PEG-SOD and Myocardial Protection

Studies in the Blood- and Crystalloid-Perfused Rabbit and Rat Hearts

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Background. Polyethylene glycol, covalently linked to superoxide dismutase (PEG-SOD), has a long plasma half-life (>30 hours) and has been proposed as an effective agent for reducing free radical-mediated injury during ischemia and reperfusion.

Methods and Results. Using an isolated rabbit heart perfused with arterial blood from a support rabbit, we have demonstrated that pretreatment with PEG-SOD (30,000 units/kg, intravenous bolus, 12–24 hours before 60 minutes of normothermic global ischemia), combined with addition of PEG-SOD to the blood perfusion circuit (30,000 units/kg to the support rabbit) and inclusion of PEG-SOD (150 μg/ml) in a cardioplegic solution, enhanced the postischemic recovery of left ventricular developed pressure (LVDP) from 51±6 to 74±9 mm Hg (p <0.05; n=9 per group). In further studies we showed that, whereas maximum protection was obtained when PEG-SOD was given as a combined pretreatment and additive to both the cardioplegic and the reperfusion solutions (postischemic LVDP recovery increased from 44±4% in the control group to 70±3% in the PEG-SOD group), the administration of PEG-SOD during pretreatment plus cardioplegia or during reperfusion alone also resulted in a significant improvement in postischemic function (62±7% and 60±3%, respectively). However, the use of PEG-SOD as a cardioplegic additive alone failed to afford protection (47±4% recovery of LVDP). In dose–response studies (with 0, 3,000, 6,000, 12,000, 30,000, or 60,000 units/kg; n=8 per group), maximum recovery of LVDP was obtained with the administration of 12,000 units/kg of PEG-SOD. Studies of the plasma activity of PEG-SOD confirmed its long half-life and showed that the treatment with PEG-SOD either 1 hour or 12–24 hours before the study resulted in similar levels of plasma activity. In an attempt to assess any involvement of blood-borne elements in the protection afforded by PEG-SOD, studies were also carried out in the crystalloid-perfused rabbit heart, and no protection was observed. Similarly, no protection was observed at any one of a variety of doses in the crystalloid-perfused rat heart.

Conclusions. PEG-SOD can afford protection in the blood-perfused rabbit heart; this protection is dose dependent and probably involves some action of PEG-SOD on blood-borne elements, possibly leukocytes. (Circulation 1992;86:672–682)

Key Words • PEG-SOD • ischemia, reperfusion • free radicals • antioxidants • myocardial protection • rabbit • rat

Recently, many studies have been directed at elucidating the role of free radicals in the pathophysiology of injury during myocardial ischemia and reperfusion. Indirect evidence for an involvement of radicals in ischemia- and reperfusion-induced injury derives from studies in which antioxidant enzymes, organic antioxidants, or agents that inhibit the production of radicals have been suggested to 1) reduce vulnerability to reperfusion arrhythmias,1–4 2) limit infarct size in regional ischemia,5–11 3) attenuate myocardial stunning,12–15 and 4) enhance postischemic recovery in models of surgical global ischemia.16–20 Because the superoxide anion is thought to be involved in radical-induced injury, much emphasis has been placed on evaluating the ability of exogenous superoxide dismutase (SOD) to reduce myocardial injury. Although many studies, both in vivo and in vitro, have shown beneficial effects,2–20 controversy still flourishes since a considerable number of studies have suggested that SOD is ineffective in comparable settings.21–27 In seeking to explain the negative results, proponents of SOD therapy have suggested differences in dose, time of administration, and experimental model as possible contributory factors. Thus, because native SOD has a very short half-life (6–10 minutes), time of administration (before, during, or after ischemia), frequency of administration, and maintenance of appropriate plasma levels may pose serious problems in both the therapeutic use of the native enzyme and the ability to compare studies from different laboratories. Furthermore, because the main sources of radicals have not been definitively identified, the use of crystalloid-perfused hearts (lacking leukocytes, which are a potential source of radicals)
might further add to the arguments surrounding the evaluation of antioxidant enzymes.

Many of the experimental difficulties of investigating the protective properties of SOD could be overcome if the half-life of the enzyme could be extended and the studies could be conducted in blood-perfused hearts with assessments of dose and temporal responsiveness. Recently, a long-acting SOD covalently linked to polyethylene glycol (PEG-SOD) has become available (Sterling Research Group, Rensselaer, N.Y.) and offers the advantage of a plasma half-life in dogs and in humans in excess of 30 hours.8,11,24,29

The objectives of the present studies were 1) to determine whether PEG-SOD could improve postischemic recovery in the isolated blood-perfused rabbit heart, 2) to ascertain whether the agent was most effective when administered as a pretreatment, as a cardioplegic additive, or as a reperfusate additive, and 3) to define any potential dose-related effects of PEG-SOD. In addition, we have sought to exploit the differences between blood- and crystalloid-perfused hearts in order to assess the possible role of blood-borne elements in injury and protection and have also compared rat and rabbit hearts to assess any possible species differences.

Methods

Animals and Reagents

New Zealand White rabbits of either sex (4–5 kg body wt for the support rabbit and 500–600 g for the donor) and male Wistar rats (250–300 g body wt) were used. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH No. 80–23, revised 1985). PEG-SOD, 10,000 µg/ml (equivalent to 30,000 units/ml) was obtained from Sterling Research Group.

Blood-Perfused Rabbit Heart Preparation

This preparation has been described previously in detail.30 Briefly, the support rabbit was premedicated with fentanyl citrate (0.015 mg/kg i.m.) and fluanisone (0.5 mg/kg i.m.) and anesthetized with sodium pentobarbital (3–6 mg/kg/hr i.v.) and anticoagulated with heparin (1,000 IU/kg/hr i.v.). Endotracheal intubation allowed the animal to breathe spontaneously with a controlled flow of 95% O2 plus 5% CO2 so as to maintain arterial Po2 and PCO2 within their physiological ranges. The femoral artery and femoral vein were cannulated for arterial blood supply to the donor heart and for the return of blood and fluids, respectively. Body temperature was maintained at 37°C with heating pads. Donor rabbits were premedicated and anesthetized as in the support animal. Heparin was administered (1,000 IU/kg i.v.), and the chest was opened; the heart was then excised and immersed in cold (4°C) saline until contraction had ceased. Depending on the required protocol, the heart was either stored in a globally ischemic state for a fixed period and then subjected to blood perfusion or was immediately subjected to blood perfusion. In either in-

stance, the aorta was rapidly cannulated and perfused with arterial blood from the support rabbit in the Langendorf mode.31 The blood was delivered to the heart by a peristaltic pump, and perfusion pressure was continuously monitored and controlled between a defined range (40–60 mm Hg). Blood was maintained at between 36.5°C and 37.5°C by a thermostatically regulated heart chamber. Blood was infused back into the support animal through a blood filter (A100 Blood Administration Set, Avon Medicals). A compliant balloon catheter, attached to a pressure transducer, was inserted into the left ventricle through the atrium and then inflated with water to maintain a constant left ventricular end-diastolic pressure (LVEDP) of 4 mm Hg. Left ventricular developed pressure (LVDP) and coronary flow were recorded throughout the experiment. Heart rate was maintained by pacing at a constant 200 beats per minute.

Crystalloid-Perfused Rat and Rabbit Heart Preparations

Rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.), and rabbits were anesthetized as described in the previous section. Heparin (1,000 IU/kg i.v.) was then administered; 30 seconds later, the chest wall was opened, and the heart was excised and placed in cold (4°C) saline until contraction had ceased. The aorta was immediately cannulated, and the heart was perfused in the nonrecirculating Langendorf mode31 at 37°C and a constant perfusion pressure of 75 mm Hg. The perfusion solution contained (in mmol/l): glucose 11.1, NaCl 118.5, KCl i.75, MgSO4 1.19, KH2PO4 1.18, NaHCO3 25.0, CaCl2 1.36, and was gassed with 95% O2 plus 5% CO2 (pH 7.4). The solution was filtered before use through a 5-µm porosity filter to remove any particulate matter. Hearts were perfused for 20 or 30 minutes, during which a compliant balloon, attached to a pressure transducer to permit recording of intraventricular pressure,32 was introduced into the left ventricle through the left atrium. The balloon was then filled with sufficient fluid to produce an end-diastolic pressure of 4 mm Hg. Assessment of cardiac function was carried out as described in the previous section.

Exclusion Criteria

In blood perfusion studies, hearts isolated from rabbits were excluded when preischemic values of LVDP were lower than 70 mm Hg or when coronary flow was <1.2 ml/min at the designated perfusion pressures. In crystalloid perfusion studies, rabbit hearts were excluded when preischemic values of LVDP were <70 mm Hg or when coronary flow was <20 ml/min.

Exclusion criteria in isolated rat hearts included preischemic values of LVDP <70 mm Hg, coronary flows <10 ml/min, and heart rates <250 beats per minute.

In some studies in which preischemic functional assessment was deliberately excluded from the protocol, it was not possible to apply the preceding exclusion criteria.

Measurement of Cardiac Function, Creatine Kinase Leakage, and Ionized Calcium

LVDP, LVEDP, heart rate, and perfusion pressure in crystalloid- or blood-perfused hearts were continuously monitored, as was the arterial pressure of the support
animal in the blood perfusion studies. Coronary flow was measured by timed collection of the effluent before ischemia and at intervals of 5 minutes during the reperfusion period. Results are expressed either as a percent of their preischemic value or in absolute terms.

In studies with crystalloid-perfused hearts, coronary effluent was collected during the total reperfusion period and was taken for the determination of total creatine kinase leakage, which was expressed as IU/40 or 60 min/heart.

Ionized calcium in blood and perfusion solutions was measured using an ion analyzer (Nova Biomedical Corp.).

Measurement of Plasma PEG-SOD Activity

Blood samples (1 ml) were obtained from donor or support rabbits at selected points in time as indicated below. Blood samples anticoagulated with heparin were centrifuged, and the plasma was aspirated and stored at −170°C in liquid nitrogen until assay.

The amount of PEG-SOD protein was determined by measuring the conversion of hydroxylamine to nitrite by superoxide in the presence of the sample. The results were compared with reference standards, and the results were computed from a calibration curve for PEG-SOD using a Cobas-Fara II analyzer. This system was validated using a cytochrome method previously described.

Experimental Protocols

Study 1: Blood-perfused rabbit heart studies with PEG-SOD given before and after ischemia. In these studies, donor rabbits received PEG-SOD (30,000 units/kg body wt i.v.) 12–24 hours before study. In addition, PEG-SOD (150 μg/ml) was added to the St. Thomas' Hospital cardioplegic solution (containing in mmol/l: NaCl 110.0, KCl 16.0, MgCl2 16.0, CaCl2 1.2, NaHCO3 10.0; pH 7.8). Finally, PEG-SOD was given (30,000 units/kg body wt i.v.) to the support animal 1 hour before study. Controls were given equivalent volumes of saline.

In this first series of studies, rabbits (n=9 per group) were pretreated with PEG-SOD or saline; 12–24 hours later, the hearts were excised and blood-perfused at a pressure of 40–60 mm Hg for 20 minutes. During this time, a balloon was inserted into the left ventricle, and preischemic control values for LVDP and coronary flow were recorded. After the 20-minute preischemic phase, hearts were immediately infused (2 minutes at a pressure equivalent to 45 mm Hg) with the St. Thomas' Hospital cardioplegic solution (with or without added PEG-SOD) and rendered globally ischemic for 60 minutes at 37°C. This was followed by reperfusion for 60 minutes; the postischemic recovery of LVDP and coronary flow were recorded at 5-minute intervals beginning after the first 10 minutes of reperfusion. Samples of blood were obtained from donor rabbits before the excision of the hearts and at the end of the experiment from the support animal. Plasma samples were frozen until assayed for PEG-SOD activity. Throughout the preischemic and postischemic periods, hearts were paced at 200 beats per minute via pacing wires attached to the right atrium.

Study 2: Blood-perfused rabbit heart study to compare the efficacy of PEG-SOD as a pretreatment and as a cardioplegic and reperfusate additive. PEG-SOD was administered to donor and/or support animals (30,000 units/kg i.v.); controls received comparable volumes of saline. In some groups, PEG-SOD (150 μg/ml) was added to the cardioplegic solution. Five groups of animals (n=8 hearts per group) were used for these studies: group A: No PEG-SOD was used at any stage; group B: PEG-SOD was administered to the donor as pretreatment 12–24 hours before study; it was also added to the cardioplegic solution and given to the support animal 1 hour before the evaluation of preischemic function in the perfused heart; group C: PEG-SOD was administered to the donor as a pretreatment and was added to the cardioplegic solution but was absent during the reperfusion phase; group D: PEG-SOD was added to the cardioplegic solution alone and was not given either as a pretreatment or during reperfusion; group E: PEG-SOD was only present during reperfusion (given to the support animal after ischemia had been induced in the perfused heart).

In this study (as in study 1), hearts were allowed to be perfused for 20 minutes before being subjected to 60 minutes of global normothermic ischemia and 60 minutes of reperfusion.

Study 3: Dose–response studies in the blood-perfused rabbit heart with administration of PEG-SOD 1 hour or 12–24 hours before study. PEG-SOD at doses of 0, 3,000, 6,000, 12,000, 30,000, and 60,000 units/kg was administered intravenously to the donor and support animals either 1 hour or 12–24 hours before study (n=8 per group).

In this study, the protocol was similar to that of studies 1 and 2 except that the preischemic phase of control perfusion was eliminated. Hearts from donor rabbits were therefore excised and immediately subjected to cardioplegic infusion before 60 minutes of normothermic global ischemia and 60 minutes of reperfusion. Absolute recoveries of contractile function were compared between groups. Blood samples were obtained from the support animal at the end of each experiment, and plasma was frozen at the temperature of liquid nitrogen until assayed for PEG-SOD activity.

Study 4: Investigation of the plasma activity of PEG-SOD during the 24-hour period after administration. In this study, 12,000 units/kg of PEG-SOD was administered intravenously to rabbits (n=4), and blood samples were obtained at 30 minutes, 1, 2, 4, 8, 16, and 24 hours. Plasma samples were then frozen until assayed for PEG-SOD activity.

Study 5: Investigation of PEG-SOD in the crystalloid-perfused rabbit heart. In this study, PEG-SOD was given as a pretreatment to the donor rabbit (30,000 units/kg i.v. 12–24 hours before the experiment), as a cardioplegic additive (150 μg/ml), and as an additive to the crystalloid perfusate fluid (150 μg/ml). Controls received equivalent volumes of saline.

Hearts (n=7 per group) were aerobically perfused in the Langendorff mode (75 mm Hg) for 20 minutes before a 2-minute period of cardioplegic infusion and 60 minutes of normothermic global ischemia; they were then reperfused, and the postischemic recovery of contractile function and coronary flow together with creatine kinase leakage were measured over 60 minutes.

Study 6: Investigation of PEG-SOD in the crystalloid-perfused rat heart. In this study, PEG-SOD was added to the perfusion fluid (300 μg/ml) both before ischemia
and during reperfusion. PEG-SOD (300 µg/ml) was also added to the St. Thomas' Hospital cardioplegic solution. Controls received equivalent volumes of saline.

Hearts (n = 8 per group) were perfused aerobically for 20 minutes, during which LVDP, coronary flow, and heart rate were recorded; after a 2-minute infusion of the cardioplegic solution, they were maintained in a state of normothermic ischemia for 40 minutes; this was followed by 40 minutes of reperfusion, during which postischemic recovery of function was measured.

Study 7: Investigations with PEG-SOD of heart rate and calcium concentration in the crystalloid-perfused rat heart. In a similar series of experiments to those described for study 5, changes in the calcium content of the crystalloid solutions were made as described in “Results.” This protocol was identical to that previously described (study 5), except that hearts were not subjected to ischemia but to various periods of continuous aerobic perfusion with left ventricular function being determined at varying rates of stimulation ranging from 230 to 350 beats per minute (n = 5 per group).

Study 8: Dose–response studies with PEG-SOD in paced, crystalloid-perfused rat hearts. In these studies, PEG-SOD was included in the perfusion fluid at one of three doses (30, 150, or 300 µg/ml) during the last 10 minutes of a 30-minute preischemic phase and during the first 15 minutes only of the reperfusion phase. PEG-SOD was excluded during the last 25 minutes of reperfusion. A similar dose of PEG-SOD was included in the cardioplegic solution. Eight hearts were studied in each group, and the results were compared with those from saline controls.

The protocol was exactly as described for study 5 except that the ischemic period was extended to 45 minutes, all hearts were paced at 280 beats per minute before and after ischemia, and the drug was absent from the perfusion fluid for the last 25 minutes of reperfusion. Because we have shown previously that a transient modification of the calcium concentration during early reperfusion does not affect the extent of recovery of function in the rat heart, we did not attempt to alter calcium concentration in the present study during the first 15 minutes of reperfusion.

Statistical Analysis

All studies were randomized and, when possible, blinded. Results are expressed as mean±SEM. The two-tailed unpaired Student’s t test was used for comparison between two means. ANOVA was used for comparison of more than two means; when a significant F value was obtained, comparisons between the untreated and each of the treated groups were carried out by the two-tailed Dunnett’s test. ANOVA for repeated measurements was used for the comparison of linear trends. A difference was considered statistically significant at a value of p < 0.05.

Results

Study 1: Blood-Perfused Rabbit Heart Studies With PEG-SOD Given Before and After Ischemia

Before the onset of ischemia, hearts in the PEG-SOD–treated group and the saline controls had comparable values for LVDP (126±9 and 107±9 mm Hg, respectively) and coronary flow (4.4±0.1 and 4.2±0.1 ml/min, respectively). Figure 1 shows that after ischemia, there was a striking difference in the recovery profile between the two study groups. Despite a similar recovery of LVDP after 10 minutes of reperfusion, the two recovery curves diverged, largely because of a progressive improvement in the PEG-SOD group in contrast to minimal recovery in the control group. ANOVA for repeated measurements showed an overall significant difference between the two groups (p < 0.001) and in particular in their linear trends (p < 0.01). Thus, after 60 minutes of reperfusion, the recovery of LVDP was 28% greater in the PEG-SOD group (74±9 mm Hg) than in the control group (51±6 mm Hg). Coronary flow recovered fully in both groups (101±2% and 97±2%, respectively).

Although the time between administration of the PEG-SOD and the measurement of plasma activity was 12–24 hours in one group and 1 hour in the other, the plasma activity of PEG-SOD was comparable in both donor (67.8±4.2 units/ml) and support animals (77.0±10.5 units/ml; NS).

Study 2: Blood-Perfused Rabbit Heart Study to Compare Efficacy of PEG-SOD as a Pretreatment, as a Cardioplegic, and as a Reperfusate Additive

The results displayed in Table 1 show that there were no significant differences in LVDP or coronary flow before the onset of ischemia in the five groups studied. Figure 2, however, shows that whereas control PEG-SOD–free hearts (group A) only recovered 44±4% of their preischemic function after 60 minutes of reperfusion, hearts given PEG-SOD as a pretreatment and also as an additive in the cardioplegic solution and during reperfusion (group B) recovered to 70±3% (p < 0.05). When PEG-SOD was given as pretreatment and as a
TABLE 1. Preischemic Contractile Function and Coronary Flow in Blood-Perfused Rabbit Hearts Subjected to Various PEG-SOD Treatment Regimens

<table>
<thead>
<tr>
<th>Group</th>
<th>PEG-SOD treatment</th>
<th>LVDP (mm Hg)</th>
<th>Coronary flow (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>101±6</td>
<td>4.2±0.1</td>
</tr>
<tr>
<td>B</td>
<td>Throughout</td>
<td>113±7</td>
<td>4.3±0.2</td>
</tr>
<tr>
<td>C</td>
<td>Pretreatment plus cardioplegia</td>
<td>96±3</td>
<td>4.1±0.3</td>
</tr>
<tr>
<td>D</td>
<td>Cardioplegia alone</td>
<td>93±3</td>
<td>4.0±0.3</td>
</tr>
<tr>
<td>E</td>
<td>Reperfusion alone</td>
<td>96±3</td>
<td>4.1±0.4</td>
</tr>
</tbody>
</table>

PEG-SOD, polyethylene glycol–superoxide dismutase; LVDP, left ventricular developed pressure.

Cardioplegic additive but was not present during reperfusion (group C), recovery was again significantly better than in control hearts (62±7% versus 44±4%; p<0.05). Similarly, when PEG-SOD was only given at the time of reperfusion (group E), a significantly improved recovery was observed (60±3% versus 44±4%; p<0.05). However, when PEG-SOD was included only in the cardioplegic phase (group D), no significant additional protection was observed (47±4% versus 44±4%; NS). Coronary flow recovered fully in all groups (98±2%, 106±3%, 102±5%, 97±4%, and 101±2%, respectively).

As seen in the previous study, comparable levels of PEG-SOD (Table 2) were shown in both donor and support animals despite the difference in time from administration.

Study 3: Dose–Response Studies in Blood-Perfused Rabbit Heart With Administration of PEG-SOD 1 Hour or 12–24 Hours Before Study

The administration of various doses of PEG-SOD 1 hour before study (Figure 3A) resulted in a bell-shaped profile for the recovery of cardiac function. The greatest improvement was observed at a dose of 12,000 units/kg, at which LVDP recovered to 83±9 mm Hg after 60 minutes of reperfusion. In the PEG-SOD–free control group, the recovery was only to 41±7 mm Hg. No benefit was obtained at doses below 6,000 units/kg or above 30,000 units/kg. The recovery of coronary flow did not vary significantly between the study groups (3.3±0.4, 3.1±0.3, 3.3±0.3, 4.1±0.5, 4.2±0.4, and 3.9±0.8 ml/min with 0, 3,000, 6,000, 12,000, 30,000, and 60,000 units/kg, respectively).

The administration of PEG-SOD 12–24 hours before study (Figure 3B) induced a similar recovery of LVDP to that observed with the 1-hour pretreatment. Significant protection was seen at all doses between 6,000 and 30,000 units/kg, with the maximal response at 12,000 units/kg (85±5 mm Hg). As in the 1-hour pretreatment studies, no effect was observed on the recovery of coronary flow (3.3±0.4, 4.2±0.3, 3.2±0.3, 4.1±0.3, 3.6±0.2, and 3.8±0.5 ml/min with 0, 3,000, 6,000, 12,000, 30,000, and 60,000 units/kg groups, respectively).

The results of the mean plasma activity of PEG-SOD (Figure 4) support the findings obtained in studies 1 and 2. Thus, the administration of PEG-SOD either 1 hour or 12–24 hours before study resulted in similar dose-dependent plasma PEG-SOD activity. Interestingly, although doses of PEG-SOD of 60,000 units/kg led to plasma activities that were twice as high as that obtained with doses of 30,000 units/kg, the recovery of contractile function was lower.

Study 4: Investigation of Plasma Activity of PEG-SOD During 24-Hour Period After Administration

As shown in Figure 5, the profile of the mean plasma activity values of PEG-SOD for the 24-hour period

TABLE 2. Plasma Activity of PEG-SOD in Donor and Support Rabbits Subjected to Various PEG-SOD Treatment Regimens

<table>
<thead>
<tr>
<th>Group</th>
<th>PEG-SOD treatment (PEG-SOD 12–24 hours before)</th>
<th>PEG-SOD treatment (PEG-SOD 1 hour before)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>Throughout</td>
<td>132.3±8.5 units/ml</td>
</tr>
<tr>
<td>C</td>
<td>Pretreatment plus cardioplegia</td>
<td>144.5±6.5 units/ml</td>
</tr>
<tr>
<td>D</td>
<td>Cardioplegia alone</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>Reperfusion alone</td>
<td>0</td>
</tr>
</tbody>
</table>

PEG-SOD, polyethylene glycol–superoxide dismutase.
after administration was characterized by relatively constant activity during the first 2 hours. This was followed by a progressive decline so that after 24 hours, the plasma activity of PEG-SOD was 49.7±8.4 units/ml, a value that represented half of the activity measured 30 minutes after administration (106.5±2.8 units/ml).

**Study 5: Investigation of PEG-SOD in Crystalloid-Perfused Rabbit Heart**

In an attempt to ascertain whether blood-borne elements play any role in the protection observed with PEG-SOD in the blood-perfused heart, a study was undertaken in which PEG-SOD (given as a pretreatment, as a cardioplegic additive, and at reperfusion) was evaluated in the crystalloid-perfused rabbit heart preparation. As shown in the blood perfusion studies before ischemia, both groups of hearts, PEG-SOD-treated and controls, exhibited comparable values for LVDP (101±4 and 109±3 mm Hg, respectively) and coronary flow (37±3 and 35±3 ml/min, respectively). However, in contrast to the blood perfusion studies, the postischemic recovery of LVDP did not differ significantly between the PEG-SOD group (72±7 mm Hg) and the control group (70±3 mm Hg). The recovery of coronary flow was almost identical in both groups (33±2 and 32±4 ml/min, respectively). In support of the functional results, no statistically significant differences were observed in the leakage of creatine kinase between PEG-SOD (125±37 IU/60 min/heart) and control (108±37 IU/60 min/heart) groups.

Although the durations of ischemia and reperfusion were identical to those in blood-perfused hearts (study 3), no attempt was made at a direct comparison between the recoveries of function because of the marked differences between preparations (e.g., perfusion pressure of 40–60 mm Hg in blood-perfused hearts versus 75 mm Hg in crystalloid-perfused hearts and much greater coronary flows in crystalloid-perfused hearts).

**Study 6: Investigation of PEG-SOD in Crystalloid-Perfused Rat Heart**

As a first step toward comparing the possible protective effects of PEG-SOD in different species, studies were undertaken in the crystalloid-perfused rat heart.
TABLE 3. Effect of PEG-SOD on Cardiac Function in the Crystalloid-Perfused Rat Heart Before and After 40-Minute Normothermic Global Ischemia

<table>
<thead>
<tr>
<th>Group</th>
<th>Preischemic value</th>
<th>Postischemic value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart rate (beats per minute)</td>
<td>LVDP (mm Hg)</td>
</tr>
<tr>
<td>Control</td>
<td>290±11</td>
<td>136±5</td>
</tr>
<tr>
<td>PEG-SOD</td>
<td>288±8</td>
<td>115±2*</td>
</tr>
</tbody>
</table>

PEG-SOD, polyethylene glycol–superoxide dismutase; LVDP, left ventricular developed pressure.
Values expressed in absolute terms and percentage of preischemic values.
*p < 0.05 compared with control value.

PEG-SOD (300 μg/ml) was added to both the perfusion fluid and the cardioplegic solution. These studies did not use pretreatment.

Table 3 shows the preischemic and postischemic values for heart rate, LVDP, and coronary flow in both control and PEG-SOD–treated hearts. When postischemic recovery of LVDP was expressed as a percentage of preischemic control function, hearts in the PEG-SOD group appeared to exhibit a greater recovery of function (79±4% versus 61±5%; p < 0.05). However, a comparison of absolute values reveals that the postischemic LVDP did not differ significantly between the two groups (91±5 versus 82±7 mm Hg). The apparent improvement in the recovery was primarily attributable to a statistically significantly lower value for LVDP during the preischemic control period in the PEG-SOD–treated hearts (115±2 versus 136±5 mm Hg, respectively; p < 0.05). This phenomenon was not observed in the blood-perfused rabbit hearts. In the crystalloid-perfused rabbit hearts, the mean value for LVDP in the PEG-SOD group (101±4 mm Hg) was lower than in the controls (109±3 mm Hg), but this difference was not statistically significant. Because the rat heart study was randomized, a chance distribution of a lower LVDP in the PEG-SOD group is unlikely; thus, the possibility was explored that PEG-SOD, at the dose used, in a crystalloid medium might in some way reduce LVDP. A possible explanation, applicable to other compounds such as creatine phosphate, might involve a reduction of ionized calcium caused by binding to the drug.

Study 7: Investigations With PEG-SOD of Heart Rate and Calcium Concentration in Crystalloid-Perfused Rat Heart

Analysis of individual preischemic values for heart rate and LVDP for all hearts entering the preceding study indicated that LVDP was particularly low in hearts with rates of contraction at the lower end of the range normally encountered with spontaneously beating isolated hearts. An additional series of experiments was therefore carried out in which the effects of PEG-SOD on pressure development in aerobically perfused rat hearts paced at different rates was undertaken. The results (Figure 6A) show that in comparison with control hearts in which the negative staircase was observed, the hearts in the PEG-SOD group were characterized by a rate-dependent negative inotropic effect with greater reductions of developed pressure at lower heart rates (particularly in the range of 230–260 beats per minute). Again, a factor that might possibly contribute to such an effect could be a reduction in the ionized calcium content of the perfusate as a consequence of the addition of PEG-SOD; in this connection, it is well known that contractile activity in the rat heart is particularly susceptible to small changes in extracellular calcium.

A PEG-SOD calcium effect was confirmed in studies in which the level of ionized calcium in the perfusion fluid with and without added PEG-SOD (300 μg/ml) was measured and found to be 1.1 and 1.2 mmol/l, respectively. The above rate–pressure studies were therefore repeated but with extra calcium (0.1 mmol/l) added to the PEG-SOD solution, so that its content of ionized calcium matched that of PEG-SOD–free perfusion fluid. As can be seen in Figure 6B, the negative inotropic effect of PEG-SOD that was previously manifest at low heart rates was now eliminated. In contrast, PEG-SOD did not induce a lowering in developed pressure in isolated rabbit hearts subjected to crystalloid perfusion.

We also investigated whether the addition of PEG-SOD to the donor in the blood-perfused heart prep-

![Figure 6](http://circ.ahajournals.org/doi/abs/10.1161/01.CIR.86.2.678)
ration in the rabbit led to any lowering of ionized calcium levels, but no detectable plasma changes were observed.

**Study 8: Dose–Response Studies with PEG-SOD in Paced, Crystalloid-Perfused Rat Hearts**

After 20 minutes of aerobic perfusion and before the infusion of PEG-SOD, hearts paced at 280 beats per minute had comparable contractile function (Table 4) in each study group. However, after 10 minutes of perfusion with buffer containing PEG-SOD, we again observed a negative inotropic effect such that LVDP declined from 135±5 mm Hg in the control group to 129±3 (NS), 113±6 (p<0.05), and 113±5 mm Hg (p<0.05) in the 30-, 150-, and 300-µg/ml PEG-SOD groups, respectively. When the recovery of function (Figure 7) in each group was compared with the preischemic value before the infusion of PEG-SOD, no additional protection in terms of postischemic recovery of function was observed in any of the PEG-SOD–treated groups. The recovery of coronary flow was similar in all groups, as was the total leakage of creatine kinase (28±1, 29±2, 21±3, and 22±4 IU/40 min/heart in the control, 30-, 150-, and 300-µg/ml PEG-SOD groups, respectively; NS).

**Discussion**

The present studies demonstrate that PEG-SOD, when administered as a 12–24-hour pretreatment, a 1-hour pretreatment, or as a reperfusate additive, can improve, in a dose-dependent manner, the postischemic recovery of function in a blood-perfused rabbit heart. We were, however, unable to demonstrate protection in crystalloid-perfused hearts from either rabbits or rats. A number of aspects of this study warrant further discussion.

**PEG-SOD Versus Native SOD**

In these studies, we used a polyethylene glycol conjugated to SOD. This covalent bonding has been reported to extend the plasma half-life of SOD activity from a few minutes to >24 hours. The present studies (Figure 5) have confirmed this in the rabbit, and, as a consequence, we have been able to achieve protection with a single bolus whether given 1 hour or 24 hours before the onset of ischemia. In addition to its long half-life, it has been reported that PEG-SOD gains access to the intracellular compartment and thereby increases endogenous antioxidant capacity. In contrast, native SOD is thought to be restricted to the vascular space.

**Dose–Response Characteristics for the Efficacy of PEG-SOD**

The protective properties of native SOD and PEG-SOD are not without controversy. Although there are numerous studies in several species with both regional and global ischemia claiming protection (e.g., reduced infarct size, enhanced postischemic function, or reduced reperfusion arrhythmias), there are also studies that question the efficacy. Thus, in a recent study in which PEG-SOD treatment was combined with catalase, no limitation of infarct size could be detected.

One important factor that might contribute to the various conflicting studies is choice of dose. In the present study (Figure 3), we demonstrated a clear, bell-shaped dose–response profile for the protective properties of PEG-SOD. This occurred with both 1-hour and 12–24-hour pretreatment, and the curve was such that, at high doses (60,000 units/kg), efficacy was lost. We and others have previously reported bell-shaped dose–response profiles for SOD and other an-

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**Table 4.** Preischemic Contractile Function and Coronary Flow in Paced, Crystalloid-Perfused Rat Hearts After 20 Minutes of PEG-SOD–Free Aerobic Perfusion Followed by 10-Minute Infusion With Various Doses of PEG-SOD

<table>
<thead>
<tr>
<th>Group</th>
<th>Before PEG-SOD</th>
<th>After PEG-SOD</th>
<th>Before PEG-SOD</th>
<th>After PEG-SOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>137±5</td>
<td>135±5</td>
<td>15.1±0.6</td>
<td>14.6±0.6</td>
</tr>
<tr>
<td>PEG-SOD (30 µg/ml)</td>
<td>134±3</td>
<td>129±3</td>
<td>14.3±0.8</td>
<td>14.2±0.7</td>
</tr>
<tr>
<td>PEG-SOD (150 µg/ml)</td>
<td>137±5</td>
<td>113±6*</td>
<td>14.0±0.4</td>
<td>13.7±0.5</td>
</tr>
<tr>
<td>PEG-SOD (300 µg/ml)</td>
<td>135±4</td>
<td>113±5*</td>
<td>14.3±0.4</td>
<td>13.4±0.5</td>
</tr>
</tbody>
</table>

PEG-SOD, polyethylene glycol–superoxide dismutase; LVDP, left ventricular developed pressure. Hearts were paced at 280 beats per minute; total aerobic perfusion was 30 minutes.

*p<0.05 compared with control value.

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**Figure 7.** Bar graph shows effect of polyethylene glycol–superoxide dismutase (PEG-SOD) on postischemic recovery of left ventricular developed pressure (LVDP) in crystalloid-perfused rat hearts. Hearts received PEG-SOD (0, 30, 150, or 300 µg/ml) as an additive to the perfusion fluid during the last 10 minutes of a 30-minute preischemic perfusion period; PEG-SOD was also added to the cardioplegic solution and was present in the perfusion fluid during the first 15 minutes of a 40-minute reperfusion period. Bars represent standard error of the mean (n=8 per group).
tioxidant interventions.\textsuperscript{39-42} It is therefore conceivable that some studies in the literature may have been negative as a consequence of excessive or insufficient SOD administration.

Although bell-shaped dose–response curves are relatively easily explained for drugs and ions in which it is the concentration that is increased, it is more difficult to explain the loss of protection at high doses for an enzyme in which it is the activity that increases. As discussed previously,\textsuperscript{42} it might be that increasing concentrations of protein may exert a deleterious effect that counteracts any benefit from the enzyme activity. Omar et al.\textsuperscript{40,41} have hypothesized that an effect of overscavenging superoxide would be the depletion of hydroperoxyl radical (the protonated lipophilic form of superoxide), thus eliminating an important step in the termination of lipid peroxidation. Another possibility has recently been advanced that proposes an interaction between peroxynitrite radicals and SOD leading to the production of a highly reactive nitronium radical that could exhaust endogenous antioxidants.\textsuperscript{43}

It is important to note that although protection is lost at high doses of PEG-SOD, recovery does not fall below that of the PEG-SOD–free controls; i.e., we have no evidence of drug-induced detrimental effects. Whatever the mechanisms involved, our results once again stress the importance of undertaking dose–response studies for any potentially therapeutic agent.

Possible Mechanisms and Sites of Action

Although it is tempting to automatically attribute the protective effects of PEG-SOD to the ability of the enzyme component to eliminate superoxide, other possibilities should not be ignored. In this connection, the effect of the complex on ionized calcium levels and the possible biological effects of the polyethylene glycol component of the molecule merit discussion. In the present study, we did observe that high doses of PEG-SOD caused a small (~0.1 mmol/l) reduction in the ionized calcium content of a crystalloid perfusion medium. Such an effect is not uncommon with proteins, metabolites, and drugs. However, we would stress that the effect was not observed in blood. Furthermore, the effect would be expected to exert only a small biological action in species such as the rat, which is typically sensitive to small changes in extracellular calcium.\textsuperscript{37}

Thus, we believe that any simple effect on extracellular calcium is unlikely to have any relevance to the protection observed in the blood-perfused rabbit heart.

Our failure to achieve protection in crystalloid-perfused preparations provides a strong indication that the protective properties of PEG-SOD may be dependent on the presence of blood-borne components. It could, therefore, be speculated that PEG-SOD might counteract the deleterious effect of some endogenous factors. A strong candidate might be the leukocyte with PEG-SOD either preventing its activation or aiding in the attenuation of leukocyte-generated radicals. The efficacy of PEG-SOD, given only at the time of reperfusion, would support an action occurring possibly during minutes of reperfusion, although with its extended half-life, PEG-SOD may well continue to act throughout the reperfusion period. These results contrast with those obtained by Omar and McCord,\textsuperscript{44} who suggest that SOD given at reperfusion has no protective effect. The same authors\textsuperscript{44} further argue that equilibration of SOD in the interstitial fluid is required to obtain protective effect. It should be noted, however, that the experiments performed by Omar and McCord\textsuperscript{44} were carried out in crystalloid-perfused hearts, whereas the protection shown in the present studies was obtained in blood-perfused hearts. Therefore, it appears that interstitial equilibration of PEG-SOD is not needed in blood-perfused hearts to obtain protection. This possibility is supported by the same authors\textsuperscript{44} who show that PEG-SOD equilibrates much slower (38±10% after 1 hour perfusion) than SOD (84±13% and 95±11% after 15 minutes of perfusion for Mn-SOD and CuZn-SOD, respectively). Un-doubtedly, another reason that might be put forward to explain our failure to obtain protection with PEG-SOD in crystalloid-perfused hearts is the inability to reach a meaningful interstitial equilibration, even after 24-hour pretreatment. The controversy can still be further expanded by the results obtained by Ambrosio et al.\textsuperscript{45} who showed that PEG-SOD could not be equilibrated with those obtained from the rabbit heart. In those studies,\textsuperscript{45} SOD improved the recovery of cardiac function and metabolism when administered at the time of reperfusion. Although we do not have a plausible explanation for the discrepancies between our results in the crystalloid perfusion studies and those from Ambrosio et al.,\textsuperscript{45} we should emphasize the difference that in our studies, PEG-SOD and longer periods of ischemia were used. It is worth noting that in the present studies, the initial recovery of contractile cardiac function after the first 10 minutes of reperfusion was similar in both PEG-SOD–treated and drug-free control groups. It was only after longer durations of reperfusion that a progressive segregation of the recovery profiles was observed. This would suggest that the protective action of PEG-SOD may be operative during late reperfusion (>10 minutes). However, we cannot rule out the possibility that such a phenomenon is the consequence, at least in part, of events initiated by the drug early in the reperfusion process.

The observation that PEG-SOD is also active as a pretreatment alone is of particular interest. In the present studies, in which animals were pretreated (12–24 hours in advance), the excised hearts were perfused with PEG-SOD–free blood from the untreated support animal, but, nonetheless, protection was still observed. Therefore, it would seem that the PEG-SOD may bind to myocardial tissue and remain active for several hours even after the complete elimination of any PEG-SOD in the plasma. Indirect support for some releasable pool of bound PEG-SOD comes from a comparison of studies 3 and 4. In the latter, we demonstrated an approximate 50% reduction in plasma activity of PEG-SOD over the 24-hour period after administration. However, in apparent contrast, in study 3, we observed similar PEG-SOD activity 1 hour or 24 hours after treatment. However, it should be noted that heparin was administered in the latter study and not in the former, and there is evidence\textsuperscript{46,47} to suggest that this can induce the release of extracellular SOD to the blood.

Protection by PEG, although currently untested with inactivated PEG-SOD, is considered unlikely in that in the present studies, PEG-SOD administered in a crystalloid perfusate did not afford any protection, suggesting that more than a mere physical interaction of the
PEG complex was involved in the protection observed in the blood perfusion experiments. Certainly, PEG has a spectrum of relevant biological effects; it prevents cell swelling\(^{48-50}\) by an osmotic mechanism and has also been postulated\(^{51}\) to stabilize membranes by interaction with their lipids. The possibility that some benefit might be attributable to the PEG component of the molecule is provided by the recent observation\(^{52}\) that the replacement of hydroxyethyl starch by PEG in the University of Wisconsin solution improves its ability to preserve isolated rabbit hearts. Against a role for PEG is the observation\(^{53}\) that SOD and PEG-SOD reduce equally the permeability and edema induced by hypertension in brain and lungs.

**PEG-SOD as a Cardioplegic Additive**

The absence of benefit when PEG-SOD was added solely to the cardioplegic solution is also of interest, and it is in agreement with previous studies\(^{16}\) in which we observed that SOD alone as an additive to a cardioplegic solution afforded no additional protection. At present, we do not know the reason for the failure of PEG-SOD to be effective as a cardioplegic additive. It cannot be explained entirely by a need for pretreatment (allowing time for access to some tissue compartment), because the drug is effective when given only at the time of reperfusion. One reason might relate to the relatively small amount of PEG-SOD trapped in the vasculature during ischemia. This volume would be washed out by PEG-SOD-free blood and rapidly diluted to a circulating dose that may be too low to exert any beneficial effect.

**Possible Clinical Interest and Limitations in Interpretation of the Present Study**

The ability of PEG-SOD to improve the functional recovery of the heart after a period of global ischemia could find valuable application during routine cardiac surgery (either as a pretreatment or as a reperfusion additive); it might also be of value in cardiac transplantation. Certainly, a number of antioxidants and antioxidant enzymes have been shown to be effective in this respect.\(^{16-20,54,55}\) However, it must be stressed that the present studies have all been carried out under conditions of normothermic ischemic arrest; for surgical application, benefit would have to be demonstrated under conditions of hypothermia. There is, nevertheless, every reason to expect the appearance of protection under these circumstances, because we have demonstrated beneficial effects when PEG-SOD was used during reperfusion alone (i.e., after any hypothermic phase).

**Conclusions**

The present studies have shown that PEG-SOD can exert a potent dose-dependent protective effect in the blood-perfused rabbit heart. It would seem that by conjugation with SOD, PEG confers important properties on the molecule; it may even be that PEG is responsible for some of the protective effects. Further extensive studies will be required to determine whether the protective action of PEG-SOD involves an action on leukocytes and/or endothelium to which a molecule such PEG-SOD might bind.

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