Cerebellar stimulation reduces inducible nitric oxide synthase expression and protects brain from ischemia

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Galea, Elena, Eugene V. Golanov, Douglas L. Feinstein, Keith A. Kobylarz, Sara B. Glickstein, and Donald J. Reis. Cerebellar stimulation reduces inducible nitric oxide synthase expression and protects brain from ischemia. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H2035–H2045, 1998.—A focal infarction produced by occlusion of the middle cerebral artery (MCAO) in spontaneously hypertensive rats induced expression of inducible nitric oxide synthase (iNOS) mRNA, measured by competitive reverse transcription-polymerase chain reaction. The mRNA appeared simultaneously in the ischemic core and penumbra at 8 h, peaked between 14 and 24 h, and disappeared by 48 h. At 24 h, inducible nitric oxide synthase (iNOS)-like immunoreactivity was present in the endothelium of cerebral microvessels and in scattered cells, probably representing leukocytes or activated microglia. Electrical stimulation of the cerebellar fastigial nucleus (FN) for 1 h, 48 h before MCAO, reduced infarct volume by 45% by decreasing cellular death in the ischemic gial nucleus (FN) for 1 h, 48 h before MCAO, reduced infarct volume by 45% by decreasing cellular death in the ischemic gial nucleus (FN). Electrical stimulation of the cerebellar fastigial nucleus (FN) (12, 23, 24, 30, 31). The area of salvage is confined to the ischemic penumbra, which is salvaged, but not in the core, which is not. A preliminary report of this study has been made in abstract form (24).

In this study we have investigated whether a conditioning stimulation of the FN will modify the expression of iNOS. Specifically, we have examined the time course, magnitude, and topographic and cellular distribution of iNOS mRNA, protein, and catalytic activity in the regions of the focal ischemic infarction produced by MCAO without or with FN stimulation. We report that MCAO induces iNOS mRNA and protein in immune-related cells and in the endothelium of cerebral microvessels restricted to the ischemic lesion and that stimulation of the FN suppresses this accumulation within the ischemic penumbra, which is salvaged, but not in the core, which is not. A preliminary report of this study has been made in abstract form (24).

TERMINOLOGY
In this context of this study, the terms “irretrievable zone” and “ischemic core” are used interchangeably to refer to the topographically identical portions of the focal ischemic infarction produced by MCAO. The term “irretrievable zone” was defined by us (23) as the area of the infarct that cannot be salvaged by stimulating FN. The ischemic core has been traditionally defined by the fact that, within it, rCBF and rCGU are at the lowest values and neuronal death cannot be prevented by treatments.

For convenience, we also use the terms “retrievable zone” and “ischemic penumbra” interchangeably, also on the basis of nearly identical topography. The term “retrievable zone” or “retrieved zone” was introduced by us to identify the territory of an infarction produced by MCAO that can be salvaged by FN stimulation (23). In contrast, the term “ischemic penumbra,” of widespread usage, was at first defined as the area of the infarction surrounding and encircling the core, in which the affected cerebral cortex is electrically unstable and subjected to recurrent waves of depolarization. The penumbra has also been further characterized as the area in which rCBF is partially reduced and rCGU often elevated, as the zone in which neuronal death is...
delayed, and also the territory in which neurons may be salvaged by appropriate pharmacological interventions. Because few studies analyze electrical events and measure rCBF and/or rCGU autoradiographically, it is reasonable to define the penumbra by its topography because this is usually described in all studies.

**MATERIALS AND METHODS**

**Surgical Procedures: FN Stimulation and MCAO**

The procedures for instrumentation, stimulation of FN, production of focal ischemia, and estimation of lesions have been described in detail elsewhere (23, 30). Studies were performed on adult male Wistar-Kyoto rats of the spontaneously hypertensive rat (SHR) strain. These rats have been used because the volume of lesions elicited by MCAO are more uniform than in other strains (2), thereby reducing the numbers of animals needed to achieve statistical significance. Moreover, FN stimulation comparably reduces lesion volumes in rats of the Sprague-Dawley strain (23, 31). Rats were anesthetized with halothane (1.8–2.5%) in 100% O2. All surgical procedures were performed under aseptic conditions with the same surgeon. The femoral arteries were cannulated to record arterial pressure (AP) and to obtain blood samples for measurement of pH, arterial PO2 (PaO2) and PCO2 (PaCO2), glucose, and hematocrit. A femoral vein was cannulated to withdraw blood during FN stimulation. Core temperature was maintained at 37°C by a thermostatically controlled infrared lamp connected to a rectal probe.

FN stimulation. Rats were placed in a stereotaxic apparatus, and the floor of the fourth ventricle was exposed by removal of the posterior lip of the occipital bone to expose the calamus scriptorius, which was used as stereotaxic zero. A hole, 1.5–2.0 mm in diameter, was drilled through the interparietal bone (1 mm lateral to the midline and 1 mm rostral to the occipital suture) for insertion of stimulating electrodes.

Stimulating electrodes were fabricated from Teflon-coated stainless steel wire (diameter 150 µm), with only the cut surface exposed and attached to a stereotaxic electrode holder. Cathodal pulses were generated by a square-wave stimulator and delivered as constant current via a photodetector stimulus isolation unit. The anode was clip-attached to the site along the track from which AP was recorded for 4 h at room temperature. The staining was visualized by the diaminobenzidine as chromogen.

Whole brains. Animals were anesthetized with pentobarbital (60 mg/kg ip) and perfused transcardially with heparinized saline followed by 50 ml of 3.75% acrolein-2% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, and 200 ml of 2% paraformaldehyde-PB. Brains were removed and post-fixed for 4 h in 2% paraformaldehyde. Coronal sections (40 µm) were made in a Lancer Vibratome and collected in PB. Adjacent sections were processed for immunohistochemistry or stained with thionine for computer-assisted infarct distribution and volume. Correction was made for contribution of edema (12). In some cases, brains were cryoprotected with 20% sucrose, frozen, and sectioned with a microtome, and sections were collected in PBS. The quality of staining using this procedure was comparable to that obtained in fresh brains. Sections were taken through the cerebellum to localize the position of the stimulating electrode.

Immunohistochemistry was carried out in free-floating sections processed in nine-well spot plates (Pirex) (1–2 sections/well). Sections were incubated 30 min in 0.5% 1% bovine serum albumin (BSA) in PBS and incubated with 0.5% H2O2 in PBS for 15 min to inactivate endogenous peroxidase activity. After two rinses in 0.1 M Tris-buffered saline (TBS, pH 7.6), sections were blocked with 0.5% bovine serum albumin (BSA) in TBS and incubated with primary antibodies in TBS containing 0.3% Triton X-100 and 0.1% BSA. Incubation was performed for 16–18 h at 4°C with gentle shaking. After several washes in TBS, sections were incubated with biotinylated anti-mouse or anti-rabbit secondary antibodies (1:400, Vector Laboratories, Burlingame, CA) for 1 h at room temperature. The staining was visualized by the biotin-avidin-peroxidase method (Elite Kit, Vector Laboratories) using diaminobenzidine as chromogen.

Endothelial cells. Assessment of the cross-reactivity of the iNOS antibodies with endothelial NOS (eNOS) was made in cultured endothelial cells of bovine pulmonary artery, which characterizedly express high amounts of eNOS (19). Cells were obtained from the American Type Culture Collection,
Table 1. Physiological variables in different experimental groups

<table>
<thead>
<tr>
<th>Physiological Variable</th>
<th>FN + MCAO 14 h (n = 5)</th>
<th>Sham (n = 3)</th>
<th>FN + MCAO 24 h (n = 5)</th>
<th>MCAO 4 h (n = 3)</th>
<th>MCAO 8 h (n = 6)</th>
<th>MCAO 14 h (n = 11)</th>
<th>MCAO 24 h (n = 8)</th>
<th>MCAO 48 h (n = 4)</th>
<th>MCAO 72 h (n = 4)</th>
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<tr>
<td>pH</td>
<td>7.40 ± 0.02</td>
<td>7.41 ± 0.03</td>
<td>7.38 ± 0.03</td>
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<tr>
<td>Pco2, mmHg</td>
<td>50 ± 2</td>
<td>57 ± 3</td>
<td>54 ± 8</td>
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<tr>
<td>Glucose, mg/dl</td>
<td>293 ± 11</td>
<td>343 ± 10</td>
<td>370 ± 124</td>
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<td></td>
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<tr>
<td>PCO2, mmHg</td>
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<td>470 ± 23</td>
<td>470 ± 99</td>
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<tr>
<td>Hct, %</td>
<td>185 ± 24</td>
<td>212 ± 19</td>
<td>177 ± 29</td>
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<td>HR, beats/min</td>
<td>176 ± 16</td>
<td>177 ± 14</td>
<td>186 ± 33</td>
<td>220 ± 10</td>
<td>206 ± 34</td>
<td>177 ± 17</td>
<td>164 ± 15</td>
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<td>Glucose, mg/dl</td>
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<td>110 ± 5</td>
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<td>110 ± 5</td>
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<td>Glucose, mg/dl</td>
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<td>110 ± 7</td>
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<td>PCO2, mmHg</td>
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<td>301 ± 25</td>
<td>334 ± 15</td>
<td>316 ± 24</td>
<td>393 ± 97</td>
<td>328 ± 40</td>
<td>334 ± 30</td>
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<tr>
<td>Glucose, mg/dl</td>
<td>276 ± 25</td>
<td>327 ± 25</td>
<td>301 ± 25</td>
<td>334 ± 15</td>
<td>316 ± 24</td>
<td>393 ± 97</td>
<td>328 ± 40</td>
<td>334 ± 30</td>
<td>313 ± 18</td>
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</table>

Values are means ± SD; n = no. of rats. Animals were stimulated in the fastigial nucleus (FN) 48 h before middle cerebral artery occlusion (MCAO). MCAO 4 h– MCAO 72 h refer to survival times after MCAO (in h). During FN, average of values before and during the last minutes of FN stimulation; during MCAO, average of values 10 min before and 10 min after MCAO; Hct, hematocrit; AP, arterial pressure; HR, heart rate.

cultured in DMEM supplemented with 20% FCS, and used between passages 15 and 20. To induce iNOS, we incubated cells for 24 h with a cytokine mixture containing interleukin-1β (IL-1β; 10 ng/ml), tumor necrosis factor-α (TNF-α; 10 ng/ml), and interferon-γ (IFN-γ; 100 U/ml). For immunohistochemistry, the cells were fixed in 4% paraformaldehyde in PB for 30 min and incubated with primary and secondary antibodies as described above for brain sections.

Antibodies. The polyclonal anti-mouse macrophage iNOS was from Upstate Biotechnology (Lake Placid, NY); the monoclonal anti-rat iNOS was generously donated by Dr. Y. Yui (Kyoto University, Kyoto, Japan) and characterized by Sato et al. (27); the monoclonal anti-ED-1, a marker of macrophages, was from Serotec (Oxford, UK); and the monoclonal anti-human eNOS was from Transduction Labs (Lexington, KY). Endothelial cells were labeled with an antibody against human factor VIII from Sigma, and astrocytes were stained with a polyclonal antibody against glial fibrillary acidic protein (GFAP) obtained from Dako (Carpinteria, CA). Antibodies were used at 1:1,000 dilution except for that against eNOS, which was used at 1:500. No staining was observed when the primary antibodies were omitted.

RNA Preparation and Quantitative Polymerase Chain Reaction Analysis

Rationale. When the FN is stimulated before MCAO, the borders of the infarction displace laterally, resulting in a smaller infarct volume (Fig. 1). The area salvaged by FN stimulation, the penumbra, can also be rescued by pharmacological interventions (see TERMINOLOGY), whereas the area surrounding the site of MCAO dies despite any treatment, including FN stimulation, thus representing the core of the infarction. Core and penumbra, therefore, are both ischemic and hence damaged areas that behave differently on FN stimulation. Such a difference suggests the prevalence of FN-elicited salvage mechanisms in the penumbra that are absent (or overrun by deadly stimuli) in the core. On the basis of this rationale, iNOS mRNA expression was analyzed in both penumbra and core. Areas were sampled in each animal according to the individual distribution of the infarction, which can be easily detected with the naked eye because it is significantly whiter than the rest of the tissue. To increase consistency, we carried out sampling in all the animals in the infarcted zone of greatest cross-sectional area (~10 mm from the interaural line). This is the level at which FN stimulation consistently elicits salvage with a variance <10%, on the basis of analyses of more than 200 brains (13, 24, 30). In nonstimulated rats, we sampled the inner borders of the infarction, containing ischemic tissue that would have been rescued by the FN. Conversely, in rats with the FN stimulated, we sampled the outer borders of the infarction corresponding to the rim of cortex that survived the ischemia.

Sampling procedure. Rats were administered an overdose of pentobarbital (60 mg/kg ip) and decapitated, and the brains were placed in 10% formalin, on ice, for 24 h. Brains were then dissected to obtain a parasagittal slice 10 mm from the interaural line. This is the level at which FN stimulation can be easily detected with the naked eye because it is significantly whiter than the rest of the tissue. To increase consistency, we carried out sampling in all the animals in the infarcted zone of greatest cross-sectional area (~10 mm from the interaural line). This is the level at which FN stimulation consistently elicits salvage with a variance <10%, on the basis of analyses of more than 200 brains (13, 24, 30). In nonstimulated rats, we sampled the inner borders of the infarction, containing ischemic tissue that would have been rescued by the FN. Conversely, in rats with the FN stimulated, we sampled the outer borders of the infarction corresponding to the rim of cortex that survived the ischemia.

Fig. 1. Areas of rat brain assayed for inducible nitric oxide synthase (iNOS). Representative brains sections were obtained 24 h after occlusion of the middle cerebral artery (MCAO) in rats without (MCAO) or with (FN + MCAO) 1 h of electrical stimulation of the fastigial nucleus (FN) 48 h earlier. Sections shown were ~10 mm from the interaural line and were stained with thionine to increase the contrast. Areas excised for analysis of iNOS mRNA expression are shown. The ischemic core (circle) is always within the lesion. The ischemic penumbra (arrows) is within the dorsomedial edge of the infarction in animals without FN stimulation and just outside of the infarction in stimulated rats. An area homologous to the penumbra in the contralateral hemisphere was control for uninjured brain.
were rapidly removed and placed in ice-cold saline. They were then placed on a chilled glass plate with the ventral surface uppermost, and the MCAs were identified. A block of tissue, ~1 mm thick, was removed by transecting the brain coronally with a razor blade bracketing the origin of the MCA at ~10 mm from the interaural line. The tissue blocks were placed flat on the dish, and samples were taken with sterile forceps from the penumbra, the core, and the contralateral hemisphere homologous to the core as described above.

Reverse transcription and competitive polymerase chain reaction. Total cytoplasmic RNA was isolated from tissue samples by homogenization in hypotonic Tris-HCl buffer, digestion in proteinase K, and extraction in phenol-chloroform. To synthesize cDNA, we mixed 0.5–1 µg of total cytoplasmic RNA with 900 ng of random primers in a total volume of 10 µl, heated the mixture at 65°C for 2 min, and placed it on ice. Reverse transcription was carried out in a final volume of 20 µl in the presence of 40 mM KCl, 2.5 mM of each deoxynucleotide 5′-triphosphate (dNTP), 10 mM dithiothreitol (DTT), 20 U RNase inhibitor, and 2 U of Superscript RNaseH reverse transcriptase (GIBCO, Gaithersburg, MD) at 37°C for 1 h. The reaction was terminated by heating at 95°C for 2 min and was diluted to 50 µl with water. Five microliters were used to assess, by competitive polymerase chain reaction (PCR), the amount of mRNAs encoding, respectively, for iNOS and for the housekeeping enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

In competitive PCR, an internal standard is coamplified with the cDNA sample, and at the end of the reaction, the relative amount of each was determined (10, 11). Because the efficiency of amplification of the standard is identical to that of the cDNA, problems associated with the lack of linearity intrinsic to PCR are circumvented. This method allows quantitation of mRNAs from minute amounts of tissue, rapidly, and with a high degree of specificity. Therefore, we considered competitive PCR the best approach for independent analysis of small portions (25–50 mg wet tissue) of brain parenchyma. As internal standards we used cDNA fragments with an internal deletion, so that although both cDNA and construct were considered competitive PCR products, the respective PCR products could be easily distinguished on an agarose gel.

To measure iNOS mRNA, we performed PCR using several concentrations of the standard (0.1–10 fg) so as to determine the absolute amounts of starting cDNA. For measurement of GAPDH mRNA, relative levels were estimated through amplification with a single concentration (100 fg) of standard (13). The PCR reaction mixture contained 200 µM of each dNTP, 50 mM KCl, 10 mM Tris-HCl, pH 8.8 at 25°C, 1.5 mM MgCl₂, 0.5 mM DTT, 0.1% Triton X-100, 400 ng each of a forward (primer A) and reverse primer (primer B), and 1.25 µCi ³²PdCTP (3,000 Ci/mmol, Amersham) in a final volume of 40 µl. The samples were heated to 88°C, and the reactions were started by the addition of 0.5 U of Taq polymerase (Promega, Madison, WI) in 5 µl of 10 mM Tris-HCl, pH 8.8. PCR conditions were denaturation at 93°C for 30 s, annealing at 60°C for 45 s, and synthesis at 72°C for 45 s. After 40 cycles, samples were maintained for 10 min at 72°C. All PCRs were carried out in a Hybrid thermal reactor controlled by tube temperature. PCR products were separated by electrophoresis through a 2% agarose gel with ethidium bromide and excised out of the gel, and the incorporated radioactivity was measured by liquid scintillation counting.

Deletion standards were generated as previously described (11). The identity of the deletion standards was confirmed by subcloning into pGEM vectors and sequencing. Amplification efficiencies for cDNA and construct were >95% identical, as revealed by the coamplification (in the same tubes) of equal amounts of cDNA and construct (0.1–100 fg, measured by OD at 260 nm) and determination of the amount of PCR products after 40 cycles (data not shown).

Sequences of NOS primers, on the basis of the rat iNOS sequence (10), were (5′–3′): 1) forward: GAC-GAG-CTG-TTC-AGG-GTC-CTC-CAC-G (bases 3,190–3,214) and 2) reverse: CAA-TAC-TAC-TTG-GTA-GGT-TAG-AGG-G (bases 3,573–3,594). Homology with other NOS sequences was <20%, as determined with the DNA alignment programs GAP and FASTA from the Pittsburgh Supercomputer Center. Sequences of GAPDH primers were (5′–3′): 1) forward: GCC-AAG-TAT-GAT-GAC-ATC-AAG-AAG (bases 781–804) and 2) reverse: TCC-AGG-GGT-TTC-TAC-TCC-TTG-GAG (bases 1,044–1,060).

Measurement of iNOS Activity

iNOS activity was assayed by measuring production of L-[³¹⁴C]citrulline from L-[¹⁴C]arginine (11). Animals were deeply anesthetized and decapitated, brains were removed, and cerebral cortices were excised from ischemic and contralateral hemispheres. Each sample was homogenized in 3 ml of 50 mM Tris·HCl buffer, pH 7.8, containing a cocktail of protease inhibitors. To remove endogenous L-arginine, we mixed homogenates with equal volumes of Dowex-50 W (Sigma) and centrifuged them briefly. Fifty microliters of the homogenates were incubated at 37°C in the presence of 50 mM Tris·HCl, pH 7.8, 0.5 mM NADPH, 5 µM FAD, 5 µM BH₄, 10 µM L-arginine, 50 nM of L-[¹⁴C]arginine (305 mCi/mmol; Amersham), and 1 mM EGTA to measure only Ca²⁺-independent activities. Blanks were the activity in the presence of the NOS inhibitor N⁵-monomethyl-L-arginine (200 µM). Incubations were carried out for 60 min at 37°C, and the reaction was stopped with 0.4 ml of 20 mM HEPES buffer, pH 5.5, mixed with 1 ml of Dowex-50 W equilibrated in this buffer. The mixture was centrifuged briefly, and the supernatant, containing L-citrulline, was mixed with scintillation fluid to determine the incorporated radioactivity.

Statistical Analysis

Values are expressed as means ± SD. Groups were statistically evaluated by one-way ANOVA followed by the Fisher’s test. Differences were considered significant when P < 0.05.

RESULTS

Expression of iNOS mRNA After MCAO

The mRNAs for iNOS and the constitutively expressed housekeeping enzyme GAPDH were measured in samples of tissue (25–50 mg wet wt) obtained from the ischemic core, ischemic penumbra, and contralateral hemisphere at various times after MCAO (see MATERIALS AND METHODS and Fig. 1). Total RNA amounted to 1–5 µg (ischemic areas) and 10–30 µg (contralateral hemisphere). The lower yield in the ischemic areas probably reflects the presence of necrotic tissue.

iNOS mRNA was not present in intact tissue. After MCAO, iNOS mRNA appeared within the ischemic core and penumbra but not in the contralateral hemisphere (Figs. 2 and 3). It first appeared by 8 h, peaked between 14 and 24 h, and returned to near-basal levels 48–72 h after MCAO (Fig. 3). The time course and magnitude of change of iNOS mRNA were comparable in core and...
penumbra. Multifactorial ANOVA indicated that “time after occlusion” and “brain area” significantly affected iNOS expression ($P < 0.05$), whereas ANOVA analysis at each postocclusion time indicated that iNOS mRNA expression in core and penumbra was significantly higher ($P < 0.05$) than in the contralateral hemisphere within 8–24 h, but that at 48–72 h only the core was significantly different ($P < 0.05$). When the two ischemic areas were compared, expression was significantly higher in core than in penumbra ($P < 0.05$) only at 48 h after MCAO, when most of the mRNA had disappeared.

In contrast to iNOS, the mRNA for GAPDH was markedly reduced throughout the infarction 14 h after MCAO, a time when iNOS mRNA was maximally expressed, whereas GAPDH mRNA was unchanged in the contralateral hemisphere (Fig. 4). The fact that the mRNAs for iNOS and GAPDH changed in opposite directions indicates that the elevations in iNOS mRNA were specific.

Cellular Localization of iNOS

To assess whether the increased expression of iNOS mRNA was associated with increased translation of iNOS protein and to establish its cellular localization, we immunostained tissue sections with either a polyclonal or a monoclonal antibody raised, respectively, against mouse and rat iNOS.

Twenty-four hours postocclusion, the noninfarcted parenchyma, either ipsilateral or contralateral to the occlusion site, was devoid of any staining (Fig. 5A). In contrast, the infarcted zone presented iNOS-like immunoreactivity localized in two cell types: microvessels (Fig. 5, B and C) and cells with round or ameboid shapes (Fig. 5, D–F). Although the two iNOS antibodies labeled both vessels and ameboid cells, they had a preference for one cell type: the polyclonal antibody produced clear images of vessels (Fig. 5B), whereas the monoclonal antibody stained vessels lightly (Fig. 5C). In contrast, the polyclonal antibody stained a significantly smaller number of ameboid cells (Fig. 5D) than did the monoclonal antibody (Fig. 5E). This cellular preference may be attributable to differences in the iNOS proteins expressed by the vessels or the ameboid cells, e.g., different posttranslational modifications or subcellular localization, which could interfere with epitope recognition.

Cells positive for the macrophage marker ED-1 (Fig. 5F) were present in sections adjacent to the ones stained with the iNOS monoclonal antibody (Fig. 5E). The strong morphological resemblance between the ED-1- and iNOS-containing cells suggests that the...
Fig. 5. iNOS immunoreactivity 24 h after MCAO. A: contralateral nonischemic hemisphere. B–F: from ischemic tissue. B: microvessels stained with a polyclonal antibody to iNOS. Some iNOS-positive immune cells appear in contact with the vascular wall. C: microvessels stained with a monoclonal antibody to iNOS. D–F: consecutive sections of ventral border of infarction stained either with polyclonal iNOS antibody (D), monoclonal iNOS antibody (E), or an ED-1 antibody (F). iNOS antibodies show cell selectivity: vessels were better labeled with the polyclonal and immune cells with the monoclonal antibody. Comparison of ED-1 and iNOS staining suggests that only a subpopulation of immune cells expresses iNOS. Magnification in all cases is ×170.
latter may be macrophages, perhaps infiltrated from
blood or derived from perivascular microglia, as the
localization in vascular walls would indicate (Fig. 5B).
Whether or not the iNOS-expressing cells are macro-
phages, the observation that the ED-1-bearing cells
outnumber the iNOS-positive ones suggests that focal
ischemia induces the appearance in brain of immune
cells that do not express iNOS.

The appearance of the microvessels as smooth and
continuous tubular structures, clearly at least with the
polyclonal antibody, suggested that the stained cell was
the endothelium, and not the glial end feet, adherent
microglia, or pericytes, which, if stained, would appear
as intermittent elements along the vessel wall. To
confirm this assumption, we stained ischemic brains
with antibodies recognizing factor VIII and GFAP,
markers for endothelial and glial cells, respectively
(Fig. 6, A and B). Profiles stained by factor VIII (Fig.
6A) closely resembled elements stained by iNOS (com-
pare with Fig. 5B), whereas those stained with GFAP
did not (Fig. 6B). This strongly supports that the
iNOS-like immunoreactivity induced in cerebral mi-
rovessels by ischemia was endothelial.

We also sought to rule out the possibility that our
antibodies were cross-reacting with the endothelial
NOS isoform (eNOS or NOS-3), which has a low
constitutive expression in vessels from normal brains
but can be upregulated after ischemia (22, 33). To
examine this possibility, we used iNOS and eNOS
antibodies to stain cultured pulmonary artery endothel-
ial cells, which, as is typical for peripheral endothel-
ium, have a high constitutive expression of eNOS (19).
Whereas in the absence of primary antibody the cells
were very lightly stained (Fig. 6C), the eNOS antibody
produced a robust staining in the cytoplasm (Fig. 6E),
thus confirming the presence of constitutive eNOS in
these cells. In contrast, the polyclonal iNOS antibody
(Fig. 6D) failed to stain the cytoplasm of the endothelial
cells, although in some cases it labeled cell nuclei,
maybe because of lack of purification. However, stain-
ing was observed when the endothelial cells were
untreated with a combination of inducers of iNOS expres-
sion like IFN-γ, TNF-α, and IL-1β (Fig. 6F). These
observations support that the iNOS antibody does
indeed recognize the iNOS isoform, whereas it does not
cross-react with the constitutive eNOS.

A comparison of the cellular distribution of iNOS
immunostaining in brains of rats killed at 8 and 24 h
after MCAO is shown in Fig. 7. At 8 h, microvessels
were lightly stained all over the infarction, whereas the
immune cells were predominantly found in the isch-
emic area close to the occlusion point. By 24 h, vessels
were more intensely stained, whereas the immune cells
were more abundant and distributed throughout the
lesion, with preference in the edges of the infarction.
These results indicate that vascular iNOS and INOS-

Fig. 6. Comparison of iNOS immunore-
activity to factor VIII and glial fibril-
lar acidic protein (GFAP) immunoreac-
tivity in ischemic brains and to
endothelial NOS (eNOS) staining in
pulmonary endothelial cells. A: immu-
nostaining of ischemic zone (24 h post-
occlusion) with an antibody to factor
VIII showing vascular profiles similar
to those stained by iNOS in Fig. 5B.
B: immunostaining with an antibody to
GFAP showing a thin patchwork of
astrocytes. Magnification in A and B,
×170. C-F: immunoreactivity of pulmo-
nary endothelial cells to INOS poly-
donal antibody (D), eNOS (E), iNOS
after incubation with iNOS inducers
(F), or in absence of primary antibodies
(C). Pulmonary endothelial cells were
immunoreactive for eNOS but not iNOS,
unless they were induced to express the
iNOS protein, suggesting that the iNOS
antibody does not cross-react with the
eNOS protein. Magnification in C-F,
×250.
expressing immune cells are not equally distributed in the infarction.

Effect of FN Stimulation

The FN was stimulated or sham stimulated 48 h before MCAO, and 14–24 h later, when the ischemia-induced expression of iNOS mRNA was maximal (see Fig. 3), rats were killed and tissues were processed for computation of infarct volume and for analysis of iNOS mRNA by competitive PCR and iNOS protein by immunohistochemistry.

To confirm that the FN stimulation was neuroprotective in the population of rats under study, we performed MCAO on groups of sham-stimulated and FN-stimulated rats 48 h after insertion of electrodes; the rats were killed 24 h later. The lesion in sham-stimulated animals was 139.8 ± 54.9 mm³ (mean ± SD), whereas that in the FN-stimulated group was 76.4 ± 21.4 mm³ (n = 5; P < 0.01; ANOVA analysis), representing a reduction in lesion size of ~45%, confirming previous results from this (12, 23, 24, 30) and another laboratory (31).

Analysis of iNOS mRNA expression revealed FN stimulation did not change the magnitude of induction of iNOS mRNA within the ischemic core, whereas it abolished expression in the ischemic penumbra (Fig. 8A).

When the brains were processed for immunohistochemistry, the infarcted zone was easily localized because it displayed a significantly whiter background than the healthy tissue. iNOS immunoreactivity was detected in both microvessels and ameboid cells, strictly localized to the infarcted areas, and absent from the ischemic penumbras localized to the outer borders of the infarctions (Fig. 8B); i.e., there was a topographical correlation between the distribution of iNOS immunoreactivity and cellular death. This correlation implies that in FN-stimulated animals the volume of tissue in which iNOS protein was upregulated was reduced proportionally to the decrease in infarction volume.

To confirm this assumption, we measured the activity of the Ca²⁺/calmodulin-independent NOS (i.e., activity attributable to iNOS) in the whole cerebral cortex of rats with MCAO who were either sham stimulated or in which the FN was stimulated 48 h before vascular occlusion. (Sham-stimulated rats were used to control for any inflammatory responses to surgery.) The concentration of L-arginine in the assays was 10 µM.

As expected, no Ca²⁺-independent NOS activity was detected in the contralateral nonischemic hemisphere. In the ischemic cortices, iNOS activity was 163.3 ± 43.5 pmol L-citrulline (mean ± SD; n = 6) in sham-stimulated rats, whereas in FN-stimulated rats activity was reduced to 92.3 ± 72.0 pmol L-citrulline, a decrease of 44% (mean ± SD; n = 6; P < 0.05 vs. sham stimulated; ANOVA analysis). The results suggest that
the iNOS protein detected immunocytochemically was catalytically active and that the FN-induced decrease in the tissue volume occupied by iNOS-expressing cells results, not surprisingly, in a decrease of the net enzyme activity.

**DISCUSSION**

Here we investigated the effects of electrical stimulation of the FN on the expression of iNOS in a focal ischemic infarction produced by permanent MCAO. Because iNOS contributes to neuronal death in ischemia (16, 17) and, by contrast, FN stimulation decreases ischemic damage (12, 23, 24, 30, 31), we seek to determine whether the FN may be neuroprotective by inhibiting iNOS expression. As a first step to answer this question, the aim of the present work was to determine whether FN stimulation altered iNOS expression in the salvaged ischemic areas (penumbra). The finding that iNOS expression was still present in the penumbra after FN stimulation would automatically rule out that inhibition of iNOS is the mechanism underlying the FN-mediated neuroprotection. In contrast, the finding that iNOS was absent from the areas rescued by the FN would support a relationship between iNOS expression and FN-stimulation, thus serving as the basis for additional studies.

Here we found that focal ischemia resulted in the transient expression of iNOS mRNA and enzyme in the endothelium of cerebral microvessels and in immune cells in both the ischemic core and penumbra. Stimulation of the FN, which reduced the volume of the infarction by 45%, markedly reduced expression of iNOS mRNA, protein, and enzyme activity.

iNOS and Ischemia

The present study confirms and extends observations by ourselves (24) and others (18) that iNOS is not detectable in normal adult rat brain. However, a focal ischemic infarction elicited by permanent MCAO initiates, by 8 h, the expression of iNOS mRNA confined to the ischemic infarction, reaching a maximum by 14–24 h before recovery. The increase in mRNA is accompanied by enhanced translation of the active enzyme protein, revealed by the appearance of immunoreactive iNOS and activity of the Ca²⁺/calmodulin-independent enzyme isoform. The increase in iNOS mRNA did not reflect widespread activation of transcription because the mRNA of the constitutively expressed GAPDH was indeed reduced.

I NOS was expressed in microvessels and in ameboid cells within the lesion. Although iNOS was expressed in arterioles and venules, capillaries constituted the bulk of the stained vascular elements. The profiles of vessels expressing iNOS were continuous, tubular, and identical in morphology to structures stained by antibodies to factor VIII but not GFAP. The finding indicates that it was the endothelium and not the vascularly associated glial end feet or microglia where iNOS was expressed. The possibility that our antibodies cross-reacted with the constitutively expressed Ca²⁺-dependent endothelial isoform eNOS, also upregulated by ischemia (22, 33), was unlikely, because antibodies to iNOS did not stain cultured endothelial cells in which eNOS was constitutively expressed in substantial amounts. The fact that iNOS is also transiently expressed in vessels during late prenatal and early postnatal development (11) and in vascular elements associated with Alzheimer’s plaques (6) suggests that NO may function in vessels undergoing dynamic changes in response to growth or disease.

I NOS was also contained in cells distributed throughout the parenchyma of the lesion. Although their phenotype was not fully established, most were conceivably macrophages, because identical cells positive for ED-1, a marker for activated members of the monocyte-macrophage lineage, appeared in adjacent sections. The labeled cells could be of hematogenous origin (21) and/or parenchymal microglia, which can be transformed from a quiescent elongated to an ameboid phenotype (20). The absence of iNOS in astrocytes was surprising, because astrocytic iNOS can be induced in vitro within a few hours of exposure to a range of cytokines or endotoxins (10). However, its appearance may have been delayed beyond the time after MCAO in which our brains were sampled. Indeed, glial iNOS is not expressed for weeks after transient global ischemia (29).

Whereas vascular iNOS was expressed throughout the infarction, immune cells first appeared just at the site of occlusion and by 24 h were preferentially recruited to the edges of the infarction. Conceivably, vascular expression might be an early response to ischemia, and, hence, it appeared simultaneously throughout the affected areas of brain. The immune cells, in turn, may initially accumulate in the core of the infarction because of the higher impact of the ischemic episode and earlier cellular death in this zone. On the other hand, the higher accumulation of immune cells by 24 h at the edges of the infarction may be facilitated by the relatively higher blood flow in this area (12). Although unlikely, surgical trauma could contribute to some of the expression of iNOS.

The results presented here confirm the conclusions of a previous study that showed an increase in iNOS enzyme activity, peaking within 24 h and remitting by 48 h, in microvessels isolated from brains after permanent MCAO (22), whereas several differences exist with another study (18). First, they reported an expression of iNOS mRNA that was maximal at 48 h and hence delayed with respect to our study, and, second, they detected iNOS protein in neutrophiles but not in microvessels. At present, the reasons for the discrepancies are not clear, although they may be caused by differences in the respective ischemia models. Also intriguing is the observation that iNOS expression after transient MCAO (15) peaks at 24 h, and it is localized to the vascular endothelium, i.e., both the cellular localization and the time course of expression of iNOS after permanent distal MCAO are akin to those produced by transient focal ischemia, suggesting that the two models trigger comparable responses.
The association of upregulation of vascular iNOS and enhanced accumulation of activated immune cells is not surprising. Activation of the iNOS gene after ischemia is probably not an isolated event but rather part of a complex and intertwined inflammatory response that includes release of such cytokines as IL-1β (3) and expression of cell adhesion molecules in endothelium, including intercellular adhesion molecule-1 (ICAM-1; 28). The latter promotes adherence and infiltration into brain of circulating immune cells (28). Most receptors for IL-1β receptors in brain are located in microvascular walls (7), indicating that microvessels are a target for this cytokine in posts ischemic brains. A potential link between ischemia and vascular inflammatory activation might be the transcription factor NF-κB, which is rapidly activated by hypoxia and IL-1β (1) and which mediates the coordinate expression of the genes encoding for iNOS (1) and cellular adhesion molecules (1).

Although the exact mechanisms by which ischemia triggers the inflammatory network are yet not well understood, it is clear that inflammation contributes to neuronal death because blockade of IL-1 (26), immune cell infiltration (32), or iNOS activity (16, 19) reduces infarct volumes. There is already evidence of neuronal injury 30 min after focal ischemia, and it is well established that the NO released by the neuronal NOS plays a role in this early damage (14). However, there is also abrupt appearance of necrotic tissue 8–12 h after focal ischemia (5). This is also the time frame of iNOS expression after permanent MCAO, suggesting that iNOS contributes to the delayed neuronal death. In support of this, aminoguanidine is effective when administered at late posts ischemic times (16, 17), when inhibition of the neuronal NOS isoform would not provide neuroprotection.

Effect of FN Stimulation

To determine whether FN stimulation could influence the time course, intranfarction distribution, and cellular localization of iNOS, MCAO was occluded 48 h after stimulating the FN. We confirmed previous observations (12, 23, 30, 31) showing that stimulation of the FN for 1 h significantly reduced, by ~45%, the volume of a focal ischemic infarction produced 48 h later by MCAO. As shown here and elsewhere (23), such salvage cannot be attributed to changes in blood gases, AP, or nonspecific effects of stimulation, because these did not differ between groups. Moreover, stimulation of other brain areas, some of which may comparably elevate rCBF, is not protective (30). Importantly, salvage cannot be attributed to a difference in the ischemic insult between nonstimulated and stimulated brains: as we have shown elsewhere (12) the reduction in rCBF in the salvaged zone is comparable in control or FN-stimulated rats. The fact that salvage occurred when the MCA was occluded 48 h after stimulation confirms our observations that protection is long-lasting (25) and supports the view that protection does not result from elevations of rCBF associated with FN stimulation. Rather, we propose that neuroprotection involves either long-term modification of existing molecules, expression of genes encoding for protective molecules, or suppression of neurotoxic genes such as iNOS.

We investigated whether conditional stimulation of the cerebellar FN would modify expression of iNOS, particularly in the area salvaged corresponding to the ischemic penumbra. The question is of interest because the extent of ischemia in the penumbra is comparable to that in the nonretrievable area (12), i.e., the penumbra is a damaged area. Therefore, it cannot be assumed a priori that, because neurons do not die in this area, an inflammatory response will not be present. Conceivably, neuronal salvage may result from processes protecting neurons from inflammation without affecting the development of the inflammatory responses.

We have shown here that FN stimulation does in fact block two signs of inflammation: expression of iNOS in blood vessels and the accumulation of iNOS-expressing immune cells within the penumbra zone. However, a critical question arises: is the reduction of iNOS expression and recruitment of immune cells the cause or the consequence of protection? That is, does the FN act directly on the microvessels to inhibit the expression of iNOS and adhesion molecules that mediate the infiltration of activated blood immune cells, or, alternatively, does the FN reduce neuronal death by other mechanisms, thereby reducing the inflammatory signals sent to the microvasculature? Although the present report does not discriminate between the two possibilities, recent observations suggest a direct anti-inflammatory effect of the FN. First, FN stimulation reduces the vascular inflammation (expression of ICAM-1 and infiltration of leukocytes) induced by injection of IL-1 in rat striata (9). Second, FN stimulation also reduces the expression of ICAM-1 and iNOS mRNAs elicited by IL-1 in brain isolated microvessels (8). This evidence, together with the absence of inflammation in the salvaged area shown in the present study, suggests that FN stimulation may render the brain microvessels refractory to inflammation, thereby eliminating the ensuing harmful effects. The mechanism by which the response is downregulated is unknown, although a role for the perivascular noadrenergic innervation (4) may be proposed on the basis of our previous observation that norepinephrine inhibits iNOS expression (10).

In conclusion, our study indicates that a source of NO within an infarction produced by permanent focal ischemia arises not only from infiltrating macrophages but also from induction of iNOS in vascular endothelium. It also indicates that the neuroprotection elicited by exciting the FN is associated with suppression of this immune response. Because others have shown that reduction in immune reactivity can contribute to salvage, it is conceivable that the long-term protection afforded by activation of FN and related neuronal networks may be mediated, in part, by inhibition of inflammatory reactions in the microvasculature. The mechanism by which a neuronal stimulus is transferred to modify immune reactivity is unknown and is a topic under investigation. Nevertheless, the study provides further evidence that neuronal systems within brain, through a process of central neurogenic neuropro-
tection, may offer long-lasting defense of this organ to ischemia and/or hypoxia.

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