Effects of Cerebral Ischemia in Mice Deficient in Neuronal Nitric Oxide Synthase

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The proposal that nitric oxide (NO) or its reactant products mediate toxicity in brain remains controversial in part because of the use of nonselective agents that block NO formation in neuronal, glial, and vascular compartments. In mutant mice deficient in neuronal NO synthase (NOS) activity, infarct volumes decreased significantly 24 and 72 hours after middle cerebral artery occlusion, and the neurological deficits were less than those in normal mice. This result could not be accounted for by differences in blood flow or vascular anatomy. However, infarct size in the mutant became larger after endothelial NOS inhibition by nitro-L-arginine administration. Hence, neuronal NO production appears to exacerbate acute ischemic injury, whereas vascular NO protects after middle cerebral artery occlusion. The data emphasize the importance of developing selective inhibitors of the neuronal isoform.

Nitric oxide has been implicated in the pathophysiology of focal cerebral ischemia on the basis of its actions as a mediator of tissue injury. For example, NO may react with superoxide to generate a peroxynitrite anion and other oxygen radicals (*1*) and may promote nitrosylation of proteins (*2*). NO concentrations increase acutely in the brain after middle cerebral artery (MCA) occlusion, from approximately 10 nM to 2.2 μM, as detected by a porphyrinic microsensor (*3*). Brain nitrite and cyclic guanosine 3′,5′-monophosphate (cGMP) levels also rise, and these increases are effectively blocked by prior administration of the NOS inhibitor nitro-L-arginine (L-NA) (*4*).

Increases, reductions, and no changes have been reported in tissue injury after the administration of nitro-L-arginine methyl ester (L-NAME) or L-NA after MCA occlusion (*5*). L-NA and L-NAME are L-arginine analogs that nonselectively block endothelial, neuronal, and inducible NOS isoforms. On the basis of results showing that the NO precursor L-arginine decreases ischemic injury (6) by enhancing blood flow in the perinfarct area by NO-mediated mechanisms (*7*), we postulated that nonselective inhibition of NO synthesis within vessels or platelets may obscure the neuroprotective effects of NOS inhibition in neurons during focal cerebral ischemia (*5–7*).

Using mutant mice that do not express the gene for the neuronal isoform of NOS (knockout mice, Kn), we documented the consequences of MCA occlusion on cerebral infarction. As previously reported, Kn displayed normal brain cytoarchitecture and microvasculature and showed no evidence of neuronal NOS gene expression when DNA, mRNA, and proteins from brain extracts were analyzed on Southern blots (DNA), Northern blots (RNA), or protein immunoblots, respectively (*8*). Moreover, brain NOS activity was less than 5% over background activity as detected by the enzymatic conversion of radiolabeled arginine to citrulline in brain homogenates, an activity presumably due to endothelial NOS (*8*, 9). Despite this gene deletion, Kn demonstrate no gross behavioral abnormalities as compared to wild type SV129 and C57Black/6 mice (*8*).

In the present study, there were no differences in the cerebrovascular anatomy of six SV129 wild-type and six mutant mice in whom the circle of Willis and major tributaries were filled with carbon black particles through the left cardiac ventricle (*10*). The C57Black/6 wild-type mice we tested showed a similar cerebrovascular anatomy upon macroscopic inspection. Moreover, we detected no significant differences in mean systemic arterial blood pressure values of SV129 wild type and C57Black/6 wild type and mutant strains [95 ± 2 and 97 ± 4 mm Hg for SV129 (n = 10) and C57Black/6 mice (n = 5), respectively, versus 90 ± 6 mm Hg in mutant mice (n = 10)], nor were there apparent differences in the ability of the mutants (n = 4) and SV129 wild-type mice (n = 8) to autoregulate over a wide range of systemic arterial blood pressures (40 to 120 mm Hg) in preliminary studies (*11*). Hence, cerebral autoregulation appeared intact.

To examine the effects of focal ischemia on infarction size, permanent ischemia was induced by occlusion of the MCA with a nylon filament inserted through the right carotid artery (*12*). Body temperature was maintained constant (36.5° to 37.5°C), and systemic arterial blood pressure was monitored continuously until 10 min after surgery. Twenty-four hours after occlusion, the SV129 mice developed an infarct that was 93 ± 5 mm^3 in volume, or 24 ± 1% of the forebrain volume (n = 13). The C57Black/6 mice developed an infarct that was 92 ± 10 mm^3 (n = 10). Smaller infarcts were observed in Kn [57 ± 5 mm^3 (n = 16), or 15 ± 6% of the forebrain volume of SV129 and 38% smaller than those in SV129 and C57Black/6 mice, respectively; $P < 0.01$)] (Fig 1A).

Significantly smaller infarct areas were evident in all 2-mm-thick coronal slices with tissue injury (Fig. 1B). Large decreases were also observed in mutants 3 to 4 days after permanent MCA occlusion [108 ± 8 mm^3 (n = 8) and 59 ± 8 mm^3 (n = 6) for SV129 and mutant groups, respectively; $P < 0.01$]. Physiological parameters determined before and during ischemia were not different among groups (*13*). Wild type and Kn showed similar regional cerebral blood flow (rCBF) reductions when flow was recorded with laser Doppler flowmetry from homologous ischemic regions after MCA occclusion (Fig. 2) (*14*). The decreases observed in infarction volume were closely paralleled by reduced neurological deficits assessed 24 hours after occlusion by an observer naïve to the identity of the groups (*15*) (Table 1). Smaller infarcts were also observed in preliminary experiments in which the MCA was occluded at its proximal segment after craniotomy and direct vessel coagulation and transection (*16*). Increased cGMP concentrations in the brain, which result from NO binding to the heme moiety of guanylate cyclase (*17*), were significantly greater in the SV129 mice after MCA occlusion (30 min) [from 151 ± 8 (base line) to 206 ± 23 (30 min) fmol per microgram of protein; $P < 0.05$] than in the mutants [from 89 ± 19 (base line) to 89 ± 19 (30 min).... fmol per microgram of protein; $P > 0.05$] (*18*). Basal levels of cGMP were also lower in mutant brain ($P < 0.01$). Hence, the capacity to generate NO is significantly decreased in the mutant brain.

We next administered L-NA to address whether inhibition of endothelial NOS activity in the mutant increased infarct volume after MCA occlusion. The adminis-

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tered dose of L-NA was sufficient to completely inhibit pial vessel dilation to topical acetylcholine application (10 μM) in SV129 and mutant (*19*). The administered L-NA produced larger infarcts in the mutant group (*20*) [38 ± 4 (vehicle, n = 10) versus 77 ± 8 mm^3 (L-NA; n = 12)]. The D-NA group (n = 8) did not differ from control ($P > 0.05$). Attendant changes in vascular hemodynamics and platelet function (*21*) probably accounted for these findings (*5*).

This study demonstrates that lack of neuronal NOS activity in brain is associated with reduced ischemic damage after MCA occlusion. The apparent resistance of knockout animals to ischemic injury cannot be due to vascular factors, because these animals have a normally developed cerebrovasculature and rCBF reductions comparable to those of the control groups upon vessel occlusion. In fact, we have recently established that Kn mice are relatively resistant to tissue injury and cell death after transient global ischemia (*22*). Fewer dead hippocampal neurons were counted 3 days after the transient occlusion of both common carotid arteries and the basilar artery than in the control experiment.

This study supports the hypothesis that parenchymal NO overproduction may lead to neurotoxicity in focal cerebral ischemia (*23*). The precise mechanism remains to be explored, but a number of possibilities have been proposed (*23–25*): the formation of NO-iron complexes with several enzymes including complexes I and II of mitochondrial electron transport, the oxidation of protein sulfhydryls, DNA deamination, the inhibition of glyceraldehyde-3-phosphate dehydrogenase through adenosine diphosphate ribosylating mechanisms, or the formation of the potent oxidant peroxynitrite. It is proposed that increased cytosolic calcium in part, owing to activation of NMDA receptors, leads to enhanced calcium-calmodulin binding and increased NOS activity during ischemia (*23–25*). Inducible NOS activity (as assessed by conversion of ^{3}H-arginine to ^{3}H-citrulline in the presence of EDTA and calcium-free buffer, in vitro) cannot be detected in rat or mouse brain before 24 hours after permanent MCA occlusion and so this activity may not affect acute ischemic events (*26*).

Previous demonstrations of the beneficial effects of NOS inhibition in in vivo ischemia models have been complicated by the multiplicity of actions, cell types, and tissue compartments that can produce NO and by the hemodynamic and related actions of NOS inhibitors (*5*). The present study provides substantial evidence to end this controversy by demonstrating (i) increased tissue survival after cerebral ischemia in mutants lacking NO production in neurons and (ii) the reversal of the resistance to cerebral ischemic injury after inhibition of endothelial NO production by L-NA administration. The former finding is consistent with preliminary data showing that 7-nitroindazole, an inhibitor with somewhat greater selectivity for the neuronal isoform in vivo, decreases infarct size after MCA occlusion in rats (*27*). The latter finding complements previous reports showing that blood flow-enhancing effects after exogenous administration reduces ischemic injury by NO-dependent mechanisms in the ischemic penumbra (*7*). Our data also suggest that the cytotoxic effects of NO in brain during focal ischemia are not sufficiently counterbalanced by the proposed cytoprotective effects of NO that are based on in vitro data (*28*).

These data support the contention that neuronal NO production contributes to the development of ischemic brain necrosis. Because endothelial NO may protect brain tissue by increasing ischemic rCBF, selective inhibition of the neuronal isoform may offer new possiblities for the treatment of cerebral ischemia.

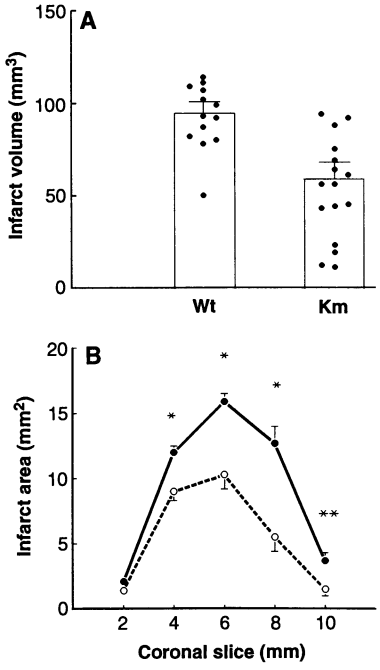

Fig. 1. Wild-type (Wt: SV-129, n = 13; Taconic Farms, Germantown, NY) and mutant (Kn, n = 16) mice subjected to MCA occlusion for 24 hours. (**A**) Each dot represents the value from an individual animal, and the data are given as mean ± SEM. (**B**) Infarction area for each of five coronal sections from rostral to caudal (2 to 10 mm) shown for SV129 (continuous line) and mutants (dotted line). Significant differences were found between the wild type and mutants in four of five sections. When the same protocol was followed with the wild-type C57Black/6 mice, infarction volumes did not differ from SV129 values and were significantly larger than those of the mutants ($P < 0.05$). Single asterisks, $P < 0.01$; double asterisk, $P < 0.05$. Physiological data are provided in (*13*).

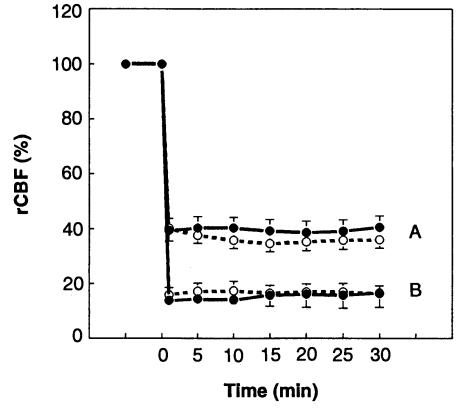

Fig. 2. Similarity in the relative changes in regional blood flow (rCBF) decrease after MCA occlusion in wild type (continuous line) and Kn (dotted line) in homologous brain regions in (**A**) the peri-infarct zone and (**B**) the MCA core territory. The rCBF level was determined simultaneously in the two regions by laser Doppler flowmetry in six animals from SV129 and mutants. In an additional four animals per group, rCBF values were recorded only in the peri-infarct zone. Time zero represents the point of MCA occlusion. Modest decreases to 39 ± 4 and 35 ± 3% of the base line in the peri-infarct zone were present in SV129 and mutant mice, respectively. More severe reductions in rCBF (15 ± 4 and 17 ± 3% of base line in SV129 and mutant mice, respectively) were present in a more deeply ischemic territory; no differences were detected between groups.

Table 1. Neurological deficit score. Neurological scoring was determined in the wild type (SV129) and knockout (Kn) mouse by the modified use of a previously published method (*15*), performed just before the mice were killed at 24 hours after MCA occlusion. The numeral abbreviations are as follows: 0, normal motor function; 1, flexion of contralateral torso and forelimb upon lifting of the whole animal by the tail; 2, circling to the contralateral side but normal posture at rest; 3, leaning to contralateral side at rest; and 4, no spontaneous motor activity.

Group	n	0	1	2	3	4	Mean ± SEM
Wt	13	0	0	4	6	3	2.92 ± 0.20
Kn	16	3	3	5	3	2	1.88 ± 0.32*

*$P < 0.05$. The data were analyzed by an analysis of variance followed by Mann-Whitney U analysis.

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10. Mice were anesthetized (as below) and perfused with 10 ml of normal saline through the left ventricle after an incision was made in the right atrium. After exsanguination, 5 ml of a 1% suspension of carbon black in India ink (particle size of 1 to 2 μm; Aldrich Chemical, Milwaukee, WI) was given through the left ventricle to enhance visualization of the cerebral arteries. R. Ward, R. L. Collins, G. Tanguay, D. Miceli, *J. Anat.* **173**, 87 (1990).
11. The mean systemic arterial pressure was varied between 9 and 154 mm Hg. Initial rCBF (laser Doppler flowmetry) was taken as 100%, and subsequent flow changes were expressed relative to this value. Systemic arterial pressure was lowered approximately 10 mm Hg every 7 min by the withdrawal of arterial blood at a rate of 30 μl/min (Harvard Apparatus, Syringe Infusion Pump, South Natick, MA). The response to hypertension was studied by the administration of 5 or 10 μg of neosynephrine intraperitoneally every 7 to 10 min. T. Dalkara, K. Irikura, Z. Huang, M. A. Moskowitz, unpublished results.
12. Wild-type and mutant male and female mice weighing 21 to 26 g were housed under diurnal lighting conditions and allowed food and water *ad libitum*. Animals were anesthetized with 2% halothane for induction and 1% halothane for maintenance in 70/30% nitrous oxide/oxygen administered by mask. Blood pressure was monitored through the femoral artery, and core temperature was measured and maintained at 36.5° to 37.5°C with a heating blanket (*6*). Occlusion of the MCA was performed by an investigator blinded to the identity of the animal groups. After occlusion of the proximal common carotid artery, a modified 6-0 nylon suture was introduced into the distal common carotid artery and advanced 10 mm distal to the carotid bifurcation. The wound was then sutured closed and the animal returned to its cage for observation over a 24-hour period. The total time for anesthesia was 40 min. Twenty-four hours later, animals were observed for behavioral changes (*15*) and killed with an overdose of pentobarbital.
13. See protocol in (*12*). Mean values for arterial pH, Pco_2, Po_2 [femoral artery; Ciba-Corning Blood Gas Analyzer 178,] 5 min before MCA occlusion were as follows (n = 12 per group; SV129 values followed by Kn values): pH, 7.25 ± 0.02 and 7.28 ± 0.02; Pco_2, 49 ± 1 and 48 ± 2; Po_2, 142 ± 9 and 138 ± 9 The values for the C57Black/6 group (n = 6) did not differ significantly from those of the SV129 group, and only the SV129 and Kn values are presented below. Physiological data (n = 5 per group) 15 min after MCA occlusion were as follows: pH, 7.27 ± 0.02 and 7.27 ± 0.02; Pco_2, 49 ± 2 and 47 ± 3; PO_2, 153 ± 7 and 171 ± 6.
14. In selected halothane-anesthetized animals, rCBF was determined by laser Doppler flowmetry (Perimed PF2B, Stockholm) and recorded on a MacLab/8 data acquisition system (ADInstruments, Milford, MA). Two flexible probe tips (Perimed PF 319:2, diameter = 0.5 mm) were secured 2 mm posterior and 3 mm lateral to bregma (peri-infarct zone) and 2 mm posterior and 6 mm lateral to bregma (MCA, deeply ischemic territory) on the ipsilateral hemisphere in animals anesthetized as above (*12*). These coordinates identify sites on the convex brain surface within the vascular territory supplied by proximal and distal segments of the middle cerebral artery, respectively. After stabilization of rCBF, the common carotid and MCA were occluded. The conlevel of rCBF was recorded continuously during occlusion and for 30 min thereafter.
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18. The concentratioon of cGMP was determined in homogenates of cortex and striatum (coronal sections 2 to 4) with a commercially available assay kit (cGMP ^{125}I scintillation proximity assay system, Amersham) (see K. Irikura, K. I. Maynard, W. S. Lee, M. A. Moskowitz, *Am. J. Physiol.* **267**, H837 (1994). Data represent the mean ± SEM of five animals per group.
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20. Mutant mice were subjected to MCA occlusion as described (*12*). Injections of L-NA, D-NA (both 2 mg/kg, intraperitoneally) or saline vehicle were given 5 min, 3 hours, and 6 hours, after MCA occlusion. The animals were killed 24 hours later, their brains were sliced into five coronal sections (2-mm-thick slices), and the tissues were stained with 2,3,5-triphenyltetrazolium chloride (*6*). The area of infarction was measured on the posterior surface of each section [BioQuant IV image analysis system (Ann Arbor, MI)], and infarct volume was calculated by numeric integration of the sequential areas. The physiological parameters of the saline-, D-NA-, and L-NA-treated groups, respectively, measured 7 hours after MCA occlusion were as follows: mean arterial blood pressure, 83 ± 2, 86 ± 3, 96 ± 5 mm Hg; arterial pH, 7.24 ± 0.2, 7.29 ± 0.3, 7.20 ± 0.02; arterial Po_2, 135 ± 10, 166 ± 10, 135 ± 14; arterial Pco_2, 50 ± 1, 37 ± 3, 49 ± 3 mm Hg.
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Laminar Comparison of Somatosensory Cortical Plasticity

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During tactile learning there is a transformation in the way the primary somatosensory cortex integrates, represents, and distributes information from the skin. To define this transformation, the site of earliest modification has been identified in rat somatosensory cortex after a change in sensory experience. Afferent activity was manipulated by clipping all except two whiskers on one side of the snout ("whisker pairing"), and the receptive fields of neurons at different cortical depths were mapped 24 hours later. Neurons in layer IV, the target of the primary thalamic pathway, were unaltered, whereas neurons located above and below layer IV showed significant changes. These changes were similar to those that occur in layer IV after longer periods of whisker pairing. The findings support the hypothesis that the layers of cortex contribute differently to plasticity. Neurons in the supragranular and infragranular layers respond rapidly to changes in sensory experience and may contribute to subsequent modification in layer IV.

The primary somatosensory cortex is reorganized during tactile learning (*1*). For example, among proficient Braille readers the fingertip used for reading elicits evoked potentials over a larger area of sensorimotor cortex than do fingertips not used for reading (*2*). The analogous observation has been made in experimental subjects. Adult owl monkeys were trained to discriminate between two frequencies of tactile stimulation of the fingertip (*3*). After several weeks of training, area 3b of the somatosensory cortex was explored with a microelectrode, and it was found that the cortical territory devoted to the stimulated skin area was expanded.

Because of the precise somatotopy of the afferent sensory pathways, the rat whisker system is an ideal model for investigating how sensory experience influences the functional organization of cortex. Each whisker on the snout projects through the thalamus to a separate cortical "barrel," a cluster of neurons in layer IV, and the barrel forms the basis of a column of neurons extending through layers II to VI (*4*). We have previously shown that receptive fields in layer IV of column D2 shift as early as 65 hours after the onset of "whisker pairing" (*5*). Whisker pairing alters the pattern of afferent activity while leaving the peripheral nerve intact: All whiskers on the right side of the snout are clipped except D2 and one of its neighbors, either D1 or D3 (Fig. 1A). Here, we carried out a laminar comparison of plasticity after just 24 hours of whisker pairing to detect the site of earliest

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