Failure of superoxide dismutase to limit size of myocardial infarction after 40 minutes of ischemia and 4 days of reperfusion in dogs

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ABSTRACT Reactive oxygen species such as the superoxide anion (·O$_2$-) have recently been implicated as important agents involved in causing cell death in the setting of myocardial ischemia and reperfusion. When superoxide anion is involved in ischemic injury the administration of superoxide dismutase (SOD) may limit infarct size by reducing the level of superoxide anions in the myocardium. The study described herein was done to determine whether SOD could limit myocardial infarct size when infarcts were produced in dogs by a 40 min occlusion of the circumflex coronary artery followed by 4 days of reperfusion. The animals in the SOD treatment group received a 1 hr intra-atrial infusion of SOD, at a rate of 250 U/kg/min starting 15 min after occlusion and ending 35 min after reperfusion; control dogs received a saline infusion over the same time frame. Infarct size was determined histologically and expressed as a percentage of the anatomic area at risk (AAR). Infarct size was similar in the two groups, averaging 26.2 ± 2.5% in the control group (n = 10) and 21.1 ± 4.8% in the SOD group (n = 11) (p = .40). Hemodynamic variables were not statistically different in the two groups during the occlusion. The transmural mean collateral blood flow at 10 min into the 40 min occlusion was 0.13 ± 0.02 ml/min/g in the controls and 0.17 ± 0.03 ml/min/g in the SOD group (p = NS); moreover, SOD did not alter collateral blood flow. In control dogs, infarct size was inversely related to collateral blood flow; analysis of covariance showed that SOD did not shift this relationship. Thus, SOD did not limit infarct size in this study. The results of the current study are consistent with our previous study in which allopurinol, a xanthine oxidase inhibitor, did not limit infarct size in this same experimental preparation. The results suggest that superoxide anions that are accessible to the infused SOD are not a major cause of myocyte death caused by 40 min of severe ischemia followed by reperfusion. Circulation 75, No. 6, 1237–1248, 1987.

REACTIVE OXYGEN SPECIES, including the superoxide anion (·O$_2$-), the hydroxyl radical (·OH), and hydrogen peroxide (H$_2$O$_2$) have been implicated as agents of cellular damage in several disease processes, including ischemia and reperfusion of the heart. Superoxide anion is produced by xanthine oxidase reaction (where hypoxanthine is oxidized to xanthine and uric acid concomitant with formation of ·O$_2$-, (3) "leaky" mitochondrial respiration, (4) enzymatic steps in arachidonic acid metabolism, and (5) autooxidation of catecholamines. Some oxygen-centered free radicals may be produced during ischemia from oxygen delivered via collateral blood flow. However, it is thought that most free radicals are likely to be produced upon reperfusion, when reactive hyperemia provides abundant supplies of oxygen to myocardium that has been "primed" by ischemia. In addition, the concentration of antioxidants, such as reduced glutathione and superoxide dismutase (SOD), may be reduced by ischemia.

The concept of a burst of free radical production upon reperfusion has led to the hypothesis that some myocytes that are still viable after an episode of ischemia may be killed by free radical-mediated dam-
age during reperfusion, so-called reperfusion injury. The concept of reperfusion injury has been given some credence by a number of studies that have reported fewer arrhythmias, decreased creatine kinase release, and smaller infarct sizes with the administration of allopurinol, a xanthine oxidase inhibitor. Similarly, administration of the free-radical scavenger SOD during and after a period of myocardial ischemia has been reported to limit infarct size and improve recovery of contractile function, ultrastructure, and subcellular function. However, we reported recently that allopurinol did not alter the size of myocardial infarcts produced by 40 min of severe ischemia followed by reperfusion. A 40 min episode of ischemia followed by reperfusion causes death of the most severely ischemic subendocardial myocytes, but results in salvage of the more moderately ischemic subepicardial region. The current study was designed to evaluate the role played by extracellular superoxide anions in causing the death of subendocardial myocytes during the early reperfusion period after a 40 min episode of ischemia. Infarct size in dogs treated with SOD, a superoxide anion scavenger, and dogs receiving saline was compared. We report here that SOD failed to limit infarct size in our 40 min ischemia/reperfusion preparation.

Materials and methods

In general, animal selection, surgical preparation, and end point analyses were conducted according to the criteria described in the multicenter AMPIM study.

Animal selection and exclusion criteria. Twenty-six adult mongrel dogs of either sex with body weight ranging from 10 to 25 kg were accepted into the study. Dogs were required to have a hematocrit of 35 or more to be accepted for study; beagles and pregnant dogs were excluded. Dogs were also rejected from the study if they had clinically evident infections or circulating heart worm filariae. None of the animals that were entered into the study were later found to have heart worms.

Experimental design. The experimental protocol is summarized in figure 1. Infarcts were produced by 40 min of coronary occlusion followed by reperfusion, and infarct size was determined after four days of reperfusion. Myocardial blood flow was measured with microspheres before occlusion and at 10 and 30 min after occlusion. Animals were randomly assigned to one of two experimental groups: (1) the treatment group (n = 14), in which dogs received SOD during occlusion and reperfusion or (2) the control group (n = 12), in which dogs received saline in the same time frame. The control group was enlarged by nine additional dogs that were studied by a similar protocol as part of a concurrent study. The only difference in protocol between these two control groups was that collateral blood flow measurements in the nine additional control dogs were made at 20 min after occlusion; this 20 min flow was assumed to be equivalent to a 10 min postocclusion flow measurement in the current protocol because there were no statistically significant differences in collateral blood flow at 10 vs 30 min after occlusion (see results). There were no statistically significant differences between the two control groups when hemodynamic variables, infarct size, area at risk, and collateral blood flow were compared.

Animals in the treatment group received bovine erythrocyte SOD (superoxide:superoxide oxidoreductase; EC 1.15.1.1, Sigma Chemical Corp.; activity approximately = 2900 units/ mg) at a dosage of 15,000 units/kg body weight. The appropriate amount of enzyme for each animal was mixed in 90 ml of normal saline and was administered through a left atrial line with a Sigmamotor model TM 20.4 infusion set. The infusion lasted for a 1 hr period beginning 15 min into the 40 min occlusion and ending 35 min after reperfusion. The control animals received an intra-atrial infusion of normal saline (90 ml) over the same time frame.

Surgical preparation. Dogs were fasted overnight before the study and then were anesthetized with 30 to 40 mg/kg of intravenous sodium pentobarbital. Additional anesthesia was administered during the experiment as needed. The dogs were intubated and ventilated at 200 ml/kg/min of room air supplemented with a low flow of oxygen. Lead II of the standard electrocardiogram was monitored continuously to verify the presence of ischemia after coronary occlusion, to detect arrhythmias during ischemia or reperfusion, and to measure heart rate at selected times. Aseptic surgical technique was used. includ-

**FIGURE 1.** Experimental protocol. The animals underwent a 40 min occlusion of the left circumflex coronary artery. Blood flow was measured directly before the coronary occlusion, 10 min after occlusion, and again at 30 min after occlusion. The infusion of SOD was started at 15 min after occlusion after baseline ischemic collateral flow had been measured. The infusion continued through the 40 min occlusion and was terminated after 35 min of reperfusion. The control animals received a saline infusion over the same time frame. The animals were then permitted to live for 4 days after which time they were reanesthetized and killed.
ing the use of sterile towels, instruments, and gloves; each dog was given 750,000 units of intramuscular penicillin before the initiation of surgery. A femoral cutdown was performed and the femoral artery and vein were cannulated. Arterial blood gases were checked and the ventilation rate or oxygen flow rate was adjusted, if necessary, to achieve a pO₂ of 80 to 140 mm Hg, a PCO₂ of 32 to 40 mm Hg., and a pH of 7.37 to 7.47. A 4 to 5 cm left thoracotomy was performed in the fourth intercostal space and the heart was suspended in a temporary pericardial cradle. The circumflex artery was isolated beneath the left atrial appendage and a No. 0 silk suture was passed around it for later temporary occlusion, which was accomplished by snaring the artery into a small plastic tube. The occlusion site was always distal to the atrial branch but proximal to the first large marginal branch of the artery. Three catheters were then passed into the left atrium for measurement of atrial pressure, injection of microspheres, and infusion of SOD or saline. Left atrial pressure, the electrocardiogram, and peripheral blood pressure were monitored on a Gould Brush 2400 recorder throughout the experiment. All dogs were allowed 15 min after completion of the surgical preparation to reach steady state before the coronary artery was occluded.

After the initial surgical preparation but before coronary occlusion, animals were randomly assigned to either the SOD treatment or the saline control group. Any animal that developed ventricular fibrillation during coronary occlusion or upon reperfusion was cardioverted, if possible, with an MRL Model 560 defibrillator with internal paddles. At the end of the experimental protocol, all catheters were removed, incisions were closed, air was evacuated from the chest, and the dogs were allowed to survive for 4 days to delineate the necrotic muscle. All dogs were then reanesthetized, given 5000 units of heparin to aid postmortem coronary perfusion, and had their hearts removed for postmortem analysis.

**Regional distribution of blood flow.** The regional distribution of myocardial blood flow was assessed with 10 ± 1 μm radioactive microspheres at three times: before occlusion, 10 min into the occlusion, and 30 min into the occlusion. The spheres were obtained from stock solutions containing 10% dextran and 0.05% Tween 80. Two or three million spheres labeled with gadolinium-153, scandium-46, or tin-113 were injected via the left atrial catheter and this was followed by a 15 ml saline flush. A reference sample of arterial blood was collected from the femoral artery at 7.75 ml/min for 2.5 min beginning just before injection of spheres.

**Postmortem studies**

**Area at risk.** To define the anatomic boundaries of the previously ischemic and nonischemic vascular beds, catheters were placed in the left main artery and in the circumflex artery at the site of previous occlusion; both vessels were perfused simultaneously with dye solutions at 120 to 140 mm Hg pressure. The perfusion fluid was sodium phosphate buffer (8.8 × 10⁻² M dibasic and 0.18 × 10⁻² M monobasic sodium phosphate, pH 8.25 to 8.6) plus approximately 8% dextran (81,500 mol wt; Sigma Chemical) to which either 1.0% triphenyl tetrazolium chloride (TTC, for the previously occluded circumflex artery) or 1.5% Super Imporse Blue B (Ciba-Geigy, left main artery) was added. The heart was then fixed by immersion in a large volume of phosphate-buffered formalin. After fixation for at least 2 days, the left ventricle was sectioned into eight transverse slices (figure 2) that were weighed; after weighing, the apical surface of each slice was photographed. The previously

![FIGURE 2](http://circ.ahajournals.org/)

**FIGURE 2.** The postmortem sampling technique for calculating area at risk, histologic infarct size, and regional blood flow. The formalin-fixed left ventricle was isolated and sliced first into four and then eight slices with a commercial meat slicer. All eight slices were then used to estimate area at risk. Histologic sections were obtained from the apical surfaces of slices 1a, 2a, 3a, 4a, and 4b for histologic infarct sizing and were assumed to be representative of composite slices 1 to 4, respectively. The remaining myocardium of composite slices 1 through 3 was further subdivided for blood flow analysis. Nonischemic and central ischemic regions were divided into inner, middle, and outer thirds. Lateral and septal border zone regions were excluded from flow analysis to avoid measurements from samples containing admixtures of ischemic and nonischemic tissue. (Reprinted from Reimer et al. 25)
occluded vascular bed (area at risk) was identified and traced from enlarged projections of the photographic slide of each ventricular slice. The area at risk was measured by “cut and weight” techniques with the use of copies of these tracings.29

Histologic analysis. The method of sampling for histologic and blood flow analysis has been described previously27 and is shown in figure 2. The apical surfaces of slices 1a, 2a, 3a, 4a, and 4b were used for histologic analysis. Two sections from each tissue sample were cut. One was stained with hematoxylin and eosin and the other with Heidenhain’s variant of Mallory’s connective tissue stain. Infarct size was determined as a percentage of each of four composite slices of the left ventricle and as a percentage of the entire left ventricle. Cut and weight techniques of infarct size analysis were used, based on copies of tracings of the necrotic and viable areas that were made from projections of the histologic sections. This postmortem analysis of necrosis was done by investigators who were “blinded” to the treatment previously given.

Regional blood flow. Flow measurements were made in the remaining portions of the first three composite slices (figure 2). The slices were divided into nons ischemic and central ischemic zones (central zone = 50% to 60% of the vascular bed at risk). The septal and lateral border zones were excluded from analysis to avoid misinterpretation of measurements from samples that might have included nons ischemic as well as ischemic myocardium. The nons ischemic and central ischemic regions were further subdivided into subendocardial, midmyocardial, and subepicardial thirds. The calculation of regional myocardial blood flow in each sample was performed with the formula:

\[ Q_m = \frac{(Q_r \times C_m)}{C_r} \]

where \( Q_m \) = myocardial blood flow (ml/min); \( Q_r \) = reference blood flow (ml/min); \( C_m \) = counts/min in each myocardial sample; \( C_r \) = counts/min in the reference sample. Myocardial blood flow was expressed relative to sample weight (ml/min/g).

 Corrections were made for apparent microsphere loss 30 if the preocclusion ischemic region/nonischemic flow ratio was less than 0.9. Values for infarct size and area at risk also were corrected when the preocclusion ischemic region/nonischemic flow ratio was less than 0.9, based on the assumption that apparent microsphere loss is a reflection of edema, inflammation, or hemorrhage causing an artifactually large infarct.30

Group comparisons of infarct size were done both with data from all surviving dogs and excluding those from four animals (three control, one SOD-treated dog; see table 1) that had subendocardial collateral blood flow greater than 0.15 ml/min/g; the latter was done to provide information on subgroups having uniformly severe subendocardial ischemia. Illustrations and group means presented in the text are based on the results from groups after exclusion of dogs with high collateral flow; however, data from all animals were included when comparing infarct size vs collateral blood flow. The effect of SOD on size of myocardial infarction was unaltered by the addition or exclusion of the four dogs with high collateral blood flow.

Data analysis. Data are expressed as the group mean ± SEM. Intergroup comparisons were made with Student’s two-tailed t test; the corresponding paired t test was used for intra-group analysis to determine the effects of SOD on intraocclusion hemodynamics and collateral blood flow. Area at risk and collateral blood flow have been recognized as important determinants of myocardial infarct size.25, 29, 31-33 Thus, infarct size is expressed as a percentage of the area at risk as well as a percentage of the left ventricle. To take into consideration the effects of collateral blood flow, infarct size was assessed versus collateral flow measured in the inner two-thirds of the central ischemic zone.28, 29 Furthermore, a simple analysis of covariance was performed with collateral blood flow as the independent variable. This analysis was performed to see if there were any statistically significant differences in infarct size in the two groups when the variability due to collateral blood flow was “removed.” A p value ≤ .05 was considered indicative of a statistically significant difference.

Results

Of the 35 dogs initially included in this study (26 randomized plus nine nonrandomized controls), three were excluded. One animal was eliminated from the treatment group due to technical difficulties with surgery and one animal was eliminated from each group due to failure to develop myocardial ischemia after coronary artery occlusion. The incidence and time of ventricular fibrillation and survival rate is summarized in table 1. Three dogs, all controls, developed ventricular fibrillation in the first 15 min of ischemia, before the initiation of placebo therapy. Two of these three dogs were initially resuscitated but neither survived the four day reperfusion period. The incidence of ventricular fibrillation in the first few minutes of reperfusion (reflow VF) was similar in the two groups. Five control dogs that survived the ischemia period without ventricular fibrillation developed reflow VF. Two were successfully resuscitated and three died. Three SOD-treated dogs also developed reflow VF. All three were successfully resuscitated. One additional control dog died approximately 36 hr after coronary occlusion. Thus, overall survival was 65% in the control group and 100% in the SOD-treated group.

It is of interest that the seven control dogs that died each had transmural mean collateral blood flows of less than 0.10 ml/min/g (data not tabulated); three control dogs and three SOD-treated dogs survived despite ischemia of this severity (transmural collateral blood flow <0.10 ml/min/g). Thus, the control group initially contained, by chance, a disproportionate number of dogs with very low collateral blood flow. The uncontrolled exclusion, due to death, of seven of these dogs from the control group served to produce two groups of surviving dogs with similar mean collateral flow values. Given that three of the seven dogs that died developed ventricular fibrillation before the onset of treatment, and that all of the dogs that died had very severe ischemia, it is very likely that the apparent

### TABLE 1

| Ventricular fibrillation (VF), death, and survival in control and SOD-treated dogs |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Group           | n   | Occlusion VF | Reflow VF | Late death | Survival        |
| Control         | 20  | 3             | 5 (29%)    | 1            | 13 (65%)        |
| SOD             | 12  | 0             | 3 (25%)    | 0            | 12 (100%)       |

Source: CIRCULATION
differential survival between the two experimental groups was unrelated to the presence or absence of SOD.

Myocardial blood flow to the circumflex region in surviving dogs is shown in table 2 and figure 3. Preocclusion flows were not significantly different between the two groups. Both groups showed a markedly reduced transmural mean flow at 10 min after occlusion: 0.13 ± .02 ml/min/g in control and 0.17 ± .03 ml/min/g in SOD-treated animals (p = NS). The collateral flow at 30 min after occlusion was similarly low at 0.18 ± .03 in SOD-treated animals (p = NS). The severity of ischemia to the inner two-thirds of the central ischemic zone (the region in which infarction occurs in this preparation) was also similar in the two groups both before and after treatment (figure 3). Thus, the experimental groups were comparable with regard to a major baseline predictor of infarct size (flow). In addition, SOD treatment was not associated with a change in collateral flow to the ischemic region.

Infarct size in each of the 25 surviving animals is listed in table 2 and these results are summarized in figure 4. Analysis of infarct size as a percentage of the left circumflex coronary (LCC) bed or the left ventricle indicated no statistically significant differences between the control and SOD groups: 26.2 ± 2.5% of the LCC bed in control dogs vs 21.1 ± 4.8% in the SOD group (p = .40). The amount of myocardium at risk, i.e., the LCC bed, also was virtually identical in the two groups. Thus, by direct group comparison no limitation of infarct size by SOD could be detected. However, direct group comparison of infarct size with the Student t test does not control for the influence of collateral blood flow, a major determinant of infarct size in control dogs. 28, 29, 31 We believe that it is essential, when evaluating the effect of an intervention on infarct size, to analyze the relationship between collateral flow and infarct size in each group. To this end, an analysis of covariance was performed with the use of collateral flow as the independent variable and infarct size as the dependent variable. The F value obtained was 1.04 (p = NS). Thus, SOD did not limit infarct size, even when the variation due to collateral flow was controlled.

In figure 5 the relationship between infarct size and collateral blood flow is illustrated. The 10 min collateral blood flow measurement was used in the illustration because it was made before the start of the SOD infusion and provides a pretherapy baseline. This illustration does show an inverse relationship between infarct size and collateral blood flow in the control group; that is, animals with low collateral flow had large infarcts and vice versa. It is noteworthy that the SOD-treated animals demonstrated more variability in this relationship than the control dogs. However, the relationship defined by the control group was not shifted downward, as would be expected if SOD had limited infarct size.

The hemodynamic data for the two experimental groups are summarized in figure 6. There were no statistically significant differences in intracoronary hemodynamics in the two groups. However, the rate-pressure product at 30 min into the occlusion in the SOD group was lower than the value at 10 min in this same group (p<.05), and the rate-pressure product in the SOD after reperfusion was significantly lower than that in the control dogs (p<.05). The statistically significant decline in the rate-pressure product reflects the cumulative effect of slight but nonsignificant decreases in both the heart rate and systolic blood pressure in the SOD group. Whether these changes are an effect of treatment with SOD is unknown; there were, however, no such changes in the control group.

Discussion

Summary and critique of present study. The design of our study has taken into account the baseline variables known to influence infarct size. In the dog, these variables include the size of the ischemic vascular bed (the area at risk), collateral blood flow to the ischemic region, and indexes of myocardial oxygen demand as estimated by the rate-pressure product. 28, 29, 32 Infarct size has been expressed as a percentage of the anatomic area at risk, thereby normalizing for variation in vascular anatomy or occlusion site. We have accounted for the effect of collateral blood flow by measuring it directly and have performed group comparisons including and excluding data from animals with high flow. Furthermore, infarct size was plotted against collateral flow to assess whether SOD altered the relationship between necrosis and flow. Hemodynamic variables were not significantly different in the groups. Results of direct group comparison and regression of infarct size vs flow indicated that SOD had no effect on infarct size. Thus, this study provides evidence against the existence of "reperfusion injury" caused by extracellular superoxide anions after a 40 min episode of ischemia in the subendocardial zone.

The failure of SOD to limit infarct size in our study indicates one of three possibilities: (1) that superoxide anions are not important agents in causing cell death in the severely ischemic subendocardial region, (2) that the -O_2^- that causes injury is not accessible to intravascularly administered SOD, or (3) that SOD alone pro-
### TABLE 2

Area at risk, infarct size, regional blood flow, and hemodynamic variables in control and SOD-treated animals

<table>
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<tr>
<th>Group</th>
<th>LCC bed (% of LV)</th>
<th>% of LCC bed of LV</th>
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<th>10 min (% of control)</th>
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Collateral blood flow was measured at 10 and 30 min into the 40 min occlusion; LV = left ventricle; I = inner (subendocardial) third; M = middle third; O = outer (subepicardial) third; Transmural mean = weighted mean transmural collateral blood flow; HR = heart rate (beats/min); SBP/DBP = systolic blood pressure/diastolic blood pressure (mm Hg); RPP = rate-pressure product (heart rate × SBP). Mean ± SEM for each group including all data is presented and a value is also given after exclusion of data from dogs without severe subendocardial ischemia (absolute I flow ≥ 0.15 ml/min/g). p values refer to nonpaired t test analyses of SOD vs corresponding control group means. Mean values reported in the text are those calculated without data from these high-flow dogs. However, no statistically significant differences between groups were detected whether data from high-flow dogs were retained or excluded.

*Data presented as RPP/1000.

*Thirty minute blood flow not measured in this dog.
TABLE 2
(Continued)

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<td>24.61</td>
</tr>
<tr>
<td>±5</td>
<td>±6/6</td>
<td>±1.51</td>
<td>±5</td>
<td>±6/5</td>
<td>±1.40</td>
</tr>
</tbody>
</table>

174 153/112 26.6 ±6  ±6/7 ±1.5 ±5 ±6/7 ±1.41

192 124/106 23.81 166 120/98 19.92
170 152/112 25.84 166 148/107 24.57
154 100/70 15.40 152 100/70 15.20
166 160/119 26.56 166 165/120 27.39
154 175/151 26.95 146 165/153 24.09
176 175/135 30.80 170 165/127 28.05
200 150/110 30.00 194 142/115 27.55
114 70/45 7.98 125 85/58 10.63
190 141/110 26.79 184 130/104 23.92
126 155/115 19.53 126 145/100 18.27
166 163/118 27.06 160 160/115 25.60
134 120/90 16.08 128 115/93 14.72
162 140/107 23.07 157 137/105 21.66
±8  ±9/8 ±2.00  ±6 ±8/7 ±1.67
NS NS NS NS NS NS
165 139/106 23.4 160 136/106 22.00
±8  ±10/9 ±2.10  ±6 ±9/8 ±1.80
NS NS NS NS NS NS

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FIGURE 3. Blood flow. Preocclusion and collateral blood flow measured at 10 min and 30 min into the 40 min occlusion were not significantly different between the two groups. Furthermore, there was little change in the collateral blood flow within each group from 10 to 30 min of occlusion. The bars represent group mean values ± SEM.

FIGURE 4. Area at risk and infarct size. Infarct size in the two groups was not statistically different when considered either as a percentage of the area at risk (AAR) or as a percentage of the left ventricle (LV). The AAR in the two groups was also comparable. The bars represent group mean values ± SEM.
TABLE 3
Infarct sizing studies with SOD

<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment regimen</th>
<th>Duration of ischemia</th>
<th>Duration of reperfusion</th>
<th>Infarct sizing</th>
<th>AAR</th>
<th>CBF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jolly et al.</td>
<td>SOD/CAT infused 15 min before reflow</td>
<td>90 min</td>
<td>1 day</td>
<td>TTC-negative tissue dissected and weighed (gravimetry)</td>
<td>Dye infusion</td>
<td>None</td>
</tr>
<tr>
<td>Werns et al.</td>
<td>SOD infused 15 min prior to occlusion</td>
<td>90 min</td>
<td>6 hr</td>
<td>TTC gravimetry and planimetry</td>
<td>Dye infusion</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>CAT 15 min prior to occlusion</td>
<td>90 min</td>
<td>6 hr</td>
<td>TTC gravimetry and planimetry</td>
<td>Dye infusion</td>
<td>None</td>
</tr>
<tr>
<td>Chambers et al.</td>
<td>SOD boluses at occl and at reflow</td>
<td>60 min</td>
<td>4 hr</td>
<td>TTC planimetry</td>
<td>Microsphere autoradiography</td>
<td>^</td>
</tr>
<tr>
<td>Ambrosio et al.</td>
<td>Recombinant human SOD bolus at reflow followed by infusion</td>
<td>90 min</td>
<td>2 days</td>
<td>Gross inspection</td>
<td>Barium angiography</td>
<td>Yes</td>
</tr>
<tr>
<td>Gallagher et al.</td>
<td>SOD infused 15 min before reflow</td>
<td>3 hr</td>
<td>1 day</td>
<td>TTC planimetry</td>
<td>Dye infusion</td>
<td>Yes</td>
</tr>
<tr>
<td>Present study</td>
<td>SOD infused 25 min before reflow</td>
<td>40 min</td>
<td>4 days</td>
<td>Histologic projections traced</td>
<td>Dye infusion</td>
<td>Yes</td>
</tr>
</tbody>
</table>

CAT = catalase; AAR = area at risk; CBF = collateral blood flow.

*aMicrosphere blood flow measured only in infarcted and noninfarcted tissue rather than by anatomic distribution.

Either the inaccessibility of SOD to \( \cdot O_2^- \) or the need for adjunctive therapy such as catalase could explain the lack of effect of SOD in the present study. However, neither scenario can explain the discrepancy between our results and other reports of the efficacy of SOD administered intravascularly in aqueous solution without addition of catalase (see below).

**Comparison with previous studies.** Many recent studies have dealt with the role of free radicals in causing cell death in the setting of myocardial ischemia and reperfusion. Both positive and negative results have been obtained. For example, Romson et al.\(^{12}\) reported that neutrophil depletion in dogs resulted in significantly smaller infarcts after 90 min of ischemia and 6 hr of reperfusion. This effect was attributed to a reduced accumulation of activated neutrophils in the ischemic/reperfused area. Mitsos et al.\(^{20}\) reported that the protective effects of neutrophil depletion could be enhanced by administration of the free-radical scavenger mercaptopropionyl glycine, a sulphydryl-containing compound, to neutrophil-depleted animals. They interpreted this to mean that both extra- and intramyocardial sources of free radicals contribute to cell death.

Production of free radicals via the xanthine oxidase reaction also has generated much interest recently. Chambers et al.\(^{11}\) reported a significant limitation of infarct size after 1 hr of ischemia and 4 hr of reperfusion in dogs that were treated with allopurinol, a xanthine oxidase inhibitor. In this same study, they reported a similar limitation of infarct size by treating dogs with SOD. Since SOD should scavenge accessible superoxide ions produced from all sources, including...
xanthine oxidase, neutrophils, etc., they expected that treatment with this enzyme would provide a greater limitation of infarct size than allopurinol alone if sources of free radicals other than the xanthine oxidase pathway are important. Since the extent of infarct size limitation was the same in the SOD- and allopurinol-treated animals, the authors concluded that xanthine oxidase is likely to be the major source of free radical-mediated injury. Werns et al.\textsuperscript{16} also reported that allopurinol limited infarct size after 90 min of ischemia and 6 hr of reperfusion.

However, we recently reported the lack of infarct size limitation with allopurinol in the same ischemia/reperfusion preparation of myocardial infarction used for the present study.\textsuperscript{22} If the xanthine oxidase pathway is indeed an important source of cytotoxic free radicals, then we would have expected to observe infarct size limitation in our preparation since there are significant quantities of hypoxanthine and xanthine in the subendocardial region after 40 min of ischemia.\textsuperscript{35} Thus, our previous results suggested that the free radicals produced via the xanthine oxidase pathway did not contribute significantly to subendocardial cell death.

Other studies have been done to assess whether the administration of SOD before or at the time of reperfusion could limit infarct size. The results of these studies are listed in table 3, along with salient methodologic features of each to facilitate comparison. Again, positive and negative results have been obtained. For example, Jolly et al.\textsuperscript{20} reported limitation of infarct size after 90 min occlusion followed by 6 hr of reperfusion by the intra-atrial infusion of SOD plus catalase. Similar protection was reported even if the infusion was not begun until 15 min before reperfusion. In a later publication from this same laboratory, Werns et

### Table 3 (Continued)

<table>
<thead>
<tr>
<th>Infarct size (% AAR)</th>
<th>Control</th>
<th>Treated</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>44 ± 4</td>
<td>22 ± 3</td>
<td>Reactive hyperemia on reflow prevented by critical stenosis</td>
<td></td>
</tr>
<tr>
<td>40 ± 3</td>
<td>19 ± 5</td>
<td>Critical stenosis</td>
<td></td>
</tr>
<tr>
<td>40 ± 3</td>
<td>30 ± 5</td>
<td>Critical stenosis</td>
<td></td>
</tr>
<tr>
<td>23 ± 4</td>
<td>5 ± 1</td>
<td>Renal vessels ligated to increase half life of SOD</td>
<td></td>
</tr>
<tr>
<td>52 ± 7</td>
<td>33 ± 2</td>
<td>Infarcts in treated group very patchy; regression of infarct size vs CBF showed SOD limited infarct size</td>
<td></td>
</tr>
<tr>
<td>32 ± 16</td>
<td>38 ± 17</td>
<td>Chronic study — closed chest, conscious dogs used; regression of infarct size vs CBF showed no effect of SOD</td>
<td></td>
</tr>
<tr>
<td>26 ± 3</td>
<td>21 ± 5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\*p<.05 when compared with corresponding value in the control group. \* p<.05 when compared with the 10 min value in the same group.

**FIGURE 6.** Hemodynamics. Intraocclusion hemodynamic parameters between the two groups were not statistically different. However, the rate-pressure product (RPP) after reperfusion was lower in the SOD-treated animals than in the control dogs (p<.05). SBP = systolic blood pressure; DBP = diastolic blood pressure; P = preocclusion; O = occlusion; R = reperfusion.

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reported that SOD alone could limit infarct size, while catalase alone had no effect. It should be noted that in these studies reactive hyperemia upon reperfusion was limited by a critical coronary stenosis, infarct size was determined by TTC staining, and collateral blood flow was not measured. As mentioned above, Chambers et al. have similarly reported that the administration of SOD as an intravenous bolus upon occlusion and again upon reperfusion limited infarct size. Collateral blood flow, measured in two samples from the center of each infarct, was comparable between the groups. Infarct sizing was again based on TTC staining. Finally, Ambrosio et al. recently reported limitation of infarct size by the administration of SOD upon reperfusion in a canine preparation involving 90 min of left circumflex coronary occlusion followed by 48 hr of reperfusion. In this study, collateral blood flow was measured and an analysis of infarct size vs collateral flow indicated that SOD limited infarct size, predominantly in animals with lower collateral flow. However, infarcts were sized by gross pathologic inspection rather than by gross histochemical or histologic methods.

In contrast, Gallagher et al. studied infarct size in conscious dogs given SOD or catalase for 1 hr beginning 15 min before reperfusion. Their preparation consisted of a 3 hr coronary occlusion followed by 24 hr of reperfusion. Collateral blood flow was measured and infarct size was based on TTC staining. A linear regression of infarct size vs collateral blood flow revealed that SOD failed to alter the control relationship between flow and necrosis, and hence failed to limit infarct size.

The reasons for the lack of infarct size limitation in the current study, when many other studies have reported dramatically positive results, are not clear. It should be noted that the SOD dosage and infusion protocol in the current study were comparable to those used in other studies in which investigators have demonstrated a beneficial effect with SOD. Moreover, Gallagher et al. have shown that bovine SOD does remain enzymatically active in dog plasma. Thus, the reasons for the disparate results must lie in differing technical aspects of the studies or in differing biology of the experimental preparations. Two aspects of our study that we believe to be important are that (1) infarct sizing was conducted on fully developed areas of necrosis and was based on histology and not on TTC staining and (2) collateral blood flow within the area at risk was measured and infarct size was analyzed relative to collateral blood flow. This method is the same as that reported previously in the preparation. Other baseline variables that influence infarct size, such as area at risk and hemodynamic variables, also were evaluated. It is possible, when small groups of dogs are used, to observe false results because of random assignment of dogs with unequal baseline characteristics to treatment and control groups.

One obvious biological variable that could account for the differing results is the duration of occlusion used to induce the infarcts. The studies reporting positive results have used occlusions of 60 or 90 min duration. Those reporting negative results have used occlusion times of 40 min or 3 hr. If the conclusions of all these studies are valid, it indicates that "reperfusion injury" is a rather transient phenomenon, appearing after more than 40 min of ischemia and disappearing after 3 hr of ischemia. This possibility may warrant further investigation. For example, 40 min of severe ischemia produces subendocardial myocyte necrosis that is not associated with marked microvascular injury. Conversely, by 90 min, microvascular injury is sufficiently severe to cause a no-reflow phenomenon in the subendocardial region. It is possible that microvascular injury contributes to the infarct produced by 90 but not by 40 min of ischemia. Administration of SOD in the 90 min preparation might be beneficial either because it limits microvascular damage in this setting or because it has better access to injured myocytes when capillary integrity has been breached. It is of interest in this regard that even in the 90 min preparation, in which SOD therapy has reportedly limited the transmural extent of infarction, SOD has not been observed to prevent necrosis in the severely ischemic subendocardial zone to which infarcts are primarily confined in our 40 min preparation.

Another biologic variable in these studies is the duration of reperfusion. The studies that were done using relatively short reperfusion times (1 to 6 hr) have yielded positive results with SOD treatment, whereas the two negative studies (the present study and that of Gallagher et al.) used reperfusion times of 1 and 4 days. This could suggest the existence of late reperfusion injury, i.e., cell death occurring at between 6 and 24 hr of reperfusion. However, the positive results of Ambrosio et al. who observed protection even after 48 hr of reperfusion, are not consistent with this hypothesis.

The negative results of the current study do not rule out the possibility that the mid and subepicardial myocardium could be salvaged after occlusion periods of longer than 40 min by free radical scavengers given during ischemia. These zones of the myocardium have higher collateral flow and, although still viable and salvageable by reperfusion after 40 min of ischemia,
undergo irreversible injury during the interval between 40 min and 3 hr of occlusion in our experimental preparation. The higher collateral flow in these zones may deliver sufficient O₂ during ischemia to produce substantial quantities of free radicals and contribute to cell injury during ischemia per se. For example, Akizuki et al. have recently reported that xanthine oxidase inhibition with allopurinol can limit infarct size in the setting of permanent coronary occlusion.

In conclusion, the role of free radicals, and especially the superoxide anion, in ischemia/reperfusion injury remains uncertain. The results of the present study are consistent with other previous results, which indicated a lack of infarct size limitation with the administration of allopurinol in this same canine preparation. Our results suggest that superoxide anions accessible to intravascularly administered SOD are not an important cause of death of subendocardial myocytes injured by 40 min of severe ischemia followed by 4 days of reperfusion.

We are grateful for the expert technical assistance provided by Jean A. Wakefield in animal surgery, blood flow measurement, and infarct sizing, and to Betty Goodfellow for preparation of histologic slides.

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