Effect of Superoxide Dismutase on Myocardial Infarct Size in the Canine Heart After 6 Hours of Regional Ischemia and Reperfusion: A Demonstration of Myocardial Salvage

Liguo Chi, Yasuo Tamura, Paul T. Hoff, Mahender Macha, Kim P. Gallagher, M. Anthony Schork, and Benedict R. Lucchesi

Available data demonstrate that oxygen free radicals and derived reactive species of oxygen are produced during myocardial ischemia as well as upon reperfusion of the ischemic tissue. The present study was designed to determine if polyethylene glycol-conjugated superoxide dismutase (PEG-SOD), with its extended plasma half-life in excess of 30 hours in contrast to the native form of the enzyme (Native-SOD), could provide protection to the ischemic myocardium subjected to a 6-hour regional ischemia followed by reperfusion for 24 hours. We hypothesized that myocardial injury due to an ischemic interval is a dynamic process involving the sustained production of cytotoxic oxygen radicals that may continue beyond the ischemic interval. The ability to demonstrate a protective effect of the free radical scavenger enzyme superoxide dismutase would require the continued presence of the antioxidant during the ischemic interval and especially during reperfusion. To test this hypothesis, 22 anesthetized, open-chest dogs underwent 6 hours of circumflex coronary artery occlusion followed by reperfusion for 24 hours. Rapid administration of either Native-SOD (1,000 U/kg), PEG-SOD (1,000 U/kg), PEG-albumin (PEG-ALB), or 0.9 % sodium chloride solution for injection (saline) was administered via the left atrium 15 minutes before occlusion of the vessel. A continuous infusion of an additional 1,000 U/kg of the respective enzyme interventions or an equivalent volume of PEG-ALB or saline was given during the 6-hour coronary artery occlusion and terminated 15 minutes after reperfusion. The animals were euthanized 24 hours after reperfusion, and the myocardial region at risk and the infarct region were quantitated by the tetrazolium method. The area of myocardium at risk of infarction, expressed as a percent of the left ventricle, did not differ among the groups: Native-SOD (n=8), 46.2±1.8%; PEG-SOD (n=6), 45.7±2.1%; PEG-ALB, 38.4±2.3% (n=4); and saline 46.0±2.1% (n=4). Hemodynamic parameters, the calculated rate-pressure-product, as well as regional myocardial blood flow (radiolabeled microsphere method) in the endocardial, midmyocardial, and epicardial segments of the risk and the nonrisk regions were comparable for all groups. Mean infarct size, determined 24 hours after reperfusion, in the group treated with PEG-SOD was 47.1±2.9 % of the area at risk (n=6), significantly smaller than that observed in each of the other treatment groups: Native-SOD, 63.5±2.2% (n=8); PEG-ALB, 64.6±2.4% (n=4); saline, 70.8±2.2% (n=4). The present studies provide support for the concept that superoxide dismutase can prevent myocardial necrosis due to oxygen radicals produced during the ischemic interval as well as the period of reperfusion. Because PEG-SOD was more effective than Native-SOD, the results suggest that the sustained presence of oxygen radical scavenger activity is necessary to prevent rather than delay myocardial necrosis. (Circulation Research 1989;64:665-675)
Reperfusion of the ischemic myocardium is essential for tissue survival. Early reperfusion will arrest the progression of cell death and reduce the total amount of tissue undergoing irreversible injury.\textsuperscript{1-4} A number of laboratories, however, have provided indirect and direct evidence for the production of oxygen derived radicals coincident with the onset of reperfusion that may lead to an extension of myocardial injury.\textsuperscript{5-8} The initial evidence for participation of reactive radical species in mediating the injury associated with myocardial reperfusion in vivo, was derived from experimental studies that documented the efficacy of radical scavengers in reducing ultimate infarct size.\textsuperscript{5,9-12} Romaschin et al\textsuperscript{13} demonstrated the production of significant amounts of hydroxyl conjugated dienes during global normothermic ischemia in the canine heart, and this was amplified significantly during reperfusion. More recently, direct determinations using spin-trapping electron-spin resonance techniques detected the presence of spin adducts during ischemia as well as during reperfusion.\textsuperscript{6,7} The formation of the reactive radical species was inhibited by superoxide dismutase. The latter findings were supported by the observations of Zweier et al,\textsuperscript{8} who used electron paramagnetic resonance techniques to demonstrate that recombinant superoxide dismutase could inhibit free radicals generated during myocardial reperfusion.

The purpose of the present study was to address whether the appearance of reactive oxygen species during myocardial ischemia and reperfusion contributed to myocyte injury and if the administration of the oxygen radical scavenger superoxide dismutase, conjugated to polyethylene glycol so as to prolong its plasma half-life, could reduce the extent of myocardial cell death resulting from a 6-hour period of regional ischemia followed by 24 hours of reperfusion. The study was designed to determine if superoxide dismutase could result in the prevention of myocardial cell death as opposed to delaying the development of myocardial necrosis.

Nearly all previous studies have involved ischemic periods lasting 3 hours or less. Przyklenk and Kloner\textsuperscript{13} reported that the infusion of superoxide dismutase plus catalase, administered before and during 6 hours of coronary occlusion, reduced myocardial infarct size determined after 6 hours of regional ischemia without reperfusion. After 6 hours of regional ischemia followed by reperfusion, the same treatment regimen was not beneficial when infarct size was determined 30–48 hours later. The authors concluded that superoxide dismutase and catalase administered only during the ischemic period delay, but do not prevent, myocyte necrosis. It was stated that some myocyte necrosis associated with permanent coronary occlusion in the dog is related to the presence of cytotoxic, oxygen-derived free radicals. A possible explanation for the inability to demonstrate a sustained protective effect of the enzyme treatment may have been related to the fact that superoxide dismutase has a limited duration of action as opposed to the progressive and dynamic nature of the infarction process.

The present study was undertaken to determine if the efficacy of superoxide dismutase is limited by its short plasma half-life. While the scavenger may provide a beneficial effect in the immediate postreperfusion period,\textsuperscript{5,6,13} it is possible that the rapid decrease in the circulating plasma enzymatic activity would allow for the delayed appearance of reperfusion injury. To address this question, we have employed the conjugated form of superoxide dismutase, which is known to have a plasma half-life in excess of 30 hours and which has been reported to become associated with vascular endothelial cells thereby increasing cellular enzyme activity in a manner that provides protection against the cytotoxic effects of superoxide anion.\textsuperscript{14-16} The results of these investigations demonstrate that polyethylene glycol-conjugated bovine superoxide dismutase (PEG-SOD), in contrast to native bovine superoxide dismutase (Native-SOD), can reduce the extent of myocardial injury in the canine heart subjected to 6 hours of regional ischemia followed by 24 hours of reperfusion. We propose that the protective effect represents salvage of jeopardized myocardial tissue rather than a delay in the appearance of cellular necrosis.

Materials and Methods

Guidelines for the Use of Experimental Animals

The procedures used in this study were in accordance with the guidelines of The University of Michigan Committee on the Use and Care of Animals. Veterinary care was provided by The University of Michigan Unit for Laboratory Animal Medicine. The University of Michigan is accredited by the American Association for Accreditation of Laboratory Animal Care, and the animal care and use program conforms to the standards in "The Guide for the Care and Use of Laboratory Animals," DHEW Publ. No. NIH 78-23, revised 1978.

According to the study protocol the canine heart was made regionally ischemic for 6 hours and then reperfused. The animals were euthanized 24 hours later (30 hours from the onset of the ischemic period), and infarct size was quantitated. The protocol is presented schematically in Figure 1.

Male, mongrel dogs, carefully selected with respect to breed and weighing between 12.0 and 17.0 kg, were anesthetized with an intravenous injection of sodium pentobarbital (30 mg/kg), intubated with auffed endotracheal tube, and ventilated with room air with a Harvard respirator (Dover, Massachusetts). The left jugular vein was cannulated for the administration of intravenous fluids while arterial blood pressure was obtained via a cannula in the left carotid artery which was connected to a calibrated Statham physiological blood pressure transducer.

A left thoracotomy was performed in the fifth intercostal space, the pericardium was opened just
Chi et al Superoxide Dismutase and Infarct Size

667

6 Hours LCX Occlusion
30 Hours
24 Hours Reperfusion

-15'
30 (Hrs. after Ischemia)
JL

Euthanize
Histochemical determination of infarct size
RMBF / Isotope counting
PEG-Albumin or Saline Solution

Treatment Groups

Native SOD or PEG-SOD Infusion (1000U/kg bolus, followed by 1000U/kg intra-atrial)

Figure 1. Protocol used to assess the cardioprotective effects of native superoxide dismutase (Native-SOD), polyethylene glycol-conjugated superoxide dismutase (PEG-SOD), polyethylene glycol-conjugated albumin (PEG-ALB), or 0.9% sodium chloride solution for injection (saline). The canine heart was subjected to 6 hours of regional ischemia by occlusion of the left circumflex coronary artery (LCX) and reperfused for 24 hours. The animals received either Native-SOD, PEG-SOD, PEG-ALB, or saline administered via the left atrial appendage starting 15 minutes before occlusion of the LCX in a dose of 1,000 U superoxide dismutase/kg or a volume of PEG-ALB that provided an equivalent volume of polyethylene glycol. The initial dose of each of the treatments was followed by a continuous infusion which delivered a total of 1,000 U/kg of the respective formulations of the enzyme or equal volumes of either PEG-ALB or saline. The rate of infusion was adjusted to permit the second dose to be administered over 6 hours and 30 minutes. Labeled microspheres for the determination of regional myocardial blood flow were administered 5 hours after occlusion of the LCX with the use of 15 µm, 131I-labeled microspheres. Infarct size was determined 24 hours after reperfusion and the heart muscle prepared for the analysis of regional myocardial blood flow (RMBF).

Inclusion and Exclusion Criteria

Predetermined exclusion criteria were: 1) the presence of heart worms upon final examination of the heart; 2) the failure to manifest electrocardiographic signs of ischemia (no ST-segment elevation) in leads II, III, or aV F of the electrocardiogram and discoloration (cyanosis) of the epicardial surface in the region of distribution of the circumflex coronary artery after left circumflex coronary artery occlusion; 3) intractable ventricular fibrillation requiring more than three attempts at cardioversion using low direct current (10 J) pulses applied directly to the surface of the heart; and 4) epicardial regional myocardial blood flow greater than 0.2 ml/min/g in the myocardial risk region during occlusion of the left circumflex coronary artery.

Histochemical Determination of Myocardial Infarct Size

After excision of the heart, histochemical determination of the anatomic area at risk and the zone below the border of the left atrial appendage, and the left circumflex coronary artery was isolated for a distance of 7–8 mm from its origin. An electromagnetic flow probe, calibrated with whole blood, was placed on the circumflex coronary artery and used for the recording of coronary artery blood flow with the aid of a Carolina Electromagnetic Square Wave Flowmeter (Carolina Medical Equipment, King, North Carolina). Two polyethylene cannulae were inserted in the left atrium and secured in place with silk sutures. One cannula was used for the administration of superoxide dismutase, while the other was used for the administration of radio-labeled microspheres for the determination of regional myocardial blood flow.

After measurement of basal coronary artery blood flow, a critical stenosis was produced by applying a silk ligature around the vessel and an 18- or 19-gauge needle. The degree of partial constriction was adjusted to reduce, by at least 50%, the reactive hyperemic response to a 10-second occlusion of the left circumflex coronary artery without altering basal coronary blood flow. The stenosis limits reperfusion hyperemia, thereby curtailing the incidence of reperfusion arrhythmias and hemorrhagic infarction. All dogs underwent occlusion of the left circumflex coronary artery for 6 hours followed by reperfusion for 24 hours. Leads II, III, aVF of the electrocardiogram, left circumflex coronary artery blood flow, and carotid artery blood pressure were monitored throughout the experimental procedure and recorded on a Grass Model 7 polygraph (Grass Instrument Company, Quincy, Massachusetts). The thoracotomy incision was closed, and the animals were returned to the postoperative recovery facilities and maintained under observation until the next day, when they were returned to the laboratory and reanesthetized. The thoracotomy incision was opened and the heart fibrillated by a 10-V, 60-Hz direct current applied to the left ventricle. Thus, euthanasia was accomplished 30 hours from the onset of regional ischemia or 24 hours from the time of reperfusion. The heart was removed and prepared for the histochemical determination of infarct size and for the determination of regional myocardial blood flow.
of infarction was accomplished with a dual perfusion technique as described previously. The aorta was perfused retrograde with 0.25% Evans blue dye, and the circumflex coronary artery was perfused with 1.5% triphenyl tetrazolium chloride in 20 mM potassium phosphate buffer (pH 7.4; 37°C). The solutions were infused simultaneously for 5 minutes under a constant pressure of 100 mm Hg with the heart suspended in a water bath (37°C). The hearts were removed from the perfusion apparatus and sectioned transversely from apex to base. Each segment was 1 cm thick. The transverse sections were weighed and then fixed in 10% formalin. Both surfaces of each ventricular section were traced onto clear plastic overlays for subsequent quantitation of the area at risk (denoted by the absence of Evans blue dye) and the infarct zone (denoted by the absence of red formazan pigment within the area at risk). The respective areas were quantitated by planimetry using an Apple Graphics Tablet and Apple IIe computer. A custom-made software program was used to calculate the masses of the infarct zone, the area at risk from the planimetered areas, and the weights of each section. Previous studies demonstrated that there is an excellent correlation between infarct size derived by the planimetric method and the direct gravimetric measurement of infarct size. The method for the determination of infarct size is illustrated in Figure 2. The infarct mass is expressed as a percent of the area at risk and the area at risk is expressed as a percent of the left ventricle. The fidelity of the tetrazolium method for the identification of irreversibly injured myocardial tissue has been verified by Vivaldi et al and by Romaschin et al, who used electron microscopy and demonstrated that ultrastructural changes of irreversible injury occurred in areas of the myocardium that were tetrazolium negative.

Determination of Regional Myocardial Blood Flow

Regional myocardial blood flow was determined with the use of 15 μm diameter microspheres labeled with 113Sn (New England Nuclear, Boston, Massachusetts). The microspheres were prepared for administration by sonication in an ultrasonic bath and agitation with a vortex mixer. An aliquot of 1-2 million microspheres was diluted in 10 ml saline (37°C) and infused via the left atrial appendage over a 30-second period, followed by two flushes with 10 ml saline. Dual reference blood samples were withdrawn simultaneously from one femoral and one carotid artery at 3.47 ml/min with a Harvard withdrawal pump beginning immediately before the injection of microspheres and ending 2 minutes later. Microspheres were injected 5 hours after occlusion of the left circumflex coronary artery. The transverse sections of the left ventricle were dissected into epicardial, midmyocardial, and endocardial thirds. A TRACOR model 1185 gamma spectrometer (TM Analytic, Elk Grove, Illinois) was used to measure the radioactivity of the tissue and blood samples. An Apple II+ computer was used to perform myocardial blood flow calculations. The reference blood sample counts were averaged for the calculation of myocardial blood flow. If the reference sample counts varied by more than 15%, the data were considered to be invalid and were discarded.

Administration of Native-Superoxide Dismutase, Polyethylene Glycol–Conjugated Superoxide Dismutase, Polyethylene Glycol Conjugated to Albumin, or Sodium Chloride Solution (0.9%) for Injection

Animals received either Native-SOD (Cu/Zn superoxide dismutase from bovine liver), PEG-SOD (Cu/ Zn superoxide dismutase from bovine liver conjugated to polyethylene glycol), or equivalent volumes of PEG-ALB conjugated to polyethylene glycol, or sodium chloride solution for injection (0.9%). The administered volume of PEG-ALB was adjusted...
so as to provide an equivalent amount of polyethylene glycol per kilogram of body weight as contained in the active enzyme conjugate. The PEG-SOD and PEG-ALB were obtained from ENZON, Inc (South Plainfield, New Jersey) and were supplied as sterile, nonpyrogenic solutions. The PEG-SOD had an enzyme activity of 2,960 U/mg. The solution contained 8.2 mg/ml with 46% of available amino groups modified by conjugation to polyethylene glycol. Native-SOD was obtained from DDI Pharmaceuticals (Mountain View, California). Native-SOD was provided as a sterile, nonpyrogenic freeze-dried solid containing 5 mg protein and 10 mg sucrose per vial. Assay of the material provided a superoxide dismutase activity of 4,300±200 U/mg protein.

The dose for each of the enzyme preparations was calculated to provide an initial enzyme activity of 1,000 U/kg. All test materials were administered as a rapid injection into the left atrium 15 minutes before occlusion of the left circumflex coronary artery. This was followed immediately by a continuous infusion which provided an additional 1,000 U/kg of the respective enzymes over a period of 6.5 hours so that the infusion of each test substance terminated 15 minutes after reperfusion was initiated. The PEG-SOD, PEG-ALB, Native-SOD, and saline solutions were administered in an identical fashion (Figure 1).

Statistics

The study compared four separate groups in which one group received Native-SOD, another received PEG-SOD and two additional groups which received either PEG-ALB or saline. The mean values for infarct size as a percent of the area at risk, infarct size as a percent of the left ventricle, and area at risk as a percent of the left ventricle were compared among the four groups using an analysis of variance (ANOVA). Hemodynamic values were compared at predrug infusion, preocclusion of the coronary artery, and at 30 and 60 minutes after occlusion and hourly (up to 6 hours) thereafter with the final measurements made 1 hour after reperfusion. The statistical comparisons were made with the use of ANOVA and Fisher’s test. The values presented are expressed as the mean±SEM and are considered to differ significantly if p<0.05.

Both the myocardial area at risk and collateral blood flow are important determinants in the extent of ischemic myocardial injury and tissue necrosis. Infarct size was assessed in relation to collateral blood flow measured in the inner two thirds of the central ischemic zone as reported elsewhere as well as being assessed with respect to the normalized epicardial collateral blood flow. In the latter approach, collateral blood flow was expressed as a percentage of flow in the nonischemic area. An analysis of covariance was performed, in which collateral blood flow was the independent variable, with the object of determining if a statistically significant difference existed in the calculated infarct size among the groups when the influence of collateral blood flow (absolute and normalized) was controlled.

Results

Group Characteristics

A total of 36 dogs was selected for study, of which 8 were excluded from the final data analysis due to the development of ventricular fibrillation (two animals assigned to the Native-SOD group, four to the PEG-SOD group, and two to the saline treatment group). Six animals did not satisfy the preestablished criteria for inclusion due to the failure to develop ST-segment changes upon occlusion of the left circumflex coronary artery and the documented presence of collateral blood flow, which exceeded 0.2 ml/min/g in the epicardial region (one from the Native-SOD group, two from the PEG-SOD group, and three from the PEG-ALB group). The study population included in the final data analysis for infarct size and hemodynamic measurements consists of 22 dogs, 19 of which received radio-labeled microspheres for regional myocardial blood flow measurements. Eight animals were assigned to the Native-SOD treatment group, six received PEG-SOD, and four dogs in each of the groups received PEG-ALB or saline. The animals in each of the groups did not differ significantly with respect to body weight: 14.8±0.6, 13.7±0.7, 15.0±0.2, and 15.8±0.9 kg; or left ventricular weight, 83.7±6.2, 83.5±7.2, 75.6±4.8, and 82.3±5.5 g for the Native-SOD, PEG-SOD, PEG-ALB, and saline groups, respectively.

Infarct Size Determinations 24 Hours After Reperfusion

The effects of the respective treatment interventions on infarct size as a percent of the area at risk are presented in Figure 3. Mean infarct size expressed, as a percent of the area at risk or as a percent of the left ventricle, was smaller in the group which had been pretreated with PEG-SOD as compared with the group that received Native-SOD. When calculated on the basis of risk region, the infarct size in the Native-SOD group was 63.5±2.2% as compared with 47.1±2.9% (p<0.001) in the group that received PEG-SOD. Similarly, infarct size expressed as a percent of the risk region was reduced significantly in the PEG-SOD-treated group when compared with the groups that received either PEG-ALB or saline. Infarct size among the three “control groups” (Native-SOD, PEG-ALB, and saline) did not differ significantly from each other. The risk region, expressed as a percentage of the left ventricle, in the Native-SOD-treated group averaged 46.2±1.8% and did not differ from the values obtained in the other three groups: PEG-SOD, 45.7±2.1%; PEG-ALB, 38.4±2.3%; saline, 46.0±2.1%. The data indicate that pretreat-
Hemodynamic Variables

The recorded hemodynamic variables included heart rate, mean arterial blood pressure, and mean circumflex coronary blood flow as recorded with an electromagnetic flow probe. A summary of the results from each group, along with the respective calculated rate-pressure product, is presented in Table 1. The preocclusion values for the measured and calculated hemodynamic parameters as well as the respective values for each of the time points during the 6-hour period of occlusion and for 1 hour into the reperfusion period did not differ when compared among groups. In each group, however, there were significant differences in each of the recorded parameters 1 hour after reperfusion, when the values were compared with the respective control periods within the groups. Thus, on the basis of the recorded hemodynamic parameters and on the calculated rate-pressure product, all four groups were similar over the initial period of the study, thereby making it unlikely that the observed differences in infarct size could be attributed to disparate hemodynamic conditions among the study groups.

Regional Myocardial Blood Flow

Determinations of regional myocardial blood flow were done in 19 dogs: six received Native-SOD, five received PEG-SOD, and four in each group were given PEG-ALB or saline. The regional myocardial blood flow determinations were done 5 hours after the onset of regional ischemia in the area of distribution of the left circumflex coronary artery. Analysis of the flow data 24 hours after reperfusion revealed no significant differences among the four treatment groups. The regional coronary blood flow data are presented in Table 2. The mean transmural coronary blood flow in the region of distribution of the left circumflex coronary artery was 0.044±0.01 compared with 0.059±0.01 ml/min/g in the Native-SOD and PEG-SOD groups respectively, indicating that the groups were similar with respect to the severity of ischemia 5 hours after left circumflex occlusion. Similarly, the analysis of the regional blood flow data in the endocardial, midmyocardial, and epicardial regions for each of the four groups demonstrated that the degree of ischemia, 5 hours after occlusion of the left circumflex coronary artery, was the same for each of the four groups. The endocardial-to-epicardial collateral blood flow ratios in the distribution of the infarct related artery did not differ significantly among the groups (ANOVA). Analysis of the coronary blood flow data from the region of distribution of the left anterior descending coronary artery showed normal values in each of the three areas, with no significant differences among the groups in any of the myocardial regions. The beneficial effects observed with PEG-SOD on myocardial infarct size could not be attributed to an improvement in regional myocardial blood flow or to the presence of collateral flow during coronary artery occlusion.

The relation between infarct size and the average collateral blood flow in the inner two thirds of the left ventricular wall of the ischemic region for each of the four groups is presented in Figure 4. An inverse relation between the extent of tissue necrosis and collateral blood flow is observed in each of the groups. The regression lines are shown for the
TABLE 1. Hemodynamic Measurements

<table>
<thead>
<tr>
<th></th>
<th>Pre-infusion</th>
<th>Pre-occlusion</th>
<th>Time after LCX occlusion</th>
<th>1 hour after reperfusion</th>
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<tr>
<td></td>
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<td></td>
<td>30 min</td>
<td>60 min</td>
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<tr>
<td>Native-SOD (n=8)</td>
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<tr>
<td>HR (beats/min)</td>
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<td>MBP (mm Hg)</td>
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<td>109.7±8</td>
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<td>CBF (ml/min)</td>
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<td>0±0</td>
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<td>R-P-P (×100)</td>
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<td>211±18</td>
<td>201±19</td>
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<td>PEG-SOD (n=6)</td>
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<tr>
<td>HR (beats/min)</td>
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<td>197±19</td>
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<td>190±19</td>
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<td>Saline (n=4)</td>
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<tr>
<td>HR (beats/min)</td>
<td>159±14</td>
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<td>167±16</td>
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<tr>
<td>MBP (mm Hg)</td>
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<td>129±7</td>
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<td>CBF (ml/min)</td>
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<td>R-P-P (×100)</td>
<td>246±26</td>
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<td>PEG-ALB (n=4)</td>
<td></td>
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<td>HR (beats/min)</td>
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<tr>
<td>R-P-P (×100)</td>
<td>229±21</td>
<td>249±25</td>
<td>189±24</td>
<td>210±25</td>
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LCX, left circumflex coronary artery; Native-SOD, native superoxide dismutase; PEG-SOD, polyethylene glycol-conjugated superoxide dismutase; Saline, sodium chloride for injection; PEG-ALB, polyethylene glycol-conjugated albumin; HR, heart rate; MBP, mean blood pressure; CBF, collateral blood flow; R-P-P, rate-pressure-product. No significant differences at any point between the four groups, as shown by one-factor analysis of variance. Mean±SEM. *p<0.05 compared with baseline value in same group.

PEG-SOD and Native-SOD treated groups. The slope for each of the four regression lines did not differ from one another making it plausible to assume a homogeneity of slopes. For any given value of collateral blood flow, infarct size is smaller (p<0.05) in those hearts from animals which received PEG-SOD treatment as compared with those that received Native-SOD. The relation between infarct size and regional collateral blood flow, as defined by the group which received Native-SOD, is shifted downward in the group that received PEG-SOD, indicating that the treatment had limited the ultimate extent of myocardial injury. Employing an analysis of covariance with collateral blood flow as the independent variable and infarct size as the dependent variable, the calculated F value was significant at p<0.05. The data are interpreted as indicating that PEG-SOD, in contrast to Native-SOD, was effective in reducing the extent of myocardial injury in the heart subjected to 6 hours of regional ischemia and reperfused for 24 hours. Similarly, calculation of the F values by an analysis of covariance for each of the groups indicated the infarct size in the PEG-SOD-treated group was significantly different from that obtained in the PEG-ALB and saline treatment groups, whereas the PEG-ALB, Native-SOD and saline treatment groups did not differ from each other with respect to ultimate infarct size.

In Figure 5, the percent myocardial necrosis is plotted against the normalized collateral blood flow. Collateral blood flow was normalized by calculating

TABLE 2. Ischemic Bed Regional Myocardial Blood Flow During Left Circumflex Coronary Artery Occlusion

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Endocardium (ml/min/g)</th>
<th>Midmyocardium (ml/min/g)</th>
<th>Epicardium (ml/min/g)</th>
<th>Mean transmural (ml/min/g)</th>
<th>Endo/Epi</th>
<th>Inner 2/3</th>
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<tr>
<td>Native-SOD (n=6)</td>
<td>0.015±0.005</td>
<td>0.032±0.012</td>
<td>0.086±0.024</td>
<td>0.044±0.013</td>
<td>0.35±0.11</td>
<td>0.023±0.008</td>
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<td>PEG-SOD (n=5)</td>
<td>0.023±0.010</td>
<td>0.045±0.019</td>
<td>0.108±0.024</td>
<td>0.059±0.017</td>
<td>0.34±0.09</td>
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<tr>
<td>PEG-ALB (n=4)</td>
<td>0.023±0.007</td>
<td>0.025±0.007</td>
<td>0.058±0.013</td>
<td>0.035±0.009</td>
<td>0.65±0.18</td>
<td>0.024±0.007</td>
</tr>
<tr>
<td>Saline (n=4)</td>
<td>0.102±0.083</td>
<td>0.027±0.022</td>
<td>0.086±0.042</td>
<td>0.071±0.048</td>
<td>0.68±0.27</td>
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</tbody>
</table>

Endo/Epi, endocardial-to-epicardial flow ratio; Native-SOD, native superoxide dismutase; PEG-SOD, polyethylene glycol-conjugated superoxide dismutase; PEG-ALB, polyethylene glycol-conjugated albumin; Saline, sodium chloride for injection. Mean±SEM. No significant differences between the four groups by one-factor analysis of variance.
Relationship between Myocardial Infarct Size and Collateral Blood Flow during Ischemia (6 Hours Occlusion / 24 Hours Reperfusion)

![Image](https://example.com/image.png)

**FIGURE 4.** Mean collateral flow in the inner two thirds of the ischemic wall, 5 hours after occlusion of the circumflex coronary artery, vs. infarct size (determined at 24 hours), normalized as a percent of the area at risk. The calculated regression equations are shown while only those for the groups that received native superoxide dismutase (Native-SOD) or polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) have been plotted. An inverse relation exists between infarct size and collateral blood flow in each of the treatment groups. The relation defined by the group which received Native-SOD, is shifted downward in the group which was treated with PEG-SOD, indicating that the latter treatment regimen had a favorable effect on ultimate infarct size. Similarly, PEG-SOD resulted in significant downward shift when compared to the groups which received polyethylene glycol-conjugated albumin (PEG-ALB) or sodium chloride solution for injection (saline). Using an analysis of covariance model, it was determined that there was no significant interaction between the covariate and the treatment. Thus, the slopes of the regression lines for each of the groups did not differ from each other. The beneficial effect of PEG-SOD on ultimate infarct size, as compared with the other treatment regimens, was independent of regional myocardial blood flow.

**Discussion**

There is increasing evidence that superoxide anion and other species of activated oxygen contribute to the cellular injury associated with myocardial ischemia and reperfusion. Reactive oxygen metabolites are believed to be capable of producing significant tissue injury by altering cellular proteins, nucleic acids, membrane lipids, cytosolic molecules, and components of the extracellular matrix. There are data, both direct and indirect, to support the concept that oxygen radicals are produced during ischemia and reperfusion of heart muscle and that interventions directed against the cytotoxic species can result in a salvage of myocardium within the area at risk. Much of the attention has focused
on the role of neutrophils as providing an extracellular source of reactive species of oxygen in myocardium subjected to reperfusion after a period of regional ischemia. Alternative hypotheses have suggested intracellular sources for the production of cytotoxic oxygen species arising during the period of ischemia as well as during reperfusion. Meerson et al.27 suggested that the shift to anaerobic metabolism in the ischemic myocardium indirectly contributes to membrane disruption by leading to the accumulation of reducing equivalents in the form of nicotinamide adenine dinucleotide reductase (NADH) due to the inhibition of the electron transport chain of aerobic mitochondrial respiration. The accumulation of NADH is thought to contribute to the decline in membrane function by participation in the endogenous generation of oxygen radicals, which lead to lipid peroxidation and membrane injury. The potential intracellular sources of oxygen-derived free radicals include mitochondria,2829 xanthine oxidase22-30-31 and perhaps other cellular oxidases such as aldehyde oxidase, and reactive species of oxygen formed during eicosanoid biosynthesis.31 It must be recognized, however, that several published studies do not support the conclusion implicating the role of oxygen radicals in myocardial ischemia/reperfusion injury.34-36

Przyklenk and Kloner13 have addressed the issue of whether free radical species generated during the ischemic period could contribute to the development of irreversible myocardial cell injury. It was suggested that the presence of collateral blood flow in the region of distribution of the occluded coronary artery renders the tissue hypoxic rather than anoxic, thereby providing a supply of oxygen to the supposedly ischemic region, and it is this source of oxygen that is sufficient to support the formation of oxygen radicals. It was suggested that residual collateral blood flow of 0.1 ml/min/g tissue could support free radical formation and that the calculated value was comparable to what is observed to occur in the midmyocardial and epicardial regions of the canine heart during coronary artery occlusion. Direct measurements of oxygen radical formation during ischemia have been provided by studies using paramagnetic resonance or electron-spin resonance techniques to detect the presence of spin adducts during the period of ischemia as well as during reperfusion6-8 and the inhibition of the oxygen radicals by superoxide dismutase. The finding that superoxide dismutase and catalase could reduce the extent of myocardial injury when administered before and throughout a permanent 6-hour period of coronary artery occlusion would support the concept that free radical production could contribute to myocardial cell death in the absence of reperfusion.13 However, long-term salvage could not be demonstrated once reperfusion was instituted, and the degree of tissue injury was quantitated 24-42 hours after reperfusion. The findings reported by Przyklenk and Kloner13 provide strong evidence for the notion that the evolution of myocardial necrosis remains a dynamic process even after 6 hours of coronary artery occlusion and that the treatment regimen employed by these investigators delayed, rather than prevented, tissue necrosis.

Recognizing the dynamic process involved in the evolution of myocardial necrosis and the possible role of oxygen radicals derived from both intracellular and extracellular sources, one might suggest that the failure to observe a sustained reduction in infarct size after a 24-hour period of reperfusion could be attributed to the relatively short half-life of both superoxide dismutase and catalase.14,15,36 Thus, the absence of the antioxidants during the prolonged period of reperfusion would leave the myocardium susceptible to free radical damage resulting from the subsequent inflammatory response to ischemic injury and the accumulation of neutrophils in the reperfused region.23-26 Przyklenk and Kloner13 suggested that a prolonged beneficial effect of the free radical scavenger might have been realized if the infusion had been maintained throughout the period of the acute inflammatory response associated with reperfusion.

The present investigation has taken advantage of the fact that the conjugation of superoxide dismutase to polyethylene glycol provides a means by which the plasma half-life of the scavenger can be extended beyond 30 hours.14,15,32 The combined presence of superoxide dismutase in the circulation would provide protection against the cytotoxic effects of superoxide anion possibly derived from extracellular sources such as the invading polymorphonuclear neutrophils which mediate the acute inflammatory response in the ischemic/reperfused heart.23-26 An alternative reasoning may involve the anti-inflammatory potential of superoxide dismutase to limit the accumulation of neutrophils in the reperfused region. An important conclusion to be drawn from this investigation is that irreversible myocardial cell injury occurs during ischemia and that the application of superoxide dismutase during the ischemic interval would provide a beneficial effect that would translate into the salvage of myocardial cells within the risk region. This conclusion is in agreement with previous observations15,32 suggesting that myocardic necrosis during the ischemic interval is related to the cytotoxic effects of oxygen free radicals or derived species of reactive oxygen. Furthermore, the persistent enzymatic activity of PEG-SOD in the circulation and its ability to adhere to the endothelial surface16 would provide a sustained protective effect, thereby allowing for the salvage of myocardial cells as opposed to simply delaying the appearance of necrosis of myocytes exposed to oxygen radicals during the period of ischemia and/or reperfusion.

The failure to observe a comparable degree of myocardial protection, 24 hours after reperfusion, in the group which received Native-SOD as compared with the group which received PEG-SOD is in
accord with the results reported by Przyklenk and Kloner.\textsuperscript{13} The latter authors reported that reperfusion (24–42 hours) after 6 hours of regional ischemia in the control group was not associated with a significant increase in infarct size when compared with the control group in which infarct size was determined after 6 hours of ischemia without undergoing reperfusion (22.7±4.4% vs. 19.8±2.2% of the total left ventricle, see Reference 13). On the basis of previous studies\textsuperscript{2–4} one could conclude that reperfusion after a 6-hour period of ischemia would offer little benefit with respect to the salvage of myocardial tissue in the area at risk. In the present study, however, pretreatment with PEG-SOD resulted in a sustained reduction in myocardial infarct size as compared with the group that received Native-SOD, suggesting that the salvaged tissue involved cells which would otherwise have been affected adversely during the ischemic interval as well as during the period of reperfusion. In contrast to Native-SOD, PEG-SOD allows for the continued presence of the free-radical scavenging enzyme which could account for the greater degree of protection obtained with the conjugated enzyme as assessed on the basis of infarct. Since the extent of irreversible myocardial cell injury is near its maximum at the end of the 6-hour period of ischemia, it is reasonable to conclude that PEG-SOD is capable of salvaging myocardial tissue placed in jeopardy during the ischemic interval as well as during the period of reperfusion. The protective effect is not simply a delay of cell necrosis as has been observed with Native-SOD.\textsuperscript{13}

The efficacy of an intervention to protect myocardial tissue subjected to ischemia and reperfusion will be dependent upon the time course of the injury process, the pharmacologic half-life of the agent used and the time at which infarct size is assessed. Previous work\textsuperscript{9} demonstrated that bovine superoxide dismutase will reduce infarct size after 6 hours of regional ischemia without reperfusion, but that the protective effect was lost when the period of observation was extended to 30–48 hours despite the presence of reperfusion. Taking into consideration the observations of Przyklenk and Kloner\textsuperscript{13} in conjunction with the results of the present study, it becomes apparent that altering the pharmacokinetic properties of superoxide dismutase by conjugation to polyethylene glycol can provide sustained protection against the injury due to myocardial regional ischemia and reperfusion. This effect could not be achieved with the native form of the enzyme, most likely due to its limited pharmacological half-life and rapid removal from the circulation.\textsuperscript{14,15,36} The relatively short half-life of Native-SOD would limit its usefulness as a free radical scavenger under conditions in which the process of tissue injury extends beyond the action or presence of the protective intervention. The failure to observe a significant degree of protection in the group treated with PEG-ALB indicates that the reduction in infarct size is attributable to the enzymatic activity of PEG-SOD and not to its glycol component.

Not all investigators\textsuperscript{34–36} have been able to detect a beneficial effect with the use of superoxide dismutase in the canine heart subjected to a period of ischemia followed by reperfusion. The dissimilarity among the various laboratories in final outcome relating to the effect of superoxide dismutase on infarct size may be related to the pharmacokinetic properties of the native enzyme as compared with its conjugated form, the duration of the ischemic interval employed, the elapsed time between the administration of the intervention and the assessment of tissue injury, and the use of awake, chronically instrumented animals.

Recently published data\textsuperscript{6–8,13} have suggested that free radical mediated myocardial cell injury can occur during the period of myocardial ischemia as well as during the time of reperfusion. We have provided an extension of the study by Przyklenk and Kloner\textsuperscript{13} by employing the polyethylene glycol conjugated form of the enzyme, superoxide dismutase, which possesses a half-life of greater than 30 hours. The PEG-SOD, in contrast to the native form of the enzyme, provided a greater degree of tissue salvage in the canine heart that was subjected to 6 hours of regional ischemia followed by 24 hours of reperfusion. The beneficial effects of the PEG-SOD or the lack of effects of Native-SOD could not be attributed to differences among groups with respect to hemodynamic parameters or to differences in regional myocardial blood flow. PEG-SOD in contrast to Native-SOD or to PEG-ALB, is able to provide sustained protection against the tissue injury induced by 6 hours of ischemia and followed by 24 hours of reperfusion. We propose that a favorable outcome with the conjugated enzyme represents a prevention of cell necrosis and not merely a delay in the process of cell death as might be inferred from previous studies which have employed the native form of the enzyme and in which assessment of tissue injury was conducted at a time which exceeded the pharmacological and or biological half-life of superoxide dismutase.\textsuperscript{13,34–36}

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