Loss of Myocardial Protection After Preconditioning Correlates With the Time Course of Glycogen Recovery Within the Preconditioned Segment

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Background. Although previous investigators have demonstrated that myocardial preconditioning reduces infarct size, the mechanisms of cardioprotection associated with preconditioning are not completely understood.

Methods and Results. To test the hypothesis that preconditioning (four 5-minute episodes of ischemia each followed by 5 minutes of reperfusion) reduces infarct size by depleting cardiac glycogen stores and attenuating the degree of intracellular acidosis during subsequent prolonged left coronary artery occlusion, preconditioned and control rats were subjected to 45 minutes of left coronary artery occlusion and 120 minutes of reflow immediately after preconditioning (groups 1P and 1C, respectively) or after 30 minutes (groups 2P+30m and 2C), 1 hour (groups 3P+60m and 3C), or 6 hours (groups 4P+360m and 4C) of nonischemic recovery after preconditioning but before prolonged ischemia. In each group, cardiotomy was performed in selected rats immediately before prolonged ischemia for cardiac glycogen assay. In selected animals, $^{31}$P magnetic resonance spectroscopy was performed to monitor intracellular pH and measure high-energy phosphate levels during ischemia and reperfusion. Group 1P rats demonstrated marked glycogen depletion after preconditioning compared with controls ($0.72\pm0.39$ [n=9] versus 5.67\pm1.73 [n=12] mg glucose/g wet wt; p<0.001 versus group 1C) that was associated with attenuation of intracellular acidosis during ischemia, as measured by $^{31}$P magnetic resonance spectroscopy ($6.8\pm0.3$ [n=11] versus 6.2\pm0.3 [n=9] pH units; p<0.01), and marked infarct size reduction ($0.3\pm0.6$% [n=7] versus 38.1\pm11.3% [n=7], infarct size divided by risk area; p<0.0001). During ischemia, there were no differences in myocardial ATP or phosphocreatine levels or in any hemodynamic determinant of myocardial oxygen demand between groups 1P and 1C. In preconditioned rats that were allowed to recover before ischemia (groups 2P+30m, 3P+60m, and 4P+360m), the time course of glycogen repletion paralleled the loss of protection from ischemic injury.

Conclusions. Glycogen depletion and the attenuation of intracellular acidosis during ischemia appear to be important factors in delaying irreversible injury and reducing infarct size in this animal model of myocardial preconditioning. (Circulation 1993;87:881–892)

Key Words • myocardial infarction • ischemia • magnetic resonance spectroscopy • proteins, stress

Current efforts to limit infarct size by pharmacological, mechanical, and surgical revascularization are limited by the relatively short time period after which irreversible myocardial injury occurs.1–3 Recently, Murry and coworkers4 demonstrated that brief, repetitive episodes of ischemia confer protection against ischemic injury from a subsequent prolonged period of coronary artery occlusion, a phenomenon known as preconditioning.

The mechanisms of the protective effect of preconditioning are not well understood. Other investigators have suggested that preconditioning may protect myocardium by preserving ATP by reduced utilization4–8 or decreased myocardial oxygen demand,8–10 by reducing the accumulation of toxic metabolites,4,8,11 and by the stimulation of adenosine A1 receptors.12,13 Previous investigators have noted that protection from ischemic injury is transient after preconditioning but have not related this to any of the above-proposed mechanisms.14

An alternative explanation for the protective effect of preconditioning may relate to the relation between myocardial glycogen levels and the degree of acidosis.
that develops during subsequent prolonged ischemia. Using 31P magnetic resonance spectroscopy (MRS) in isolated perfused rat hearts, Bailey and coworkers\textsuperscript{15} demonstrated that glycogen loading with glucose and insulin resulted in more lactate production and a greater degree of intracellular acidosis during prolonged ischemia that was proportional to the myocardial glycogen content before ischemia. Conversely, Garlik and coworkers\textsuperscript{16} demonstrated that glycogen-depleted rat hearts developed less intracellular acidosis during prolonged ischemia, presumably because of diminished substrate availability for anaerobic glycolysis. Finally, Neely and Grotjahn\textsuperscript{17} demonstrated that glycogen-depleted perfused rat hearts exhibited greater functional recovery after global ischemia and reperfusion compared with controls. These data suggest that the myocardial glycogen content has an important effect on the level of intracellular acidosis during subsequent ischemia and that the degree of acidosis may influence the amount of ischemic injury.

In the canine model, Murry and coworkers\textsuperscript{8} have reported that preconditioned cardiac tissue contains less glycogen than do control hearts. However, these investigators did not study the potential relations between cardiac glycogen stores, tissue acidosis, and ischemic injury after preconditioning. In this study, we evaluated the potential contributions of glycogen depletion, intracellular acidosis, and high-energy phosphate depletion as determinants of infarct size in preconditioned and control rats. Specifically, we tested the hypothesis that glycogen depletion and attenuation of intracellular acidosis might, in part, contribute to the enhanced myocardial salvage in preconditioned hearts after prolonged ischemia and reperfusion.

Methods

Animal Model of Acute Myocardial Ischemia and Reperfusion

A rat model of reversible myocardial ischemia and reperfusion was used, as described previously.\textsuperscript{1–3,18} Sprague-Dawley rats weighing 225–250 g were used for all experiments. After induction of anesthesia (pentobarbital, 40 mg/kg i.p.), an intravenous catheter was inserted into the tail vein. A tracheostomy was performed, and the animal was ventilated on room air with a Harvard rodent respirator (tidal volume, 0.5 to 1.5 ml; respiratory rate, 95–105 breaths per minute). A reversible snare occluder was placed around the proximal left coronary artery (LCA) via a midline sternotomy. After the coronary artery occluder was visually tested with a brief period of occlusion and reperfusion, the thoracotomy was closed. The animals were then subjected to one of the experimental protocols described below (Table 1). The protocols were approved by the animal research committee, and all experiments were conducted in accordance with the guidelines for animal research at the University of California San Francisco.

Assessment of Myocardial Glycogen Content

To determine the degree of glycogen depletion caused by preconditioning and the time course of recovery of glycogen stores after preconditioning, myocardial glycogen levels were measured in preconditioned rats and corresponding control rats immediately after preconditioning (group 1P, n=9; group 1C, n=12) and after 30 minutes (group 2P+30m, n=9; group 2C, n=11), 1 hour (group 3P+60m, n=6; group 3C, n=6), 3 hours (n=6, n=4, respectively) and 6 hours (group 4P+360m, n=8; group 4C, n=8) of nonischemic recovery. Tissue samples from the ischemic risk area of the anterior left ventricle (LV) were excised just before the 45-minute ischemic period and rapidly frozen in liquid nitrogen. The ischemic risk area was defined by briefly reoccluding the LCA for several seconds and noting the region that became cyanotic. Each sample was then pulverized with a mortar and pestle filled with liquid nitrogen, inserted in a preweighed vial containing 30% potassium hydroxide, allowed to digest for 60 minutes while heated, and then precipitated with sodium sulfate and ethanol. The glucose content in the precipitate of each sample was then assayed in duplicate with amyloglucosidase and glucose oxidase and compared with glucose standards as described previously\textsuperscript{9,20} and expressed as milligrams glucose per gram wet weight of cardiac tissue.

Experimental Protocols

The preconditioning protocol consisted of four consecutive 5-minute episodes of LCA occlusion, each followed by a 5-minute period of reperfusion. Control animals had a comparable 40-minute nonischemic period with the snare occluder open. The preconditioned and control animals were then divided into several groups (Table 1). Group 1 (P and C) animals were subjected to an immediate 45-minute period of LCA occlusion followed by 120 minutes of reperfusion (Table 1). Groups 2P+30m and 2C animals were allowed to recover for 30 minutes after the preconditioning and control periods, respectively, and then subjected to 45 minutes of LCA occlusion followed by 120 minutes of reflow (Table 1). Groups 3P+60m and 3C and groups 4P+360m and 4C rats were allowed to recover for 1 and 6 hours, respectively, before 45 minutes of LCA occlusion and 120 minutes of reperfusion (Table 1). Animals in groups 1P and 1C were subjected to either MRS or hemodynamic monitoring during LCA occlusion and reperfusion as described below. Selected animals in groups 4P+360m and 4C were also subjected to MRS during ischemia and reflow. All animals that completed 45 minutes of LCA occlusion and 120 minutes of reperfusion were assessed for infarct size as described below.

Infarct Sizing

At the end of the experimental protocols, the infarct size and the size of the ischemic risk area were measured as described previously.\textsuperscript{2,3} The thoracotomy was reopened, the LCA was reoccluded, and phthalocyanine blue dye was infused into the LV cavity, causing the dye to perfuse the nonischemic regions of the LV, leaving the ischemic region unstained. The heart was then excised, rinsed of excess blue dye, and sliced transversely into sections 2 mm thick. After the right ventricle and atria were dissected away, the LV was incubated in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) until viable noninfarcted myocardium stained brick red. The LV sections were fixed in 10% formalin and weighed (Mettler LAE, 200 balance, Mettler Inc., Hightstown, N.J.). Color photographs were taken of
Table 1. Experimental Groups and Data Collected in Each Group

<table>
<thead>
<tr>
<th>Group</th>
<th>Precondition</th>
<th>Recovery time (minutes)</th>
<th>Ischemic time (minutes)</th>
<th>Reflow time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1P</td>
<td>+</td>
<td>0</td>
<td>45</td>
<td>120</td>
</tr>
<tr>
<td>1C</td>
<td>-</td>
<td>0</td>
<td>45</td>
<td>120</td>
</tr>
<tr>
<td>2P+30m</td>
<td>+</td>
<td>30</td>
<td>45</td>
<td>120</td>
</tr>
<tr>
<td>2C</td>
<td>-</td>
<td>30</td>
<td>45</td>
<td>120</td>
</tr>
<tr>
<td>3P+60m</td>
<td>+</td>
<td>60</td>
<td>45</td>
<td>120</td>
</tr>
<tr>
<td>3C</td>
<td>-</td>
<td>60</td>
<td>45</td>
<td>120</td>
</tr>
<tr>
<td>4P+360m</td>
<td>+</td>
<td>360</td>
<td>45</td>
<td>120</td>
</tr>
<tr>
<td>4C</td>
<td>-</td>
<td>360</td>
<td>45</td>
<td>120</td>
</tr>
</tbody>
</table>

+ Positive; −, negative; MRS, magnetic resonance spectroscopy; inf, infarct sizing; MRS only, rat died after MRS protocol was completed but before infarct sizing (2 hours after reflow); hemo, hemodynamics; hemo only, rat died after hemodynamic protocol was completed but before infarct sizing (2 hours after reflow); inf only, infarct size only was determined; died, rat died before any protocol was completed, no data collected. For description of groups, see text.

The infarct size as a percentage of total LV mass was expressed as

\[
\frac{\text{infarct size/LV mass}}{\text{total LV weight}} \times 100
\]

The risk area was expressed as a percentage of total LV mass as

\[
\frac{\text{risk area/LV mass}}{\text{total LV weight}} \times 100
\]

The infarct size determined as a percentage of the ischemic risk area was expressed as

\[
\frac{\text{infarct size/risk area}}{\text{risk area weight in each slice}} \times 100
\]

In previous studies with this rat model of ischemia followed by 2 hours of reperfusion, the presence or absence of histological evidence of infarction correlated with the presence or absence of infarction assessed by TTC staining. Furthermore, infarct size correlated closely with the degree of phosphocreatine (PCr) recovery assessed by \(^3\)P MRS.

Some sections did not stain adequately or the photographic images were not of sufficient quality to assess infarct size accurately, so these were not used in the analysis. Hearts from groups 1P (n=22), 1C (n=21), 2P+30m (n=13), 2C (n=14), 3P+60m (n=15), 3C (n=10), 4P+360m (n=11), and 4C (n=10) were suitable for infarct sizing. In groups 1P and 1C, risk area measurements were performed on only seven animals in each group. Phthalocyanine blue dye and TTC staining were not performed on hearts that were used for myocardial glycogen assay.

Selected animals in each group were killed just before the 45-minute ischemic period, and the ischemic risk zone was assayed for myocardial glycogen content as described previously.

Magnetic Resonance Spectroscopy

MRS experiments were performed in selected animals in groups 1P (n=11), 1C (n=9), 4P+360m (n=5), and 4C (n=6) with a modified solenoid coil implanted around the heart as previously described. \(^3\)P MR spectra were acquired at 95.6 MHz on a 5.6-T superconducting magnet with a 6.6-cm horizontal bore (Nalorac Cryogenics Corp., Concord, Calif.) interfaced with a homebuilt spectrometer using a Nicolet 1180-293 B data system. Spectra were acquired for a total of 224 scans over a period of 5 minutes with a 10-μsec pulse width and a 1-second interpulse interval. In selected animals (n=3), fully relaxed spectra were acquired for a total of 100 scans with a 10-μsec pulse width and a 10-second interpulse interval. Data were collected in 4,000 data points and Gaussian-multiplied to give a line broadening of 20 Hz after Fourier transformation.
Intracellular pH was determined from the position of the inorganic phosphate (P_i) resonance relative to that of PCR. It should be noted that, although the resonance of 2,3-diphosphoglycerate (2,3-DPG) overlies the P_i region, a definite P_i resonance was visible above that of 2,3-DPG, especially during ischemia and reperfusion. In spectra with multiple P_i peaks, the resonance corresponding to the lowest pH was selected. After baseline correction with a convolution algorithm, spectral peak areas were measured with the NTCCAP simulation routine (Nicolet, Fremont, Calif.). Spectra from group 1P preconditioned animals (n=11) were obtained at baseline, every 5 minutes during the preconditioning period, immediately after the onset of LCA occlusion, every 15 minutes during ischemia, immediately after reflow, and every 15 minutes during the first hour of reperfusion. Spectra from the control group (group 1C) (n=9) were obtained at baseline, at the conclusion of the 40-minute nonischemic control period (baseline 2), immediately after LCA occlusion, every 15 minutes during ischemia, immediately after reflow, and every 15 minutes during the first hour of reperfusion. Spectra from group 4P+360m preconditioned (n=5) and group 4C control animals (n=6) were obtained before ischemia, immediately after coronary artery occlusion, every 15 minutes during ischemia, immediately after reperfusion, and every 15 minutes during the first hour of reperfusion.

**Hemodynamic Measurements**

Hemodynamic measurements were obtained in group 1P and 1C animals as described previously. A micro-manometer (Millar) catheter was introduced into the left atrium via a small incision in the left atrial appendage and advanced to the LV. After the thoracotomy was repaired and the animal had stabilized for 20 minutes, baseline measurements of LV systolic pressure, heart rate, and maximum rate of LV pressure development (LV dp/dt) were recorded on a Gould RS-3200 physiological recorder (Gould, Inc., Cleveland, Ohio). The LV rate–pressure product was calculated as the heart rate multiplied by the LV systolic pressure. Preconditioned (group 1P) animals (n=10) and control (group 1C) animals (n=10) were subjected to 45 minutes of LCA occlusion and 120 minutes of reperfusion as described in the “Experimental Protocols” section. Hemodynamic measurements of preconditioned animals (group 1P) were obtained at baseline, 4 minutes after the conclusion of preconditioning, 1 minute after the onset of the 45-minute LCA occlusion, at 15-minute intervals during LCA occlusion, 1 minute after reflow, and at 30-minute intervals during reflow. Hemodynamic measurements of group 1C control animals were recorded at the beginning and end of the 40-minute control period, immediately after LCA occlusion, at 15-minute intervals during ischemia, immediately after reperfusion, and at 30-minute intervals during the reflow period.

**Statistics**

All values are expressed as mean±SD (in tables) or mean±SEM (in figures). Comparisons of hemodynamic and MRS data between preconditioned and control groups at specific time points were assessed for significance by one-way ANOVA. Comparisons between cardiac glycogen content or infarct size in preconditioned animals and their individual control groups were assessed for significance by one-way ANOVA. Comparisons of infarct size and glycogen content between different treatment groups were made by one-way ANOVA with the Student-Newman-Keuls test. Within a specific group, comparisons of ATP and PCR levels and of hemodynamic parameters at various time points were assessed for significance with a one-factor ANOVA with repeated measures by the Scheffe test. Statistical significance was defined as p<0.05.

**Results**

**Myocardial Glycogen Content**

The effect of preconditioning on myocardial glycogen stores and the time course of glycogen repletion are summarized in Figure 1. Immediately after preconditioning (group 1P rats), cardiac glycogen content was significantly reduced compared with controls (group 1C) (0.72±0.39 [n=9] versus 5.67±1.73 [n=12] mg glucose/g wet wt; p<0.001). Preconditioned rats that had recovered for only 30 minutes (group 2P+30m) had partial repletion of myocardial glycogen stores that was significantly greater than group 1P rats (p<0.05) but still significantly less than controls (group 2C) (2.21±0.69 [n=9] versus 5.54±0.99 [n=11] mg glucose/g wet wt; p<0.001) and groups 3P+60m and 4P+360m rats (p<0.05). Preconditioned rats that had recovered for 60, 180, or 360 minutes had fully repleted myocardial glycogen stores that were similar to their respective control groups (Figure 1).

**Infarct Size**

The effect of preconditioning and recovery after preconditioning on infarct size is displayed in Table 2 and Figure 2. Rats that underwent LCA occlusion immediately after preconditioning (group 1P) had greater than 10-fold reduction in infarct size compared with controls (group 1C). In contrast, rats that had recovered for 60 and 360 minutes after preconditioning before LCA occlusion (groups 3P+60m and 4P+360m) had an infarct size that was similar to corresponding controls (groups 3C and 4C) (Table 2, Figure 2). Preconditioned rats that were allowed to recover for 30 minutes before LCA occlusion (group 2P+30m) demonstrated an infarct size that was significantly greater than group 1P rats but still significantly less than controls (group 2C) (Table 2, Figure 2).

Thus, in preconditioned rats that were allowed to recover before prolonged ischemia, the loss of cardioprotection paralleled the time course of glycogen repletion (Figures 1 and 2).

**Relation Between Glycogen Levels and Infarct Size**

As summarized in Figures 1 and 2, there was a relation between myocardial glycogen levels and infarct size. Group 1P rats that had the lowest glycogen content before ischemia (0.72±0.39 mg glucose/g wet wt of cardiac tissue, n=9) had the smallest infarct size (0.3±0.6% infarct size divided by LV mass, n=7). In contrast, groups 1C, 2C, 3P+60m, 3C, 4P+360m, and 4C had the highest glycogen levels, ranging from 5.02 to 6.12 mg glucose/g wet wt of tissue, and had the largest infarcts, ranging from 35.5% to 43.6% of ischemic risk area. Group 2P+30m rats, which had been allowed to partially replete cardiac glycogen stores for 30 minutes...
after preconditioning (2.21±0.69 mg glucose/g wet wt, n=9), had an intermediate infarct size (24.1±22.0%, infarct size divided by risk area, n=13).

**Intracellular pH Values**

Relative changes in intracellular pH in preconditioned (group 1P) and control (group 1C) rats are summarized in Figure 3A. During ischemia, intracellular pH fell dramatically in control (group 1C) rats, reaching 6.2±0.3 (n=9) after 43 minutes of LCA occlusion. In contrast, the degree of intracellular acidosis was significantly attenuated in preconditioned (group 1P) rats, with pH reaching only 6.8±0.33 (n=11) (p<0.01 versus group 1C) after 43 minutes of ischemia. In both preconditioned and control rats, pH recovered to baseline value after reperfusion.

As summarized in Figure 3B, the changes in intracellular pH during ischemia were similar and more pronounced in preconditioned rats that had recovered for 6 hours before LCA occlusion (group 4P+360m) and control rats (group 4C). The intracellular pH became markedly acidic in both groups during the 45-minute ischemic period and recovered after 30 minutes of reperfusion.

**Magnetic Resonance Spectroscopy**

To assess the potential impact of preconditioning on high-energy phosphates during ischemia, 31P MRS was

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**Table 2.** Infarct Sizes of Preconditioned Rats in All Groups After 45 Minutes of Ischemia and 120 Minutes of Reperfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>Infarct size/LV mass</th>
<th>Risk area/LV mass</th>
<th>Infarct size/risk area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1P (n=7)</td>
<td>2.0±3.4 (p&lt;0.0001 vs. control group 1C)*</td>
<td>48.9±13.1 (p=NS vs. control group 1C)</td>
<td>0.3±0.6 (p&lt;0.0001 vs. control group 1C)</td>
</tr>
<tr>
<td>1C (n=7)</td>
<td>22.0±12.4†</td>
<td>52.7±10.6</td>
<td>38.1±11.3</td>
</tr>
<tr>
<td>2P+30m (n=13)</td>
<td>9.2±8.3 (p=0.034 vs. control group 2C, p&lt;0.05 vs. group 1P)</td>
<td>37.5±9.1 (p=NS vs. control group 2C)</td>
<td>24.1±21.9 (p=0.037 vs. control group 2C, p&lt;0.05 vs. group 1P)</td>
</tr>
<tr>
<td>2C (n=14)</td>
<td>16.4±8.4</td>
<td>38.1±10.6</td>
<td>43.6±23.9</td>
</tr>
<tr>
<td>3P+60m (n=15)</td>
<td>17.5±8.5 (p=NS vs. control group 3C)</td>
<td>46.9±14.4 (p=NS vs. control group 3C)</td>
<td>35.8±17.7 (p=NS vs. control group 3C)</td>
</tr>
<tr>
<td>3C (n=10)</td>
<td>20.4±10.4</td>
<td>50.9±8.6</td>
<td>40.7±19.9</td>
</tr>
<tr>
<td>4P+360m (n=11)</td>
<td>17.9±14.5 (p=NS vs. control group 4C)</td>
<td>43.8±5.7 (p=NS vs. control group 4C)</td>
<td>40.4±30.6 (p=NS vs. control group 4C)</td>
</tr>
<tr>
<td>4C (n=10)</td>
<td>16.0±14.0</td>
<td>50.0±13.3</td>
<td>35.5±31.0</td>
</tr>
</tbody>
</table>

LV, left ventricular. Infarct size measured by absence of triphenyltetrazolium chloride staining. Risk area measured by absence of phthalocyanine blue staining. All values expressed as mean±SD. All p values by ANOVA. For description of groups, see text.

*n=22, †n=21.
performed on selected rats in groups 1P and 4P+360m and their respective control rats (groups 1C and 4C). At baseline, PCr/ATP ratios were similar in preconditioned (group 1P) and control (group 1C) rats (2.14±0.40, n=11 versus 2.26±0.30, n=9; p=NS; ratios corrected for partial saturation). Figure 4 summarizes the changes in cardiac ATP and PCr levels during preconditioning, during 45 minutes of LCA occlusion, and after reflow in group 1P rats. During the preconditioning protocol, myocardial PCr was significantly depleted during the 5-minute episodes of LCA occlusion but recovered significantly during reperfusion and was 96.1±10.2% of

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{Bar graph showing infarct size of preconditioned and control rats that were subjected to left coronary artery (LCA) occlusion immediately after preconditioning (None=no recovery) or after a 30-minute, 1-hour, or 6-hour recovery period. Note that hearts undergoing LCA occlusion immediately after preconditioning (group 1P) had a marked reduction in infarct size compared with control hearts (group 1C). In contrast, preconditioned rats that had recovered for 1 and 6 hours before LCA occlusion (groups 3P+60m and 4P+360m) had an infarct size similar to their respective control groups (groups 3C and 4C). Preconditioned rats that were allowed to recover for 30 minutes after preconditioning (group 2P+30m) had an infarct size that was significantly greater than group 1P rats (p<0.05) but significantly less than the control rats (group 2C) (p=0.037). Inf/RA, infarct size as a percentage of risk area.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Panel A: Graph showing effect of left coronary artery (LCA) occlusion and reperfusion on intracellular pH in preconditioned (group 1P) and control (group 1C) rat hearts. There was a significant reduction in intracellular pH in both groups during ischemia that recovered to baseline value during reperfusion. The decline in intracellular pH was significantly attenuated in preconditioned rats compared with control rats during the ischemic period. *Significant difference between groups by ANOVA with p≤0.01. Panel B: Graph showing effect of 45 minutes of LCA occlusion and reperfusion on intracellular pH in preconditioned (group 4P+360m) and control (group 4C) rat hearts. There was a significant reduction in intracellular pH during the ischemic period that was similar in both groups, pH recovered to baseline values after reperfusion in each group. *Significant reduction from baseline value by ANOVA with p≤0.05.}
\end{figure}
baseline just before the 45-minute episode of LCA occlusion (n=11, p=NS versus baseline value) (Figure 4). Myocardial ATP levels did not vary significantly during the preconditioning protocol and were 88.0±12.1% of baseline value just before prolonged ischemia (n=11, p=NS versus baseline value).

Figures 5A, 5B, 6A, and 6B compare ATP and PCr levels during ischemia and reperfusion in preconditioned (group 1P) and control (group 1C) rats. During the ischemic period, there was a gradual decline in myocardial ATP levels in preconditioned rats that was similar to that observed in the control group (group 1C)
Myocardial PCr levels were also similar during the prolonged ischemic period in both preconditioned and control groups (Figure 6B). After 60 minutes of reperfusion, although ATP and PCr recovered significantly from 43 minutes of ischemia in preconditioned animals (p<0.05), there was no significant recovery in control rats (Figures 5A, 5B, 6A, and 6B). Furthermore, myocardial ATP levels in preconditioned rats were significantly greater than in control rats after 60 minutes of reperfusion (82.2±10.9%, n=11, versus 63.6±28.0%, n=9; p<0.001). Myocardial PCr levels in preconditioned rats were significantly greater than in control rats throughout the reperfusion period (p<0.001).

Figures 7A and 7B summarize changes in ATP and PCr levels during ischemia and reperfusion in preconditioned and control rats that had recovered for 6 hours after preconditioning but before the prolonged episode of ischemia (groups 4P+360m and 4C). Before ischemia, the PCr/ATP ratios (corrected for partial saturation) in preconditioned (group 4P+360m) and control (group 4C) animals were 2.53±0.67 (n=5) and 2.28±0.42 (n=6), respectively (p=NS). During the 45-minute period of LCA occlusion, there was a gradual and parallel decline in ATP levels in both preconditioned (group 4P+360m) and control (group 4C) rats (Figure 7A). During reflow, ATP failed to recover in both groups. There was a similar decline in PCr levels in preconditioned (group 4P+360m) and control (group 4C) rats during LCA occlusion (Figure 7B) and no PCr recovery in either group after reperfusion.

**Hemodynamic Data**

To determine whether the cardioprotection noted in preconditioned (group 1P) rats might be associated with alterations in the hemodynamic determinants of myocardial oxygen supply or demand, LV hemodynamics were recorded in selected group 1P and 1C rats during ischemia and reperfusion, as tabulated in Table 3. There were no significant differences in heart rate, LV systolic pressure, rate–pressure product, or LV dp/dt between preconditioned and control animals before and during the period of LCA occlusion. After 120 minutes of reperfusion, there was significant preservation of systolic LV function, as indicated by greater LV systolic pressure in preconditioned animals compared with controls (113±19 [n=10] versus 95±19 [n=10] mm Hg, p<0.05). However, there were no differences in heart rate, rate–pressure product, or LV dp/dt at any time during the 120 minutes of reperfusion in either the preconditioned or control groups.

**Discussion**

This study demonstrates four important findings. First, the cardioprotective effect of preconditioning was related to the degree of myocardial glycogen depletion before prolonged ischemia. Second, this protective effect was associated with attenuation of intracellular acidosis during ischemia in preconditioned rats. Third, the protective effect was independent of both the hemodynamic determinants of myocardial oxygen demand and the degree of ATP depletion during ischemia.
Fourth, in animals that were allowed to recover after preconditioning, the time course of glycogen recovery paralleled loss of protection from ischemic injury. The results support the hypothesis that glycogen depletion and the attenuation of intracellular acidosis during subsequent prolonged ischemia may be important factors responsible for delaying irreversible ischemic injury in preconditioned hearts. We noted that preconditioned hearts contained very little glycogen (Figure 2) and did not become markedly acidic during subsequent prolonged coronary artery occlusion (Figure 6A). In animals allowed to recover after preconditioning, the loss of protection closely paralleled the depletion of glycogen stores (Figures 1 and 3) and was associated with marked intracellular acidosis during subsequent ischemia (Figure 6B). Finally, there was an association between the degree of protection and the degree of glycogen depletion before ischemia. These observations are consistent with those of others who used both isolated perfused rat hearts and intact animals. In Langendorff-perfused rat hearts, Garlick et al.23 and Wolfe et al.22 noted that glycogen depletion results in less intracellular acidosis during subsequent prolonged ischemia, presumably because of diminished substrate available for anaerobic glycolysis. The importance of the amount of lactic acid production in ischemic injury was further emphasized by Neely and Grotyohann,17 who noted that, in isolated perfused rat hearts that were glycogen-depleted before prolonged global ischemia, recovery of ventricular function after reperfusion was inversely related to the level of tissue lactate during the ischemic episode. In the canine model, Murry et al.8 demonstrated that preconditioning caused a reduction in glycogen content and a decrease in lactate production during ischemia in preconditioned animals compared with controls. However, they did not measure intracellular pH and did not correlate the degree of myocardial protection with glycogen levels before prolonged coronary artery occlusion.

Since glycogen is a major substrate for anaerobic glycolysis during ischemia, depletion of glycogen could potentially have a detrimental effect on myocardial viability. If the capacity of the ischemic myocyte to produce ATP were limited by loss of substrate for anaerobic glycolysis, one might anticipate an acceleration of ATP depletion and an increase in ischemic injury in preconditioned hearts. Consistent with this hypothesis are the observations of Bailey and coworkers,15,24 who demonstrated that inhibiting the activity of phosphorylase b with deoxyglucose 6-phosphate attenuated the degree of acidosis and accelerated ATP depletion during global ischemia in isolated perfused rat hearts. In the present study, the fact that preconditioned hearts became less acidic during ischemia suggests that glycolysis was limited by substrate depletion, because anaerobic glycolysis is the major contributor of H+ production during myocardial ischemia.22 However, preconditioned myocardial tissue appears to be characterized not only by reduced glycolytic activity but also by reduced ATP utilization. Murry and

**Figure 7.** Panel A: Graph showing effect of 45 minutes of left coronary artery (LCA) occlusion and reperfusion on myocardial ATP levels in preconditioned (group 4P+360m) and control (group 4C) rats that had been allowed to recover for 6 hours before prolonged ischemia. During the ischemic period, there was a similar reduction in ATP levels in both groups that failed to recover after reperfusion. Significant reduction from baseline value by ANOVA with p<0.05. Panel B: Graph showing effect of 45 minutes of LCA occlusion and reperfusion on myocardial phosphocreatine (PCr) levels in preconditioned (group 4P+360m) and control (group 4C) rats. During the ischemic period, there was a significant reduction in PCr in both groups that failed to recover after reperfusion. Significant reduction from baseline value by ANOVA with p<0.05.
TABLE 3. Hemodynamic Changes in Preconditioned (Group 1P) (n=10) and Control Rats (Group 1C) (n=10) During Ischemia and Reperfusion

<table>
<thead>
<tr>
<th></th>
<th>Baseline 1</th>
<th>Baseline 2</th>
<th>1</th>
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<tbody>
<tr>
<td><strong>HR (bpm)</strong></td>
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<tr>
<td>P</td>
<td>371±50</td>
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<td>370±35</td>
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<tr>
<td>C</td>
<td>381±38</td>
<td>392±50</td>
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<td><strong>LVSP (mm Hg)</strong></td>
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<tr>
<td>P</td>
<td>131±60</td>
<td>137±60</td>
<td>107±38</td>
<td>123±50</td>
<td>122±44</td>
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<td>C</td>
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<td>137±47</td>
<td>107±41</td>
<td>116±25</td>
<td>115±28</td>
<td>108±35</td>
</tr>
<tr>
<td><strong>HR×LVSP (bpm×mm Hg/1,000)</strong></td>
<td>48.0±21.8</td>
<td>50.1±22.8</td>
<td>39.3±13.9</td>
<td>50.0±18.6</td>
<td>45.4±16.1</td>
<td>46.2±13.6</td>
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<tr>
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<td>C</td>
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<tr>
<td><strong>LV dP/dt (mm Hg/sec)</strong></td>
<td>5.062±1.853</td>
<td>5.019±1.679</td>
<td>4.382±2.065</td>
<td>4.791±1.825</td>
<td>5.064±1.701</td>
<td>5.099±1.597</td>
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<tr>
<td>P</td>
<td>5.062±1.853</td>
<td>5.019±1.679</td>
<td>4.382±2.065</td>
<td>4.791±1.825</td>
<td>5.064±1.701</td>
<td>5.099±1.597</td>
</tr>
<tr>
<td>C</td>
<td>5.624±1.882</td>
<td>5.927±1.853</td>
<td>4.636±1.572</td>
<td>5.218±860</td>
<td>5.227±759</td>
<td>4.779±980</td>
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</table>

HR, heart rate; bpm, beats per minute; LVSP, left ventricular systolic pressure; LV dP/dt, left ventricular change in pressure per second; P, preconditioned group 1P; C, control group 1C (see text). Baseline 1 refers to hemodynamic recordings obtained at the onset of the protocol. Baseline 2 refers to hemodynamics recorded after preconditioning or after a corresponding nonischemic control period. There were no significant differences in any hemodynamic parameter between groups at baseline or during coronary artery occlusion by ANOVA. There were no significant differences within either group at any time compared with the initial baseline value by ANOVA. All values are expressed as mean±SD.

*p<0.05 compared with the control group by ANOVA.

coworkers demonstrated that in the canine model of coronary artery occlusion and reperfusion, the rates of glycogen breakdown and ATP use during ischemia were significantly reduced in preconditioned hearts compared with controls. This resulted in slightly less ATP depletion in preconditioned hearts after 10 minutes of coronary artery occlusion but no net sparing of ATP after 20 and 40 minutes of ischemia. Further evidence for reduced ATP degradation in preconditioned hearts comes from the work of Swain et al., Reimer et al., Murry et al., and Kida et al., who demonstrated that, after preconditioning, the rate of overall ATP depletion was reduced during subsequent ischemia. In the present study, the similarity of ATP depletion in ischemic preconditioned and control hearts, despite reduced glycolytic activity in preconditioned hearts, suggests that the rate of ATP utilization was reduced in preconditioned hearts during ischemia. Thus, although the capacity for ATP synthesis may be limited by loss of available substrate for glycolysis, ATP levels appear to be maintained, to a degree, by decreased ATP utilization in preconditioned hearts.

Another potential mechanism of protection in preconditioned hearts is myocardial stunning, which potentially reduces the metabolic demands of the ischemic tissue. Previous investigators have reported that short episodes of coronary artery occlusion followed by reperfusion result in significant reduction in myocardial contractility, which could potentially reduce myocardial oxygen demand. Although it is possible that myocardial stunning may account for the reduction in ATP utilization noted above, our results and those of others suggest that stunning does not totally account for the protection afforded by preconditioning. Murry and coworkers noted a dissociation between stunned myocardium and cardioprotection secondary to preconditioning. In that study, preconditioned animals that were allowed to recover for 2 hours before prolonged ischemia had marked loss of protection even though myocardial stunning persisted. Although we did not measure segmental contractility in our rat model of preconditioning, we found no differences in the global myocardial determinants of oxygen demand (LV dP/dt, LV systolic pressure, LV rate-pressure product; Table 3) in preconditioned animals compared with control animals.

The results of this study and those of others suggest that intracellular acidosis may play an important role in the development of irreversible cellular injury secondary to sustained myocardial ischemia. As in the current study, Kida et al. also reported that preconditioned hearts develop less intracellular acidosis than control hearts during prolonged ischemia. Jennings and coworkers recently reported that the irreversible phase of myocardial injury was characterized by ATP and PCr depletion, low pH, and high lactate concentrations. The importance of avoiding marked reductions in pH is also suggested by the resistance of cardiac tissue to the development of acidosis. Previous work from our laboratory demonstrated that the intrinsic buffering capacity of rat myocardium increases at lower pH values, thus limiting the degree of intracellular acidosis that develops during ischemia. The results of the present study suggest that, by depleting glycogen stores, preconditioning may promote cellular viability during subsequent ischemia by further limiting the degree of intracellular acidosis that develops.

In addition to limiting the degree of intracellular acidosis, glycogen depletion may protect preconditioned myocardium by reducing lactate production per se, thereby reducing the osmotic load on injured cells after reperfusion. Sanz et al. recently reported that pig hearts subjected to preconditioning followed by 48 minutes of coronary artery occlusion and 30 minutes of...
reperfusion exhibited a 35% reduction in myocardial edema compared with controls. These investigators suggested that preconditioning may reduce irreversible ischemic injury by limiting reperfusion edema. Potentially, by reducing lactate production during ischemia, prior glycogen depletion might reduce reperfusion edema.

Although our data indicate the importance of glycogen depletion and attenuating the degree of intracellular acidosis in limiting irreversible injury in preconditioned hearts, this is unlikely to be the sole explanation. Other investigators have implicated potential roles for generation of free radicals,30 reduction in activated neutrophils,12 stimulation of A1 adenosine receptors,11,12,31 opening of potassium channels,32 altered fatty acid metabolism,33 and induction of stress protein34,35 in mediating the cardioprotection imparted by preconditioning. The effect of preconditioning on subsequent ischemic injury is a complex phenomenon that probably results from an interplay of multiple factors. Further work is necessary to define how these various factors interact to reduce ischemic injury in preconditioned hearts.

This study has several limitations. First, the myocardial glycogen levels were measured in groups of rats separate from those that underwent prolonged coronary artery occlusion, reflow, and subsequent infarct sizing. Thus, although infarct size correlated directly with myocardial glycogen levels, no direct cause-and-effect relation can be established. However, it should be emphasized that the animals killed for cardiac glycogen determination were subjected to protocols identical to those that were used for infarct sizing. Second, because myocardial ATP and PCr determinations were measured from the entire heart (including both ischemic and nonischemic segments), differences in ischemic risk area could potentially mask differences in high-energy phosphate levels within the ischemic territory between groups. That the ischemic risk areas were similar in preconditioned (group 1P) and control (group 1C) animals that underwent 31P MRS is evidenced by the similarity of the degree of PCr depletion during LCA occlusion in the two groups (Figure 6B). A previous study in our laboratory2 demonstrated a good correlation between the degree of PCr reduction and the size of the ischemic risk area in this rat model of coronary artery occlusion and reperfusion. Third, because the P1 resonance was contaminated by that of 2,3-DPG, this could potentially limit one’s ability to accurately measure intracellular pH. However, as stated previously, the P1 resonance was clearly visible in most cases, especially during ischemia and reflow. Fourth, it should be noted that since we did not measure myocardial blood flow in our preparation, one cannot exclude the possibility that preconditioning caused an increase in collateral blood flow. However, collateral blood flow to the ischemic zone is minimal in the ischemic rat preparation.1 Furthermore, there were no significant differences in the degree of ATP or PCr depletion between preconditioned animals and control animals during the ischemic period. If preconditioning resulted in significant induction of collateral blood flow, one would expect sparing of high-energy phosphates during subsequent prolonged ischemia. Finally, Murry and coworkers,4,8,9 Li and coworkers,36 and Hale and Klone37 have demonstrated that, in the canine and rabbit models, preconditioning results in significant infarct size reduction without increasing collateral blood flow to the ischemic area.

Conclusions

This study demonstrates that myocardial protection in preconditioned animals was correlated with the degree of glycogen depletion before prolonged ischemia resulting in attenuation of the degree of intracellular acidosis during subsequent prolonged coronary artery occlusion. The potential importance of glycogen depletion in reducing infarct size was further suggested by the parallel time courses of glycogen resynthesis after preconditioning and the loss of protection from ischemic injury. Although glycogen depletion limited the glycolytic activity in ischemic preconditioned hearts, the reduced capacity for ATP synthesis appears to be balanced by reduced ATP utilization, resulting in no net differences in ATP depletion between preconditioned and control hearts during ischemia. These results suggest that glycogen depletion, limitation of the severity of

<table>
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<th>TABLE 3. Continued</th>
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<tr>
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<tr>
<td>HR (bpm)</td>
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<tr>
<td>P</td>
<td>376±38</td>
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<tr>
<td>C</td>
<td>386±41</td>
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<td>LVSP (mm Hg)</td>
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<tr>
<td>P</td>
<td>123±38</td>
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<tr>
<td>C</td>
<td>96±32</td>
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<td>HR×LVSP (bpm×mm Hg/1,000)</td>
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<tr>
<td>P</td>
<td>46.1±13.6</td>
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<tr>
<td>P</td>
<td>4,269±1,553</td>
</tr>
<tr>
<td>C</td>
<td>3,573±1,157</td>
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</table>
intracellular acidosis, and reduced ATP utilization may be important factors in delaying the onset of irreversible ischemic injury in this animal model of myocardial preconditioning.

Acknowledgments
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Loss of myocardial protection after preconditioning correlates with the time course of glycogen recovery within the preconditioned segment.
C L Wolfe, R E Sievers, F L Visseren and T J Donnelly

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