Conjugated Superoxide Dismutase Reduces Extent of Caudate Injury After Transient Focal Ischemia in Cats

Naoki Matsumiya, MD, PhD; Raymond C. Koehler, PhD; Jeffrey R. Kirsch, MD; and Richard J. Traystman, PhD

We tested the efficacy of preischemic and postischemic systemic treatment with 30,000 units polyethylene glycol–conjugated superoxide dismutase in a reperfusion model of focal cerebral ischemia. Forty-one anesthetized cats underwent 2 hours' occlusion of the left middle cerebral artery and both common carotid arteries followed by 4 hours of reperfusion. Cats were blindly assigned to one of three groups: treatment with vehicle (10% polyethylene glycol in saline, n=17), pretreatment with drug 3 hours before ischemia (n=12), and posttreatment with drug at the time of reperfusion (n=12). Size of the ischemic injury was calculated from 2,3,5-triphenyltetrazolium chloride staining. Injury in the caudate nucleus was significantly reduced with pretreatment (28±6% of ipsilateral caudate volume, mean±SEM) compared with the vehicle (56±8%). Posttreatment did not significantly ameliorate caudate injury (46±10%). Between the first and second hours of ischemia ipsilateral caudate blood flow determined using microspheres increased significantly from 11±4 to 16±5 ml/min/100 g with pretreatment, but blood flow remained constant throughout ischemia with vehicle (8±2 ml/min/100 g) and posttreatment (10±3 ml/min/100 g). The size of cortical injury (vehicle, 17±5%; pretreatment, 11±3%; posttreatment, 17±5% of hemispheric volume) did not differ significantly among groups. Somatosensory evoked potential recovery did not differ among groups. We conclude that pretreatment with conjugated superoxide dismutase can ameliorate the extent of injury in an end-artery region, such as the caudate nucleus, in a reperfusion model of focal ischemia. Because posttreatment was less effective and pretreatment led to increased blood flow during ischemia, superoxide dismutase may operate via a vascular mechanism during ischemia. 

(Stroke 1991;22:1193–1200)

Oxygen radicals may contribute to reperfusion injury, depending on the severity and duration of ischemia.1 In heart and intestines, intravenous administration of free radical scavenger enzymes such as superoxide dismutase (SOD) can ameliorate damage.2,3 In brain, positive evidence for protection with administration of these enzymes after global ischemia is less compelling,4,5 although improved cerebral oxygen consumption has been reported in postasphyxic lambs.6 However, with focal ischemia, Liu et al7 reported a 24% amelioration of infarct volume after 24 hours' occlusion of the middle cerebral artery (MCA) combined with 90 minutes' occlusion of both common carotid arteries in rats pretreated with polyethylene glycol (PEG)-conjugated SOD and catalase. Conjugation with PEG extends the half-life of circulating SOD beyond 1 day.2 Similar reductions in infarct size have been reported after pretreatment with liposome-entrapped SOD in rats with permanent occlusion of the right MCA and right carotid artery plus 1 hour's occlusion of the left carotid artery.8

We examined the efficacy of PEG-SOD in a model of focal ischemia that permits full reperfusion and hence provides ample oxygen for the potential generation of superoxide anions. The model uses 2 hours' unilateral MCA occlusion combined with bilateral common carotid artery occlusion in cats. We have previously reported consistently low levels of cerebral blood flow (CBF) in the MCA territory and partial recovery of somatosensory evoked potentials in this model,9 and Bose et al10 reported significant

From the Department of Anesthesiology and Critical Care Medicine, The Johns Hopkins Medical Institutions, Baltimore, Md.

Supported in part by United States Public Health Service National Institutes of Health grants NS20020, NS24394, and CIDA NS01225.

Address for reprints: Raymond C. Koehler, PhD, Department of Anesthesiology and Critical Care Medicine, The Johns Hopkins Hospital, 600 North Wolfe Street, Baltimore, MD 21205.

Received November 12, 1990; accepted May 22, 1991.
infarcts. We examined the effects of PEG-SOD administration both prior to ischemia and prior to reperfusion to help dissociate SOD's action during ischemia versus that during reperfusion. In addition to measuring size of the ischemic neuronal injury and recovery of the evoked potentials, we measured CBF to determine whether treatment exerted a beneficial effect via a vascular mechanism.

Materials and Methods

Forty-one cats of either sex weighing 2.7–4.0 kg were induced with 50 mg/kg i.m. ketamine and 1.1 mg/kg i.m. acepromazine. Anesthesia was maintained by continuous infusion of 21.5 μg/hr i.v. fentanyl and mechanical ventilation with 70% N₂O/30% O₂. A 0.3 mg/kg i.v. bolus of pancuronium bromide was used as a muscle relaxant. Femoral arteries and veins were catheterized. A left thoracotomy was performed, and a catheter was inserted into the left atrium for injection of radiolabeled microspheres. The left MCA was exposed by a transorbital approach using microsurgical techniques. The MCA was reversibly occluded near its origin from the intracranial carotid artery using a microvascular clip, and both common carotid arteries were occluded with vascular ligatures. Occlusion lasted 2 hours and was followed by 4 hours of reperfusion.

Arterial pH and blood gases were measured with a Radiometer electrode system (ABL 3; Copenhagen, Denmark), and arterial O₂ content was measured with a CO-Oximeter (Instrumentation Laboratories, Lexington, Mass.). Rectal temperature was maintained at 37.5±0.5°C with a warmed water blanket. Plasma samples were analyzed for SOD activity by the cytochrome c reduction assay. Units of activity are as defined by McCord and Fridovich.

A multichannel signal averager (Med-80, Nicolet Instrument Corp., Madison, Wis.) was used to measure somatosensory evoked potentials as previously described. Silver ball electrodes were placed bilaterally in burr holes in the skull, and stimulatory needle electrodes were placed percutaneously on the volar surface of both forelegs. At selected times 256 stimuli were delivered at a rate of 5.9/sec, and the evoked responses were time-averaged after bandpass filtering between 30 and 1,500 Hz. The amplitude to the peak of the first major negative wave (12–14 msec latency) was measured from the peak of the preceding positive wave.

At the end of the protocol the cats were killed with KCl. The brain was removed and cut into 12 uniform coronal sections 2.5 mm thick. The freshly cut sections were immersed in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC; Sigma Chemical Co., St. Louis, Mo.) in normal saline at 37°C for 40 minutes and were turned over every 10 minutes. Viable brain areas appear dark red as a result of reduction of TTC by mitochondrial enzymes, whereas nonviable areas appear white. Both sides of each section were photographed, and the areas of ischemic injury of the cerebral cortex and caudate nucleus were measured by digital planimetry. The area of the anterior and posterior surfaces of each section were averaged. The product of this averaged area and the section thickness was calculated for each section, and the volume of injury was obtained by summing these products from all 12 sections. Several laboratories have used TTC to measure the size of ischemic injury with MCA occlusion and have reported a good correlation of TTC-determined infarct size with that determined using traditional histological techniques.

Regional CBF was measured with radiolabeled microspheres (16±0.5 μm diameter; Du Pont-New England Nuclear Products, Boston, Mass.) using six radiolabels (gadolinium-153, indium-114m, tin-113, ruthenium-103, niobium-95, and scandium-46). Approximately 1.5–2×10⁶ microspheres were injected into the left atrium while aortic blood was withdrawn for 2 minutes at 1.94 ml/min for calculation of CBF by the reference sample technique. Formalin-fixed coronal sections were dissected on the basis of their TTC staining. We analyzed 12 regions, the left injured cortex and its homologous area in the right cortex, the left noninjured cortex in the MCA territory and its homologous area in the right cortex, the left and right subcortical white matter, the left and right caudate nucleus, the left and right posterior cerebral artery territories of the gray matter, and the left and right anterior cerebral artery territories of the gray matter. The anterior cerebral artery territory was dissected from the dorsomedial gray matter of the three most anterior coronal sections, and the posterior cerebral artery territory was dissected from the dorsomedial gray matter of the three most posterior sections. Red-stained areas of the fourth through the ninth coronal sections (starting anteriorly) were assumed to be the noninjured area of the MCA territory.

The investigators were blinded to treatment until the entire study was completed and all measurements including injury volume were calculated. The pretreatment group (n = 12) received PEG-SOD immediately after the placement of a venous catheter (i.e., 3 hours before ischemia) and received vehicle just before release of the vascular occluders at the end of ischemia. The posttreatment group (n = 12) received vehicle approximately 3 hours before ischemia and PEG-SOD just before reperfusion. The vehicle group (n = 17) received vehicle both 3 hours prior to ischemia and just prior to reperfusion. The dose of PEG-SOD was 30,000 units (Sigma), and the vehicle was 2 ml of a 10% (by weight) solution of PEG (molecular weight 3,350) in phosphate buffered saline (pH 7.4).

Data are presented as mean±SEM. For repeated measurements, two-way analysis of variance (ANOVA) was performed. If two-way ANOVA indicated an effect of treatment or a treatment×time interaction, one-way ANOVA was performed among groups at each time and the two treatment groups were compared with the vehicle group by using
Dunnett’s test. If two-way ANOVA indicated an effect of time or a treatment x time interaction, one-way ANOVA with repeated measures and Dunnett’s test were used to test for changes over time within each group. Injury volume was compared among groups by one-way ANOVA and Dunnett’s test. A probability value of less than 0.05 was regarded as significant except where noted.

Results

There were no significant differences in arterial blood gases or arterial blood pressure among groups (Table 1). With carotid occlusion, mean arterial blood pressure increased significantly during ischemia in all groups. Pressure remained partially elevated through 120 minutes of reperfusion in the pretreatment group.

Plasma SOD activity was not detectable in the vehicle group (assay sensitivity 5 units/ml). In six cats in the pretreatment group, SOD activity was 209±9 and 174±10 units/ml at 30 and 240 minutes of reperfusion, respectively. In two cats in the posttreatment group, SOD activity was 263 and 260 units/ml at 240 minutes of reperfusion.

Size of the caudate nucleus injury was 56±8% of the ipsilateral caudate nucleus volume (249 mm³) in the vehicle group. With drug pretreatment this value was reduced by half (*p<0.05) (Table 2). Posttreatment had no significant effect (Figure 1).

Size of the cortical injury was not significantly reduced with either pretreatment or posttreatment (Table 2). However, there was a wide range of values and most cats in each group had small injuries (<15% of hemispheric volume) (Figure 1). The incidence of large cortical injury (>15% of hemispheric volume) was 35% (six of 17 cats) in the vehicle group and 17% (two of 12 cats) in the pretreatment group.

During ischemia, regional CBF in the cortical gray matter destined for injury was <10 ml/min/100 g in
every cat in the study and averaged 3±0.3 ml/min/100 g (Figure 2). In the noninjured MCA territory, regional CBF averaged 22±4 ml/min/100 g and did not differ among groups. In the total ipsilateral caudate nucleus, regional CBF at 60 minutes of ischemia was 8±2 and 10±3 ml/min/100 g in the vehicle and posttreatment groups, respectively, and it remained unchanged at 120 minutes of ischemia. However, in the pretreatment group, caudate regional CBF increased from 11±4 to 16±5 ml/min/100 g during this time. Within this group, two-way ANOVA using the two CBF measurements during ischemia revealed a significant interaction between ipsilateral brain regions and time. Paired t test with the Bonferroni correction indicated significant increases in regional CBF from 60 to 120 minutes of ischemia in the caudate nucleus and the noninjured MCA, the anterior cerebral artery, and the posterior cerebral artery territories but not in the injured MCA territory. In the vehicle and posttreatment groups, there was no significant increase in regional CBF during ischemia. Subcortical white matter regional CBF (not shown) at 120 minutes of ischemia was 30±3%, 36±5%, and 35±4% of the preischemic control value in the vehicle, pretreatment, and posttreatment groups, respectively. These values did not differ from those at 60 minutes.

Upon reperfusion, regional CBF in the ipsilateral caudate nucleus and noninjured MCA territory increased transiently above the control values in all groups (Figure 2). However, in the cortex destined for ischemic injury, the hyperemia was significant only in the pretreatment group. No significant postischemic hyperperfusion occurred in any region.

In the contralateral hemisphere, CBF generally remained unchanged (Table 3). However, in the area homologous to the injured cortical region, a modest decrease in regional CBF was detected in the vehicle group, possibly due to transcallosal diaschisis. In addition, subcortical white matter regional CBF decreased approximately 25% during ischemia in all groups, presumably reflecting less autoregulatory ability in the white matter with carotid occlusion. There was no regional CBF decrease in the contralateral anterior or posterior cerebral artery territories (Table 3), in contrast to that in the ipsilateral territories (Figure 2). Thus, the regional CBF decrease in the ipsilateral anterior and posterior cerebral artery territories likely represents a redistribution of blood flow into the occluded MCA territory and not solely the effect of carotid occlusion.

With right foreleg stimulation, somatosensory evoked potential amplitude over the left cortex was suppressed equivalently in all groups at 15 minutes of ischemia (Figure 3). Amplitude remained suppressed during ischemia and recovered only partially during reperfusion. There were no differences among groups. With left foreleg stimulation, somatosensory evoked potential amplitude over the right cortex at 90, 120, 180, and 210 minutes of reperfusion in the pretreatment group was greater than that in the vehicle group. Right somatosensory evoked potential amplitude in the vehicle and posttreatment groups decreased at 240 minutes of reperfusion, when right white matter regional CBF had decreased by 19±6% and 22±6% from control, respectively. The potential recorded at the level of the second cervical vertebra remained at the control level throughout the experiment with both right and left foreleg stimulation. Recovery of left somatosensory evoked potential amplitude at 240 minutes of reperfusion was correlated with cortical injury volume (r=-0.59, p<0.005; Figure 4) and with caudate nucleus injury volume (r=-0.49, p<0.005; not shown).

Discussion

Four major findings were made in this study concerning the efficacy of PEG-SOD treatment with temporary focal ischemia. First, PEG-SOD can ameliorate the extent of caudate injury when given 3 hours before ischemia; however, administration prior to reperfusion was not significantly effective. Second, PEG-SOD pretreatment improved residual caudate regional CBF during ischemia. Third, treatment neither before nor after ischemia significantly reduced the extent of cortical injury. Fourth, neither pretreatment nor posttreatment improved the recovery of somatosensory evoked potentials.
This model of focal ischemia is sufficiently severe and widespread to affect not only the cortical territory of the MCA, but also the caudate nucleus, where regional CBF was reduced to 8 ml/min/100 g. We anticipated that amelioration of injury would be greater in the cortical gray matter, where a collateral blood supply is more available than in an end-artery region such as the caudate nucleus. Therefore, 50% amelioration of caudate injury was unexpected. Our results with PEG-SOD pretreatment show that caudate regional CBF increased between the first and second hour of ischemia from 11 to 16 ml/min/100 g; though not large, this increase may have been sufficient to preserve viability. This observation, together with the finding that treatment just prior to reperfusion had no significant effect, suggests that the primary ameliorative effect of PEG-SOD pretreatment was mediated by a vascular mechanism during ischemia. Furthermore, the fact that regional CBF also increased in the ipsilateral noninjured MCA, anterior cerebral artery, and posterior cerebral artery territories indicates that the increase in caudate regional CBF was not a “steal phenomenon.” Rather, there was probably vasodilation in large cerebral arteries, permitting a generalized increase in hemispheric CBF. One action of SOD is prolongation of the half-life of endothelium-dependent relaxing factor. Thus, SOD may promote CBF in our model by inhibiting the inactivation of relaxing factors. Furthermore, SOD may prevent the formation of toxic peroxynitrite from superoxide and nitric oxide. The source of superoxide anions may include leukocytes as well as vascular elements. Alternatively, it is possible that SOD improves metabolism and that the increase in CBF is secondary to metabolic coupling. However, this mechanism is less likely because PEG-SOD does not appear to penetrate into brain parenchyma.

Arterial blood pressure remained elevated during early reperfusion in the pretreatment group (Table 1), which could have promoted increased CBF. However, this explanation of SOD protection is unlikely because caudate regional CBF at 10 minutes of reperfusion in the pretreatment group (145±21 ml/min/100 g) was not greater than that in the vehicle group (160±18 ml/min/100 g).

Liu et al reported a 24% reduction in cortical infarct volume in rats pretreated with PEG-SOD and PEG-conjugated catalase. Imaizumi et al reported similar reductions with liposomal SOD pretreatment. The 33% reduction that we observed, although not statistically significant, is comparable in magnitude. The lack of significance of differences in cortical injury size in our study may be due in part to the low injury volume in a large portion of the vehicle-treated cats. Liu et al and Imaizumi et al used permanent MCA occlusion, which apparently yields large areas of injury more consistently. In addition, the use of PEG-conjugated catalase by Liu et al to scavenge hydrogen peroxide may have further decreased the likelihood of hydroxyl radical formation by Fenton chemistry.

The incidence of large cortical injury (>15% of hemispheric volume) in the PEG-SOD pretreatment group was half that in the vehicle group. We also found that regional CBF in the MCA territory not destined for injury rose during ischemia in the pretreatment group. These two observations suggest that PEG-SOD may act to limit the progression of cortical injury by increasing collateral blood flow in a time-dependent fashion.

The lack of a definitive effect on the extent of cortical injury and the recovery of evoked potentials with PEG-SOD pretreatment or posttreatment does not exclude the possibility that superoxide generation contributes to tissue injury during reperfusion. Superoxide anion has been indirectly detected on the cortical surface of newborn piglets after global ischemia by the nitro blue tetrazolium technique,
TABLE 3. Regional Cerebral Blood Flow of Right Brain (Contralateral to Ischemia) in Cats

<table>
<thead>
<tr>
<th>Group</th>
<th>Ischemia (min)</th>
<th>Reperfusion (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>60</td>
</tr>
<tr>
<td>Homologous to MCA injured territory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>82±11</td>
<td>55±4*</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>73±10</td>
<td>56±6</td>
</tr>
<tr>
<td>Posttreatment</td>
<td>76±10</td>
<td>62±7</td>
</tr>
<tr>
<td>Homologous to MCA noninjured territory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>89±10</td>
<td>79±11</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>102±15</td>
<td>79±8</td>
</tr>
<tr>
<td>Posttreatment</td>
<td>83±10</td>
<td>73±7</td>
</tr>
<tr>
<td>Caudate nucleus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>114±7</td>
<td>98±5</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>116±16</td>
<td>98±7</td>
</tr>
<tr>
<td>Posttreatment</td>
<td>111±13</td>
<td>90±10</td>
</tr>
<tr>
<td>ACA territory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>102±11</td>
<td>88±9</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>91±15</td>
<td>90±8</td>
</tr>
<tr>
<td>Posttreatment</td>
<td>88±11</td>
<td>91±9</td>
</tr>
<tr>
<td>PCA territory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>86±9</td>
<td>91±12</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>87±10</td>
<td>91±9</td>
</tr>
<tr>
<td>Posttreatment</td>
<td>88±12</td>
<td>95±12</td>
</tr>
</tbody>
</table>

Values are mean±SEM ml/min/100 g; vehicle group, n=17; pretreatment group, n=12; posttreatment group, n=12. MCA, middle cerebral artery; ACA, anterior cerebral artery; PCA, posterior cerebral artery.

*p<0.05 different from control.

is thought to be sensitive to extracellular concentrations. Circulating PEG-SOD may not gain access to the interstitial space. Although blood–brain barrier permeability may increase transiently after focal ischemia, the increase may not be sufficient or it may occur too late for obtaining adequate and timely interstitial SOD levels. Furthermore, prevention of injury may require intracellular scavenging. Significant uptake of PEG-SOD into cultured endothelial cells takes hours, not minutes, and recent data in newborn piglets failed to detect enrichment of whole brain tissue SOD activity 2 hours after achieving similar plasma SOD activity with PEG-SOD. With liposomal SOD, significant enrichment of tissue SOD levels is achieved within 1 hour. Treatment with α-tocopherol, 21-aminosteroids, and other lipophilic antioxidants may be more efficacious than treatment with PEG-SOD in preventing parenchymal lipid peroxidation. Furthermore, we cannot exclude that PEG-SOD treatment at the time of reperfusion did not exert some protective effect obscured by the variability in injury size.

In the present model, regional CBF in the subcortical white matter was severely reduced and was not increased by PEG-SOD pretreatment. Thus, it is possible that full recovery of somatosensory evoked potentials was limited by impaired white matter function and that PEG-SOD was not effective in this vascular bed. With permanent MCA occlusion, evoked potential impairment generally corresponds to the extent of hemispheric injury. With reperfusion, we found that evoked potential recovery correlated with injury volume in that animals with large injuries had poor evoked potential recovery, whereas cats with partial evoked potential recovery had small injuries (Figure 4). However, the correspondence was not one-to-one in that some animals with poor evoked potential recovery had small injuries. Thus, somatosensory evoked potential recovery is not a precise predictor of the extent of injury. It is possible that longer reperfusion times might have allowed progression of injury as detected by TTC staining in this subpopulation with poor recovery of evoked potentials. It is also possible that discrete injury in white matter of the foreleg somatosensory pathway is responsible for the imprecise correlation. Finally, TTC staining may overestimate the extent of irreversible injury.
With multiple microsphere injections over 6 hours in 10 nonischemic cats, the coefficient of variation within each cat was 16%. The CBF value of the last 210 minutes of reperfusion are omitted for clarity.

In conclusion, we found that pretreatment with PEG-SOD in a model of focal ischemia and reperfusion ameliorates infarction in the caudate nucleus. Despite the fact that this is an end-artery region with few collaterals, PEG-SOD appeared to be acting by a vascular mechanism on cerebral arteries throughout the hemisphere because CBF increased in most ipsilateral regions during the 2 hours of occlusion.

Acknowledgments

The authors express appreciation to Dr. Steven E. Haun for performing the SOD assay and to Mary North for her excellent technical assistance.

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**Key Words**: cerebral blood flow • evoked potentials • superoxide dismutase • cats
Conjugated superoxide dismutase reduces extent of caudate injury after transient focal ischemia in cats.
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Stroke. 1991;22:1193-1200
doi: 10.1161/01.STR.22.9.1193

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