Lecithinized Superoxide Dismutase Improves Outcomes and Attenuates Focal Cerebral Ischemic Injury via Antiapoptotic Mechanisms in Rats

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Background and Purpose—Recent studies have shown the antiapoptotic neuroprotective effects of lecithinized superoxide dismutase (PC-SOD) in different forms of brain injury. We tested the effects of PC-SOD in focal cerebral ischemia in the rat middle cerebral artery occlusion model (MCAO).

Methods—Adult male Sprague-Dawley rats were treated with PC-SOD (0.3, 1.0, and 3.0 mg/kg) administered intravenously after 90 minutes of occlusion (beginning of reperfusion). Physiological parameters, neurological score, and infarct volume were assessed at 24 and 72 hours in 3 groups of animals: sham-operated (n=18), MCAO treated with vehicle (n=26), and MCAO treated with PC-SOD (n=37). Oxidative stress was evaluated by malondialdehyde assay, and the apoptotic mechanisms were studied by Western blotting.

Results—PC-SOD treatment significantly reduced infarct volume and improved neurological scores at different time points compared with the vehicle-treated group. PC-SOD treatment decreased malondialdehyde levels, cytochrome c, and cleaved caspase 3 expression and increased mitochondrial Bcl-2 expression.

Conclusions—Inhibition of oxidative stress with PC-SOD treatment improves outcomes after focal cerebral ischemia. This neuroprotective effect is likely exerted by antiapoptotic mechanisms. (Stroke. 2007;38:000-000.)

Key Words: cerebral ischemia ■ lecithinized superoxide dismutase ■ neuronal apoptosis ■ oxidative stress

Superoxide dismutase (SOD) has been reported to be neuroprotective in different forms of brain injury, including focal cerebral ischemia.\textsuperscript{1–3} However, the practical use of unmodified form of SOD is hindered because of its short half-life in vivo and low tissue affinity.\textsuperscript{3} PC-SOD, the lecithinized form of SOD, is a derivative of human Cu,Zn-SOD conjugated with 4 covalently bound molecules of lecithin.\textsuperscript{5} PC-SOD has a longer half-life in blood, higher tissue affinity, and greater pharmacological activity than unmodified SOD.\textsuperscript{4,6} Furthermore, lecithinization has been suggested to improve the drug delivery of SOD to the central nervous system.\textsuperscript{4}

Studies with transgenic mice and in vivo administration of unmodified SOD formulations have shown beneficial effects after cerebral ischemia mainly attributed to the antioxidative and antiapoptotic properties of SOD.\textsuperscript{2,3} Protective effects of PC-SOD have been reported in spinal cord and traumatic brain injuries.\textsuperscript{7–9} However, a potential protective role for PC-SOD in cerebral ischemic injuries has not been addressed before. Therefore, the present study was designed to study whether PC-SOD protects against neuronal apoptotic cell death in a rat model of transient focal ischemia.

Materials and Methods

Experimental Animals
All experiments were approved by the institutional Animal Care and Use Committee of Loma Linda University. Seventy-eight adult male Sprague-Dawley rats (Harlan, Indianapolis, Ind) weighing between 280 and 330 g were divided randomly into 3 groups: sham-operated (n=18), middle cerebral artery occlusion (MCAO) treated with vehicle (n=26), and MCAO treated with PC-SOD (n=37).

MCAO Model
Rats were weighed before surgery. General anesthesia was induced with ketamine (80 mg/kg, IP) and xylazine hydrochloride (8 mg/kg IP) followed by atropine (0.1 mg/kg SC). The rats were then intubated and ventilated with an animal ventilator (Harvard Apparatus). A heating pad and a heating lamp were used to maintain the core body (rectal) temperature at 36.5±0.5°C. Rats were subjected to MCAO as described previously, with minor modifications.\textsuperscript{10} In brief, the left common carotid artery, internal carotid artery, and external carotid artery were surgically exposed. The external carotid artery was then isolated and coagulated. A 4–0 silicone-coated nylon suture (Doccol Co, Albuquerque, NM) was inserted into the internal carotid artery through the external carotid artery stump and gently advanced to occlude the MCA. Mean arterial blood pressure (via the left femoral artery), heart rate, arterial blood gases, and blood core body (rectal) temperature at 36.5±0.5°C. Rats were subjected to MCAO as described previously, with minor modifications.\textsuperscript{10} In brief, the left common carotid artery, internal carotid artery, and external carotid artery were surgically exposed. The external carotid artery was then isolated and coagulated. A 4–0 silicone-coated nylon suture (Doccol Co, Albuquerque, NM) was inserted into the internal carotid artery through the external carotid artery stump and gently advanced to occlude the MCA. Mean arterial blood pressure (via the left femoral artery), heart rate, arterial blood gases, and blood temperature at 36.5±0.5°C. Rats were subjected to MCAO as described previously, with minor modifications.\textsuperscript{10} In brief, the left common carotid artery, internal carotid artery, and external carotid artery were surgically exposed. The external carotid artery was then isolated and coagulated. A 4–0 silicone-coated nylon suture (Doccol Co, Albuquerque, NM) was inserted into the internal carotid artery through the external carotid artery stump and gently advanced to occlude the MCA. Mean arterial blood pressure (via the left femoral artery), heart rate, arterial blood gases, and blood temperature at 36.5±0.5°C. Rats were subjected to MCAO as described previously, with minor modifications.\textsuperscript{10} In brief, the left common carotid artery, internal carotid artery, and external carotid artery were surgically exposed. The external carotid artery was then isolated and coagulated. A 4–0 silicone-coated nylon suture (Doccol Co, Albuquerque, NM) was inserted into the internal carotid artery through the external carotid artery stump and gently advanced to occlude the MCA. Mean arterial blood pressure (via the left femoral artery), heart rate, arterial blood gases, and blood...
glucose levels before, during, and after ischemia were analyzed. After 90 minutes of MCAO, the suture was removed to restore blood flow (reperfusion). The rats were allowed to recover after incision closure and housed individually until euthanization. All animals had free access to food and water.

Administration of Drugs
The tail vein was isolated and cannulated with a PE-10 tube filled with phosphate-buffered saline (PBS, Sigma-Aldrich, St. Louis, Mo). PC-SOD was dissolved in distilled water. The PC-SOD treatment group was injected with a single dose of 0.3, 1.0, and 3.0 mg/kg PC-SOD (LTT Bio-Pharma, Japan) via tail vein immediately after 90 minutes of occlusion (beginning of reperfusion). The MCAO with vehicle treatment group was injected with the same volume of distilled water.

Neurological Scoring

<table>
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<tr>
<th>Symptom</th>
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<th>Score 1</th>
<th>Score 2</th>
<th>Score 3</th>
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<td>Spontaneous Activity, 3 min</td>
<td>No movement</td>
<td>Slight movement</td>
<td>Touches 1–2 sides of the cage</td>
<td>Touches 3–4 sides of the cage</td>
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<tr>
<td>Symmetry of movement, right forelimb and hind limb</td>
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<td>Near-total asymmetry</td>
<td>Mild asymmetry</td>
<td>Complete symmetry</td>
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<tr>
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<td>No walking</td>
<td>Walks only in circles</td>
<td>Curvilinear path</td>
<td>Straight path</td>
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<tr>
<td>Beam walking</td>
<td>Falls off the beam</td>
<td>Hugs the beam</td>
<td>Weak response</td>
<td>Symmetrical response as compared to unaffected side</td>
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<tr>
<td>Response to vibrissae touch</td>
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<td>Weak response</td>
<td>Symmetrical response as compared to unaffected side</td>
<td></td>
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<tr>
<td>Side stroking</td>
<td>No response</td>
<td>Weak response</td>
<td>Symmetrical response as compared to unaffected side</td>
<td></td>
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Determination of Oxidative Stress

The level of lipid peroxidation products (malondialdehyde [MDA]) was measured with an LPO-586 kit (OxisResearch, Portland, Ore) as previously described. Samples from 3 groups (n=4 for each group) were used for experiments. The animals were anesthetized and humanely killed at 24 hours by transcardial perfusion with cold PBS. Left cerebral cortices were homogenized in 20 mmol/L phosphate buffer (pH 7.4) and 0.5 mol/L butylated hydroxytoluene in acetone–trile. The homogenates were centrifuged at 3000g for 10 minutes at 4°C. Protein concentration was measured by DC protein assay (Bio-Rad). Equal amounts of proteins in each sample were reacted with a chromogenic reagent at 45°C for 60 minutes. The samples were centrifuged at 15 000g for 10 minutes at 4°C, and supernatants were measured spectrophotometrically at 586 nm. The level of MDA was calculated in picomoles per milligram protein based on a standard curve.

Subfractionation of Cellular Proteins for Western Blotting

Animals were humanely killed 24 hours after reperfusion for Western blotting (n=4 for each group). Brains were stored at

Fluorescence Immunohistochemical Staining

Samples from 3 groups (n=4 for each group) were used for experiments. At 24 hours after reperfusion, the rats were anesthetized and transcardially perfused with PBS and 10% paraformaldehyde as described previously. The brains were quickly removed and postfixed in 10% paraformaldehyde followed by 30% sucrose (wt/vol) for 3 days. Ten-micron-thick coronal sections were cut on a cryostat (Leica Microsystems LM3050S) and mounted on poly-l-lysine–coated slides (Richard Allen, Kalamazoo, Mich). Double-fluorescence labeling was performed as described previously. The following primary antibodies were used: oxidized (ox) oxLDL and NeuN (Chemicon, 1:200 dilution). Appropriate fluorescein isothiocyanate– or Texas red–conjugated secondary antibodies (Jackson Immuno Research, 1:100 dilution) were used. The sections were visualized with a fluorescence microscope (Olympus), and pictographs were recorded and analyzed with MagnaFire SP 2.1B software. For negative controls, either the primary or the secondary antibodies were omitted, and the same staining procedures were followed.

Figure 1. PC-SOD provides neuroprotection in a concentration-dependent manner after focal cerebral ischemia. Figure shows quantification of infarct volume by TTC staining 24 hours after focal ischemia. All concentrations of PC-SOD (0.3, 1.0, and 3.0 mg/kg) significantly decreased infarct volume compared with vehicle treatment (*P<0.05 vs vehicle, ANOVA). The PC-SOD dose of 1 mg/kg, however, showed a maximal decrease in infarct volume (49.4% compared with vehicle treated) and also when compared with other doses (#P<0.05 vs PC-SOD 0.3 mg/kg, ANOVA). N=6 for each group.
−80°C until analysis. Western blot analysis was performed as described previously. In brief, whole-cell lysates were obtained by gently homogenizing the left cerebral cortices with a homogenizer in 5-mL volume of buffer (20 mmol/L HEPES, 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 250 mmol/L sucrose, 0.1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L dithiothreitol, and proteinase inhibitor cocktail tablets; pH 7.9). Samples were further centrifuged at 14,000 g at 4°C for 15 minutes to separate the sample into a supernatant and pellet. The supernatant was used as the cytosolic fraction, and the mitochondrial fraction was used after resuspension in buffer. Protein concentration was determined by the DC protein assay (Bio-Rad).

Western Blot Analysis

Equal amounts of protein (50 μg) were loaded in each lane of polyacrylamide—sodium dodecyl sulfate gels. The gels were electrophoresed, followed by a transfer of the protein to a nitrocellulose membrane. The membrane was then blocked with a blocking solution and probed with primary antibodies overnight at 4°C. The primary antibodies and concentrations used in Western blot analysis were as follows: Bcl-2, cytochrome c (1:200, Santa Cruz Biotechnology), and cleaved caspase-3 (1:300, BD Pharmingen Inc). Immunoblots were next processed with appropriate secondary antibodies (1:2000, Santa Cruz Biotechnology) for 1 hour at room temperature. Bands were detected with a chemiluminescence reagent kit (ECL Plus, Amersham Bioscience). Blot bands were quantified by densitometry with Image J software (Image J 1.33u, NIH). β-Actin (1:2000, Santa Cruz Inc) and COX IV (1:5000, Abcam) were blotted on the same membrane as a loading control for the cytosolic and mitochondrial fractions, respectively. The quantified values were expressed as a percentage of sham (100%).

Statistical Analysis

Data are expressed as mean±SEM. Statistical differences between the various groups was assessed with a 1-way ANOVA followed by a post hoc test. Comparisons between 2 groups were assessed by unpaired t test. A value of P<0.05 was considered statistically significant. A χ² test was done to analyze mortality data.

Results

Physiological Data

No statistical differences were observed between the vehicle-treated and PC-SOD–treated groups with regard to mean arterial blood pressure, heart rate, arterial blood gases, or glucose levels before, during, or after ischemia (data not shown). No statistical differences were observed between the vehicle-treated and PC-SOD groups for mean body weight at 24 hours after reperfusion.
Mortality
The mortality rate was as follows: 8% (2 of 26 rats) in the vehicle-treated group and 3% (1 of 37 rats) in the PC-SOD-treated group. No rats in the sham group died. There was no statistical difference in mortality between the groups on analysis.

PC-SOD–Induced Neuroprotection Is Dose and Time Dependent
PC-SOD treatment in incremental doses (0.3, 1.0, and 3.0 mg/kg) resulted in a significant decrease in infarct volume 24 hours after MCAO (Figures 1, 2A, and 2B). Maximal beneficial effects were observed at the 1.0 mg/kg dose (49.4% reduction in infarct size). This dose also significantly decreased infarct volume at 72 hours after MCAO (Figures 2C and 2D). Similar changes were observed in neurological scores. A significant improvement in neurological score was observed at both 24 and 72 hours after MCAO with the 1.0 mg/kg dose of PC-SOD (Figures 2C and 2D). Similar changes were observed in neurological scores. A significant improvement in neurological score was observed at both 24 and 72 hours after MCAO with the 1.0 mg/kg dose of PC-SOD (Figures 2C and 2D).

Oxidative Stress Is Attenuated by PC-SOD Treatment
Oxidative stress was assessed qualitatively as well as quantitatively by immunostaining with an oxLDL antibody (marker for lipid peroxidation) and evaluating the products of lipid peroxidation with an MDA assay.13 MCAO caused a significant increase in MDA levels in cortical areas of the brain. Treatment with the 1 mg/kg dose of PC-SOD, however, significantly reduced MDA levels compared with those in the vehicle-treated group (Figure 3B). The neurons in the peri-infarct zone (left anterior cerebral artery–MCA border area as shown in Figure 3A, region of interest) showed oxidative stress as indicated by colocalization of immunoreactivities of NeuN (neuronal marker) and oxLDL (lipid peroxidation marker).

PC-SOD Reduces Neuronal Apoptosis
Neuronal cell death was evaluated quantitatively by studying the expression of apoptotic markers such as cleaved caspase-3 and cytochrome c by Western blotting analyses. In addition, mitochondrial Bcl-2 levels were also estimated in the ischemic cortex area (Figure 4). The vehicle-treated group showed reduced expression of mitochondrial Bcl-2. PC-SOD...
treatment, however, significantly increased the expression of mitochondrial Bcl-2 compared with that in the vehicle-treated group (Figure 4A). The mitochondrial and cytosolic expressions of cytochrome c were significantly decreased and increased, respectively, in the vehicle-treated group compared with the sham group (Figures 4B and 4C). This cytochrome c release was significantly reversed by PC-SOD treatment (1 mg/kg). Cleaved caspase-3 expression was dramatically increased in the vehicle-treated group compared with the sham group, which was significantly reversed by PC-SOD treatment (1 mg/kg; Figure 4D).

**Discussion**

The present study shows that PC-SOD, the lecithinized form of SOD, decreased infarct volume and improved neurological outcomes at different time points after focal cerebral ischemic injury. PC-SOD decreased oxidative stress and provided neuronal protection through antiapoptotic mechanisms.

Previous studies have highlighted that unmodified SOD plays an important role in attenuating different forms of brain injury, including cerebral ischemia. However, its short in vivo half-life and low tissue affinity have hampered the practical use of unmodified SOD formulations. The enzymatic activity of PC-SOD is comparable to that of unmodified SOD. The in vitro activity of unmodified SOD by the xanthine–xanthine oxidase method was 3467 U/mg, whereas that of PC-SOD was 2876 U/mg. Therefore, the activity of PC-SOD was equivalent to 83% of unmodified SOD. PC-SOD, however, has many advantages, such as longer in vivo half-life, greater tissue affinity, and better drug delivery, resulting in pharmacological potency 100 to 200 times more than that of unmodified SOD. We observed that PC-SOD treatment significantly decreased oxidative stress after cerebral ischemia. Use of ox-LDL, an oxidative stress marker, indicated that PC-SOD specifically attenuated neuronal oxidative stress (Figure 3).

PC-SOD has been shown to protect endothelial cells in vitro studies and it also has anti-inflammatory properties. The results of the present study suggest that PC-SOD provides neuronal protection by antiapoptotic mechanisms. PC-SOD increased the mitochondrial levels of antiapoptotic Bcl-2 protein and decreased the subsequent cytosolic translocation of cytochrome c, thus attenuating the downstream caspase activation.

Modifications of SOD such as conjugation with polyethylene glycol and liposome-entrapped SOD have been shown to have neuroprotective roles in cerebral ischemia. There are concerns regarding blood-brain barrier penetration of polyethylene glycol–SOD, whereas liposome-entrapped SOD can penetrate the blood-brain barrier and cell membranes.
efficiently. The lecithinized form of SOD used in this study had been previously shown to penetrate the blood-brain barrier in rats.\textsuperscript{15} A 1-ng/kg dose of PC-SOD resulted in brain tissue concentrations of 14 ng/g wet weight 2 hours after injection into the tail vein.

PC-SOD may also provide beneficial effects when used as an adjunctive treatment to conventional clinical treatments, such as recombinant tissue-type plasminogen activator.\textsuperscript{22} Future studies can be designed to test such combination therapies in addition to evaluating the long-term effects of PC-SOD after cerebral ischemia. The present study emphasized apoptotic mechanisms after focal cerebral ischemia, and the neuroprotective effects of PC-SOD were studied at relevant time points accordingly. It would be interesting to study whether PC-SOD affects upstream cell-survival targets, such as PI3K/AKT and HIF-1.\textsuperscript{2,7,23} Recently, lecithinized SOD has also been used in human subjects for corneal ulcers.\textsuperscript{24} PC-SOD is a promising therapeutic option against neuronal cell death after transient focal ischemia by blocking activation of the Bad cell death signaling pathway. J Neurosci. 2003;23: 1710–1718.


