Myocardial Protection With Preconditioning

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Myocardial preconditioning with brief coronary artery occlusions before a sustained ischemic period is reported to reduce infarct size. To determine the number of occlusive episodes required to produce the preconditioning effect, we performed single or multiple occlusions of the left circumflex coronary artery (LCx) followed by a sustained occlusion (60 minutes) of the LCx. Anesthetized dogs underwent one (P1), six (P6), or 12 (P12) 5-minute occlusions of the LCx. Each occlusion was followed by a 10-minute reperfusion period. A 60-minute occlusion of the LCx followed the preconditioning sequences. A control group received a 60-minute occlusion of the LCx without preconditioning. All groups were subjected to 6 hours of reperfusion after which the heart was removed for calculating infarct size (IS), area at risk (AR), and left ventricular mass (LV). The IS/AR ratio for the control group was 29.8±4.4% (n=17), which was substantially greater (p<0.001) than that of the preconditioned groups: P1, 3.9±1.3% (n=14); P6, 0.4±0.3% (n=5); and P12, 2.9±2.8% (n=5). There were no significant differences in the IS/AR ratio among the three preconditioned groups. The AR/LV ratio was comparable among all groups and did not differ statistically: control, 40.4±1.3%; P1, 36.2±1.7%; P6, 36.1±1.7%; and P12, 37.3±2.1%. Collateral blood flow to the inner two thirds of the risk region determined with radiolabeled microspheres during ischemia did not differ significantly between the control group (0.03±0.01 ml/min/g, n=8) and single occlusion group (0.06±0.02 ml/min/g, n=8), indicating that the marked disparity in infarct size could not be attributed to differences in collateral blood flow. The data indicate that preconditioning with one brief ischemic interval is as effective as preconditioning with multiple ischemic periods. (Circulation 1990;82:609-619)

Timely reperfusion of the ischemic heart in experimental animals limits the extent of irreversible myocardial injury1,2 and enhances the long-term prognosis for survival in patients with coronary artery disease.3 Such findings have prompted extensive efforts directed at salvage of jeopardized myocardial tissue by altering the wavefront progression of irreversible injury due to ischemia.4 A notable recent development is the demonstration that repeated brief episodes of ischemia, not long enough to culminate in tissue necrosis, can render the heart more resistant to ischemic injury.

For example, Geft et al5 noted that exposure of the canine heart to intermittent regional ischemic intervals followed by reperfusion was frequently unaccompanied by evidence of necrosis. Swain et al6 compared the effects of three 12-minute ischemic periods with one 12-minute ischemic episode in terms of high-energy phosphate depletion and observed no difference, which suggested that the reduction in contractile function observed after reperfusion may protect the heart from subsequent ischemia by reducing myocardial oxygen requirements and ATP turnover.7 Reimer et al8 calculated adenine nucleotide concentrations and myocardial necrosis in dogs 4 days after four 10-minute periods of ischemia. They confirmed that intermittent ischemia and reperfusion minimized cumulative metabolic deficits. They also demonstrated the striking finding that myocardial necrosis was reduced significantly. The investigators attributed the protective effects of repetitive reperfusion to the capacity of myocardial tissue to replenish high-energy phosphates or removal of deleterious catabolites.

More recently, Murry et al9 coined the term "preconditioning" to describe the effect of brief coronary occlusions on infarct size produced by subsequent, prolonged occlusion. They were the first to directly test the hypothesis that repeated, brief ischemic episodes modify infarct size. Canine hearts were preconditioned with four 5-minute occlusions of the
circumflex coronary artery, each separated by 5 minutes of reperfusion. Then, a 40-minute sustained occlusion was performed, and infarct size was measured 4 days later. The preconditioned hearts were characterized by a 75% reduction in infarct size compared with hearts of control animals that underwent a 40-minute occlusion without preconditioning.

The present study tested the hypothesis that preconditioning provides myocardial protection during 60 rather than 40 minutes of regional ischemia, a time period associated with more irreversible injury than 40 minutes of ischemia. The second objective was to evaluate the potential efficacy of single, as opposed to multiple, preconditioning occlusions in terms of protection from myocardial injury. The results of the present study confirm that preconditioning is effective, even with the more intense ischemic insult produced by 60 minutes of coronary occlusion. In addition, we observed that a single preconditioning occlusion of 5 minutes in duration was as effective as six or 12 5-minute occlusions.

Methods

General Surgical Preparation

The experiments that form the basis of this report were conducted according to the guidelines of the American Physiological Society regarding the use of laboratory animals and 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 for 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,” DHEW Publ. No. NIH 78-23.

Study Population and Surgical Procedures

Male mongrel dogs, weighing between 13.0 and 20.0 kg, were anesthetized with an intravenous injection of diallyl barbituric acid and urethane (0.6 ml/kg). The animals were intubated with a cuffed endotracheal tube and ventilated with room air on a Harvard respirator (Harvard Apparatus, South Natick, Mass.) at a tidal volume of 20 ml/kg and a rate of 10 cycles/min. The left jugular vein was isolated and cannulated with a polyethylene catheter for the administration of intravenous fluids. A polyethylene catheter was placed in a femoral artery for measurement of blood pressure by a calibrated Statham physiological pressure transducer (Gould-Statham, Cleveland, Ohio). In experiments where regional myocardial blood flow was measured with radioactive microspheres, the left carotid and left femoral arteries were cannulated for the simultaneous withdrawal of reference blood samples.

The heart was exposed by means of a thoracotomy in the fifth intercostal space. The pericardium was opened just below the border of the left atrial appendage, and the heart was supported in a pericardial cradle. The proximal left circumflex coronary artery (LCx) was dissected away from the underlying tissue at the border of the left atrial appendage. A calibrated electromagnetic flow probe (Carolina Electromagnetic Flowmeter, Carolina Medical Electronics, King, N.C.) was placed on the LCx for the continuous recording of coronary blood flow. A Silastic snare occluder, pulled through a short piece of polyethylene tubing and secured with a hemostat, was used to obstruct the artery. A mechanical screw occluder was placed proximal to the snare and was adjusted to produce a critical stenosis. This approach has been shown to attenuate the hyperemic response on reperfusion and to reduce the severity and frequency of reperfusion arrhythmias and the development of hemorrhagic infarction.

On reperfusion, the mechanical screw occluder was released gradually throughout a 5-minute period during the preconditioning occlusions and during a 30-minute period after the 60-minute occlusion. The reperfusion blood flow was increased to a value not to exceed the basal flow rate that existed before interruption of the LCx blood flow. A polyethylene catheter was inserted into the left atrial appendage, secured in place with a single suture, and was used for the injection of radioactive microspheres. Leads II, III, and aVF of the electrocardiogram, LCx blood flow, and arterial blood pressure were monitored and recorded on a polygraph (model 7, Grass Instruments, Quincy, Mass.) throughout the experiment.

Experimental Protocol

The design of the experimental protocol is diagrammed in Figure 1. The animals were allocated to one of three groups subjected to three different preconditioning protocols or to a control group that did not undergo preconditioning. A preconditioning protocol consisted of a 5-minute occlusion of the LCx, followed by a 10-minute reperfusion period. Three different preconditioning sequences were used. Dogs received either one (P1), six (P6), or 12 (P12) preconditioning sequences in which the respective cumulative ischemic times were 5, 30, or 60 minutes with a 10-minute period of reperfusion between each 5-minute period of ischemia. The final preconditioning episode was followed by a 10-minute period of reperfusion after which the LCx was occluded for 60 minutes and then reperfused for 6 hours. Then, the heart was fibrillated with 10-V, 60-Hz square-wave pulses applied directly to the left ventricle. Thereafter, the heart was excised and prepared for histochemical determination of infarct size (and for determination of regional myocardial blood flow in a subset of the control and P1 animals).

Determination of Myocardial Infarct Size

After excision of the heart, the size of the anatomic area at risk and extent of irreversible injury were determined with an ex-vivo dual perfusion technique as described previously. A cannula was inserted
into the aorta and secured with a suture. The proximal LCx was ligated, and a catheter was inserted and fixed in place immediately distal to the point of ligation. The aorta was perfused retrograde with solution containing 0.25% Evans blue dye, and the LCx was perfused with 1.5% triphenyl tetrazolium chloride (TTC) in 20 mM potassium phosphate buffer adjusted to pH 7.4. The solutions and heart were maintained at a temperature of 37°C. The solutions were infused simultaneously for 5 minutes at a constant pressure of 100 mm Hg. The heart was removed from the perfusion apparatus and cut into four to six transverse sections approximately 1 cm thick. The transverse sections were weighed, and both surfaces were traced onto clear plastic overlays for subsequent calculation of infarct size. The anatomic area at risk was demarcated by the absence of Evans blue dye, and the TTC negative region representing irreversibly injured myocardium was indicated by the absence of red formazan precipitate. The respective regions corresponding to the left ventricle, anatomic area at risk, and infarct zone were calculated with a Kurta digitizing board (Kurta Corporation, Phoenix, Ariz.), an Apple Macintosh Computer and appropriate software (MacDraft, Innovative Data Design, Inc., Concord, Calif.) for area determination of the digitized tracing. A custom software program, with the respective areas and weight of each transverse ventricular section, was used to calculate the weight of the infarct zone, anatomic area at risk, and left ventricle. Previous studies demonstrated that there is an excellent correlation between infarct size derived by the planimetric method and the direct gravimetric measurement of infarct size. The infarct region is expressed as a percentage of the area at risk or as a percentage of the total left ventricular weight. The fidelity of the TTC method for identifying irreversibly injured myocardial tissue has been verified by Vivaldi et al and by Romaschin et al, demonstrating that microscopic and ultrastructural changes of irreversible injury occurred in areas of the myocardium that were TTC negative.

**Determination of Regional Myocardial Blood Flow**

Regional myocardial blood flow in a subset of control and P1 dogs was determined with radioactive microspheres (15-μm diameter, New England Nuclear, Boston, Mass.) with the reference withdrawal method. The microspheres labeled with cesium 141, ruthenium 103, or scandium 46 were prepared for administration by sonication in an ultrasonic bath and agitation with a vortex mixer. One milliliter of the suspended microspheres (1–2 million microspheres) was diluted in warm saline (37°C) and injected into the left atrium during a 30-second period, followed by two flushes with 10 ml warm saline. Dual reference blood samples were withdrawn simultaneously from one femoral and one carotid artery at 3.47 ml/min with a Harvard withdrawal pump beginning immediately before injection of the microspheres and continuing for a period of 2 minutes. 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.

Regional myocardial blood flow measurements were made at baseline, 2.5 minutes into the 5-minute preconditioning occlusion, and 55 minutes into the 60-minute occlusion in eight of the P1 dogs. In eight of the control animals, microspheres were injected at baseline and 55 minutes into the sustained 60-minute occlusion period. Transverse sections of the heart from the central ischemic and nonischemic zones as demarcated by the dual perfusion method with Evans blue dye and TTC were dissected into endocardial, midmyocardial, and epicardial thirds weighing 0.5 to 1.0 g. Tissue samples from the ischemic and control areas of four of the left ventricular slices were used.
Samples were obtained 1 cm or more from the perfusion boundaries delineated with TTC and Evans blue dye. Each heart tissue specimen and the withdrawal blood samples were assayed for radioactivity using a Tracor model 1185 gamma counter (TM Analytic, Elk Grove, Ill.). Regional myocardial blood flow was calculated with the equation: \( Q_m = (C_m \times Q_i) / (C_i \times G) \), where \( Q_m \) is myocardial blood flow (ml/min/g), \( C_m \) is counts/min in tissue samples, \( Q_i \) is withdrawal rate of the reference arterial sample (ml/min), \( C_i \) is counts/min in the reference arterial sample, and \( G \) is tissue sample weight. Endocardial, midmyocardial, and epicardial blood flows in each dog were calculated by averaging blood flow from the four ischemic and four control tissue samples.

**Exclusion Criteria**

Experimental animals were excluded from additional data analysis according to the following criteria: 1) failure to manifest electrocardiographic signs of ischemia (no ST segment elevation) in leads II, III, or aVF and epicardial cyanosis and akinesia in the region of distribution of the LCx after occlusion of the coronary artery, 2) intractable ventricular fibrillation requiring more than three attempts at defibrillation with low-energy DC (10-J) pulses applied directly to the surface of the heart, 3) presence of heart worms on final examination of the heart.

In experiments that included regional myocardial blood flow, the data were excluded from analysis if endocardial blood flow, determined 55 minutes into the period of sustained LCx occlusion, was greater than 0.15 ml/min/g of tissue.9 The data also were analyzed with a more stringent criteria with subendocardial collateral blood flow of greater than 0.10 ml/min/g of tissue being excluded from analysis. One dog in the P1 group died 3 hours into reperfusion and was included in the data analysis.

**Statistics**

The data are expressed as mean±SEM. Differences were considered significant if \( p \) was less than 0.05. Infarct size, hemodynamic, and collateral blood flow data were evaluated with analysis of variance and Scheffe’s multiple comparison confidence intervals to detect group differences in collateral blood flow, rate-pressure product (heart rate multiplied by mean arterial blood pressure), area at risk/left ventricle ratio, infarct/area at risk ratio, and infarct/left ventricle ratio. For comparison of values within each group over time points, repeated measures and the Scheffe’s test were used. Where ischemic collateral blood flow data were available at two time points in the P1 group, a paired \( t \) test (two tailed) was used for data analysis. Analysis of covariance, by regional collateral blood flow in the inner two thirds of the ventricular wall as the covariate, was used to account for the effect of collateral blood flow on infarct size expressed as a percentage of the risk region.

**Results**

This study was performed in two phases. In the first phase of the study, one group of animals (designated as P12) was subjected to twelve 5-minute preconditioning occlusions of the LCx, each separated by a 10-minute period of reperfusion. Two additional groups with one (P1) or six (P6) preconditioning ischemic periods also were studied. The second phase of this study was a repetition of the P1 protocol with the addition of regional myocardial blood flow measurements by the microsphere technique. The control group of animals was managed in the same manner except the hearts were not preconditioned with short periods of regional ischemia. In each phase, control and preconditioned animals were assigned randomly to the study groups.

Seventy dogs were used in this study, of which 27 were randomized to the control group. Ten control dogs were excluded from analysis: Eight dogs developed intractable ventricular fibrillation and two animals failed to manifest ischemic ST segment elevation. One dog in the control group was successfully resuscitated with less than three defibrillation attempts and was included in the data analysis.

There were 20 dogs in the P1 group. Six dogs were excluded from analysis: Three dogs developed intractable ventricular fibrillation, and three dogs failed to exhibit ischemic ST segment elevation. In the P6 group, eight animals were studied, and three animals were excluded from analysis: Two dogs developed intractable ventricular fibrillation, and one dog did not have ischemic ST segment changes. In the P12 group, very high mortality was encountered. Fifteen animals were randomized to this group, and nine dogs developed intractable ventricular fibrillation, and one dog was excluded because of a failure to manifest electrocardiographic changes consistent with regional ischemia. Thus, the final data analysis included 17 control, 14 P1, five P6, and five P12 animals.

**Hemodynamics**

The rate-pressure product and other hemodynamic parameters are presented in Table 1. The data are presented at baseline, at the end of the preconditioning sequences just before the application of the sustained 60-minute occlusion, at the end of the 60-minute occlusion, and during reperfusion at 1, 3, and 6 hours after the release of the LCx occlusion. There were no differences in mean values compared with control values at each respective time point except in the P6 group in which the mean arterial pressure during each reperfusion time point differed significantly from the corresponding times in the control group. The hemodynamic parameters at each of the time points were analyzed with repeated measures ANOVA. Significant decreases in mean arterial blood pressure, the rate-pressure product, and coronary blood flow were noted in the control group during sustained regional ischemia and reperfusion. Significant reductions in mean arterial pressure and coro-
Table 1. Hemodynamic Data

<table>
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<th>Occlusion 60 min</th>
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</table>

Values are mean±SEM.

*p<0.05 ANOVA repeated measures compared with baseline; †p<0.05 ANOVA factorial compared with control.

Coronary blood flow were recorded in the P1 group at the end of reperfusion. Coronary blood flow at the end of reperfusion was reduced significantly compared with baseline values in the control and P1 groups. The rate-pressure product, an estimate of myocardial oxygen consumption, was not significantly different in the preconditioned groups compared with the control group or over time compared with baseline, although there was a trend downward during preconditioning, sustained LCx occlusion, and early reperfusion. The only significant intergroup difference occurred between the control and P6 groups in the postreperfusion values for mean arterial blood pressure. Overall, the hemodynamic parameters were comparable across groups, making it unlikely they could account for differences in infarct size.

Infarct Size

The effects of single and multiple preconditioning occlusions on infarct size are presented in Figure 2. Infarct size expressed as a percentage of the anatomic area at risk was reduced significantly (p<0.0001) in each preconditioned group compared with the control group (n=17). Infarct size in the control group as a percentage of the area at risk was 29.8±4.4%, in marked contrast to infarct size that averaged 3.9±1.3% in the P1 group, 0.4±0.3% in the P6 group, and 2.9±2.8% in the P12 group. Similarly, infarct size as a percentage of the left ventricle for the preconditioned groups was reduced significantly (p<0.0001) compared with control hearts. Infarct size as a percentage of the left ventricle for the control group was 12.4±2.0% compared with 1.5±0.6%, 0.1±0.1%, and 1.0±1.0% in the P1, P6, and P12 groups, respectively. Infarct size compared among the three preconditioned groups did not differ significantly. The anatomic area at risk as a percentage of the left ventricle was not significantly different across groups: control, 40.4±1.4%; P1, 36.2±1.7%; P6, 36.1±1.7%; and P12, 37.3±2.1%.

Because infarct size in the dogs with one ischemic preconditioning occlusion was not significantly different from the dogs with six and twelve 5-minute preconditioning episodes, we performed experiments in a subset of control (n=8) and P1 (n=8) dogs in which regional myocardial blood flow determinations were made with radiolabeled microspheres [microsphere (+)] to determine whether differences in regional blood flow could account for the effects of preconditioning on infarct size. Data from these animals were included in the pooled analysis presented above. Nine dogs in each group completed the protocol, but one animal in each group was excluded from the microsphere (+) analysis because of technical problems. Figure 3 summarizes the infarct size results of control (n=8) and P1 (n=8) dogs with regional myocardial blood flow measurements. Infarct size (percentage of area) was 35.8±7.7% and 4.7±1.6% (p<0.005) in the control and P1 subsets, respectively. Infarct size (percentage of left ventricle) was 14.3±3.4% in the control subset and 1.8±0.6% (p<0.005) in the P1 subset. Risk areas were not significantly different (control, 39.4±1.5%; P1, 36.7±2.5%). All control and P1 dogs with regional myocardial blood flow measurements had...
subendocardial flows less than 0.15 ml/min/g tissue. When infarct size was analyzed using dogs with subendocardial blood flows less than 0.10 ml/min/g tissue, only one animal from the P1 group had to be eliminated (subendocardial blood flow of 0.133 ml/min/g tissue). All control animals had subendocardial blood flows less than 0.10 ml/min/g tissue. Infarct size in the P1 (n=7) subset of hearts as a percentage of risk region was 5.3±1.7% and as a percentage of left ventricle was 2.0±0.7%, which are values also significantly smaller from those characterizing the control group. The risk area was 37.0±3.0% of the left ventricle for the smaller subset of P1 and was not significantly different from that of the control group.

No difference in infarct size was observed between the groups with [microsphere (+)] and without [microsphere (-)] regional myocardial blood flow determination. Six of the 14 dogs in the P1 group did not receive microsphere injections for regional blood

**Figure 2.** Bar graph comparing effects of the three separate preconditioning protocols on myocardial infarct size with respect to each other as well as to a control group that was not subjected to preconditioning. All hearts underwent 60 minutes of regional ischemia followed by 6 hours of reperfusion. Infarct size in each preconditioned group was less than that in the unconditioned control group. Comparison of infarct sizes among the three preconditioned groups did not show any significant differences. Area at risk of infarction did not differ among the four groups, therefore, suggesting that the protective effect of preconditioning was independent of this baseline predictor of infarct size. Bars represent group mean±SEM. IZ, ischemic zone; AR, area at risk; LV, left ventricle.

**Figure 3.** Bar graph of the effect of preconditioning on myocardial infarct size in a subset of control and P1 animals in which regional myocardial blood flow was determined with radiolabeled microspheres. Infarct size expressed as a percentage of the risk region (IZ/AR) or as a percentage of the left ventricle (IZ/LV) is smaller in hearts that were preconditioned (P1) with one 5-minute episode of regional ischemia compared with control group that was not subjected to preconditioning; control vs. P1 IZ/AR, 35.8±7.7% vs. 4.7±1.6%. Area at risk expressed as a percentage of the left ventricle (AR/LV) did not differ between groups; control vs. P1 AR/LV, 39.4±1.5% vs. 36.7±2.5%. Transmural mean myocardial blood flow (ml/min/g tissue) determined 55 minutes into the 60-minute occlusion period did not differ between groups and averaged 0.053±0.012 in the control group and 0.085±0.023 in the P1 group (p=0.258). Two major baseline determinants of infarct size, risk region size, and collateral blood flow being similar in both groups, could not account for the observed decrease in infarct size in the P1 group.
flow determinations. The infarct sizes as a percentage of risk area and left ventricle were 2.9±2.3% and 1.2±1.0%, respectively, and did not differ significantly from microsphere (+) dogs with subendocardial flows below 0.15 or 0.10 ml/min/g tissue. The risk area as a percentage of left ventricle was 35.5±2.4%, similar to microsphere (+) groups (p=NS).

When a similar analysis of infarct size with control animals was performed, there was no significant difference between microsphere (−) and microsphere (+) dogs. Infarct size as a percentage of the risk region was 24.5±4.5% in microsphere (−) dogs and 35.7±7.7% (p=NS) in microsphere (+) dogs. Infarct size as a percentage of the left ventricle was 10.7±2.3% and 14.3±4.4% for microsphere (−) and microsphere (+) dogs, respectively (p=NS). The sizes of the risk regions were also similar, averaging 41.3±3.2% in the microsphere (−) and 39.4±1.5% (p=NS) of the left ventricle in the microsphere (+) dogs.

**Regional Myocardial Blood Flow Measurements**

Regional myocardial blood flow in the subendocardial, midmyocardial, and subepicardial thirds, as well as average flow in the inner two thirds and across the entire myocardial wall of the left ventricle, are summarized in Table 2 for the subsets of control and P1 animals in which radiolabeled microspheres were used. Baseline values are presented for both the control and P1 groups of animals. A microsphere flow determination was made in the P1 group at the time of the 5-minute preconditioning episode. Both groups had regional coronary blood flow determinations obtained 55 minutes after occlusion of the LCx. As noted above, animals entered into the final data analysis had to satisfy the criterion that subendocardial flow did not exceed 0.15 ml/min/g. A previous study suggested that regional blood flows below this value were associated with irreversible myocardial injury. A more stringent cutoff value for subendocardial collateral blood flow, less than 0.10 ml/min/g, also was used to analyze infarct size, but only one animal from the P1 group had to be eliminated.

<table>
<thead>
<tr>
<th>Table 2. Regional Myocardial Blood Flow</th>
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<tr>
<td><strong>Group</strong></td>
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</tr>
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<td>Control (n=8)</td>
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<td>Baseline</td>
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<tr>
<td>Occlusion 55 min</td>
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<tr>
<td>P1 (n=8)</td>
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<td>Baseline</td>
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<td>Preconditioning occlusion</td>
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<td>Occlusion 55 min</td>
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</table>

Values are mean±SEM (ml/min/g).

LCx, left circumflex coronary artery; LAD, left anterior descending coronary artery; Endo, subendocardium; Mid, midmyocardium; Epi, subepicardium; Inner 2/3, subendocardium and midmyocardium; Trans, transmural.

*p<0.05 by two-tail paired t test (P1 occlusion compared with Occlusion 60 min); †p<0.05 compared with control by ANOVA factorial Scheffe’s F test.

Regional myocardial blood flow was not altered significantly in any of the myocardial layers of the left anterior descending coronary artery perfused territory (control area) compared with baseline blood flows. The mean subendocardial and transmural collateral blood flows in the LCx region during the 5-minute P1 preconditioning occlusion were 0.03±0.01 and 0.06±0.01 ml/min/g, respectively. During the sustained (60-minute) ischemic period in the control group, subendocardial and transmural collateral blood flows were 0.02±0.01 and 0.05±0.01 ml/min/g, respectively, in the LCx region. Collateral blood flow during the sustained ischemic period in the P1 group was 0.05±0.02 ml/min/g in the subendocardium and 0.09±0.02 ml/min/g transmurally, which are values not significantly different from the control group values. Therefore, differences in regional myocardial blood flow in the control and P1 groups during the 60-minute period of LCx occlusion could not account for the marked discrepancy in infarct size observed between the control and P1 groups. An increase in collateral blood flow was observed in the P1 group between the measurements obtained midway through the 5-minute preconditioning occlusion and those obtained after 55 minutes of the 60-minute period of ischemia (Table 2). The flow values, however, remained lower than our exclusion criterion, supporting the view that they had limited biological significance. More important is the observation that there were no significant differences between the control and P1 groups in terms of regional myocardial blood flow in any of the myocardial layers or combinations of layers (Table 2).
covariate and the treatment \((F=0.004, p=0.948)\). The regression lines for the control and preconditioned group (P1) were significantly different by analysis of covariance \((F = 18.9, p=0.001)\). The regression line relating infarct size to collateral blood flow in the P1 group was shifted markedly downward compared with the control infarct-flow relation. Therefore, for a given collateral blood flow (below approximately 0.07 ml/min/g), infarct size as a percentage of the risk area was dramatically smaller in the P1 group.

Because randomization alone does not ensure equivalent distribution of independent variables, covariate analysis for several known covariates (area at risk, regional collateral blood flow, and rate-pressure product) was also performed, and it demonstrated comparability among these determinants of infarct size. The difference in infarct size observed between the two subset groups of control and P1 animals could not be explained by an unequal distribution of one or more of the independent variables that might have biased the final results.

**Discussion**

The model of ischemic preconditioning we used involved either one, six, or 12 sequential episodes of 5-minute LCx occlusions, each followed by 10 minutes of reperfusion. Infarct size due to a subsequent prolonged coronary occlusion was drastically reduced by the preconditioning regimens compared with control hearts that were not preconditioned, confirming the results of Murry et al., who first reported the salutary effects of preconditioning in terms of infarct size modification. Our findings also extend those of Murry et al. in several respects. Infarct size expressed as a percentage of the risk region or as a percentage of the left ventricle was similar in each of the groups subjected to preconditioning stimuli regardless of the number of preconditioning events. A single, 5-minute ischemic episode before a sustained coronary occlusion of 60 minutes in duration was sufficient to precondition the myocardium. Multiple, repetitive ischemic periods did not add to the protective effect, nor did they diminish the extent of the protection. Furthermore, the occlusion time over which preconditioning was demonstrated to be effective was 60 rather than 40 minutes. In our view, the most notable finding is the observation that intermittent ischemia is not required to induce the preconditioning effect. Only a brief 5-minute prior ischemic period is necessary.

The major predictors of myocardial necrosis in the canine heart include the size of the anatomic risk area, collateral blood flow, and myocardial oxygen consumption. There were no significant differences in area at risk. Rate-pressure product, an indirect measure of myocardial oxygen consumption, also did not differ significantly among the groups. The exclusion criteria used in this laboratory assist in preventing entry of animals with high collateral blood flow into the experimental protocol. Dogs without ST segment elevation, epicardial cyanosis, and akinesia are excluded from further consideration. In the subset of control and P1 dogs, all subendocardial collateral blood flows were below 0.15 ml/min/g tissue, a value proposed as ensuring ischemia. Collateral blood flow was measured in the central risk zone, which is representative for the risk area given the abrupt nature of the interface between ischemic and nonischemic myocardium produced by acute coronary occlusion.

The extremely low value of collateral blood flow to the subendocardium and inner two thirds of the left ventricular wall (Table 2 and Figure 4) indicates that factors other than collateral blood flow were responsible for protecting ischemic myocardium from progressing to the point of irreversible injury. There was a slight, but statistically significant, rise in ischemic LCx collateral flow between the preconditioning occlusion and the sustained occlusion in P1 animals. The extent of regional collateral blood flow during
the sustained ischemic episode, however, remained very low and was not statistically different from that in the control group. The possibility exists that myocardial blood flow was significant and reduced ischemic injury in the P6 and P12 groups because radioactive microspheres were not used. However, we believe that with the use of our other exclusion criteria, only dogs with low collateral blood flows were likely to have been included. Another limitation is the small number of animals in the P6 and P12 groups. Because there were only five animals in each group, a larger number of dogs may show a significant difference between preconditioned groups. The power between the P1 and P6 is low (<10%) and would require a large number of dogs to detect a significant difference (α=0.05) with acceptable power (80%). The difference in infarct size among the preconditioned groups is small (<5%), which we believe is not physiologically significant.

In rats, preconditioning24 with four 5-minute periods of regional ischemia and reperfusion followed by 45 minutes of sustained ischemia was accompanied by a reduction in infarct size (22.3±3.7% in controls to 3.0±3.9% in preconditioned hearts). After reflow, preconditioned hearts were characterized by significant recovery of phosphocreatine and ATP (measured with in vivo phosphorous 31 nuclear magnetic resonance imaging) compared with control hearts, suggesting that preservation or accelerated repollication of high-energy phosphate stores contribute to the beneficial effects of preconditioning. Myocardial myeloperoxidase activity also was determined in these experiments and was found to be lower in preconditioned than in control hearts. Because the polymorphonuclear leukocyte is a principal source of the enzyme, the data support the possibility that a reduction of neutrophil-mediated injury may contribute to the preconditioning effect. On the other hand, fewer neutrophils might have accumulated simply because the infarcts were smaller in the preconditioned group. It is not apparent at present, however, by what mechanism(s) preconditioning stimuli could influence the accumulation of polymorphonuclear leukocytes, development of local inflammatory responses, or tissue injury in reperfused myocardium.

In addition to modification of infarct size, preconditioning is reported to influence the electrophysiological effects of ischemia. In a rodent model of preconditioning, Shiki and Hearsne22 demonstrated a reduction of reperfusion-induced arrhythmias. Rats were subjected to 5 minutes of left anterior descending coronary artery occlusion and reperfused for 10, 20, 30, 60, 120 minutes, 1 day, or 3 days. The hearts were exposed to another 5 minutes of ischemia and reperfusion. There was a significant reduction of reperfusion-induced ventricular fibrillation up to a reperfusion period of 60 minutes and significant reduction of reperfusion-induced ventricular tachycardia up to a reperfusion period of 30 minutes. It was concluded that preconditioning reduces the incidence of reperfusion arrhythmias, but the protective effects degenerate in a time-dependent manner. Miyazaki and Zipes23 studied autonomic denervation in a canine model of preconditioning, using four brief ischemic periods, followed by a 3-hour sustained ischemic period. Efferent sympathetic and vagal responses were preserved in preconditioned dogs compared with control dogs that underwent 3 hours of left anterior descending coronary artery occlusion alone. There was no reduction of infarct size, however, in preconditioned dogs after 3 hours of sustained ischemia, an observation consistent with the study by Murry et al.9

In a number of studies, the relation between repetitive coronary artery occlusions and myocardial high-energy phosphate stores was examined.6-8,24 The observations from several groups are in agreement and indicate that repetitive occlusion followed by intermittent reperfusion are not associated with a cumulative metabolic deficit. There is no further decrement in high-energy phosphate stores beyond that which is observed to occur with the initial brief occlusion, and there is a decrease in the rate of high-energy phosphate utilization or a “sparing” effect with each subsequent brief ischemic episode. Furthermore, intermittent periods of reperfusion between episodes of myocardial ischemia provide a protective effect in terms of preventing a cumulative deterioration of myocardial ultrastructure so that four 10-minute periods of coronary artery occlusion when separated by 20-minute periods of reperfusion produce no more ultrastructural damage than a single 10-minute occlusion.25

The reduction in regional function, or myocardial stunning, that results from the initial ischemic insult may account for the reduced rate of energy utilization, thereby protecting the myocardium during subsequent brief ischemic periods. Dissociation between myocardial stunning and the preconditioning effect, however, is suggested by the preliminary report of Murry et al.,26 who demonstrated that 120 minutes of reperfusion reduced the effects of preconditioning on infarct size but did not reverse myocardial stunning. Washout of catabolites has also been proposed9,27 as a possible mechanism for preventing or minimizing the cumulative effects of multiple ischemic events, and it may contribute to the phenomenon of preconditioning. The accumulation of catabolites such as lactate, hydrogen ion, and NADH during the period of ischemia could lead to the transition from reversible to irreversible damage. Therefore, intermittent reperfusion could remove harmful products of anaerobic metabolism. Neely and Grotyohann27 showed that functional recovery correlated closely with tissue lactate concentration rather than adenosine nucleotide concentrations and functional improvement could be abolished by the addition of lactate to the perfusion medium. The investigators concluded that the accumulation of glycolytic intermediates rather than the depletion of high-energy phosphate stores was responsible for functional deterioration of the heart. Depletion of glycogen stores during repetitive occlu-
sions and the failure to support the production of lactate during prolonged anaerobiosis could contribute to the protective effects of preconditioning when the heart is subjected to a more prolonged insult. Whether or not such a mechanism explains the beneficial effect of a single preconditioning ischemic insult of 5 minutes in duration is not known.

The mechanism by which preconditioning protects the heart is unknown and clearly warrants further investigation. It has not been elucidated by this present study. We have shown the mechanism does not depend on multiple ischemic periods, is induced rapidly, and is effective over occlusion periods of at least 1 hour. Whether preconditioning exists in humans is speculative, but its demonstration in the dog,9,23,26 rat,21,22 pig,28 and rabbit29 suggests that this intriguing laboratory observation extends to the clinical situation in which patients experience repetitive ischemic episodes of brief duration. Patients with unstable angina or chest pain in the hours to days before a myocardial infarction might have a longer time period to reperfusion with thrombolytic therapy or angioplasty and may experience less myocardial necrosis. Similarly, patients who have an abrupt reoclusion after thrombolytic therapy or angioplasty may suffer less myocardial necrosis or have a longer period available for effective reperfusion with “rescue angioplasty.” Patients with failed thrombolysis or angioplasty may also have an increased time frame for successful emergency bypass surgery and revascularization. Preconditioning may also play a role in open heart surgery with intermittent perfusion during cardioplectic arrest.

The practical value of preconditioning is difficult to assess at this early stage of investigation. Because the preconditioning effect implies that myocardial cells are capable of rapidly adapting to brief stresses in a manner that is protective, however, the potential biological importance of the effect may be substantial.

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Myocardial protection with preconditioning.
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