**Laboratory Investigation**

**Postischemic Recovery of Mitochondrial Adenine Nucleotides in the Heart**

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**Background.** Adenine nucleotides (AdNs) are lost from the mitochondrial fraction of the heart cell during ischemia. It is unknown whether this pool of AdNs can be replenished after reperfusion. The purpose of this study was to evaluate the postischemic recovery of the mitochondrial AdN pool.

**Methods and Results.** The left anterior descending coronary artery (LAD) of the canine heart was occluded for 30 minutes followed by either no reflow, 30-minute reflow, 1-day reflow, or 7-day reflow. Systolic shortening in the LAD-supplied region was absent during occlusion but recovered to approximately 30% of preocclusion values during early reperfusion. Mitochondrial and tissue AdNs (ATP, ADP, and AMP) were determined in the LAD-supplied and left circumflex-supplied (control) regions of the heart. The AdN content (expressed as percent of control values) of mitochondria from the LAD region was 55±10% (p<0.002), 64±7% (p<0.001), 81±6% (p<0.03), and 94±8% for the no-reflow, 30-minute-reflow, 1-day-reflow, and 7-day-reflow groups, respectively. The AdN content (expressed as percent of control values) of tissue samples from the LAD region was 52±9% (p<0.002), 48±12% (p<0.02), 68±5% (p<0.002), and 70±9% for the no-reflow, 30-minute-reflow, 1-day-reflow, and 7-day-reflow groups, respectively. There was a good correlation between mitochondrial and tissue AdN (r=0.95). Using initial exchange rates, adenine nucleotide translocase activities of mitochondria from the LAD and control regions were not significantly different. State 3 respiration of LAD mitochondria was depressed (approximately 25%, p<0.05) only in the no-reflow group. Accent control ratios of the LAD mitochondria were not significantly different from control values in any group.

**Conclusions.** After 30 minutes of regional ischemia, postischemic restoration of the mitochondrial AdN pool occurs between 1 and 7 days; this restoration is preceded by recovery of respiratory and adenine nucleotide translocase functions. Although the abnormally low levels of AdN persist in the mitochondrial compartment during the early reperfusion period, postischemic contractile dysfunction cannot be explained by depressed mitochondrial respiratory activity. (*Circulation* 1992;85:2212–2220)

**KEY WORDS** • myocardial ischemia • reperfusion • adenine nucleotide translocase • mitochondria

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During ischemia, when energy usage exceeds energy production, the adenine nucleotide content (ATP, ADP, and AMP) of the heart decreases as a result of enzymatic degradation to nucleosides and purine bases. Approximately 80% of the adenine nucleotide pool is located in the cytosolic compartment; the remaining 20% is located in the mitochondrial compartment. Although both pools of adenine nucleotides decrease during myocardial ischemia, mitochondrial adenine nucleotides are not catabolized to adenosine and inosine because 5' nucleotidase activity is not present in the mitochondrial compartment. Therefore, loss of mitochondrial adenine nucleotides during ischemia is probably caused by direct transport of ATP, ADP, or AMP out of the mitochondria into the cytosolic space, where enzymatic degradation to nucleosides and purine bases will occur.

Provided that the ischemic period is not severe enough to cause cell death, reperfusion will eventually result in the restoration of the adenine nucleotide pool to normal values. Although the heart can synthesize adenine nucleotides from nucleosides and purine bases by salvage pathways, washout of these precursor molecules after ischemia limits the degree of recovery of adenine nucleotides during early reperfusion. Complete recovery of adenine nucleotides requires de novo synthesis and may take a week or longer, depending upon the severity of the ischemic episode. Although myocardial adenine nucleotides will eventually recover to normal values, it is not known whether the cytosolic and mitochondrial pools recover at the same rate and to the same extent. Recovery of mitochondrial adenine nucleotides would require transport of the nucleotides across the mitochondrial membrane, because the enzymes responsible for de novo synthesis and salvage are localized only in the cytosolic compartment. The purpose of the present study was to determine whether the mitochondrial adenine nucleotide pool can recover to normal values after a moderate ischemic episode in the heart.
Methods

Surgical Procedures

Adult, heartworm-free mongrel dogs (18–22 kg) were fasted overnight, anesthetized with sodium thiopental (25 mg/kg i.v.), intubated, and placed on positive-pressure ventilation with a mixture of 1–2% isoflurane–nitrous oxide–oxygen to maintain anesthesia. Ventilation was adjusted to maintain arterial blood gases (PO2, 110–150 mm Hg; PCO2, 32–40 mm Hg) and pH (7.32–7.42) within physiological limits. A left thoracotomy was done through the fifth intercostal space, and a pericardial cradle was created. A catheter-tip pressure transducer (Camino Laboratories, San Diego, Calif.) was placed in the left ventricle through a stab incision in the apex of the heart. The left anterior descending coronary artery (LAD) was exposed by blunt dissection just below the first diagonal branch. A Doppler flow probe (Triton Technology, Inc., San Diego, Calif.) was placed around the LAD to measure coronary blood flow. A No. 1 silk snare was placed proximal to the flow probe to occlude the vessel. Two 2-mm, 5-MHz ultrasonic segment-length crystals (SL 5-2, Triton Technology, Inc.) were placed in the subendocardium of the anterior left ventricular wall supplied by the LAD. The left ventricular pressure waveforms were used to determine end systole and end diastole to calculate percent change in systolic segment shortening. The change in segment shortening during occlusion was used to verify the relative degree of ischemia; when segment shortening was greater than 5% of preoclusion value, results were not included in this study. All hemodynamic measurements were continuously monitored with an 80386-based computer. Data were stored and analyzed using the Cardiology Data Acquisition and Analysis System software package (Symbolic Logic Inc., Dallas, Tex.).

Experimental Protocol

The animal was allowed to stabilize for 30 minutes after instrumentation. The LAD was occluded for 30 seconds to verify the placement of the crystals and to indicate the severity of ischemia. Ten minutes after this test occlusion, the LAD was occluded for 30 minutes. Lidocaine (1.5 mg/kg i.v.) was administered after 25 minutes of occlusion. The animals were divided into four groups. In three groups, blood flow to the LAD region was restored by removal of the snare. The four experimental groups were no reflow (n=5), 30-minute reflow (n=5), 1-day reflow (n=6), and 7-day reflow (n=6). Animals were excluded from the study if ventricular fibrillation occurred during the occlusion period. When fibrillation occurred during reperfusion, normal rhythm was restored with direct countershock. The number of hearts that experienced ventricular fibrillation upon reperfusion was two in the 30-minute-reflow group, two in the 1-day-reflow group, and three in the 7-day-reflow group.

For the 1- and 7-day-reflow groups (conducted under sterile conditions), the flow probe and pressure transducer were removed from the heart after 30 minutes of reflow. The sonomicrometer crystals were left in place with the leads encased in a latex pouch. The chest was closed, and the animal was allowed to recover. All surgical animals were standing, had normal rectal temperatures, and were eating 1–2 hours after surgery. One day or 7 days later, the dog was anesthetized and the heart was instrumented as described above. After 30 minutes of stabilization, hemodynamic measurements were obtained. Subendocardial placement of the crystals was routinely confirmed postmortem.

After all hemodynamic data were collected, all instrumentation was removed. Core biopsies (3 mm) were obtained from the LAD-supplied and contralateral control regions. Transmural cores were obtained with an air-driven biopsy drill (Alko Diagnostic Corp., Holliston, Mass.). The cores were pulled through the drill by suction and caught on an in-line filter holder and were immediately rinsed with ice-cold saline. Each core was removed and cut in half, and the subendocardial half of each core was frozen in liquid nitrogen. The samples were stored in liquid nitrogen for later analysis of tissue metabolites. The time from drilling the core to freezing the sample was approximately 10 seconds. The values for tissue metabolites are comparable to other published values.11,12 The hearts were excised, cut with scissors from base to apex through the left ventricle (between the LAD and control regions), and immediately packed in ice. Transmural sections of muscle were cut from the LAD (within the boundary of the sonocrystals) and control regions. The sonocrystals were purposely left in the chronic animal preparations to ensure comparable measurements after 1 and 7 days of reflow and to obtain tissue samples from the specific region where ischemic and postischemic contractile changes were occurring. The epicardial and endocardial surfaces of these sections were approximately 2×2 cm. The sections were placed in ice-cold KE buffer (described below). Two grams of tissue from the subendocardial half of the sections was used for mitochondrial isolation. No infarct was observed by visible inspection in any of the samples used.

Isolation of Mitochondria

The muscle was cut into thin slices with scissors and placed in cold buffer consisting of 150 mM KCl, 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 (KE buffer). The tissue was finely minced by hand with razor blades affixed to a spring-loaded piston. The minced tissue was rinsed several times with KE buffer to remove excess blood. The tissue was then incubated for 2 minutes with a proteinase (1 mg/g wet heart; Nagarse, Enzyme Development, New York). The tissue was homogenized with a tissue homogenizer and a motor-driven Teflon pestle. The suspension was centrifuged at 16,000g for 10 minutes. The pellet was suspended in KE buffer and centrifuged at 500g for 5 minutes. The supernatant was saved. The pellet was suspended in 10 ml KE buffer and centrifuged at 500g for 5 minutes. The two supernatants were pooled and centrifuged at 10,000g for 10 minutes. The pellets were suspended in 5 ml of 150 mM KCl, 10 mM MOPS, and 0.5% bovine serum albumin, pH 7.4 (KA buffer). The suspensions were centrifuged again at 10,000g. The pellets were suspended in 2 ml of KA buffer. The protein concentration was determined by the method described by Lowry.15 The mitochondrial yields (expressed as mg protein/g wet heart) for the groups were as follows (the first and second values
indicate the mitochondrial yields from the control and LAD regions, respectively: no reflow (n=5), 20±1.2 and 18.9±1.3; 30-minute reflow (n=6), 17.3±0.9 and 16.9±1.1; 1-day reflow (n=6), 20.9±1.6 and 19.4±1.7; 7-day reflow (n=6), 16.0±1.2 and 14.2±0.6. There were no significant differences in the mitochondrial yields from control and LAD regions within each group.

Adenine Nucleotide Translocase Activity

Mitochondria were incubated for 5 minutes at a final concentration of 1 mg/ml in a buffer consisting of 150 mM KCl, 10 mM NaF, 10 mM Tris, and 10 mM α-ketoglutarate, pH 7.4. Temperature was maintained at 20°C. Inorganic pyrophosphate (5 mM final concentration) was added to initiate the exchange reaction (extramitochondrial pyrophosphate for endogenous ATP or ADP; see References 16–19). The reaction was stopped at 15 seconds, 30 seconds, 1 minute, and 5 minutes by addition of carboxyatractyloside (20 µM final concentration) to 0.2-ml aliquots of the reaction mixture. Background was run for each mitochondrial preparation as described above except that carboxyatractyloside was present initially. The aliquots were centrifuged at 13,000g for 30 seconds in an Eppendorf microcentrifuge. The supernatants were assayed for adenine nucleotides by the high-performance liquid chromatography method described below. Background values were subtracted to determine the carboxyatractyloside-sensitive exchange activity.

Other investigators who have reported translocase activity in heart mitochondria initiated the exchange reaction with extramitochondrial ATP or ADP.20,21 We are unable to consistently observe linear rates of exchange using this method, however, presumably because of the extremely rapid exchange in heart mitochondria (even at 4°C). Pyrophosphate/ATP (ADP) exchange via the adenine nucleotide translocase occurs at one tenth the activity of the ADP/ATP exchange. For this reason, we chose the pyrophosphate exchange method.

Mitochondrial Respiration

Mitochondria (≈1 ng) were assayed in a buffer (pH 7.4) having the following millimolar composition: sucrose 225, KCl 15, KH2PO4, 10, Tris 10, and EDTA 1. The assay was performed with either 5 mM glutamate/5 mM malate or 10 mM succinate (with 1 µg of rotenone). State 3 respiration was initiated with the addition of 400 nmol of ATP. When succinate was used as the respiratory substrate, dinitrophenol (100 µM final concentration) was also added during the state 4 phase to determine the uncoupled rate of respiration. The acceptor control ratio was determined by dividing the state 3 rate by the rate of oxygen consumption before addition of ADP.

Mitochondria and Tissue Core Extractions and Analyses

Two milligrams of mitochondrial protein was extracted in ice-cold 1N perchloric acid (PCA); final volume, 2 ml. After centrifugation to remove the denatured protein, 1.5 ml of the supernatant was neutralized (pH 7.0–7.2) with 6N KOH; if pH was above 7.2, adjustment was made with phosphoric acid. After remaining on ice for 20 minutes, the samples were centrifuged to remove the potassium perchlorate precipitate.

The frozen core samples were ground to a fine powder in liquid nitrogen with a mortar and pestle. One milliliter of 1N PCA was added to the powdered sample, which was still in liquid nitrogen. After the liquid nitrogen boiled off, the PCA suspension was allowed to thaw and then was kept on ice. The extracts were centrifuged to remove the denatured protein. The protein pellets were extracted a second time in 1 ml of cold 1N PCA, and the centrifugation process was repeated. The two supernatants were combined and neutralized as described for the mitochondrial extracts. The protein pellets were suspended in 6N KOH and incubated at 30°C for 6 hours. The dissolved pellet was diluted with water and assayed for protein content.

Adenine nucleotides (ATP, ADP, and AMP) of the extracts were determined by reverse-phase high-performance liquid chromatography using a modification of a previously described method.3 Samples (50 µl) were injected onto an Altex Ultrasphere ODS column (5-µm particle size, 25 cm×4.6 mm). The samples were eluted at a flow rate of 1 ml/min with a buffer consisting of 30 mM KH2PO4, 19% acetonitrile, and 15 mM tetrabutylammonium hydrogen sulfate, pH 6.75. Effluents were monitored with a Beckman 160 absorbance detector (254 nm) interfaced to a Spectra-Physics 4270 integrator. Chromatographic peaks were identified by determining the retention times of known standards added to sample extracts. In addition, extracts were treated with hexokinase plus glucose, myokinase, and 5'-nucleotidase for the enzymatic removal of ATP, ADP, and AMP. The relation between the peak area and concentration of the standards was linear between 0.2 and 5 µM for ATP, ADP, and AMP (r=0.98 for each nucleotide). The retention times for ATP, ADP, and AMP were approximately 12.5, 6.5, and 4.0 minutes, respectively. The minimum reliably detectable amounts of ATP, ADP, and AMP were approximately 3, 2, and 1 pmol, respectively. Reproducibility of triple injections of ATP, ADP, and AMP standards (2.5 µM each) is indicated by standard errors (expressed as a percent of the mean areas) of ±3.0, ±4.0, and ±5.7%, respectively. Quantification was determined by relating peak areas of samples to the areas of the known standards. In addition, neutralized core extracts were assayed for creatine phosphate (CP) and ATP by the enzymatic assay as previously described.

Statistical Analyses

Within each group, the paired t test was used to compare values from the control and LAD regions. When values between groups were compared, ANOVA was used to determine whether there were any significant differences between any of the groups. If indicated by ANOVA, a modified t test (Bonferroni) was used to determine which groups differed significantly. A value of p<0.05 was considered significant.

Results

Hemodynamic and Regional Wall Motion Data

Table 1 summarises the hemodynamic data of the four groups studied. The baseline heart rate, left ventricular systolic pressure, and left ventricular diastolic pressure did not differ among the groups with the exception of slightly lower left ventricular systolic pres-
Table 1. Hemodynamics

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<th>Preoclusion</th>
<th>15-Minute occlusion</th>
<th>30-Minute occlusion</th>
<th>15-Minute reflow</th>
<th>30-Minute reflow</th>
<th>1-Day reflow</th>
<th>7-Day reflow</th>
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<tr>
<td>No reflow (n=5)</td>
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<td>Systolic LVP (mm Hg)</td>
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<td>112±7</td>
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<td>91±7</td>
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<td>1-Day reflow (n=6)</td>
<td>94±6</td>
<td>91±6</td>
<td>86±5*</td>
<td>89±5</td>
<td>90±6</td>
<td>102±10</td>
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<td>87±4*</td>
<td>94±5</td>
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<td>Diastolic LVP (mm Hg)</td>
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<tr>
<td>No reflow (n=5)</td>
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<td>16±2</td>
<td>15±2</td>
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<td>11±5</td>
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<td>14±3</td>
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<td>11±2</td>
<td>13±2</td>
<td>16±3</td>
<td>11±3</td>
<td>12±3</td>
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</table>

bpm, Beats per minute; LVP, left ventricular pressure.
Values are mean±SEM. Number of hearts in each group is given in parentheses.

Comparing the 1- and 7-day-recovery groups, postischemic recovery of segment shortening was significantly (p<0.005) greater after 7 days than 1 day. The data suggest that despite an immediate improvement of contractility upon reperfusion, a decline in function occurs by 1 day. Farber et al22 reported a similar observation in the postischemic dog heart. The cause of this postischemic decline in contractility is not known. However, our data suggest that the depression does not persist; see Figure 1, 1 and 7 days.

Tissue Metabolites and Energy Charge

Table 2 shows that high-energy phosphate (CP and ATP), total adenine nucleotides, and energy charge (ATP+0.5 ADP/ATP+ADP+AMP) were depressed in the ischemic myocardium (no-reflow group). With the exception of ATP and total adenine nucleotides, control and LAD values were not significantly different in the reperfused groups. The data indicate that, whereas CP and energy charge of the myocardium recovered to normal values upon reperfusion, only partial recovery of ATP and adenine nucleotides occurred in the heart even after 7 days of reperfusion.

Postischemic Recovery of Mitochondrial and Tissue Adenine Nucleotides

Figure 2 shows the mitochondrial (Figure 2A) and tissue (Figure 2B) adenine nucleotides (ATP, ADP, and AMP) from control and LAD-supplied regions of the four groups studied. The mitochondrial adenine nucleotide content of the LAD-supplied region was significantly lower than control values in all groups except the 7-day-reflow group (Figure 2A). The data suggest that the mitochondrial adenine nucleotide content of the LAD region progressively increased during reperfusion; however, a significant difference (p<0.03) from the no-reflow group was observed only in the 7-day-reflow group (Figure 2A). The tissue adenine nucleotide content of the LAD-supplied region was significantly lower than control values in all groups studied. The tissue adenine nucleotides of the LAD region tended to increase with increasing time of reperfusion (Table 2 and Figure 2B); however, ANOVA indicated no significant differ-
The data as a percent of control values in each group. In general, the mitochondrial and tissue adenine nucleotide levels in the LAD region were similar within each experimental group; however, the differences were significant after 1 and 7 days of reperfusion (Figure 3). Although mitochondrial adenine nucleotides may recover faster than the cytosolic adenine nucleotides (assuming that most of the tissue levels are cytosolic), the data suggest a close relation between the sizes of the two pools.

**Adenine Nucleotide Translocase and Respiratory Activity**

Figure 4 shows the adenine nucleotide translocase exchange activity of mitochondria from control and LAD regions of each group. The sizes of exchangeable pools of the mitochondrial preparations can be estimated by the 5-minute values. The exchangeable adenine nucleotide pool of mitochondria from the LAD

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**Table 2. Myocardial Metabolites and Energy Charge**

<table>
<thead>
<tr>
<th>Group</th>
<th>CP (nmol/mg)</th>
<th>ATP (nmol/mg)</th>
<th>ADP (nmol/mg)</th>
<th>AMP (nmol/mg)</th>
<th>TAdN (nmol/mg)</th>
<th>EC (nmol/mg)</th>
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<tr>
<td>No reflow (n=5)</td>
<td></td>
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<tr>
<td>Control area</td>
<td>53.3±7.7</td>
<td>40.6±3.1</td>
<td>6.3±0.4</td>
<td>0.3±0.1</td>
<td>47.1±3.2</td>
<td>0.93±0.01</td>
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<td>Ischemic area</td>
<td>13.5±4.3†</td>
<td>15.5±4.4†</td>
<td>7.8±0.4*</td>
<td>1.1±0.2†</td>
<td>24.4±4.4†</td>
<td>0.77±0.05†</td>
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<tr>
<td>30-Minute reflow (n=5)</td>
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<tr>
<td>Control area</td>
<td>61.7±4.8</td>
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<td>7.0±1.4</td>
<td>0.6±0.2</td>
<td>48.6±6.0</td>
<td>0.92±0.01</td>
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<tr>
<td>Ischemic area</td>
<td>75.4±8.3</td>
<td>18.9±3.7†</td>
<td>4.3±0.3</td>
<td>0.3±0.1</td>
<td>23.5±3.5*</td>
<td>0.89±0.02</td>
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<td>1-Day reflow (n=6)</td>
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<tr>
<td>Control area</td>
<td>44.6±3.1</td>
<td>38.0±2.8†</td>
<td>5.3±0.2</td>
<td>0.4±0.1</td>
<td>43.7±2.9</td>
<td>0.93±0.01</td>
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<td>Ischemic area</td>
<td>51.0±5.3</td>
<td>25.0±2.8†</td>
<td>4.5±0.4</td>
<td>0.4±0.1</td>
<td>29.9±3.1†</td>
<td>0.91±0.01</td>
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<tr>
<td>7-Day reflow (n=6)</td>
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<tr>
<td>Control area</td>
<td>64.5±6.3</td>
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<td>6.6±0.4</td>
<td>0.9±0.4</td>
<td>52.1±4.6</td>
<td>0.91±0.01</td>
</tr>
<tr>
<td>Ischemic area</td>
<td>51.4±5.3</td>
<td>30.3±4.1*</td>
<td>5.7±0.7</td>
<td>0.6±0.1</td>
<td>36.6±4.0*</td>
<td>0.89±0.03</td>
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</table>

Values are mean±SEM. Number of hearts in each group is given in parentheses. CP, creatine phosphate; TAdN, total adenine nucleotides; EC, energy charge.

*p<0.05 vs. control area.

†p<0.01 vs. control area.
region was depressed (compared with values in the control region) in the no-reflow and 30-minute-reflow groups. There were no significant differences between the control and LAD regions in the size of the exchangeable pools in the 1- and 7-day-reflow groups; however, in the 1-day-reflow group, the exchangeable pool of mitochondria from the control region was significantly lower \((p<0.05)\) than control values of the other three groups. The exchange activity can be ascertained from the early incubation time points (Figure 4). No significant differences between mitochondria from the control and LAD regions were seen within any group at 15 seconds. The data indicate that adenine nucleotide translocase activity of mitochondria from the LAD region was not significantly depressed after ischemia and reperfusion.

Figure 5 shows state 3 respiration of the mitochondria from control and LAD regions of the hearts in each group; mitochondrial respiration was determined with glutamate/malate and succinate as respiratory substrates. In the no-reflow group, state 3 respiration of the LAD region was depressed (compared with the control region) 25% and 21% with glutamate/malate and succinate, respectively. There were no significant differences in state 3 respiration between control and LAD regions in any of the reperfused groups, including 30-minute reflow. Respiratory coupling, as indicated by the acceptor control ratio, was not different between the control and LAD regions in any of the groups (Table 3). These data imply that the mitochondrial lesion responsible for depressed state 3 respiration was readily reversed upon reperfusion, such that by 30 minutes of reperfusion, the mitochondrial fraction of the heart had normal respiratory capacity.

**Discussion**

We previously reported that ischemia-induced loss of mitochondrial adenine nucleotides is not reversed by 60 minutes of reperfusion in the isolated rat heart.\(^3\) The major conclusion of the present study is that restoration of blood flow to the heart muscle after 30 minutes of ischemia results in the progressive replenishment of the mitochondrial adenine nucleotide pool, reaching normal levels between 1 and 7 days of reperfusion (Figure 2).

Although the hemodynamic values (Table 1) of the 1- and 7-day-reflow groups tended to be lower than the values of the no-reflow group, Katz et al.\(^29\) have shown that over a threefold-to-fourfold change in rate-pressure product, no significant changes in the intracellular contents of CP, ATP, ADP, or cytosolic inorganic phosphate \((P_i)\) occur in the canine heart in vivo. Therefore, the possibility of somewhat lower MVO\(_2\) values due to a lower rate-pressure product would not account for...
the higher mitochondrial and tissue adenine nucleotide levels in the LAD regions of 1- and 7-day-reflow groups compared with the no-reflow group. Therefore, these higher values are probably due to replenishment of both pools of adenine nucleotides. From the average values for the mitochondrial adenine nucleotides in the LAD region after 30 minutes of occlusion and 1 day of reflow (Figure 2A), the rate of replenishment can be calculated to be 1.87 nmol/mg mitochondrial protein per day. Assuming that 9.5 nmol/mg mitochondrial protein (average value from all control groups) is the normal adenine nucleotide value and the rate of replenishment is constant, full recovery of mitochondrial adenine nucleotides after 30 minutes of myocardial ischemia would require approximately 2.5 days.

Previous reports from this laboratory have focused on understanding the mechanism of loss of mitochondrial adenine nucleotides during myocardial ischemia. Because the 5' nucleotidase enzyme is not found in the mitochondrial matrix, degradation of mitochondrial adenine nucleotides to adenosine cannot explain the depletion of mitochondrial adenine nucleotides during ischemia. Alternatively, the loss must occur by direct transport of at least one of the nucleotides (AMP, ADP, or ATP) through the inner membrane into the cytosol, where degradation can occur. We have previously suggested that an increase in Pi during ischemia may trigger the release of mitochondrial adenine nucleotides. This is supported by the observations that the intracellular concentration of Pi increases threefold to fivefold during myocardial ischemia and that Pi can cause a rapid release of adenine nucleotides from isolated heart mitochondria. In vitro, Pi-induced efflux of adenine nucleotides from isolated heart mitochondria is inhibited by atractyloside, a specific inhibitor of the adenine nucleotide translocase. Although observations suggest that Pi can induce net transport of adenine nucleotides out of the matrix via the adenine nucleotide translocase, we have shown that extramitochondrial ATP can inhibit the Pi-induced release of adenine nucleotides from isolated heart mitochondria. Moreover, we have evidence that suggests the cytosolic concentration of ATP does not fall to sufficiently low levels during ischemia to account for the loss of mitochondrial adenine nucleotides by the phosphate-induced, atractyloside-sensitive pathway.

It is likely that the mitochondrial adenine nucleotide pool remains constant under normal conditions because a slow efflux of adenine nucleotides is counterbalanced by a slow influx of adenine nucleotides. Aprille has proposed a model to explain net change in the size of the mitochondrial adenine nucleotide pool in liver. In this model, the inner membrane is permeable to ATP-Mg complex (specificity may be due to an atractyloside-insensitive carrier in the inner membrane), and the net movement (in or out) is determined by the relative concentrations of intramitochondrial and extramitochondrial ATP-Mg. (It is important to distinguish this activity from the adenine nucleotide translocase activity, which facilitates a one-for-one exchange of adenine nucleotides resulting in no net change in the size of the adenine nucleotide pool.) Equilibration of the cytosolic and mitochondrial pools by this model can explain the loss of mitochondrial adenine nucleotides during ischemia (cytosolic ATP concentration decreasing) and replenishment during reperfusion (cytosolic ATP concentration increasing; see Table 2). This is consistent with the observation in this study that parallel changes in tissue and mitochondrial adenine nucleotides were observed.

### Table 3. Acceptor Control Ratios

<table>
<thead>
<tr>
<th></th>
<th>Glutamate/malate</th>
<th>Succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>No reflow (n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control region</td>
<td>22.2±3.7</td>
<td>7.3±0.8</td>
</tr>
<tr>
<td>LAD region</td>
<td>22.1±4.5</td>
<td>6.8±1.0</td>
</tr>
<tr>
<td>30-Minute reflow (n=5)</td>
<td>21.7±1.3</td>
<td>7.0±0.4</td>
</tr>
<tr>
<td>Control region</td>
<td>20.8±1.8</td>
<td>7.6±0.9</td>
</tr>
<tr>
<td>LAD region</td>
<td>21.7±1.8</td>
<td>7.2±0.3</td>
</tr>
<tr>
<td>1-Day reflow (n=6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control region</td>
<td>15.1±1.1</td>
<td>7.0±0.5</td>
</tr>
<tr>
<td>LAD region</td>
<td>18.8±1.7</td>
<td>7.2±0.3</td>
</tr>
<tr>
<td>7-Day reflow (n=6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control region</td>
<td>18.7±0.7</td>
<td>6.7±0.6</td>
</tr>
<tr>
<td>LAD region</td>
<td>21.3±2.3</td>
<td>7.4±0.4</td>
</tr>
</tbody>
</table>

LAD, left anterior descending coronary artery.

Values are mean±SEM. Number of hearts in each group is given in parentheses.
otide levels occur during ischemia and reperfusion (Figures 2 and 3).

Replenishment of the mitochondrial adenine nucleotide pool in the heart is analogous to the postnatal increase in the adenine nucleotide pool in liver mitochondria. Although the two processes may occur by the same mechanism, the rates of accumulation differ by an order of magnitude. Postischemic restoration of the mitochondrial pool in the heart takes several days (this study), whereas in the liver, maturation from =3 nmol/mg protein to adult levels (=10 nmol/mg protein) requires only 5 hours and is not associated with an increase in cytosolic adenine nucleotides. These observations suggest that in addition to the cytosolic ATP (or total adenine nucleotide) concentration, other factors may influence uptake of mitochondrial adenine nucleotides. It is also possible that mitochondrial adenine nucleotide recovery did not occur to the extent indicated by our data because of selective isolation of a pool with normal adenine nucleotide levels. This seems unlikely, however, because mitochondrial yields were not significantly different from control values (see “Methods”).

Whereas cytosolic ATP is absolutely required for cardiac muscle contraction, intramitochondrial ATP is absolutely required to maintain the cytosolic ATP pool during normal contractile activity. The rapid interaction of the two pools is the result of the one-for-one exchange of cytosolic ADP (resulting from hydrolysis of ATP) for intramitochondrial ATP facilitated by the adenine nucleotide translocase. A depressed exchange rate may be associated with a depressed rate of ATP synthesis by the mitochondria. Regitz et al reported that the adenine nucleotide translocase activity and the mitochondrial adenine nucleotide pool size were depressed to similar levels after 3 hours of coronary occlusion in the dog heart. In the present study, we found that the exchangeable adenine nucleotide pool (as indicated by the 5-minute incubation value, Figure 4) of the mitochondria was significantly depressed after 30 minutes of ischemia and remained depressed after 30 minutes of reperfusion. This is probably because of the differences in the total adenine nucleotide pools of the mitochondria from the LAD and control regions (Figure 2A). The reasons for the depressed levels of exchangeable adenine nucleotides in control mitochondria after 1-day reflow remain unclear (Figure 4). Although the total pool was less than the control mitochondria of the other groups, the differences were not significant (Figure 2A).

Interpretation of the effect of ischemia and reperfusion on translocase activity (rate of exchange) is dependent upon the sampling time of the measurement (Figure 4). Initial exchange rates (after 15 seconds) indicate no significant effect on adenine nucleotide translocase activity. However, the rate of ADP/ATP exchange by the adenine nucleotide translocase is proportional to the size of intramitochondrial adenine nucleotide pool in liver mitochondria, in which more accurate measurements of exchange rate can be determined because of the relatively slow activity compared with heart mitochondria. This relation between the intramitochondrial pool size and adenine nucleotide translocase activity implies that the concentration of intramitochondrial ATP is near the K_m for the internal binding site of the adenine nucleotide translocase. Therefore, loss of mitochondrial adenine nucleotides during ischemia and subsequent failure to replenish this pool after reperfusion could result in the depressed rate of exchange of cytosolic ADP and mitochondrial ATP. This could potentially limit the rate of energy production in the postischemic heart, which in turn could result in depressed contractile activity. Our results, however, indicate that adenine nucleotide translocase activity was not adversely affected.

State 3 respiration was depressed 20–25% after 30 minutes of ischemia. Normal respiratory activity returned by 30 minutes of reperfusion, however, and remained at normal levels at 1 and 7 days of reperfusion. The loss of mitochondrial respiration cannot be explained by the loss of adenine nucleotide translocase activity, because uncoupling did not stimulate respiration above state 3 levels (data not shown). This observation is in disagreement with Shug et al, who reported that uncoupled respiration was significantly higher than state 3 respiration of mitochondria isolated from ischemic heart muscle. The reason for these different observations is unknown. However, the return of the state 3 respiration to normal levels upon reperfusion (this study) implies that the energy demand of the heart could be met by oxidative phosphorylation, although the mitochondrial adenine nucleotide pool was abnormally low.

In summary, after 30 minutes of ischemia, energy charge and mitochondrial respiration returned to normal after 30 minutes of reperfusion. Adenine nucleotide translocase activity returned to normal by 1 day, and mitochondrial adenine nucleotides returned to normal by 7 days. In contrast, tissue adenine nucleotides and contractile function remained depressed after 7 days, indicating severe “stunning.” Myocardial stunning after short (5–15-minute) periods of coronary occlusion cannot be explained by depressed energy production, because normal contractile reserve of the affected regions of these hearts has been demonstrated in the presence of inotropic agents. Although mitochondrial abnormalities are likely to become more severe as the duration of ischemia is extended, the present study shows that depressed mechanical function after 30 minutes of coronary artery occlusion cannot be explained, even in part, by depressed mitochondrial function, although depressed levels of mitochondrial adenine nucleotides and adenine translocase activity persist during the immediate postischemic period.

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References

Postischemic recovery of mitochondrial adenine nucleotides in the heart.
G K Asimakis, J B Zwischenberger, K Inners-McBride, L A Sordahl and V R Conti

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