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

Paul J. Simpson, Joseph C. Fantone, Judith K. Mickelson, Kim P. Gallagher, and Benedict R. Lucchesi

Prostacyclin and iloprost (a chemically stable analogue of prostacyclin) are protective in a number of experimental models of myocardial ischemia. Recent studies provide substantial evidence demonstrating that polymorphonuclear leukocyte (neutrophil) infiltration during an evolving myocardial infarction contributes to irreversible myocardial injury. Since prostacyclin and iloprost inhibit in vitro production of cytotoxic oxygen free radicals and the release of degradative lysosomal enzymes, it was hypothesized that the cytoprotective effect of prostacyclin and iloprost in...
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.
3. Ilo-48. Control 3 included 10 dogs treated with iloprost (100 ng/kg/min; 0.5 ml/min) beginning 5 minutes after occlusion and continuing for 1 hour into the period of reperfusion. Then, the dose was decreased to 25 ng/kg/min (0.05 ml/min), which was maintained for 48 hours with a tether apparatus.

4. Antibody. Group 4 consisted of 11 dogs given sheep-derived antibody (antibody) directed against canine neutrophils (0.3 ml/kg i.v.) 30 minutes before occlusion and at half-hour intervals during the initial surgical preparations until 2 hours after reperfusion to maintain a neutrophil-depleted state. Additional single doses of antibody were administered to these dogs 24 and 48 hours after reperfusion.

**Histological Assessment of Myocardial Tissue**

Midventricular transmural sections from hearts from each of the four treatment groups were examined by light microscopy. The relative degree of neutrophil infiltration was assessed in a semiquantitative manner by a pathologist in a blinded fashion on hematoxylin and eosin stained sections of left ventricle.

**Determination of Regional Myocardial Blood Flow**

Regional myocardial blood flow was determined with tracer-labeled microspheres (15 μm diameter; New England Nuclear, Boston, Massachusetts) by the reference withdrawal method as described previously.1 Injections of microspheres (labeled with 51Cr, 85Sr, 141Ce, 103Ru, or 89Sc) were made in each experiment, with the order of the isotopes being randomized. Reference arterial blood samples were obtained simultaneously from both the femoral and carotid arteries at a constant rate with a withdrawal pump (Harvard Apparatus, South Natick, Massachusetts), beginning immediately before the injection of microspheres into the left atrium and ending 2 minutes later. The reference sample counts were averaged for calculation of myocardial blood flow. If the reference sample counts varied by more than 15%, the data were discarded. Each bottle of microspheres was placed in an ultrasonic bath with subsequent vortex agitation before injection to ensure that adequate dispersal of the microsphere suspensions was achieved before being administered. Regional myocardial blood flow was calculated by the formula: 

\[ Q_m = \frac{Q_r}{\text{counts/min}} \text{ in myocardial sample} \times \frac{\text{counts/min}}{\text{counts/min}} \text{ in reference sample} \times \frac{C_m}{C_r} \text{ where } Q_m \text{ is myocardial blood flow (ml/min), } Q_r \text{ is reference blood flow (ml/min), } C_m \text{ is counts/min in myocardial sample, and } C_r \text{ is counts/min in reference blood sample.} \]

Adjustments were made in blood flow to the ischemic region for apparent microsphere loss due to tissue edema.19-21

Regional myocardial blood flows were determined with microspheres before occlusion, 5 minutes after occlusion (before drug infusion), and 80 minutes after occlusion. Tissue samples weighing 0.5–1.0 g were dissected from the subepicardium, midmyocardium, and subendocardium of the perfusion bed supplied by the LCX and from the nonischemic region. At least 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.22 The myocardial content of myeloperoxidase activity has been correlated with histological evidence of neutrophil infiltration.23-24 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 antplatelet 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 Dunnetts 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 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 ioprost 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. In the present study, dogs that were given ioprost 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 ioprost 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 ioprost for only 2 hours. Administration of antiserum against neutrophils over

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. Values are mean±SEM for each treatment group. Solid circles represent the mean±SEM for each treatment group. Ilo-48 received ioprost for 48 hours after reperfusion; Ilo-2 received ioprost only for the initial 2 hours of reperfusion, antibody group received neutrophil antibody (neutrophil depletion). *p<0.05 compared with control or Ilo-2.

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 ioprost for 48 hours, and one dog that was treated with ioprost 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
TABLE 1. Hemodynamic Values

<table>
<thead>
<tr>
<th></th>
<th>Heart rate</th>
<th>Mean arterial pressure</th>
<th>Rate pressure product</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>baseline</td>
<td>15' occ</td>
<td>30' occ</td>
</tr>
<tr>
<td>1. Control</td>
<td>150 ± 5</td>
<td>151 ± 7</td>
<td>152 ± 6</td>
</tr>
<tr>
<td>2. Ilo-2</td>
<td>155 ± 5</td>
<td>131 ± 2</td>
<td>142 ± 10</td>
</tr>
<tr>
<td>3. Ilo-48</td>
<td>143 ± 11</td>
<td>146 ± 10</td>
<td>150 ± 10</td>
</tr>
<tr>
<td>4. Antibody</td>
<td>151 ± 7</td>
<td>158 ± 9</td>
<td>150 ± 8</td>
</tr>
</tbody>
</table>

No significant differences in heart rate.

<table>
<thead>
<tr>
<th></th>
<th>baseline</th>
<th>15' occ</th>
<th>30' occ</th>
<th>60' occ</th>
<th>90' occ</th>
<th>1 hr rep</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>107 ± 2</td>
<td>95 ± 6</td>
<td>97 ± 4</td>
<td>101 ± 4</td>
<td>101 ± 3</td>
<td>97 ± 5</td>
</tr>
<tr>
<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>
<td>73 ± 4†</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>
<td>84 ± 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>
<td>104 ± 8</td>
</tr>
</tbody>
</table>

*p < 0.01, †p < 0.05

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).

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. Least-squares estimates of the linear regressions for the three groups are as follows:

control (n = 7), IN/AR = 83.3 - 324.6 × blood flow, r = 0.91, p < 0.01; Ilo-48 (n = 6), IN/AR = 23.1 - 26.9 × blood flow, r = 0.32, p < 0.10; antibody (n = 6), IN/AR = 46.6 - 131.3 × blood flow, r = 0.80, p < 0.25.

Although only the linear regression of the control group had a significant correlation, the relations among the various groups are such that for a given severity of ischemia, especially under conditions of low collateral blood flow (e.g., < 0.10 ml/min/g), the resulting infarct size in the Ilo-48 group was smaller than that in either the antibody-treated or control groups. In addition, for a given severity of ischemia, the infarct size in the antibody-treated group was smaller than that of the control group. Thus, in the present study, collateral blood flow is not the factor responsible for infarct size reduction with iloprost treatment or with neutrophil depletion (antibody).

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-
extent of inflammatory cell infiltration: the group with 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. 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. 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>4. Antibody (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></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>
</tr>
<tr>
<td>Subepicardial</td>
<td>0.71±0.05</td>
<td>0.62±0.11</td>
<td>0.55±0.07</td>
</tr>
<tr>
<td><strong>Early occlusion</strong></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><strong>Late occlusion</strong></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>
</tr>
<tr>
<td>Midmyocardial</td>
<td>1.00±0.07</td>
<td>0.83±0.14</td>
<td>1.00±0.16</td>
</tr>
<tr>
<td>Subepicardial</td>
<td>0.80±0.06</td>
<td>0.67±0.12</td>
<td>0.70±0.10</td>
</tr>
</tbody>
</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.1,7 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.1

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,24 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](image-url)  
**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](image-url)  
**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 hours 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.
reduce 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. 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, 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 pros- tacyclin or iloprost have been suggested. These include prevention of destruction of adrenergic nerve terminals within the myocardium or a direct membrane stabilizing effect or preventing the loss of phospholipids during ischemia.
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 after ischemia and reperfusion. The inhibition of neutrophil activation and accumulation in the myocardium over a prolonged period of time (<48 hours) is necessary to ensure a beneficial effect on the ultimate extension of irreversible myocardial injury associated with ischemia and reperfusion.

Acknowledgments

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

References

11. Fantone JC, Kinnes DA: Prostaglandin E1 and prosta
glandin 12 modulation of superoxide production by human neutro
specificity of prostaglandin inhibition of rabbit polymorphono
cuclear leukocyte lysosomal enzyme release and superoxide anion production. Am J Pathol 1984;115:9–16
13. Lucchesi B, Burnmester W, Lomas T, Abrams G: Ischemic changes in the canine heart as affected by the dimethyl
quaternary analogue of propranolol, UMT 272 (SC 27761). J Pharmacol Exp Ther 1976;199:310–328
20. Reimer K, Jennings R: The "wavefront phenomenon" of myocardial ischemic cell death. Trends in the progression of necrosis within the framework of ischemic bed size (myocardium at risk) and collateral flow. Lab Invest 1979;40:633–644
29. Smith EF, Gallenkamper W, Beckman R, Thomsen T, Mannesmann, Schror K: Early and late administration of a

**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.
P J Simpson, J C Fantone, J K Mickelson, K P Gallagher and B R Lucchesi

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
Copyright © 1988 American Heart Association, Inc. All rights reserved.
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:
http://circres.ahajournals.org/content/63/6/1070

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/