Rapid Cerebral Ischemic Preconditioning in Mice Deficient in Endothelial and Neuronal Nitric Oxide Synthases

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Background and Purpose—The purpose of this study was to test the hypothesis that nitric oxide is required for preconditioning in an intact animal model of focal ischemia using neuronal and endothelial nitric oxide synthase (nNOS and eNOS) knockout mice.

Methods—Cerebral blood flow was measured in wild-type, nNOS knockout, and eNOS knockout mice by hydrogen clearance (absolute) and laser Doppler flowmetry (relative). Mice were preconditioned by three 5-minute episodes of transient middle cerebral artery occlusion (MCAO) and subjected to permanent MCAO. Neurological deficit and infarct size were determined 24 hours later.

Results—Although wild-type mice showed protection from ischemic preconditioning, neither eNOS nor nNOS knockout mice showed protection. Laser Doppler measurements indicated that the relative blood flow decreases in core ischemic areas were the same in all groups.

Conclusions—Neither eNOS nor nNOS knockout mice show protection from rapid ischemic preconditioning, suggesting that nitric oxide may play a role in the molecular mechanisms of protection. (Stroke. 2003;34:1299-1303.)

Key Words: cerebral ischemia ■ ischemic preconditioning ■ mice, knockout ■ models, animal

Cerebral ischemic preconditioning (IPC) is a phenomenon in which brief episodes of ischemia protect the brain from subsequent, more severe ischemic insult.1,2 IPC has been divided into rapid and delayed forms. Rapid IPC occurs when the preconditioning stimuli precede the severe ischemic insult by a short time interval (minutes to several hours); delayed IPC requires a longer time interval (hours to days). In many models of delayed IPC, new protein synthesis is required, suggesting that subsequent changes in gene expression may underlie delayed IPC. However, it is clear that some changes in gene expression occur extremely rapidly, so there may be considerable overlap in the mechanisms of rapid and delayed IPC.

The molecular mechanisms of IPC are not fully understood, although several potential protective mechanisms have been identified. These include alterations in cell death genes,3 heat shock proteins,4 lipid peroxidation,5 inflammation,6 and mitochondrial metabolism.7 It is likely that there are multiple protective mechanisms, and the precise molecular mechanisms may depend on the nature of the preconditioning stimuli and the specific model.

Nitric oxide (NO) has been implicated in several models of cerebral preconditioning. Gidday et al8 found that hypoxic preconditioning of newborn rats induced protection against subsequent hypoxia 6 days later. Protection was blocked by the nonselective nitric oxide synthase (NOS) inhibitor l-nitro-arginine (L-NA) but not 7-nitro indazole (7-NI) or amino guanidine, which block neuronal (nNOS) and inducible (iNOS) NOS, respectively, suggesting that endothelial NOS (eNOS) might be responsible. Puisieux et al9 found that infarct size from middle cerebral artery occlusion (MCAO) was reduced by predministration of lipopolysaccharide (LPS) and that this effect was blocked by the nonspecific NOS inhibitor 7-nitro-l-arginine methyl ester (L-NAME). Furthermore, LPS administration resulted in upregulation of eNOS in cerebral blood vessels. However, in this same model system, preconditioning by ischemia itself did not appear to be mediated by NO. Thus, the precise role of NO in IPC is unclear.

The present study was designed to test whether eNOS or nNOS is required for preconditioning in an intact animal model of rapid cerebral IPC using intraluminal MCAO. In this model, 3 episodes of ischemia for 5 minutes each significantly reduce infarct size in wild-type mice from ipsilateral permanent MCAO 30 minutes later.10 We applied this model to eNOS and nNOS knockout mice and determined infarct size and neurological deficits. We measured baseline absolute cerebral blood flow (CBF) to determine...
whether there were differences between genotypes at baseline. We used laser Doppler flowmetry to monitor relative CBF over the core ischemic area to verify that IPC and MCAO caused equivalent reductions in blood flow in the 3 genotypes.

Materials and Methods

Mice
nNOS knockout mice\(^\text{11}\) and eNOS knockout mice\(^\text{12}\) were generated from J1 embryonic stem cells of SV129 origin.\(^\text{13}\) To avoid genetic background effects, each knockout mouse strain was backcrossed to the C57BL/6 strain (Jackson Laboratories) for 10 generations to obtain congenic animals.\(^\text{14}\) C57BL/6 wild-type mice were used as control animals. Mice 8 to 12 weeks of age weighing 19 to 28 g were used for all experiments. All experiments were approved by the Institutional Animal Care and Use Committee. Mice were euthanized according to the recommendations of the American Veterinary Medical Association Panel on Euthanasia.

Absolute Blood Flow Measurements
Quantitative regional CBF (rCBF) was measured in the parietal cortex and striatum by the hydrogen clearance method\(^\text{15}\) in wild-type, eNOS knockout, and nNOS knockout mice (n=10 for each group). Mice were anesthetized with urethane (0.8 g/kg IP), paralyzed with pancuronium bromide (0.1 mg/kg IV), and mechanically ventilated with 30% oxygen/balance nitrogen using a small animal respirator (Edco) with settings of tidal volume at 0.4 mL and rate at 110/min. The mice were placed in a stereotaxic frame (David Kopf Instruments), and after craniotomy, H\(_2\)-sensitive electrodes were inserted into the striatum using stereotaxic coordinates (AP, +1.25 mm; ML, +3.0 mm; V, 1.0 mm lateral to bregma). Electrodes were made from platinum wire 100 \(\mu\)m in diameter insulated with epoxy except for 1.0-mm length at the pointed tip (15 to 20 \(\mu\)m) covered by Nafion membrane to protect the electrode from fouling.

Mean arterial pressure and temperature were monitored continuously, and temperature was maintained at 37°C with a heating blanket (FHC). For rCBF measurements, hydrogen gas (2.5% in air) was added via a ventilating pump for 60 seconds. The hydrogen-containing gas was then switched to the base breathing gas, and washout curves were recorded. Absolute values of rCBF (mL per 100 g tissue per minute) were calculated by the initial slope method using Mathematica 3.0 software (Wolfrom Research, Inc) with minor modifications.\(^\text{15}\)

IPC and MCAO
Mice were anesthetized with 1.5% isoflurane in a mixture of 30% oxygen and 70% nitrous oxide. Surgery was conducted on spontaneously breathing mice. A flexible 0.5-mm fiberoptic probe was affixed to the skull over the brain cortex supplied by the MCA (2 mm posterior and 7 mm lateral to bregma) for relative CBF measurements by laser Doppler flowmetry (Perimed). Baseline CBF values measured before internal carotid artery (ICA) occlusion were defined as 100% flow. An incision was made in the external carotid artery, and a silicon-covered 8-0 nylon monofilament was advanced through the ICA to the origin of the anterior cerebral artery to occlude the MCA and posterior communicating artery. MCAO was documented by laser Doppler flowmetry over the lateral parietal cortex during IPC (core ischemic zone) is presented in Figure 1. Baseline CBF recorded before ICA ligation under steady-state conditions was defined as 100% flow. After ligation of the ICA, CBF decreased to 66±15% in wild-type mice, 78±17% in eNOS knockout mice, and 76±11% in nNOS knockout mice. The decrease in relative CBF after ICA ligation was significantly greater in wild-type mice compared with eNOS and nNOS knockout mice (P<0.05). Ischemia was confirmed when the laser Doppler signal was reduced to <20% of control values.

Cerebral Blood Flow during Reperfusion
During reperfusion, CBF returned to preischemic levels by the 10th minute of each reperfusion cycle. CBF levels at the 30th minute between preconditioning stimuli and permanent MCAO were not significantly different between groups: 51±31% of preischemic level in wild-type mice, 55±24% in eNOS knockout mice, and 50±23% in nNOS knockout mice.
In mice that underwent IPC without subsequent MCAO, CBF was restored to preischemic levels within 60 minutes after the third IPC stimulus (data not shown).

Mean arterial blood pressure was 97±15 mm Hg before MCAO and did not differ significantly after MCAO in all groups.

**Infarct Size**

IPC induced significant neuroprotection against damage from subsequent permanent MCAO in wild-type mice, as shown in Figure 2. Cerebral infarct volumes were significantly reduced in wild-type mice treated with IPC (92.5±30.2 mm$^3$) compared with unpreconditioned mice (119.4±20.0 mm$^3$, $P<0.05$). There were no significant differences in cerebral infarct volumes between eNOS knockout mice subjected to IPC (105.0±13.1 mm$^3$) and those not subjected to IPC (116.5±26.6 mm$^3$). The infarct volumes of nNOS knockout mice without IPC (81.1±30.9 mm$^3$) were significantly less than in wild-type animals without IPC ($P<0.05$). There was no neuroprotection in preconditioned nNOS knockout mice (94.2±25.5 mm$^3$) compared with unpreconditioned nNOS knockout mice.

The infarct areas in 6 coronal slices from rostral to caudal are presented in Figure 3. There were significant decreases in infarct area in preconditioned wild-type mice compared with unpreconditioned mice at the third and fourth coronal sections, located 6 mm and 8 mm from the rostral surface of brain ($P<0.05$). Neither eNOS or nNOS knockout mice showed differences in infarct areas between preconditioned and unpreconditioned mice.
Neurological Deficit

The reduction in infarct size in preconditioned wild-type mice is paralleled by functional changes in neurological deficits, as shown in the Table. However, eNOS knockout mice showed no significant difference with or without preconditioning. The neurological deficit score of un preconditioned nNOS knockout mice was decreased compared with un preconditioned wild-type mice, and preconditioned nNOS knockout mice did not show a reduction in deficit score. Sham-operated animals showed minor deficits (data not shown), similar to mice treated with IPC alone.

Discussion

We previously described a mouse model of rapid preconditioning in focal cerebral ischemia. Three episodes of MCAO preconditioning, each lasting 5 minutes, protect the brain against damage from subsequent permanent MCAO performed 30 minutes later. Infarct size measured 24 hours later is reduced in preconditioned animals. In this study, we applied this rapid IPC model of focal ischemia to eNOS and nNOS knockout animals. We found that although wild-type mice demonstrate a reduction in infarct size after 3 cycles of IPC, neither eNOS knockout mice nor nNOS knockout mice do. Baseline absolute blood flow measurements by the hydrogen clearance method are the same in the 3 genotypes, so differences in the baseline absolute rCBF do not account for the results. Relative blood flow measurements by laser Doppler flowmetry confirm effective MCAO with each preconditioning episode in each of the 3 genotypes.

To date, there have been 2 studies on NO in IPC in intact animal models. Both suggest that eNOS may mediate protection against ischemia. In the newborn rat hypoxia model, hypoxic preconditioning protection against subsequent hypoxia was blocked by the nonselective inhibitor L-NA but not 7-NI (nNOS-specific inhibitor) or amino guanidine (iNOS-specific inhibitor). In a rat model of MCAO, Puisieux et al found that both transient MCAO and LPS administration could precondition. LPS was associated with increased eNOS expression, and its effects were blocked by L-NAME, implicating eNOS in the protection. However, L-NAME had no effect on IPC. The possible reasons for this discrepancy include differences in species (rat versus mouse), differences in models (1 episode of 3 minutes of transient MCAO versus 3 episodes of 5 minutes of MCAO), and incomplete blockade of eNOS expression by L-NAME. This last possibility is highlighted by the lack of effect of L-NAME on unconditioned infarct size in this study. One potential mechanism for eNOS would be augmentation of blood flow, eg, vasodilation of cerebral vessels supplying the ischemic penumbra. Other potential mechanisms involve leukocyte-endothelial interactions, or platelet-endothelial interactions, that might be modulated by eNOS.

We should note some potential limitations to this study, related to the blood flow measurements, a possible ceiling effect, and the preconditioning model. First, although we confirmed that the preconditioning stimuli and MCAO resulted in an equivalent reduction in CBF in the core ischemic zone by laser Doppler flowmetry, this does not rule out differences in blood flow in the ischemic penumbra in preconditioned wild-type animals. Further work using techniques that allow simultaneous measurements of spatial and temporal blood flow is needed to test this possibility. Our CBF measurements were all made in anesthetized animals, which may decrease the likelihood of finding CBF differences among different groups of mice. Second, in this study, infarct sizes in eNOS knockout mice and wild-type mice were equivalent, although the infarcts in our wild-type mice were larger than previously reported (possibly because of operator-dependent effects). These results raise the possibility of a ceiling effect—ie, that the model we used results in a maximal infarct volume, making it difficult to show enlargement of infarct size in the eNOS knockout mice or protection by preconditioning in these same animals. Third, in our preconditioning model, ICA ligation resulted in a greater decrease in CBF in wild-type mice (to 65.8% of baseline) compared with both eNOS knockout (78.3% of baseline) and nNOS knockout mice (75.8% of baseline). We do not know whether these differences might account for the lack of preconditioning in the knockout animals. When we measured neurological deficits, we found that IPC alone and sham-operation resulted in similar minor deficits in all groups. Although there were no significant differences between the wild-type, nNOS knockout, and eNOS knockout groups, the minor deficits do raise the possibility that repeated insertions of the filament may itself induce vascular injury. Finally, we measured infarct size at 24 hours but did not measure whether the protection extended to later periods. Thus, we do not know whether this model results in lasting benefit or whether there is only an early reduction in infarct size.

### Neurological Deficit Scores

<table>
<thead>
<tr>
<th>Genotype</th>
<th>3 IPC Alone (n=5, each group)</th>
<th>MCAO (n=10, each group)</th>
<th>3 IPC+MCAO (n=10, each group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.4±0.5</td>
<td>3.1±0.6*</td>
<td>2.7±0.5</td>
</tr>
<tr>
<td>eNOS knockout</td>
<td>1.6±1.3</td>
<td>2.7±1.1</td>
<td>2.8±0.4</td>
</tr>
<tr>
<td>nNOS knockout</td>
<td>1.4±0.5</td>
<td>1.7±1.1</td>
<td>2.2±1.6</td>
</tr>
</tbody>
</table>

Neurological scores, on a 5-point scale, ranged from 0 (no deficit) to 4 (severe deficit) in mice treated with three 5-minute episodes of IPC alone (3 IPC alone), MCAO alone (MCAO), or 3 episodes of IPC followed 30 minutes later by MCAO. Scoring was done 24 hours after MCAO by observers blinded to genotype and procedure of the animals. Values are mean±SE. *p<0.05 vs wild-type mice treated with 3 IPC alone and nNOS knockout mice treated with MCAO alone.
In summary, to the best of our knowledge, these are the first studies to apply an intact animal model of rapid IPC to eNOS and nNOS knockout animals. Our results indicate that eNOS and nNOS knockout mice fail to show protection from a rapid model of IPC. Further work is necessary to delineate the biochemical and molecular mechanisms by which eNOS and nNOS may mediate IPC. The possibilities include (1) a requirement for NO in the mechanisms of vascular protection, and (3) a role for NO in mediating innate neuronal resistance to ischemia. NO interacts with at least 2 signaling pathways important to neuronal survival: the Ras/Raf/MEK/ERK cascade20,21 and the PI3 kinase/Akt pathway.22,23 Future work will focus on the oxidative stress mechanisms for IPC, possible changes in gene transcription resulting from these stresses, and whether NO acts at the vascular or neuronal level or both.

Acknowledgments

This work was supported by PHS grants 2R01-NS33335 and 5PS0-NS10828 from the NINDS, NIH.

References

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*Stroke*. 2003;34:1299-1303; originally published online April 3, 2003; doi: 10.1161/01.STR.0000066870.70976.57
*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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