Transient Focal Ischemia Increases Endothelial Nitric Oxide Synthase in Cerebral Blood Vessels

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Background and Purpose—Production of NO by endothelial NO synthase (eNOS) plays a protective role in cerebral ischemia. We studied the effects of transient focal ischemia on eNOS expression.

Methods—Wistar rats (n=72) underwent reversible filament occlusion of the right middle cerebral artery for 75 minutes. After 6, 24, 72, or 168 hours of reperfusion, brains were removed and coronal sections cut for eNOS immunohistochemistry, eNOS–alkaline phosphatase costaining, and hematoxylin-eosin staining. Samples for eNOS immunoblots were taken from corresponding striatum and overlying parietal cortex bilaterally.

Results—eNOS protein occurred in virtually all blood vessels and was consistently increased in microvessels in the ischemic striatum after 24 to 168 hours of reperfusion but not at 6 hours. eNOS upregulation in the parietal cortex was only present in animals with evidence of cortical infarcts documented on adjacent HE-stained sections. Costaining of endogenous alkaline phosphatase and eNOS demonstrated eNOS expression in all segments of cerebral microvessels. Quantitative analysis of eNOS immunostaining and immunoblots showed no attenuated increase in animals that were treated with indomethacin (5 mg/kg IP), NS398 (20 mg/kg IP), or L-arginine-methyl ester (10 mg/kg IP). In contrast to eNOS, levels of brain NOS did not increase after ischemia.

Conclusion—eNOS protein is upregulated in pre- and postcapillary microvessels and upregulation appears slower after transient compared with permanent ischemia. Cyclooxygenase and NOS products do not play a major role in postischemic eNOS induction. (Stroke. 2002;33:2704-2710.)

Key Words: cerebral ischemia ■ gene expression ■ nitric oxide ■ reperfusion ■ rats
in cerebral blood vessels after global ischemia/reperfusion in piglets and that COX products may affect eNOS expression. However, we have only investigated this interaction in the early postischemic period.

In the present study, we therefore investigated (1) the time course of eNOS expression after 75 minutes of middle cerebral artery occlusion using complementary immunostaining and immunoblotting techniques, (2) the localization of eNOS protein in the cerebral microvasculature after focal ischemia, (3) the distribution of increased eNOS in relation to ischemia-induced histologic damage, (4) the effect of COX or NOS inhibition on postischemic eNOS expression, and (5) the effect of ischemia on nNOS.

**Materials and Methods**

**Surgical Preparation**

All experiments were performed on male Wistar rats (n=72; Harlan Sprague-Dawley, Inc) weighing 280 to 325 g. All experimental procedures were approved by the Institutional Animal Care and Use Committee. Focal cerebral ischemia was induced using the filament model introduced by Koizumi et al23 with some modifications as previously described. Briefly, after anesthesia was induced with 5% halothane in O2, the trachea was intubated and the lungs were mechanically ventilated. Halothane was reduced to 0.5% to 1.2% in 70% N2/30% O2. A temperature probe was inserted beneath the temporalis muscle. During surgery, ischemia, and the early reperfusion period, pericranial temperature was held between 37.0°C and 38.0°C. The tail artery was cannulated for blood pressure recording and to provide samples for glucose and blood gas measurements. The major branches of the external carotid artery (ECA) were ligated and cut. The ECA was cut between 2 ligations leaving a stump attached to the common carotid artery. A suture loop was put around the proximal end of the ECA stump. A 0.25-mm-diameter nylon monofilament with a rounded tip coated with poly-L-lysine (Sigma) was introduced via the ECA stump into the proximal internal carotid artery and advanced until a resistance was felt ~20 mm from the carotid bifurcation. The filament was held in place by tightening the loop around the proximal ECA stump. After closure of the neck, anesthesia was discontinued and animals were ventilated with 100% O2. After awakening, rats were extubated and a brief behavioral assessment was performed ~10 minutes after introduction of the filament. In rats without left forelimb paresis, the occlusion of the MCA was regarded as unsuccessful, and these animals were excluded from further study. Rats with left forelimb paresis were rapidly transferred into a chamber, which allowed continuous arterial blood pressure and pericranial temperature measurement. Animals were briefly reanesthetized with halothane, and the filament was removed 75 minutes after introduction. The pericranial temperature probe and the arterial line were removed, and the surgical wounds were closed. Animals were awakened in an oxygen-enriched environment and subsequently transferred to their cages. Rectal temperature was measured 4 and 24 hours after ischemia. Additional sham-operated animals (n=3) underwent identical procedures except for only brief introduction of the filament into the ECA stump.

**Time Course of eNOS Expression**

Animals were anesthetized with halothane 5% in O2, perfused with ice-cold saline, and then decapitated at 6 hours (n=6), 24 hours (n=10), 72 hours (n=6), or 168 hours (n=6) after ischemia. Brains were rapidly removed and cut coronally at the level of the optic chiasm. The anterior part of the brain was immersed in 10% formalin and then embedded in paraffin. Coronal sections of 8-μm thickness were cut for hematoxylin-eosin (HE) staining and immunohistochemistry (see below). The adjacent posterior part of the brain was used for eNOS immunoblots. Samples for immunoblots were taken from corresponding striatum and overlying parietal cortex bilaterally, frozen in isopentane, and stored at −80°C.

**Immunohistochemistry**

Paraffin-embedded sections were hydrated and washed in 10 mmol/L PBS. Slides were incubated in 10% normal goat serum (NGS; Vector Laboratories) in 10 mmol/L PBS for 120 minutes before incubation with monoclonal eNOS antibody (Transduction Laboratories) overnight. After rinsing the slides in NGS, endogenous peroxidase was blocked using 3% H2O2, 10% methanol in PBS for 30 minutes. After washing, sections were incubated for 2 hours in biotinylated goat anti-mouse IgG (Vector Laboratories), which was diluted 1:1000 in 2% NGS. Subsequently, sections were washed and ABC Reagent (Vector Laboratories) was added for 30 minutes. Finally, diaminobenzidine (DAB) was added for 5 minutes, and slides were dehydrated, dried, and covered with a coverslip.

**Quantitative Analysis of Immunostaining**

eNOS-stained sections were digitized using a camera mounted on a BH2 microscope (Olympus) and the NIH image analysis system (NIH Image, version 1.60). To quantify side differences, 3 corresponding visual fields in the striatum were acquired as a grayscale image for each side. We primarily quantified eNOS expression in the striatum because it is the most consistently infarcted area in this model (ie, 75 minutes of reversible MCA occlusion) and because eNOS expression was most prominent inside the infarct borders (see below). A mosaic of these 6 visual fields was assembled for each animal (Figure 1). To account for differences in staining intensity, the mosaic was adjusted using the same threshold for both hemispheres. The semiquantitative parameter “area above threshold,”
which reflects both extent and density of eNOS staining, was calculated for each side (Figure 1).

**eNOS Immunohistochemistry/HE Staining**
Sections adjacent to eNOS-stained sections were stained with HE to detect infarcted tissue or selective cellular damage. Areas of infarct were superimposed on thresholded and contrast-enhanced images of eNOS staining. To ensure sufficient resolution for this purpose, eNOS-stained sections were acquired in a mosaic pattern using the semiautomatic Axioplan Z microscope (Zeiss) and KS 4000 image acquisition software (Zeiss). Percentages of eNOS staining within and outside the infarcted area of the ischemic hemisphere were calculated for a subset of animals.

**Immunohistochemistry: Alkaline Phosphatase/eNOS Double Staining**
In additional rats (n=5), eNOS–alkaline phosphatase costaining was performed to examine the distribution of eNOS staining in various cerebral microvessels. The brain was rapidly removed after 24 hours of reperfusion, frozen in methylbutane at −80°C, and stored at −60°C. Coronal sections were taken at a thickness of 50 µm with a cryotome and desiccated in a cold room overnight. For endogenous alkaline phosphatase staining, the sections were washed in PBS for 5 minutes. The sections were incubated in nitroblue tetrazolium/BCIP in Tris-HCl for 5 minutes and then washed again with PBS. Stained sections were lightly postfixed in 4% formalin for 10 minutes. eNOS immunostaining was performed as described above except for the concentration of eNOS primary antibody (1:750). Finally, double-stained sections were dehydrated, dried, and covered with a glass coverslip.

**Western Analysis**
Protein was extracted from frozen whole-brain tissue (see above) in boiling lysis buffer (1% 1 mol/L Tris and 1% SDS). The samples were sonicated, heated at 95°C for 5 minutes, and centrifuged for 5 minutes at 12 000 rpm at 4°C. An aliquot of the supernatant was removed for protein concentration determination.

**Statistical Analysis**
All values are expressed as mean±SD. For comparison of pixels above threshold in immunostained sections and optical density in immunoblots among groups, 1-way ANOVA was used. When indicated by a significant F ratio, post hoc between-group testing was performed using the Student-Newman-Keuls test using the SigmaStat and SigmaPlot software (Jandel Scientific). A probability value <0.05 was regarded as statistically significant.

**Results**
Under optimal conditions, virtually all blood vessels of various sizes were immunopositive for eNOS. eNOS protein immunostaining was consistently increased in the ischemic striatum compared with the nonischemic side after 24 to 168 hours of reperfusion (Figures 1 and 2). This increase appeared in blood vessels of all sizes, including relatively large arteries as well as arterioles and veins. In contrast, increased eNOS expression on the ischemic side was only detected in half of the animals euthanized 6 hours after ischemia, and side differences of immunostaining were not very prominent in most of these animals (Figure 2). Importantly, eNOS Western blots performed on subcortical tissue samples taken 24 hours after ischemia confirmed consistently enhanced eNOS expression in the ischemic striatum (Figure 3). Even though there is variation among different animals, eNOS protein

![Figure 2. Time course of eNOS immunoreactivity in ischemic (I) and nonischemic (N) striatum after 75 minutes of ischemia. Immunostaining was quantified by calculation of area above threshold using thresholded grayscale images of corresponding immunostained section from both hemispheres (see text for details). Significant differences between hemispheres were observed at 24 (n=10), 72 (n=6), and 168 (n=6) hours, but not at 6 hours (n=6) after reperfusion (*P<0.05).](image)

![Figure 3. Striatal eNOS levels 24 hours after middle cerebral artery occlusion (MCAO) on ischemic (I) and nonischemic (N) side. Shown are representative eNOS immunoblot from 4 rats. Mean optical density (O.D.) of eNOS blots was ~2-fold higher on the ischemic compared with the nonischemic side.](image)
bands on the ischemic side were always greater than on the control side when comparisons were made within animals. eNOS upregulation in the overlying parietal cortex was less consistent, most likely as a result of variable ischemic damage of the cortex (see below). However, in animals with cortical infarcts on adjacent HE-stained sections, Western blots demonstrated eNOS upregulation (data not shown). All eNOS Western blots showed only a single band at the appropriate molecular weight range, supporting specificity of monoclonal antibody binding to eNOS. Duplicate running of selected protein samples within 1 blot demonstrated excellent reproducibility of band intensities as well as treatment effects. Comparison of eNOS Western blots from samples taken from the nonischemic hemisphere of MCA-occluded rats and of pharmacologically treated rats and from naïve as well as sham-operated rats suggested no substantial effect of contralateral ischemia either of NOS or COX inhibitors or of the surgical procedure on eNOS expression.

Superimposing of adjacent HE- and eNOS-stained sections revealed increased immunostaining largely in infarcted areas. In areas with evidence of only limited or selective cellular damage, however, eNOS was not substantially increased on the ischemic compared with the nonischemic side at any examined time point. Semiquantitative analysis of the brains of several animals revealed that 92% of above-threshold eNOS staining in the ischemic hemisphere was located within the infarct. Infarcts in these animals comprised 64% of the ischemic hemisphere.

Costaining for endogenous alkaline phosphatase and eNOS demonstrated a characteristic pattern. Alkaline phosphatase and eNOS immunostaining was colocalized in many of the cerebral microvessels, whereas other microvessels only immunostained for eNOS alone (Figure 4). Thus, eNOS was present in arterioles and capillaries as well as in venules and veins. Our methods did not allow us to differentiate between changes in eNOS immunostaining between alkaline phosphatase–positive and –negative microvessels. Nonetheless, review of the eNOS-stained sections indicates that ischemia appears to result in an increase in immunostaining for all segments of microvessels.

Analysis of eNOS immunostaining 3 days after focal ischemia showed no significant difference between animals that were either untreated or pretreated with L-NAME (Figure 5). There was a trend suggesting some attenuation of the eNOS-side difference after pretreatment with indomethacin. Percentage difference in immunostaining intensity (ischemic versus nonischemic side difference in pixels/ischemic side in pixels×100) was 83±8% in nontreated animals and 66±27% in indomethacin-treated animals (P=0.17, n=6 for each group). To further examine this potential effect of COX inhibition on postsischemic eNOS expression, we administered indomethacin repetitively and also studied the selective COX-2 inhibitor NS398 using Western blots 24 hours after ischemia. No significant effect on eNOS-side difference among animals that were either untreated or pretreated with COX inhibitors was detected (Figures 6 and 7).

In contrast to eNOS levels on Western blots, nNOS protein levels were consistently reduced at 24 hours in infarcted compared with normal tissue. The magnitude of this reduction was ~40%.
Discussion

Production of NO by constitutive eNOS represents an important protective mechanism against cerebral ischemia.6,8,9,11 The time course, microvascular distribution, and mechanisms involved in eNOS expression after transient focal ischemia have not been investigated. Our experiments produced 4 major new findings, as follows: (1) eNOS expression is upregulated in cerebral microvessels after transient focal ischemia. (2) Consistent upregulation of eNOS occurs at a later time point than previously reported for permanent focal ischemia. (3) Substantial upregulation of eNOS takes place only in areas undergoing severe ischemic tissue damage. (4) COX and NOS metabolites do not play a major role in eNOS upregulation after focal ischemia in rats.

Brain levels of NO and overall NOS activity are increased after focal ischemia.8,9,20,29 This may be due to changes of enzyme levels, substrate availability, or other yet-undefined factors. Postischemic changes of NOS protein in brain have been shown for all 3 isoenzymes, but spatial and temporal expression patterns differ among NOS subtypes.8,10 Vascular eNOS protein expression is believed to increase very rapidly after focal ischemia.10 However, except for 2 studies using assays without NOS isoenzyme selectivity,16,17 this concept is based on a single study using eNOS-specific immunostaining in a permanent focal ischemia model.20 In this study, eNOS expression was higher in the ischemic compared with the nonischemic hemisphere. eNOS protein appeared increased as early as 1 hour after onset of ischemia, peaked at 24 hours, and remained upregulated for at least 7 days.20 A disadvantage of this important pioneer study is that it failed to provide a second method such as immunoblotting to demonstrate antibody specificity and improve quantification of immunostaining findings. Our study examined cerebrovascular NOS expression after focal ischemia using 2 complementary eNOS isoenzyme–specific assays. Only 3 of 6 animals showed increased eNOS expression 6 hours after ischemia, but eNOS was consistently upregulated after 24 to 168 hours of reperfusion on immunostained sections and Western blots. At 1 day after ischemia when eNOS levels were substantially increased, nNOS levels had decreased. Differences between experimental paradigms, ie, transient ischemia in our study versus permanent ischemia in the study of Zhang et al.,20 may account for the different latencies of eNOS upregulation. Indeed, differences of temporal expression between permanent and transient focal ischemia models have been reported for other proteins such as iNOS.23,30 However, it should be emphasized that both studies found prolonged eNOS upregulation after focal ischemia for at least 7 days. Whether sustained eNOS upregulation occurs in preexisting microvessels only or also in neovascularizations remains to be shown. Specificity of the enhanced expression of eNOS is apparent when comparing this response with the decrease in protein levels of brain NOS after ischemia.

To our knowledge, our study is the first to address the question of which cerebral vessels express increased levels of eNOS after ischemia. We used alkaline phosphatase staining, which allows reliable differentiation of staining precapillary microvessels and capillaries versus nonstaining postcapillary microvessels in the brain of rodents and other species, including humans.31 Sections were performed on frozen brains to avoid enzyme inactivation by formalin fixation. Our observations demonstrate that eNOS staining occurs in microvessels of various sizes. Previous studies using the NADPH diaphorase method reported NOS to be primarily expressed in cerebral arteries and arterioles and only to a much lesser extent in capillaries and veins.32,33 In contrast, postcapillary vessels stained for eNOS on the ischemic and, to a lesser extent, on the nonischemic side in our study. This suggests that increases in eNOS levels probably occur in all microvessels after ischemia. Potential implications of enhanced eNOS levels in venules could be antiadhesive and antiaggregating effects of NO on leukocytes and platelets, respectively.

Our experimental approaches did not allow us to directly evaluate the role of angiogenesis in elevated eNOS levels after ischemia. However, it is unlikely that angiogenesis was an important component of the response for at least the first 24 hours after ischemia. Other studies have shown that little or no detectable angiogenesis occurs for up to 24 hours after transient or permanent ischemia in rats or humans,34,35 and afterward angiogenesis is limited to the border of the infarct. In addition, enhanced immunostaining for eNOS at 24 hours after ischemia occurs in relatively large arteries and arterioles and veins in our study. It is unlikely that these large blood
vessels would form during early angiogenesis. Lastly, we compared immunostaining at locations in the striatum in ischemia and nonischemia hemispheres and not in the border of the infarct where angiogenesis is known to occur. Nonetheless, is seems likely that a component of the increase in total eNOS protein levels at 72 and 168 hours is due to newly formed blood vessels.

The cellular sources and the molecular mechanisms of the stimuli involved in postischemic eNOS upregulation are unknown. However, these stimuli could include local hypoxia; acute intraluminal shear stress; and production of growth and/or inflammatory agents by cerebral tissues, blood vessels, and blood cells.\(^{36-38}\) Although several regulatory sites in the eNOS promoter have been identified, the molecular machinery controlling eNOS expression after ischemia is only partially understood, but may involve activation of kinase signaling pathways and activation of transcription factors such as hypoxia-inducible factor-1 and activator protein-1.\(^{1,37-40}\) Although we could not identify the mediator responsible for augmented eNOS levels after ischemia, our data provide some interesting clues. First, increased eNOS levels largely were restricted to infarcted areas. Thus, there is no evidence to suggest that substances diffusing from infarcted tissue or neurally mediated signaling to noninfarcted tissues were involved. Second, the comparatively late upregulation of eNOS after ischemia in our study (between 6 and 24 hours) widens the number of potential trigger mechanisms that could be involved. Immediate and delayed effects of events associated with reperfusion on endothelial cells such as acute shear stress, free oxygen radical generation, leukocyte adhesion, production of cytokines, or other substances induced by ischemia may be involved in increased eNOS expression.\(^{15,18,36-40}\) Previous reports indicated that products of COX and NOS isoforms could induce eNOS expression after focal ischemia. For example, Beasley et al.\(^{15}\) showed induction of COX-2 protein in large cerebral vessels 6 hours after global ischemia/reperfusion in piglets. Iadecola et al.\(^{23}\) reported postischemic iNOS expression after 12 hours of permanent ischemia. The doses of NOS and COX inhibitors used in this study were based on efficacy data in the literature, and we cannot rule out that the inhibition of enzyme activity was incomplete. Also, the aggravating effect of L-NNAME or COX inhibitors on ischemic outcome or drug-induced increases of arterial blood pressure may have counteracted the inhibition of eNOS synthesis in the present study. Nevertheless, our data do not support a major role of either NO or the superoxide anion produced by COX on postischemic eNOS induction in rats. Although COX and NOS are important sources of reactive oxygen species after ischemia, radicals produced by other sources (e.g., mitochondria) may still be involved in eNOS upregulation.

In conclusion, a sustained increase of eNOS protein levels in pre- and postcapillary cerebral blood vessels begins between 6 and 24 hours after transient focal ischemia. The latency of endogenous postischemic eNOS protein upregulation may explain the favorable effect of prophylactic eNOS augmentation by drugs such as statins.\(^{12}\) Although the functional significance of enhanced postischemic eNOS levels to the cerebral circulation is not well defined, increased capacity for NO production may promote dilation of resistance vessels, inhibit platelet aggregation, and lead to angiogenesis in border areas of reduced blood flow. Future studies are needed to further investigate the mechanisms of postischemic eNOS upregulation.

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