Intermittent Ischemia Produces a Cumulative Depletion of Mitochondrial Adenine Nucleotides in the Isolated Perfused Rat Heart

Gregory K. Asimakis, Gulzar S. Sandhu, Vincent R. Conti, Louis A. Sordahl, and Joseph B. Zwischenberger

The purpose of the present study was to determine if repetitive myocardial ischemia would result in the cumulative loss of mitochondrial adenine nucleotides. Isolated perfused rat hearts were subjected to continuous or intermittent ischemia. A single 5-minute period of continuous ischemia did not result in a significant decrease in the mitochondrial adenine nucleotide pool; a single 10-minute period of ischemia resulted in a decrease of approximately 17%. Next, the adenine nucleotide content of mitochondria from preischemic and 30-minute continuous ischemic hearts was compared with two groups of hearts undergoing intermittent ischemia (both groups receiving a total of 30 minutes of ischemia). One group received three 10-minute episodes of ischemia interrupted by 5-minute periods of reperfusion (3×10-minute intermittent ischemia); the other intermittent ischemic group received six 5-minute episodes of ischemia interrupted by 5-minute periods of perfusion (6×5-minute intermittent ischemia). The mitochondrial adenine nucleotide content (expressed as nanomoles per nanomole cytochrome a) for the preischemic and 30-minute continuous ischemic hearts was 14.7±0.6 and 8.0±0.4, respectively. The mitochondrial adenine nucleotide content of the 3×10-minute intermittent ischemia group (8.5±0.5) was not significantly different from the 30-minute continuous ischemic group. The mitochondrial adenine nucleotide content of the 6×5-minute intermittent ischemia group (11.0±0.6) was significantly larger than that of the 30-minute continuous and the 3×10-minute intermittent ischemia groups (p<0.05). Postischemic reperfusion for up to 1 hour did not result in levels of mitochondrial adenine nucleotides significantly above those observed at the end of ischemia. Moreover, isolated rat heart mitochondria were unable to accumulate adenine nucleotides when incubated in 2 mM ATP; conversely, the adenine nucleotide content of isolated liver mitochondria increased approximately fivefold within 60 minutes when incubated under the same conditions. The study suggests that short, repetitive episodes of ischemia result in the cumulative loss of mitochondrial adenine nucleotides. The results also indicate that heart mitochondria may not have a mechanism to replenish the lost pool of adenine nucleotides, indicating that short episodes of ischemia interrupted by long periods of perfusion could also result in the cumulative loss of mitochondrial adenine nucleotides. (Circulation Research 1990;66:302-310)

The human myocardium can be subjected to recurrent, closely spaced episodes of ischemia due to coronary vasospasm or from intermittent occlusions arising during coronary thrombus formation. Although the duration of any single ischemic episode may be insufficient to cause irreversible injury, metabolic derangements (such as depressed levels of adenine nucleotides) may persist. In this case, repetitive episodes of ischemia could lead to cumulative metabolic abnormalities that may result in irreversible damage.

The adenine nucleotide content (ATP+ADP+AMP) of the heart decreases during ischemia. Adenine nucleotide loss occurs both in the cytosol and mitochondrial matrix.1-3 During ischemia, aerobic ATP synthesis ceases and there is net degradation of cytosolic ATP to ADP and AMP, which can further be converted to nucleosides and purine bases. Although mitochondrial adenine nucleotides can be interconverted,4 mitochondrial AMP is not accessible

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to 5'-nucleotidase activity, which is located in sarcolemmal membrane and cytosol. Therefore, the loss of adenine nucleotides from the mitochondrial space is not likely due to degradation of AMP to adenosine. Instead, the loss is most readily explained by the direct movement of at least one of the nucleotide species through the inner membrane, possibly in response to elevated levels of inorganic phosphate.\(^7\)\(^-\)\(^9\) The loss of both mitochondrial and cytosolic adenine nucleotides could severely limit posts ischemic contractile recovery by limiting the availability of ATP for energy-requiring processes of the myocardial cell.

The posts ischemic, reversibly injured heart can salvage purine bases and nucleosides to nucleotides and ultimately to ATP. However, only partial recovery of nucleotides occurs early after reperfusion due to washout of the nonphosphorylated precursors.\(^10\)\(^,\)\(^11\) Recovery of the total myocardial adenine nucleotide pool to normal levels requires de novo synthesis and may take a week or longer even after a brief period of ischemia.\(^12\) It has not been established if the mitochondrial adenine nucleotide pool of the posts ischemic heart can be restored.

Several investigators\(^13\)\(^-\)\(^15\) have reported that repetitive episodes of myocardial ischemia do not result in the cumulative loss of whole-tissue adenine nucleotides beyond that observed after the initial ischemic episode. However, other investigators\(^16\)\(^,\)\(^17\) have reported contradictory results. In all of these reports, only the adenine nucleotide content of the whole heart (and not the intracellular compartments) was measured. In the present study, we evaluated the effect of repetitive ischemia and reperfusion on the adenine nucleotide content of the mitochondrial compartment relative to the whole myocardium.

Materials and Methods

Male, Sprague-Dawley rats (200–250 g) were injected intraperitoneally with sodium heparin (160 units). Each animal was anesthetized with an injection of 25 mg i.p. sodium pentobarbital. The hearts were excised and placed in ice-cold Krebs-Henseleit buffer (KHB). For each heart, the aorta was cannulated, and the heart was perfused with oxygenated (95% O\(^2\)-5% CO\(_2\)) KHB (37° C) at a perfusion pressure of 75 mm Hg. KHB had the following millimolar composition: KCl 4.7, CaCl\(_2\) 2.5, MgSO\(_4\) 2.5, KH\(_2\)PO\(_4\) 1.2, EDTA 0.5, NaHCO\(_3\), 25,NaCl 118, and glucose 5. The pulmonary artery was cut to ensure that coronary flow was not restricted. Each heart was perfused for 15 minutes before ischemia was induced (unless the heart was to be used as a preischemic control).

Ischemic Groups

Continuous ischemia. After 15 minutes of preliminary perfusion, coronary perfusion was stopped by cross-clamping the buffer line leading to the aortic cannula. The ischemic hearts were maintained at 37° C in a water-jacketed chamber. Unless stated otherwise, the duration of ischemia was 30 minutes.

 intermittent ischemia (3×10-minute). After the initial perfusion period, these hearts underwent three 10-minute episodes of ischemia (37° C) interrupted by two 5-minute periods of perfusion. Time of total ischemia was 30 minutes.

 intermittent ischemia (6×5-minute). After the initial perfusion period, these hearts underwent six 5-minute episodes of ischemia (37° C) interrupted by five 5-minute periods of reperfusion. Total time of ischemia was 30 minutes.

At the end of the ischemic episode (the last ischemic episode of the intermittent ischemic groups), the hearts were homogenized for the isolation of mitochondria; freeze clamped at liquid nitrogen temperature for the determination of whole-heart metabolites; or reperfused for a specified length of time, after which mitochondria were isolated or the hearts were freeze clamped.

Hemodynamic measurements. For those hearts that were reperfused, preischemic and posts ischemic hemodynamics were determined. In each of these hearts, the atrial appendage was opened and a latex balloon was inserted through the mitral valve into the left ventricle. A piece ofstuff, small-bore plastic tubing was used to facilitate balloon insertion and to inflate the balloon with water. The balloon was interfaced to a pressure transducer (Statham model P23, Gould Instruments, Cleveland, Ohio) and a 12-channel linear recorder (Mark IV, Watanabe, Japan). During the initial perfusion periods, the balloon was inflated to a volume to give a diastolic pressure of approximately 10 mm Hg. Pressure measurements were made just before the initiation of ischemia. Before the initiation of ischemia, the balloon was deflated, and the balloon remained deflated during the time of ischemia. Postischemic pressure measurements were made after 5 minutes and 60 minutes of reperfusion. At these times, the balloon was reinfated to the original volume. Preischemic and posts ischemic coronary flow rates were directly measured by collecting aliquots into a graduated cylinder.

Isolation of Heart Mitochondria

Each heart was placed in cold buffer consisting of 250 mM sucrose, 10 mM 3-(N-morpholino) propanesulfonic acid (MOPS), and 1 mM ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA), pH 7.4 (SE buffer). The tissue was cut into slices with scissors and minced with a specially designed tissue mincer (razor blades affixed to a spring-loaded piston). The minced tissue was rinsed several times with SE buffer. The rinsed tissue was incubated for 30 seconds with proteinase at 1 mg/g tissue (Nagarse, Enzyme Development, New York). The suspension was then centrifuged at 16,000g for 10 minutes. The pellet was resuspended and centrifuged at 500g for 5 minutes. The supernatant was saved, and the pellet was resuspended in 10 ml SE buffer and centrifuged at 500g for 5 minutes. The supernatant was pooled with the supernatant from the previous centrifugation and centrifuged at...
10,000g for 10 minutes. The pellets were resuspended in 5 ml of 250 mM sucrose, 10 mM MOPS, and 0.5% bovine serum albumin, pH 7.4 (SA buffer). The suspensions were centrifuged again at 10,000g. Each pellet was resuspended in 1 ml SA buffer. Mitochondrial yields and purity (as determined by cytochrome a content) were not affected by ischemia or reperfusion. The mitochondrial yield was 20.5±0.7 mg protein/g wet heart and the cytochrome a was 0.86±0.01 nmol/mg mitochondrial protein (n=11).

**Uptake of Adenine Nucleotides**

In one experiment, the ability of isolated heart and liver mitochondria to accumulate adenine nucleotides was compared. Heart mitochondria were isolated from freshly excised hearts by the method described above. It was necessary to pool mitochondria from four hearts to obtain enough protein for the experiment. Liver mitochondria were isolated by a previously described method. Mitochondria (1 mg/ml) were incubated at 30°C in a buffer with a millimolar composition of KCl 120, KH2PO4 10, MgCl2 10, α-ketoglutarate 10, ATP 2, and Tris 10, pH 7.4. During the incubation, 15-ml aliquots were taken at 5, 15, 30, and 60 minutes and centrifuged at 10,000g for 10 minutes. The pellets were resuspended in 15 ml SA (or 250 mM sucrose and 10 mM Tris, pH 7.4, for liver mitochondria) and centrifuged again at 10,000g for 10 minutes. The pellets were suspended in approximately 1 ml buffer.

**Assays**

Mitochondrial protein was determined by the biuret assay as previously described by Jacobs et al. Cytochrome a content of the mitochondria was determined by a modification of the method of Williams using a dual beam spectrophotometer (Kontron Instruments, Everett, Massachusetts). Mitochondrial oxygen consumption was determined using a Clark electrode. Mitochondria (0.5 mg) were assayed in a buffer with a millimolar composition of sucrose 225, Tris 10, KCl 15, EDTA 1, rotenone (1 µg/mg protein), and KH2PO4 10 at pH 7.4 (final volume, 1.0 ml at 30°C). After 1 minute, approximately 400 nmol ADP was added to initiate state 3 respiration. The acceptor control ratio was determined by dividing the state 3 rate by the rate of oxygen consumption before the addition of ADP. Mitochondrial and whole-heart adenine nucleotides of perchloric acid extracts were estimated by reverse-phase high-performance liquid chromatography as previously described. Inorganic phosphate was determined by the method of Baginski et al. ATP, creatine phosphate, glucose 6-phosphate and lactate were determined by standard enzymatic analysis of the neutralized extracts. For tissue ATP levels, the values used were from the enzymatic analysis and not the high-performance liquid chromatography analysis.

**Figure 1.** Decrease in the mitochondrial adenine nucleotide pool of the heart during ischemia. The hearts were perfused with Krebs-Henseleit buffer for 15 minutes followed by a period of normothermic, global ischemia as indicated. At the end of the ischemic period, mitochondria were isolated and assayed for ATP, ADP, and AMP. The mitochondrial adenine nucleotides (MAdN) is the sum of these three nucleotides. Each point represents the mean±SEM of six to eight hearts. *p<0.05 compared with zero-time group.

**Statistical Analysis**

Analysis of variance (ANOVA) was used to determine significant differences within groups. If indicated by ANOVA, a modified t test (Bonferroni method) was used to determine differences between groups. A value of p<0.05 was considered significant.

**Results**

**Effect of Continuous and Intermittent Ischemia on the Mitochondrial Adenine Nucleotide Content and Respiratory Activity**

The change in the mitochondrial adenine nucleotide content (ATP+ADP+AMP) during continuous ischemia in the isolated rat heart is shown in Figure 1. Although the mitochondrial adenine nucleotide content decreased on average approximately 18% after 10 minutes of ischemia, ANOVA indicated no significant differences among the preischemic, the 5-minute, and 10-minute ischemic groups. After 30 minutes of ischemia, the mitochondrial adenine nucleotide content was significantly lower than the preischemic (p<0.0001) and 10-minute ischemic (p<0.005) groups. We next determined if short, repetitive episodes of ischemia would be cumulative with respect to the loss of mitochondrial adenine nucleotide. Based on the information shown in Figure 1, we used repetitive 5- and 10-minute episodes of ischemia, each followed by 5 minutes of reperfusion; the total time of ischemia was 30 minutes in both groups. The groups receiving 5-minute and 10-minute intermittent ischemic episodes are designated 5×5 and 3×10, respectively. There was no significant difference between the continuous and the 3×10-minute intermittent ischemic groups (Figure...
The mitochondrial adenine nucleotide content of both of these groups was approximately 50% of preischemic levels. The mitochondrial adenine nucleotide content of the 6\times5-minute intermittent group was also significantly lower than that of the preischemic group. However, mitochondrial adenine nucleotide content of the 6\times5-minute intermittent group was significantly higher than either the continuous or 3\times10-minute intermittent groups (Figure 2).

State 3 respiration of mitochondria from the continuous ischemic group was depressed by 27% with succinate and 14% with the NADH-linked substrate pair glutamate/malate (Table 1); the difference with glutamate/malate was not statistically significant. State 3 was not significantly depressed from control values after either 3\times10- or 6\times5-minute intermittent ischemia. With glutamate/malate as the respiratory substrate, the average state 3 values were elevated (although the differences were not statistically significant) above normal levels. Respiratory coupling (as indicated by the acceptor control ratios) was not significantly affected by continuous or intermittent ischemia compared to controls.

**Effect of Reperfusion on the Mitochondrial Adenine Nucleotide Content and Respiratory Functions After Intermittent Ischemia**

In the experiment shown in Table 2, mitochondria were isolated from three groups of hearts: preischemic, 3\times10-minute intermittent ischemia, and 3\times10-minute intermittent followed by 60 minutes of reperfusion. The total mitochondrial adenine nucleotide content was 64% of the nonischemic values at the end of the ischemic period and 48% of the preischemic values after 60 minutes of reperfusion. The additional loss of adenine nucleotides during reperfusion was not the result of prolonged perfusion; in a separate experiment, the mitochondrial adenine nucleotide content of hearts perfused for 15 and 45 minutes was 14.7\pm0.8 nmol/nmol cytochrome a and 15.9\pm0.7 nmol/nmol cytochrome a, respectively (n=6 for both groups). State 3 respiration was not significantly affected by intermittent ischemia or reperfusion (Table 2). At the end of the ischemic period, the acceptor control ratio with succinate was lower than preischemic levels, and the ratio remained depressed following reperfusion. With glutamate/malate, the control ratio at the end of ischemia and reperfusion tended to be lower than preischemic values; however, the differences were not statistically significant.

The results in Table 2 show that the mitochondrial adenine nucleotide pool was not restored after 60 minutes of reperfusion. While liver mitochondria can increase the adenine nucleotide pool of the matrix by direct transport of ATP across the inner membrane, it is not known if a similar process can occur in heart mitochondria. We compared the abilities of isolated liver and heart mitochondria to

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**Table 1. Effect of Continuous and Intermittent Ischemia on Mitochondrial Respiration**

<table>
<thead>
<tr>
<th></th>
<th>Succinate</th>
<th>Glutamate/malate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>State 3*</td>
<td>ACR</td>
</tr>
<tr>
<td>Preischemic</td>
<td>7</td>
<td>586\pm48</td>
</tr>
<tr>
<td>Continuous ischemic</td>
<td>8</td>
<td>430\pm24†</td>
</tr>
<tr>
<td>Intermittent ischemic (3\times10 min)</td>
<td>8</td>
<td>549\pm67</td>
</tr>
<tr>
<td>Intermittent ischemic (6\times5 min)</td>
<td>6</td>
<td>521\pm20‡</td>
</tr>
</tbody>
</table>

ACR, acceptor control ratio; n, number of hearts.

*tp<0.05 compared with preischemic.
‡p<0.05 compared with continuous ischemic.
increase their adenine nucleotide pools when incubated in the presence of 2 mM ATP. While the adenine nucleotide pool of the liver mitochondria increased fivefold over 60 minutes, no change was observed for heart mitochondria (Figure 3). Incubation of heart mitochondria in ADP or AMP also did not result in an increase in the adenine nucleotide content (data not shown).

**Whole Heart Metabolites: Effect of Continuous and Intermittent Ischemia**

In a parallel experiment to that described in Figure 2, the hearts were freeze clamped for the determination of whole-heart metabolites (Table 3). An additional group of hearts were freeze clamped after 10 minutes of continuous ischemia. For all of the ischemic groups, tissue ATP and creatine phosphate were significantly lower than nonischemic values. After 10 minutes of ischemia, approximately 50% of the ATP content was lost. After 30 minutes of ischemia (continuous and intermittent), the ATP levels were depressed approximately 90%. There were no significant differences between the ATP levels of the 30-minute continuous and the two intermittent ischemic groups. The total adenine nucleotide content of the 30-minute continuous and the intermittent ischemic groups was similarly depressed from preischemic and 10-minute ischemic levels. The total adenine nucleotide content of the 3 × 10-minute intermittent ischemic group was approximately 20% lower than both the 30-minute continuous and the 6 × 5-minute intermittent ischemic groups; the difference between the 30-minute continuous and the 3 × 10-minute intermittent ischemic groups was due to the difference in the AMP and ADP levels (Table 3). Following 30 minutes of ischemia (continuous or intermittent), the inorganic phosphate (P_i) levels were elevated approximately threefold. Lactate levels of the 10- and 30-minute continuous ischemic groups were approximately 40- and 50-fold greater, respectively, than the preischemic group. The lactate levels of the 3 × 10-minute intermittent ischemic group was only 15-fold (compared with 40-fold for the 10-minute continuous ischemic group) greater than preischemic levels. The glucose 6-phosphate levels increased fivefold and 17-fold after 10 and 30 minutes of continuous ischemia, respectively. Glucose 6-phosphate levels of the 3 × 10-minute intermittent ischemic group was not significantly different than nonischemic values. Lactate and glucose 6-phosphate levels were not determined for the 6 × 5-minute intermittent ischemic group.

**Metabolic and Functional Recovery After Continuous and Intermittent Ischemia**

The effect of reperfusion after continuous and intermittent ischemia on the levels of whole-heart metabolites is shown in Table 4. Three ischemic groups were compared: 30-minute continuous ischemia, 3 × 10-minute intermittent ischemia, and 6 × 5-minute intermittent ischemia. For each group, the hearts were reperfused for 5 or 60 minutes. ATP levels were significantly higher in the groups subjected to intermittent ischemia compared with the continuous ischemic group. The total adenine nucleotide levels were not significantly different within the groups, indicating that little if any extra washout of adenine nucleotide precursors occurred during the

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**Table 2. Effect of Reperfusion on Mitochondrial Adenine Nucleotides and Respiration After Intermittent Ischemia**

<table>
<thead>
<tr>
<th></th>
<th>Succinate</th>
<th>Glutamate/malate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAdN*</td>
<td>State 3†</td>
</tr>
<tr>
<td>Preischemic</td>
<td>13.9±0.1</td>
<td>683±39</td>
</tr>
<tr>
<td>Intermittent ischemic (3×10 min)</td>
<td>8.9±0.4‡</td>
<td>689±28</td>
</tr>
<tr>
<td>Ischemic (3×10 min) + 60 minutes reperfusion</td>
<td>6.0±0.4§</td>
<td>672±34</td>
</tr>
</tbody>
</table>

ACR, acceptor control ratio; n = 5 for each group.

*Mitochondrial adenine nucleotides (MAdN) expressed as nmol/nmol cytochrome a.

†ng atoms 0/min/nmol cytochrome a.

‡p < 0.001 compared with preischemic.

§p < 0.001 compared with intermittent ischemic.

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**Figure 3. Accumulation of adenine nucleotides by isolated liver and heart mitochondria.** Both mitochondrial preparations were incubated in 2 mM ATP. At the times indicated, samples were taken and "washed" twice. The "washed" samples were assayed for ATP + ADP + AMP. The values represent the average of the two experiments. Details of the experiments are given in "Materials and Methods." MAdN, mitochondrial adenine nucleotides.
TABLE 3. Effect of Continuous and Intermittent Ischemia on Myocardial Metabolites

<table>
<thead>
<tr>
<th></th>
<th>AMP</th>
<th>ADP</th>
<th>ATP</th>
<th>TAdN*</th>
<th>P&lt;sub&gt;i&lt;/sub&gt;</th>
<th>CP</th>
<th>Lactate</th>
<th>G-6-P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol/mg myocardial protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preischemic</td>
<td>2.2±0.3</td>
<td>8.6±0.7</td>
<td>31.8±1.0</td>
<td>42.1±1.8</td>
<td>46.6±0.6</td>
<td>41.6±2.9</td>
<td>4.6±1.2</td>
<td>0.82±0.14</td>
</tr>
<tr>
<td>Cont. isch (10 min)</td>
<td>10.4±0.8†</td>
<td>11.0±0.7†</td>
<td>17.2±1.7†</td>
<td>38.4±0.6</td>
<td>94.9±6.4†</td>
<td>0.40±0.4†</td>
<td>171.±14†</td>
<td>0.40±0.9†</td>
</tr>
<tr>
<td>Cont. isch (30 min)</td>
<td>18.2±1.6†</td>
<td>4.4±0.5†</td>
<td>3.3±0.3†</td>
<td>27.6±2.4†</td>
<td>152±11†</td>
<td>4.4±0.6†</td>
<td>229±8†</td>
<td>14.2±0.9†</td>
</tr>
<tr>
<td>Intermittent isch (3×10 min)</td>
<td>14.2±0.5†</td>
<td>3.8±0.4†</td>
<td>3.8±0.3†</td>
<td>21.9±0.8†</td>
<td>152±7†</td>
<td>5.3±0.2†</td>
<td>68.2±0.8†</td>
<td>0.7±0.3§</td>
</tr>
<tr>
<td>Intermittent isch (6×5 min)</td>
<td>14.6±0.8†</td>
<td>5.9±1.0</td>
<td>4.7±0.5†</td>
<td>24.9±0.8†</td>
<td>152±7†</td>
<td>5.8±0.7†</td>
<td>nd§</td>
<td>nd§</td>
</tr>
</tbody>
</table>

CP, creatine phosphate; G-6-P, glucose 6-phosphate.
*Total adenine nucleotides (TAdN) (AMP+ADP+ATP); AMP and ADP were not determined in all samples; therefore, the sum of the means of the three individual nucleotides may not equal the mean TAdN.
†p<0.05 compared with preischemic.
‡p<0.05 compared with continuous ischemic (30 minutes).
§Not determined.

n=5 or 6 for each group.

perfusion periods in the intermittent ischemic groups. However, P<sub>i</sub> levels were lower in the intermittent ischemic groups than the continuous ischemic group. The energy charge after reperfusion was significantly higher in the intermittent ischemic groups compared with the continuous ischemic groups; preischemic energy charge was 0.83±0.02 (n=6). Following 5 minutes of reperfusion, creatine phosphate levels tended to be higher in the intermittent ischemic groups compared with continuous ischemic group, although the differences were statistically significant only in the 6×5-minute intermittent ischemic group. In the continuous ischemic group, the creatine phosphate levels decreased significantly between 5 and 60 minutes of reperfusion. In the 3×10-minute intermittent ischemic group, there was no significant difference in the creatine phosphate levels after 5 and 60 minutes of reperfusion; the 6×5-minute intermittent ischemic group was reperfused for 5 minutes only.

Postischemic recovery of hemodynamic functions is shown in Table 5. In general, the intermittent ischemic hearts had better recovery of contractile function than the continuous ischemic group. For the 30-minute continuous ischemic group, three out of 11 hearts did not show any recovery of mechanical function after reperfusion. For the 3×10-minute intermittent ischemic group, one heart out of 11 failed to recover any functional activity. For the 6×5-minute intermittent ischemic group, all hearts (n=7) exhibited partial recovery after reperfusion. Average recovery of heart rate, developed pressure, and dP/dt was greater in the intermittent ischemic groups compared with the continuous ischemic group. Severe ischemic contracture was evident in the continuous ischemic hearts as indicated by the relatively large increase in left ventricular end-diastolic pressure. Restoration of coronary flow was significantly higher in the intermittent ischemic groups compared with the continuous ischemic group.

**Discussion**

**Intermittent Ischemia and Mitochondrial Adenine Nucleotides**

Although the adenine nucleotide pool of the mitochondrial fraction of the normal heart cell represents...
TABLE 5. Recovery of Hemodynamic Functions During Reperfusion After Continuous and Intermittent Ischemia

<table>
<thead>
<tr>
<th></th>
<th>Coronary flow</th>
<th>Heart rate</th>
<th>dP/dt</th>
<th>Developed pressure</th>
<th>LVEDP* (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min ischemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min rep</td>
<td>31.0±5.5</td>
<td>23.1±8.3</td>
<td>5.5±2.8</td>
<td>10.6±3.9</td>
<td>50.4±8.0</td>
</tr>
<tr>
<td>60 min rep</td>
<td>40.4±3.8</td>
<td>40.7±14.6</td>
<td>21.4±10.5</td>
<td>26.1±13.7</td>
<td>24.4±14.0</td>
</tr>
<tr>
<td>Intermittent ischemia (3×10 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min rep</td>
<td>84.0±8.2†</td>
<td>63.7±11.3†</td>
<td>67.3±14.0†</td>
<td>68.6±16.0†</td>
<td>13.0±3.3†</td>
</tr>
<tr>
<td>60 min rep</td>
<td>62.1±6.3‡</td>
<td>69.6±17.6</td>
<td>63.4±16.8‡</td>
<td>51.0±14.8</td>
<td>10.0±3.5‡</td>
</tr>
<tr>
<td>Intermittent ischemia (6×5 min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min rep</td>
<td>75.5±12.6†</td>
<td>87.4±5.1†</td>
<td>84.5±8.6†</td>
<td>95.2±6.9†</td>
<td>10.0±3.1†</td>
</tr>
</tbody>
</table>

*Left ventricular end diastolic pressure.
†p<0.05 compared with 30 min continuous isch−5 min rep.
‡p<0.05 compared with 30 min continuous isch−60 min rep.
n=5−8 for each group.

only 15–20% of the cellular adenine nucleotide pool,26 this pool is essential for ATP synthesis. The purpose of the present study was to determine if repetitive, short episodes of myocardial ischemia would result in cumulative depletion of the mitochondrial adenine nucleotide pool. Ten minutes of continuous ischemia resulted in an average decrease of 15% in the mitochondrial adenine nucleotide pool (Figure 1). Three 10-minute episodes of ischemia, interrupted by 5-minute periods of reperfusion, resulted in a 50% loss of the mitochondrial adenine nucleotide pool (Figure 2). This loss was not different than that observed for one 30-minute continuous episode of ischemia. Five minutes of continuous ischemia resulted in no significant decrease in the adenine nucleotide pool (Figure 1). However, six 5-minute episodes of ischemia, interrupted by five periods of perfusion, resulted in approximately 25% loss of mitochondrial adenine nucleotides (Figure 2). The results demonstrate that the ischemia-induced loss of mitochondrial adenine nucleotides was cumulative. Additional adenine nucleotide loss occurred during reperfusion (Table 3), although, the rate of loss was less during reperfusion than during ischemia. The loss of mitochondrial adenine nucleotides occurred only during ischemia and reperfusion, since prolonged perfusion of nonischemic hearts did not result in the loss of adenine nucleotides.

We have previously reported that P flow can induce the efflux of mitochondrial adenine nucleotides.7–9 In the myocardium, the P flow level doubles within 1 minute after the onset of ischemia.27–29 We observed that P flow increases in a biphasic manner with a rapid rise that coincides with the decrease in creatine phosphate and a slower rise that coincides with the decrease in adenine nucleotides (data not shown). Kubler and Katz27 estimated that during myocardial ischemia, the P flow concentration can increase eightfold, from 5.6 to 48 mM. In our study, the P flow levels were elevated approximately threefold over normal levels after 30 minutes of ischemia (continuous or intermittent) and the levels remained elevated (approximately twofold above nonischemic levels) after 60 minutes of reperfusion (Tables 3 and 4). These elevated P flow levels may contribute to the loss of adenine nucleotides from the mitochondrial compartment during ischemia and reperfusion. An alternative explanation is that mitochondrial membrane damage occurs during ischemia and the integrity of the membrane is not restored (or the damage is exacerbated) during reperfusion resulting in the adenine nucleotide loss during isolation of the mitochondria. However, mitochondria isolated from ischemic hearts do not lose adenine nucleotides during isolation,1 and the membrane integrity of mitochondria from postischemic hearts appears intact as judged by the acceptor control ratios (Table 2).

Another observation of this study is that repetitive ischemic episodes result in the cumulative loss of tissue ATP and adenine nucleotides. This appears to conflict with the reports of other investigators.13–15 The discrepancy may be due to the different ischemic models and sampling techniques. We used global ischemia in the rat heart, whereas the other investigators used regional ischemia in the dog heart. With regional ischemia, some collateral flow, retrograde perfusion, and autonomic “shunting” are possible, especially in the dog heart. These factors make it possible to maintain some level of energy production either through the stimulation of glycolysis or through residual oxidative phosphorylation. The globally ischemic heart does represent a model of total, acute myocardial ischemia, and it is likely that similar changes occur in areas of total ischemic insult in the regionally ischemic heart.

**Postischemic Recovery of Adenine Nucleotides**

Postischemic recovery of whole-heart adenine nucleotides occurs in two phases and can take a week or longer even after brief periods of ischemia.11,12 The early phase of resynthesis takes place by salvage of adenine nucleotide degradation products. The later phase requires de novo synthesis. Both phases or pathways occur in the extramitochondrial space.30

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Therefore, the whole-tissue adenine nucleotide pool may eventually be completely restored, but restoration to normal levels within the intracellular compartments during reperfusion can occur only if a mechanism of net uptake of adenine nucleotides into the mitochondrial compartment exists. Although extramitochondrial ADP or ATP can be transported across the mitochondrial inner membrane by the adenine nucleotide translocase, there is no net change in the mitochondrial adenine nucleotide pool size since the adenine translocase facilitates a one-for-one exchange of intramitochondrial ATP (or ADP) for extramitochondrial ADP (or ATP).31,32 In our study, the intermittent ischemic hearts were metabolically and functionally better than the continuous ischemic hearts (Tables 4 and 5). The inability of the mitochondrial adenine nucleotide content to recover after 60 minutes of reperfusion (Table 2) was due to either lack of a mechanism for adenine nucleotide uptake in heart mitochondria or a net efflux in the early reperfusion period (up to 60 minutes). The lack of an uptake mechanism implies that the adenine nucleotide pool of the mitochondrial fraction of the postischemic heart may not recover, regardless of length of the reperfusion period. If a mechanism of uptake exists, the mitochondrial adenine nucleotide pool would eventually recover once the metabolic, regulatory factors (e.g., P, and ATP) return to normal levels such that the rate of accumulation surpasses the rate of efflux. Our results with isolated heart mitochondria indicate that, unlike liver mitochondria, they may not be able to accumulate adenine nucleotides (Figure 3). If a mechanism of net uptake does exist, it is slow compared with that of liver mitochondria. However, caution should be taken when extrapolating in vitro results to the in vivo situation. For example, Haynes et al23 have reported that Ca++ stimulates the net uptake of adenine nucleotides in isolated liver mitochondria. Therefore, intracellular conditions that trigger net uptake of adenine nucleotides into cardiac mitochondria may occur at some point during reperfusion. However, the results of the present study indicate that recovery of the mitochondrial adenine nucleotide pool does not occur rapidly. Therefore, repetitive episodes of myocardial ischemia separated by relatively long periods of perfusion can lead to the cumulative loss of mitochondrial adenine nucleotides.

Depressed Mechanical Recovery and Mitochondrial Function

Postischemic depression of myocardial contractile function may be due either to depressed ATP utilization or depressed ATP synthesis. Using 20–60 minutes of continuous, normothermic ischemia in the isolated ferret heart, Neubauer et al28 determined that although postischemic ATP utilization was depressed (as measured by creatine kinase reaction velocity), the maximum rate of ATP synthesis (as determined by myocardial oxygen utilization) was depressed to an even greater degree. On the basis of these data, these investigators28 postulated that recovery of postischemic contractile function in the heart is limited by the rate of ATP synthesis. In our study, the energy charge of the continuous ischemic hearts was abnormally low following reperfusion (Table 4), indicating depressed ATP synthesis. This is more than likely due to the depressed coronary flow rates (Table 5) and impaired oxygen delivery after reperfusion. These results are consistent with other reports demonstrating a “no-reflow” phenomenon (particularly in the subendocardium) during reperfusion of hearts severely injured by ischemia.33,34 Moreover, injury to the mitochondrial fraction during ischemia may further decrease postischemic energy production. However, 30 minutes of continuous ischemia resulted in at most a 27% reduction in the maximal rate (state 3) of mitochondrial respiration (Table 1). Therefore, it is unlikely that this limited degree of mitochondrial injury would be the major cause of the 80–95% reduction in postischemic contractile function (Table 5).

Postischemic contractile dysfunction, similar to that observed with our intermittent, ischemic hearts, has been reported in hearts receiving 5–15 minutes (too little time to significantly affect mitochondrial respiration) of ischemia.35–37 These hearts maintain normal contractile reserve in the presence of inotropic agents,37,38 implying that energy production is not the limiting factor in postischemic recovery of the “stunned” heart. In our study, mitochondrial respiration following intermittent ischemia was not depressed at the end of ischemia or reperfusion (Tables 1 and 2). Moreover, the energy charge returned to normal upon reperfusion (Table 4). Therefore, postischemic dysfunction of the intermittent ischemic hearts cannot readily be attributed to depressed respiration or limiting oxygen delivery. Tissue lactate levels at the end of ischemia (Table 3) may have contributed to depressed, postischemic function of the intermittent ischemic hearts; Neely and Grotyohann39 reported that postischemic ventricular function was inversely correlated with end-ischemic lactate levels. In addition, elevated phosphate levels during reperfusion (Tables 3 and 4) may have contributed to postischemic dysfunction by effectively lowering the intracellular calcium concentration27 or by inhibiting the interaction of the myosin and actin filaments.40

In summary, the results of this study indicate that repetitive episodes of myocardial ischemia can lead to the cumulative loss of mitochondrial adenine nucleotides. Moreover, in vitro studies indicate that heart mitochondria lack a mechanism for the net accumulation of adenine nucleotides from the extramitochondrial space. Therefore, even long-term reperfusion may not be sufficient to restore the mitochondrial adenine nucleotide pool before subsequent ischemic episodes occur, which would result in the further depletion of the pool.
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References


Key Words • myocardial ischemia • adenine nucleotides • mitochondria • reperfusion
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