Cerebral Endothelial Nitric Oxide Synthase Expression After Focal Cerebral Ischemia in Rats

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**Background and Purpose:** The purpose of this study was to measure the temporal profile of expression of the endothelial nitric oxide synthase (NOS) in cerebral microvessels after middle cerebral artery occlusion in the rat.

**Methods:** Middle cerebral artery occlusion was performed on 24 male Wistar rats by extracranial insertion of a 4-0 nylon monofilament into the internal artery. Three additional rats were used as controls. Animals were killed at 1, 2, 4, 6, 24, 48, 72, and 168 hours after middle cerebral artery occlusion (n=3 per time point). Rat brains were perfused with buffer, frozen, sectioned, and stained with a monoclonal antibody against endothelial NOS. Adjacent sections were stained with hematoxylin and eosin for evaluation of neuronal damage.

**Results:** The endothelial NOS in the cerebral vessels was upregulated at 1 hour after induction of ischemia throughout the ischemic region. The induction of the endothelial NOS progressively increased up to 24 hours of ischemia. In the periphery of the area of necrosis in the cortex, a delayed (24-hour) upregulation of the endothelial NOS remained constant throughout the duration of ischemia.

**Conclusions:** The rapid and intense differential expression of the endothelial NOS in the core and peripheral areas of the lesion indicates a role for endothelial NOS in ischemic cell damage and suggests that the increased expression of NOS may mediate changes in the cerebral blood flow. (Stroke. 1993;24:2016-2022.)

**KEY WORDS** • endothelium • middle cerebral artery occlusion • nitric oxide • nitric oxide synthase • rats

Oclusion of the middle cerebral artery (MCA) causes severe reduction of blood flow and progressive impairment of the microcirculation in the ischemic region. Possible mechanisms for microvascular obstruction include endothelial luminal membrane tags, endothelial cell swelling, and polymorphonuclear leukocyte adherence to endothelium. Studies are now emerging that suggest that the production of endothelium-derived relaxing factor/nitric oxide (NO) by endothelial cells may significantly influence the basal tone of cerebral vessels, and the modulation of NO synthesis can lead to cerebral vascular endothelial changes and alteration of cerebral blood flow.

Using a NO-sensitive microelectrode, we have recently measured an increase in NO in rat brain during and after transient MCA occlusion. NO is synthesized from l-arginine by NO synthase (NOS). The changes of NO occurring in cerebral ischemia may be related to changes in NOS expression. There are at least three isoforms of NOS, Type I NOS, from neurons, and type III NOS, from endothelium, are calcium dependent and generate low levels of NO under physiological conditions. Type II NOS, from macrophages, is inducible by cytokines and generates high levels of NO. Genes for type I NOS, type II NOS, and type III NOS have recently been cloned. They share approximately 55% of the same amino acid sequences. Endothelial cells may express constitutive and inducible NOS.

Although alteration in NOS expression and thereby NO may have important physiological effects on ischemic brain tissue, to our knowledge there have been no investigations on the changes in NOS expression induced by focal ischemia. The aim of the present study was, therefore, to measure the temporal profile of endothelial expression of NOS in the brain by using a specific monoclonal antibody to type III NOS in a rat model of focal cerebral ischemia produced by occlusion of the MCA.

**Materials and Methods**

Experiments were performed on 27 male Wistar rats weighing 260 to 300 g. Focal cerebral ischemia was induced as described. Brieﬂy, rats were anesthetized with halothane (1% to 3.5% in a mixture of 70% nitrous
oxide and 30% oxygen) using a face mask. The rectal temperature of the animal was maintained at 37°C with a heating pad. The femoral artery was cannulated for sampling blood before ischemia to determine pH, Po2, and PCO2. The right common carotid artery, external carotid artery, and internal carotid artery were isolated via a ventral midline incision. A 4-0 nylon monofilament, with its tip rounded by heating near a flame, was introduced into the external carotid artery lumen through a puncture at the external carotid artery and was advanced into the internal carotid artery to block the origin of the MCA.

The rats were randomly assigned to a control group (n=3), in which the animals were not subjected to any surgical procedure, or to an experimental group, in which rats were subjected to MCA occlusion and were killed at various time points after the onset of MCA occlusion (1, 2, 4, 6, 24, 48, 72, and 168 hours; n=3 per time point).

Rats were anesthetized with intraperitoneal ketamine (44 mg/kg) and xylazine (13 mg/kg) and were transcannally perfused with heparinized 0.05 mol/L phosphate-buffered saline (PBS) followed by ice-cold 15% sucrose in PBS. The brains were rapidly removed and frozen in 2-methylbutane (Fisher) cooled on dry ice. Coronal brain sections (8-μm thick) were cut on a cryostat and were thaw-mounted on gelatin-coated slides. After blocking in 5% horse serum, sections were incubated for 60 minutes at room temperature with monoclonal antibody (MAb) H32 against endothelial NOS28,29 at a 1:1000 dilution. The sections were washed in PBS and were incubated at room temperature in biotinylated horse anti-mouse immunoglobulin G (IgG) (absorbed in rat serum, Vector) at a 1:200 dilution for 30 minutes and in avidin-biotin complex (ABC kit, Vector) for 60 minutes. Peroxidase was demonstrated with 3-amino-9-ethylcarbazole (Sigma, St Louis, Mo) for 5 minutes at room temperature. After immunocytochemical staining, the sections were counterstained with Mayer’s hematoxylin and placed on coverslips. Negative control sections received identical treatment but were incubated in nonimmune mouse serum IgG (Becton Dickinson) at a 1:10 000 dilution, which represents the same IgG concentration as the primary antibody. Preabsorption was performed by preincubating MAb H32 at a 1:1000 dilution with equal volumes of excess antigen (approximately 10 to 15 times more than the primary antibody). Absorption was carried out for 1 hour at 37°C followed by overnight incubation at 4°C. Absorbed MAb H32 was tested by immunohistochemistry and was compared with unabsorbed MAb H32. A section centered in the lesion, corresponding to the coronal section at interaural 8.2 mm, bregma 0.8 mm,27 was selected from each animal for evaluation of endothelial NOS expression. Adjacent coronal sections were stained with hematoxylin and eosin and were examined at high magnification by light microscopy for neuronal and inflammatory cell morphology. The slides were evaluated by two people, one of whom was blinded to the experiment.

Scales were used to provide a semiquantitative evaluation of NOS-like immunoreactivity. Both the percentages of positive-stained microvessels and the intensity of these vessels were accounted for. The intensity of microvasculature immunoreactivity was graded as either faint (1) or intense (2). The percentages of the microvascular immunoreactivity were graded as follows: <2%=0; 2% to 10%=1; 11% to 50%=2; and >50%=3.

In nonischemic control animals, in which large vessels stain positive for NOS, therefore, a value of “0” is provided where <2% of the vessels are immunoreactive.

**Results**

Arterial blood gas values were within normal physiological ranges in all animals. MCA occlusion was confirmed at autopsy in all ischemic rats by observing that the tip of the filament was located in the proximal segment of the anterior cerebral artery. Immunoreactivity was absent with nonimmune mouse serum IgG and with the absorbed MAb H32 in all rats.

In the control animals neuronal damage was not detected, and type III NOS-like immunoreactivity was localized only to the endothelial layers of large vessels (Fig 1) and the choroid plexus.

In animals subjected to MCA occlusion, neuronal shrinkage and swollen neurons were observed 1 to 2
hours after MCA occlusion. The scattered microvessels showed faint type III NOS staining throughout the area of neuronal damage (Fig 2).

By 4 to 6 hours after MCA occlusion, the ischemic lesion involved the supraoptic area, the basal ganglia, and the cortex. Neuronal shrinkage and swollen neurons were prominent in the lesion (Fig 3A). At this time numerous microvessels exhibited intense type III NOS staining throughout the damaged region (Fig 3B).

Eosinophilic neurons became evident and progressed to ghost cells from 24 to 48 hours throughout the ischemic region (Fig 4A). Morphological changes in the microvasculature occurred, and intense type III NOS-like immunoreactivity was present in the distorted ves-

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Fig 3. Photomicrographs. a. Angular and swollen neurons in the ipsilateral cortex at 4 hours of ischemia. b. Numerous microvessels exhibit type III nitric oxide synthase–like immunoreactivity in the ipsilateral cortex 4 hours after middle cerebral artery occlusion. Bars=50 μm.

Fig 4. Photomicrographs. a. Ghost cells (arrows) in the necrotic cortex at 48 hours of ischemia (hemotoxylin and eosin). b. An adjacent section, stained for type III nitric oxide synthase (NOS), shows intense type III NOS–like immunoreactivity present in the microvessels. c. An adjacent section with preabsorption was used as control. d. Type III NOS–like immunoreactivity was absent from the contralateral cortex. Bars=50 μm.
sels in the necrotic core at 24 to 48 hours of ischemia (Fig 4B and 4C). Type III NOS–like immunoreactivity was absent from the contralateral hemisphere. Fig 4D indicates the absence of type III NOS–like immunoreactivity in the contralateral cortex. In the cortex, at the periphery adjacent to the necrotic region of cortex, type III NOS–positive microvessels appeared at 24 hours and persisted up to 168 hours of ischemia.

Necrotic cells were present, and inflammatory cells infiltrated the core of the lesion at 72 hours. The entire necrotic lesion was clearly demarcated and macrophages were prominent in the lesion at 168 hours of ischemia. Type III NOS–like immunoreactivity was not detected in the morphologically identified macrophages or neuronal cells (Fig 5). The distorted and fragmented vessels displayed dense type III NOS staining throughout the infarcted zone through 168 hours of ischemia (Fig 6).

Fig 7 shows the semiquantitative scores of type III NOS–like immunoreactivity contained within the ipsilateral hemisphere. An increase in the percentage of microvessels exhibiting NOS reactivity occurred with...
time. By 24 hours the increase had reached a plateau, and the percentage of positive-stained microvessels remained constant for the duration of the experiment. The intensity of NOS staining increased at 4 hours of ischemia and remained elevated through 168 hours of ischemia.

Discussion

Our data indicated that type III NOS is expressed in the endothelial layers of large vessels and the choroid plexus in the normal rat brain. This is consistent with data from previous reports that basal production of NO is dependent on cerebral vessel size and is greatest in the large arteries and that NO has an important modulating effect on basal perfusion of the choroid plexus.

The most important finding in the present study was that after focal cerebral ischemia type III NOS was rapidly upregulated in the cerebral microvasculature throughout the ischemic region as well as in the periphery of the area of necrosis in the cortices at 24 hours of ischemia. The induction of type III NOS is progressive, with increasing duration of ischemia in the ischemic core region. NO released by endothelial cells mediates vascular relaxation. Severe reduction of ipsilateral cerebral blood flow after MCA occlusion is present in this model. Overexpression of type III NOS in the microvessels may increase the synthesis of NO in order to dilate blood vessels and thereby to compensate for the reduction of cerebral blood flow. This is consistent with the recent report that the administration of L-arginine increases regional cerebral blood flow and decreases the infarct size caused by MCA occlusion in spontaneously hypertensive rats. The increased expression of type III NOS in the ischemic and peripheral zones of the lesion may mediate changes in the cerebral blood flow.

Degenerative change of endothelial cells has been documented in animal models of focal ischemia. Upregulation of type III NOS may also represent a mechanism of cerebral vascular endothelial damage. Recently, Palmer et al have shown that NO synthesized by an inducible NOS in endothelium reduced the viability of the endothelial cells.

Cytokines may induce the expression of NOS in endothelial cells. An in vitro study has demonstrated that, 8 hours after the addition of interferon, in combination with tumor necrosis factor, interleukin-1, or endotoxin, to murine brain endothelial cells, NO was released in these cells. Although it is possible that cytokines are responsible for the induction of NOS in endothelial cells in vitro, the time course of NOS expression in vivo, which occurs as early as 1 hour after ischemia, is inconsistent with the delayed (8-hour) expression of NOS in vitro evoked by cytokines. It is possible, however, that a distinct isoform of NOS is expressed in response to cytokines that was not recognized by our antibody (MAB H32). Further study is needed to identify those factors involved in the upregulation of NOS gene expression after ischemia.

Endotoxin-treated murine macrophages synthesize nitrite and nitrate. Macrophage NOS mRNA is inducible. We failed to detect type III NOS–like immunoreactivity in the activated macrophages. This finding is in agreement with our previous studies, in which MAb H32 against endothelial NOS did not cross-react with other known isoforms of NOS.

In summary, our data suggested a role for endothelium-derived NO in cerebral vascular endothelial function after focal ischemia in the rat.

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References

Nitric oxide (NO) or endothelium-derived relaxing factor is rapidly emerging as a major regulator of cerebral circulation. Cerebral blood vessels are unique in that they are regulated by at least three different sources of NO. The first is the endothelial cell, which expresses the NO-forming enzyme NO synthase (NOS). The other two sources, which are unique to the cerebral circulation, include the NOS-containing cerebral perivascular nerve fibers, which mediate nonaden-nergic, noncholinergic vasodilation of cerebral blood vessels, and NOS-containing neurons and astrocytes, which may regulate local cerebral blood flow in an activity-dependent fashion. Another potential source for NO under pathological conditions may be the induction of NOS in a variety of cell types including astrocytes, microglia, macrophages, and endothelial cells. The inducible NOS differs from the constitutive NOS of neurons and endothelial cells in that it is calcium independent, produces NO for extended periods, probably mediates most of the toxic actions of NO, and is induced on stimulation with a variety of cytokines. The constitutive enzymes produce NO in a calcium-calmodulin-dependent fashion, and the regulation of the constitutive enzymes are thought to be posttranslational through processes such as phosphorylation. Three different isoforms of NOS, designated neuronal, endothelial, and macrophage NOS, have been purified to homogeneity and molecularly cloned; they represent three distinct gene products. It is imperative to consider all potential sources of NO when examining the function of this unique molecule in normal and pathological conditions related to the central nervous system. In this issue, Zhang et al take an important step in examining the distribution and expression of endothelial NOS after occlusion of the middle cerebral artery in rats. They report an upregulation of endothelial NOS after 1 hour of occlusion, with the highest levels occurring after 24 hours of occlusion. This upregulation of endothelial NOS most likely represents the constitutive endothelial NOS since their monoclonal antibody does not cross-react with macrophage NOS. These findings add another level of complexity to the regulation of endothelial NOS. In addition to posttranslational modifications of NOS, the amount of the enzyme can be upregulated. The factors responsible have yet to be identified. What are the implications of these findings? NO may mediate some of the neuronal damage after middle cerebral artery occlusion, as low doses of NOS inhibi-
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