Brain and Tissue Distribution of Polyethylene Glycol-Conjugated Superoxide Dismutase in Rats

Kenshi Yoshida, MD; Gregory F. Burton, PhD; Jerry S. McKinney, BS; Harold Young, MD; and Earl F. Ellis, MD

Background and Purpose: The purpose of this study was to determine the distribution of polyethylene glycol-conjugated superoxide dismutase in the brain, cerebrospinal fluid, and various organs.

Methods: Distribution of iodine-125-labeled polyethylene glycol-conjugated superoxide dismutase was determined in three groups of male Sprague-Dawley rats: a normotensive sham control group (n=9) and groups given 125I-labeled polyethylene glycol-conjugated superoxide dismutase either 30 minutes before (n=10) or 30 minutes after (n=7) norepinephrine-induced hypertensive injury.

Results: In the first 30 minutes after intravenous administration, polyethylene glycol-conjugated superoxide dismutase plasma activity declined to 70% of the initial value and then decreased negligibly between 30 and 90 minutes. Levels of 125I-labeled polyethylene glycol-conjugated superoxide dismutase in normotensive animals were low in the brain and cerebrospinal fluid and highest in kidney. Brain levels of polyethylene glycol-conjugated superoxide dismutase were elevated only in those rats that received it before hypertensive injury, however, cerebrospinal fluid levels were elevated in animals receiving the drug either before or after hypertensive injury.

Conclusion: Our results suggest that the blood-brain barrier becomes more permeable to polyethylene glycol-conjugated superoxide dismutase only during the hypertensive period but that the blood-cerebrospinal fluid barrier sustains more permanent injury. We suggest that the therapeutic effectiveness of polyethylene glycol-conjugated superoxide dismutase in hypertensive brain injury is due to its action in the vascular wall or to its extracellular activity in the cerebrospinal fluid.

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KEY WORDS • blood-brain barrier • free radicals • neuronal damage • rats

We and other investigators have attempted to therapeutically use an antioxidant agent or a free radical scavenging enzyme such as superoxide dismutase (SOD) because there is strong evidence supporting the involvement of oxygen free radicals in the pathogenesis of various kinds of brain injury.5-9 When SOD is injected into the circulation, it is thought to be the site of generation of free radicals.5-7,9-11 However, SOD is a large, water-soluble molecule that is therefore unlikely to cross many barriers, including the blood-brain barrier.12,13 Additionally, native SOD has a circulating plasma half-life of only 6-10 minutes.13,14 To increase the circulating half-life, SOD has been conjugated with polyethylene glycol (PEG), and studies indicate that PEG-SOD has a half-life of approximately 40 hours in the circulation.15,16 While conjugation increases half-life, it also increases the molecular weight and may present increased problems in terms of extravascular distribution. This large molecular weight may not be as much of a hindrance to tissue access after injury because the integrity of various barriers may be broached, resulting in an increased tissue distribution of PEG-SOD. We therefore surmise that PEG-SOD may be able to enter the brain more readily after various kinds of insults, such as traumatic brain injury,17,18 cerebral ischemia,19,20 or acute hypertension.21,22

A few studies have examined the pharmacokinetics of SOD in the central nervous system after its peripheral administration.14,16 Chan et al7 have reported that SOD does not enter the brain when given peripherally. Very recently Haun et al23 reported that intravenous PEG-SOD did not increase SOD activity in the brain despite global ischemia and reperfusion. Although the report of Haun et al clearly shows that there are not large increases in PEG-SOD after global ischemia and reperfusion, the lower limit of sensitivity for their SOD assay may have prevented them from detecting very small increases in activity.24-26 The aim of the present study was to address the tissue distribution of iodine-125-labeled PEG-SOD. It was hoped that by radioactively labeling the PEG-SOD we would increase the lower limit of sensitivity for detection of its tissue distribution. We also investigated whether distribution of 125I-PEG-SOD was altered by acute norepinephrine-induced hypertension.
Materials and Methods

Iodination of PEG-SOD (Sigma Chemical Co., St. Louis, Mo.) was performed using a modification of the procedure of Beckman et al. Briefly, 15 μg PEG-SOD (in 100 μl Dulbecco’s phosphate-buffered saline) was mixed with 2 mCi 125I (Amersham, Arlington Heights, Ill.) and added to a glass culture tube previously coated with 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (Iodo-Gen, Pierce Chemical Co., Rockford, Ill.). The reaction mixture was incubated at room temperature for 20 minutes, and the reaction was stopped by removing the liquid phase. Unbound iodine was removed by adding the iodine–protein mixture to an Eppendorf tube containing Sephadex G-25 (Pharmacia, Piscataway, N.J.), centrifuging for 30 seconds at 900g, and collecting the supernatant. The PEG-SOD was labeled to a specific activity of 21–28 μCi/μg protein. The iodinated PEG-SOD retained 78% of its superoxide scavenging capacity, as determined by the cytochrome C reduction assay for superoxide.

The material was stored at 4°C until used.

A total of 26 male Sprague-Dawley rats weighing 300–400 g were used. After anesthesia was induced with 50 mg/kg i.p. pentobarbital, the animals were tracheotomized and ventilated with room air. The ventilatory rate and volume were adjusted so that the end-expiratory CO2 was maintained at approximately 30 mm Hg. A femoral artery and vein were cannulated for blood sampling and intravenous infusion. Blood gases and pH were analyzed to ensure adequate and consistent ventilation. The temperature of all animals was maintained at 37°C with a heating pad. The animals were placed in a prone position, and a rostralcaudal midline incision was made in the scalp and a needle inserted to collect cerebrospinal fluid (CSF) from the cisterna magna. Samples showing any evidence of blood were discarded.

Hypertension was produced by constant intravenous administration of norepinephrine using an infusion pump. The norepinephrine stock solution was in 0.9% saline, and the infusion speed was varied from 0.068 to 180 mm Hg for 5 minutes. It took approximately 30 μg/kg per minute to maintain this pressure for the period. The total infusion volume for the 5 minutes of hypertension did not exceed 1.2 ml.

125I-PEG-SOD (5 μCi/kg) plus unlabeled PEG-SOD (2,000 IU/kg) was given in 0.2 ml saline through the femoral vein. The total weight of PEG-SOD to achieve this dose was less than 1 mg per animal. Therefore, there is no significant drug effect on osmolality. Plasma samples (0.1 ml) were collected through the arterial line at 1, 5, 30, 60, and 90 minutes after PEG-SOD infusion. All animals were killed at 90 minutes after administration of PEG-SOD.

The animals were divided into three experimental groups (Figure 1). One group received only 125I-PEG-SOD. Another group received 125I-PEG-SOD 30 minutes after hypertensive injury, and a third group received 125I-PEG-SOD 30 minutes before hypertensive injury. At 90 minutes after injection of PEG-SOD, a CSF sample was collected by cisternal puncture. The CSF sample was examined visually, and any sample with evidence of blood was discarded because the presence of blood would cause abnormally high 125I-PEG-SOD levels. Animals were then perfused with saline through the left ventricle at a pressure of 100 mm Hg to eliminate intravascular 125I-PEG-SOD. The brain and various organs were then removed and the 125I counted with a gamma counter. Exogenous PEG-SOD concentration in each sample was estimated from the radioactive counts in the tissue by use of the specific activity of total PEG-SOD infused. Results were examined by analysis of variance followed by a Fisher least significant difference test. Data are expressed as mean±SEM. A value of p<0.05 was considered to be statistically significant.

Results

The control physiological parameters shown in Table 1 indicate that the three groups of rats were not significantly different. Changes in plasma PEG-SOD concentration in the control, preinjury, and postinjury groups are shown in Figure 2. Although the average plasma levels of PEG-SOD in the control group are lower than those of the other groups, there was no statistically significant difference in the plasma levels between groups over the 90-minute period. The plasma concentration of PEG-SOD fell more dramatically in the first 30 minutes and was relatively stable between 30 and 90 minutes after injection. At 90 minutes after injection, the plasma PEG-SOD level was approximately 60–70% of that at 1 minute after injection.

Figure 3 shows the tissue concentration of PEG-SOD in various organs at 90 minutes after intravenous injec-
TABLE 1. Control Physiological Parameters in Rats by Group

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (PEG-SOD, no HT) (n=9)</th>
<th>Preinjury (PEG-SOD→HT) (n=10)</th>
<th>Postinjury (HT→PEG-SOD) (n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean arterial pressure (mm Hg)</td>
<td>100.6±8.7</td>
<td>102.4±5.4</td>
<td>102.7±8.5</td>
</tr>
<tr>
<td>PaO2 (mm Hg)</td>
<td>94.4±4.1</td>
<td>96.1±4.1</td>
<td>90.5±2.7</td>
</tr>
<tr>
<td>PacO2 (mm Hg)</td>
<td>32.4±0.6</td>
<td>31.8±0.4</td>
<td>30.6±0.8</td>
</tr>
<tr>
<td>Blood pH</td>
<td>7.399±0.02</td>
<td>7.406±0.01</td>
<td>7.392±0.01</td>
</tr>
</tbody>
</table>

Physiological variables measured at baseline in control, preinjury, and postinjury groups. Values are mean±SEM. PEG-SOD, polyethylene glycol-superoxide dismutase; HT, 5-minute duration of norepinephrine-induced hypertension.

Figure 2 shows that the brain PEG-SOD concentration was increased only in the group that received PEG-SOD before the hypertensive injury. It also shows that, unlike the brain response, CSF PEG-SOD was elevated in both groups that received hypertensive injury. Table 2 shows the brain-to-plasma and CSF-to-plasma ratios for PEG-SOD in the control, preinjury, and postinjury groups. Again, it can be clearly seen that the brain-to-plasma ratio is similar in the control and posttreatment groups and elevated approximately threefold in the group given radiolabeled PEG-SOD before hypertensive injury. The ratios also show that the permeability of the blood-cerebrospinal fluid barrier to PEG-SOD increased approximately eightfold in the group treated before injury and 11-fold in the group treated after injury.

Discussion

Odlind et al reported the distribution pattern of 125I-labeled bovine SOD in rats after intravenous infusion using whole-body autoradiography. They showed that SOD, with a half-life of approximately 6 minutes, rapidly disappears from the circulation, with subsequent dominant localization of 125I-SOD in the kidney and urinary tract. Our current results are in agreement with those of Beckman et al and show that conjugation of SOD with PEG results in a dramatic increase in circulating half-life of SOD. Our study showed a marked decline in the concentration of PEG-SOD within 30 minutes after intravenous injection, which likely represents its distribution and equilibrium with the various systemic organs. After 30 minutes, this first phase of distribution was complete, and plasma levels remained relatively stable between 30 and 90 minutes after infusion.

Our results for distribution of PEG-SOD are similar to those of Odlind et al in that we also show dominant localization of PEG-SOD to the kidney. Although the kidney is less than 2% of the rat body weight, it appears to be the major organ for distribution of PEG-SOD after intravenous injection. However, the role of the liver cannot be discounted because its mass and percent body weight are relatively high compared with those of the kidney.
In agreement with previous studies, our current results show that in control animals there is no major uptake of PEG-SOD into the brain or CSF. In animals in which iodinated PEG-SOD was present during the hypertensive challenge, analysis of the brain showed an approximate 2-fold increase in the brain level of PEG-SOD. This increase in PEG-SOD level in the brain in the pretreatment but not the posttreatment group is similar to the results that we have previously reported with 123I-labeled human serum albumin and hypertensive challenge; that is, permeability increases only during the hypertensive phase. Therefore, when PEG-SOD, like human serum albumin, is administered after the hypertensive challenge, there is no increase in brain content of these two substances.

The results with CSF PEG-SOD levels offer a different interpretation with respect to the effect of hypertension on the blood-CSF barrier. Figure 4 shows that PEG-SOD activity at 90 minutes after administration was increased in the CSF sample irrespective of whether the PEG-SOD was given before or after hypertensive injury. This implies that unlike the blood-brain barrier, the blood-CSF barrier is more lastingl injured by the acute hypertension. This results in a dramatic increase in PEG-SOD sampled at the cisterna magna in both the pretreatment and posttreatment groups. From these results we infer that the tissue that forms the CSF we sampled is injured and compromised beyond the time of the hypertensive insult.

These findings shed light on the interpretation of one of our previous studies, wherein we examined the effect of PEG-SOD on brain edema induced by acute hypertension. We reported that treatment after acute hypertension with PEG-SOD, at the same dose used in the current study, was able to reduce hypertension-induced brain edema. It should be emphasized that edema caused by acute hypertension is minor, causing less than 1% increase in water content. Whether PEG-SOD would substantially reduce edema in more severe models of edema, such as that caused by cold injury, is less certain. The results of the current study suggest that if the site of therapeutic action of PEG-SOD is extravascular, the PEG-SOD may enter the CSF by crossing the choroid plexus. Alternatively, of course, it may be suggested that the therapeutic site of action is entirely intravascular and that any effect of PEG-SOD to reduce brain edema is due to the action of PEG-SOD on the vessel wall.

The main source of oxygen radical formation after injury is thought to be intracellular; however, we have previously provided evidence that after experimental fluid percussion brain injury or acute hypertension, oxygen radicals enter the extracellular space through an anion channel. In reality, it is not known whether

### Table 2: Brain-to-Plasma and CSF-to-Plasma Ratios of PEG-SOD in Rats by Group

<table>
<thead>
<tr>
<th>Group</th>
<th>Control (PEG-SOD only)</th>
<th>Preinjury (PEG-SOD → HT)</th>
<th>Postinjury (HT → PEG-SOD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IU per gram brain</td>
<td>1.51 ± 0.26 (n=8)</td>
<td>3.35 ± 0.65* (n=7)</td>
<td>1.07 ± 0.24 (n=6)</td>
</tr>
<tr>
<td>IU per milliliter plasma</td>
<td>2.30 ± 0.20 (n=5)</td>
<td>18.2 ± 3.93† (n=7)</td>
<td>25.9 ± 7.19† (n=5)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. CSF, cerebrospinal fluid; PEG-SOD, polyethylene glycol-superoxide dismutase; HT, 5-minute duration of norepinephrine-induced hypertension.

Brain-to-plasma ratio in control and postinjury groups were not significantly different. Brain-to-plasma ratio in preinjury group shows significant elevation compared with those in other group; *p < 0.05 versus control or postinjury group. CSF-to-plasma ratio in preinjury and postinjury groups shows significant elevation compared with control group; †p < 0.05 versus control.
radical damage to cells is greater intracellularly or extracellularly. The possibility exists that when radicals leave the cell, they attack the outer cell membrane, where defense mechanisms may be more limited. Since PEG-SOD is not likely to readily enter cells its main site of action may be on radicals which transiently exist outside the cell.

Another factor to consider is the specific cellular site at which brain 125I-PEG-SOD is located. Our studies show that very small amounts of PEG-SOD are found in the brain when considered on a whole-brain basis. However, we do not know exactly where the 125I-PEG-SOD is localized. If the 125I-PEG-SOD were concentrated exclusively in one particular tissue, its local concentration might reach a more effective scavenging level. If, for example, the PEG-SOD were only in the vascular wall or endothelium, its local concentration might effectively be 100-fold greater. This is an important question to be resolved in future studies.

Our results using iodinated PEG-SOD confirm the work of Haun et al\(^1\) indicating that at least for global ischemia/reperfusion and acute hypertensive injury, PEG-SOD does not appear to enter the brain in large quantities. However, with the current radiolabel technique we were also able to sample CSF and show that the level of SOD activity in CSF was significantly elevated. This points out the importance of sampling both brain and CSF for SOD activity. In addition, our study suggests that finding little or no increase in whole-brain SOD activity does not necessarily mean that SOD is not increased in the CSF bathing the brain. Although the current studies indicate little passage of PEG-SOD across the blood–brain barrier after hypertensive injury, this does not rule out the possibility that more dramatic types of brain injury, such as traumatic injury, this does not rule out the possibility that SOD into the brain parenchyma.

**References**

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