Protective effects of N-2-mercaptopropionyl glycine against myocardial reperfusion injury after neutrophil depletion in the dog: evidence for the role of intracellular-derived free radicals

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ABSTRACT Reperfusion of the previously ischemic myocardium is associated with the production of oxygen free radicals and their metabolites, which contribute to the ultimate extent of irreversible myocardial injury. The relative importance of polymorphonuclear leukocytes vs intracellular-derived oxygen metabolites has remained uncertain. We evaluated the effectiveness of a free-radical scavenger, N-2-mercaptopropionyl glycine (MPG), in limiting infarct size after ischemia/reperfusion in dogs that were depleted of neutrophils with specific antisera. Twenty-four anesthetized open-chest dogs were subjected to 90 min of ischemia by occlusion of the left circumflex coronary artery followed by 6 hr of reperfusion. Dogs were randomly assigned to receive nonimmune serum, neutrophil antiserum, or neutrophil antiserum plus MPG (20 mg/kg intra-atrially 15 min before reperfusion was initiated and for 45 min after reperfusion). Infarct size, as a percent of the area at risk, was reduced by 33% in the neutrophil antiserum group as compared with the nonimmune group (30.7 ± 2.7% vs 45.6 ± 3.7%, p < .01). The combined administration of neutrophil antiserum plus MPG reduced the size of infarction by 63% of the area at risk compared with that in the nonimmune group (17.0 ± 2.7% vs 45.6 ± 3.7%, p < .01). The reduction in infarct size with neutrophil antiserum plus MPG was significantly greater than that with the neutrophil antiserum alone (p < .01). The areas at risk did not differ among the groups. Myocardial protection could not be explained on the basis of hemodynamic differences. The observation that MPG enhances the protective effects of neutrophil depletion suggests that both extramyocardial- and intramyocardial-derived oxygen free radicals contribute significantly to reperfusion-induced myocardial injury.

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Experimental Observations have provided evidence that early reperfusion of the ischemic myocardium can salvage tissue before it becomes irreversibly injured.1,2 Since cell death is not instantaneous, and the early pathophysiologic changes associated with myocardial ischemia are reversible, the effort to salvage ischemic myocardium by early reperfusion is a worthwhile endeavor. In the clinical setting, current therapeutic strategies involve thrombolytic therapy with agents such as streptokinase3, 4 or tissue-type plasminogen activator,5, 6 by percutaneous transluminal coronary angioplasty,7 or by surgical revascularization.8 In light of the current use of these interventions it has become increasingly important to gain insight into the physiologic and biochemical effects of myocardial reperfusion.

While progressive cellular injury will ensue if ischemia is prolonged, and ultimate cell survival is dependent on the restoration of blood flow, it is now clear that reperfusion of the ischemic myocardium is associated with a number of potentially deleterious effects as well.9, 10 Recent observations have indicated that oxygen-derived free radicals may play a central role in reperfusion-induced cell injury. Myocardial reperfusion has been shown to be accompanied by the release of free radicals from the myocardium,11 with the result-
ing generation of lipid peroxide intermediates. In addition, decreases in myocardial antioxidant concentrations, including superoxide dismutase, catalase, and glutathione peroxidase, have been observed. The hypothesis that oxygen-derived free radicals are important mediators of myocardial injury associated with reperfusion is supported further by the ability of antioxidant enzymes and free-radical scavengers to limit myocardial reperfusion injury.

However, uncertainty exists as to the primary source of these reactive oxygen species. One potential extracellular source is the activated neutrophil, which is known to mediate tissue injury through the release of oxygen radicals and their subsequent metabolites. The proposed role of the neutrophil in myocardial reperfusion injury is strengthened by the knowledge that neutrophils accumulate in ischemically injured tissue during reperfusion and that neutrophil depletion is associated with a significant reduction in the extent of irreversible myocardial injury. Myocardial salvage has also been demonstrated with nonsteroidal anti-inflammatory agents that interfere with neutrophil function or migration.

Alternatively, there are several potential intracellular sources that may contribute to the formation of oxygen-derived free radicals during reperfusion. Ischemic injury to the myocardium is associated with the enhanced catabolism of ATP to purine nucleotide precursors, hypoxanthine and xanthine, and the proteolytic conversion of the enzyme xanthine dehydrogenase to xanthine oxidase. It has been proposed that xanthine oxidase plays an important role in reperfusion-induced tissue injury, since the reintroduction of molecular oxygen to cells containing high concentrations of hypoxanthine and xanthine would theoretically result in the release of superoxide anion and hydrogen peroxide. Various hemoproteins, including myoglobin, in the presence of H₂O₂ have been shown to generate an oxidant capable of initiating lipid peroxidation. Acute myocardial ischemia is accompanied by the release of myoglobin from injured myocardial cells. Thus, free myoglobin present in areas of evolving myocardial infarction also could contribute to free radical–mediated myocardial injury. In addition, reactive oxygen metabolites may be generated at the level of the mitochondria as a result of alterations in the redox state of the cell that occur during ischemia.

In an effort to assess the relative importance of intracellular- vs neutrophil-derived oxygen metabolites in myocardial reperfusion injury, we evaluated the effectiveness of a free-radical scavenger, N-2-mercaptopropionyl glycine (MPG), in protecting against reperfu-

**Methods**

**Oclusion-reperfusion preparation of myocardial infarction.** Male mongrel dogs (13 to 17 kg) were anesthetized with Dial-Urethane (20% ethyl urea, 20% allobarbital, 0.5% urethane; 0.6 ml/kg iv), intubated, and ventilated with room air via a Harvard respirator. Lead II of the electrocardiogram and hemodynamics were monitored continuously throughout the course of the experiment. A catheter was inserted into the left carotid artery and advanced into the left ventricle for the continuous recording of left ventricular pressure. A second catheter was inserted into the right femoral artery for measurements of arterial blood pressure.

A left thoracotomy was performed at the fifth intercostal space, the heart was suspended in a pericardial cradle, and the left circumflex coronary artery (LCX) was isolated distal to its atrial branch and proximal to any major ventricular branches. An electromagnetic flow probe was placed on the artery for the determination of basal LCX blood flow. Initially, the LCX was constricted partially with a ligature to an extent that did not change resting flow, but the peak flow increment (reactive hyperemic response) after a 10 sec complete occlusion was decreased by more than 70% (critical stenosis). After 15 min of partial constriction, the LCX was occluded completely with a second ligature. Total occlusion was maintained for 90 min, followed by 6 hr of reperfusion with the critical stenosis in place for the initial 30 min of reperfusion. The critical stenosis limits the reperfusion hyperemia and therefore reduces the severity of reperfusion arrhythmias, the extent of hemorrhagic myocardial infarcts, and the potential for ventricular fibrillation. After 6 hr of reperfusion, the heart was fibrillated electrically and removed rapidly for postmortem quantification of infarct size.

**Postmortem quantification of size of infarction.** Size of myocardial infarction was determined with a dual-perfusion staining technique ex vivo. Cannulas were inserted into the aorta above the coronary ostia and into the LCX at the site of the previous occlusion. The LCX bed was perfused with 1.5% triphenyl tetrachloride hydrochloride (TTC) in 20 mM potassium phosphate buffer (pH 7.4, 38°C). The aorta was perfused in a retrograde manner with 0.25% Evans blue dye. Both the LCX region and the remainder of the heart were perfused with their respective stains at a constant pressure of 100 mm Hg for 10 min. The heart was cut into six equal sections approximately 1.0 cm thick perpendicular to the apical-basal axis. The staining technique readily delineates the area of left ventricle at risk of infarction and infarcted myocardium within the area at risk from the area of left ventricle that is not dependent on the LCX for blood flow and that is stained with Evans blue dye. Viable myocardium within the area at risk is stained red due to the conversion of the colorless TTC to a red formazan precipitate by tissue dehydrogenase enzymes. Infarcted myocardium within
the area at risk remains unstained due to the loss of dehydrogenase enzymes from the irreversibly injured tissue.

The transverse ventricular sections were trimmed of right ventricular muscle and valvular and fatty tissue and then traced onto a clear plastic overlay for the planimetric determination of the size of infarction. Infarct size was expressed as a percent of the area at risk and as a percent of the total left ventricle.

Preparation and administration of antiserum to canine polymorphonuclear leukocytes. Canine polymorphonuclear leukocytes were isolated from heparinized whole canine blood by use of Ficoll-Hypaque discontinuous gradient. Erythrocytes contaminating the cell pellets were lysed with NH4Cl and the isolated neutrophils were subsequently washed twice and resuspended in Hanks balanced salt solution. Microscopic examination of the purified neutrophil suspension revealed less than 2% contamination with other cells. Sheep were inoculated by subcutaneous injection of 1 × 10^8 canine neutrophils suspended in complete Freund’s adjuvant. Ten days later, the sheep received a second challenge of 2.5 × 10^7 canine neutrophils in incomplete Freund’s adjuvant. The sheep were bled 25 days after the initial exposure to canine neutrophils (i.e., 15 days after the second challenge) and the serum samples were pooled and heat inactivated. Nonimmune serum, which was administered to dogs in the control group, was prepared by bleeding nonchallenged sheep.

Dogs were assigned randomly to three groups that received either nonimmune serum, neutrophil antiserum, or neutrophil antiserum plus MPG. An initial 4 ml dose of serum was administered by slow intravenous injection 60 min before occlusion. The initial administration of the neutrophil antiserum was sometimes associated with a brief (2 to 3 min) vasodepressor response of 15 to 20 mm Hg. After completion of the surgical preparation dogs in all groups were allowed to equilibrate for 60 min until the reestablishment of baseline hemodynamic values before complete occlusion of the LCX for 90 min. Sixty minutes into the occlusion phase an additional 2 ml of serum was administered. In addition, 0.75 ml of serum was administered at 30 min intervals throughout the 6 hr of reperfusion. In the group that received the combination of neutrophil antiserum plus MPG, the MPG (20 mg/kg) was given as a constant infusion into the left atrium for a period of 3 hr beginning 15 min before reperfusion and continuing throughout 45 min of reperfusion.

The time course of occlusion, reperfusion, and treatment is shown in figure 1.

Circulating neutrophil counts were assessed by total peripheral white cell counts in conjunction with differential counts. Blood samples were taken at 1 hr intervals before and after the administration of the serum. The neutrophil antiserum specifically lowered circulating polymorphonuclear leukocytes and did not alter circulating lymphocyte or monocyte counts.

Histologic examination. Tissue blocks, representing the area dependent on the LCX for blood flow, were taken from a midventricular transverse section of the heart. The tissue blocks were cut so that the specimen extended from endocardium to epicardium, covering the full thickness of the ventricular wall, and included the entire area at risk. The blocks were embedded in paraffin, cut to a thickness of 6 μm, and stained with hematoxylin and eosin. Light microscopy was used to assess the presence of hemorrhage, the uniformity of necrosis from endocardium to epicardium, and the extent of the leukocyte infiltration associated with the irreversibly injured myocardium. Leukocyte infiltration was assessed on a semiquantitative basis with the use of an arbitrary scale of 0 to +++, a score of +++ being assigned to tissue samples with the most dense and diffuse leukocyte infiltration. Repetitive blind trials of the same tissue samples confirmed that the results with the grading system were reproducible. All sections were coded and examined by a pathologist (G. D. A.) who was unaware of the treatment regimens.

Statistics. All values are expressed as mean ± SEM. Hemodynamic parameters were analyzed by profile analysis. Data on myocardial infarct size (percentage of area at risk infarcted, percentage of total left ventricle infarcted, and percentage of left ventricle at risk) were compared by analysis of variance followed by the Newman-Keuls multiple-range test. Differences were considered statistically significant when p < .05. Statistical analysis was performed by computer program (Midas Statistical Program, University of Michigan Statistical Research Laboratory).

Results

Thirty-two dogs were subjected to 90 min of LCX occlusion followed by 6 hr of reperfusion. Four experi-
ments were terminated early due to intractable ventricular fibrillation during occlusion/reperfusion. Criteria were established to ensure that all dogs included in the data analysis were subjected to comparable degrees of ischemia. Criteria for inclusion in the study were: ST segment elevation on occlusion of the LCX, epicardial cyanosis for the duration of occlusion, and the development of arrhythmias on reperfusion. Three dogs did not meet two or more of the criteria and were excluded from the study during the experiment. One dog had an abnormally high peripheral white blood cell count and was excluded from the study because the administration of the antiserum did not result in a decrease in circulating neutrophil levels. Thus, a total of 24 dogs was distributed in three groups: those receiving nonimmune serum (n = 8), those receiving neutrophil antiserum (n = 8), and those receiving neutrophil antiserum plus MPG (n = 8).

Time course for depletion of circulating polymorphonuclear leukocytes. Circulating neutrophil counts for the three groups are illustrated in figure 2. In both the neutrophil antiserum and neutrophil antiserum plus MPG groups circulating neutrophil counts were decreased markedly throughout the course of the experiment. Sixty minutes after the initial administration of the neutrophil antiserum, circulating neutrophil counts were decreased by 80% in each of these groups. In the neutrophil antiserum group, circulating neutrophils were depleted by an average of 85 ± 1% (range 80% to 90%) over the course of the experiment. Similarly, for the dogs that received neutrophil antiserum plus MPG, circulating neutrophil counts were decreased by an average of 86 ± 1% (range 80% to 90%). In contrast, in control dogs that received nonimmune serum there was a progressive increase in the circulating neutrophil counts. At 6 hr of reperfusion, circulating neutrophils for the nonimmune group had risen threefold, from 77 ± 12 × 10⁴ to 209 ± 21 × 10⁴ per ml. This was most likely in response to the myocardial injury associated with the ischemic episode and in part attributable to the surgical procedure.

Cardiovascular parameters. Changes in heart rate, mean arterial blood pressure, rate-pressure product, peak left ventricular systolic pressure, and coronary blood flow are presented in table 1. During the period of occlusion, mean arterial pressure and peak left ventricular systolic pressure declined progressively, whereas heart rate remained unchanged in each of the three groups. The rate-pressure product (calculated from heart rate and peak systolic arterial pressure) tended to decrease slightly during the period of occlusion as a result of the decrease in blood pressure. Over the 6 hr of reperfusion mean arterial blood pressure and peak left ventricular systolic pressure decreased further and heart rate increased progressively. The rate-pressure product tended to return toward baseline values. There were no significant interactions or group differences with respect to these parameters (profile analysis; p > .05). Mean LCX blood flow also did not differ significantly among the groups throughout the course of the experiment. However, dogs that received neutrophil antiserum or neutrophil antiserum plus MPG appeared to maintain higher coronary arterial blood flow. After 6 hr of reperfusion, left circumflex coronary blood flow averaged 19 ± 2 and 20 ± 3 ml/min compared with initial values of 25 ± 2 and 25 ± 2 ml/min in the neutrophil antiserum and neutrophil antiserum plus MPG groups, respectively. In dogs that received nonimmune serum LCX blood flow declined from 26 ± 2 to 16 ± 3 ml/min after 6 hr of reperfusion.

Leukocyte infiltration into infarcted myocardium. Histologic examination of infarcted myocardium from the nonimmune serum and neutrophil antiserum groups confirmed that the extent of irreversible myocardial injury was reduced in the neutrophil antiserum and neutrophil antiserum plus MPG treatment groups. Similar morphologic changes in the foci of myocardial necrosis were observed. In all groups myocardial tis-
TABLE 1
Mean cardiovascular parameter values in each group

<table>
<thead>
<tr>
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<th>Before serum</th>
<th>After serum</th>
<th>Occlusion</th>
<th>Reperfusion</th>
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<tbody>
<tr>
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<tr>
<td>Nonimmune serum (n = 8)</td>
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<tr>
<td>Heart rate (beats/min)</td>
<td>120 ± 4</td>
<td>120 ± 8</td>
<td>121 ± 10</td>
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<td></td>
<td>138 ± 13</td>
<td>143 ± 15</td>
<td>160 ± 15</td>
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<td>MAP (mm Hg)</td>
<td>102 ± 7</td>
<td>98 ± 6</td>
<td>85 ± 4</td>
<td>83 ± 4</td>
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<td>Rate × pressure/10^3</td>
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<td>15.3 ± 0.9</td>
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<td>15.6 ± 1.8</td>
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<tr>
<td>LV peak pressure (mm Hg)</td>
<td>125 ± 8</td>
<td>118 ± 6</td>
<td>100 ± 6</td>
<td>93 ± 5</td>
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<td>Coronary blood flow (ml/min)</td>
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<td>22 ± 2</td>
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<td>Neutrophil antiserum</td>
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<tr>
<td>Heart rate (beats/min)</td>
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<td>130 ± 5</td>
<td>127 ± 8</td>
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<tr>
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<td>134 ± 7</td>
<td>144 ± 9</td>
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<tr>
<td>MAP (mm Hg)</td>
<td>111 ± 8</td>
<td>108 ± 8</td>
<td>103 ± 9</td>
<td>95 ± 7</td>
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<td>97 ± 5</td>
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<td>Rate × pressure/10^3</td>
<td>17.3 ± 1.6</td>
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<td>16.9 ± 1.6</td>
<td>16.4 ± 1.2</td>
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<tr>
<td>LV peak pressure (mm Hg)</td>
<td>129 ± 9</td>
<td>128 ± 9</td>
<td>119 ± 7</td>
<td>109 ± 6</td>
</tr>
<tr>
<td>Coronary blood flow (ml/min)</td>
<td>25 ± 2</td>
<td>24 ± 3</td>
<td>—</td>
<td>24 ± 2</td>
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<tr>
<td>Neutrophil antiserum + MPG (n = 8)</td>
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<tr>
<td>Heart rate (beats/min)</td>
<td>137 ± 9</td>
<td>130 ± 12</td>
<td>128 ± 5</td>
<td>119 ± 7</td>
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<tr>
<td></td>
<td>124 ± 7</td>
<td>137 ± 9</td>
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<tr>
<td>MAP (mm Hg)</td>
<td>108 ± 4</td>
<td>104 ± 5</td>
<td>99 ± 4</td>
<td>91 ± 5</td>
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<tr>
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<td>Rate × pressure/10^3</td>
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<td>15.8 ± 1.1</td>
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<td>14.8 ± 1.3</td>
<td>14.7 ± 2.0</td>
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<td>LV peak pressure (mm Hg)</td>
<td>131 ± 6</td>
<td>130 ± 6</td>
<td>118 ± 5</td>
<td>113 ± 6</td>
</tr>
<tr>
<td>Coronary blood flow (ml/min)</td>
<td>25 ± 2</td>
<td>22 ± 1</td>
<td>—</td>
<td>25 ± 3</td>
</tr>
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</table>

The data are expressed as mean ± SEM. No significant differences were observed among the groups throughout the time course of the experiments (profile analysis).

MAP = mean arterial pressure; LV = left ventricular.

The extent of myocardial injury arising from ischemia and reperfusion was significantly less in dogs that received neutrophil antiserum. Neutrophil depletion resulted in a 33% reduction in the size of infarction expressed as a percent of the area at risk compared with the size of infarction in the nonimmune group (30.7 ± 2.7% vs 45.6 ± 3.7%, p < .01). These results are in accord with previous findings from this laboratory in which neutrophil depletion was associated with a 43% reduction in the size of infarction (from 47.1 ± 7.5% in dogs receiving nonimmune serum to 27.0 ± 4.5% in dogs receiving neutrophil antiserum). The combined administration of neutrophil antiserum plus MPG reduced infarct size by 63% in comparison with that in the nonimmune serum group (17.1 ± 2.7% vs 45.6 ± 3.7% of the area at risk, p < .01). The reduction in the size of infarction observed with neutrophil depletion plus MPG was significantly greater than that with neutrophil depletion alone (p < .01). When infarct size was expressed as a percentage of the left ventricle a similar trend was observed. When infarct size was expressed as a percentage of the left ventricle, neutrophil depletion resulted in a 36% reduction (13.0 ± 1.4% vs 20.4 ± 1.6%, p < .01) and neutrophil depletion plus MPG resulted in a 61% reduction (7.9 ± 1.6% vs 20.4 ± 1.6%, p < .01) relative to that in the nonimmune serum group. The observed differences in myocardial salvage could not be explained on the basis...
of differences in the extent of left ventricle rendered at risk of infarction. Overall size of the region at risk expressed as a percentage of the left ventricle was 45.0 ± 1.5%, 43.0 ± 2.9%, and 46.0 ± 2.1% for the nonimmune serum, neutrophil antiserum, and neutrophil antiserum plus MPG groups, respectively.

**Discussion**

The ability to limit the extent of irreversible injury associated with myocardial ischemia and reperfusion by selective depletion of polymorphonuclear leukocytes has been demonstrated previously. The results of this study are in accord with previous findings and support the concept that neutrophils play a fundamental role in the pathogenesis of acute myocardial infarction associated with occlusion and reperfusion.

In addition, we were able to demonstrate that the administration of a free-radical scavenger, MPG, just before the onset of reperfusion enhances the protective effects of neutrophil depletion, suggesting that reactive oxygen species derived from myocardial tissue are in part responsible for cellular injury that occurs during reperfusion. Since neutrophil-derived oxygen metabolites have been shown to initiate tissue injury at sites of inflammation, these observations suggest that oxygen-derived metabolites from both neutrophils and myocardial sources contribute significantly to cellular injury resulting from reperfusion of the previously ischemic myocardium.

The mechanism by which neutrophil depletion provides cardioprotection appears to be related to its ability to suppress the inflammatory response. The administration of the neutrophil antiserum decreased circulating neutrophil counts by an average of 85 ± 1% and 86 ± 1% in the neutrophil antiserum and neutrophil antiserum plus MPG groups, respectively. In each of these groups depletion of the circulating neutrophils was associated with a substantial decrease in the extent of leukocytic infiltration into the infarcted myocardium. In contrast, dogs that received nonimmune serum showed a progressive increase in circulat-

![Graphs showing leukocyte infiltration score](image)

**FIGURE 3.** Effect of neutrophil depletion on the inflammatory response in infarcted myocardium. Results of histologic assessment of leukocyte infiltration into infarcted myocardium for nonimmune serum, neutrophil antiserum, and neutrophil antiserum plus MPG treatment groups. The letters are the sequential coded designations for the dogs used in the study. Leukocyte infiltration was assessed on a semiquantitative basis using a scale of 0 (absence of leukocytes) to + + + (assigned to specimen with the most extensive accumulation of leukocytes).

![Graphs showing % of area at risk](image)

**FIGURE 4.** Effect of neutrophil depletion and neutrophil depletion plus MPG on the extent of irreversible myocardial injury after 90 min of LCX occlusion followed by 6 hr of reperfusion. Size of myocardial infarction is expressed as a percent of the area at risk and as a percent of the total left ventricle. When expressed as a percent of the area at risk, infarct size is reduced by 33% in the neutrophil antiserum group and by 63% in the neutrophil antiserum plus MPG groups. A similar trend is observed when infarct size is expressed as a percent of the total left ventricle. The size of areas at risk did not differ significantly among the groups. * p < .05 compared with nonimmune serum group. ** p < .05 compared with neutrophil antiserum group.
ing neutrophil counts over the course of the experiment and marked accumulation of neutrophils in the infarcted tissue. The observed elevation in circulating neutrophil counts is in response to the ischemic myocardial injury and is in part attributable to the surgical procedure, rather than an effect of the nonimmune serum, since similar increases in neutrophil counts have been observed in saline-treated dogs subjected to the identical protocol.\textsuperscript{2} However, we must call attention to the fact that our ability to accurately quantitate the extent of neutrophil infiltration in the reperfused myocardium by light microscopic examination of hematoxylin and eosin–stained sections is at best a semiquantitative approach and above all does not measure intravascular margination of neutrophils, as reported by Engler et al.\textsuperscript{40}

The observed differences in myocardial salvage could not be explained on the basis of hemodynamic differences. The three groups were similar with respect to heart rate, mean arterial pressure, and left ventricular pressure, indicating that there were no favorable alterations in myocardial oxygen consumption. LCX blood flow also did not differ significantly among the groups, but dogs treated with the neutrophil antiserum or neutrophil antiserum plus MPG maintained slightly better LCX blood flow in the later stages of reperfusion. This appeared to be a result of the myocardial salvage, in that the dogs in the neutrophil antiserum plus MPG group, which had the smallest infarcts, maintained the highest coronary blood flow. In addition, the three groups maintained similar LCX blood flow throughout the initial phase of reperfusion. It has been reported that leukocytes adhere to vascular endothelium, causing capillary obstruction and thus impaired reperfusion of the previously ischemic region.\textsuperscript{41} Therefore, the possibility that neutrophil depletion salvages jeopardized myocardium in part by influencing reperfusion or the regional distribution of coronary blood flow should also be considered.

Measurements of collateral blood flow were not included in the present study protocol. Previous studies with MPG given according to the same dosing regimen along with the assessment of regional myocardial blood flow showed no alterations that could be interpreted as an improvement in vascular supply to the jeopardized myocardium.\textsuperscript{37} However, in the future it would be beneficial to assess the effects of both neutrophil depletion and MPG with respect to changes in the distribution of regional myocardial blood flow since it has been demonstrated that intravascular margination of neutrophils on reperfusion may not only serve to impair regional myocardial blood flow but may also exert a deleterious effect on the vascular endothelium via their ability to release reactive oxygen species.\textsuperscript{40} It is conceivable, therefore, that a part of the protective effect achieved with MPG may be derived from the ability of the thiol-containing compound to modulate the activity of the polymorphonuclear leukocyte. This interpretation is supported by the recent observation that MPG is able to scavenge HOCl formed by myeloperoxidase from H\textsubscript{2}O\textsubscript{2} and chloride ions. In addition, it was noted that MPG, but not cysteine, was capable of inhibiting myeloperoxidase itself.\textsuperscript{41} The failure of the neutrophil antiserum to produce a total depletion of the circulating PMNs might also account for the added protective effect that was obtained on the subsequent addition of MPG to the treatment regimen. It may be possible that MPG provides all of its protective effects via extracellular mechanisms, but in our previous study\textsuperscript{37} the thiol compound given in the absence of neutropenia failed to provide the same degree of protection observed in the present study when the thiol scavenger was administered to the neutropenic animal. Thus, our previous\textsuperscript{37} and present observations provide suggestive evidence that MPG is acting to protect the reperfused myocardium by a mechanism(s) beyond that involving the destructive properties of the neutrophil.

Sulfhydryl compounds, including MPG, have been shown to protect against cellular injury in pathophysiological processes involving inflammation,\textsuperscript{41-43} hyperoxigenation,\textsuperscript{44} and irradiation.\textsuperscript{45} The protective properties of sulfhydryl compounds are believed to be derived from their ability to scavenge oxygen free radicals and thus to prevent the deleterious effects of these reactive oxygen species.

Oxygen radicals and their metabolites can interact with a variety of cell components and therefore initiate cell injury through multiple mechanisms.\textsuperscript{36, 46} Many of the deleterious effects of oxygen free radicals are produced through the oxidation of polyunsaturated fatty acids, with a resulting generation of lipid hydroperoxides that can lead to losses in cell membrane integrity and ultimately cell death.\textsuperscript{47} These reactive oxygen species also interact with structural and functional proteins, particularly those that are rich in sulfhydryl-containing amino acids, subsequently oxidizing important sulfhydryl groups.\textsuperscript{48} Such interactions may interfere with cellular metabolic processes by inactivating key enzymes as well as contribute further to cell membrane damage. Additionally, oxygen free radicals induce DNA strand scission and base pair transformation, a proposed mechanism for cell injury resulting from ionizing radiation.\textsuperscript{48}
MPG, which contains a reduced sulphydryl group, is able to scavenge both $O_2^-$ and 'OH, two important reactive species generated from molecular oxygen. The proposed reactions are as follows:

$$2RSH + O_2^- \rightarrow 2RS + H_2O_2$$
$$RSH + \cdot OH \rightarrow RS^- + H_2O$$
$$RS^- + RS^- \rightarrow RSSR$$

The main end product is a relatively nontoxic disulfide and its formation terminates the free-radical reaction.

MPG could exert its protective influence through multiple mechanisms, including reaction with these toxic oxygen products. By providing a reduced sulphydryl group, MPG may be preferentially oxidized, preventing the oxidation of sensitive sulphydryl groups of enzymes and transmembrane proteins. In addition, by scavenging toxic oxygen metabolites it may prevent the initiation of lipid peroxidation as well as function to maintain the glutathione/glutathione peroxidase system. MPG could also provide protection by directly reacting with intermediates of lipid peroxidation.

MPG most likely interferes with free-radical mechanisms at a number of intracellular sites. At the present time we can only speculate as to the intracellular site(s) of action of MPG. Subcellular distribution studies in the liver with $^35$S-labeled MPG have shown a maximal localization of MPG in the mitochondria. Mitochondria can generate $O_2^-$, $H_2O_2$, and 'OH, particularly under conditions of oxidative stress, when there is an accumulation of reduced cofactors in the cell. Mitochondria are not only a potential source of these reactive oxygen species, but are highly susceptible to free radical attack. MPG could therefore exert its protective effect by interfering with generation of oxygen free radicals and/or damage at this site. It has been reported that MPG prevents structural and functional alterations of mitochondria from isolated hearts subjected to hypoxia and reoxygenation. MPG has also been reported to inhibit lipid peroxidation and enzyme release from mitochondria in vitro.

Alternatively, MPG has been demonstrated to be a scavenger in vitro of $O_2^-$ generated by the xanthine/xanthine oxidase reaction. Although MPG does not inhibit xanthine oxidase directly, it could potentially function in vivo as a scavenger of oxygen metabolites that are generated by the xanthine oxidase enzyme. The role of xanthine oxidase as a potential source of oxygen free radicals in myocardial ischemia/reperfusion injury is supported by recent studies indicating the protective effects of allopurinol. It is recognized, however, that not all investigators have been able to identify a protective role of allopurinol in the canine preparation of ischemia/reperfusion injury.

Hemoproteins such as hemoglobin and myoglobin have been shown to react with $H_2O_2$, forming a complex that is capable of initiating the oxidation of lipids. Thus, a potent oxidant (possibly ferryl ion) is apparently produced from the interaction of myoglobin with $H_2O_2$, and this oxidant has the potential to promote cell injury by initiating lipid peroxidation. Ischemia results in the release of myoglobin from irreversibly injured cells. During reperfusion injury of the ischemic myocardium, phagocytic cell- or intracellular-derived $H_2O_2$ may react with myoglobin and initiate lipid peroxidation reactions independent of hydroxyl radical ion formation, leading to cell injury. We have demonstrated that MPG and other compounds containing a reduced -SH group (reduced glutathione, N-acetyl cysteine, cysteine) can inhibit hemoprotein-$H_2O_2$-mediated peroxidation of arachidonic acid in vitro. The cardioprotective effects of MPG and other sulphydryl-containing compounds during reperfusion injury may be attributed at least in part to their ability to inhibit the myoglobin-$H_2O_2$-mediated lipid peroxidation that may occur both within and without the cellular compartment of the myocardial cell. To date our studies on the role of myoglobin have been limited to those in vitro and additional efforts are needed to define more completely the role of myoglobin as a mediator of myocardial ischemia/reperfusion injury in vivo. It is of importance that MPG, which is able to act both intracellular and extracellular, is able to prevent the radical-mediated events resulting from the myoglobin-$H_2O_2$-mediated peroxidation reactions.

The observations reported in this study expand our understanding of the mechanisms responsible for reperfusion-induced myocardial injury. It appears that oxygen free radicals derived from both extracellular and intracellular sources contribute significantly to the deleterious effects of reperfusion, and that myocardial salvage can be enhanced by free-radical scavengers and/or by altering leukocyte function. The development of therapeutic strategies to maximize the extent of myocardial salvage by reperfusion has potential clinical application in coronary artery bypass surgery, open heart surgery with cardioplegic arrest, and acute myocardial infarction with early reperfusion, placing high priority on continued efforts to optimize the conditions under which the restoration of blood flow occurs. It must be recognized, however, that the results obtained in this study pertain to an experimental animal preparation of acute myocardial ischemia and re-
perfusion injury. The protective effects observed with the induction of neutropenia and/or the addition of MPG may not be realized in experiments of longer duration. Conclusions regarding the long-term benefits from such interventions must await additional studies designed to specifically answer this question.

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