Human Copper-Zinc Superoxide Dismutase Transgenic Mice Are Highly Resistant to Reperfusion Injury After Focal Cerebral Ischemia

Guoyuan Yang, MD; Pak H. Chan, PhD; Jun Chen, MD; Elaine Carlson, BS; Sylvia F. Chen, PhD; Philip Weinstein, MD; Charles J. Epstein, MD; Hideyuki Kamii, MD, PhD

Background and Purpose We have demonstrated in a previous study that superoxide radicals play a role in the pathogenesis of cerebral infarction, using a transgenic mouse model of distal middle cerebral artery occlusion, permanent ipsilateral cerebral carotid artery occlusion, and 1-hour contralateral cerebral carotid artery occlusion that produced infarction only in the cortex. However, the role of superoxide radicals in reperfusion injury in transgenic mice overexpressing superoxide dismutase (SOD) is unknown. Using a mouse model of intraluminal blockade of middle cerebral artery that produced both cortical and striatal infarction, we now further examined the role of superoxide radicals in ischemic cerebral infarction after reperfusion in transgenic mice overexpressing human CuZn-SOD activity.

Methods Transgenic mice of strain Tg HS/SF-218, carrying human SOD-1 genes, and nontransgenic littermates were anesthetized with chloral hydrate (350 mg/kg IP) and xylazine (4 mg/kg IP). Physiological parameters were maintained at a normal range using a 30% O2/70% N2O gas mixture inserted via an inhalation mask. Body temperature was maintained at 37±0.5°C by using a heating pad throughout the studies. The middle cerebral artery occlusion was achieved with a 5-0 rounded nylon suture placed within the internal cerebral artery for 3 hours followed by the removal of the suture to allow reperfusion for another 3 hours. Cerebral infarct size in brain slices and infarct volume, neurological deficit, cortical blood flow, and glutathione levels were measured in both transgenic and nontransgenic mice.

Results Compared with the nontransgenic mice, the infarcted areas were significantly decreased in coronal slices from transgenic mice. The infarct volume (in cubic millimeters) was reduced by 26% in transgenic mice after ischemia and reperfusion. This decrease in the infarct volume in transgenic mice closely paralleled the reduced neurological deficits. Introduction of the suture to block blood supply to the middle cerebral artery territory produced a rapid decrease in the relative surface blood flow in the ipsilateral core and the peri-ischemic (penumbra) areas. There were no significant differences in the local cerebral blood flow in the ischemic core or the penumbra areas between the transgenic and nontransgenic groups. However, the level of reduced glutathione in the penumbra area was significantly higher in transgenic mice than in nontransgenic mice, whereas there was no difference in the reduced glutathione levels in the ischemic core between these two groups.

Conclusions Our study demonstrated that superoxide radicals play a major role in the pathogenesis of cerebral infarction in reperfusion injury after a focal stroke. The reduction in infarct volume and neurological deficits is not dependent on the changes in cerebral blood flow but rather correlate with reduced oxidative stress in the ischemic brain tissue, which was indicated by the relatively high levels of endogenous reduced glutathione in transgenic mice. (Stroke. 1994;25:165-170.)

Key Words: cerebral ischemia, focal • free radicals • reperfusion • mice

Oxygen free radicals have been proposed to be involved in central nervous system injury that is produced by trauma and ischemia.1-5 Because of the transient nature of oxygen radicals and the technical difficulties inherent in accurately measuring their brain levels, experimental strategies have been focused on the use of pharmacological agents and antioxidants, seeking a correlation between an exogenous supply of specific free radical scavengers (ie, superoxide dismutase [SOD], catalase) and the subse-

Received July 8, 1993; final revision received August 26, 1993; accepted September 14, 1993.

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quent protection of cerebral tissues from ischemic injury.6-9 However, this strategy entails problems that cannot not be easily resolved. These problems include the hemodynamic, pharmacokinetic, and possible toxic side effects of the drugs, as well as their blood-brain barrier permeability properties. An alternate and more direct method for the study of oxidative stress in ischemia and reperfusion injury is to use genetically modified animals that overexpress antioxidant enzymes. Transgenic (Tg) mice overexpressing human copper-zinc-superoxide dismutase (SOD-1) activity have been successfully developed and maintained in our laboratory.10,11 These SOD-1 Tg mice have been proven to be highly effective in ameliorating vasogenic edema and cerebral infarction after cold injury.12 Using a rat model of cortical infarction that is produced by permanent occlusions of the ipsilateral middle cerebral artery (MCA) distally and the cerebral carotid artery (CCA) and 1 hour occlusion
of the contralateral CCA, we have demonstrated that the infarcted area in brains of Tg mice was significantly smaller than that in nontransgenic (nTg) mice. However, this previous study did not address the role of reperfusion in the ipsilateral hemisphere in the pathogenesis of cerebral infarction in Tg mice. The successful development of the intraluminal suture for MCA occlusion and reperfusion in mice now allows us to address this question. Although oxygen radicals have been widely recognized as being factors responsible for tissue injury after reperfusion, their exact role in the pathogenesis of infarction after cerebral ischemia and reperfusion has not been clearly established, despite the achievement of some recent advances in this research area. The aim of this study is to test the hypothesis that cerebral injury and infarction are significantly reduced in SOD-1 Tg mice, since oxygen radicals play a role in the pathogenesis of ischemic infarct after reperfusion. The present study now reports the changes in local cerebral blood flow (LCBF), cerebral infarction, neurologic deficits, and antioxidant glutathione after cerebral ischemia and reperfusion in both Tg mice and nTg littermates.

Materials and Methods

Transgenic Mice

Heterozygous Tg mice of the strain Tg HS/SF-218, carrying human CuZn-SOD genes (SOD-1) were derived from the founder stock described by Epstein et al10 and were bred on a CD-1 mouse background. Tg mice were identified by qualitative demonstration of human CuZn-SOD using nondenaturing gel electrophoresis followed by nitroblue tetrazolium staining.10,12 There were no observable phenotypic differences between Tg mice and nTg mice.

Transient Middle Cerebral Artery Occlusion

Transgenic mice and nTg littermates, weight 25 to 35 g, were anesthetized with chloral hydrate (350 mg/kg IP) and xylazine (4 mg/kg IP). The mouse's body temperature was maintained at 37 ± 0.5°C with a heating pad throughout the studies. Animals were given a 30% O2/70% N2O gas mixture via an inhalation mask. The left femoral artery was cannulated with an arterial catheter (PE-10, Clay Adams) for measurement of mean arterial blood pressure (MABP), Pao2, Paco2, and pH during and after MCA occlusion. The MABP was measured by a pressure transducer (p23Db, Statham). The MCA occlusion13 was achieved as described previously for rats (using an intraluminal suture).15,19 The left common carotid artery was exposed, and the external carotid artery and its branches were isolated and coagulated. A 5-0 monofilament nylon suture, blunted at the tip, was introduced into the internal carotid artery through the external carotid artery stump and advanced to the anterior cerebral artery. The distance from the tip of the suture to the bifurcation of the common cerebral artery was 11 ± 0.5 mm in each mouse. After 3 hours of proximal MCA occlusion, blood flow was restored by removal of the nylon suture for 3 hours. This timing (3 hours of ischemia plus 3 hours of reperfusion) was selected because it produced a reproducible infarction in our preliminary studies. These surgical procedures were conducted by one of us (G.Y.) who was blinded to the type of mice being used.

Measurement of Infarct Size and Infarct Volume

At the end of reperfusion, the brains were removed and the forebrain was cut coronally 1 mm, 3 mm, and 7 mm distal from the frontal pole by using the mouse Brain Matrix (Harvard Apparatus). The brain slices were then stained with 2% (wt/vol) 2,3,5-triphenyltetrazolium chloride in Dulbecco's phosphate buffer (pH 7.4) at 37°C.8 Triphenyltetrazolium chloride, which stains mitochondrial dehydrogenase, accurately delineates the infarct area compared with a measurement achieved through the use of hematoxylin-eosin staining.20,21 The infarcted areas of the brain slice were quantitated by an image-analysis system. The infarct volume was calculated by the sum of the infarct area of different brain slices times the thickness of the slices. Edema volume that contributes to infarct volume was subtracted by comparison with the total volume of the contralateral hemisphere.21

Measurement of Neurological Deficits

Mice were allowed to recover for 3 hours from anesthesia during the reperfusion period. They were then tested for neurologic deficits by an investigator (J.C.) blinded to the experiment. The neurologic deficit score assignment of 0 to 4 was based on Bederson et al:20 0, no observable neurologic deficits; 1, failure to extend right forepaw; 2, circling to the right; 3, falling to the right; and 4, cannot walk spontaneously.

Local Cerebral Cortical Blood Flow Determination

Local cerebral cortical blood flow was measured in anesthetized mice using a BFM 403 A LDF monitor, equipped with a small-caliber probe of 0.7-mm diameter (TSI, Inc). Three cortical sites were chosen: ischemic core, anteroposterior (distance away from bregma toward anterior pole), 1 mm and lateral, 4.5 mm from the midline; peri-ischemic (pnenbra), anteroposterior, 1 mm and lateral, 2 mm; and contralateral (control), anteroposterior, 1 mm and lateral, 4.5 mm. The probe was held in a micromanipulator and was stereotaxically advanced to gently touch the infarct skull to bring the desired cortical sites. Warm saline (37°C) was slowly rinsed around the probe during the measurement. Laser Doppler flowmetry values in a continuous digital display were averaged over 5-second intervals and were recorded every 20 minutes during the MCA occlusion and reperfusion.

Reduced Glutathione Measurement

After the reperfusion, mice were anesthetized with pentobarbital, and their brains were quickly removed for reduced glutathione (GSH) measurements. A brain cortical slice (350 μm in thickness, 100 mg in weight) was dissected along the MCA territory (infarcted area). Another cortical slice (60 mg) of the peri-ischemic area of the anterior cerebral artery was also dissected out. The contralateral cortex of the origin of the MCA (100 mg) was dissected to serve as a control. GSH was measured according to the previous method.22

Statistical Analysis

In most experiments (cerebral infarction, GSH levels), when comparisons are made between Tg and nTg mice, a one-way ANOVA was used, followed by the Student's t test. For analysis of cortical blood flow, a repeat analysis of ANOVA was used. An ANOVA followed by Mann-Whitney U analysis was used for neurologic deficit scores analysis between Tg and nTg groups. Significance was denoted for P < 0.05.

Results

The physiological parameters (mean ± SE) were determined before ischemia for MABP (nTg, 91.8 ± 7.5 mm Hg; Tg, 86.8 ± 13.5 mm Hg), Pao2 (nTg, 49.9 ± 3.4 mm Hg; Tg, 49.3 ± 3.5 mm Hg), Paco2 (nTg, 94.9 ± 3.7 mm Hg; Tg, 92.8 ± 3.1 mm Hg), and pH (nTg, 7.32 ± 0.03; Tg, 7.33 ± 0.01) for both Tg (n = 4) and nTg (n = 4) mice. There were no differences between the physiological parameters for Tg and nTg mice. Furthermore, the levels of the physiological parameters used during MCA occlusion and at the end of reperfusion were maintained at normal physiological conditions and were not significantly different from the preischemic values (data not shown). After 3 hours of ischemia and 3 hours of reperfusion, the infarcted
areas were significantly decreased in coronal slices 1, 3, and 7 mm distal from the frontal pole in Tg mice (Fig 1). Furthermore, the infarct volume was reduced by 26% in Tg mice compared with that in nTg mice (Table 1). The neurological deficits were significantly reduced in Tg mice when compared with their nTg littermates (Table 2). These data suggest reduced neuropathology and improved functional outcome in Tg mice that display high levels of CuZn-SOD activity after cerebral ischemia and reperfusion.

Introduction of the suture to block blood supply to the MCA territory produced a rapid decrease of the relative surface blood flow in the ipsilateral core and the peri-ischemic (penumbra) areas. The LCBF of the ischemic core was reduced to 11% to 14% and 10% to 12% of the baseline (100%) in Tg and nTg groups, respectively. The peri-ischemic area (penumbra) was reduced to 30% to 35% and 30% to 40% of the baseline in Tg and nTg groups, respectively, after 3 hours of ischemia and reperfusion (Fig 2). After reperfusion, LCBF of the core was increased only to 40% to 50% and 37% to 60% and in the penumbra was increased to 48% to 83% and 70% to 91% of the contralateral baseline in the Tg and nTg mice, respectively (Fig 2). There were no significant differences in absolute value in baseline as well as the values under experimental conditions between the Tg and nTg groups. The levels of GSH in the ischemic core (MCA territory) were significantly decreased in both Tg and nTg mice (Fig 3). However, no differences were observed in the ischemic core between Tg and nTg mice. The GSH levels in the histologically normal peri-ischemic region (anterior cerebral artery) were higher in Tg mouse brains than the levels found in the same anatomic area in nTg mice (Fig 3).

**Discussion**

This study demonstrates that Tg mice containing a threefold increase in CuZn-SOD activity are resistant to injury induced by cerebral ischemia and reperfusion, since both infarct volume (Table 1) and neurological deficits (Table 2) are significantly reduced in Tg mice. Although numerous studies have demonstrated that exogenously supplied SOD offers neuronal protection against ischemic brain injury,49 this study is believed to be the first to demonstrate that elevating the levels of endogenous antioxidant enzyme by molecular genetic manipulation can ameliorate reperfusion injury. Since the cellular constituents (neurons, glia, endothelial cells) all expressed cytosolic CuZn-SOD activity in Tg mice, this animal thus offers a unique model for the study of antioxidant effects on brain cell injury without incurring the problems often encountered in pharmacological approaches. On the other hand, since increasing levels of CuZn-SOD activity are expressed in many cells, including leukocytes and macrophages, and since decreasing levels of superoxide radicals are likely to be produced in those inflammatory cells, it is not clear whether the lack of superoxide production in these inflammatory cells in Tg mice may contribute to the observed effects in ischemic resistance in Tg mice.

The physiological and biochemical mechanisms underlying the reduction of ischemic infarction in reperfused Tg animals were further investigated. It was reported that exogenously supplied antioxidants, such as SOD, may improve the histological and functional outcome in ischemic animals by increasing the cerebral blood flow.23 Our data have shown that the LCBF in the ischemic core and the peri-ischemic penumbra area in both Tg and nTg mice after ischemia and reperfusion are very similar (Fig 2). These data suggest that alterations in the LCBF would not account for the reduction of ischemic neuronal injury observed in Tg mice. Recently, Barone et al26 reported a significant difference...
TABLE 2. Reduced Neurological Deficit in Transgenic Mice After Focal Cerebral Ischemia and Reperfusion

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Neurological Deficit Score</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nontransgenic (n=12)</td>
<td>0 1 2 3 4</td>
<td>2.42±0.79</td>
</tr>
<tr>
<td>Transgenic (n=12)</td>
<td>1 6 3 2</td>
<td>1.50±0.91*</td>
</tr>
</tbody>
</table>

Data are mean±SD where indicated. Mice were subjected to ischemia (3 hours) followed by 3 hours of reperfusion. Mice were tested for neurological deficits by investigators blinded to the experiment. *P<.05, using ANOVA followed by Mann-Whitney U analysis.

between mouse strains in their sensitivity to focal cerebral ischemia, related to the structural and functional differences in the potency of the circle of Willis. Although the functional vascular anatomy was not examined in either Tg or nTg mice, the relatively similar LCBF in Tg and nTg mice might suggest that functional vascular anatomy does not play a role in ischemia/reperfusion injury. Also, the differences in the circle of Willis may not have occurred at all since the same strain of Tg and nTg littersmates (CD-1) was used for the present study.

Under normal physiological conditions, a relatively higher level of H$_2$O$_2$ is produced in the brains of Tg mice than in nTg mice. Further, it was also shown in a recent study that a rapid increase in H$_2$O$_2$ is generated during reperfusion in rats. Thus, it is logical to predict that increased H$_2$O$_2$ levels may put ischemic tissue under oxidative stress that results in tissue infarction. However, this prediction appears to be unlikely. First, the increased level of H$_2$O$_2$ alone is relatively low to elicit the ischemic injury. In our preliminary studies, we observed that 10 minutes of focal ischemia did not cause neuronal injury, measured at 7 days after reperfusion, despite a relatively high level of H$_2$O$_2$ being produced (H.K., P.H.C., unpublished data, 1993). Although the activities of H$_2$O$_2$ scavenging enzymes, glutathione peroxidase and catalase, were not studied, the highly stable glutathione peroxidase would reduce H$_2$O$_2$ to a lower, nontoxic level. On the other hand, the level of GSH in the penumbra area of Tg mouse brain that contains no histological injury is significantly higher than that in the same area with histological infarction in nTg mouse brain, suggesting that maintenance of a higher level of antioxidants in Tg mouse brains may underlie the ischemic resistance mechanism. Second, most of the toxicity of H$_2$O$_2$ is suggested to be due to its reduction to highly reactive hydroxyl radicals by the iron-catalyzed, superoxide-dependent Haber-Weiss reaction. The lack of superoxide radicals in the brain of Tg mouse would make the generation of hydroxyl radicals unlikely.

We have demonstrated previously that the levels of GSH and ascorbate were spared in the ischemic penumbra, which is the brain region that can be rescued...
from further development into infarction.\textsuperscript{14} Depletion of brain GSH through the use of buthionine sulfoximine enhances cerebral ischemic infarction in rats, again suggesting that an adequate level of endogenous antioxidants is required to protect from cerebral ischemic brain injury.\textsuperscript{22} The levels of GSH in the ischemic core (MCA territory) were significantly decreased in both Tg and nTg mice (Fig 3). Such a significant reduction of the antioxidant levels correlates with the irreversible ischemic infarction in the MCA core area. However, there were no significant differences in GSH levels in the MCA core area between Tg and nTg mice. On the other hand, the ischemic penumbra, the anterior cerebral artery area that was histologically normal in Tg mice, was found to contain higher levels of GSH than the levels found in the same anatomic area that exhibits ischemic infarction in nTg mice (Fig 3).

Thus, the relatively high levels of GSH that are found in the penumbra of Tg mice may prevent oxidative stress-induced neuronal death after ischemia and reperfusion injury. Since astroglial cells contain GSH levels that are an order of magnitude higher than those found in neurons,\textsuperscript{29,30} the high level of GSH in the penumbra may imply that glial function is better maintained within this region. It is possible that this increased functional integrity of astroglial cells offers neuronal protection such as through the active reuptake of extracellular glutamate (which is known to participate in ischemic neuronal death) by astroglial cells.\textsuperscript{23-31,33}

Another possible mechanism underlying reduced infarction in Tg mice after reperfusion injury may involve nitric oxide (NO) radicals. It has been demonstrated that superoxide radicals react instantly with NO\textsubscript{•} radicals when produced by brain nitric oxide synthase,\textsuperscript{24,34,35} forming peroxynitrite ONOO\textsuperscript{-}.\textsuperscript{36,37} Peroxynitrite decays rapidly at neutral pH, forming highly reactive radicals, including hydroxyl (-OH) radicals and nitrogen dioxide that can cause endothelial cell injury. Thus, increased CuZn-SOD activity in the Tg mouse brain would eliminate the formation of peroxynitrite during reperfusion. On the other hand, depletion of superoxide radicals might increase the LCBF by increasing the level of NO.\textsuperscript{•} The latter is known to be a potent vasodilator.\textsuperscript{38}

Although the SOD-1 Tg mice offer ischemic tolerance and resistance after reperfusion, they nevertheless did not come away from ischemia/reperfusion unscathed. These data suggest that increased endogenous CuZn-SOD activity alone cannot be sufficient to offer total neuronal protection against ischemia/reperfusion. Other mechanisms, including LCBF, excitotoxins, and other unknown factors, may also account for the cerebral infarction observed in Tg mice. At the present time, we can speculate only that the functional integrity of glial cells, along with the lower level of peroxynitrite, alone or combined with other unknown factors, would reduce oxidative stress in the ischemic brain by providing neuronal protection from reperfusion injury in SOD-1 Tg mice.

Acknowledgments

This study was supported by grants NS-25372, NS-14543, and AG-08938 from the National Institutes of Health, Bethesda, Md. We thank Dr Glenn Gobbel for his critical review of this manuscript and Ms Julie Weigel for her editorial assistance.

References

Considerable evidence has been gathered in the last few years supporting the hypothesis that oxygen-derived free radicals are mediators of central nervous system injury in ischemia/reperfusion. Most of the experimental evidence supporting the role of oxygen-derived radicals in stroke has relied on the demonstration that the administration of antioxidant(s) provides significant protective effect. The accompanying article by Yang and colleagues offers additional strong evidence supporting the role of oxygen-derived radicals in ischemia/reperfusion in the rat brain.

Yang and his colleagues in the study reported above showed that transgenic mice that have elevated levels of endogenous superoxide dismutase activity are resistant to injury associated with focal stroke. The authors measured cerebral infarct size and volume, neurological deficit, cortical blood flow, and glutathione levels in transgenic mice and in their nontransgenic littermates in response to transient middle cerebral artery occlusion. The experimental design was well planned to achieve reproducible results in inducing cerebral infarction. The 3-hour duration of focal ischemia followed by 3 hours of reperfusion mimics very well many human strokes. Equally important was the fact that the experimental results were evaluated impartially by blinding some of the investigators who analyzed the end points.

It should be noted that the increased endogenous superoxide dismutase activity did not provide total neuronal protection against cerebral ischemia/reperfusion in transgenic mice. There are several reasons for this. It is possible, for example, that mechanisms other than oxygen-derived radicals are involved. Another possibility is that the increased antioxidant activity was not sufficient to completely eliminate oxygen radicals under the circumstances of the experiment and that further increases in superoxide dismutase activity might be more effective. Clearly, Yang and his colleagues have provided strong direct evidence that oxygen-derived free radicals play a major role in the pathogenesis of cerebral infarction induced by focal ischemia/reperfusion.

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Human copper-zinc superoxide dismutase transgenic mice are highly resistant to reperfusion injury after focal cerebral ischemia.

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*Stroke*. 1994;25:165-170
doi: 10.1161/01.STR.25.1.165

*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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