

## MICE OVEREXPRESSING EXTRACELLULAR SUPEROXIDE DISMUTASE HAVE INCREASED RESISTANCE TO FOCAL CEREBRAL ISCHEMIA

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**Abstract**—Transgenic mice, which had been transfected with the human extracellular superoxide dismutase gene, causing an approximate five-fold increase in brain parenchymal extracellular superoxide dismutase activity, were used to investigate the role of extracellular superoxide dismutase in ischemic brain injury. Transgenic ( $n=21$ ) and wild-type ( $n=19$ ) mice underwent 90 min of intraluminal middle cerebral artery occlusion and 24 h of reperfusion. Severity of resultant hemiparesis and cerebral infarct size were measured. Wild-type mice had larger infarcts (cortex: wild type =  $37 \pm 14$  mm<sup>3</sup>, transgenic =  $27 \pm 13$  mm<sup>3</sup>,  $P=0.03$ ; subcortex: wild type =  $33 \pm 14$  mm<sup>3</sup>, transgenic =  $23 \pm 10$  mm<sup>3</sup>,  $P=0.02$ ). Neurological scores, however, were similar ( $P=0.29$ ). Other mice underwent autoradiographic determination of intra-ischemic cerebral blood flow. The volume of tissue at risk of infarction (defined as volume of tissue where blood flow was  $<25$  ml/100 g/min) was similar between groups (cortex: wild type =  $51 \pm 15$  mm<sup>3</sup>, transgenic =  $47 \pm 9$  mm<sup>3</sup>,  $P=0.65$ ; subcortex: wild type =  $39 \pm 16$  mm<sup>3</sup>, transgenic =  $37 \pm 17$  mm<sup>3</sup>,  $P=0.81$ ).

These results indicate that antioxidant scavenging of free radicals by extracellular superoxide dismutase plays an important role in the histological response to a focal ischemic brain insult. © 1998 IBRO. Published by Elsevier Science Ltd.

**Key words:** extracellular superoxide dismutase, brain, cerebral blood flow, mouse, transgenic, Circle of Willis.

Increased free radical formation is a known consequence of cerebral ischemia.<sup>4,21</sup> Natural defenses against oxidative stress include the antioxidant enzymes glutathione peroxidase, catalase and superoxide dismutase (SOD). Three isoforms of SOD are known to exist in the mammalian brain.<sup>1</sup> Copper-zinc superoxide dismutase (CuZn-SOD) is found in the cytosol and nucleus of cells. Increased activity of CuZn-SOD found in mice carrying the transgene for this isoform results in a reduction of both focal and global ischemic brain injury.<sup>13,24</sup> Conversely, targeted disruption of the CuZn-SOD coding sequence causes increased edema and enlarged infarcts after murine middle cerebral artery occlusion (MCAO).<sup>8</sup> The manganese superoxide dismutase (Mn-SOD) isoform, located in mitochondria, has been less thoroughly characterized. However, a preliminary report indicates that mice deficient in Mn-SOD have enlarged infarcts resulting from MCAO.<sup>12</sup> This information cumulatively implicates a crucial role for

intracellular SOD in modulating free radical injury associated with cerebral ischemia.

The final isoform of SOD, extracellular superoxide dismutase (EC-SOD), is located in the extracellular matrix. In the brain, EC-SOD is normally found in substantially smaller concentrations than either CuZn-SOD or Mn-SOD.<sup>17</sup> For this reason, little attention has been directed towards understanding the role of this SOD isoform in the response to cerebral ischemia. Consequently, there is also little known about the relative importance of extracellular versus intracellular superoxide in the ischemic brain. This experiment utilized a strain of mice overexpressing EC-SOD to investigate the role EC-SOD plays in the pathogenesis of ischemia/reperfusion injury. We hypothesized that an increase in EC-SOD activity would result in an attenuation of injury resulting from an episode of transient focal cerebral ischemia.

### EXPERIMENTAL PROCEDURES

This study was approved by the Duke University Animal Care and Use Committee. The EC-SOD transgenic mice used in this experiment have been described previously.<sup>17</sup> Briefly, transgenic mice were generated by microinjecting nuclei of fertilized oocytes from (C57Bl/6 × C3H)F<sub>1</sub> female mice with the cDNA of human EC-SOD containing a  $\beta$ -actin promoter. Mice carrying the transgene were identified by polymerase chain reaction amplification of the

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**Abbreviations:** CBF, cerebral blood flow; CuZn-SOD, copper-zinc superoxide dismutase; EC-SOD, extracellular superoxide dismutase; MCAO, middle cerebral artery occlusion; Mn-SOD, manganese superoxide dismutase; SOD, superoxide dismutase.

human EC-SOD gene and backbred into (C57BL/6 × C3H)F<sub>1</sub> mice. Overexpression of EC-SOD in the brain of transgenic mice was verified by SOD activity analysis, using the xanthine/xanthine oxidase cytochrome C assay. Transgenic mice had a five-fold increase in EC-SOD activity. The levels of other antioxidant enzymes were unchanged.<sup>17</sup> Litter mates not containing the EC-SOD transgene were used as wild-type controls.

#### *Outcome from transient focal cerebral ischemia*

Animals were prepared for MCAO using modifications of previously described techniques.<sup>5,9</sup> Male mice (eight to 10 weeks of age) were fasted from food for 12–14 h, but allowed free access to water. With the surgical operator blinded to genetic identity, mice were anesthetized with 1.0–1.5% halothane in 50% O<sub>2</sub>/balance N<sub>2</sub> delivered via a snout mask and allowed to breathe spontaneously. Via a midline cervical skin incision, the right common carotid artery was identified. The external carotid artery was ligated and transected. The internal carotid artery was dissected distally until the origin of the pterygopalatine artery was visualized. Following surgical preparation, a 15-min interval was allowed for physiological stabilization. Rectal temperature was monitored continuously and servoregulated with surface heating/cooling at 37.0°C throughout the procedure.

A 6-0 nylon monofilament was blunted at the tip in a flame and then lightly coated with silicone. The filament was inserted into the proximal external carotid artery stump and advanced ≈11 mm into the internal carotid artery to occlude the middle cerebral artery. Inspired halothane concentration was reduced to 0.9% at the time of filament insertion. After 90 min, the filament was removed and the skin incision closed with suture. Ten minutes after filament removal, halothane was discontinued and the mice were allowed to awaken. The mice were placed in an oxygen-enriched environment (FIO<sub>2</sub>=50%) for 1 h and then returned to their cages.

After 24 h of reperfusion, all animals underwent neurological evaluation. Each mouse was assigned a score of 0–4, where 0=no observable neurological deficit, 1=failure to extend the left forepaw, 2=circling to the left, 3=falling to the left and 4=cannot walk spontaneously.<sup>24</sup> Neurological examination was performed by one observer blinded to mouse genotype.

Following neurological evaluation, animals were anesthetized with halothane and decapitated. The brain was removed and frozen at –20°C. Using a cryotome, six 20-μm-thick coronal sections were taken at 320-μm intervals over the rostral-caudal extent of the infarct. The sections were dried and stained with Hematoxylin and Eosin.

Infarct volume was measured by digitally sampling stained sections with a video camera controlled by an image analyser (M2 Turnkey System, Imaging Research, Inc., St Catharines, Ontario, Canada). The image of each section was stored as a 1289 × 960 matrix of 210 μm<sup>2</sup> pixel units. The digitized image was then displayed on a video screen. With the observer blinded to the experimental condition, infarct borders in both the cortex and subcortex were individually outlined (corpus callosum excluded) using an operator-controlled cursor. The area of infarct (mm<sup>2</sup>) was determined automatically by counting pixels contained within the outlined regions of interest. Infarct volumes (mm<sup>3</sup>) were computed as running sums of infarct area multiplied by the known interval (e.g., 320 μm) between sections over the extent of the infarct expressed as an orthogonal projection.

#### *Measurement of physiological parameters*

Because the blood volume required for analytical assays was potentially great enough to cause hypovolemia, a parallel study was performed to define the probable physiological state of mice studied above. Both EC-SOD

transgenic and wild-type mice were examined (*n*=5 per group). These animals underwent a surgical procedure and MCAO insult identical to that described above. In addition, a pericranial needle thermistor was placed adjacent to the skull beneath the temporalis. Servoregulation of body temperature was performed via the rectal probe. Therefore, pericranial temperature only was monitored. A right femoral arterial catheter was placed to monitor blood pressure and to withdraw blood. Blood chemistries were measured at 10 min before and 45 min after onset of ischemia. The animals were then killed by an overdose of halothane anesthesia.

#### *Measurement of cerebral blood flow*

Male EC-SOD (*n*=7) and wild-type (*n*=10) mice (eight to 10 weeks of age) were fasted from food for 12–14 h, but allowed free access to water. Mice underwent an anesthetic and surgical protocol identical to that described for Experiment 1. In addition, catheters were placed via surgical incision in the right femoral artery and left femoral vein. The middle cerebral artery was then occluded by intraluminal insertion of a nylon monofilament, as described above. Arterial blood gases were analysed 20 min after initiation of MCAO. Twenty-five minutes after onset of MCAO, 5 μCi of 4-iodo-n-methyl-[<sup>14</sup>C]antipyrine in 100 μl saline (specific activity 55.4 mCi/mmol, American Radiolabeled Chemicals, Inc., St Louis, MO, U.S.A.) was infused i.v. over 65 s via a ramp infusion pump. The infusion was a 65-step ramp from 0.1 to 200 μl/min designed to produce a rising arterial concentration of 4-iodo-n-methyl-[<sup>14</sup>C]antipyrine. During the infusion, 12 10-μl arterial blood samples were collected (Unopette<sup>®</sup> capillary pipettes, Becton Dickinson Co., Franklin Lakes, NJ, U.S.A.) for later determination of arterial <sup>14</sup>C activity. The samples were collected every 5 s, beginning 10 s after the infusion was initiated. Between sample collections, the arterial catheter was allowed to bleed freely. The blood volume lost was ≈50 μl greater than the volume infused. In preparing for the study, venous (3 cm PE-10 affixed to 1 cm PE-50) and arterial (2 cm PE-10) catheters were standardized for length and volume. Using these catheters and the ramp infusion, the lag time for the infusion to reach the blood vessels was found to be 15 s. For this reason, the baseline blood sample was obtained 10 s after the infusion was initiated. Simultaneous with the last blood sample and completion of isotope infusion, the animals were decapitated and brains rapidly frozen in 2-methylbutane (–20°C). Arterial blood samples were placed on filter paper, dried for 24 h, and then eluted for an additional 24 h in 1 ml normal saline and 10 ml liquid scintillation cocktail (CytoScint<sup>®</sup>, ICN, Costa Mesa, CA, U.S.A.). Radioactivity was determined via liquid scintillation counting using an external quench correction.

The frozen brains were coronally sectioned (20 μm thick) at –20°C on a cryostat. The brain was sectioned serially with every third section mounted. Eight sections were mounted per glass slide, dried for 5 min on a hot plate (36.0°C) and exposed to Kodak SB-5 autoradiographic film for five days in an X-ray cassette, along with [<sup>14</sup>C]methyl methacrylate standards (ranging from 0 to 35.0 nCi/mg; American Radiolabeled Chemicals, Inc.).

A representative section from each slide was scanned and converted to a digital optical density image on a scanning microdensitometer. There were 12 images per animal. The image of each section was stored as a 1280 × 960 matrix of calibrated pixel units (14 μm × 15 μm). Digital optical densities from these autoradiographic images, standard radioactivity values derived from <sup>14</sup>C standards and timed arterial blood <sup>14</sup>C activity were entered into a computerized image analyser (MCID-M2, version 3.0, revision 1.2; Imaging Research Inc.). Radioactivity values were converted to cerebral blood flow (CBF) values ( $\lambda=0.8$ ) using equations developed by Reivich *et al.*<sup>18</sup> and modified by Sakurada *et al.*<sup>19</sup> and Jay *et al.*<sup>6</sup> The digitized image was

then displayed on a video screen. An observer, blinded to the experimental group, outlined the cerebral hemispheres. Both the cortex and subcortex (corpus callosum excluded) were individually delineated in the right cerebral hemisphere (ipsilateral to MCAO) using an operator-controlled cursor. The area of brain at risk of infarction ( $\text{mm}^2$ ) was determined automatically by a preset target scan limited to counting pixels contained within each region of interest, where CBF was less than or equal to  $25 \text{ ml}/100 \text{ g}/\text{min}$  (arbitrarily defined). This area is reported as cortical, subcortical and total hemispheric area. At risk volumes ( $\text{mm}^3$ ) were computed as running sums of at risk area multiplied by the known interval (e.g.,  $480 \mu\text{m}$ ) between sections over the extent of the at risk region expressed as an orthogonal projection. Values from this population of pixels were averaged to provide an estimate of the distribution of flow values within the tissue at risk of infarction. Blood flow in the left (contralateral) cerebral hemisphere was measured similarly.

#### Circle of Willis anatomy

With the surgical operator blinded to genetic identity, male EC-SOD ( $n=16$ ) and wild-type ( $n=14$ ) mice (10–12 weeks of age) were anesthetized with 3% halothane in  $\text{O}_2$  and endotracheally intubated (20-gauge catheter). The lungs were ventilated mechanically with a delivered gas mixture of 1.0–1.5% halothane in 50%  $\text{O}_2$ /balance  $\text{N}_2$ . Via a midline cervical neck incision, the common carotid arteries were isolated and encircled with sutures. Via thoracotomy, an 18-gauge blunt-tipped needle was introduced into the left ventricle and fixed in the ascending aorta. The right atrium was opened. Normal saline (5 ml) was infused intra-aortically via a syringe pump at a constant rate ( $0.5 \text{ ml}/\text{min}$ ). Upon completion, the common carotid arteries were ligated. A second infusion of 4 ml of a 1:1 mixture of gelatin (7 g Knox unflavored gelatin dissolved in 100 ml water; Nabisco Inc., East Hanover, NJ, U.S.A.) and India ink (Black India Ultradraw 3085-F; Koh-i-noor, Bloomsbury, NJ, U.S.A.) was delivered at  $0.5 \text{ ml}/\text{min}$ . Upon completion, brains were harvested and fixed in 4% neutral buffered formaldehyde.

Cerebrovascular anatomy was visualized by imaging the blackened blood vessels via a video-linked dissecting microscope controlled by an image analyser (MCID-M2, version 3.0, revision 1.2; Imaging Research Inc.). The image of each section was stored as a  $1280 \times 960$  matrix of calibrated pixel units ( $14 \mu\text{m} \times 15 \mu\text{m}$ ). The digitized image was then displayed on a video screen. An observer blinded to experimental group assessed the integrity of the Circle of Willis.

#### Statistical analysis

Neurological scores were compared between groups by the Mann-Whitney  $U$  statistic. Infarct volumes were correlated with neurological scores by the Spearman rank correlation coefficient. Physiological and CBF values and volumes of tissue at risk of infarction were compared by the unpaired two-tailed Student  $t$ -test. Parametric values are expressed as mean  $\pm$  S.D. Significance was assumed with  $P < 0.05$ .

## RESULTS

Three wild-type and two EC-SOD transgenic mice died during recovery. Neurological scores of surviving mice (EC-SOD,  $n=19$ ; wild type,  $n=16$ ) are shown in Fig. 1A. Although neurological score correlated with cerebral infarct volume ( $P=0.01$ ; Fig. 1B), no difference was present between groups ( $P=0.29$ ).

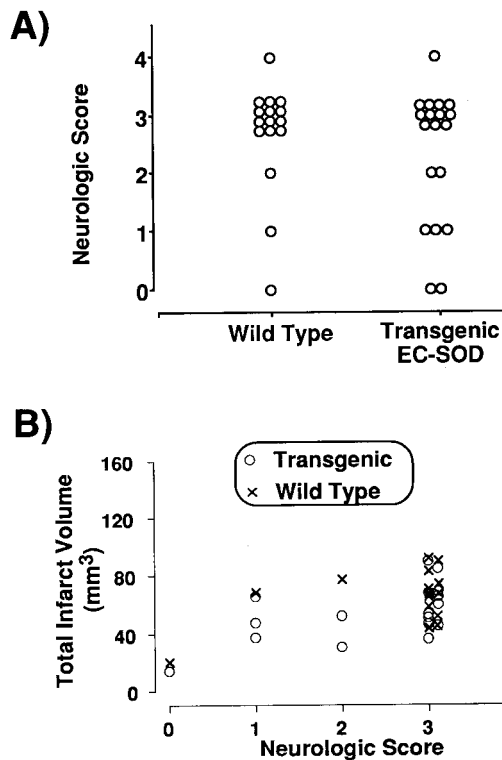


Fig. 1. (A) Neurological scores at 24 h after 90 min MCAO in wild-type and transgenic EC-SOD mice. There was no difference between groups ( $P=0.29$ ). (B) For each mouse, total infarct volume and neurological score as measured 24 h after 90 min of MCAO is depicted. A correlation was noted for these two variables (Spearman rank correlation coefficient:  $r=0.42$ ;  $P=0.01$ ). 0=no deficit, 4=cannot walk spontaneously.

Cortical (EC-SOD =  $27 \pm 14 \text{ mm}^3$ , wild type =  $37 \pm 13 \text{ mm}^3$ ,  $P=0.03$ ), subcortical (EC-SOD =  $23 \pm 10 \text{ mm}^3$ , wild type =  $33 \pm 14 \text{ mm}^3$ ,  $P=0.02$ ) and total (EC-SOD =  $51 \pm 22 \text{ mm}^3$ , wild type =  $70 \pm 27 \text{ mm}^3$ ,  $P=0.02$ ) cerebral infarct volumes are depicted in Fig. 2. For each region, infarct size was decreased in the EC-SOD transgenic mice. Overall, a 27% reduction in total infarct size was present in the EC-SOD transgenic mice.

There were no significant physiological differences between EC-SOD and wild-type mice exposed to the MCAO procedure (Table 1). Although rectal temperature was controlled as intended at  $37.0 \pm 0.1^\circ\text{C}$ , pericranial temperature was decreased by approximately  $2^\circ\text{C}$  in both groups, indicating a mild cerebral hypothermia.

Results from the CBF study are illustrated in Table 2. Physiological values in this group of mice were similar to those reported in Table 1. In particular, there were no differences between groups for  $p_a\text{CO}_2$  (EC-SOD =  $46 \pm 7 \text{ mmHg}$ , wild type =  $43 \pm 3 \text{ mmHg}$ ) or mean arterial pressure (EC-SOD =  $72 \pm 6 \text{ mmHg}$ , wild type =  $73 \pm 9 \text{ mmHg}$ ). There were no differences between groups for either absolute CBF in the cortex

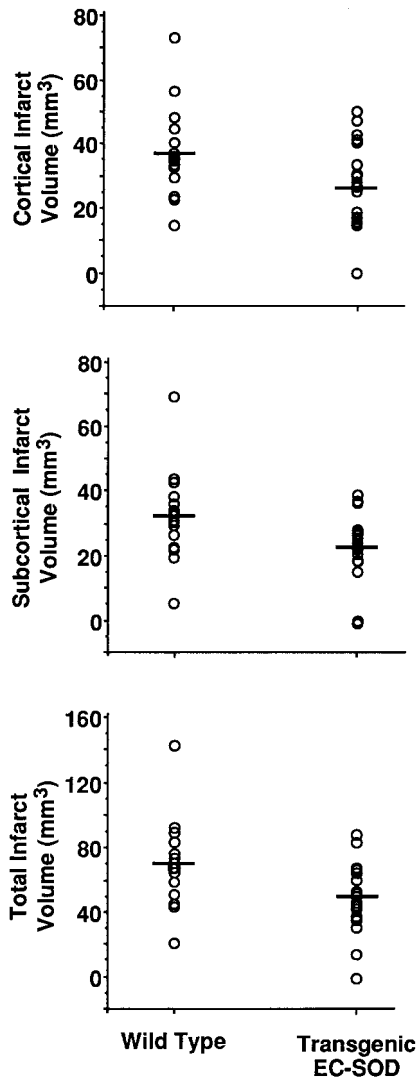


Fig. 2. Cortical, subcortical and total cerebral infarct volumes in individual (open circle) wild-type and transgenic EC-SOD mice. Values from individual animals measured 24 h after 90 min of MCAO are depicted. In all three regions, infarct size was significantly less in the transgenic EC-SOD mice. Horizontal bars denote mean values for each group.

or subcortex ipsilateral to the MCAO, or for the volume of tissue where CBF values were  $<25$  ml/100 g/min (Table 2).

Table 3 provides summarized data from the examination of the Circle of Willis. In both groups, the predominant anomaly was atresia of the posterior communicating arteries. The frequency of this anomaly was similar between groups.

#### DISCUSSION

The results of this investigation demonstrate that increased brain EC-SOD activity reduces the cerebral infarct size resulting from temporary MCAO. This effect is not attributable to differences between trans-

Table 1. Physiological values obtained in surrogate animals

	Wild type ( <i>n</i> =5)	EC-SOD ( <i>n</i> =5)
Pre-ischemic body weight (g)	21 ± 4	22 ± 3
10 min pre-ischemia		
MAP (mmHg)	68 ± 6	66 ± 4
Rectal temperature (°C)	37.0 ± 0.1	37.0 ± 0.1
Pericranial temperature (°C)	35.0 ± 0.5	35.4 ± 0.4
45 min after onset of ischemia		
pH <sub>a</sub>	7.24 ± 0.06	7.19 ± 0.02
<i>p</i> <sub>a</sub> CO <sub>2</sub> (mmHg)	42 ± 13	40 ± 6
<i>p</i> <sub>a</sub> O <sub>2</sub> (mmHg)	208 ± 19	196 ± 10
MAP (mmHg)	64 ± 5	70 ± 7
Glucose (mg/dl)	97 ± 17	118 ± 42
Hematocrit (%)	41 ± 4	42 ± 1
Rectal temperature (°C)	37.0 ± 0.1	37.0 ± 0.1
Pericranial temperature (°C)	35.4 ± 0.5	35.4 ± 0.6

All values are mean ± S.D. There were no significant differences between groups. MAP, mean arterial pressure.

genic and wild-type animals for either intra-ischemic volume of tissue at risk of infarction or congenital anomalies in intracranial large vessel anatomy. We therefore presume that EC-SOD reduced damage by scavenging superoxide in the extracellular space. The magnitude of the neuroprotective effect of EC-SOD was relatively large. Total infarct volume was reduced by 27% in mice overexpressing EC-SOD, which is similar to the 25% reduction of infarct volume observed in transgenic mice overexpressing CuZn-SOD.<sup>24</sup> Oury *et al.*<sup>17</sup> have reported that EC-SOD activity is increased by approximately five-fold in the transgenic animals investigated in the current report (i.e. transgenic =  $33.9 \pm 0.3$  and wild type =  $7.0 \pm 0.3$  units per gram of tissue). This information indicates that EC-SOD may play an important and direct role in defining the outcome of a cerebral ischemic insult.

EC-SOD has been thought to be a minor contributor to the scavenging of free radicals which result from an ischemic brain insult. It is plausible that this is true in wild-type animals. Analysis of the relative expression of the three SOD isoforms in human tissue indicates that, in most organs, including the brain, the concentration of EC-SOD is appreciably less than that of the other two isoforms.<sup>10</sup> Furthermore, a principal site of ischemia/reperfusion-mediated generation of reactive oxygen species is thought to be mitochondria within the intracellular compartment. Because EC-SOD has a positively charged heparin-binding domain, the enzyme remains localized in the extracellular matrix of tissue following its secretion.<sup>14</sup> Therefore, the superoxide scavenged by EC-SOD would also have to be present in the extracellular matrix. The extent to which superoxide contributes to ischemic injury in the extracellular space has not been defined. The fact that a five-fold increase in EC-SOD activity caused such a large effect on infarct

Table 2. Cerebral blood flow and volume of tissue at risk of infarction in wild-type and extracellular superoxide dismutase transgenic mice undergoing right middle cerebral artery occlusion

	Wild type (n=10)	EC-SOD (n=7)	P value
<b>Ipsilateral cortex</b>			
At risk blood flow (ml/100 g/min)	6 ± 4	4 ± 2	0.27
Volume at risk (mm <sup>3</sup> )	51 ± 15	47 ± 9	0.65
Volume (mm <sup>3</sup> )	63 ± 9	58 ± 9	0.32
<b>Ipsilateral subcortex</b>			
At risk blood flow (ml/100 g/min)	11 ± 5	8 ± 4	0.25
Volume at risk (mm <sup>3</sup> )	39 ± 16	37 ± 17	0.81
Volume (mm <sup>3</sup> )	66 ± 8	60 ± 10	0.24
<b>Contralateral hemisphere</b>			
Average blood flow (ml/100 g/min)	121 ± 51	125 ± 44	0.89
Volume (mm <sup>3</sup> )	113 ± 9	118 ± 11	0.39

Values are mean ± S.D. At risk blood flow: average flow for pixels having flow <25 ml/kg/min.  
Volume at risk: tissue within the specified region where flow was <25 ml/100 g/min.

Table 3. Number of animals with anomalies in the Circle of Willis

	Wild type (n=14)	EC-SOD (n=16)
Normal MCA	14 (100)	17 (100)
Normal ACA	14 (100)	7 (100)
Normal ACOM	14 (100)	7 (100)
Atretic PCOM	3 (21)	3 (18)
Normal PCOM	11 (79)	14 (82)
Number of minor right ICA branches (0/1/2)	3/1/10	2/9/6

MCA, middle cerebral artery; ACA, anterior communicating artery; ACOM, anterior communicating artery; PCOM, posterior communicating artery; ICA, internal carotid artery. Values in parentheses are percentage of the group with anomalies. Number of minor right internal carotid artery branches is depicted as number of animals with no, one or two branches, respectively.

size suggests that extracellular superoxide concentrations are important. Future studies using mice with targeted disruptions of the EC-SOD coding sequence may be helpful in further defining the role of extracellular superoxide in brain recovering from an ischemic insult.

There is another consideration which suggests that the extracellular compartment is an important site for formation/action of superoxide in the ischemic brain. Histological damage was reduced by a five-fold increase in EC-SOD activity. Because normal expression of EC-SOD in the brain is far less than that of either CuZn-SOD or Mn-SOD, the increase in EC-SOD activity present in transgenic animals still constitutes a very small fraction of total brain SOD. This suggests a disproportionately large contribution of extracellular superoxide to ischemic injury.

There is evidence, in addition to that provided by our experiment, which supports the contention that

extracellular free radical activity exists and is important. A marked increase in conversion of salicylate to 2,3- and 2,5-dihydroxybenzoic acid during early reperfusion from global cerebral ischemia has recently been demonstrated by use of microdialysis techniques.<sup>4,7</sup> This indicates the presence of hydroxyl radicals in the extracellular space. The fact that brain temperature modulation of dihydroxybenzoic acid production closely parallels the effects of temperature on histological injury is additional evidence that extracellular free radical activity plays a role in ischemic insults.

The source of superoxide in the extracellular space following an ischemic insult is incompletely defined, but may be segregated into two probable categories. Superoxide could be generated during the early stages of post-ischemic reperfusion from oxidative stress. Alternatively, cellular mediators of inflammation (e.g., neutrophils and macrophages) are recognized sources of superoxide.<sup>23</sup> Because recruitment of inflammatory cells into brain parenchyma requires time, EC-SOD-mediated reduction of damage from this source of superoxide would be expected to occur at later stages of reperfusion.<sup>11,25</sup> Discrimination of the relative contribution of EC-SOD to scavenging of superoxide derived from early versus later reperfusion would be difficult in transgenic mice, where an increase in EC-SOD is constitutively expressed. Should pharmacological mimetics of EC-SOD be developed which penetrate the blood-brain barrier,<sup>3</sup> administration of such compounds at different intervals after ischemia might allow definition of the relative importance of these reactive oxygen species.

EC-SOD is a predominant source of SOD activity in vascular tissue.<sup>15</sup> A postulated mode of regulation of the contractile state of cerebral vasculature is the interaction between superoxide and nitric oxide.<sup>22</sup> Superoxide serves as a potent substrate for neutralization of nitric oxide, resulting in the formation of

peroxynitrite.<sup>2</sup> Increased concentrations of EC-SOD would be expected to result in a reduction of superoxide and therefore an increase in nitric oxide.<sup>16</sup> Increased concentrations of nitric oxide would be expected to result in vasodilation and an increase in CBF. Cerebrovasodilation achieved by administration of nitric oxide donors or intra-arterial administration of L-arginine has been identified as a mechanism of neuroprotection during focal cerebral ischemia and the acute recovery interval.<sup>20</sup> To examine the possibility that an increase in EC-SOD reduced infarct volume because of an indirect vasodilatory effect, we measured intra-ischemic blood flow. A flow threshold for definition of tissue at risk of infarction was defined arbitrarily as 25 ml/100 g/min. Given this criterion, more than 80% of the ipsilateral cerebral hemisphere was ischemic, with no difference between transgenic and non-transgenic mice. The fact that average blood flow in cortical tissue having CBF values <25 ml/100 g/min was 4–6 ml/100 g/min in both groups demonstrates that intra-ischemic blood flow was severely reduced and similar for both strains of mice. This indicates that the effect of EC-SOD on neurological outcome is not attributable to differential effects on nitric oxide and regulation of CBF during ischemia. However, we did not measure blood flow at various phases of

reperfusion, leaving open the possibility that overexpression of EC-SOD altered blood flow during that interval.

#### CONCLUSION

Both transgenic mice overexpressing EC-SOD and wild-type litter mate controls were subjected to 90 min of temporary intraluminal MCAO. Following 24 h of reperfusion, cerebral infarct volume was reduced by 27% in the EC-SOD transgenic group. Intra-ischemic physiological values and CBF distribution were similar between groups. These results indicate that extracellular superoxide plays an important role in the pathogenesis of ischemic brain damage. Whether this reflects extracellular superoxide produced during the early stages of reperfusion or inducible superoxide generated by later inflammatory responses to ischemia is yet to be defined. Development of pharmacological compounds which mimic the actions of EC-SOD may be of value in limiting injury resulting from ischemia and reperfusion.

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