Polyethylene Glycol–Conjugated Superoxide Dismutase Fails to Augment Brain Superoxide Dismutase Activity in Piglets

Steven E. Haun, MD; Jeffrey R. Kirsch, MD; Mark A. Helfaer, MD; Kenneth L. Kubos, PhD; and Richard J. Traystman, PhD

We studied the effect of intravenously administered polyethylene glycol–conjugated superoxide dismutase (8,000 units/kg) on brain superoxide dismutase activity in 44 1–2-week-old piglets in the absence and presence of global cerebral ischemia and reperfusion. Four groups (n=6 each) of piglets not exposed to ischemia were studied. Enzyme administration increased plasma superoxide dismutase activity from <5 to 142±8 units/ml (mean±SEM) without increasing brain activity (e.g., activities in the caudate were 7.9±0.5 and 8.1±0.4 units/mg protein) for up to 2 hours following administration. Four additional groups (n=5 each) of piglets were given either enzyme or polyethylene glycol 5 minutes prior to 10 minutes of global cerebral ischemia induced by aortic cross-clamping followed by either 5 or 45 minutes of reperfusion. Enzyme administration increased plasma superoxide dismutase activity from <5 to 144±5 units/ml but failed to increase brain activity even after 45 minutes of reperfusion (e.g., activities in the caudate were 8.5±0.3 and 8.6±0.6 units/mg protein). We conclude that intravenous polyethylene glycol–conjugated superoxide dismutase does not increase superoxide dismutase activity in the brain despite global ischemia and reperfusion. (Stroke 1991;22:655–659)

Oxygen-derived free radicals have been implicated as mediators of reperfusion injury following ischemia in many different organs including the heart, intestine, and brain. Copper-zinc (CuZn) superoxide dismutase (SOD) has been proposed as a therapeutic agent for reperfusion injury because of its ability to scavenge oxygen-derived free radicals. SOD has one major drawback as a therapeutic agent. A large, water-soluble molecule (molecular mass=32 kd), CuZn SOD therefore cannot readily penetrate cell membranes or cross the blood–brain barrier in significant quantities following intravenous administration. Thus, if access to intracellular compartments is required for therapeutic efficacy, it appears unlikely that CuZn SOD would provide a protective effect. Indeed, pretreatment with CuZn SOD and catalase failed to improve neurological outcome after global ischemia in dogs, and pretreatment with CuZn SOD alone failed to prevent delayed hypoperfusion following global ischemia in rats.

Investigators have attempted different approaches to increase the intracellular access of SOD. The administration of liposome-entrapped SOD has been shown to increase brain SOD activity and reduce infarct volume in a rat model of focal cerebral ischemia. The conjugation of polyethylene glycol (PEG) monomers to SOD results in enhanced uptake of SOD into cultured endothelial cells following a 24-hour incubation, and rats pretreated with PEG-conjugated SOD had a 24% reduction in infarct volume in a model of focal cerebral ischemia. Pretreatment with PEG-SOD and PEG-conjugated catalase has also been shown to prevent delayed hypoperfusion following asphyxia in newborn lambs. There are no published reports of the therapeutic efficacy of PEG-SOD in a piglet model of transient global cerebral ischemia.

In contrast to liposome-entrapped SOD, the effect of PEG-SOD administration on brain SOD activity has not been described. Conjugation to PEG increases SOD's molecular mass from 32 kd to approximately 100 kd. In light of the size of PEG-SOD, we hypothesized that it would not increase brain SOD activity within 2 hours following intravenous administration. Two hours was chosen based on the finding that oxygen-derived free radical production appears to...
occur early after the onset of reoxygenation in vascular endothelium14 and reperfusion in brain.1 We studied the effect of intravenously administered PEG-SOD on brain SOD activity in piglets with and without subsequent exposure to global ischemia and reperfusion.

Materials and Methods

Bovine xanthine oxidase was obtained from Calbiochem Corp., San Diego, Calif. Coumassie protein assay reagent was obtained from Pierce Chemical Co., Rockford, Ill. PEG-SOD, cytochrome c (type III), xanthine, potassium phosphate, ethylene diaminetetraacetic acid (EDTA), and potassium cyanide were obtained from Sigma Chemical Co., St. Louis, Mo. We used 44 1–2-week-old mixed-breed piglets (Thomas D. Morris, Inc., Reisterstown, Md.) weighing 2.5–3.5 kg.

The 24 piglets not exposed to ischemia were sedated with ketamine (10 mg/kg i.m.) and anesthetized with pentobarbital (30 mg/kg i.v. bolus, 4–6 mg/kg/hr i.v. infusion). Mechanical ventilation via a tracheostomy was adjusted to maintain arterial carbon dioxide tension (Paco2) between 35 and 40 mm Hg, and arterial oxygen tension (Pao2) was maintained between 100 and 200 mm Hg with supplemental O2. A femoral vein and artery were cannulated for the administration of fluids and drugs and for the monitoring of arterial blood pressure and gases, respectively. Rectal temperature was maintained at 37.5 ± 0.5°C with the use of heat lamps and a heating pad.

The 20 piglets used in the ischemia and reperfusion protocol were anesthetized with pentobarbital (50 mg/kg i.p., 4–6 mg/kg/hr i.v. infusion). Pancuronium (0.1 mg/kg i.v.) was administered prior to thoracotomy. These animals were surgically prepared and monitored as previously described.15 Briefly, the piglets were mechanically ventilated via a tracheostomy and instrumented for the administration of fluids and drugs, the monitoring of arterial blood pressure, and the induction of transient global ischemia by aortic cross-clamping. Arterial blood gases and mean arterial blood pressure were monitored at preischemic values throughout reperfusion by adjusting the ventilation and infusing epinephrine intravenously, respectively.

Plasma and brain tissue were obtained and prepared for SOD assay in the following manner. Arterial blood was collected in a test tube containing EDTA and centrifuged at 500g for 20 minutes. At the conclusion of each experiment, the piglet was killed with KCl and its brain was rapidly perfused with 1,000 ml 0.9% sterile saline in each carotid artery. The brain was removed, and the caudate and hippocampus and a portion of the parietal cortex were dissected. These brain regions were immediately homogenized (Tissumizer, Tekmar Co., Cincinnati, Ohio) in 3.0 ml of 50 mM potassium phosphate, 0.1 mM EDTA (pH = 7.8) at 4°C and centrifuged at 4,500g for 20 minutes.16 Plasma and brain supernatants were frozen at −70°C until enzyme and protein assays were performed.

The SOD activity was assayed by the enzyme's ability to inhibit xanthine oxidase–mediated cytochrome c reduction.17 Units of activity are defined as originally assigned by McCord and Fridovich.4 Prior to enzyme assay, brain supernatants were dialyzed (Spectra/Por membrane, molecular weight cutoff: 10,000; Fisher Scientific, Pittsburgh, Penn.) in 50 mM potassium phosphate, 0.1 mM EDTA (pH = 7.8) for 18–20 hours to remove low-molecular-weight reduc-tants of cytochrome c. For the determination of total SOD activity, the final reaction mixture contained 10 μM potassium cyanide to inhibit tissue cytochrome c oxidase. For the determination of manganese (Mn) SOD activity, the final reaction mixture contained 3.0 mM potassium cyanide to inhibit CuZn SOD activity. The CuZn SOD activity was derived by subtracting the Mn SOD activity from the total SOD activity. Protein concentration of the brain supernatant was determined using the method described by Bradford.18 Plasma SOD activity is expressed as units per milliliter, and brain SOD activity is expressed as units per milligram protein.

Four groups of piglets that were not exposed to ischemia (n = 6 each) were studied. The control group received no PEG-SOD, and blood and brain specimens were obtained 30 minutes after the completion of surgery. The three PEG-SOD–treated groups received 8,000 units/kg i.v. PEG-SOD immediately after surgery was completed, and brain samples were obtained at 15 (T15 group), 60 (T60 group), or 120 (T120 group) minutes. Blood was obtained prior to PEG-SOD administration, 15 minutes after PEG-SOD administration, 15 minutes after PEG-SOD administration (for calculation of the volume of distribution), and immediately before brain specimens were obtained. Physiological variables (pH, Paco2, PaO2, hemoglobin content, blood glucose concentration, and mean arterial blood pressure) were measured immediately before brain samples were obtained. Four groups of piglets exposed to ischemia and reperfusion (n = 5 each) were also studied. These piglets received either 8,000 units/kg i.v. PEG-SOD or an equal volume of PEG 30 minutes after the completion of surgery. Five minutes after drug administration, the piglets underwent 10 minutes of global cerebral ischemia followed by either 5 (PEG5 and PEG-SOD5 groups) or 45 (PEG45 and PEG-SOD45 groups) minutes of reperfusion. Blood was obtained for SOD measurement, and physiologic variables were recorded prior to drug administration and at the end of reperfusion. Brain specimens were obtained at the end of each protocol.

Each variable is expressed as mean ± standard error of the mean (SEM). Physiological variables and brain SOD activities between groups of piglets not exposed to ischemia were compared by one-way analysis of variance. Caudate, hippocampus, and cortex SOD activities of the control piglets were also compared using one-way analysis of variance. Post-hoc comparisons were made with Scheffe's
test. Plasma SOD activities at the end of the protocol were compared by the Wilcoxon-Mann-Whitney test. In piglets exposed to ischemia and reperfusion, planned orthogonal comparisons were used to test for differences in physiological variables and brain SOD activity between PEG-treated and PEG-SOD-treated groups exposed to the same duration of reperfusion. Plasma SOD activities at the end of the protocol were compared by the Wilcoxon-Mann-Whitney test. Statistical significance was assumed when \( p < 0.05 \).

### Results

In piglets not exposed to ischemia, physiological variables did not differ in the groups receiving PEG-SOD compared with the control group (pH, 7.37±0.02; Paco\(_2\), 38±2 mm Hg; Pao\(_2\), 193±17 mm Hg; hemoglobin content, 10.7±0.6 g/dl; blood glucose concentration, 71±9 mg/dl; and mean arterial blood pressure, 82±4 mm Hg). Likewise, there were no differences in physiological variables between matched groups of piglets exposed to ischemia and reperfusion (PEG5 versus PEG-SOD5 and PEG45 versus PEG-SOD45) at any time during the protocol. Both groups exposed to 5 minutes of reperfusion had a mild respiratory acidosis (e.g., for PEG5 pH was 7.29±0.05 and Paco\(_2\) was 48±5 mm Hg). However, in all groups exposed to ischemia and reperfusion Pao\(_2\) was maintained above 100 mm Hg, hemoglobin content above 10 g/dl, and mean arterial blood pressure above 60 mm Hg (not different from preischemic values).

Plasma SOD activity was less than the lower limit of sensitivity of the SOD assay (5.0 units/ml) in all groups of piglets not receiving PEG-SOD. Plasma SOD activity increased more than 25-fold in all groups receiving PEG-SOD (T15: 160±8, T60: 157±8, and T120: 142±8 units/ml) despite subsequent exposure to ischemia and reperfusion (PEG-SOD5: 141±6 and PEG-SOD45: 144±5 units/ml). The volume of distribution of PEG-SOD was 51±2 ml/kg (n=18).

In the control piglets, there was no difference in total SOD activity (Figure 1) or CuZn SOD activity (caudate, 5.2±0.4; cortex, 5.3±0.3; and hippocampus, 4.9±0.6 units/mg protein), but Mn SOD activity was significantly higher in the caudate than in the hippocampus (caudate, 2.8±0.2; cortex, 2.1±0.3; and hippocampus, 1.5±0.1 units/mg protein). The effect of intravenous PEG-SOD on regional brain SOD activity in piglets not exposed to ischemia is shown in Figure 1. There was no difference for total SOD activity in the caudate, cortex, or hippocampus among the groups.

Brain SOD activity from piglets exposed to ischemia and reperfusion is shown in Figure 2. Again, there was no difference in the caudate, cortex, or hippocampus SOD activity when comparing the PEG-SOD5 and PEG5 groups or the PEG-SOD45 and PEG45 groups. Brain SOD activities of the T60 and PEG-SOD45 groups were also compared to determine the effect of ischemia and reperfusion on brain SOD activity following PEG-SOD administration. There were no differences between groups.

### Discussion

The intravenous administration of PEG-SOD caused a dramatic rise in the plasma SOD activity but...
failed to increase the brain SOD activity during 2 hours. In addition, global cerebral ischemia and reperfusion did not facilitate an increase in brain SOD activity after the intravenous administration of PEG-SOD. We speculate that PEG-SOD has limited transport across the blood–brain barrier so that if this agent is protective early during reperfusion against free radical–induced injury, it is unlikely to be via a direct antioxidant effect within the brain parenchyma.

Endogenous plasma SOD activity in our study was <5.0 units/ml, which is consistent with that found by Marklund et al in adult swine. The increase in plasma SOD activity after PEG-SOD administration is similar to that reported in rats. The volume of distribution of PEG-SOD (51 ± 2 ml/kg) is very similar to the plasma volume of young swine (62.5 ml/kg), suggesting that PEG-SOD does not enter other compartments quickly. This is also consistent with our finding that the intravenous administration of PEG-SOD had no effect on brain SOD activity in the regions studied.

Endogenous brain SOD activity for piglets is similar to that reported for rats and substantially lower than that reported for humans. The CuZn SOD activity is approximately 2–3 times greater than the Mn SOD activity, which is similar to that reported for both rats and humans. The low endogenous activity of Mn SOD in the hippocampus is consistent with this brain region's selective vulnerability to injury from ischemia and reperfusion.

We did not specifically look for the presence of PEG-SOD in brain. Therefore, it is possible that PEG-SOD accumulated in the brain but was inactivated during the process of crossing the blood–brain barrier. We believe this is an unlikely explanation for our data. SOD is a relatively stable enzyme, but it is susceptible to degradation by proteases in an acidic pH. Conjugation to PEG increases the resistance of an enzyme to proteolysis. Beckman and coworkers demonstrated that PEG-SOD, unlike native SOD, is resistant to degradation by endothelial cells. Thus, it is unlikely that PEG-SOD was inactivated when crossing the blood–brain barrier.

Our data cannot exclude the possibility that a small quantity of PEG-SOD entered the brain vascular endothelium. Beckman and coworkers demonstrated that conjugation of PEG monomers to SOD facilitated uptake of the enzyme into cultured endothelial cells. After 24 hours of incubation in media containing approximately 500 units/ml PEG-SOD, endothelial cell SOD activity increased approximately 300%. Our plasma SOD activity ranged from 142 to 160 units/ml, which is approximately one third the SOD activity of the incubation media in that report. Thus, a significantly lower concentration gradient for the movement of PEG-SOD into brain capillary endothelium would be expected under our experimental conditions. In our longest experiment, brain SOD activity was measured only 2 hours after PEG-SOD administration. After 2 hours of incubation, endothelial cell SOD activity in the study of Beckman et al had increased no more than 35% (increase was not specified as being statistically significant). Even if brain vascular endothelium SOD activity had increased by 35%, it is unlikely that we would have detected an increase in total brain SOD activity because brain capillary endothelium is a relatively small portion of the total brain weight. Therefore, our results are not inconsistent with those of Beckman and coworkers and do not exclude the possibility that small quantities of PEG-SOD were taken up into the brain vascular endothelium during the time studied.

Intravenous PEG-SOD administered prior to transient focal ischemia in cats decreases infarct volume, and pretreatment with both PEG-SOD and PEG-catalase has been shown to reduce infarct volume following permanent focal ischemia in rats. PEG-catalase is an even larger molecule (molecular mass of unconjugated catalase = 242 kd) than PEG-SOD and therefore is also unlikely to cross the blood–brain barrier in significant quantities. Our data with global ischemia cannot be directly transferred to the situation of focal ischemia since the mechanisms of brain injury for each may be quite different. For example, with transient focal ischemia there may be rapid disruption of the blood–brain barrier, which permits entry of PEG-conjugated enzymes into the parenchyma. However, recent data with transient focal ischemia suggest that pretreatment with PEG-SOD reduces infarct volume by improving blood flow to brain regions with poor collateral circulation during ischemia, without an independent effect on parenchymal reperfusion injury. Although the effect of PEG-SOD on any measure of outcome following global cerebral ischemia is unknown, pretreatment with PEG-SOD and PEG-conjugated catalase prevented delayed hypoperfusion following severe asphyxia in newborn lambs. As in our study, blood–brain barrier permeability was not quantified by Rosenberg and colleagues. In addition, since the brain activities of SOD and catalase were not measured following asphyxia, it is not possible to determine if brain protection is dependent on increased antioxidant activity in the parenchyma. We speculate that improved blood flow following asphyxia is the result of an antioxidant effect on the vascular endothelium rather than a direct effect on the brain parenchyma. Alternatively, these PEG-conjugated enzymes may exert a protective effect on the vascular endothelium by scavenging oxygen-derived free radicals produced in the intra-vascular space.

In summary, the intravenous administration of PEG-SOD failed to increase brain SOD activity in piglets. In addition, global cerebral ischemia and reperfusion failed to augment brain SOD activity following PEG-SOD administration. If PEG-SOD exerts a protective effect following global cerebral ischemia, it may do so by ameliorating free radical–induced injury to the brain vascular endothelium.
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References


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