Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning

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Zhao, Zhi-Qing, Joel S. Corvera, Michael E. Halkos, Faraz Kerendi, Ning-Ping Wang, Robert A. Guyton, and Jakob Vinten-Johansen. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol 285: H579–H588, 2003. First published April 3, 2003; 10.1152/ajpheart.01069.2002.—Ischemic preconditioning (Pre-con) is an adaptive response triggered by a brief ischemia applied before a prolonged coronary occlusion. We tested the hypothesis that repetitive ischemia applied during early reperfusion, i.e., postconditioning (Post-con), is cardioprotective by attenuating reperfusion injury. In anesthetized open-chest dogs, the left anterior descending artery (LAD) was occluded for 60 min and reperfused for 3 h. In controls (n = 10), there was no intervention. In Pre-con (n = 9), the LAD was occluded for 5 min and reperfused for 10 min before the prolonged occlusion. In Post-con (n = 10), at the start of reperfusion, three cycles of 30-s reperfusion and 30-s LAD reocclusion preceded the 3 h of reperfusion. Infarct size was significantly less in the Pre-con (15 ± 2%, P < 0.05) and Post-con (14 ± 2%, P < 0.05) groups compared with controls (25 ± 3%). Tissue edema (% water content) in the area at risk was comparably reduced in Pre-con (78.3 ± 1.2%, P < 0.05) and Post-con (79.7 ± 0.6%, P < 0.05) versus controls (81.5 ± 0.4%). Polymorphonuclear neutrophil (PMN) accumulation (myeloperoxidase activity, absorbance-min1-g tissue−1) in the area at risk myocardium was comparably reduced in Post-con (9.8 ± 5.5, P < 0.05) and Pre-con (13.4 ± 3.4, P < 0.05) versus controls (47.4 ± 15.3). Basal endothelial function measured by PMN adherence to postischemic LAD endothelium (PMNs/mm2) was comparably attenuated by Post-con and Pre-con (15 ± 0.6 and 12 ± 0.6, P < 0.05) versus controls (37 ± 1.5), consistent with reduced expression of P-selectin on coronary vascular endothelium in Post-con and Pre-con. Endothelial function assessed by the maximal vasodilator response of postischemic LAD to acetylcholine was significantly greater in Post-con (104 ± 6%, P < 0.05) and Pre-con (109 ± 5%, P < 0.05) versus controls (71 ± 8%). Plasma malondialdehyde (µM/ml), a product of lipid peroxidation, was significantly less at 1 h of reperfusion in Post-con (2.2 ± 0.2, P < 0.05) versus controls (3.2 ± 0.3) associated with a decrease in superoxide levels revealed by dihydroethidium staining in the myocardial area at risk. These data suggest that Post-con is as effective as Pre-con in reducing infarct size and preserving endothelial function. Post-con may be clinically applicable in coronary interventions, coronary artery bypass surgery, organ transplantation, and peripheral revascularization where reperfusion injury is expressed.

IN THE LAST TWO DECADES, considerable effort has focused on limiting infarct size and other manifestations of posts ischemic injury. In the long run, clinical as well as preclinical results using various cardioprotective strategies to attenuate reperfusion injury have been rather unsatisfactory (13, 32). The inconsistent results among different species (including humans) and the difficulty in translating these cardioprotective strategies into clinical practice have dampened the enthusiasm for such therapeutic approaches. For strategies specifically targeting reperfusion injury, pharmacological and mechanical interventions must be implemented at the time of reperfusion. Although intracoronary delivery of drugs is clinically applicable, the medical community has not enthusiastically embraced these approaches for clinical practice.

In 1986, Murry et al. (21) first introduced the concept of ischemic preconditioning in which repetitive brief periods of ischemia protected the myocardium from a subsequent longer ischemic insult. Preconditioning succeeded in significantly reducing infarct size at a time when other pharmacological strategies were inconsistent in their effect. Preconditioning has been reported to reduce infarct size (21), preserve vascular endothelial function (8, 28), decrease polymorphonuclear neutrophil (PMN) accumulation (22), and reduce apoptosis (22). Although preconditioning has been clinically successful in attenuating the physiological effects of serial balloon inflations during percutaneous transluminal coronary angioplasty (9, 29), its use as a clinical cardioprotective strategy to attenuate the pathophysiological consequences (i.e., arrhythmias and infarction) of ischemia-reperfusion injury is limited by the inability to predict the onset of ischemia. However, the implementation of cardioprotective therapy at the time of reperfusion is clinically feasible because the onset of reperfusion is more predictable and is under the clinician’s control.

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IN THE LAST TWO DECADES, considerable effort has focused on limiting infarct size and other manifestations of posts ischemic injury. In the long run, clinical as well as preclinical results using various cardioprotective strategies to attenuate reperfusion injury have been rather unsatisfactory (13, 32). The inconsistent results among different species (including humans) and the difficulty in translating these cardioprotective strategies into clinical practice have dampened the enthusiasm for such therapeutic approaches. For strategies specifically targeting reperfusion injury, pharmacological and mechanical interventions must be implemented at the time of reperfusion. Although intracoronary delivery of drugs is clinically applicable, the medical community has not enthusiastically embraced these approaches for clinical practice.

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It has been previously reported that reperfusion injury can be significantly reduced by modifying the conditions and the composition of the initial reperfusion (1, 2, 5). Relevant to the modification of the conditions (hydrodynamics or flow rate) of reperfusion, we have reported that gradual restoration of coronary blood flow (CBF) during the first 30 min of reperfusion following a period of coronary occlusion reduced infarct size and postischemic myocardial blood flow defects (26, 34). In the present study, we used an open-chest canine model of left anterior descending artery (LAD) occlusion and reperfusion to test the hypothesis that three cycles of 30-s reperfusion followed by 30-s reocclusion during the initial moments of reperfusion (i.e., postconditioning) would attenuate reperfusion-induced myocardial injury. This strategy was compared with conventional preconditioning. We found that infarct size, tissue edema, and postischemic coronary artery endothelial dysfunction in the area at risk (AR) myocardium were reduced with postconditioning to the same extent as with preconditioning.

MATERIALS AND METHODS

Surgical preparation. The experimental animals were handled in compliance with the Guiding Principles in the Use and Care of Animals published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). The Institutional Animal Care and Use Committee of Emory University approved the study protocol.

Heartworm-free adult dogs of either sex weighing 25–35 kg were initially medicated with an intramuscular injection of morphine sulfate (4 mg/kg) followed by continuous inhalation of isoflurane (0.5–2%) after endotracheal intubation. Dogs were ventilated with a volume-cycled respirator using oxygen-enriched room air. The left femoral artery was cannulated with a polyethylene catheter for blood sampling. The chest was opened by a left lateral thoracotomy, and the heart was suspended in a pericardial cradle. Millar catheter-tipped pressure transducers were inserted into the aorta via the internal mammary artery and into the left ventricle through an apical incision, respectively. A pair of 5-MHz ultrasonic crystals was implanted in the anterior midmyocardium and connected to a sonomicrometer (model 120, Triton Technology; San Diego, CA) to measure regional contractile function. A Doppler flow probe was placed on the proximal LAD coronary artery and connected to a pulsed Doppler flowmeter (Triton Technology) for the measurement of instantaneous CBF. A catheter was inserted into the left atrium for injection of colored microspheres to measure regional myocardial blood flow. A 2-0 silk suture was passed below the first diagonal branch of the LAD distal to the flow probe to occlude the LAD. All dogs were then systemically heparinized with 300 U/kg heparin sodium before the start of the experiment.

Experimental protocol. All animals were randomly assigned to one of three groups (Fig. 1): 1) control (n = 10), the LAD was reversibly occluded for 60 min followed by 3 h of reperfusion; 2) preconditioning (n = 9), the LAD was occluded for 5 min followed by 10 min of reperfusion before the 60 min of prolonged occlusion; 3) postconditioning (n = 10), after 60 min of LAD occlusion, reperfusion was initiated for 30 s followed by 30 s of reocclusion, repeated for three cycles (3 min total intervention). Reperfusion was continued for a total of 3 h in all experiments.

Steady-state baseline hemodynamic measurements were acquired in duplicate, and a blood sample was withdrawn to measure plasma creatine kinase (CK) activity and malondialdehyde (MDA) level. The LAD was reversibly occluded by gently pulling up on the snare to produce a zone of regional ischemia in the anterior free wall of the left ventricle. After 60 min of LAD occlusion, colored microspheres were injected to quantify collateral blood flow in the area at risk, and lidocaine (2 mg/kg) was administered intravenously just before release of the ligature. The ligature was then loosened, and the ischemic myocardium was reperfused for 3 h. Data were again collected at 15 min and 3 h of reperfusion. At the end of reperfusion, the heart was excised to evaluate infarct size, tissue edema, myocardial blood flow, tissue myeloperoxidase activity, and superoxide level in the AR. To determine postischemic coronary artery endothelial vasoreactivity and PMN adherence to coronary endothelium, a 1.0-cm long segment of LAD and left circumflex coronary arteries (LCX) was harvested and placed in cold physiological buffer.

Hemodynamics and regional contractile function. Hemodynamic data were acquired during a 12-s period of respiratory apnea by using an analog-to-digital converter and acquisition software (Spectrum, Wake Forest University). Heart rate (HR), mean aortic pressure (MAP), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), the maximum and minimum first derivative of LVSP (+dP/dtmax and −dP/dt), LAD CBF, end-diastolic segment length, end-systolic segment length, percent segmental shortening, and segment work in ischemic zone were determined as described previously (39). Hemodynamic and cardiodynamic data were collected at baseline, at 60 min of ischemia, and after 15 min, 1, 2, and 3 h of reperfusion.

Determination of area at risk and infarct size. After the heart was harvested, the LAD was religated in the original position, and diluted Unisperse blue dye was injected into the aortic root under 80-mmHg perfusion pressure to stain the nonischemic region blue and thereby outline the AR. After excision of the coronary vessels under cold buffer, the left ventricle was cut into transverse slices. The AR was separated from the nonischemic zone and incubated in a 1% solution of triphenyltetrazolium chloride (TTC) at 37°C to differentiate the area of necrosis from the nonnecrotic AR. The AR was expressed as a percentage of the left ventricular mass (AR/LV), and the area of necrosis, as a percentage of the AR (AN/AR), and they were calculated by tissue weight as reported previously (39).
Regional myocardial blood flow. Regional myocardial blood flow (expressed as ml·min⁻¹·g tissue⁻¹) in the ischemic subepicardium and subendocardium was determined by colored microspheres (Triton Technology) at baseline, at the end of ischemia, and at 15 min and 3 h of reperfusion using the reference sampling method as previously described (39).

**Determination of CK and MDA from plasma.** Arterial blood samples were withdrawn at baseline, at the end of ischemia, and at 1, 2, and 3 h of reperfusion for measuring CK (Sigma Diagnostic, St. Louis, MO) and MDA (Lipid Peroxidation Assay, Calbiochem), an index of lipid peroxidation reflecting oxygen free radicals. All samples were centrifuged, and the plasma was drawn off. The extracted supernatants after reaction of plasma with tested reagents in CK and MDA detection kits were analyzed spectrophotometrically (SPECTRAmax, Molecular Devices; Sunnyvale, CA) at 340-nm and 586-nm absorbance, respectively. CK activity was expressed as units per gram protein, and MDA values were given as micromoles per milliliter of plasma.

**Edema from AR myocardium.** Tissue samples (0.3 g) were taken from subepicardial and subendocardial regions of the AR, weighed. The samples were desiccated in an 80°C oven for 24 h and reweighed. The percent tissue edema was calculated as 100-[1 – (dry weight/wet weight)]

**PMN isolation and adherence to coronary artery endothelium.** After induction of anesthesia, arterial blood was withdrawn from the femoral artery. PMN isolation and adherence to ischemic-reperfused coronary artery segments were performed as described previously (40). Briefly, the LAD and LCX segments were cut into 3-mm segments, carefully opened, and placed in round cell culture dishes. Fluorescently labeled and unstimulated PMNs (4 × 10⁶ cells/ml) were added to the culture dishes containing oxygenated Krebs Henseleit buffer at 37°C and allowed to incubate with coronary artery segments for 20 min. After incubation, the segments were removed, rinsed of nonadherent PMNs, and placed on glass slides. The number of PMNs adhering to the endothelial surface in six separate microscopic fields was counted under epifluorescent microscopy.

**Coronary artery endothelial function.** Vasoreactivity in posts ischemic epicardial macrovessels was studied as described previously (40). Briefly, LAD and LCX coronary artery segments were cut into 3-mm segments and hung in organ chambers containing oxygenated Krebs-Henseleit buffer at 37°C. After 60 min of stabilization, the coronary artery rings were preconstricted with the thromboxane A2 mimetic U-46619. Endothelial function was assessed by quantifying the vasorelaxation responses to incremental concentrations of endothelium-dependent vasodilator acetylcholine, whereas smooth muscle function was assessed with incremental concentrations of the endothelium-independent vasodilator sodium nitroprusside. Vascular responses were monitored using a video graphics program (Spectrum; Winston-Salem, NC).

**Tissue myeloperoxidase activity.** After we determined infarct size, tissue samples from the nonischemic and AR zones were saved for analysis of myeloperoxidase (MPO) activity, an enzyme used as a marker of neutrophil accumulation. The samples were frozen and stored at −70°C until assayed. The samples were homogenized in hexadecyltrimethyl ammonium bromide dissolved in phosphate buffer. After centrifugation, supernatants were collected and mixed with o-dianisidine dihydrochloride and hydrogen peroxide in phosphate buffer. The activity of MPO was measured spectrophotometrically at 460 nm absorbance (abs) (SPECTRAmax, expressed as Aabs·min⁻¹·g tissue⁻¹) (39).

**P-selectin expression on vascular endothelium.** Transmural tissue samples from nonischemic and ischemic myocardium were processed for immunohistochemistry as reported previously (39). Briefly, the tissue blocks were embedded in optimal cutting temperature (OCT, Miles Laboratories) compound after fixation in paraformaldehyde, frozen in liquid nitrogen, and stored at −70°C. Cryosections of the tissue (7 μm) were cut using a Hacker-Bright cryostat, thaw-mounted on Fisher-Plus (Fisher Scientific) slides and stored at −70°C with desiccant until use. After being fixed in acetone and air dried, the tissue sections were initially blocked with gelatin in PBS to inhibit nonspecific binding. The sections were then incubated with polyclonal rabbit anti-human CD 62P (P-selectin, BD PharMingen) for 1–2 h at a concentration of 1 μg/ml. The slides were then washed in PBS and incubated with 1:500 dilution of biotinylated goat anti-rabbit IgG (Vector Laboratories). The sections were then counterstained with hematoxylin and dehydrated in graded dilutions of alcohol. Controls for immunohistochemical single or double labeled sections were made by either eliminating the primary antibody or incubating the tissue with a nonimmune IgG.

**In situ detection of superoxide.** Dihydroethidium (DHE, Molecular Probes) staining for superoxide was carried out as described previously (20). Briefly, transmural tissue samples from nonischemic and ischemic myocardium were harvested, placed in cold saline, and embedded in OCT for cryosectioning. The tissue sections (20 μm) were cut using a Hacker-Bright cryostat, thaw-mounted on Fisher-Plus (Fisher Scientific) slides and stained with 10 μM DHE at 37°C for 30 min. The image of ethidium staining was obtained by using an imaging system (Image-Pro Plus, Media Cybernetics; Silver Spring, MD) with a 585-nm long-pass filter. Generation of superoxide by tissue was demonstrated by red fluorescent labeling.

**Statistical analysis.** A one-way ANOVA followed by Duncan’s post-hoc test was used to analyze group differences in a single data such as PMN adherence, infarct size, and CK activity data. Hemodynamic data and other time-dependent determinations were analyzed by repeated measures analysis of variance followed by post-hoc analysis with Student-Newman-Keuls multiple comparisons. Dose-response curves of agonist-induced vascular relaxation were calculated as a percentage of the decrease of U-46619-induced isometric force. A P < 0.05 was considered significant. Results are reported as means ± SE.

**RESULTS**

Thirty-nine dogs were initially entered into the study. Three were excluded due to a small AR (<15% of the left ventricle); four were excluded for unstable hemodynamics (HR > 120 beats/min at baseline) and ventricular fibrillation during reperfusion; and three were excluded because of high transmural blood flow (> 0.15 ml·min⁻¹·g⁻¹) during coronary occlusion as determined by microspheres. Data from 29 dogs are included in the final analysis: 10 in the control group, 9 in the preconditioning group, and 10 in the postconditioning group.

**Hemodynamics and regional contractile function.** Hemodynamics and regional contractile function during the experiments are summarized in Table 1. In all three groups, HR increased during ischemia; however, these increases were not significant compared with...
their respective baseline value. HR was only significantly increased at some time points during reperfusion in control and preconditioning groups. During and after 15 min of reperfusion, LVSP in the control and preconditioning groups was significantly less compared with the respective baseline values but showed no significant group-related differences throughout the procedure. There were nonsignificant trends in the changes in LVEDP, +dP/dt, and −dP/dt compared with the respective baseline value during the experiment in the three groups. During coronary occlusion, all the groups exhibited a rightward shift in the pressure-length relationship and a significant decline in segmental shortening and segmental shortening (Table 1). Wall motion remained dyskinetic or severely hypokinetic throughout the entire reperfusion period in all three groups with no group differences.

Area at risk and infarct size. The AR was comparable among the three groups, averaging between 25 and 31% (Fig. 2). Infarct size in the postconditioning group was 44% less than that in the control group (Fig. 2). Similarly, infarct size in preconditioning was 40% less than that in the control group (P < 0.05). There was no statistical difference in infarct size between the preconditioning and postconditioning groups.

Regional myocardial blood flow in area at risk. Regional myocardial blood flow in the nonischemic myocardium remained unchanged during the experiment (Table 2). LAD occlusion reduced regional myocardial blood flow in the ischemic subepicardium and subendocardium to the same extent in all three groups. Transmural blood flow (in ml·min⁻¹·g⁻¹) in the AR at the end of ischemia was comparable among all three groups, averaging 0.06 ± 0.01 in the control, 0.07 ± 0.01 in the preconditioning, and 0.09 ± 0.02 in the postconditioning groups. Release of the coronary artery snare resulted in a significant increase in regional myocardial blood flow in the AR subepicardium and subendocardium in the control group at 15 min of reperfusion relative to values during ischemia. The reactive hyperemic response in the subepicardial region of the AR at 15 min reperfusion was significantly

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**Table 1. Hemodynamics and regional contractile function during the experiment**

<table>
<thead>
<tr>
<th>Time</th>
<th>HR</th>
<th>MAP</th>
<th>LVSP</th>
<th>LVEDP</th>
<th>+dP/dt</th>
<th>−dP/dt</th>
<th>CBF</th>
<th>SS</th>
<th>SW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
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</tr>
<tr>
<td>Control</td>
<td>73 ± 2</td>
<td>82 ± 4</td>
<td>101 ± 2</td>
<td>10 ± 0.4</td>
<td>1,599 ± 109</td>
<td>−1,568 ± 37</td>
<td>6.2 ± 0.5</td>
<td>25 ± 1</td>
<td>217 ± 17</td>
</tr>
<tr>
<td>Post-con</td>
<td>78 ± 6</td>
<td>84 ± 3</td>
<td>100 ± 3</td>
<td>10 ± 0.8</td>
<td>1,576 ± 86</td>
<td>−1,561 ± 68</td>
<td>6.4 ± 0.4</td>
<td>22 ± 1</td>
<td>175 ± 25</td>
</tr>
<tr>
<td>Pre-con</td>
<td>73 ± 3</td>
<td>86 ± 2</td>
<td>103 ± 3</td>
<td>10 ± 0.5</td>
<td>1,582 ± 55</td>
<td>−1,665 ± 44</td>
<td>7.5 ± 1.3</td>
<td>23 ± 2</td>
<td>210 ± 12</td>
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<tr>
<td>Ischemia</td>
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<tr>
<td>Control</td>
<td>92 ± 5</td>
<td>77 ± 3</td>
<td>91 ± 2</td>
<td>12 ± 0.5</td>
<td>1,359 ± 58</td>
<td>−1,301 ± 22</td>
<td>−5 ± 0.6*</td>
<td>17 ± 5*</td>
<td></td>
</tr>
<tr>
<td>Post-con</td>
<td>90 ± 6</td>
<td>85 ± 3</td>
<td>100 ± 3</td>
<td>13 ± 0.4</td>
<td>1,550 ± 56</td>
<td>−1,441 ± 51</td>
<td>−3 ± 0.3*</td>
<td>30 ± 3*</td>
<td></td>
</tr>
<tr>
<td>Pre-con</td>
<td>94 ± 5</td>
<td>82 ± 3</td>
<td>96 ± 2</td>
<td>11 ± 0.8</td>
<td>1,453 ± 50</td>
<td>−1,463 ± 46</td>
<td>−4 ± 0.7*</td>
<td>28 ± 8*</td>
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</tr>
<tr>
<td>Control</td>
<td>101 ± 4*</td>
<td>69 ± 3</td>
<td>82 ± 2*</td>
<td>13 ± 0.8</td>
<td>1,105 ± 44</td>
<td>−1,126 ± 38</td>
<td>13.9 ± 1.2*</td>
<td>2 ± 1*</td>
<td>50 ± 11*</td>
</tr>
<tr>
<td>Post-con</td>
<td>86 ± 6</td>
<td>83 ± 5</td>
<td>96 ± 4</td>
<td>12 ± 1</td>
<td>1,366 ± 68</td>
<td>−1,441 ± 51</td>
<td>6.4 ± 0.9†</td>
<td>5 ± 1*</td>
<td>81 ± 12*</td>
</tr>
<tr>
<td>Pre-con</td>
<td>98 ± 7*</td>
<td>75 ± 2</td>
<td>89 ± 2*</td>
<td>11 ± 0.9</td>
<td>1,267 ± 69</td>
<td>−1,385 ± 69</td>
<td>11.3 ± 1.3</td>
<td>5 ± 2*</td>
<td>101 ± 14*</td>
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<td>R2 h</td>
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</tr>
<tr>
<td>Control</td>
<td>97 ± 4</td>
<td>67 ± 2</td>
<td>84 ± 4*</td>
<td>11 ± 0.7</td>
<td>1,281 ± 65</td>
<td>−1,185 ± 26</td>
<td>12.6 ± 1.4*</td>
<td>2 ± 1*</td>
<td>30 ± 8*</td>
</tr>
<tr>
<td>Post-con</td>
<td>97 ± 6</td>
<td>72 ± 3</td>
<td>91 ± 3</td>
<td>11 ± 0.4</td>
<td>1,376 ± 48</td>
<td>−1,325 ± 43</td>
<td>9.4 ± 3.3</td>
<td>3 ± 2*</td>
<td>34 ± 10*</td>
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<tr>
<td>Pre-con</td>
<td>107 ± 8*</td>
<td>72 ± 3</td>
<td>86 ± 3*</td>
<td>9 ± 0.8</td>
<td>1,315 ± 99</td>
<td>−1,209 ± 58</td>
<td>10.8 ± 1.4</td>
<td>3 ± 2*</td>
<td>34 ± 18*</td>
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<td>R3 h</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>106 ± 5*</td>
<td>67 ± 3</td>
<td>85 ± 1*</td>
<td>11 ± 0.9</td>
<td>1,283 ± 41</td>
<td>−1,183 ± 22</td>
<td>11.5 ± 1.6</td>
<td>1 ± 1*</td>
<td>23 ± 6*</td>
</tr>
<tr>
<td>Post-con</td>
<td>98 ± 8</td>
<td>71 ± 4</td>
<td>92 ± 1</td>
<td>11 ± 0.6</td>
<td>1,433 ± 33</td>
<td>−1,349 ± 54</td>
<td>10.5 ± 3.5</td>
<td>6 ± 2*</td>
<td>58 ± 11*</td>
</tr>
<tr>
<td>Pre-con</td>
<td>112 ± 7*</td>
<td>72 ± 3</td>
<td>86 ± 3*</td>
<td>8 ± 0.9</td>
<td>1,381 ± 92</td>
<td>−1,218 ± 88</td>
<td>10.9 ± 1.2</td>
<td>3 ± 2*</td>
<td>45 ± 18*</td>
</tr>
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</table>

All values are means ± SE. HR, heart rate (beats/min); MAP, mean aortic pressure (mmHg); LVSP, left ventricular (LV) peak systolic pressure (mmHg); LVEDP, LV end-diastolic pressure (mmHg); +dP/dt (mmHg); −dP/dt (mmHg) first derivative of LV pressure; CBF, mean CBF (ml/min); SS, systolic shortening (%); SW, segmental work (mmHg·mm); R1, R2, 3 h; 15 min, 1, 2, and 3 h of reperfusion, respectively. *P < 0.05 vs. baseline value; †P < 0.05 vs. control group.

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**Fig. 2.** Bar graph shows determination of infarct size by triphenyl-tetrazolium chloride (TTC) staining. Post-con significantly reduced area of necrosis (AN)-to-area at risk (AR) ratio by 48% compared with the control group, showing equivalent cardioprotection to that of Pre-con. *P < 0.05 vs. control. Values are group means ± SE.
Table 2. Regional myocardial blood flow during the experiment

<table>
<thead>
<tr>
<th>Time</th>
<th>Normal Zone</th>
<th>Isch-Epi</th>
<th>Isch-Endo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline Control</td>
<td>0.89 ± 0.08</td>
<td>0.86 ± 0.07</td>
<td>0.72 ± 0.03</td>
</tr>
<tr>
<td>Pre-con</td>
<td>0.88 ± 0.06</td>
<td>0.86 ± 0.03</td>
<td>0.76 ± 0.05</td>
</tr>
<tr>
<td>Post-con</td>
<td>1.0 ± 0.06</td>
<td>0.88 ± 0.08</td>
<td>0.7 ± 0.05</td>
</tr>
<tr>
<td>Ischemia Control</td>
<td>0.83 ± 0.09</td>
<td>0.09 ± 0.02*</td>
<td>0.03 ± 0.01*</td>
</tr>
<tr>
<td>Pre-con</td>
<td>0.85 ± 0.04</td>
<td>0.11 ± 0.01*</td>
<td>0.04 ± 0.01*</td>
</tr>
<tr>
<td>Post-con</td>
<td>1.1 ± 0.1</td>
<td>0.14 ± 0.02*</td>
<td>0.04 ± 0.01*</td>
</tr>
<tr>
<td>R15 Control</td>
<td>0.75 ± 0.1</td>
<td>1.2 ± 0.19‡</td>
<td>1.55 ± 0.2‡</td>
</tr>
<tr>
<td>Pre-con</td>
<td>0.89 ± 0.09</td>
<td>0.72 ± 0.09‡‡</td>
<td>1.7 ± 0.18‡‡</td>
</tr>
<tr>
<td>Post-con</td>
<td>0.85 ± 0.08</td>
<td>0.62 ± 2‡‡</td>
<td>2.1 ± 0.27‡‡</td>
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<tr>
<td>R3 h Control</td>
<td>1.1 ± 0.1</td>
<td>0.98 ± 0.13</td>
<td>1.38 ± 0.2</td>
</tr>
<tr>
<td>Pre-con</td>
<td>1.1 ± 0.1</td>
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<td>1.3 ± 0.09</td>
</tr>
<tr>
<td>Post-con</td>
<td>1.3 ± 0.09</td>
<td>1.1 ± 0.09</td>
<td>1.54 ± 0.2</td>
</tr>
</tbody>
</table>

All values are means ± SE (ml·min⁻¹·g tissue⁻¹). Isch-Epi and Isch-Endo, ischemic epi- and endomyocardium, respectively. *P < 0.05 vs. baseline value; †P < 0.05 vs. ischemic value; ‡P < 0.05 vs. control group.

Reduced in both preconditioning and postconditioning groups. There was a similar marked hyperemic response in LAD CBF measured by the flow probe in the control group at the start of reperfusion that was significantly blunted in the postconditioning group. There were no group differences in regional myocardial blood flow in the subepicardium at 3 h of reperfusion. In addition, there were no group differences in the AR subendocardial blood flow at 15 min and 3 h of reperfusion.

To exclude the possibility that the smaller infarct size in the preconditioning and postconditioning groups was due to higher collateral blood flow during coronary occlusion, transmural AR myocardial blood flow was plotted against the size of infarction as covariate. As shown in Fig. 3, there was an inverse relationship between infarct size and collateral blood flow. However, at any given value of collateral blood flow, the infarct size in the preconditioning and postconditioning groups was smaller than that in the control group. Therefore, the reduction in infarct size in these two intervention groups was not determined by changes in collateral blood flow during coronary occlusion.

**Plasma CK activity during ischemia and reperfusion.** CK activity was used to confirm infarct size quantified by TTC staining. Coronary occlusion only slightly increased CK values, with no group differences. However, CK activity was significantly increased during reperfusion relative to ischemia, reaching a final value of 16 ± 3 IU/g protein at 3 h of reperfusion in the control group. Preconditioning and postconditioning were associated with significantly lower CK activity at 3 h of reperfusion, with values averaging 7 ± 1 and 8 ± 1 IU/g protein, respectively. Group differences in CK activity were consistent with those observed for infarct size.

**Tissue edema in ischemic myocardium.** Ischemia and reperfusion significantly increased tissue edema in the AR myocardium compared with the nonischemic myocardium in the control group (Fig. 4). Tissue edema in the ischemic subendocardial region in the preconditioning and postconditioning groups was significantly higher than that in the nonischemic myocardium. However, tissue edema in these two groups was significantly less compared with the respective region in the control group. There was no significant difference between preconditioning and postconditioning in tissue edema in any regions.

**Tissue MPO activity in AR myocardium.** MPO activity (Δabs·min⁻¹·g tissue⁻¹) in the nonischemic tissue was low and comparable among the three groups. Ischemia-reperfusion significantly increased MPO activity in the AR in the control group (47 ± 15) compared with that in the nonischemic tissue (3 ± 1). However, MPO activity in the AR myocardium was significantly reduced in the preconditioning (13 ± 3) and postconditioning (11 ± 6) groups relative to the control group.

Fig. 3. Relationship between infarct size (AN/AR) and collateral blood flow measured in transmural ischemic zone during 60 min of ischemia. There was an inverse relationship between the AN/AR and collateral blood flow in each group. It is apparent that the reduction in infarct size in Post- and Pre-con groups was independent of collateral blood flow. Each symbol represents one animal.

Fig. 4. Bar graph showing myocardial edema after ischemia and reperfusion. Normal, nonischemic zone; Isch-ei, ischemic subepimyocardium; Isch-endo, ischemic subendomyocardium. Post-con significantly reduced tissue water content compared with the control. *P < 0.05 vs. normal zone. †P < 0.01 vs. control group. Values are group means ± SE.
PMN adherence to postischemic endothelium. PMN adherence to postischemic coronary artery endothelium is a marker of endothelial dysfunction linked to attenuated basal release of nitric oxide (19). There was no difference among groups in PMN adherence to the LCX coronary artery segments. In the control group, there was a significantly augmented PMN adherence (in mm² endothelium) to the LAD coronary artery segments at 3 h of reperfusion (37 ± 2) compared with adherence to the LCX (13 ± 0.8). PMN adherence to the ischemic-reperfused LAD was significantly reduced in both postconditioning and preconditioning groups (12 ± 0.6 and 15 ± 0.6) with no difference between groups. PMN adherence to LAD in the preconditioning and postconditioning groups was comparable to that in the nonischemic LCX.

Postischemic vasorelaxation. Endothelial function in isolated coronary arteries was assessed by relaxation responses to incremental concentrations of the vasodilators in postischemic vessels. Figure 5 shows vasodilator response to acetylcholine in the isolated postischemic coronary artery rings from LCX and LAD. The concentration-response curve of the LAD in the control group was shifted downward and to the right relative to those of the LCX, i.e., relaxation responses were less than those in the LCX. Both preconditioning and postconditioning reversed this blunted relaxation response to acetylcholine to the same extent. There were no significant differences in the responses to nitroprusside in the LAD and LCX in the three groups (data not shown).

P-selectin expression in vascular endothelium. P-selectin is expressed on the surface of activated endothelium. Immunohistochemical staining of the endothelial surface of vessels in tissue sections obtained from nonischemic and AR myocardium is shown in Fig. 6. There was no detectable immunoreactivity to P-selectin on the endothelial surface of vessels in nonischemic myocardium (Fig. 6A). After 3 h of reperfusion, the immunoreactivity to P-selectin was markedly increased along the endothelial surface of vessels in the AR myocardium in the control group (Fig. 6B). However, less P-selectin immunoreactivity was observed in sections from AR myocardium from both preconditioning and postconditioning groups (Fig. 6, C and D).

Plasma MDA levels during ischemia and reperfusion. Appearance of MDA-reactive products in plasma has been used as an indicator of lipid peroxidation. Coronary occlusion was associated with slightly increased MDA values with no group difference compared with baseline values (Fig. 7). However, in the blood samples withdrawn at 1 h of reperfusion in the control group, the concentration of MDA-reactive products was significantly increased relative to coronary occlusion, and higher values was maintained until the end of reperfusion. However, in the blood samples withdrawn at this time point in the postconditioning group, MDA-reactive products were significantly lower than that in the control group. No group difference was detected at end of reperfusion.

DHE staining in the AR myocardium. DHE reacts with superoxide anions to form ethidium bromide, which in turn intercalates with DNA to provide nuclear fluorescence as a marker of superoxide anion generation. As shown in Fig. 8, the intensity of DHE staining in ischemic-reperfused myocardium was markedly enhanced in vascular endothelial cells and in myocytes in the control group compared with staining intensity in the nonischemic myocardium. However, the intensity of fluorescent staining in the postconditioning group was markedly reduced in the ischemic myocardium to a comparable level in the nonischemic myocardium.

DISCUSSION

Timely reperfusion salvages myocardium from tissue injury after prolonged ischemia. However, there is convincing evidence that sudden restoration of blood flow to ischemic myocardium may paradoxically exaggerate injury that is not present at the end of ischemia (15, 18, 33). This reperfusion injury is primarily expressed as contractile and coronary vascular endothelial dysfunction, upregulation of adhesion molecules on the endothelium, and transendothelial emigration of inflammatory cells into the parenchyma, edema, infarction, and apoptosis (13, 14, 35). Modifying the hydrodynamic conditions (i.e., blood flow and intracoronary perfusion pressure) during the early period of reperfusion has been reported to reduce the extent of reperfusion injury in the AR (23, 24, 26, 34). For example, gradually increasing the perfusion rate and intracoronary pressure during the early minutes of reperfusion have been associated with a reduction in necrosis, attenuation in the release of CK and lactate dehydrogenase, and tissue edema (24, 26, 34). Therefore, mechanical manipulation of the early phase of reperfusion may reduce the extent of postischemic injury.
In the present study, we present evidence that repetitive cycles of briefly interrupted reperfusion applied at the onset of coronary reflow (i.e., postconditioning) significantly reduces infarct size. This reduction in infarct size was corroborated by a similar decrease in plasma CK activity and attenuation in tissue edema. The comparable collateral blood flow during ischemia among the three groups suggests that the protective effect of postconditioning on infarct size was not due to differences in coronary collateral blood flow. In addition, postconditioning reduced neutrophil accumulation in the AR myocardium and preserved postischemic coronary artery endothelial function assessed by vasodilatory responses to acetylcholine and adherence of unactivated neutrophils to the endothelial surface. However, neither postconditioning nor preconditioning improved regional contractile function. Postconditioning was associated with a significant decrease in MDA-reactive products of lipid peroxidation and with less intensity of DHE staining of endothelium and myocytes in the AR myocardium. These observations suggest a reduction in the generation of reactive oxygen species and less oxidant-mediated injury by postconditioning. The protection achieved with postconditioning was equivalent to the benefits gained by ischemic preconditioning in all physiological end points studied. Therefore, strategically modifying early reperfusion events provides powerful cardioprotection by reducing reperfusion injury comparable to a pretreatment strategy such as preconditioning. However, because preconditioning triggers cardioprotective pathways before ischemia while postconditioning alters events after ischemia, the mechanisms and the timing of those mechanisms are likely quite different between the two maneuvers.

Generation of abundant oxygen free radicals during early reperfusion has been implicated as a major player in the pathogenesis of tissue injury. The burst of oxygen-derived free radicals occurs within the first minute and peaks at 4 to 7 min after reperfusion; increased free radical generation is still detectable during later periods of reperfusion (4). Superoxide anions have been implicated in lipid peroxidation of biological membranes, triggering adhesion molecule expression on endothelium, and subsequent initiation of neutrophil and endothelial cell interactions (10, 25). Both in vivo and in vitro studies have shown that oxygen free radicals are potent stimuli for the rapid upregulation of P-selectin and ICAM-1 on endothelium as well as initiation of acute inflammation and subsequent recruitment of neutrophils in ischemic myocardium.
dium (3, 10, 11). Our observations are consistent with this oxygen-mediated process. However, the source of oxidants [i.e., PMNs, endothelium via NAD(P)H oxidase, or myocytes] have not yet been elucidated. In addition, it is not clear whether postconditioning blocks a single pathway or etiology of endothelial dysfunction (i.e., endothelial superoxide generation), or multiple signals (i.e., endothelial oxidants, PMN oxidants, and proteasas, etc.). Further studies are needed to clarify the source and the species of free radicals attenuated by postconditioning and the major pathways of endothelial dysfunction that are interdicted by this cardioprotective maneuver.

The brief repetitively interrupted reperfusion with postconditioning applied during the onset of reperfusion was associated with lower levels of MDA-reactive products and DHE-staining intensity, suggesting attenuated lipid peroxidation and reduced generation of superoxide anions in the AR myocardium. Postconditioning may have limited delivery of oxygen substrate and thereby directly attenuated the generation of oxygen-derived free radicals. In the case of oxygen free radicals may have reduced endothelial activation (P-selectin expression) and dysfunction (PMN adherence and vasorelaxation responses) and potentially the pathogenesis of infarction. We did not measure MDA-reactive products or DHE fluorescence in the preconditioning group; however, previous studies have shown that ischemic preconditioning has been associated with reduced generation of oxygen free radicals and attenuated oxidant-related injury to endothelium and myocytes (31, 36). In vitro studies using isolated cardiomyocytes and endothelial cells have reported that the generation of superoxide radicals and cytokines at reoxygenation was significantly attenuated by hypoxic preconditioning (31, 36). These authors suggest that a transient low-level stimulation of oxygen radical generation by preconditioning may act as a signal that awakens an antiinflammatory-type "cardiomyocyte and endothelial preconditioning," which then reduces responsiveness of these cells to inflammatory mediators.

In addition to potentially attenuating oxidant injury, postconditioning may have favorably altered mechanical events during reperfusion independent of altering oxidant-mediated injury. For example, the increase in perpendicular and shear stresses caused by abruptly elevated blood pressure in the reperfused vessels has been reported to induce tissue injury and edema by physically overstretching vascular endothelium and increasing fluid extravasation in the parenchyma (12). As has been reported, an abrupt increase in blood pressure in canine coronary arteries selectively reduced endothelium-dependent vascular relaxation, resulting in enhanced vascular responses to vasoconstrictor agents (7). In previous studies from our laboratory and that of others (26, 34), the gradual restoration of perfusion pressure during early reperfusion was associated with a reduction in microvascular injury, infarct size, and tissue edema. Postconditioning may have modulated these hydrodynamic forces during early reperfusion and thereby attenuated edema and endothelial injury. However, there are notable differences in the protection afforded by postconditioning compared with gradually restoring perfusion. Gradually, reperfusion was associated with increased neutrophil accumulation, whereas in contrast postconditioning was associated with a decrease in neutrophil
acquisition in the AR. In addition, there was significant preservation in maximal endothelium-dependent vascular relaxation with postconditioning, which was not observed with gradual reperfusion (26). The differences in neutrophil accumulation and postischemic endothelial function between gradual reperfusion and postconditioning suggest that different mechanisms are, in part, involved.

Regional contractile dysfunction is a consequence of both reversible and irreversible injury after ischemia-reperfusion. Short periods (<15 min) of coronary artery occlusion cause contractile dysfunction in the absence of infarction; this recovery of myocardial stunning takes at least 6 h (16). However, contractile dysfunction in irreversibly damaged (infarcted) myocardium persists, and recovery may require days or months (6, 16, 38). In the present study, contractile dysfunction was observed in the AR of untreated hearts, as has been abundantly reported previously (17, 34, 38). Although pre- and postconditioning reduced infarct size significantly, they did not improve postischemic function in the AR. The ultrasonic crystals were placed in the subendocardial region of the AR, which was infarcted to some extent in both groups. Acute recovery of function would not be expected regardless of the mechanism involved in myocardial salvage, unless the overlying noninfarcted myocardium could acutely compensate for the contractile failure of the underlying subendocardial (infarcted) tissue. However, few studies have reported functional recovery even in the presence of persistent albeit diminished infarction (27, 30).

In conclusion, although experimental studies demonstrate that ischemic and pharmacological preconditioning attenuate ischemia-reperfusion injury, these interventions have to be applied before the prolonged “index” ischemia. In the clinical setting, however, pre-treatment is rarely an option. The results of the present study show that salvage of the coronary endothelium and cardiomyocyte can be achieved when the heart is postconditioned by cycles of briefly interrupted perfusion during the early moments of reflow. The degree of myocardial salvage with postconditioning was comparable to that observed with preconditioning, although the intervention is applied at opposite ends of the index ischemia. The observations from the present study provide compelling evidence demonstrating a new mechanism of myocardial protection from reperfusion injury specifically, which is in contradistinction to the mechanism of ischemic preconditioning. Future studies are necessary to determine the optimal algorithm of perfusion-occlusion sequences, as well as other mechanisms involved in postconditioning. It is possible that postconditioning may delay the washout of endogenous anti-inflammatory autacoids such as adenosine (37) and maintain cellular high-energy phosphate stores in ischemic myocardium (24).

The postconditioning procedure is very simple, and therefore, may be clinically applicable. For example, cycles of briefly interrupted reperfusion could be achieved during percutaneous transluminal coronary angioplasty by using rapid reiterative balloon inflation and deflation or by using controllable intracoronary flow devices such as delivery pumps and appropriately sized catheters. This procedure can also be applied after completion of vascular grafts in surgical revascularization, as a final procedure before removal of the aortic cross-clamp in on-pump cardiac surgery, upon removal of the target vessel ligature during off-pump coronary artery bypass graft surgery, or during organ transplantation.

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DISCLOSURES

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REFERENCES


