Identification of a Time Window for Therapy to Reduce Experimental Canine Myocardial Injury: Suppression of Neutrophil Activation During 72 Hours of Reperfusion

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The cardio-protective effects of neutrophil depletion or inhibition of neutrophil activation early in the course of myocardial reperfusion has been established. Whether these treatments would be effective during extended periods of reperfusion has not been ascertained. Open-chest anesthetized dogs were subjected to left circumflex artery (LCX) occlusion for 90 minutes followed by 72 hours of reperfusion. Dogs were randomized into one of four groups: 1) control; 2) Ilo-2 (iloprost 100 ng/kg/min administered via the left atrium beginning 10 minutes after LCX occlusion and continuing 2 hours into reperfusion); 3) Ilo-48 (iloprost 100 ng/kg/min administered as above until 1 hour after reperfusion then 25 ng/kg/min for 48 hours of reperfusion; or 4) antibody (neutrophil antibody administered before occlusion and hourly for 2 hours of reperfusion and then every 24 hours). Myocardial infarct size, as a percentage of the area at risk assessed after 72 hours of reperfusion, was significantly smaller in the antibody-treated group (32.1±5.0% mean±SEM) or Ilo-48 (22.6±4.0%) treatment group compared with control (48.7±5.6%) or Co-2 (57.6±5.2%) groups. Regional myocardial blood flow studies demonstrated that all groups developed similar degrees of ischemia. The iloprost-treated groups had lower mean arterial blood pressures during occlusion and reperfusion than groups 1 and 4 (p<0.05). Circulating neutrophil counts were increased in groups 1 and 2 at 24 and 48 hours after reperfusion compared to groups 3 and 4 (p<0.001). Analysis of histological sections demonstrated a direct association between the extent of inflammatory cell infiltration and the size of myocardial infarcts (p<0.05). Thus, iloprost or neutrophil depletion effectively reduces ultimate myocardial infarct size detected after prolonged reperfusion, and there is a direct relation between infarct size and neutrophil accumulation within the myocardium risk region. In addition, a "time window for therapy" has been defined. Iloprost treatment during the first 2 hours of reperfusion reduces the infarct size that results after 6 hours of reperfusion, but not after 72 hours of reperfusion. However, iloprost treatment for 48 hours or prolonged neutrophil depletion reduces the ultimate extent of myocardial injury. The delayed development of myocardial injury after acute short term iloprost infusion suggests that therapeutic interventions with limited pharmacological half-lives should be administered up to 48 hours after reperfusion to effectively reduce the ultimate extent of irreversible myocardial injury. (Circulation Research 1988;63:1070–1079)
canine regional ischemia and reperfusion is due primarily to inhibition of neutrophil activation.

Ischemic myocardium was protected by iloprost and prostacyclin, and tissue injury was reduced, as determined by the resultant infarct size after a 90 minute left circumflex coronary occlusion and 6 hours of reperfusion. Based on these results, we proposed that inhibition of neutrophil migration and accumulation in myocardial region at risk during reperfusion, as well as the ability of these agents to inhibit neutrophil activation, were the relevant mechanisms of protection afforded by these agents.

A pertinent question remained to be answered after the completion of these initial studies: Does prostacyclin or its analogues merely delay the development of infarction or is ultimate infarct size limited by such interventions? The present series of experiments was designed to answer this question: Can iloprost be of benefit in limiting myocardial ischemia and reperfusion injury after prolonged reperfusion (72 hours)? Insight into the time course of neutrophil activation during the process of myocardial infarction was of interest in this study and was monitored by determining circulating neutrophil counts and the accumulation of neutrophils in reperfused myocardial region at risk.

Materials and Methods

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

General Surgical Preparation

Details of the methods have been published. Adult, male, mongrel dogs (12-17 kg) were anesthetized with 30 mg/kg sodium pentobarbital and ventilated with room air (tidal volume 30 ml/kg at a rate of 12/min). Aseptic surgical methods were employed. A left thoracotomy was performed and the proximal left circumflex coronary artery (LCX) was isolated and instrumented for continuous blood flow measurement with a calibrated electromagnetic flow probe. Arterial blood pressure, heart rate, the standard limb lead II electrocardiogram and LCX blood flow were recorded continuously. Regional myocardial ischemia was produced by occlusion of the LCX for 90 minutes followed by reperfusion in the presence of a critical stenosis. The critical stenosis prevents the development of hemorrhagic infarction and reduces the incidence of reperfusion-induced ventricular fibrillation. The thoracotomy incision was closed, and the animals were allowed to recover from surgical anesthesia. Seventy-two hours after reperfusion was initiated, the animals were reanesthetized, the chest was reopened, and the heart was fibrillated electrically. Infarct size was assessed by the ex vivo dual perfusion histochemical method previously described by Romson et al. The histochemical assessment of viable and irreversibly injured myocardium involves perfusion of the cannulated LCX with a 1.5% triphenyltetrazolium chloride solution buffered with 20 mM potassium phosphate (pH 7.4), with simultaneous perfusion of the remainder of the coronary circulation with Evan's blue dye (0.25%) introduced into the aorta. Both solutions were delivered to the respective vascular distributions under a constant pressure of 100 mm Hg and at a temperature of 39°C for a period of 5 minutes. The hearts were cut into five or six thick transverse sections, 1 cm in thickness, and infarct size, region at risk, and total left ventricle were determined planimetrically. The method of quantitating infarct size with triphenyltetrazolium chloride has been validated by a number of investigators and has been shown to demarcate viable from nonviable myocardial tissue as determined by the histochemical reaction between triphenyltetrazolium chloride and myocardial dehydrogenase enzymes.

Exclusion Criteria

To assure that all treatment groups were comparable with respect to the degree of regional myocardial ischemia, predetermined exclusion criteria were established. Animals were excluded from the final data analysis if they manifested one of the following: a) the failure to demonstrate electrocardiographic signs of regional ischemia (i.e., no ST segment change upon coronary occlusion); b) ventricular fibrillation that was not converted with fewer than four attempts at low amperage direct current cardioversion (<20 joules applied to the heart surface); or c) the presence of heartworms in the heart determined by postmortem examination.

Treatment Groups

Dogs were assigned randomly to one of four treatment groups as follows:

1. Control. The control group consisted of 13 dogs that received 0.9% sodium chloride solution given as an infusion at a rate of 0.5 ml/min via a left atrial infusion cannula beginning 5 minutes after LCX occlusion and continuing during the first hour of reperfusion. At this time (1 hour of reperfusion), the infusion was switched to the jugular infusion cannula and administered via a fluid tether and swivel (Alice King Chatham Medical Arts, Los Angeles, California) apparatus at a rate of 0.05 ml/min for 48 hours.

2. Ilo-2. Group 2 consisted of five dogs treated with iloprost at a dose of 100 ng/kg/min infused at a rate of 0.5 ml/min beginning 10 minutes after coronary occlusion and terminated after 2 hours of reperfusion.
three sections from each heart were used so that blood flows to each region represent the average of three or four samples for each experiment.

**Evaluation of Neutrophil Accumulation in Myocardial Tissue: Myeloperoxidase Assay**

Samples of myocardium (50–200 mg) were taken from the central infarct region, the noninfarcted tissue within the area at risk, the endocardial to epicardial border zone between infarct region and area at risk, and also from normal noninfarcted and unstained myocardium (Figure 4). The tissue samples were homogenized and assayed for myeloperoxidase activity as described by Bradley et al. The myocardial content of myeloperoxidase activity has been correlated with histological evidence of neutrophil infiltration. The assays involve the extraction of myeloperoxidase by homogenization of (Polytron) the tissue in buffer containing 50 mM phosphate with 0.5% hexadecyltrimethylammonium bromide and 5 mM EDTA (pH 6.0). After centrifugation, the supernatants were assayed in 50 mM phosphate pH 6.0 with 0.167 mg/ml o-dianisidine and 0.005% hydrogen peroxide. The rate of decomposition of hydrogen peroxide by myeloperoxidase is determined by measurement of the change in absorbance at 460 nm. One unit of myeloperoxidase activity is defined as that amount of enzyme that decomposes 1 μmol of hydrogen peroxide per minute at 25°C under the conditions of the assay.

**Preparation of Antiserum to Canine Neutrophils**

Canine neutrophils were isolated from venous blood of untreated and unanesthetized dogs by Ficoll-Hypaque gradient separation techniques. Red blood cell lysis was achieved by treatment of the blood with buffered ammonium chloride. The resulting neutrophil pellets were washed twice with 0.9% NaCl at 4°C. The purity of the preparation was assured by differential staining of the cell suspension. Viability of the cells in the preparation was determined with the Trypan blue exclusion method. Neutrophil purity was greater than 95%. Approximately 100 million neutrophils were suspended in 3 ml of 0.9% saline. An emulsion was then prepared by mixing this suspension with an equal volume of Freund’s complete adjuvant. The emulsion was injected intradermally and subcutaneously into 10 to 15 sites along the back of the sheep. Ten days later, the sheep was inoculated with an emulsion consisting of 50 million cells and an equal volume of Freund’s incomplete adjuvant. After an additional 15 days, blood was collected from the sheep, and serum was tested for the ability to produce neutropenia in a pentobarbital-anesthetized dog. When the antibody titer was considered high enough, the sheep was exsanguinated and blood allowed to clot for 3 hours at room temperature and then stored at 4°C overnight. After centrifugation, the serum was collected and stored frozen (−60°C) until used for the experiments.
Adsorption of antiplatelet serum from the preparation. Initial tests in two anesthetized dogs indicated that the neutrophil antiserum was contaminated with antibodies that resulted in thrombocytopenia after serum injection into the dog. Before antibody injection, normal circulating platelet counts were noted and after injection of the antiserum, platelet counts decreased to less than 10% of the initial values. Thus, it was deemed necessary to adsorb the platelet antibodies with washed canine platelets. This was accomplished by incubation of the sheep serum with washed canine platelets for 30 minutes at 37°C and then removal of the platelets by centrifugation. After the antiserum had been treated as described, intravenous injection of antiserum into dogs did not affect circulating platelet counts.

Statistical Analyses

All data are expressed as the arithmetic mean±SEM. Data were compared among treatment groups by analysis of variance when there were more than two treatment groups. Subsequent comparisons were made using Dunnett’s method for multiple comparisons to a control group. All other data were compared with the respective control group by Student’s t test using Bonferroni’s method to control for experimental error when making multiple comparisons to the same control group. Profile analysis (MIDAS Statistical Programs, the University of Michigan Statistical Research Laboratory) was used for repeated hemodynamic measurements over time. The χ-square test for binomial distributions was used to test for differences among treatment groups in the incidence of ventricular fibrillation. Values of p<0.05 were considered significant.

Results

Myocardial Infarct Size

A total of 52 dogs was included in this study to determine the effectiveness of iloprost or neutrophil depletion on the infarct size which results after 90 minutes of regional ischemia and 72 hours of reperfusion (Figure 1). The ultimate size of myocardial infarct expressed as a percentage of the area at risk (IN/AR) that resulted under control conditions in the 72 hour reperfusion experiments reported herein (1. control [n=13], 48.7±5.6% IN/AR) was the same as the infarct size which resulted after 6 hours of reperfusion in a previous study.1 In the present study, dogs that were given iloprost for the initial 2 hours of reperfusion and then allowed to reperfuse for a total of 72 hours without additional drug therapy developed myocardial infarct sizes (2. Ilo-2 [n=5], 57.6±5.2% IN/AR) which were similar to control infarct sizes. However, if iloprost infusions were continued at a dose of 25 ng/kg/min over the first two days of reperfusion, myocardial infarct size was significantly reduced (3. Ilo-48, [n=10] 22.6±4.0% IN/AR; p<0.01) compared with control dogs or with dogs receiving iloprost for only 2 hours. Administration of antiserum against neutrophils over

\[
\begin{align*}
\text{FIGURE 1. Myocardial infarct size: 72-hour reperfusion experiments. Myocardial infarct size that resulted after 90 minutes of regional ischemia and 72 hours of reperfusion are expressed as a percentage of the area at risk. Points represent the infarct size for individual experiments. Solid circles represent the mean±SEM for each treatment group.} \\
\end{align*}
\]

3 days (4. antibody, n=11) resulted in an infarct size that was 32.1±5.0% of the area at risk. This was also a significant reduction compared with control (p<0.01). The amount of myocardium that was rendered ischemic was estimated by the area at risk as a percentage of the total left ventricle (AR/LV). There were no significant differences among the treatment groups with respect to AR/LV. The values of AR/LV were as follows: 1. control (n=13), 42.5±1.5%; 2. Ilo-2 (n=5), 47.6±2.0%; 3. Ilo-48 (n=10), 44.6±2.2%; 4. antibody (n=11), 44.1±2.3%.

Exclusions

Of the total number (52) of dogs used in the study, 13 were excluded from the final analysis. One control dog failed to demonstrate ST segment changes on the electrocardiogram and was excluded. Twelve dogs were excluded due to premature death from ventricular fibrillation including four control dogs, five antibody-treated dogs, two dogs that were to be treated with iloprost for 48 hours, and one dog that was treated with iloprost for 2 hours of reperfusion.

Incidence of Ventricular Fibrillation

There were no differences among the four groups with respect to the incidence of ventricular fibrillation as analyzed with the χ-square test for binomial distributions (χ-square, 1.12; p>0.05).

Hemodynamic Data

Hemodynamic values (Table 1) demonstrated that there were no differences in heart rate or rate pressure product at any time point measured during these experiments when analyzed by ANOVA for

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TABLE 1. Hemodynamic Values

<table>
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<td></td>
<td>baseline</td>
<td>15' occ</td>
<td>30' occ</td>
<td>60' occ</td>
<td>90' occ</td>
</tr>
<tr>
<td>1. Control</td>
<td>150±5</td>
<td>151±7</td>
<td>152±6</td>
<td>155±6</td>
<td>157±6</td>
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<tr>
<td>2. Ilo-2</td>
<td>155±5</td>
<td>131±2</td>
<td>142±10</td>
<td>152±12</td>
<td>160±9</td>
</tr>
<tr>
<td>3. Ilo-48</td>
<td>143±11</td>
<td>146±10</td>
<td>150±10</td>
<td>149±9</td>
<td>152±8</td>
</tr>
<tr>
<td>4. Antibody</td>
<td>151±7</td>
<td>158±9</td>
<td>150±8</td>
<td>156±9</td>
<td>159±8</td>
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No significant differences in heart rate.

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<th>Mean arterial pressure</th>
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<td>15' occ</td>
<td>30' occ</td>
<td>60' occ</td>
<td>90' occ</td>
</tr>
<tr>
<td>1. Control</td>
<td>107±2</td>
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<td>97±4</td>
<td>101±4</td>
<td>101±3</td>
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<td>2. Ilo-2</td>
<td>97±8</td>
<td>68±12*</td>
<td>64±11*</td>
<td>66±5*</td>
<td>74±7*</td>
</tr>
<tr>
<td>3. Ilo-48</td>
<td>109±3</td>
<td>80±5</td>
<td>75±5*</td>
<td>74±5*</td>
<td>75±5*</td>
</tr>
<tr>
<td>4. Antibody</td>
<td>112±8</td>
<td>95±8</td>
<td>98±7</td>
<td>101±9</td>
<td>102±9</td>
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</table>

*p<0.01, †p<0.05

TABLE 2. Left Circumflex Coronary Artery Blood Flow (ml/min)

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>1-Hour reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>29±3 (13)</td>
<td>25±2 (13)</td>
</tr>
<tr>
<td>2. Ilo-2</td>
<td>29±3 (6)</td>
<td>25±4 (6)</td>
</tr>
<tr>
<td>3. Ilo-48</td>
<td>24±2 (10)</td>
<td>23±2 (10)</td>
</tr>
<tr>
<td>4. Antibody</td>
<td>27±2 (11)</td>
<td>28±2 (11)</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SEM (number of experiments). There were no significant differences among the four treatment groups or within groups with time. Ilo-2, iloprost treatment for 2 hours; Ilo-48, iloprost treatment for 48 hours.

Each time point. However, iloprost did reduce mean arterial blood pressure during occlusion (p<0.01) and early reperfusion (p<0.05) in those two treatment groups. LCX blood flow (Table 2) was similar among the four treatment groups before occlusion and after one hour of reperfusion.

Regional Myocardial Blood Flow

Regional myocardial blood flow was determined in three of the four treatment groups (Tables 3 and 4): 1. control, 3. Ilo-48, and 4. antibody. There were no differences among groups in regional myocardial blood flow to the ischemic (Table 3) or the nonischemic (Table 4) regions of the myocardium after coronary artery occlusion. Therefore, the protective effect of iloprost or antibody treatment was not due to increased collateral blood flow to the ischemic myocardium.

Since collateral blood flow is an important factor affecting myocardial infarct size, the data are plotted as a function of collateral blood flow (Figure 2).

Circulating Neutrophil Counts

Compared with the control group at baseline (Figure 3), the antibody-treated group demonstrated >90% neutrophil depletion (p<0.001). Prior to the dose of antibody on days 1 and 2, there was an increase in the circulating neutrophil count at 24 and 48 hours, which declined significantly after antibody treatment. Neutrophil antibody or prolonged iloprost treatment (Ilo-
The extent of inflammatory cell infiltration when analyzed on the basis of heart tissue. The group that was given antibody (4. antibody) and the group that received iloprost over 48 hours (3. Ilo-48) had the least myeloperoxidase activity detected in the heart tissue, reflecting perhaps the accumulation of fewer neutrophils in the reperfused myocardial region.

Comparison Between Infarct Size and Extent of Inflammatory Cell Infiltration

The extent of inflammatory cell infiltration at the border region between infarcted tissue and noninfarcted tissue was assessed histologically by a pathologist (J.C.F.) who was unaware of the treatment regimen. Samples from each heart were evaluated and assigned to one of three groups (Figure 5) according to the extent of inflammatory cell infiltration. The three groups were A. Scant numbers of neutrophils present; B. moderate numbers of inflammatory cells present (intermediate infiltration); and C. marked infiltrate present (acutely inflamed). There were no significant correlations between the extent of neutrophil infiltration when analyzed on the basis of treatment groups. However, when the results were grouped irrespective of the original treatment (control, antibody, Ilo-2, or Ilo-48), there was a clear association between infarct size (IN/AR) and extent of inflammatory cell infiltration: the group that had the largest infarcts also had the greatest cell infiltration score (p<0.01).

Discussion

The extent of myocardial damage that develops in a canine model during 90 minutes of regional ischemia and 72 hours of reperfusion was limited by a sustained infusion of iloprost, a stable prostacyclin analogue. The data suggest that the mechanism of protection is independent of collateral blood flow to the ischemic myocardium (measured with radiolabeled microspheres) and is independent of the effects of iloprost on myocardial oxygen demand. These experiments provide evidence that prolonged neutrophil depletion also reduces myocardial infarct size that results after an extended period of reperfusion (72 hours). As before, the mechanism of myocardial protection is independent of myocardial oxygen demand (rate pressure product) or oxygen supply (collateral blood flow).

Previous studies have shown that short-term iloprost treatment reduces myocardial infarct size after 6 hours of reperfusion.\(^1\) The present experiments expand on the previous studies and demonstrate for the first time that short-term iloprost infusion does not effectively reduce the myocardial damage when determined after 72 hours of reperfusion. Thus, short-term therapy (terminated after 2 hours of reperfusion) delays the development of myocardial cell death, possibly as a result of the transient suppression of the inflammatory response during the initial hours of reperfusion. However, up to 48 hours of sustained infusion of iloprost is necessary to demonstrate myocardial protection after 72 hours of reperfusion.

One potential mechanism by which iloprost may inhibit myocardial ischemia and reperfusion injury is through an action whereby it inhibits neutrophil activation.\(^1\) The extent of myocardial injury after ischemia/reperfusion is in part dependent on neutrophils. Iloprost or prostacyclin has been shown to inhibit neutrophil activation in vitro and alter the pattern of neutrophil accumulation in dermal lesions.
TABLE 4. Nonischemic Myocardium: Regional Myocardial Blood Flow (ml/min/g tissue)

<table>
<thead>
<tr>
<th></th>
<th>1. Control (n=7)</th>
<th>2. Iloprost (n=6)</th>
<th>3. Antibody (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subendocardial</td>
<td>0.90±0.08</td>
<td>0.78±0.14</td>
<td>0.81±0.08</td>
</tr>
<tr>
<td>Midmyocardial</td>
<td>0.85±0.07</td>
<td>0.77±0.12</td>
<td>0.74±0.07</td>
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<tr>
<td>Subepicardial</td>
<td>0.71±0.05</td>
<td>0.62±0.11</td>
<td>0.55±0.07</td>
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<tr>
<td>Early occlusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subendocardial</td>
<td>0.93±0.07</td>
<td>0.92±0.16</td>
<td>0.81±0.12</td>
</tr>
<tr>
<td>Midmyocardial</td>
<td>0.90±0.06</td>
<td>0.89±0.16</td>
<td>0.76±0.13</td>
</tr>
<tr>
<td>Subepicardial</td>
<td>0.76±0.05</td>
<td>0.73±0.16</td>
<td>0.59±0.05</td>
</tr>
<tr>
<td>Late occlusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subendocardial</td>
<td>1.02±0.07</td>
<td>0.79±0.12</td>
<td>1.08±0.14</td>
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<tr>
<td>Midmyocardial</td>
<td>1.00±0.07</td>
<td>0.83±0.14</td>
<td>1.00±0.16</td>
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<tr>
<td>Subepicardial</td>
<td>0.80±0.06</td>
<td>0.67±0.12</td>
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</table>

No significant differences among treatment groups with ANOVA.

Iloprost treatment group represents only those dogs treated with iloprost for 48 hours (Ilo-48). Regional myocardial blood flow was not measured in the Ilo-2 group.

when administered systemically at a dose identical to that which reduces myocardial infarct size.\(^1\)\(^2\) The reduction in ultimate infarct size by iloprost is associated with a decrease in neutrophil accumulation within the infarcted region, especially in the border zone between irreversibly injured and viable myocardium when measured 6 hours after reperfusion.\(^3\)

Circulating neutrophil counts (Figure 3) in the Ilo-48 hour group at 24 and 48 hours after reperfusion were lower than that in the controls or the group of animals treated with iloprost for 2 hours (Ilo-2) after reperfusion. These data suggest that the sustained anti-inflammatory effect of continuous iloprost infusion prevents the rise in circulating neutrophil counts during myocardial infarction and ultimately, may be responsible for the observed beneficial effects on myocardial salvage.

Although neutrophils have been shown to be an important cellular component of the inflammatory response during the first 24 hours after ischemia and reperfusion myocardial injury,\(^2\) their role at later times is less clear. Myocardial myeloperoxidase activity, which has been correlated with histological evidence of neutrophil accumulation, was determined in myocardial tissue after 72 hours of reperfusion (Figure 4). Myeloperoxidase activity was not significantly different among the treatment groups in each of the three myocardial regions. When one compares the amount of myeloperoxidase present within the hearts after 72 hours of reperfusion to the activity within the hearts after 6 hours of reperfusion,\(^1\) it is apparent that the myocardial myeloperoxidase activity is greater after six hours of reperfusion than after 72 hours of reperfusion. Since iloprost effectively

![Figure 2](http://circres.ahajournals.org/)

**Figure 2.** Infarct size vs. collateral blood flow. Individual values are plotted for control (●), ilo-48 (□), and antibody (ab; ○), to demonstrate the relation between collateral blood flow (which is the subepicardial 1/3 myocardial blood flow in the ischemic region 5 minutes after coronary occlusion) and infarct size that results (expressed as a percentage of the area at risk [in/ar]). Linear regression analysis (equations in text) demonstrates that each of the three groups scribes a different line with the control group scribing the line with the steepest negative slope. This analysis suggests that for a given severity of ischemia the infarct size in the control group is larger than with ilo-48 or with antibody treatment.

![Figure 3](http://circres.ahajournals.org/)

**Figure 3.** Circulating neutrophil counts. Neutrophil counts were determined manually with a hemacytometer at the indicated times after baseline. Ilo-48 denotes dogs that received iloprost for 48 hours; ilo-2 denotes dogs that received iloprost for only the initial 2 hours after reperfusion. The antibody group received anti-neutrophil antibody at half-hour intervals until 2 hours of reperfusion and then at 24 and 48 hours. Occ, occlusion; 1 hr to 72 hr, time after reperfusion. *p<0.005, ilo-48 and antibody compared with control and ilo-2; **p<0.001, antibody compared with the other three treatment groups. Symbols represent the mean±SEM.
reduces neutrophil accumulation in the myocardium when determined after 6 hours of reperfusion, the most obvious explanation for the observed decrease in myeloperoxidase activity after 72 hours of reperfusion is that neutrophils that accumulate over three days of reperfusion have undergone autolysis. In addition, the myeloperoxidase that had been released would be inactivated or removed from the tissue.27 Thus, the myeloperoxidase activity of heart tissues after 72 hours of reperfusion may not provide an accurate measure of the cumulative amount of neutrophil infiltration. The results from the histological assessment of these tissues confirm this observation (Figure 5). While there is no clear-cut qualitative difference among the groups with regard to the intensity of the inflammatory cell infiltration in these tissues, there is a direct relation between the size of the infarct and the extent of cell infiltration. This is important since the variability of infarct size could not be related to the extent or degree of ischemia, that is, myocardial oxygen supply or demand was similar in all groups, yet infarct sizes were significantly smaller when fewer neutrophils were present in the tissue.

These experiments offer evidence that the reduced myocardial oxygen demand (decreased blood pressure) produced by iloprost is not responsible for the myocardial protection during temporary regional ischemia and reperfusion. Although both iloprost groups exhibited a similar reduction in blood pressure during occlusion, the group treated with iloprost during ischemia and the initial two hours of reperfusion (Ilo-2) had an infarct size essentially identical to that in the control group (Figure 1). In contrast, the group treated with iloprost for 48 hours had a reduced infarct size. Hemodynamic data were not recorded after 2 hours of reperfusion, and there is the possibility that myocardial oxygen demand was reduced over the first 48 hours of reperfusion in the group that received iloprost for 2 days. However, reduction of myocardial oxygen demand 45 minutes after the onset of ischemia does not result in reduced infarct size,28 therefore any decrease in blood pressure that occurred after 2 hours in the Ilo-48 treatment group probably did not contribute to limiting ultimate infarct size. Furthermore, the three groups in which regional myocardial blood flow was measured (control, antibody, Ilo-48), had similar myocardial blood flows (Tables 3 and 4) before coronary occlusion and during early and late occlusion. Thus, both myocardial blood supply and demand were similar among treatment groups.

Alternative mechanisms of protection by prostacyclin or iloprost have been suggested. These include prevention of destruction of adrenergic nerve terminals within the myocardium29 or a direct membrane stabilizing effect29 or preventing the loss of phospholipids during ischemia.30

**FIGURE 4.** Myocardial myeloperoxidase. On the top right of the figure is a schematic of a heart section demonstrating the typical patterns of infarcted and noninfarcted myocardium and the area at risk of infarction. Tissue biopsies from the hearts were taken after 72 hours of reperfusion from the central infarct region (top panel), from the interface between the infarcted and noninfarcted tissue (middle panel), and from the left ventricle within the area at risk but not infarcted (bottom panel). There were no statistically significant differences among the treatment groups in any of the three regions (ANOVA).

**FIGURE 5.** Comparison between infarct size and extent of inflammatory cell infiltration in heart tissues. Individual experiments are depicted as well as the mean±SEM for each group. A: Minimal infiltration, few neutrophils and monocytes; B: Intermediate infiltration, a few neutrophils present and some monocytes; C: Acutely inflamed, marked neutrophil infiltrate present. *p<0.01 compared with other two groups.
The most important observation obtained from these studies is that the ultimate size of myocardial infarction can be affected. Chambers and coworkers\(^3\) have suggested that some interventions do not salvage myocardium, but instead only delay the necrosis that “inevitably” occurs. This concept is based on the belief that if an intervention reduces infarct size after 6 hours of reperfusion, but not after 48 hours, the agent will not be of value in a clinical situation because the ultimate infarct size that determines clinical prognosis will not be smaller. The data support this concept in that iloprost treatment for 2 hours was previously reported to show a reduction in the extent of myocardial damage after 6 hours of reperfusion. However, in the present study this mode of treatment had no effect on the infarct size after 72 hours of reperfusion. More importantly, the data extend these observations and go on to define a critical time “window” (between 2 and 48 hours of reperfusion) for limiting the progression of myocardial tissue injury which occurs after reperfusion. Both prolonged neutrophil depletion and iloprost treatment for 48 hours reduce the infarct size determined after 72 hours of reperfusion. These studies demonstrate that anti-inflammatory treatment with modalities other than glucocorticoids will reduce the ultimate infarct size of Paul Hoff, Karl Lee, and Thomas McClanahan.

**Acknowledgments**

The authors acknowledge the technical assistance of Paul Hoff, Karl Lee, and Thomas McClanahan.

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**KEY WORDS** • myocardial infarction • ischemia • iloprost
Identification of a time window for therapy to reduce experimental canine myocardial injury: suppression of neutrophil activation during 72 hours of reperfusion.

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Circ Res. 1988;63:1070-1079
doi: 10.1161/01.RES.63.6.1070

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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