Marked Reduction in Myocardial Infarct Size Due to Prolonged Infusion of an Antioxidant During Reperfusion

Lawrence D. Horwitz, MD; Paul V. Fennessey, PhD; Robert H. Shikes, MD; Yinong Kong, MD

Background  There has been controversy about whether early reperfusion of myocardial infarcts causes further necrosis mediated by reactive oxygen species or other mechanisms. Unequivocal evidence that therapeutic agents given only during reperfusion can prevent, rather than delay or modify, injury has been sparse. Failure to account for variables, such as collateral blood flow, that influence infarct size independently and attempts to measure infarct size too early in reperfusion may have limited the sensitivity and specificity of some previous studies.

Methods and Results  After 90 minutes of coronary occlusion and 48 hours of reperfusion in a canine model, we examined the effect on infarct size of intravenous infusion of N-(2-mercaptopropionyl)-glycine (MPG), a diffusible antioxidant. Infarct size and region at risk were measured by postmortem dual perfusion with triphenyl tetrazolium chloride and Evans blue dyes, and regional myocardial blood flow was measured with radioactive microspheres. Infusion of MPG 100 mg·kg·h⁻¹, beginning either 15 minutes before the onset of reperfusion or 30 minutes after the onset of reperfusion and continued until 4 hours of reperfusion and followed by an intramuscular dose, reduced infarct size, normalized for both region at risk and the level of collateral blood flow, by 60% and 45%, respectively. When infusion of MPG was limited to the last 15 minutes of ischemia and the first hour of reperfusion only, the normalized infarct size was reduced by 26%. Heart rate, blood pressure, and their product did not differ among the four groups studied. The plasma half-time of MPG was <10 minutes. In in vitro experiments MPG was a scavenger of hydrogen peroxide but not of superoxide radical.

Conclusions  After 90 minutes of coronary ligation, infusion of the diffusible hydrogen peroxide scavenger, MPG, for several hours, beginning as late as 30 minutes after the onset of reperfusion, substantially reduced infarct size measured 48 hours later. In this model, necrosis caused by processes during reperfusion may be more extensive than necrosis caused by ischemia alone. Since infusion of this agent for only the first hour of reperfusion was considerably less effective, it appears that most of the oxidant injury leading to necrosis occurred after the first 60 minutes but within the first 4 hours of reperfusion. (Circulation. 1994;89:1792-1801.)

Key Words  • ischemia  • free radicals  • oxygen

I t has been proposed that early reperfusion of acute myocardial infarctions prevents further damage caused by ischemia but may itself cause injury, resulting in suboptimal myocardial salvage.1,2 This process during reoxygenation has been attributed to excessive accumulation of reactive oxygen metabolites in reperfused regions through release from invading leukocytes or other mechanisms.2-5 However, there has been considerable controversy about the importance of "reperfusion injury."

Studies of animal models in which there was apparent salvage resulting from administration of antioxidants have generally used administration of these agents before the ischemic period and evaluation of myocardial damage after reperfusion periods limited to a few hours.2,6 Only a limited number of studies have been done after 24 hours or more of reperfusion and have also been normalized for critical independent determinants of infarct size, including size of the anatomic region at risk and the magnitude of collateral coronary flow.7-14 Most of these failed to demonstrate reductions in infarct size with antioxidant interventions.7,9,10,12,13 An exception was a study in which there was modest benefit from administration of the diffusible antioxidant dimethylthiourea at the onset of reperfusion, with measurements made 48 hours later.14 However, questions remain about the magnitude and importance of reperfusion injury. In addition, little is known about the timing of oxidant reactions during reperfusion and the optimum duration of treatment.

N-(2-mercaptopropionyl)-glycine (MPG) is a low-molecular-weight synthetic analogue of glutathione, an important endogenous antioxidant.15 In a study in cultured cardiac myocytes, we found that MPG was more effective than dimethylthiourea in preventing cytotoxicity caused by hydrogen peroxide and was not toxic in high concentrations.16 Therefore, we decided to test the efficacy of this diffusible antioxidant for limiting infarct size. In a canine model subjected to 90 minutes of regional ischemia, we measured infarct size at 48 hours of reperfusion to ensure that we were detecting myocardial salvage rather than delay or temporary modification of necrosis. Care was taken to normalize for independent variables that influence infarct size, and histology was performed to confirm that there was accurate delineation of necrosis by the tetrazolium dye

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used for this purpose. The dosage and duration of MPG infusions were considerably greater than have been used previously in studies of myocardial infarction. We found that we could substantially reduce infarct size, even with initiation of MPG administration 30 minutes after reperfusion began, provided that the drug was given for several hours. Scavenging of hydrogen peroxide may be an important mechanism of this protective effect.

Methods

Instrumentation

Forty-one male mongrel dogs weighing 24±0.4 kg (mean±SEM) were anesthetized with pentobarbital sodium (30 mg/kg IV). The dogs were intubated and ventilated with a respirator (Harvard Apparatus, South Natick, Mass.) using a mixture of 35% oxygen and 65% nitrogen, which permits maintenance of arterial blood gases in the usual physiological range at sea level despite Denver's altitude. Under sterile technique, a thoracotomy was performed in the left fifth intercostal space. The pericardium was opened. Catheters were inserted into the ascending aorta and left atrial appendage. A 1-0 silk ligature was placed around the left anterior descending coronary artery (LAD) just distal to the origin of the first diagonal branch. The ligature was left loosely in place, and the ends were pulled through a 4-cm length of polyethylene tubing. Arterial blood gases were measured, and ventilation was adjusted to maintain normal physiological levels.

Aortic pressure was recorded with a Statham P23Db transducer (Gould, Cleveland, Ohio). A lead II ECG was recorded with subcutaneous needle electrodes. Aortic pressure and ECG were recorded continuously on an oscillograph (model R612, Beckman Instruments, Inc, Fullerton, Calif). The heart rate–blood pressure product was calculated by multiplying heart rate (in beats per minute) times mean aortic pressure.

Preparation of MPG

Powdered MPG was purchased from Sigma Chemical Co, St Louis, Mo. MPG is highly soluble in aqueous solutions, and 0.5 g/kg dog weight was dissolved in 30 mL of lactated Ringer's solution and 5% dextrose injection, which was removed from a 500-mL sterile infusion bag (purchased from Baxter Healthcare Corp, Deerfield, Ill). Because an aqueous solution of MPG is highly acidic (pH approximately 2.0), the pH was adjusted to 7.3 to 7.4 by addition of sodium hydroxide pellets (approximately 0.275 g NaOH/g MPG). The pellets were purchased from MCB Manufacturing Chemists Inc, a division of E. Merck, Darmstadt, Germany. The neutralized MPG solution was then passed through a 0.2-μm sterile filter to remove bacteria before administration. The filtered, neutralized MPG was reinjected into the 500-mL infusion bag from which the aliquot had originally been obtained. The MPG solution or vehicle was infused at 100 mL/h. For intramuscular doses, 200 mg/kg MPG was dissolved in 15 mL saline, and pH was adjusted to 7.3 to 7.4 with sodium hydroxide.

Experimental Protocol

After a baseline microsphere injection for regional myocardial blood flow determination, LAD occlusion was performed by tightening and clamping of the ligature for 90 minutes. Epicardial cyanosis was apparent in all dogs. Seventy minutes into the occlusion period, a second batch of radioactive microspheres was injected. At the end of the 90-minute occlusion period, the ligature was gradually loosened over approximately 5 minutes and then was removed. One hour later, the left atrial and aortic lines were externalized at the back of the neck through scubutaneous tunnels, and the chest was closed. Appropriate doses of morphine sulfate were given intramuscularly by the investigators or by a supervising veterinarian to relieve discomfort during the first 24 hours after surgery. The dogs were returned to the kennel after spontaneous respirations were resumed and infusions of experimental drugs had been completed. During the next 2 days they were fed a standard diet. The wounds were cleaned and the catheters flushed with heparinized saline each day. Forty-eight hours after reperfusion had begun, the dogs were returned to the laboratory and again anesthetized with pentobarbital sodium through the left atrial catheter. The thoracotomy incision was reopened, heparin (5000 IU IV) was administered, and the heart and proximal aorta were removed.

The protocols are outlined in Fig 1. Four groups of dogs were studied: The control group received vehicle (Ringer's lactate and 5% dextrose injection) intravenously starting at the beginning of surgery and continuing until 4 hours of reperfusion. MPG group 1 was switched from vehicle alone to MPG at a rate of 100 mg·kg\(^{-1}\)·h\(^{-1}\) beginning 15 minutes before the end of the occlusion period and continuing for 4 hours and 15 minutes (ending at 4 hours of reperfusion). An intramuscular dose of 200 mg/kg MPG was given at the end of the intravenous infusion. MPG group 2 was switched from vehicle alone to MPG at a rate of 100 mg·kg\(^{-1}\)·h\(^{-1}\) beginning 30 minutes after the end of the occlusion period and continuing for 3 hours and 30 minutes (ending at 4 hours of reperfusion). An intramuscular dose of 200 mg/kg was given at the end of the intravenous infusion. MPG group 3 was switched from vehicle alone to MPG at a rate of 100 mg·kg\(^{-1}\)·h\(^{-1}\) beginning 15 minutes before the end of the occlusion period and continuing for 1 hour and 15 minutes (ending at 1 hour of reperfusion). Vehicle
alone was given subsequently for 3 additional hours. No intramuscular dose of MPG was given.

Measurement of Infarct Size

The heart and aorta were connected to a dual perfusion apparatus. The aortic root and the LAD immediately distal to the site of coronary occlusion were cannulated. The proximal LAD was ligated at the site of the prior occlusion. This allowed perfusion of the region subserved by the circumflex coronary artery to be perfused with a 0.25% solution of Evans blue dye through the aortic root cannula, and the region subserved by the LAD was perfused through the LAD cannula with a 1% solution of 2,3,5-triphenyl tetrazolium chloride (TTC) at 37°C. Perfusion pressure in both cannulas was equalized at 100 mm Hg.

After 2 minutes, the heart was removed from the perfusion apparatus. The left ventricle was isolated and weighed. Six transverse sections approximately 1 cm thick were obtained by sectioning parallel to the atrioventricular groove. The slices were weighed and traced onto clear acetate sheets to demarcate (1) a remote normal region (blue stain); (2) an ischemic, noninfarcted region (TTC-positive, red stain); and (3) an ischemic, infarcted region (TTC-negative, unstained).

Regional Myocardial Blood Flow

Regional myocardial blood flow was assessed using 15-μm diameter microspheres labelled with 48Sc or 48Sr (Dupont, Wilmington, Del). Approximately 1.5 million microspheres with one of these labels in a 0.1% Tween 80 suspension (in 10% dextran) were diluted to a final volume of 2 mL with 0.9% NaCl. After vigorous agitation, the microspheres were injected into the left atrial catheter and flushed in with 6 mL of 0.9% NaCl. Starting 10 seconds before injection of the microspheres and continuing until 3 minutes after the injection, a reference sample was withdrawn from the aortic catheter at a rate of 4 mL/min. This procedure was done before occlusion and 70 minutes into the occlusion period, using a different isotope each time.

After the hearts were perfused with dyes and sliced, each slice was divided into regions of normal, ischemic but noninfarcted, and infarcted regions. Sections from each zone were sectioned into epicardial, midwall, and endocardial samples. Samples were weighed and counted in a gamma spectrophotometer (model 5000, Packard Instrument Co, Inc, Meriden, Conn) using appropriate energy windows. Background and crossover counts from the other isotope were accounted for and blood flow measurements in mL·100 g-1·min-1 were obtained as described previously. To avoid errors from microsphere leakage or tissue edema, flows to the infarct zone during the ischemic period were multiplied by a correction factor. This factor was calculated as the ratio of the preclosure blood flow in nonischemic myocardial zones to preclosure blood flow in the zone that was subsequently infarcted.

Histopathology

The ability of the TTC stain method to distinguish viable, previously ischemic tissue from infarcted tissue was assessed by histopathology. Sections of myocardium from TTC-positive (red), TTC-negative (unstained), and Evans blue-stained sections were selected from three control group and three MPG group 1 dogs and stored in 10% buffered formalin. The segments were embedded in paraffin, and serial sections were stained with hematoxylin and eosin. The sections were semi-quantitatively assessed for coagulative necrosis, contraction band necrosis, interstitial edema, hemorrhage, and interstitial neutrophil infiltration.

All sections were reviewed in a blinded manner by a cardiac pathologist (R.H.S.). Presence of coagulative necrosis characterized by changes in staining qualities and loss of myocyte subcellular structural detail was scored as 0, none (normal); 1, mild; 2, moderate; and 3, severe. Presence of contraction band necrosis characterized by myocyte contraction bands accompanied by myocytolysis was scored as 0, none (normal); 1, occasional; 2, moderate; and 3, frequent. Intersitial edema was scored as 0, normal; 1, focal and mild loosening of interstitial connective tissue stroma; 2, diffuse and moderate loosening of interstitial connective tissue stroma; and 3, diffuse and severe loosening of interstitial connective tissue stroma. Hemorrhage was scored as 0, absent; 1, focal with mild extravasation of erythrocytes; 2, focal or diffuse but moderate extravasation; and 3, diffuse with severe extravasation. Neutrophil infiltration was scored as 0, absent; 1, present in a few high-power fields; 2, present in approximately half of high-power fields; and 3, present in most high-power fields.

Measurement of Plasma MPG Levels

A technique that combined gas chromatography with mass spectrometry (GC/MS) was used to measure plasma levels of MPG. Plasma samples (0.1 mL) were acidified with 0.1 mL of 1.0N hydrochloric acid. MPG was extracted with ethyl acetate, which was then dried under nitrogen at 60°C. The dried extract was derivatized with 15% N-methyl-N-(tert-butyldimethyl)silyl trifluoroaceticamide (Pierce, Rockford, Ill) for 1 hour at 100°C to produce a di-(tert-butyldimethylsilyl) derivative. The samples were analyzed with an HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, Calif) fitted with a 15M DB1 fused silica column (J&W Columns, Rancho Cordoba, Calif). The initial temperature was 140°C and was increased to 200°C at a rate of 10°C per minute. Injection port and transfer line temperatures were maintained at 250°C. The gas chromatograph was interfaced to an HP 5970 mass selective detector (Hewlett-Packard) operated in the selected ion monitoring mode. Ions monitored were m/z 376, 334, and 274. The ion at m/z 334, which corresponded to loss of one of the t-butyl moieties from the derivatized MPG, was used for quantitative analysis. The other two ions were used for confirmation.

Measurement of Hydrogen Peroxide Scavenging Capacity

Using a modification of the method of Thurman et al, which we have described previously, we measured the capacity of MPG to scavenge H2O2 in a cell-free solution. The method uses the conversion of ferrous to ferric ion by hydrogen peroxide. Ferric ion is detected by addition of thiocyanate. This results in formation of ferric thiocyanate, which is measured by spectrophotometry. MPG concentrations of 10 and 100 μg/mL were tested at a hydrogen peroxide concentration of 0.15 mmol/L.

Measurement of Superoxide Radical Scavenging Capacity

Using the method of McCord and Fridovich, we measured the capacity of MPG to scavenge superoxide radical (O2-) in a cell-free solution. The assay was performed with samples in 2 mL of 0.05 mol/L potassium phosphate buffer (pH 7.8) containing 10-4 mol/L EDTA at a temperature of 25°C. The reaction mixture contained ferricytochrome c (1 × 10-3 mol/L), xanthine (5 × 10-3 mol/L), and sufficient xanthine oxidase to produce a rate of reduction of ferricytochrome c at 550 nm of 0.025 absorbance units per minute. Inhibition of the rate of reduction of ferricytochrome c by 0.0125 units per minute was defined as 1 unit of scavenging activity. MPG was added at concentrations of 0.5, 5.0, 10.0, 326, and 653 μg/mL.

Statistical Analysis

Data are reported as mean±SEM. All analyses of differences among groups were done by one-way ANOVA using the Scheffé test of significance. Intragroup analyses over time were done by two-way ANOVA with the Scheffé test. Comparisons

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of normal and ischemic regions within each animal were done by the paired \( t \) test. Regression coefficients were calculated by the least-squares method. Collateral ischemic blood flow versus infarct size as a percent of risk area (MI/RISK) was assessed by ANCOVA,\(^ {19,20} \) with transmural flow in the ischemic region assigned as the independent variable and MI/RISK as the dependent variable. For all analyses, a value of \( P<.05 \) was considered to be significant. To avoid bias from dogs in which the severity of ischemia was insufficient to ensure likelihood of an infarct of sufficient size so that effects of interventions could be meaningfully analyzed, a transmural regional myocardial blood flow \( \leq 30 \text{mL} \cdot 100 \text{~g}^{-1} \cdot \text{min}^{-1} \) in at least one ischemic segment was preselected as necessary for inclusion of an animal in the statistical analysis.

**Results**

**Study Groups**

Initially it was planned to study only two groups: controls and the one designated MPG group 1. However, after 4 dogs in each group had been studied and the efficacy of the MPG had become apparent, it was decided to add two other MPG groups so that the effectiveness of less lengthy MPG regimens could be compared with MPG group 1. Twenty-nine dogs were included in the final analysis (8 in the control group, 8 in MPG group 1, 6 in MPG group 2, and 7 in MPG group 3). Six dogs were excluded because of ischemic zone endocardial collateral flow \( > 30 \text{mL} \cdot 100 \text{~g}^{-1} \cdot \text{min}^{-1} \) (4 in MPG group 1 and 2 in MPG group 2). Five dogs did not survive the procedure because of fatal ventricular tachyarrhythmias during the ischemic or reperfusion periods (3 in MPG group 1, 1 in MPG group 2, and 1 in MPG group 3). An additional dog in MPG group 1 died because a mechanical respirator failed, leaving the animal unventilated for several minutes. During the reperfusion period, ventricular fibrillation requiring defibrillation once or twice occurred in 7 dogs that were included in the analysis (3 in MPG group 1, 1 in MPG group 2, and 3 in MPG group 3).

**Regional Myocardial Blood Flow**

There were no differences among the groups in transmural or endocardial blood flows in the normal zones. There were also no statistically significant differences among the groups in transmural or endocardial flow in the ischemic regions. Mean endocardial blood flows during occlusion in the ischemic zones were 15.2 ± 2.11 ± 3, 16.2 ± 3, and 7 ± 1 \text{mL} \cdot 100 \text{~g}^{-1} \cdot \text{min}^{-1} \) for control and MPG groups 1, 2, and 3, respectively. Within each group there were substantial and significant \( (P<.01 \) in all cases) reductions in transmural and endocardial flows in the ischemic zones compared with the normal zones in the same dogs. Transmural flows for each group in the normal and ischemic zones are shown in Fig 2.

There were no significant differences among the groups in endocardial-to-epicardial regional flow ratios in the normal or ischemic zones during ischemia, although there was a trend for a lower ratio in MPG group 3. Endocardial-to-epicardial ratios in the ischemic zones were 0.49 ± 0.09, 0.41 ± 0.06, 0.56 ± 0.15, and 0.32 ± 0.06 for control and groups 1, 2, and 3, respectively. Within each group there were substantial and significant \( (P<.01 \) in all cases) reductions in endocardial-to-epicardial ratios in the ischemic zones compared with the normal zones in the same dogs.

**Hemodynamics**

There were no significant differences among the groups in heart rate, mean aortic blood pressure, or the product of heart rate and mean aortic blood pressure during ischemia or reperfusion. The mean values for heart rate–blood pressure product are plotted in Fig 3.

**Myocardial Infarct Size**

There were no differences among the four groups in the ratio of the weight of the risk region to the total left
ventricular weight (RISK/LV) (Fig 4, top). The mean values for RISK/LV were 25±3%, 22±3%, 20±3%, and 19±2% for the control group and MPG groups 1, 2, and 3, respectively.

However, the infarct region as a function of the region at risk (MI/RISK) differed substantially among the groups (Fig 4, bottom). In the control group, MI/RISK was 58±6%, a value similar to the measurements we reported in saline-treated control dogs in a previous study using this technique and timing.\textsuperscript{14} In MPG group 1 (MPG treatment began intravenously 15 minutes before the onset of reperfusion and continued for 4 hours and 15 minutes followed by an intramuscular injection), MI/RISK was 29±6%. The difference between the control group and MPG group 1 was significant at \(P<.01\). In MPG group 2 (MPG begun intravenously at 30 minutes of reperfusion and continued for 3.5 hours followed by an intramuscular injection), MI/RISK was 29±7%. There was a statistically significant difference between the control group and MPG group 2 (\(P<.05\)). In MPG group 3 (MPG begun 15 minutes before the onset of reperfusion and continued for 1 hour and 15 minutes without intramuscular administration of drug), MI/RISK was 52±4%. There was no significant difference between the control group and MPG group 3.

Individual values of MI/RISK plotted against the transmural ischemic zone blood flow during coronary occlusion are shown in Fig 5. Each panel plots the control values and one of the treatment groups. Within each group there was an inverse relation between transmural blood flow in the ischemic zone and myocardial infarct size. A linear fit was approximated by the least-squares method within each group. The negative \(r\) values were .71, .54, .83, and .71 for the control, MPG group 1, MPG group 2, and MPG group 3, respectively. The slopes of the relations were similar among the groups. At comparable flow levels, MI/RISK was consistently less in MPG group 1 and MPG group 2 than in the control group. MI/RISK was also lower at comparable flow levels in MPG group 3 compared with the control group, although the differences were less marked than in the other treated groups.

Mean values for MI/RISK normalized for transmural blood flow by the method of Wright\textsuperscript{19} are shown in Fig 6. This gives a more accurate picture of the influence of interventions on infarct size than Fig 3, since it corrects for both the size of the region at risk and the intensity of the ischemia during occlusion—variables that independently determine infarct size.\textsuperscript{20} Adjusted mean values were 62±4% in the control group and 25±4%, 34±5%, and 46±4% for MPG group 1, MPG group 2,
and MPG group 3, respectively. There were significant differences between the control group and all the treated groups (MPG group 1, P<.001; MPG group 2, P<.001; MPG group 3, P<.01). However, there was also a significant difference (P<.001) between MPG group 1, which received more than 4 hours of treatment beginning before the onset of reperfusion, and MPG group 3, in which treatment began at the same time but ended after the first hour of reperfusion. There was no significant difference between MPG group 1 and MPG group 2, which also received a prolonged infusion, although it was begun 30 minutes after reperfusion began. The magnitude of the reductions in infarct size with the various treatment arms varied. There was a 60% mean reduction in the normalized infarct size in MPG group 1, a 45% reduction in MPG group 2, and a 26% reduction in MPG group 3. There were 4 dogs in MPG group 1 and 2 dogs in MPG group 2 that were not included in the analysis because they failed to meet the predetermined criteria for reduction in regional myocardial blood flow during coronary occlusion. In 1 of these dogs there was no detectable infarct, and in the other 5 dogs the infarcts were small. MI/RISK was 0%, 12%, 13%, and 14% in the 4 dogs excluded from MPG group 1 and 2% and 29% in the 2 dogs excluded from MPG group 2. If these results had been included in the analysis, they would not have altered the statistical comparisons among groups and would have had only a slight effect on the normalized INFARCT/RISK mean values in MPG group 1 and MPG group 2. Therefore, treatment with MPG for 4 hours or more during reperfusion substantially and significantly altered normalized infarct size, whereas treatment for only the first hour of reperfusion had a small but also statistically significant effect.

**Histopathology**

The results of the histopathological scoring in sections obtained at 48 hours of reperfusion are shown in Table 1. Three dogs from the control group and 3 dogs from MPG group 1 were studied. There was no evidence of vascular obstruction from leukocytes or other causes in any dogs. Within the infarct zones all dogs in both groups had substantial necrosis of both the coagulative and contraction type. In all the dogs there was neutrophil infiltration in the infarct zones. The neutrophil infiltration appeared to be more marked in the infarcts in the MPG-treated dogs. There was hemorrhage and edema in the infarct zone in all 3 of the MPG dogs and 1 of the 3 control dogs. There were no abnormalities in any of the remote regions not exposed to ischemia in either group. In one of the MPG-treated dogs there were occasional necrotic cells, moderate neutrophil infiltration, and minimal interstitial hemorrhage and edema in the ischemic-viable (TTC-positive) region, although there were many normal-appearing myocardial cells as well. The infarct region (TTC-negative) in the same dog had more uniform necrosis and more severe neutrophil infiltration, interstitial edema, and hemorrhage. In the other two MPG dogs and all the control dogs, the ischemic-viable zones were entirely normal. Therefore, the TTC staining method appeared to accurately delineate the infarct region in both control and MPG-treated dogs, and MPG did not prevent leukocyte accumulation in the infarct region.

**MPG Blood Levels**

Fig 7 is a semilogarithmic plot of plasma MPG values obtained from multiple aortic blood samples obtained after termination of a 75-minute intravenous infusion of MPG (100 mg·kg⁻¹·h⁻¹) in an MPG group 3 dog. The data are well described by first-order kinetics. The half-time for elimination (t₁/₂) was approximately 7 minutes. The plasma MPG concentration during infusion was approximately 100 μg/mL.

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<th>Zone</th>
<th>Coagulative Necrosis</th>
<th>Contraction Band Necrosis</th>
<th>Interstitial Edema</th>
<th>Hemorrhage</th>
<th>Neutrophil Infiltration</th>
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Values are mean±SEM.
Capacity of MPG to Scavenge Hydrogen Peroxide and Superoxide Radical

MPG, at a concentration of 100 μg/mL, inactivated 48.0% of a 0.15 mmol/L solution of hydrogen peroxide in vitro. At an MPG concentration of 10 μg/mL, inactivation was 31%. Therefore, MPG was an excellent scavenger of hydrogen peroxide at the same drug concentration (100 μg/mL) as measured during the infusions in our protocols in vivo.

However, at a range of doses from 0.5 to 653 μg/mL, MPG had no effect on reduction of ferricytochrome c by superoxide radical. In the absence of MPG the rate of reduction was $4.27 \times 10^{-4}$ absorbance units/s, and at the highest concentration of MPG it was $3.45 \times 10^{-4}$ absorbance units/s. Therefore, MPG is not a scavenger of superoxide radical even at more than a sixfold greater concentration than occurred during our infusions.

Discussion

Because of discrepancies in the results of studies of effects of superoxide dismutase on myocardial infarct size in animal models of ischemia and reperfusion, there has been a reevaluation of the reliability of estimations of this measurement. The high cost and considerable time required to estimate infarct size by histological methods has led to use of tetrazolium dyes, which form colored precipitates in tissue with intact dehydrogenase enzyme systems. The use of TTC, the dye employed in this study, has been carefully validated for detection of infarct size in dogs. It has been recognized that discrepancies between untreated and treated groups of animals in several physiological variables that influence infarct size must be accounted for by a satisfactory normalization process to ascertain whether a treatment is effective. Postmortem injections of dye through occluded and nonoccluded coronary arteries have been used frequently for estimation of the volume of myocardium at risk from ischemia, an important determinant of infarct size. Heart rate and blood pressure and their product are important hemodynamic determinants of myocardial oxygen consumption that must be taken into account if there are differences in these measurements among groups being compared. Recently, there has been evidence that the level of collateral blood flow to the ischemic region during coronary occlusion is also an important determinant of infarct size, and the advisability of normalizing for variations in this measurement by ANCOVA has been stressed. Failure to normalize for collateral flow level variation may account for many of the discrepancies in results of studies of effects of superoxide dismutase on myocardial infarct size.

Although the majority of studies of infarct size during ischemia and reperfusion have performed measurements within the first 6 hours of reperfusion, it has been suggested that this may be misleading, because some interventions may delay necrosis but not prevent it. The studies that avoided this problem by waiting at least 24 hours after reperfusion began before measuring infarct size and also normalized for size of the risk region and, by ANCOVA, for collateral blood flow during ischemia are shown in Table 2. Although two studies with different forms of superoxide dismutase reported reductions in infarct size, four others concluded that this agent alone or together with catalase had no benefit. Possible reasons for the ineffectiveness of superoxide dismutase include its high molecular weight, which makes it unlikely that it can readily enter myocardial cells, and the possibility that scavenging of superoxide radical alone may not prevent injury from other reactive oxygen species. In one study from our laboratory, dimethylthiourea, a diffusible scavenger of hydrogen peroxide and hydroxyl radical with a half-life of 43 hours, was used in our canine model. When administered at the onset of reperfusion after 90 minutes of regional myocardial ischemia, a bolus of dimethylthiourea modestly but significantly reduced infarct size after 48 hours of reperfusion. Because of our success with dimethylthiourea, we hypothesized that antioxidants that readily enter myocardial cells and can be administered in a manner that allows effectiveness for several hours were most likely to offer optimal protection against reperfusion injury. In studies of cultured cardiac myocytes, we observed that MPG, a low-molecular-weight synthetic analogue of glutathione with little

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<tr>
<td>Tamura et al</td>
<td>90 min</td>
<td>4 d</td>
<td>PEG SOD</td>
</tr>
<tr>
<td>Carrea et al</td>
<td>90 min</td>
<td>48 h</td>
<td>DMTU</td>
</tr>
<tr>
<td>Uraizee et al</td>
<td>40 min</td>
<td>4 d</td>
<td>SOD</td>
</tr>
<tr>
<td>Richard et al</td>
<td>90 min</td>
<td>4 d</td>
<td>SOD+CAT</td>
</tr>
<tr>
<td>Nejima et al</td>
<td>90 min</td>
<td>7 d</td>
<td>SOD</td>
</tr>
<tr>
<td>Tanaka et al</td>
<td>90 min</td>
<td>4 d</td>
<td>PEG SOD+CAT</td>
</tr>
<tr>
<td>Forman et al</td>
<td>90 min</td>
<td>24 h</td>
<td>N-Acetylcysteine</td>
</tr>
</tbody>
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SOD indicates superoxide dismutase; PEG SOD, polyethylene glycol superoxide dismutase; DMTU, dimethylthiourea; and CAT, catalase.
apparent toxicity in humans or animals, was more effective than dimethylthiourea in preventing injury from exposure to hydrogen peroxide. Therefore, we decided to test its efficacy in preventing myocardial necrosis in intact dogs.

Previous studies of MPG in animals subjected to myocardial infarctions have had mixed results. Tsao et al. found minimal benefit in attenuating coronary endothelial injury and no effect on myocardial injury after a 40-minute infusion of MPG at a rate of 8 mg · kg⁻¹ · h⁻¹ intravenously for 30 minutes to a cat model of regional myocardial ischemia and reperfusion. Mitsos et al. administrated MPG at a rate of 20 mg/kg for 1 or 2 hours of reperfusion to a canine model of regional myocardial ischemia and 6 hours of reperfusion. They reported that if infusions were begun before or at the onset of reperfusion, there was a 35% reduction in infarct size measured at 6 hours of reperfusion, but there was no benefit when an infusion was begun 45 minutes after reperfusion began. In a second study by this group, a similar dose of MPG enhanced the benefits of a neutrophil antiserum in the same model. Neither of the studies by Mitsos et al normalized for collateral flow during ischemia. In a study of myocardial stunning in dogs exposed to 15 minutes of coronary occlusion, MPG infused at 50 mg · kg⁻¹ · h⁻¹ for 4 hours into the left ventricle markedly attenuated left ventricular dysfunction. We considered the possibility that the length or magnitude of the MPG infusions was suboptimal in the previous studies of myocardial infarction.

Our study differed markedly from previous studies of MPG in myocardial infarction in the manner in which MPG was given. We administered a much higher dose for a much longer infusion period (100 mg · kg⁻¹ · h⁻¹ for either 3 hours and 30 minutes or 4 hours and 15 minutes, plus an intramuscular dose at the end of the intravenous infusion) in two of the treatment groups. To facilitate use of this higher dose without causing acidois, we neutralized the highly acidic aqueous solution of MPG to a pH of 7.3 to 7.4 before administration, a precaution not taken in the previous cardiovascular studies. Finally, we compared results after prolonged infusions with those caused by infusion during the first hour of reperfusion only. We demonstrated that this antioxidant is highly efficacious in reducing the extent of myocardial necrosis when administered during most or all of the first 4 hours of reperfusion but was only modestly effective when administration was limited to the first hour of reperfusion.

Although another mechanism cannot be excluded, it is probable that MPG exerted its beneficial effect through prevention of myocardial injury by reactive oxygen metabolites. Specifically, we demonstrated that MPG, in the same concentration as we measured in plasma, is a scavenger of hydrogen peroxide but not of superoxide radical. Although it is difficult to precisely extrapolate results of in vitro scavenging assays to the in vivo setting, neutralization of hydrogen peroxide is a plausible mechanism of action for MPG. On the basis of reports that there is a burst of reactive oxygen species during the first 15 to 30 minutes of reperfusion after myocardial ischemia or hypoxia, it has often been assumed that antioxidant measures for a relatively brief time early in reperfusion would suffice to prevent oxidative injury. The most likely sources of excessive quantities of reactive oxygen metabolites in the first 15 to 30 minutes of reperfusion are generation by myocardial cells through the mitochondrial electron transport or P450 system or generation by endothelial cells through the xanthine oxidase system. Leukocytes do not appear to be a factor in myocardial stunning. However, although early generation of reactive oxygen species from nonleukocyte sources probably plays a role in reversible injury, such as "myocardial stunning," it may not be a cause of myocardial necrosis. We achieved excellent protection by beginning a prolonged infusion of MPG after the first 30 minutes of reperfusion but could detect only modest benefit when we limited our infusion to the last 15 minutes of ischemia and the first hour of reperfusion.

However, there is evidence that during cardiac reperfusion after ischemia of 1 hour or more, there is an inflammatory response that results in considerable accumulation of polymorphonuclear leukocytes in the myocardium for the first 4 to 6 hours of reperfusion. It has been reported that once invading polymorphonuclear leukocytes become bound to myocardial integrin adhesion sites, hydrogen peroxide is released. Myocardial oxidant injury from polymorphonuclear leukocytes requires a series of complex processes, including adhesion of leukocytes to endothelial cells, transmigration into the extravascular space, adhesion to myocardial cells, release of reactive oxygen species, and intracellular myocardial oxidation reactions. We propose that completion of this series of processes requires sufficient time so that most of the myocardial necrosis that results from this mechanism occurs after the first hour of reperfusion. When the infusion of MPG was limited to the first hour of reperfusion, reduction in normalized infarct size was much less than occurred with longer infusions. Therefore, although antileukocyte measures administered for only 2 hours of reperfusion after 90 minutes of ischemia were ineffective, whereas administration of the same agents for 48 hours reduced myocardial infarct size. From our data with three different MPG infusion protocols, the most critical period for suppression of reactive oxygen metabolites is probably between 30 minutes and 4 hours after reperfusion begins.

We found no evidence that MPG reduced leukocyte accumulation in the reperfused myocardium. On the contrary, there was a trend toward increased leukocyte infiltration in the MPG-treated dogs. It has been reported that MPG has chemotactic and chemokinetic effects that enhance migration of polymorphonuclear leukocytes. Therefore, if MPG prevents myocardial cytotoxicity caused by leukocytes, it is probably through protection it affords against hydrogen peroxide or other reactive oxygen species released by neutrophils. In this study we determined that MPG scavenges hydrogen peroxide, and in a previous study we demonstrated that it markedly attenuates cytotoxicity caused by exposure to this oxidant. Although we also established in this study that it does not scavenge superoxide radical, we cannot exclude the possibility that it inhibits neutrophil release of superoxide radical, as has been suggested by others.
This study appears to resolve the issue of whether injury caused by processes during reperfusion can be an important component of myocardial necrosis. Administration of MPG, a diffusible, low-molecular-weight scavenger of hydrogen peroxide with a circulating half-life of approximately 7 minutes, throughout the first 4 hours of reperfusion reduced normalized infarct size by 60% from values in untreated dogs. We conclude that under certain circumstances involving early reperfusion of infarcted myocardium, necrosis caused by processes during reperfusion may be more extensive than necrosis caused solely by ischemia. The processes that occur during reperfusion appear to take several hours and occur mostly after the first 30 minutes of reperfusion.

There are limitations to this study that should be taken into account. Infarct size is a nonspecific measurement that probably has limited sensitivity because of factors such as intermingled islands of normal and necrotic tissue that limit ability to precisely delineate margins of necrotic regions. The relation between collateral flow and MI/RISK is only roughly linear, and it is difficult to normalize infarct size precisely for variability because of local flow heterogeneity during ischemia. Other unrecognized variables may influence infarct size independently of interventions. Investigators were not blinded. Use of small numbers of dogs may give misleading results from random variation. Although these problems decrease confidence in our ability to detect subtle alterations, the infarct size reductions that occurred in this study were large, and the conclusions above appear to be justified.

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