direct regional NOS assays also have demonstrated greater activity in the cortex and up to 50% higher striatal NOS activity than other brain regions.7,47 In addition, other regions within the rat MCA distribution (hypothalamus, amygdala, and nucleus accumbens)48 also have greater NOS concentrations than cortical structures.7 Overall, the available data suggest that brain regions supplied by the MCA that form the ischemic core have greater NOS activity than the penumbra.

A related concern is the certainty of our assignment of core and penumbral regions during dissection. Use of a novel “sandwich” technique allowed us to measure NOS activity in areas predesignated as core and penumbra under control, sham, and ischemic conditions as well as to determine the degree of ischemic injury after MCAO with the use of TTC staining. In our control and sham groups, MCAO was not performed, and the dissection of the brain into core and penumbral regions was based on earlier pharmacological and histopathologic studies. Primarily on the basis of studies of pharmacological rescue, it is clear that in the SHR a definable penumbra exists,16 and as shown in our TTC-stained prepa-

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**Figure 5. Effects of cofactors on NOS activity.** Data are presented as the log transformation of the ratios of NOS activity observed in the absence of cofactors relative to that observed after cofactor addition. The top panel shows the ratio data by region for the control (CTL) and sham groups (see "Materials and Methods") and reflects the fractional activation of NOS supported by endogenous levels of cofactors. No significant differences were observed when we compared the core versus penumbral regions or left versus right hemispheres. The bottom panel (control and MCAO groups) shows no differences at the end of occlusion (R0) or early during reperfusion (R0.5). However, after 24 hours of reperfusion, values of fractional activation of NOS were dramatically depressed in both the core and penumbral regions of the infarcted left side compared with similar regions from the right hemisphere (*) or with similar regions from the left hemisphere of the sham controls at 24 hours (+). Abbreviations are as in Figure 4.
rations, the area of ischemic mitochondrial injury clearly progressed over the three time periods included in the present study.

In the ischemic groups, TTC staining was used to substantiate the presence of ischemic injury before inclusion for core and penumbral NOS assays. Our measurements of TTC injury are similar to those reported previously by other investigators who used both immersion and perfusion techniques. Our data demonstrate a progression of the area of ischemic injury with time that reflected the maturation of the ischemic injury into an irreversible infarct. However, because of the limitations of the TTC (or any) method for accurate measurement of infarct volume within the first hours after occlusion, we did not attempt to compare the relation between infarct volume and core or penumbral NOS activity.

Another issue relates to substrate availability. The marked pathological demarcation between core and penumbra observed in many experimental studies has been striking to many observers. Because NO synthesis relies on substrate availability, it is the core-penumbral interface that theoretically would have relatively higher concentrations of oxygen, arginine, NADPH, and other necessary cofactors. Thus, higher NOS concentrations in the core, coupled with elevated pericore substrate and cofactor availability, could create a “leading edge” of NO formation and, depending on local CBF and perfusion pressure, could have neurotoxic or neuroprotective effects. The striking increases in NOS activity observed with cofactor addition in all regions at all times support this possibility.

Effects of Cofactor Addition on NOS Activity

Not unexpectedly, our data demonstrate that cofactor addition increased NOS activity in all groups. The preservation of the hierarchy of core over penumbra, even after cofactor addition, lends additional support to the notion that intrinsic differences in the concentration of the NOS enzyme or its specific activity are present in these two regions. To control for the effects of differences in NOS concentration or specific activity, we normalized NOS activity values relative to the maximum values observed in the presence of saturating concentrations of cofactors and thus quantified the fractional activation of NOS supported by endogenous cofactor concentrations. This analysis revealed that the fractional activation of NOS supported by endogenous cofactor concentrations is relatively constant at all time points and regions in sham animals, as well as at the end of occlusion and early during reperfusion in ischemic animals. Thus, at these times differences in total NOS activity can be attributed only to differences in NOS concentration or enzyme specific activity. In contrast, after 24 hours of reperfusion, the fractional activation of NOS supported by endogenous cofactor concentrations was dramatically depressed, indicating that cofactor availability is a major determinant of NOS activity in post-ischemic infarct regions. Additional studies, measuring FAD, FMN, and particularly BH4, which is uniquely necessary for NOS, are needed to clarify exactly which of these cofactors are most limited in availability.

Core and Penumbral NOS Activity in Isoflurane Sham Groups

Earlier in vitro investigations have shown either an inhibitory effect (up to 85%) or no effect of isoflurane and other volatile anesthetics on brain NOS activity. However, in the studies that used rat brain homogenates, isoflurane was added to the incubation mixture rather than given in vivo to the intact animal. In the present study (Figure 2, top panel) we did not find evidence of inhibition of NOS using a sham protocol when isoflurane was given for 1.5 or 2.0 hours. Surprisingly, after cofactor addition, significant increases in NOS activity were observed in the 1.5-hour and 24-hour sham groups. These findings, as well as others in the literature, suggest that certain anesthetics might augment as well as inhibit NOS synthesis under different experimental conditions and point out the need to include appropriate control groups.

Conclusions

The observation that NOS activity is greater in core regions than in penumbra in control, sham, and ischemia/reperfusion groups suggests an additional explanation for the vulnerability of the core to ischemic and reperfusion injury. Higher core NOS activity may also contribute to infarct maturation. In addition, the effects of cofactors on restoring NOS activity in both core and penumbral regions suggest that regulation of cofactors could provide an alternative approach to the treatment of stroke.

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25. Hatfield RH, Mendelow AD, Perry RH, Alvarez LM, Modha P. Triphe-
The brain produces relatively large amounts of nitric oxide under normal conditions. The two sources of this constitutively produced NO are endothelial and neuronal isofoms of NO synthase (eNOS and nNOS, respectively).

The role of NO in cerebral ischemia is complex, with the potential for both protective and detrimental effects. NO produced by eNOS appears to play a protective role during and after cerebral ischemia. Potential protective effects include vasodilatation, inhibition of aggregation of platelets, and inhibition of expression of redox-sensitive genes such as vascular cell adhesion molecule-1 (an endothelial-leukocyte adhesion molecule).2

In contrast, nNOS appears to contribute to injury following ischemia. Cytotoxic effects of NO may be mediated by the reaction of NO with superoxide anion, resulting in the formation of peroxynitrite, a potent oxidant that can nitrosylate proteins and damage DNA.1,2

The production of NO and L-citrulline from L-arginine by NOSs requires the presence of L-arginine (the substrate), molecular oxygen, NADPH, tetrahydrobiopterin, FAD, and FMN. Interestingly, NOSs can generate superoxide and hydrogen peroxide in the absence of adequate levels of L-arginine or tetrahydrobiopterin.1,5

The present study suggests that cerebral ischemia reduced activity of NOSs (measured as the conversion of L-arginine to L-citrulline in brain homogenates in vitro) in both the ischemic core and penumbra of chronically hypertensive rats. The addition of enzyme cofactors (tetrahydrobiopterin, FAD, FMN) to the assay significantly increased activity of NOS in both brain regions, particularly after 24 hours of reperfusion. Thus, the availability of enzyme cofactors may be limiting for production of NO. The authors speculate that higher levels of NOS activity in the ischemic core may contribute to increased vulnerability of this region and suggest that regulation of availability of NOS enzyme cofactors may be an alternative therapy to stroke.

Although the suggestion of regulation of intracellular levels of NOS cofactors is attractive initially, the effect of manipulation of these cofactors on brain injury after ischemia is difficult to predict. If cofactor availability is restricted, nNOS may produce more superoxide, which could be detrimental. Alternatively, if cofactor availability is increased, nNOS may produce more NO, which could be protective under some conditions (by inactivation of superoxide by NO if subsequent degradation products are nontoxic) but might also be maladaptive, since nNOS is generally thought to contribute to injury following ischemia.1,3 In addition, enzyme cofactors could have additional effects unrelated to NOS. For example, high concentrations of exogenous tetrahydrobiopterin can produce superoxide directly through auto-oxidation.6

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Core and Penumbral Nitric Oxide Synthase Activity During Cerebral Ischemia and Reperfusion
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