Stroke Outcome in Double-Mutant Antioxidant Transgenic Mice

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Background and Purpose—Both NO and superoxide cytotoxicity are important in experimental stroke; however, it is unclear whether these molecules act within parallel pathological pathways or as coreagents in a common reaction. We examined these alternatives by comparing outcomes after middle cerebral artery occlusion in male and female neuronal NO synthase (nNOS)-deficient (nNOS−/−) or human CuZn superoxide dismutase–overexpressing (hSOD1+/−) mice and a novel strain with both mutations.

Methods—Permanent middle cerebral artery occlusion was performed by use of the intraluminal filament technique (18 hours). Neurological status was scored, and tissue infarction volume was determined by 2,3,5-triphenyltetrazolium staining and image analysis.

Results—Hemispheric infarction volume was reduced in each transgenic strain relative to the genetically matched, wild-type, control cohorts (WT mice): nNOS−/− (80±6 mm3) and double-mutant (49±6 mm3) mice versus WT mice (114±7 mm3) and hSOD1+/− mice (52±7 mm3) versus WT mice (95±5 mm3). Human CuZn superoxide dismutase had a larger effect on mean infarction volume (30% of contralateral hemisphere) than did nNOS deficiency (46%). Although infarction volume was less in double-mutant mice compared with nNOS−/− mice, injury was not improved relative to hSOD1+/− mice. There was no difference in histological damage by sex within each strain; however, female nNOS−/− mice were not protected from ischemic injury, unlike male mutants.

Conclusions—Superoxide generation contributes to severe ischemic brain injury in vivo to a greater extent than does neuronal derived NO. In vivo, significant superoxide scavenging by CuZn superoxide dismutase occurs within cellular compartments or through biochemical pathways that are not restricted to, and may be distinct from, neuronal NO/superoxide reaction and peroxynitrite synthesis. (Stroke. 2000;31:2685-2691.)

Key Words: cerebral ischemia ■ gender ■ middle cerebral artery occlusion ■ nitric oxide synthase ■ stroke ■ superoxide dismutase ■ mice

Outcome from experimental stroke is linked to a complex intertwining schema of pro-oxidant mechanisms. Both NO and superoxide anion are thought to be essential actors in oxidative injury during cerebral ischemia. Neuronal overproduction of NO has been consistently postulated to occur in the ischemic brain, leading to the death of neighboring cells. In part, NO toxicity is explained as a consequence of its propensity to react with superoxide at extremely fast rates and form the potent oxidant peroxynitrite (ONOO−). ONOO− is lipid soluble and has a wide assortment of potential oxidation targets, including proteins, RNA, and DNA.1,2 Some of the neurotoxicity of ONOO− results from depression of mitochondrial respiration3,4 and from DNA strand breakage, which stimulates energy-consuming DNA repair processes involving poly(ADP-ribose) polymerase.5,6 Pharmacological inhibitors of NO synthase (NOS) or genetic deficiency of the neuronal enzymatic isofrom (nNOS) reduces NO/ONOO− toxicity and improves cell survival in many experimental paradigms (References 7 and 8; for review, see Reference 9). In turn, superoxide anion has long been recognized as a key oxidant species in brain injury. Numerous sources of enhanced superoxide production have been identified, including anoxic mitochondria, activated microglia and neutrophils, and membrane-bound oxidases. Therapeutic maneuvers de-

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Received March 27, 2000; final revision received June 26, 2000; accepted July 20, 2000.
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Under an agreement between the Johns Hopkins University and Guilford Pharmaceuticals, Drs T.M. Dawson and V.L. Dawson are entitled to a share of sales royalty received by the University from Guilford. Dr T.M. Dawson and the University also own Guilford stock, and the University stock is subject to certain restrictions under University policy. The terms of this arrangement are being managed by the University in accordance with its conflict-of-interest policies.
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Stroke is available at http://www.strokeaha.org

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signed to limit intraischemic increases in intracellular superoxide concentration also achieve neuroprotection (for review, see References 10 and 11). Compared with nontransgenic mice, mice overexpressing human CuZn superoxide dismutase (hSOD1) sustain less brain injury after middle cerebral artery occlusion (MCAO)12; extracellular superoxide dismutase (SOD)–overexpressing strains are protected in a similar manner.13 Furthermore, overexpression of manganese SOD, the mitochondrial isof orm, reduces NO and ONOO− toxicity in vitro.14,15

Although it is clear that reactions involving NO or superoxide are activated in the ischemic brain, it is not known whether these molecules act within parallel pathological pathways or as coreagents in a common toxic reaction. Furthermore, it has been difficult to independently manipulate these agents in the intact brain or to determine relative contributions of NO release and superoxide generation in an animal model of neuroinjury. We hypothesized that both NO and superoxide must be present in sufficient amounts and in proximate tissue compartments if ONOO− synthesis is to be fueled. If either NO or superoxide concentration is largely ablated, then lower levels of ONOO− could result, with decreased injury to intracellular targets. Under these conditions, salvage of tissue from injury would not be different, regardless of whether NO, superoxide, or both are reduced.

Alternatively, enhanced SOD1 activity through non-NO overlapping mechanisms could directly benefit the injured brain. Under these conditions, enhanced superoxide scavenging might benefit the brain in a manner additive or synergistic to that of the nNOS+/− mice. The aim was to determine whether nNOS−/−,hSOD1+/− mice exhibit improved function and smaller tissue injury after MCAO compared with mice with a single mutation.

**Materials and Methods**

The present study was conducted in accordance with the National Institutes of Health guidelines for the care and use of animals in research and under protocols approved by the Animal Care and Use Committee of the Johns Hopkins University. Three age- and weight-matched transgenic strains were studied, and each was compared with its genetically matched wild-type strain (WT). The animal groups were as follows: (1) homozygous nNOS-null mice (nNOS−/−, founder stock14; n=33 total, 22 males and 11 females); (2) heterozygous hSOD1 overexpressors (hSOD1+/− mice), and a novel strain in which both mutations were combined (nNOS−/−,hSOD1+/− mice). The aim was to determine whether nNOS−/−,hSOD1+/− mice exhibit improved function and smaller tissue injury after MCAO compared with mice with a single mutation.

Transgenic mice had been backcrossed to the WT mice >8 times at the time of the present study. Because nNOS−/− mice were therefore >99% genetically identical to WT mice, the C57Bl/6 mice from Charles River were used as the nNOS−/− control cohort. The hSOD1-overexpressing mice were produced as previously described in 1995.15 These animals were originally produced in the C57Bl/6J×Hel hybrid strain, initially backcrossed to this same hybrid, and then subsequently bred to the C57Bl/6J strain (Charles River). The level of hSOD1 to endogenous mouse SOD1 activity in brain is 8:1, with a transgene product distribution quite similar to that of endogenous mouse enzyme.17 The hSOD1 transgenic mice have been extensively backcrossed to the C57Bl/6J WT mice, resulting in near genetic confluence. Therefore, this WT mouse was used in control comparisons with the hSOD1 mutant.

To develop the novel double mutants, hSOD1+/− mice were bred to WT C57Bl/6 mice, and the colony was screened by use of one PCR primer common for mouse and human SOD (5′-GTT ACA TAT AGG GGT TTA CTT CAT AAT CGT-3′) and human/mouse SOD primers (5′-CAG CAG TCA CAT TGC CCA (A/GGT CTC CAA CAT G-3′). The presence of mouse SOD on PCR served as an internal PCR control, because all mice expressed mouse CuZn SOD. Female hSOD1+/− mice were then bred to nNOS−/−, male mice, creating nNOS heterozygotes, some of which also overexpressed SOD (nNOS−/−,SOD+/−/− mice). These double heterozygotes were then bred to produce nNOS-null SOD-overexpressing mice (nNOS−/−,SOD+/−/− mice). This new colony was screened by using PCR primers for nNOS and SOD as described above. The PCR primers were designed to confirm the absence of nNOS (5′-CTT TCA TCT CTG CTT TGG CTG GTG ATC CTA G-3′) and the presence of neomycin (5′-CAC CAT GAT ATC CGA C-3′), 5′-TGG AGA GGC TAT CGT ATG AC-3′).

**Ischemic Model**

Mice were anesthetized with 1% to 1.2% halothane in oxygen-enriched air by face mask. The femoral artery was cannulated for measurement of arterial blood gases and blood pressure. Rectal temperature was controlled at near 37°C throughout the experiment with heating lamps and water pads for all animals. After baseline arterial blood gas measurements, intracarotid MCAO was performed with use of an intraluminal filament-insertion technique. The proximal common carotid artery was ligated, and a 6-0 nylon monofilament was inserted and advanced into the internal carotid artery to a distance of 6 mm from the internal carotid/pterygopalatine artery bifurcation to the suture tip. Intraischemic arterial blood pressure and blood gases were determined after occlusion, then the catheters removed, and anesthesia was discontinued. Neurological deficit was confirmed in each animal at 1 hour of occlusion. Neurological deficit was scored as follows: 0, no deficit; 1, forelimb weakness; 2, circling to affected side; 3, unable to bear weight on affected side; and 4, no spontaneous motor activity. If no deficit was observed, the animal was removed from the study cohort. After 18 hours of MCAO, neurological status was again scored, and the brain was harvested for analysis. Infarction volume was determined by 2,3,5-triphenyltetrazolium staining in five 2-mm slices and evaluated via digital planimetry.

**Statistical Analysis**

All data are expressed as mean±SE. Physiological variables and histology were analyzed by 1-way ANOVA with a post hoc Newman-Keuls test to correct for multiple comparisons between transgenic strains. Postischemic neurological scores were analyzed by the Mann-Whitney U test. Statistical comparisons were made between each transgenic strain and its appropriate WT control group. Because SOD1 overexpressors were originally developed from C57Bl/6J mice, this additional cohort was incorporated into the study design. For simplicity, we have presented the data analysis so that the nNOS−/− and double-mutant mice were compared with their commonly held WT background. Alternatively, when data from the nNOS−/− and double-mutant mice were statistically compared with either WT cohort of the study, the same
result was obtained. Last, animals of both sexes were used in each group. Accordingly, a post hoc analysis of histology was carried out to determine whether there were sex differences in infarction within each genetic group and whether the effect of the mutation was the same in both males and females.

Physiological Measurements

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight, g</th>
<th>Time</th>
<th>pH</th>
<th>PaO2, mm Hg</th>
<th>PaCO2, mm Hg</th>
<th>Rectal Temperature, °C</th>
<th>MAP, mm Hg</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT NOS</td>
<td>24±0.8</td>
<td>Baseline</td>
<td>7.36±0.01</td>
<td>142±6</td>
<td>30±1</td>
<td>37.2±0.2</td>
<td>82±2</td>
</tr>
<tr>
<td>nNOS−/−</td>
<td>23±0.7</td>
<td>Baseline</td>
<td>7.34±0.01</td>
<td>124±4†</td>
<td>35±1†</td>
<td>37.8±0.2</td>
<td>86±1</td>
</tr>
<tr>
<td>nNOS−/−, SOD1+−</td>
<td>23±0.7</td>
<td>Baseline</td>
<td>7.35±0.01</td>
<td>145±4</td>
<td>32±1†</td>
<td>37.3±0.2</td>
<td>84±1</td>
</tr>
<tr>
<td>WT SOD1</td>
<td>25±0.8</td>
<td>Baseline</td>
<td>7.34±0.01</td>
<td>115±4</td>
<td>36±1</td>
<td>37.7±0.2</td>
<td>84±1</td>
</tr>
<tr>
<td>SOD1+−</td>
<td>23±0.5</td>
<td>Baseline</td>
<td>7.34±0.01</td>
<td>123±4†</td>
<td>36±1†</td>
<td>37.5±0.2</td>
<td>81±1</td>
</tr>
</tbody>
</table>

Values are mean±SE. MAP indicates mean arterial pressure. Groups are as follows: WT controls for nNOS−/− and double mutants, n=20; nNOS−/− nNOS-deficient transgenics, n=33; nNOS−/−, SOD1+− double mutants, n=25; WT for SOD1+− human CuZn SOD overexpressors, n=34; and SOD1+− heterozygotes overexpressing human CuZn SOD, n=18.

Results

Arterial blood pressure, body temperature, and blood gas composition before and during MCAO remained within physiological range in all groups (Table). In general, intraschismic values were comparable among transgenic groups. Figure 1 summarizes total hemispheric infarction volume in each animal cohort, expressed as a percentage of the contralateral hemisphere. First, each genetic modification reduced stroke relative to the WT mouse. Infarction volume was smaller in nNOS−/− mice (80±6 mm³) and in the double-mutant mice (49±6 mm³) than in the WT control mice (114±7 mm³). Similarly, injury was reduced in hSOD1+− mice (52±7 mm³) compared with their background WT mice (95±5 mm³). Neurological scores at 18 hours reflected these histological differences. Consistent with the severity of the ischemic insult, distinct functional deficits were observed in all animals, except for 2 females within the hSOD1+− group, who were accorded a score of 0. Outcome score was improved in nNOS−/− (2.5±0.2) and double-mutant (1.9±0.1) mice compared with WT mice (3.1±0.2). Similarly, outcome was improved in hSOD1+− (1.6±0.2) compared with WT (3.0±0.1) mice. Second, hSOD1 overexpression had a larger effect on infarction volume than did nNOS deficiency. Mean infarction volume was 46% of the contralateral hemisphere in nNOS−/− mice compared with 30% in hSOD1+− transgenic mice. However, neurological scores were not different between the nNOS−/− and hSOD1 groups. Third, damage in the double-mutant mice was not reduced relative to each single mutation. Although infarction volume in double-mutant mice was less than that observed in mice with the single mutation (nNOS−/−), the injury in double-mutant mice was not different from that in hSOD1+− mice. The neurological outcome score again paralleled improvements in tissue injury. Although double-mutant animals scored better than did the nNOS−/− animals after MCAO, the outcome scores were the same in double-mutant (1.9±0.1) and hSOD1+− mice (1.6±0.2) animals.

We also evaluated the effect of each mutation separately in male and female animals on a post hoc basis to identify potential sex bias in the study. There was no absolute difference in infarction between males and females within any genetic group. However, there was one large sex difference when the protection provided by the mutation was assessed relative to WT control animals. Whereas male
nNOS−/− animals exhibited a clear reduction in histological damage compared with male WT animals (Figure 2A), the female animals did not benefit from nNOS deficiency (Figure 2B). However, the females did benefit from hSOD1 overexpression or the double mutation. In hSOD1 overexpressors, infarction volume was reduced in both sexes relative to their respective WT (Figure 3), although the effect was less robust in male hSOD1+/− animals (Figure 3B). Both male and female nNOS−/−,hSOD1+/− mice sustained equivalent total infarction volumes, and each sex was protected compared with its respective WT (Figure 4).

In view of a potential for sex bias in the nNOS−/− group, we repeated the analysis of infarction volume for this group when limited to only male animals. Infarction volume remained smaller in male nNOS−/− mice (76±8 mm³, n=22) compared with WT control mice (125±11 mm³).

Discussion
The present study presents 2 main findings. First, double-mutant mice of both sexes that are nNOS deficient and overexpress hSOD1 sustain reduced tissue infarction relative to their genetically matched WT counterparts. However, each single mutation is not equipotent in reducing ischemic injury in vivo, because the efficacy of reducing nNOS can be increased by hSOD1 availability. Conversely, SOD1 overexpression is equally efficacious in the presence or absence of neuronal NO generation, suggesting that superoxide generation contributes more to damage than does NO toxicity after severe ischemia. Therefore, CuZn SOD may scavenge superoxide within cellular compartments or through biochemical pathways that are not restricted to, and may be distinct from, neuronal NO/superoxide reaction and ONOO− synthesis. Second, these experiments demonstrate a novel sex-based difference in stroke pathophysiology. Whereas nNOS null mice are protected in experimental stroke, as previously reported, this protection appears to be limited to the male and is not demonstrated in the female. Furthermore, sex bias in stroke outcome was not generalized to all transgenic strains in the present study. This apparent specificity could suggest that fundamental neuronal NO mechanisms of ischemic injury are redirected in the female mutant during cerebral ischemia.
The double-mutant strain was constructed to evaluate conditions in which both neuronal NO and superoxide levels are theoretically optimized for ischemic neuroprotection. We reasoned that if both NO and superoxide were requisite as coreagents in ONOO− formation, then no additional effect would be observed by a combined reduction of both radical species. For example, a single previous study used the nNOS inhibitor 7-nitroindazole as a means of further reducing injury within the 18 hours of occlusion.19 We did not measure cerebral blood flow in these experiments and so cannot comment on blood flow–associated mechanisms of protection. Furthermore, although the middle cerebral artery was permanently occluded in all animals, leading to a distinct neurological deficit, residual ischemic blood flow could have been different among the 3 transgenic species at any point within the 18 hours of occlusion.

The present investigation was not constructed to prospectively evaluate sex differences in the various transgenic strains. However, in view of increasing reports of sex-linked differences in outcome from neuroinjury, we studied large cohort sizes to ensure a reasonably equivalent male-to-female animal ratio. Female animals have been shown to sustain reduced stroke damage relative to males in rats,30 gerbils,31 and mice,32 and this protection is linked to female sex steroids, particularly estrogen (for review, see Reference 33).

**Figure 4.** Stroke damage in male (A) and female (B) nNOS−/−, SOD1+/− mice. Hemispheric infarction by brain slice is shown as percentage of contralateral nonischemic side (contra hemi). *P<0.05 vs transgenic mice.
The present finding that female mice do not benefit from nNOS deficiency is one of the first demonstrations of a sex-based difference in a fundamental cellular mechanism of cell injury. Further experiments are needed to fully understand this observation and to determine whether the finding is replicated in reversible MCAO or other ischemic models. If estrogen (or progesterone) is implicated in this surprising result, it remains to be shown. If so, then the lack of nNOS-mediated neuroprotection in females may suggest that female sex steroids alter ischemic injury cascades at a point upstream from nNOS activation. Alternatively, estrogen and/or progesterone may protect the brain by a mechanism that parallels the genetic “blockade” of neuronal NO toxicity. Recent work indicates that female mice are also not protected by genetic deficiency in inducible NOS after permanent MCAO, unlike male mice. Interactions between sex steroids and NO toxicity could be quite complex because estrogen alters the expression of endothelial NOS and inducible NOS.

In contrast, both male and female mice benefit from hSOD1 overexpression or the double mutation during permanent focal ischemia. These results confirm many previous reports in male mutants with global cerebral ischemia and with reversible, but not permanent, MCAO. There are a large number of methodological differences between the present experiments and this earlier report, including differing WT backgrounds (C57Bl/6 versus CD-1) and methods of transgenic strain development, duration of MCAO (18 versus 24 hours), size of occlusive monofilament (5.0 versus 6.0), and analytic methods for correction of edema. It should also be noted that the largest difference is that the study of Chan et al was conducted in all male animals. Even in the present study, in which a significant effect could be seen, the protection in male hSOD1 mice was not striking compared with that observed in female hSOD1 mice. It may be that native SOD1 activity is lower in female mice; hence, overexpression is highly beneficial in the female. Further studies are needed for exploration of this issue and for comparative quantification of hSOD1 transgene expression products in male versus female mutants.

In conclusion, stroke damage in a novel strain of nNOS-deficient hSOD1-overexpressing mice was equivalent to that achieved with the single hSOD1-overexpression mutation. SOD1 overexpression reduces acute stroke damage by mechanisms not restricted to nNOS generation.

Acknowledgments

This study was funded by US Public Health Service grants NS-33668, NR-03521, NS-20020, and NS-37090 (Dr T.M. Dawson) and NS-37460 (Dr V.L. Dawson). Dr T.M. Dawson is an Established Investigator of the American Heart Association. Dr V.L. Dawson is a Mary Lou Mcllhany Scholar and a Stulgin Music Festival NARSAD Investigator.

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Both superoxide anion and nitric oxide (NO) have been implicated in the pathogenesis of ischemic brain injury. Genetically engineered mice have been used to delineate the function of single genes that alter superoxide anion or neuronal NO formation in cerebral ischemia. Thus, mice overexpressing CuZn superoxide dismutase (SOD) with enhanced capacity to scavenge superoxide anion were more resistant to ischemic insult.1 Similarly, mice deficient in neuronal nitric oxide synthase (nNOS) sustained lesser degrees of ischemic brain injury.2 Superoxide anion and NO interact to form peroxynitrite, a highly reactive and toxic free radical species that causes tissue damage.3 Sampei and coworkers applied a novel mouse strain harboring double mutations characterized by CuZn SOD overexpression and nNOS deletion to explore possible synergistic or additive neuroprotective effects of reducing superoxide anion and neuronal NO generation. An important observation in the present study is the comparison of the effectiveness of the 2 types of mutations, namely, nNOS deletion versus CuZn SOD overexpression, in mice with otherwise identical genetic backgrounds. This study is the first to show CuZn SOD overexpression to be more potent than nNOS deletion in protecting the brain from ischemia. The findings are also interesting in that mice with double mutations were better protected than mice deficient in nNOS. In contrast, CuZn SOD overexpression alone was as effective as double mutations in reducing ischemic brain injury. These results strongly suggest a broader action of superoxide anion, beyond its interaction with NO to form peroxynitrite, in the pathogenesis of ischemic brain injury.

The senior author of the present study, Dr Hurn, is a leading authority in gender differences in brain vulnerability to ischemic insult.4 It is interesting to note a gender effect of nNOS deletion such that male but not female mice benefited from nNOS deletion. A possible gender difference is also noted in mice overexpressing CuZn SOD. The gender effects disclosed by gene manipulations provide important insights into the interactions of female hormones with ischemia-induced brain injury cascades.

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Stroke. 2000;31:2685-2691
doi: 10.1161/01.STR.31.11.2685

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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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