Superoxide Dismutase Conjugated to Polyethylene Glycol Provides Sustained Protection Against Myocardial Ischemia/Reperfusion Injury in Canine Heart

Yasuo Tamura, Liguo Chi, Edward M. Driscoll Jr., Paul T. Hoff, Bruce A. Freeman, Kim P. Gallagher, and Benedict R. Lucchesi

Disagreement regarding the cardioprotective role of superoxide dismutase may relate to the use of different durations for induction of ischemic injury and reperfusion. The present study employed superoxide dismutase conjugated to polyethylene glycol (PEG-SOD), which has a half-life greater than 30 hours. Two protocols differing in the mode of administration and the duration of the reperfusion interval were used. Dogs were subjected to occlusion of the circumflex coronary artery for 90 minutes, then reperfused for 6 hours (Protocol A) or 4 days (Protocol B). The dogs received either polyethylene glycol conjugated to albumin (PEG-ALB) or PEG-SOD (1,000 U/kg). In Protocol A, treatment was administered starting 15 minutes before coronary occlusion and continued for 2 hours, terminating 15 minutes after reperfusion. Infarct size was determined 6 hours later. In Protocol B, the conjugated proteins were given 15 minutes before reperfusion and ended simultaneously with reperfusion. Infarct size was measured after 4 days. Infarct size (percentage of area at risk) in control (n = 9) and treated (n = 9) dogs in Protocol A differed between groups: 46.7±3.5% versus 28.3±2.9%, respectively (p<0.005); risk regions did not differ: 42.8±1.5% versus 43.8±2.1%, respectively. Myocardial salvage also was observed in Protocol B. Infarct size in control (n = 13) and treated (n = 13) groups was 44.2±2.6% versus 29.2±1.6%, respectively (p<0.005), with risk regions being 44.4±1.4% versus 46.0±1.6% (p=NS). Hemodynamic variables did not differ during the period of coronary artery occlusion. The respective collateral blood flows to the inner two thirds of the ischemic myocardium determined 60 minutes after occlusion were 0.05±0.01 ml/min/g and 0.06±0.04 ml/min/g (p=0.806) for the PEG-ALB and PEG-SOD treated groups, respectively. Infarct size was related inversely to collateral blood flow in the PEG-ALB treated group. This relation shifted downward (analysis of covariance, p=0.017). Plasma SOD activity in Protocols A sustained for 6 hours. Significant enzymatic activity was present after 4 days in Protocol B. Previous negative studies with native SOD may be related to the short half-life of its free-radical scavenging capacity, which compromises the chances of observing a protective effect after 4 days of reperfusion. The present results support our previous observations, as well as those of other investigators, demonstrating that superoxide dismutase can reduce that component of myocardial injury associated with reperfusion. (Circulation Research 1988;63:944-959)
Efforts to reperfuse ischemic myocardium have prompted considerable interest to be focused on the issue of reperfusion injury due to the generation of intracellular or extracellular cytotoxic oxygen free radicals. Data from recent experimental studies suggest that one or more cellular sites may be associated with the generation of cytotoxic oxygen species. Among the proposed intracellular sources for reactive oxygen species is the xanthine oxidase–mediated conversion of hypoxanthine to xanthine and uric acid in which molecular oxygen serves as the electron acceptor leading to the formation of superoxide anion. In mitochondria, approximately 1% of the electrons that pass down the electron transport chain leak off to molecular oxygen by reaction with semiquinone, resulting in the production of superoxide anion in the mitochondrion. The rate of production of the superoxide anion is related directly to the partial pressure of oxygen in the mitochondrion. Various subcellular organelles other than the mitochondria, such as nuclei and microsomes, have the capacity to form reactive oxygen species that can induce myocyte injury when intracellular defense mechanisms become depleted. Under normal conditions, cells possess several antioxidant enzymes to protect against the endogenously generated reactive oxygen species. The major intracellular protective antioxidant enzymes include: superoxide dismutase, glutathione peroxidase, and catalase. The antioxidants of tissues can also become inhibited or depleted during prolonged periods of oxidative stress.

Perhaps the most important extracellular source for the generation of oxygen free radicals and cytotoxic oxygen metabolites is the polymorphonuclear leukocyte, which, by virtue of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase–mediated conversion of molecular oxygen to superoxide anion and the myeloperoxidase-catalyzed production of hypochlorous acid, can lead to tissue injury in an organ undergoing reperfusion after a period of ischemia. On reperfusion of the ischemic myocardial tissue, the polymorphonuclear leukocyte, under the influence of adherence-promoting cell surface glycoproteins, forms an attachment to the vascular endothelium and can cause damage to the vessel wall by forming cytotoxic products of oxygen in the microenvironment established between the neutrophil and the endothelial cell. The extracellular space, unlike the intracellular compartment, is not well endowed with antioxidants able to protect against cytotoxic oxygen metabolites generated by the invading polymorphonuclear leukocytes.

Hill and Ward were among the first to recognize the role of the polymorphonuclear neutrophil as a contributor to myocardial ischemic injury. From their study, we and others made the observation that reperfusion injury was mediated in large part by the formation of oxygen-derived free radicals from one or more sources with the most likely site of production being the infiltrating neutrophils.

The reported reduction in ultimate infarct size by administration of superoxide dismutase, a free radical scavenger, especially when given immediately before instituting reperfusion, has provided indirect evidence for the role of the superoxide anion as a contributor to the phenomenon of reperfusion injury. The concept of reperfusion injury is based on the belief that a population of myocardial cells that are viable at the end of the ischemic period become irreversibly injured by the act of reperfusion itself. Not all laboratories, however, have had the same experience with the beneficial effects of superoxide dismutase in the limitation of myocardial infarct size. Consequently, the role of the superoxide anion in mediating myocardial injury associated with reperfusion has been questioned.

The present study was designed specifically to determine whether the efficacy of superoxide dismutase was limited by its short plasma half-life. Although the scavenger may exert a beneficial effect in the immediate postreperfusion period, it is possible that the rapid decrease of enzymatic activity in the circulating plasma allows a delayed development of reperfusion injury and cell death to occur between 2 and 4 days after reperfusion. To investigate the potential of superoxide dismutase to provide a prolonged protective effect in the reperfused heart, we employed the conjugated form of the enzyme, 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, potentially increasing cellular enzyme activity in a manner that provides protection against the cytotoxic effects of superoxide anion. The results of the present study provide evidence that polyethylene glycol–conjugated superoxide dismutase (PEG-SOD), administered before coronary artery reperfusion, can provide prolonged (>4 days) plasma concentration of antioxidant enzyme activity as well as protection of the heart as defined by a reduction in ultimate infarct size when quantitated 4 days postreperfusion.

Materials and Methods

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 Publication No. NIH 78-23, revised 1978).

Two separate study protocols, each employing a 90-minute period of regional myocardial ischemia followed by reperfusion (6 hours or 4 days), were used in this series of investigations to document the effect of PEG-SOD on ultimate infarct size in the canine heart. Polyethylene glycol conjugated to
bovine serum albumin (PEG-ALB) was used as a placebo control. The design of the two protocols is depicted schematically in Figure 1.

Effects of PEG-SOD on Myocardial Infarct Size
6 Hours After Reperfusion

Detailed methods have been published previously.4,18,19,30,31 The dogs used in this protocol were male mongrels, carefully selected with respect to breed, weighing between 13 and 18 kg. The dogs were anesthetized with sodium pentobarbital 30 mg/kg i.v. After induction of anesthesia, the dogs were ventilated with room air with a Harvard respirator (South Natick, Massachusetts). A left thoracotomy was performed, and the proximal segment of the left circumflex coronary artery was isolated and an electromagnetic flow probe was attached. The left atrial appendage was cannulated to allow for the administration of drug infusions. After measurement of basal coronary 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 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 90 minutes, followed by gradual reperfusion for 30 minutes. Leads II, III, and aV_{F} of the electrocardiogram, left circumflex coronary artery blood flow, and carotid artery pressure were monitored throughout the experimental procedure. The experiment was terminated 6 hours after the occlusive ligature was removed from the left circumflex coronary artery and perfusion was reestablished, but the critical stenosis was left in place.

Exclusion and Inclusion Criteria

Predetermined exclusion criteria were 1) presence of heart worms on final examination of the heart; 2) 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) failure to develop arrhythmias on reperfusion; and 4) intractable ventricular fibrillation requiring more than three attempts at cardioversion using low DC current (10 J) pulses applied directly to the surface of the heart. In those experiments in which regional myocardial blood flow was determined with radiolabeled microspheres, an added criterion for inclusion in the final data analysis was the need to demonstrate a subendocardial collateral blood flow of less than 0.15 ml/min/g of tissue at 60 minutes after occlusion of the vessel.

Administration of PEG-SOD and PEG-ALB

Animals were randomly assigned to receive either polyethylene glycol-conjugated bovine liver superoxide dismutase, 1,000 units/kg (supplied as 2,960 units/mg, 5.3 mg/ml, 15,600 units/ml, with 46% of available amino groups modified) or bovine serum albumin fraction V conjugated to polyethylene glycol.4,27,28 Both the conjugated enzyme formulation and the conjugated albumin were obtained from ENZON, Inc., South Plainfield, New Jersey. Appropriate dilutions were prepared daily shortly before the time of administration. The PEG-ALB was selected as a placebo control. PEG-ALB, in addition to controlling for the administered polyethylene glycol, also provided the nonspecific protein for any osmotic effects of the enzyme, and the dose selected provided an amount of protein equivalent to that contained in the administered dose of PEG-SOD (0.337 mg protein/kg body wt). The PEG-SOD or PEG-ALB solutions were infused via the left atrium at 0.5 ml/min over a period of 2 hours beginning 15 minutes before and ending 15 minutes after a 90-minute occlusion of the left circumflex coronary artery (Figure 1, Protocol A). Reflow through the left circumflex coronary artery was increased gradually over 30 minutes by slowly releasing the silastic occluder.

Determination of Myocardial Infarct Size and Area at Risk

After 6 hours of reperfusion, the hearts were excised immediately after electrical induction of ventricular fibrillation by application of a 60 Hz, 10 V, square wave train of pulses to the ventricular apex with bipolar platinum electrodes. Histochemical determination of the anatomic area at risk and the zone of infarction was accomplished with a dual perfusion technique previously described.4,18,19,30,31 The aorta was perfused retrograde with 0.25% Evans blue dye, and the circumflex coronary artery was perfused with 1.5% 2,3,5-triphenyltetrazolium chloride (TTC) 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 at 37° C. The heart was cut into 1-cm-thick transverse sections and fixed in 10% formalin solution. 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) 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 and the area at risk from the planimetered areas and the weights of each section. Previous studies demonstrate that there is an excellent correlation between infarct size derived by this planimetric method and the direct...
Gravimetric measurement of infarct size. 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.

**Effect of PEG-SOD on Myocardial Infarct Size 4 Days After Reperfusion**

The study population employed for the assessment of myocardial infarct size after 4 days of reperfusion consisted of 11 dogs (four in the PEG-SOD group and one in the PEG-ALB group). Five dogs (one in the PEG-SOD group and one in the PEG-ALB group) were excluded before the administration of treatment because of the failure to meet the inclusion criteria of ST segment elevation and completion of the 4-day study and were not included in the final analysis of infarct size. Among these latter eight dogs, four (one in the PEG-ALB group and three in the PEG-SOD group) died of intractable ventricular fibrillation; two PEG-ALB-treated animals died 4 hours after reperfusion, and one PEG-SOD-treated animal died overnight within the first 24-hour period. In addition, one PEG-ALB- and one PEG-SOD-treated dog had to be excluded because of the need for more than three attempts at cardioversion for the management of ventricular fibrillation. The final experimental group consisted of 13 PEG-ALB-treated and 13 PEG-SOD-treated animals.

The animals were subjected to the same experimental surgical procedure as described above for the induction of myocardial ischemia by occlusion of the left circumflex artery for 90 minutes followed by reperfusion in the presence of a critical stenosis. PEG-SOD or PEG-ALB was administered according to the same regimen as indicated previously with the exception that the full dose was administered within 15 minutes, commencing 15 minutes before reperfusion of the left circumflex coronary artery.

The surgical incisions were closed and the animals were returned to the Unit for Laboratory Animal Medicine to be cared for under the direction of the veterinary staff. As indicated above, blood samples were obtained on the day of surgery and daily for the next 4 postoperative days for plasma determinations of superoxide dismutase enzymatic activity. Four days later, the animals were returned to the laboratory and anesthetized, and the heart was removed for further analysis. The dual perfusion method using Evans blue and TTC was employed for the histochemical demarcation of the risk region, infarct region, and total left ventricle.
The heart was cut into transverse sections 1 cm thick, and the outlines of the histochemically defined regions were traced onto clear acetate sheets and subsequently quantitated as described above.

Treatment Groups

Animals were randomly assigned to either the PEG-SOD treatment group or to the PEG-ALB control group. The animals in the treatment group received PEG-SOD, 1,000 units/kg supplied as 2,960 units/mg in solution containing 5.3 mg/ml or 15,600 units/ml as provided by the ENZON Corporation. The animals randomly assigned to the control group received PEG-ALB. The amount of protein contained in the administered dose of PEG-SOD and in the administered dose of PEG-ALB were the same (0.337 mg protein/kg body wt). The total PEG-SOD and PEG-ALB doses were diluted to 60 ml with 0.9% sodium chloride solution for injection and infused at a rate of 4 ml/min over a period of 15 minutes starting 15 minutes before the onset of reperfusion. The method of drug administration differed from that used in Protocol A presented in the previous section in which the enzyme was given over a period of 2 hours starting 15 minutes before the onset of ischemia and ending 15 minutes after the start of reperfusion. In the Protocol B series of studies, the drug or placebo solutions were administered 15 minutes before the onset of reperfusion to approximate the conditions that might exist in a clinical setting in which coronary thrombolysis or coronary artery angioplasty is preceded by the administration of a therapeutic intervention for the limitation of myocardial reperfusion injury. The solutions were administered via a cannula in the left atrium to permit mixing in the left ventricular blood pool and thus maximize the amount of drug gaining access to the coronary circulation at the time of reperfusion.

Determination of Regional Myocardial Blood Flow With Radiolabeled Microspheres

Regional myocardial blood flow was determined with tracer-labeled microspheres (15 μm diameter, New England Nuclear, Boston, Massachusetts) by the reference withdrawal method described previously. In Protocol B, 10 animals were given two injections of microspheres (labeled with 141Ce and 103Ru) with the order of the isotopes randomized. Reference arterial blood samples were obtained simultaneously from both the femoral and carotid arteries at a constant rate with a Harvard withdrawal pump, beginning immediately before the injection of microspheres into the left atrium and ending 2 minutes later. The reference sample counts were averaged for calculation of myocardial blood flow. If the reference sample counts varied by more than 15%, the data were discarded. Each bottle of microspheres was placed in an ultrasonic bath with subsequent vortex agitation before injection to ensure that adequate dispersal of the microsphere suspensions was achieved before being administered. Microspheres were administered when baseline flows were determined before occlusion of the left circumflex coronary artery and when regional flow determinations were made for a second time 60 minutes after occlusion of the coronary artery (Figure 1, Protocol B). Tissue samples weighing 0.5–1.0 g were dissected from the subepicardial, midmyocardial, and subendocardial sections of the heart in the regions of distribution of the left circumflex coronary artery and from the nonischemic region of the left ventricle in the region of distribution of the left anterior descending coronary artery. At least three sections from each heart were used so that blood flow to each region represents the average of three to four samples for each experiment. Corrections were made for apparent microsphere loss as described previously.

Plasma Superoxide Dismutase Activity

Venous blood samples (2 ml) used for the determination of superoxide dismutase enzymatic activity were collected in heparinized tubes from each dog treated with PEG-SOD or PEG-ALB. Blood samples were collected for the acute studies in Protocol A 1) immediately before occlusion of the coronary artery and 2) 60, 120, 240, 360, and 450 minutes after the administration of the respective treatment regimens. In Protocol B, blood samples were collected 1) immediately before the administration of PEG-SOD or PEG-ALB during the phase of regional myocardial ischemia; 2) 15, 60, 120, and 360 minutes after the administration of the respective treatment regimens; and 3) 24, 48, 72, and 96 hours after the administration of the respective treatment regimens. The blood samples were centrifuged immediately to obtain the plasma, which was separated and stored frozen. Plasma samples were incubated at −20° C were sent to the University of Alabama at Birmingham to the laboratory of Dr. Bruce Freeman, who conducted the assay for plasma superoxide dismutase enzymatic activity on the samples provided.

Plasma superoxide dismutase was assayed by the method of McCord and Fridovich, using the superoxide-mediated reduction of cytochrome c as a measure of free radical formation and quantitating the superoxide dismutase activity in the plasma based on the ability of an aliquot of the plasma sample to inhibit the reduction of cytochrome c. Inhibition of cytochrome c reduction by plasma from untreated dogs was due to amounts of superoxide dismutase that were at the lower limit of detection by this assay system and to reaction or interception of superoxide anion by serum components. Except for the rare case, the nonenzymatic activity of plasma never approached 1 unit/ml.

Statistics

All data are expressed as the mean ± SEM. Paired or group t test analyses were applied where appropriate. Differences were considered significant for
p<0.05. When comparisons among more than two means were made (infarct/area at risk; infarct/left ventricle; area at risk/left ventricle) the data were compared by analysis of variance and Scheffe's multiple comparison confidence intervals.

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. An analysis of covariance was performed, in which collateral blood flow was the independent variable, with the object of determining if there existed a statistically significant difference in the calculated infarct size between the two groups when the influence of collateral blood flow was controlled.

Results

Protocol A: Effect of PEG-SOD on Ultimate Myocardial Infarct Size—90-Minute Occlusion and 6 Hours of Reperfusion

A total of 28 dogs (14 in the PEG-ALB group and 14 in the PEG-SOD group) were used in the first series of experiments (Protocol A). Six dogs (two in the PEG-ALB group and four in the PEG-SOD group) died of intractable ventricular fibrillation and could not be included in the final analysis of the data with respect to infarct size. Two PEG-ALB dogs were excluded because they failed to satisfy the criteria for inclusion in the study as they did not develop objective evidence of regional myocardial ischemia on left circumflex coronary artery occlusion (failure to develop ST segment elevation and epicardial cyanosis). In addition, two dogs (one PEG-ALB and one PEG-SOD) were excluded because more than three attempts at cardioversion were required to reverse ventricular fibrillation. Thus, the final groups consisted of nine PEG-ALB–treated and nine PEG-SOD–treated animals.

Group Characteristics of Acute Infarct Studies

Body weight. Comparison of the two groups of animals with respect to body weight failed to demonstrate a significant difference (15.9±0.4 vs. 15.6±0.4 kg for PEG-ALB–treated vs. PEG-SOD–treated groups).

Heart size and risk region. Total left ventricular mass was the same for both groups (84.6±6.0 vs. 84.5±6.7 g for PEG-ALB– vs. PEG-SOD–treated groups). The area at risk as determined with the triphenyltetrazolium/Evan’s blue dual-staining method and expressed as a percent of the left ventricle did not differ between the control and the enzyme-treated groups (42.8±1.5% vs. 43.8±2.1% for PEG-ALB– vs. PEG-SOD–treated groups). The overall area at risk for the 18 experimental animals was 43.3±1.2% of the total left ventricle. Due to the small variability in the size of the risk region, the dependence of infarct size on the size of the area at risk was minimized as a variable, thus making it possible to assess the potential of a pharmacologic agent to reduce ultimate infarct size.

Ultimate infarct size. Infarct size expressed as a percent of the anatomic area at risk in the PEG-SOD group was significantly smaller than that observed in the PEG-ALB–treated group: 46.7±3.5% versus 28.3±2.9% for PEG-ALB versus PEG-SOD groups, respectively (p<0.005). The data for infarct size expressed as a percent of the area at risk and a comparison of the risk regions, expressed as a percentage of the total left ventricle, are presented in Figure 2. The size of the risk region for each group did not differ. The similarity between groups with respect to the size of the area at risk facilitates statistical comparison of the infarct size data from each of the experimental groups as the size of the risk region is one of the major determinants of ultimate infarct size.

A significant reduction in infarct size with PEG-SOD treatment also was observed when the infarct zone was expressed as a percent of the total left ventricle: 19.9±1.5% versus 12.4±1.5% for PEG-ALB versus PEG-SOD groups, respectively (0.0005<p<0.005).

Assessment of infarct size as a percentage of area at risk demonstrated that pretreatment with PEG-SOD resulted in a 39% reduction in ultimate infarct size when the circumflex coronary artery was occluded for 90 minutes followed by 6 hours of reperfusion. The results with PEG-SOD are equivalent to those obtained in previous studies from this laboratory24 in which the native bovine copper, zinc superoxide dismutase was found to reduce ultimate infarct size to a similar extent in a closely related model of regional myocardial ischemia followed by reperfusion.

Hemodynamic measurements. Heart rate, mean arterial blood pressure, rate/pressure product, mean left circumflex coronary artery blood flow, and aortic dP/dt did not change significantly during the initial 15 minutes of PEG-ALB or PEG-SOD infusion preceding occlusion of the left circumflex coronary artery. There were no significant intergroup differences in these measurements before, during, or after left circumflex coronary artery occlusion (Table 1). Within each group, mean left circumflex coronary artery blood flow and mean arterial blood pressure after reperfusion were significantly lower, and heart rate in the PEG-ALB group 6 hours after reperfusion was significantly greater than the preocclusion control value. However, there were no significant differences between the groups at any of the time points at which measurements were made.

Plasma superoxide dismutase activity. Enzymatic activity for superoxide dismutase was determined in plasma samples obtained from two groups of 14 dogs. In each of the groups, nine dogs were employed for the study of infarct size at the end of the 6-hour reperfusion period. The plasma samples were obtained at 0, 60, 120, 240, 360, and 450 minutes after injection of either the conjugated PEG-SOD or PEG-ALB. A relatively long plasma
MYOCARDIAL INFARCT SIZE
(90 MINUTES OCCLUSION AND 6 HOURS REPERFUSION)
PEG-ALBUMIN vs PEG-SOD - INFARCT SIZE

TABLE 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Infarct Size (Area % of Total Left Ventricle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-ALB</td>
<td>40</td>
</tr>
<tr>
<td>PEG-SOD</td>
<td>30</td>
</tr>
</tbody>
</table>

FIGURE 2. Bar graph showing myocardial infarct size expressed as a percent of the area at risk (left panel). Analysis of results indicates that administration of polyethylene glycol conjugated to superoxide dismutase (PEG-SOD) results in significant reduction in ultimate myocardial infarct size in the heart that has been subjected to 90 minutes of regional ischemia followed by 6 hours of reperfusion. Administration of polyethylene glycol conjugated to albumin (PEG-ALB) to control animals had no apparent effect on ultimate infarct size when compared with data from studies in which the same durations of ischemia and reperfusion were employed. Right panel shows data for size of area at risk expressed as percent of total left ventricle. Since ultimate infarct size is related directly to size of risk region, comparable risk regions in each experimental group makes it possible to compare effects of one variable, that is, treatment with PEG-SOD, on the results being measured.

Protocol B: Effect of PEG-SOD on Ultimate Myocardial Infarct Size—90 Minutes of Occlusion and 4 Days of Reperfusion

Group Characteristics of Acute Infarct Studies

Body weight. The two groups of animals did not differ with respect to body weight. The mean body weight ± SEM for the PEG-ALB-treated group was 16.0 ± 0.4 kg (n = 13) as compared with the PEG-SOD-treated group, which was 15.7 ± 0.5 kg (n = 13; 0.1 < p < 0.375).

Heart size and risk region. The left ventricular mass did not differ between the two treatment groups. Left ventricular weight in the PEG-ALB group was 90.7 ± 3.9 versus 92.8 ± 4.6 g for the PEG-SOD-treated group. The area at risk for each group, expressed as a percentage of the left ventricle, was 46.4 ± 1.4% versus 46.0 ± 1.6% for the PEG-ALB and PEG-SOD groups, respectively. The overall area at risk for the 26 experimental animals included in the data analysis for myocardial infarct size was 46.8 ± 1.4% of the total left ventricle.

Myocardial infarct size. Twenty-six dogs were entered into the infarct size data analysis for Protocol B. Infarct size in the group of 13 animals that received PEG-SOD was reduced significantly (p < 0.005) when compared with the group of 13 control animals that received PEG-ALB. The normalized infarct size, expressed as a percentage of the anatomic area at risk, was 29.2 ± 1.6% in the PEG-SOD-treated group (n = 13) as compared with 44.2 ± 2.6% in the PEG-ALB-treated group (n = 13) when determined after the induction of experimental myocardial ischemic injury resulting from a 90-minute occlusion of the left circumflex coronary artery followed by reperfusion for 4 days. The data in Figure 4 show the risk region and infarct mass for each of the groups analyzed separately according to whether radiolabeled microspheres were employed for regional myocardial blood flow determinations.
TABLE 1. Hemodynamic Measurements in Acute Myocardial Ischemia and Reperfusion (90-Minute Occlusion of the Left Circumflex Coronary Artery and 6 Hours of Reperfusion)

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>15 Minutes post-infusion</th>
<th>End occlusion</th>
<th>Reperfusion (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG-ALB group (n = 9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>169 ±6</td>
<td>169 ±6</td>
<td>176 ±7</td>
<td>173 ±7 179 ±5 186 ±6*</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>107 ±2</td>
<td>108 ±2</td>
<td>98 ±3*</td>
<td>95 ±5 87 ±5 85 ±5*</td>
</tr>
<tr>
<td>R/P P (× 1,000)</td>
<td>20.4 ±0.7</td>
<td>20.8 ±0.8</td>
<td>19.6 ±0.9</td>
<td>19.2 ±1.1 18.0 ±1.3 18.5 ±1.2</td>
</tr>
<tr>
<td>LCX flow (ml/min)</td>
<td>27 ±2</td>
<td>27 ±2</td>
<td>0</td>
<td>17 ±2* 14 ±2* 14 ±1*</td>
</tr>
<tr>
<td>Ao dP/dt (mm Hg/sec)</td>
<td>345 ±60</td>
<td>359 ±61</td>
<td>379 ±64</td>
<td>361 ±53 322 ±42 339 ±51</td>
</tr>
<tr>
<td>PEG-SOD group (n = 9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>167 ±6</td>
<td>166 ±6</td>
<td>166 ±6</td>
<td>168 ±5 174 ±8 173 ±9</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>98 ±5</td>
<td>100 ±5</td>
<td>91 ±5</td>
<td>87 ±4 88 ±3 87 ±3*</td>
</tr>
<tr>
<td>R/P P (× 1,000)</td>
<td>18.5 ±1.0</td>
<td>19.2 ±1.0</td>
<td>17.5 ±1.0</td>
<td>17.2 ±0.7 17.8 ±0.9 17.5 ±1.1</td>
</tr>
<tr>
<td>LCX flow (ml/min)</td>
<td>22 ±2</td>
<td>22 ±2</td>
<td>0</td>
<td>16 ±2* 15 ±2* 14 ±1*</td>
</tr>
<tr>
<td>Ao dP/dt (mm Hg/sec)</td>
<td>356 ±70</td>
<td>364 ±70</td>
<td>332 ±61</td>
<td>339 ±64 315 ±56 359 ±78</td>
</tr>
</tbody>
</table>

PEG-ALB, polyethylene glycol conjugated to albumin; MAP, mean arterial blood pressure; R/P, rate/pressure product; LCX, left circumflex coronary artery; Ao dP/dt, change in aortic pressure over time; PEG-SOD, polyethylene glycol conjugated to superoxide dismutase.

All values are mean ± SEM.

* p ≤ 0.05 compared with control or preischemic levels.

Significant differences did not exist within the groups when myocardial infarct size or size of the region at risk were compared between those animals that received microspheres and those that did not.

Regional myocardial blood flow data were obtained with the use of radiolabeled microspheres in 11 dogs that completed the study protocol. Infarct size in the animals in which regional blood flow was determined with the use of radiolabeled microspheres, expressed as a percentage of the area at risk, was 44.4 ±5.8% versus 28.8 ±2.1% for the group treated with PEG-ALB (n = 5) or PEG-SOD (n = 6), respectively (0.01 < p ≤ 0.025). The myocardial risk regions expressed as a percent of the left ventricle were 45.2 ± 3.2% for the group treated with PEG-ALB (n = 5) and 45.7 ± 1.5% for the group treated with PEG-SOD (n = 6) (p = NS). Fifteen dogs did not receive radiolabeled microspheres for regional blood flow determinations. Myocardial infarct size expressed as a percent of the risk region was 44.2 ± 2.7% for the group treated with PEG-ALB (n = 8) as compared with 29.5 ± 2.4% for the group treated with PEG-SOD (n = 1) (0.0005 < p ≤ 0.005). The myocardial risk...
region expressed as a percent of the left ventricle was 47.1 ± 1.3% for the animals treated with PEG-ALB (n = 8) and 46.4 ± 2.8% for the animals treated with PEG-SOD (n = 7) (p = NS).

Since the two PEG-SOD and two PEG-ALB groups did not differ from each other in infarct size and region measurements, the data were pooled. Each group consisted of 13 animals that completed the protocol involving a 90-minute period of regional occlusion and reperfusion for 4 days. Infarct size, expressed as a percentage of the area at risk, differed significantly between the two groups: 44.2 ± 2.5% versus 29.2 ± 1.6% for the PEG-ALB and PEG-SOD groups, respectively (p < 0.005). Risk region size was similar in both groups: 44.4 ± 1.4% versus 46.0 ± 1.6% (p = NS). The data presented in Figure 5 indicate that for any given mass of risk region, the amount of tissue that had undergone irreversible injury was less in the PEG-SOD–treated group as compared with the control group, which received PEG-ALB.

Hemodynamic measurements. Table 2 contains summaries of the hemodynamic data obtained in each of the two groups of animals used for the assessment of the effects of PEG-SOD and PEG-ALB on ultimate myocardial infarct size. There were no significant intergroup differences in any of the measurements recorded. Thus, the observed reduction in infarct size in the group that received PEG-SOD could not be attributed to a favorable hemodynamic alteration that resulted in the reduction of myocardial oxygen consumption or myocardial work. The hemodynamic measurements were determined at specific intervals starting with the control period and repeated at 30, 60, 75, and 90 minutes after coronary artery occlusion and at 30 minutes after reperfusion.

Plasma superoxide dismutase activity. The animals that were studied for a period of 4 days after myocardial infarction were given either PEG-ALB or PEG-SOD (1,000 units/kg) as a slow infusion over a period of 15 minutes starting 15 minutes before reperfusion of the left circumflex coronary artery. Thus, the infusions of PEG-ALB and PEG-SOD were completed coincident with the onset of reperfusion. Plasma samples were obtained at specific time intervals over the next 4 days and later analyzed for superoxide dismutase activity. The data are presented graphically in Figure 6 and show that the administration of the PEG-SOD is associated with a prolonged increase in plasma superoxide dismutase activity as compared with the enzyme activity in the plasma from the PEG-ALB–treated animals. The dosage regimen employed ensured that a peak enzyme activity would be present at the time of reperfusion. The increase in plasma superoxide dismutase activity was maintained for the first 4 days after the administration of the enzyme at a
Tamura et al  Conjugated Superoxide Dismutase on Myocardial Infarct Size  953

Infarct Mass In Relation to Myocardium at Risk

FIGURE 5. Infarct mass is plotted in relation to mass of myocardial region at risk for animals treated with polyethylene glycol conjugated with albumin (PEG-ALB) and polyethylene glycol conjugated with superoxide dismutase (PEG-SOD). Significant reduction in infarct size was obtained with PEG-SOD as compared with PEG-ALB-treated controls. For a given mass of tissue constituting the risk region, there was less tissue mass that had undergone irreversible injury in the PEG-SOD-treated group, as indicated by relative position of points on graph. There is a minimal degree of variability in risk region mass between the two groups.

value significantly above that found in the plasma of dogs that received PEG-ALB.

Indexes of myocardial ischemia and its relation to ultimate infarct size. One of the inclusion criteria for the entry of an animal in the final data analysis relies on changes in the ST segment as being indicative of regional myocardial ischemia and lack of a significant coronary collateral circulation that would otherwise limit the extent of myocardial ischemic injury. We have analyzed the data relating to infarct size as a percentage of the area at risk from three groups of animals in which ST segment elevation was either present or absent during the 90-minute period of left circumflex coronary artery occlusion. The animals in each of the two control groups had received PEG-ALB, and each of the animals met the electrocardiographic requirements (ST segment elevation) for inclusion in the study. The animals included for infarct analysis in the third group did not manifest ST segment elevation on occlusion of the circumflex coronary artery. The respective infarct size for the two control groups that had ST segment elevation were 46.7 ± 3.5% (n = 9) and 44.2 ± 2.7% (n = 8) of the area at risk (p = NS) as compared with 3.2 ± 0.7% (n = 7) of the risk region in the group of animals that did not have ST segment elevation. The consistency of the area at risk among the groups and the consistency of the ultimate infarct size within groups would suggest that the use of ST segment elevation as an inclusion criterion is capable of ensuring that only those animals with a sustained and significant degree of regional myocardial ischemia are included in the final data analysis. Random assignment of the experimental animals to either the PEG-ALB or PEG-SOD treatment served as an additional measure to guard against biasing the data by inclusion of animals with high collateral blood flow in the PEG-SOD-treatment group.

Regional myocardial blood flow was determined with the use of radiolabeled microspheres in 11 animals (five in the PEG-ALB group and six in the PEG-SOD group). The data for preischemic and ischemic (60 minutes after occlusion) regional myocardial blood flows are presented in Table 3. No significant differences were observed between the groups with respect to myocardial blood flow in the endocardial, midmyocardial, and epicardial regions before or during coronary artery occlusion in the region of distribution of the left circumflex coronary artery.

TABLE 3. Hemodynamic Measurements in Acute Myocardial Ischemia Reperfusion (90-Minute Occlusion of the Left Circumflex Coronary Artery and 4 Days of Reperfusion)

<table>
<thead>
<tr>
<th></th>
<th>PEG-ALB group (n = 13)</th>
<th>PEG-SOD group (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preischemia</td>
<td>Time after LCX occlusion (minutes)</td>
<td>30 Minutes after reperfusion</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>148 ± 5</td>
<td>154 ± 5</td>
</tr>
<tr>
<td>Mean BP (mm Hg)</td>
<td>115 ± 5</td>
<td>107 ± 4</td>
</tr>
<tr>
<td>R/P P (× 1,000)</td>
<td>20 ± 4</td>
<td>19 ± 4</td>
</tr>
</tbody>
</table>

LCX, left circumflex coronary artery; PEG-ALB, polyethylene glycol conjugated to albumin; BP, blood pressure; R/P P, rate/pressure product; PEG-SOD, polyethylene glycol conjugated to superoxide dismutase.

All values are mean ± SEM.

*p < 0.05 compared with baseline preischemic or control value within group.
artery or in the myocardial region perfused by the left anterior descending coronary artery. Similar values were obtained in each of the groups with respect to the endocardial-to-epicardial blood flows and the mean transmural collateral flow determined 60 minutes after coronary artery occlusion (0.05 ± 0.02 ml/min/g in the PEG-ALB group as compared with 0.05 ± 0.01 ml/min/g in the PEG-SOD-treated group; \( p = 0.8 \)). Blood flows to the inner two thirds of the ischemic left ventricle were 0.03 ± 0.01 versus 0.04 ± 0.01 ml/min/g for the PEG-ALB and PEG-SOD groups, respectively.

Infarct size (percentage of the area at risk) versus collateral blood flow is plotted in Figure 7. The normalized infarct size is expressed in relation to collateral blood flow in the inner two thirds of the ischemic left ventricular myocardium. Limitation of infarct size in the group of animals that received PEG-SOD is evidenced by a downward shift in the regression line describing the relation between the extent of irreversible myocardial injury and collateral blood flow. Using the mean collateral blood flow to the inner two thirds of the ischemic myocardium as a covariate, infarct size expressed as a percentage of the area at risk was smaller for the PEG-SOD-treated group than for the PEG-ALB-treated group (\( p<0.05 \)).

**Discussion**

**Critique of Methods Used in the Present Study**

A crucial determinant affecting the ultimate mass of myocardial tissue that undergoes irreversible

**TABLE 3. Regional Myocardial Blood Flow Before and 60 Minutes After Left Circumflex Coronary Artery Occlusion**

<table>
<thead>
<tr>
<th></th>
<th>LCX bed (ml/min/g)</th>
<th></th>
<th>LAD bed (ml/min/g)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endocardium</td>
<td>Midmyocardium</td>
<td>Epicardium</td>
<td>Endocardium</td>
</tr>
<tr>
<td><strong>PEG-ALB (n = 5)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preischemia</td>
<td>0.88 ± 0.14</td>
<td>0.83 ± 0.12</td>
<td>0.74 ± 0.09</td>
<td>1.18 ± 0.13</td>
</tr>
<tr>
<td>Postischemia</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.09 ± 0.07</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td><strong>PEG-SOD (n = 6)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preischemia</td>
<td>0.94 ± 0.12</td>
<td>0.90 ± 0.12</td>
<td>0.87 ± 0.12</td>
<td>1.09 ± 0.06</td>
</tr>
<tr>
<td>Postischemia</td>
<td>0.03 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.05 ± 0.01</td>
</tr>
</tbody>
</table>

LCX, left circumflex coronary artery; LAD, left anterior descending coronary artery; PEG-ALB, polyethylene glycol conjugated to albumin; PEG-SOD, polyethylene glycol conjugated to superoxide dismutase.

Values are mean ± SEM.
Thus, time is a major determinant in the recruitment to achieve electrocardiographic (ST segment elevation) evidence of regional myocardial ischemia. The indirect relation between the presence of collateral vessels and ultimate infarct size is known\cite{37,39} and it is the immediate availability of coronary collateral vessels at the time of and during the period of coronary artery occlusion that is the important determinant of ultimate infarct size.

In Protocol B of the present study, direct regional myocardial blood flow determinations were made with the use of radiolabeled microspheres. Comparison of regional myocardial blood flow data in the endocardial, midmyocardial, and epicardial regions of the risk area during the period of coronary artery occlusion provides assurance that the severity of the ischemic insult was identical in both treatment groups. Taken together with the fact that all groups had similar risk regions expressed as a percent of the left ventricle, we have controlled two of the most important baseline variables that determine myocardial infarct size, the anatomical area at risk, and collateral blood flow.

Another important determinant of ultimate infarct size is that of myocardial oxygen consumption as influenced by heart rate and mean arterial pressure. No significant differences between groups were observed at any of the time points with respect to the measured hemodynamic data. The mean values for heart rate and blood pressure indicate that random assignment of the experimental animals resulted in groups with comparable baseline myocardial oxygen demand as determined from the rate/pressure product. Random assignment should also result in groups with ischemia of equivalent severity because all animals used in the data analysis had to meet the same inclusion criteria. It is unlikely, therefore, that differences in baseline collateral flow or myocardial oxygen use would account for the difference in infarct size in the two control groups as compared with the two treatment groups.
Another question is whether the absence of TTC staining is an adequate index of tissue necrosis. Vivaldi et al.,22 and more recently Romaschin et al.,23 performed electron microscopic examination of TTC-demarcated areas of myocardium from hearts subjected to a period of regional ischemia followed by reperfusion. Ultrastructural evidence of irreversible injury was detected only in those specimens obtained from regions that failed to assume the deep red color of the formazan precipitate and thus were considered TTC negative. Several studies have documented an excellent correlation between the quantitative evaluation of infarct size by the TTC method and histopathology.32,33,40 Our laboratory has demonstrated a close correlation between infarct size quantitation with nitro blue tetrazolium, a dehydrogenase histochemical reagent, and depletion of myocardial creatine kinase activity.41

Previous Studies With Superoxide Dismutase on Myocardial Infarct Size

Ambrosio et al.,25 using human recombinant copper, zinc superoxide dismutase (h-SOD), demonstrated a 35.6% reduction in ultimate infarct size in the canine heart under identical conditions of regional ischemia (90 minutes occlusion) followed by 48 hours of reperfusion. This differed from our previous study24 in two respects: 1) they administered h-SOD immediately at the time of reperfusion and 2) the duration of reperfusion was 48 hours. Our earlier study by Jolly et al. demonstrated that bovine superoxide dismutase produced significant reductions in infarct size regardless of whether the enzyme was administered 15 minutes before occlusion of the left circumflex coronary artery or 15 minutes before the onset of reperfusion (after 75 minutes of ischemia). Thus, three separate dosage regimens using superoxide dismutase have demonstrated the potential of the antioxidant enzyme to limit the extension of myocardial injury during reperfusion in a canine model of regional myocardial ischemia. In each of these instances, the duration of regional myocardial ischemia was maintained for 90 minutes followed by reperfusion for periods of 6 to 48 hours. From the study by Ambrosio et al.,25 we conclude that the observed benefit obtained with superoxide dismutase is not due solely to a delay in the development of myocardial necrosis because reduction in the size of the ultimate infarct was observed when quantitated 48 hours after reperfusion.

The beneficial effects of superoxide dismutase are not confined to canine models of myocardial ischemia and reperfusion, as demonstrated by the studies of Naslund et al.,26 who reported that native superoxide dismutase plus catalase reduced ultimate infarct size in a porcine model of coronary artery occlusion (60 minutes) followed by 5 hours of reperfusion. The pig, unlike the dog, is not endowed with a native collateral coronary vascular bed,42 thus diminishing the possibility that the results reported by Naslund et al.26 are blemished by failure to include determinations of regional myocardial blood flow.

Not all studies have demonstrated beneficial effects of superoxide dismutase in models of myocardial ischemia and reperfusion. Uraizee et al. employed a canine model in which the circumflex coronary artery was occluded for 40 minutes followed by reperfusion, and infarct size was determined 4 days later. They failed to observe a beneficial effect of the therapy in reduction of infarct size. The results, which diverge from those cited above, may be due to the relatively short duration of occlusion employed by these investigators, resulting in a relatively small area of myocardial infarction.42 In addition, it is also possible that there was a recurrence of the reperfusion phase of injury after a prolonged period of reperfusion that would not be prevented by the administration of native superoxide dismutase, which has a plasma half-life of less than 10 minutes. The presence of resident inflammatory cells within the reperfused region could account for the continuous production of cytotoxic derivatives of oxygen, thereby allowing for the progressive extension of tissue injury. Quantitation of infarct size at an earlier time point in the phase of reperfusion, such as that used by Ambrosio et al.,25 may have permitted the investigators to detect a reduction in the extent of tissue injury. That a recrudescence of myocardial injury can occur has been recently reported by us in a study in which a delayed development of myocardial injury was observed due to the limited pharmacological half-life of iloprost, the therapeutic intervention used to protect the ischemic heart.

The present study demonstrates that PEG-SOD can achieve a reduction in ultimate infarct size when quantitation of myocardial injury is done after 4 days of reperfusion, a time when the plasma superoxide dismutase activity is maintained well above that found in the PEG-ALB control group. The previous studies of Uraizee et al. and Ambrosio et al. suggest that the effectiveness of native superoxide dismutase in limiting myocardial reperfusion injury may not persist beyond 48 hours, whereas PEG-SOD may have an extended duration of effectiveness because of its continued presence in the circulation and because of its ability to adhere to the surface of the vascular endothelium and to augment cellular antioxidant activities in a manner that can provide protection from both intracellular (xanthine oxidase and other possible free radical loci) and extracellular (neutrophils) sources of superoxide and hydrogen peroxide.37-39,44-46 Ambrosio et al. determined collateral blood flow with the use of radiolabeled microspheres, and the analysis of infarct size versus collateral blood flow indicated that protection with human recombinant superoxide dismutase was greatest in animals with the lowest collateral flows. The latter authors used a 90-minute period of regional myocardial ischemia followed by
48 hours of reperfusion in a protocol that called for the administration of superoxide dismutase coincident with the onset of reperfusion.

The failure on the part of Uraizee et al.\textsuperscript{23} to detect a beneficial effect of superoxide dismutase on ultimate infarct size in the canine heart might be related to the model of myocardial ischemic injury employed by these investigators. The ischemic insult was for a duration of 40 minutes followed by 4 days of reperfusion. Eng et al.\textsuperscript{47} provide interesting insights into the relevance of the duration of coronary artery occlusion in a study designed to assess interventions for the protection of the ischemic heart. These investigators employed a unique model of myocardial ischemia in which the risk region was made severely ischemic, but despite the severity of flow depletion, necrosis only began with occlusion periods of 30 minutes or longer, with no evidence of necrosis occurring with ischemia of 20 minutes’ duration. The actual level of regional myocardial blood flow at the initial 20-minute period of ischemia appears to have no influence on cell viability, leading to the suggestion that myocardial metabolism is the sole determinant of cell viability during the first 20 minutes of an ischemic insult. Thus, modifications of blood flow below a presumed critical value would not alter the inevitable metabolic determinants of cell viability. Furthermore, the extent of necrosis after 40 minutes in a region of severe ischemia was only 10% of the risk region. Eng and coworkers\textsuperscript{47} demonstrated that an “explosive” increase occurs in the extent of necrosis when reperfusion is instituted between a narrow 50- to 60-minute period of ischemia. In their study, approximately 70% of the region at risk became necrotic after 90 minutes of severe blood flow deprivation followed by reperfusion. Thus, studies that use short periods of ischemia (e.g., 40 minutes) may not be able to detect a component of injury due to reperfusion because most of the cellular damage associated with the ischemic interval may be related to myocardial metabolism. According to Eng et al.\textsuperscript{47}, beginning at 30 minutes of blood flow deprivation, necrosis progressed from the endocardium toward the epicardium in a wave front pattern so that 90 minutes of ischemia resulted in irreversible injury to 70% of the risk region.

The second study that failed to detect a beneficial effect with superoxide dismutase plus catalase was that of Gallagher et al.\textsuperscript{22} in which occlusion of the coronary artery was maintained for 3 hours in the conscious, chronically instrumented canine heart followed by reperfusion for 24 hours. The lack of reported efficacy with superoxide dismutase and catalase could have been because infarct size in the risk region becomes near maximal within 3 hours of ischemia, thereby leaving little opportunity for an expression of tissue injury secondary to reperfusion.\textsuperscript{48} Because superoxide dismutase and catalase are directed toward preventing the free radical–dependent component of injury associated with reperfusion, experimental protocols that employ a 3-hour period of coronary artery occlusion are less likely to reveal a beneficial effect of an intervention on ultimate infarct size. Recently, Jolly et al.\textsuperscript{31} reported that neutrophil depletion is able to reduce ultimate infarct size in the canine heart that is subjected to 90 minutes of regional ischemia, but not when the duration of the ischemic insult is extended to 4 hours. The results suggest that there are limits beyond which the reperfusion injury is either too small to be detected or does not occur because the injury has developed to its full extent as a result of the ischemic insult alone.

Another reason why previous investigators\textsuperscript{22} were unable to detect a protective effect of antioxidant enzymes may be related to the use of chronic instrumentation of the canine heart. Bloor\textsuperscript{49} and Murdock et al.\textsuperscript{30} demonstrated that instrumentation of the canine heart increases the number and caliber of anatomically demonstrable collateral blood vessels that may alter the rate and extent of infarct development. This may explain in part the large scatter in baseline values that reveal a linear correlation between infarct size and collateral blood flow.\textsuperscript{22,23}

The observations in the present study suggest that PEG-SOD, with its extended pharmacological half-life, may be of benefit over the native enzyme. This may not only be due to its prolonged duration in the circulation, but may also be related to the observation that polyethylene glycol-conjugation of superoxide dismutase or catalase enhances cell association and uptake of the enzymes in a manner that renders vascular endothelium more oxidant resistant.\textsuperscript{29,45} The vulnerability of the endothelium to injury from reactive species of oxygen may be related to a number of factors, among which are anatomical proximity to activated and invading inflammatory cells and localization of xanthine oxidase activity within endothelial cells.\textsuperscript{51} Thus, PEG-SOD offers a degree of protection against the damage induced by the oxygen free radicals generated by neutrophils, which are marginalized on the endothelial cell surface.\textsuperscript{14–17} The parenteral administration of polyethylene glycol–conjugated enzymes may augment cellular antioxidant enzyme activities in vivo by accumulation in vascular-associated cells.\textsuperscript{29,45} The pharmacokinetics of cell uptake and turnover of polyethylene glycol–conjugated enzymes may be more complicated than reflected by the determination of plasma activities and clearance of the enzyme.

The ability of superoxide dismutase to provide protection against superoxide anion–induced tissue injury and to modulate the inflammatory reaction in response to tissue injury suggests that the conjugated form of the enzyme is of greater protective benefit by virtue of its longer plasma half-life. The covalent attachment of monomethoxypolyethylene glycol to superoxide dismutase markedly increases the blood-circulating life of the enzyme.\textsuperscript{44–46} The
unmodified superoxide dismutase has a half-life of 6 minutes and is cleared from the circulation within 1 hour. By contrast, PEG-SOD given intravenously to the rat resulted in an increase in superoxide dismutase enzymatic activity that was detectable in the circulation for 8 days. Our own data in the dog (unpublished observations) show the presence of a statistically significant increase in plasma superoxide dismutase enzymatic activity for up to 7 days after the administration of the conjugated enzyme in a dose of 1,000 units/kg.

Regardless of the cellular site for their generation (neutrophil, endothelial cell, or other cellular loci), the role of reactive species of oxygen, and especially that of superoxide anion, in mediating cellular damage is now recognized. The experimental data make a strong case that similar mechanisms occur in clinical situations wherein myocardial tissue becomes ischemic and subsequently undergoes reperfusion. Such a situation exists in the patient in whom an acute myocardial infarction is evolving and who is selected for thrombolytic therapy or coronary angioplasty, or both. The literature has not always agreed with the concept that reperfusion of the ischemic myocardium may result in an extension of injury beyond that which has resulted from the ischemic insult itself. The data and discussion presented in this report suggest that the inconsistent observations among laboratories are related to the important differences in the models employed with respect to the instrumentation of the heart, the duration of occlusion, and the duration of reperfusion. It is our hope that this report will provide a better insight into some of the methodological problems associated with different experimental designs. Whether myocardial reperfusion injury is of major clinical importance remains to be determined. In the meantime, a better understanding of the biological mechanisms involved in the progression of events leading to cell injury and death and the potential involvement of oxygen radicals in this process can only be of benefit to all interested in the pathophysiology of myocardial ischemic injury.

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**KEY WORDS** • regional myocardial blood flow • infarct size • myocardial ischemia • myocardial reperfusion injury • oxygen radicals

*Tamara et al* Conjugated Superoxide Dismutase on Myocardial Infarct Size 959

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