Liposome-Entrapped Superoxide Dismutase Reduces Cerebral Infarction in Cerebral Ischemia in Rats

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We studied the role of superoxide radicals in the pathogenesis of ischemic brain injury using a model of focal cerebral ischemia in 102 rats and liposome-entrapped CuZn-superoxide dismutase, which can penetrate the blood–brain barrier and cell membranes efficiently. The bolus intravenous administration of 25,000 units of liposome-entrapped CuZn-superoxide dismutase elevated superoxide dismutase activities in the blood and brain 1, 2, 8, and 24 hours later as well as in the ischemic hemisphere and contralateral cortex. Determined 24 hours after right middle cerebral and bilateral common carotid artery occlusion by the lack of staining for mitochondrial dehydrogenase activity with 2,3,5-triphenyltetrazolium chloride, infarct sizes were reduced by 33%, 25%, and 18% in the anterior, middle, and posterior brain slices, respectively, by treatment with liposome-entrapped CuZn-superoxide dismutase. Our data demonstrate that superoxide radicals are important determinants of infarct size following focal cerebral ischemia and that liposome-entrapped CuZn-superoxide dismutase may have pharmacologic value for the treatment of focal cerebral ischemic injury. (Stroke 1990;21:1312-1317)

Oxygen radicals have been postulated to be involved in brain injury and cell death secondary to ischemia and traumatic injury.1-5 Due to their transient nature and the technical difficulties inherent in accurately predicting their brain levels, the involvement of oxygen radicals in injured brain has been identified by most investigators indirectly through 1) measuring the levels of antioxidants (enzymes, thio compounds, vitamins E and C, etc.)6-10 2) measuring the concentrations of lipid peroxidative products,11-15 3) spin-trapping free radicals,16 and 4) inhibiting oxygen reactions using antioxidants.1-3-5 The last strategy is most often used to identify specific oxygen radicals involved in ischemic brain injury. As specified free radical scavengers, superoxide dismutase (SOD) and catalase have been used extensively to identify superoxide radicals and hydrogen peroxide as injurious factors in ischemic brain injury. Although free native antioxidant enzymes and iron chelators have been used to ameliorate brain injury in models of cardiac arrest and focal ischemia,17,18 the extremely short half-lives of native SOD and catalase in circulating blood and their inability to pass the blood–brain barrier (BBB) may limit their therapeutic usefulness in treating ischemic brain injury.19 These inherent technical difficulties can be circumvented by using chemically modified antioxidative enzymes20 or liposome-entrapping techniques.19,21-23 Using a rat model of focal cerebral ischemia that reproducibly causes extensive cortical infarcts, Liu et al20 clearly demonstrated the therapeutic effectiveness of polyethylene glycol–SOD and polyethylene glycol–catalase in the treatment of ischemic brain injury. However, the question remains whether polyethylene glycol–SOD or polyethylene glycol–catalase alone have beneficial effects. It is also not clear whether the observed beneficial effects are due to elevated enzyme levels in ischemic brain tissue following treatment. We and other investigators have demonstrated that positively charged liposomes can deliver active SOD through the BBB19,23 and that elevated brain SOD levels correlate well with the reduction of hyperoxia-induced brain injury.24 Increased brain SOD levels also correlate strongly with the amelioration of posttraumatic brain edema and BBB permeability to iodine-125–labeled bovine serum albumin.23 Therefore, our aim was to examine the effects of positively charged liposome-entrapped SOD on ischemic brain injury, using a rat...
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normal & 1H group

surgical site

FIGURE 1. Schematic drawing of rat brain regions for CuZn-superoxide dismutase activity measurements. Infarcted cortex (A) was identified visually by surface color change 2 hours after focal cerebral ischemia. Pale gray cortex and corresponding area in contralateral cortex were sampled as A and C, respectively, in rats killed 2, 8, and 24 hours after ischemia. Subcortical tissues under A and C were sampled as B and D, respectively. Since ischemic regions were not well defined in rats killed before and 1 hour after ischemia, 8-mm-wide area from surgical site was sampled as A; corresponding area in contralateral cortex was sampled as C, and subcortical tissues under A and C were sampled as B and D, respectively.

model of focal cerebral ischemia that produces an extensive but reproducible infarct.20,25

Materials and Methods

We used 102 adult male Sprague-Dawley rats weighing 300–400 g, 67 in the control groups and 35 in the treated groups that received liposome-entrapped SOD. Rats were anesthetized with 100 mg/kg i.p. chloral hydrate. A femoral artery was catheterized for blood pressure recording and blood sampling for gas and pH analysis. Body temperature was maintained at 37°C with a heating pad controlled by a rectal probe. Blood pressure was maintained above 90 mm Hg, and blood gases were analyzed twice during surgery. Rats were given 30% O2 in 70% N2 via an inhalation mask if $\text{PacO}_2$ fell below 90 mm Hg. Focal cerebral ischemia was produced in 57 rats by the method of Chen et al,25 with minor changes. Briefly, the right middle cerebral artery (MCA) was explored 1–2 mm under the junction of zygomatic bone and skull to identify the pyriform branch,26 ligated proximal to it with a single 10-0 suture, and then electrically coagulated at a low power setting. Immediately following coagulation, the common carotid arteries (CCAs) were exposed with care taken to avoid damage to the vagus nerve. The right CCA was ligated at two sites, and the left CCA was occluded for 1 hour using an aneurysm clip. Both CCAs were occluded ≤5 minutes after the right MCA was occluded. In five rats, the MCA and CCAs were exposed but not occluded; these rats were killed immediately as the preischemia group.

Control rats and those in the preischemia group received 2.5 ml saline and treated rats received 25,000 units liposome-entrapped SOD/kg body wt in saline (total volume 2.5 ml) injected into the right jugular vein 10–20 minutes before CCA occlusion. Injection of liposomes alone (10 rats) does not affect physiologic parameters (mean arterial blood pressure, blood gases, and body temperature)23 compared with injection of liposome-entrapped SOD. Positively charged, unilamellar liposomes with a large internal aqueous space and high capture were prepared based on the method of Szoka and Papahadjopoulos.21 Lipids (L-α-dipalmitoyl phosphatidylcholine [Avanti, Polar-Lipids, Inc., Pelham, Ala.], cholesterol [Sigma Chemical Co., St. Louis, Mo.], and sterylamine [Sigma]) at a molar ratio of 14:7:4 (170 μmol:85 μmol:47.5 μmol) were dissolved in chloroform with warming and then dried in a rotary evaporator. An aliquot of lipid containing 31.2 mg L-α-dipalmitoyl phosphatidylcholine was dissolved in 5 ml diethyl ether by mixing and warming at 37°C. Recombinant human CuZn-SOD (Chiron, Emeryville, Calif.) was dissolved in 4 mM N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid (HEPES) buffer (pH 7.4), and 1.5 ml of this SOD solution (approximately 60 mg/ml) was added to the lipid aliquot in ether and then sonicated. The SOD/lipid mixture was then evaporated using a rotary evaporator at 37°C under a vacuum of 100–200 mm Hg until the ether was removed. The SOD/lipid mixture was then diluted with 10 volumes of isotonic HEPES buffer and centrifuged at 27,000g for 15 minutes. The supernatant was removed, and the pellet was resuspended in a small amount of isotonic HEPES buffer. Liposome-entrapped SOD was obtained by passing the suspension through a 27-gauge needle several times for homogenization. Before enzyme activity was measured, 20 μl of the liposome-SOD suspension was sonicated in the presence of 20 μl of 1% Triton X (Sigma) and diluted with phosphate-buffered saline. Entrapment, measured by SOD activity and lipid phosphorus, ranged from 200 to 500 units SOD per milligram lipid.

We measured CuZn-SOD activities in four brain regions (right infarcted cortex, A; right surrounding subcortex, B; left cortex, C; and left subcortex, D; Figure 1) and in blood from the right atrium in the preischemia group and in control and treated rats killed by decapitation (upon release of the left CCA.
After anesthesia with 35 mg/kg i.p. pentobarbital, the rats were perfused via the aorta with 250 ml of 0.9% saline to eliminate intravascular blood from the brain. The four brain regions were quickly removed, homogenized in 2 ml of 50 mM potassium phosphate buffer (pH 7.8) in the presence of 1 mM ethylenediaminetetraacetic acid, and centrifuged at 4,000 g for 20 minutes. The supernatants were stored at −70°C for enzyme assay within 3 days. The CuZn-SOD activity was assayed by its ability to inhibit superoxide anion–dependent reactions. The assay medium contained 30 nmol cytochrome c and 150 nmol xanthine and brain homogenate in 50 mM phosphate buffer (pH 7.8), with a final volume of 3 ml. The reaction was initiated by adding 10 μl xanthine oxidase, and the rate of light absorbance at 550 nm was recorded for 5 minutes. One unit of SOD was defined as the amount in brain homogenate needed to inhibit the rate of cytochrome c reduction by 50%. For the assay of total SOD activity, 10 μM KCN was added to the assay medium to inhibit cytochrome c oxidase. For the assay of Mn-SOD activity, 1 mM KCN was added to the assay medium to inhibit CuZn-SOD. The CuZn-SOD activity was calculated by subtracting the Mn-SOD activity from the total SOD activity. Plasma CuZn-SOD activity was determined in both control and liposome-entrapped SOD treated groups. Heparinized blood was centrifuged at 1,000 g to obtain plasma. Approximately 0.5 ml plasma was used for the determination of CuZn-SOD activity in the control group.

For the measurement of infarct size, we killed 27 control and 10 treated rats 24 hours after occlusion. Their brains were removed immediately without perfusion. Slices 2 mm thick at the site of division of the olfactory nerve (anterior slice), at the MCA trunk (middle slice), and at the rostral end of the basilar artery (posterior slice) were obtained using a tissue slicer and then immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride (Dulbecco's phosphate buffer, pH 7.4). Cover glasses were placed over the slices to prevent distortion, and the solution was kept at 40°C by a heating pad to amplify staining. After 20 minutes, the brain slices were placed in 10% buffered formalin in the dark and refrigerated until photographed. The rostral surface of each slice was photographed using color slide film (Kodak Ektachrome, ASA 64-Daylight, EPR 135-36; Rochester, N.Y.) within 4 days. Slides of the TTC-stained brain slices were projected and traced. Infarct size was quantified by cutting out and weighing the traced normal and infarcted areas. Infarct size is expressed as a percentage of the entire brain slice.

All data are given as mean±SEM. For CuZn-SOD activity in blood, we compared groups using analysis of variance followed by Student's t test. For CuZn-SOD activity in brain and infarct size, we compared groups using analysis of variance followed by Scheffé's F test for multiple comparisons. A probability value of <0.05 was considered to indicate a significant difference.

Results

The CuZn-SOD activity in plasma of the control rats did not differ from that in the preischemia group during the experiment (Figure 2). In the treated rats, activity at 1 hour was significantly greater than that in the preischemia group and it remained significantly higher at 2 and 8 hours; activity was nonsignificantly higher at 24 hours (Figure 2).

Figure 3 compares CuZn-SOD activities in the four brain regions of treated rats and the preischemia group. In the preischemia group, activities in regions A, B, C, and D did not differ significantly. In the treated rats, activity in region C was significantly greater than that in the preischemia group at 2 and 24 hours, whereas activity was significantly greater in...
FIGURE 4. Bar graph comparing mean±SEM infarct size as percentage of area of entire brain slice between control (black bars, n=27) and treated (white bars, n=10) groups determined by staining with 2,3,5-triphenyltetrazolium chloride. *p<0.005, **p<0.05 different from control by analysis of variance and Scheffé's F test for multiple comparisons. Anterior, at level of olfactory nerve; middle, at middle cerebral artery trunk; posterior, at rostral end of basilar artery.

region A at all times. Activity in region B was also significantly greater at 1 and 2 hours. Mean±SD infarct sizes in treated and control rats 24 hours after occlusion are compared in Figure 4.

FIGURE 5. Photographs of representative middle slices (from middle cerebral artery trunk region) in control (top) and treated (bottom) rats following focal cerebral ischemia. Staining with 2,3,5-triphenyltetrazolium chloride revealed infarct as unstained area. Note significant reduction of infarcted area in slice from treated rat brain.

Administration of liposome-entrapped SOD significantly reduced infarct size in the anterior and middle sections; there was no significant effect in the posterior section. Liposomes alone or liposomes containing denatured enzyme were not effective (data not shown). Figure 5 shows the effects of liposome-entrapped SOD on TTC staining in representative middle slices from control and treated rats.

Discussion

Our major aim was to determine the involvement of oxygen radicals in focal cerebral ischemia and to investigate the pharmacologic efficacy of liposome-entrapped SOD in brain injury. To achieve this goal, a model of focal cerebral ischemia causing an extensive and reproducible infarct was required. Many reliable models of focal cerebral ischemia have been developed that we might have used.25-27-29-35 We chose the model of right MCA and bilateral CCA occlusion, taking advantage of the fact that this model provides a standard-sized infarct in the cortex without evidence of subcortical necrosis.25 We also paid special attention to the anatomic variations of the pyriform branch of the MCA in rats, as reported by Rubino and Young.26 Our studies indicate that extensive, reproducible infarcts could be obtained using this model if the MCA is occluded proximal to the pyriform branch. Since the pyriform MCA branch supplies blood to the anterior region of the rat brain and is an end-artery, the anterior region is prone to ischemic insult. Our data show that this is clearly the case since the infarct was largest in the anterior slice and smaller in the middle and posterior slices (Figure 4).

Oxygen radicals and lipid peroxidation have been proposed to be factors involved in cerebral ischemia and reperfusion injury. This has been shown in studies of ischemia and reperfusion injury in the heart, intestine, and other systemic organs, as reviewed by McCord30 and Parks and Granger.31 However, recent studies indicate that the xanthine dehydrogenase/xanthine oxidase system may not be fully operational during ischemia and reperfusion in brain capillaries; thus, the levels of oxygen radicals are low and cannot account for oxygen radical-dependent reperfusion injury in brain.32 Other strategies are required to elucidate oxygen radical-dependent ischemic and reperfusion injury in the brain. In previous studies, we examined the levels of endogenous antioxidant enzymes as indicators of oxidative stress in ischemic brain injury. Our previous results indicate that CuZn-SOD activity decreased significantly immediately following both MCA and CCA occlusion. There was a reversible change in CuZn-SOD activity in the contralateral hemisphere following reperfusion of the CCA, indicating a profound but transient change in CuZn-SOD activity.10 On the other hand, CuZn-SOD activity in the MCA territory and corresponding subcortical area was reduced during CCA occlusion and remained low during reperfusion of the right CCA, suggesting that these areas suffer irreversible changes in CuZn-SOD
activity. In our current study, a single bolus injection of liposome-entrapped SOD 10–20 minutes before bilateral CCA occlusion immediately and significantly elevated plasma SOD activity (Figure 2) and SOD activity in the MCA territory (region A) and corresponding subcortical area (region B); activity remained high for up to 24 hours in region A. SOD activity in region B was significantly increased 1 and 2 hours after occlusion. Our current data indicate that liposome facilitates the delivery of SOD into the brain, not only in the infarct but also in the noninfarcted, subcortical area as well. It is not known whether the consistently higher SOD activity in the infarct was due to the increased transport of liposome-entrapped SOD into it, where the BBB is likely to break down.

Since we were able to increase brain levels of SOD by injecting liposome-entrapped SOD, we also studied the effect of SOD on infarct size following focal cerebral ischemia. Our data indicate that liposome-entrapped SOD reduced infarct size as determined by TTC staining by 33% (p<0.005), 25% (p<0.05), and 18% (p>0.05), respectively, for the anterior, middle, and posterior slices (Figure 4). In four of 10 rats, treatment with liposome-entrapped SOD rescued tissue adjacent to the MCA occlusion (Figure 5). This finding is somewhat surprising and interesting and may be affected by collateral circulation from the posterior or anterior cerebral arteries. In three of 10 rats, infarction distal to the MCA occlusion was reduced by liposome-entrapped SOD treatment. Infarction in three rats was not affected by treatment with liposome-entrapped SOD. These results clearly demonstrate the role of superoxide radicals in the pathogenesis of an ischemic infarct. However, the mode of action and the target sites of liposome-entrapped SOD are not clear at present. Several questions arise that need to be addressed. First, if neurons and glia are the primary generators of superoxide radicals, does intravenous SOD pass the endothelial cell barrier to reach the brain parenchyma? Second, even if liposome-entrapped SOD passes the endothelial cells through an unlikely series of events including endocytosis and exocytosis, do neurons or glia take up extracellular liposome-entrapped SOD or free SOD? The answers to these questions are unclear at present since liposomal lipid components are likely to be degraded by endothelial lysosomes; furthermore, free enzymes, even after exocytosis to the brain’s extracellular fluid space, could not be taken up by neurons or astrocytes to exert intracellular actions (as demonstrated in primary cell cultures12). An alternative explanation is that some liposome-entrapped SOD penetrates the leaky BBB in the ischemic zone (MCA territory) and is subsequently taken up by adjacent neurons and glia, as demonstrated by our in vitro studies using primary cell cultures.12 Another explanation is that superoxide radicals readily cross the cell membrane in either direction via the anion channel.23 In addition, the release of superoxide radicals through the anion channel of endothelial cells following brain injury has been demonstrated by Kontos et al.24 Thus, liposome-entrapped SOD could exert its action on extracellular superoxide on the luminal side of capillaries without penetrating the BBB. In any case, it seems likely that endothelial cells per se are the target site of liposome-entrapped SOD. We speculate that a combination of these processes is required to achieve the beneficial effects of liposome-entrapped SOD on infarct size that we observed.

An important question that remains is whether postischemic treatment with liposome-entrapped SOD is beneficial. In our previous studies, postischemic treatment reduced the severity of vasogenic brain edema and protected BBB permeability in cold-induced brain injury.23 We speculate that liposome-entrapped SOD might have therapeutic potential in ameliorating the oxidative stress associated with ischemic brain injury. Furthermore, the usually delayed development of infarction following focal and global cerebral ischemia suggests that it may be possible to take advantage of a time window that would allow for therapeutic intervention using antioxidant agents.

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References


17. Davis RJ, Bulkley GB, Traystman RJ: Role of oxygen-free radicals in focal brain ischemia (abstract). J Cereb Blood Flow Metab 1987;7(S1)


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