Enhancement of Mitochondrial Oxidative Phosphorylation Capability by Hypoperfusion in Isolated Perfused Rat Heart

Peter C.D. Pelikan, James T. Niemann, Guangzhi Xia, Gale Jagels, and John Michael Criley

During ischemia, limitation of oxygen delivery reduces mitochondrial oxidative phosphorylation and results in a reduction of intracellular adenosine triphosphate (ATP) content.1-4 After severe ischemia, isolated mitochondria show defects in structure and in the kinetics of oxidative phosphorylation, predominantly a reduction in state 3 respiration and an uncoupling of ATP synthesis.5-8 These changes may play a role in defining the extent of ischemia-induced loss of tissue viability and may limit cell viability during reperfusion.9

The function and metabolism of myocardium subjected to a reduction in but not complete cessation of, coronary perfusion have not been studied extensively. After coronary occlusion, coronary flow is low but persistent in parts of the area at risk.10-12 In these areas, there is less necrosis,13 and the time of onset of irreversible tissue injury occurs later.10,11,14 Hypoperfused myocardium, therefore, may have a greater likelihood of survival during reperfusion and deserves further study.

The purpose of this study was to examine the effects of myocardial hypoperfusion on left ventricular function and on the kinetics of mitochondrial oxidative phosphorylation. The isolated, perfused, beating rat heart was used as the model. Systolic and diastolic left ventricular pressures, coronary flow rate, myocardial oxygen consumption, myocardial lactate production, and creatine kinase release were monitored in control and hypoperfused hearts. After perfusion, the rates of mitochondrial oxidative phosphorylation were measured. These experiments demonstrate an unexpected increase in the maximum rate of ATP synthesis (state 3 respiration) in the mitochondria isolated from hypoperfused hearts. This may represent an adaptive mechanism that maximizes ATP synthesis during myocardial oxygen supply/demand imbalance. The biochemistry of hypoperfused myocardium may be important in the rational development of therapeutic strategies designed to increase myocardial salvage during posts ischemic reperfusion.

Materials and Methods

Isolated, Perfused Rat Heart

Randomly paired, male Sprague-Dawley rats (300-400 g) were anticoagulated with heparin and then anesthetized with intraperitoneal pentobarbital (15-30 mg/kg) and maintained on a constant infusion of the drug. Aortic pressure was measured by a radio-transmitter placed around the aorta. Intravenous fluid replacement was administered to maintain aortic pressure above baseline levels. Ventricular function was monitored by aortic pressure and left ventricular end-diastolic pressure. Coronary flow rate and myocardial oxygen consumption were measured as described.15-17 Heart rate and oxygen saturation of mixed venous blood were monitored continuously. The hearts were harvested at the end of the experiment and stored at -70°C until analyzed.

Received October 7, 1986; accepted June 9, 1987.

From the Division of Cardiology, Department of Medicine, and the Department of Emergency Medicine, Harbor-UCLA Medical Center, Torrance, Calif.

Supported in part by a grant from the Rosenhaus Peace Foundation, by the Research and Education Institute, Inc., Harbor-UCLA Medical Center, and by grant RR00425 (CLINFO).

Address for correspondence: Peter Pelikan, MD, Division of Cardiology, Harbor-UCLA Medical Center, 1000 West Carson Street, Torrance, CA 90509.
mg/kg). Under anesthesia, hearts were removed and immediately immersed in cooled perfusate. Within 30–60 seconds, the ascending aorta was cannulated distal to the coronary arteries and perfusion established by a modified Langendorff method. Perfusate contained (in mM) NaCl 140, KCl 5, CaCl2 1.5, MgCl2 1.2, HEPES 4, and glucose 10 at 37°C. The pH of the perfusate was adjusted to 7.40 with NaOH; perfusate was continuously bubbled with 100% oxygen, with measured Po2 approximately 500 mm Hg.

Aortic and, therefore, coronary perfusion was gravity regulated via a perfusate reservoir positioned 110 cm above the isolated hearts. Perfusion pressure was strictly controlled by in-line constriction and measured with a side-arm of the aortic perfusion catheter connected to a Statham P23ID transducer (Gould Statham Instruments, Inc., Hato Rey, Puerto Rico). All hearts were paced at 375 beats/min by a Grass pacing stimulator (Grass Medical Instruments, Inc., Quincy, Mass.). The left ventricle was vented with an apical stab incision. A thin-walled rubber balloon attached to a fluid-filled length of PE 190 tubing was inserted into the left ventricular cavity through the left atrium and held in place with a purse-string ligature. The PE tubing was connected to a pressure transducer by a three-way stopcock to allow recording of left ventricular systolic and diastolic pressures. After beginning perfusion, intraventricular balloon volume was increased in increments of 0.05 ml until a maximum developed pressure was achieved and was left at that volume throughout the experiment.

A second PE 190 catheter was inserted into the right ventricle through the dissected pulmonary artery remnant. In preliminary studies, the Po2 of perfusate withdrawn from the right atrium was found to be the same as that of perfusate withdrawn from the right ventricle. Since catheter position was more stable in the right ventricle than the right atrium, perfusate effluent was withdrawn through this tubing to measure coronary sinus Po2. Because oxygen diffusion through the plastic tubing could have altered the O2 content, the Po2 measured with this system was compared with that obtained with a metal catheter/glass syringe assembly. The measured Po2 using the plastic assembly was almost identical to that obtained with the metal/glass assembly (n=6, R=1.0, R2=0.999, p<0.001). A Corning Model 165 blood gas analyzer (Medfield, Mass.) was used for measurement of perfusate and effluent Po2. Myocardial oxygen consumption (MV02) was calculated as follows:

$$MV_{O2} = k \times \text{coronary flow rate} \times (P_{O2 \text{, aorta}} - P_{O2 \text{, coronary sinus}})$$

where k is O2 solubility constant (3.25 x 10^-4 ml O2/mm Hg Po2).

The coronary effluent was also utilized to assess the rates of myocardial lactate production and creatine kinase (CK) release to determine whether hypoperfusion resulted in anaerobic metabolism and/or myocardial damage. Myocardial lactate production was calculated as follows: myocardial lactate production = coronary flow rate x coronary sinus lactate concentration. Coronary effluent lactate was measured every 10 minutes during perfusion with an automated lactate analyzer (model 23L, Yellow Springs Instrument Co., Yellow Springs, Ohio).

Myocardial CK release was also monitored every 10 minutes. Coronary effluent creatine kinase was measured using an Abbott Biochromatic Analyzer (Abbott Laboratories, Diagnostics Division, Irving, Tex.), and myocardial CK release was calculated using the following formula: myocardial CK release = coronary flow rate x coronary sinus CK concentration.

Myocardial oxygen consumption, lactate production, and CK release were not normalized for cardiac weight because the left ventricular weights were similar in the hypoperfused and control hearts (1.2 ± 0.02 versus 1.1 ± 0.02, respectively; p<0.05) and because the relative contributions of the atria and right ventricle to coronary flow, oxygen consumption, and lactate and CK production were not evaluated separately.

**Experimental Protocol**

**Hypoperfusion.** All experiments were performed on pairs of hearts. After establishing perfusion with a coronary perfusion pressure of approximately 80 mm Hg in both hearts, the intraventricular balloon volume was adjusted to produce maximum left ventricular developed pressure (systolic minus diastolic pressure). After a 20–30-minute baseline stabilization period in both hearts, baseline measurements of left ventricular systolic and diastolic pressures, coronary perfusion pressure, coronary flow rate (measured effluent volume), and coronary sinus Po2 were recorded. One heart from each pair was then randomly selected to undergo 30 minutes hypoperfusion (coronary perfusion pressure reduced to 18–22 mm Hg) by partial clamping of the perfusion line. During hypoperfusion, the temperature was maintained at 37°C with constant temperature water jackets in the perfusion line and heart bath, and pacing was continued at 375 beats/min. The coronary perfusion pressure in the control heart was not changed after randomization, and perfusion in this heart was continued for 30 minutes, simultaneously with the experimental heart. In both hearts, left ventricular systolic and diastolic pressures and coronary perfusion pressure were recorded every 5 minutes. Coronary flow rate and coronary sinus Po2, CK, and lactate levels were measured every 10 minutes. The aortic perfusate Po2 was measured at the end of perfusion. At the end of this 30-minute experimental period, perfusion was discontinued, and each heart was chilled separately in mitochondrial isolation medium as described below.

**Global ischemia.** Five hearts were isolated and perfused as described above. After the baseline stabilization period, during which left ventricular end-diastolic and developed pressures were measured, the coronary perfusion lines were totally clamped, reducing the coronary flow rate to zero. Thereafter, hearts were maintained at 37°C, paced at 375 beats/min, for 30 minutes with no alteration in the intraventricular...
balloon volume. At the end of 30 minutes of ischemia, mitochondria were isolated and studied as described below.

**Mitochondrial Isolation**

Mitochondrial isolation medium contained 70 mM sucrose, 210 mM mannitol, 0.5 mM EGTA [ethylene glycol bis-(aminoethyl ether) N,N',N'-tetra-acetic acid], 5 mM HEPES, and 1 mg/ml bovine serum albumin, at pH 7.20 and temperature 4° C. Mitochondria were isolated separately and simultaneously from the experimental and control hearts using a modification of the technique previously described by Jacobus. Briefly, after removal of the atria and the right ventricle, the left ventricle was weighed and then finely minced. After trypsin digestion and homogenization with a Teflon tissue homogenizer, cellular debris was separated by differential centrifugation at 480g for 10 minutes at 2° C. The supernatant solution containing the mitochondrial fraction was decanted and filtered. Three further centrifugations at 7,700g × 10 minutes with subsequent washings were performed to isolate a final mitochondrial pellet. This was resuspended in 50 μl of isolation medium. Protein determination was by the biuret method. The final concentration was 30–50 mg mitochondrial protein/ml.

**Oxgraph Determinations**

To measure the rate of oxidative phosphorylation, Clark oxygen electrode studies were conducted in a 1.8-ml chamber at 37° C. The oxygen medium contained (mM) KCl 130, KH₂PO₄ 2, MgCl₂ 1, K₂-EGTA 0.5, HEPES 5, and either glutamate 10 plus malate 10 or succinate 5 (with 5 μM rotenone) as respiratory substrates. The oxygen graph medium was adjusted to pH 7.20 and 37° C. The rates of resting and stimulated oxygen uptake of 0.5 mg mitochondrial protein with glutamate/malate as substrate or 0.25 mg protein with succinate as substrate were measured with standard techniques.

The rate of endogenous respiration (state 2) was measured in the presence of phosphate ion, substrate, and oxygen before the addition of ADP to the oxygraph chamber. Thereafter, the maximum ADP stimulation rate (state 3) and recovery rate (state 4) of oxygen consumption were measured after the pulsed addition of ADP with a final concentration of 195.9 μM. Oxygen consumption rates were determined as nanogram atoms oxygen per minute per milligram mitochondrial protein. To quantify the rate of mitochondrial respiration in the absence of ADP to that in the presence of ADP, the acceptor control ratio (state 3/state 2) was calculated. In addition, the respiratory control ratio (state 3/state 4) and ADP/O ratio were calculated for each heart (modified from Chance and Williams and Lehninger). All 17 pairs of hearts perfused were thus studied with glutamate and malate as substrates, and 11 of the 17 pairs were also studied with succinate as substrate.

To define the kinetics of oxidative phosphorylation, one or two separate determinations of state 3 respiration were made after the addition of each of 6 different ADP pulses with final concentrations approximately 10, 20, 40, 75, 110, and 196 μM. The rate of state 3 respiration increases with increasing ADP concentration and asymptotically approaches Vₘₐₓ (the maximum rate of ATP production). Therefore, a double-reciprocal plot was used to calculate the Vₘₐₓ as well as the apparent Kₘ (ADP concentration at ½ Vₘₐₓ) for ADP for mitochondria isolated from each heart. Six of the 17 pairs of hearts were used to measure Vₘₐₓ and the apparent Kₘ with glutamate and malate as substrates. In 6 of the 11 pairs of hearts additionally studied with succinate (see paragraph above), Vₘₐₓ and the apparent Kₘ with succinate as the respiratory substrate were determined.

To explicate further the alterations in mitochondrial function induced by perchfusion, mitochondrial fractions were also exposed to 2,4-dinitrophenol (DNP, 80 nmol pulsed addition) in the oxygraph chamber to measure uncoupled rates of electron transport. Correlation of Vₘₐₓ with the DNP uncoupled rate of electron transport allowed an assessment of the relation between alterations in the kinetics of oxidative phosphorylation and the rate of electron transport.

**Mitochondrial Malate Dehydrogenase Activity**

When rates of mitochondrial respiration are expressed relative to mitochondrial protein, the inclusion of fragmented, nonfunctional mitochondria would increase the protein content but not the rate of respiration of the isolated fraction. Thus, decreased purity would falsely underestimate the actual mitochondrial activity. To exclude this possibility, the activity of mitochondrial malate dehydrogenase (MDHm), a mitochondrial enzyme that is stable during ischemia, was assayed in selected mitochondrial fractions spectrophotometrically. This procedure allowed the expression of the rates of respiration relative to this marker enzyme rather than to the nonspecific protein concentration of the mitochondrial fraction.

**Statistics**

Statistical analyses were performed on a VAX 11/750 computer using the CLINFO program. Data from control hearts were compared with those from experimental hearts using unpaired t tests and the Wilcoxon rank sum test when data were not normally distributed. Linear regression was used to derive the equation of the double-reciprocal plot relating 1/state 3 to 1/[ADP], from which Vₘₐₓ and the apparent Kₘ were calculated. Data are presented as mean ± SEM.

**Results**

**Cardiac Function and Coronary Perfusion**

In both the hypoperfused and control hearts, left ventricular function and coronary flow (Table 1) were similar before the reduction in coronary perfusion pressure in the experimental group.

At the beginning of the period of experimental hypoperfusion, the coronary perfusion line for the randomly selected experimental heart was partially
Pelikan et al  Hypoperfusion Enhances Mitochondrial Function

Table 1. Cardiac Function and Coronary Perfusion Prior to Hypoperfusion

<table>
<thead>
<tr>
<th></th>
<th>Control hearts</th>
<th>Hypoperfused hearts</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of hearts</td>
<td>17</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Developed pressure (mm Hg)</td>
<td>80.9 ± 2.9</td>
<td>81.6 ± 3.1</td>
<td>NS</td>
</tr>
<tr>
<td>End-diastolic pressure (mm Hg)</td>
<td>3.4 ± 0.8</td>
<td>3.1 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Coronary perfusion pressure (mm Hg)</td>
<td>79.1 ± 1.2</td>
<td>77.8 ± 1.2</td>
<td>NS</td>
</tr>
<tr>
<td>Coronary flow rate (ml/min)</td>
<td>20.0 ± 0.9</td>
<td>20.1 ± 1.0</td>
<td>NS</td>
</tr>
</tbody>
</table>

clamped. The coronary perfusion pressure was reduced to low but stable levels and after 30 minutes was 20.2 ± 1.8 mm Hg. As expected, the coronary flow rate in these hearts was also reduced and after 30 minutes was 4.9 ± 0.3 ml/min. In comparison, the control group coronary perfusion pressure was 75.6 ± 1.2 mm Hg, and the coronary flow rate was 19.4 ± 1.2 ml/min (p < 0.0001 versus experimental).

In Figure 1, the marked drop in left ventricular developed pressure in the experimental hearts after reduction in coronary perfusion pressure is shown in contrast to pressure in control hearts. After 30 minutes hypoperfusion, the developed pressure was only 20.5 ± 1.5 mm Hg in the experimental group, whereas in control hearts it was 74.8 ± 3.3 (p < 0.0001). End-diastolic pressure showed a slight initial fall after the onset of hypoperfusion and then rose. At the end of the perfusion protocol, the end-diastolic pressure was 3.5 ± 2.0 mm Hg in the experimental hearts and 3.2 ± 0.9 in the controls (p = NS).

The 5 hearts selected to undergo 30 minutes of ischemia also had similar left ventricular function prior to cessation of coronary flow. The left ventricular developed pressure was 80.4 ± 3.6 (p versus control = NS), and the end-diastolic pressure was 2.6 ± 2.4 (p versus control = NS). After clamping the coronary perfusion line, the coronary perfusion pressure and the coronary flow rate both fell to zero. There was a concomitant fall in developed pressure, which became unmeasurable within 1–2 minutes.

Global Myocardial Metabolism

MVo2 was similar in the experimental hypoperfused (0.19 ± 0.01 ml/min) and control (0.18 ± 0.01) groups of hearts prior to the onset of hypoperfusion (p = NS). However, accompanying the reduction in coronary perfusion pressure and the resultant decreases in coronary flow rate and left ventricular pressure development, there was a fall in MVo2. After 10 minutes hypoperfusion, the MVo2 reached a low, stable level. After 30 minutes, the MVo2 was 0.06 ± 0.01 ml O2/min in the hypoperfused group compared with 0.17 ± 0.01 in controls (p < 0.0001). The changes in MVo2 in the two groups of hearts are shown in Figure 2.

Both the hypoperfused and control hearts (n = 5 pairs) produced similar levels of lactate during the baseline period (0.0 ± 0.0 μmol/min versus 0.5 ± 0.5, respectively, p = NS). As shown in Figure 3, myocardial lactate production increased to 3.0 ± 0.6 μmol/min after 30 minutes hypoperfusion and remained low in controls, 0.7 ± 0.5 (p < 0.02).

CK release was assessed in 4 pairs of hearts and was not found to be increased by cardiac hypoperfusion. During the baseline period, myocardial CK release was 121.1 ± 23.3 mIU/min in the experimental hypoperfused hearts and 59.4 ± 19.7 in the controls (p = NS). At the end of 30 minutes hypoperfusion, the myocardial CK release was 51.1 ± 20.6 compared with 39.1 ± 16.4 in the controls (p = NS).

Mitochondrial Oxidative Phosphorylation

When glutamate and malate were used as substrates (17 pairs of hearts), mitochondria isolated from hypoperfused hearts showed a marked increase in the rate of state 3 respiration compared with simultaneously perfused control hearts. Table 2 summarizes the data on oxidative phosphorylation when state 3 respiration was stimulated by addition of ADP at a final concentration of 195.9 μM. Accompanying the augmentation of state 3, there was an increase in state 4 respiration. However, there was no evidence of uncoupling of oxidative phosphorylation. In fact, the acceptor control ratio actually was higher in the hypoperfused group. The ADP/O ratio in the experimental, hypoperfused hearts was similar to that in the controls.

Global Myocardial Metabolism

MVo2 was similar in the experimental hypoperfused (0.19 ± 0.01 ml/min) and control (0.18 ± 0.01) groups of hearts prior to the onset of hypoperfusion (p = NS).

However, accompanying the reduction in coronary perfusion pressure and the resultant decreases in coronary flow rate and left ventricular pressure development, there was a fall in MVo2. After 10 minutes hypoperfusion, the MVo2 reached a low, stable level. After 30 minutes, the MVo2 was 0.06 ± 0.01 ml O2/min in the hypoperfused group compared with 0.17 ± 0.01 in controls (p < 0.0001). The changes in MVo2 in the two groups of hearts are shown in Figure 2.

Both the hypoperfused and control hearts (n = 5 pairs) produced similar levels of lactate during the baseline period (0.0 ± 0.0 μmol/min versus 0.5 ± 0.5, respectively, p = NS). As shown in Figure 3, myocardial lactate production increased to 3.0 ± 0.6 μmol/min after 30 minutes hypoperfusion and remained low in controls, 0.7 ± 0.5 (p < 0.02).

CK release was assessed in 4 pairs of hearts and was not found to be increased by cardiac hypoperfusion. During the baseline period, myocardial CK release was 121.1 ± 23.3 mIU/min in the experimental hypoperfused hearts and 59.4 ± 19.7 in the controls (p = NS). At the end of 30 minutes hypoperfusion, the myocardial CK release was 51.1 ± 20.6 compared with 39.1 ± 16.4 in the controls (p = NS).

Mitochondrial Oxidative Phosphorylation

When glutamate and malate were used as substrates (17 pairs of hearts), mitochondria isolated from hypoperfused hearts showed a marked increase in the rate of state 3 respiration compared with simultaneously perfused control hearts. Table 2 summarizes the data on oxidative phosphorylation when state 3 respiration was stimulated by addition of ADP at a final concentration of 195.9 μM. Accompanying the augmentation of state 3, there was an increase in state 4 respiration. However, there was no evidence of uncoupling of oxidative phosphorylation. In fact, the acceptor control ratio actually was higher in the hypoperfused group. The ADP/O ratio in the experimental, hypoperfused hearts was similar to that in the controls.

Global Myocardial Metabolism

MVo2 was similar in the experimental hypoperfused (0.19 ± 0.01 ml/min) and control (0.18 ± 0.01) groups of hearts prior to the onset of hypoperfusion (p = NS). However, accompanying the reduction in coronary perfusion pressure and the resultant decreases in coronary flow rate and left ventricular pressure development, there was a fall in MVo2. After 10 minutes hypoperfusion, the MVo2 reached a low, stable level. After 30 minutes, the MVo2 was 0.06 ± 0.01 ml O2/min in the hypoperfused group compared with 0.17 ± 0.01 in controls (p < 0.0001). The changes in MVo2 in the two groups of hearts are shown in Figure 2.

Both the hypoperfused and control hearts (n = 5 pairs) produced similar levels of lactate during the baseline period (0.0 ± 0.0 μmol/min versus 0.5 ± 0.5, respectively, p = NS). As shown in Figure 3, myocardial lactate production increased to 3.0 ± 0.6 μmol/min after 30 minutes hypoperfusion and remained low in controls, 0.7 ± 0.5 (p < 0.02).

CK release was assessed in 4 pairs of hearts and was not found to be increased by cardiac hypoperfusion. During the baseline period, myocardial CK release was 121.1 ± 23.3 mIU/min in the experimental hypoperfused hearts and 59.4 ± 19.7 in the controls (p = NS). At the end of 30 minutes hypoperfusion, the myocardial CK release was 51.1 ± 20.6 compared with 39.1 ± 16.4 in the controls (p = NS).

Mitochondrial Oxidative Phosphorylation

When glutamate and malate were used as substrates (17 pairs of hearts), mitochondria isolated from hypoperfused hearts showed a marked increase in the rate of state 3 respiration compared with simultaneously perfused control hearts. Table 2 summarizes the data on oxidative phosphorylation when state 3 respiration was stimulated by addition of ADP at a final concentration of 195.9 μM. Accompanying the augmentation of state 3, there was an increase in state 4 respiration. However, there was no evidence of uncoupling of oxidative phosphorylation. In fact, the acceptor control ratio actually was higher in the hypoperfused group. The ADP/O ratio in the experimental, hypoperfused hearts was similar to that in the controls.
In comparison to the augmentation in state 3 respiration observed in hypoperfused heart mitochondria, 30 minutes of ischemia resulted in a reduction in state 3 respiration to 124.6 ± 25.9 ng atoms O/min/mg mitochondrial protein (p versus control <0.0001) with glutamate and malate as the respiratory substrates. The acceptor control index was also reduced to 4.4 ± 1.2 (p versus control <0.02).

In 11 of the 17 pairs of hearts, rates of oxidative phosphorylation were also studied using succinate as the respiratory substrate. The data for these studies are shown in Table 3. These experiments were performed in the presence of 5 μM rotenone and utilized a final concentration of ADP of 195.9 μM to stimulate state 3 respiration. The marked increase in the rate of glutamate/malate-supported state 3 respiration induced by hypoperfusion, described above, was also apparent in this selected subgroup. Nevertheless, with succinate as the substrate, no differences in states 2, 3, and 4 respiration were found when these rates were compared in experimental and control hearts. In addition, there were no alterations in the acceptor control, respiratory control, and ADP/O ratios.

With succinate as substrate, 30 minutes of ischemia reduced state 3 respiration to 135.3 ± 42.9 ng atoms O/min/mg mitochondrial protein (p versus control <0.0001) and similarly reduced the acceptor control ratio to 2.8 ± 0.5 (p versus control <0.005).

Kinetics of Oxidative Phosphorylation
A differential effect of the substrates on the Vₘₐₓ and apparent Kₘ of state 3 respiration was also present (Table 4). The mean Vₘₐₓ with glutamate and malate in the experimental, hypoperfused hearts was almost twice as high as that measured in control hearts. In addition, the apparent Kₘ for ADP, with glutamate and malate as substrate, was also twice as high in the experimental hearts as in controls. In contrast, with succinate as substrate, the Vₘₐₓ and apparent Kₘ for ADP were both similar in control and experimental hearts. In the 6 pairs of hearts in which the kinetics of oxidative phosphorylation were measured with succinate as substrate, the rates of states 3 and 4 respiration were also determined with glutamate and malate, using a final ADP concentration of 195.9 μM, and were found to be increased in the hypoperfused hearts, similar to the increase observed in all 17 pairs of hearts.

Uncoupled Mitochondrial Respiration
The rates of uncoupled respiration, measured after the pulsed addition of 80 nmol DNP, were compared with the rates of state 3 respiration for both glutamate/malate and for succinate as the respiratory substrates. As shown in Table 5, with a given substrate, the rates of state 3 respiration and uncoupled electron transport were similar in each group of hearts. Therefore, the hypoperfusion-induced alterations in the rate
of ATP synthesis occurred in parallel with the alteration in the rates of uncoupled electron transport.

Mitochondrial Yield and Purity

Because differences in mitochondrial fragility could result in the isolation of different amounts of mitochondria from control and experimental hearts, mitochondrial yield was quantified directly. In 5 pairs of hearts, the total mitochondrial protein isolated, as well as the MDHm activity, was measured in addition to the rates of oxidative phosphorylation using glutamate/malate and succinate as respiratory substrates. In this subgroup of hearts, reductions in coronary flow and left ventricular developed pressure that resulted from the reduction in coronary perfusion pressure were similar to those observed in all pairs of hearts. As shown in Table 6, there was no difference in the amount of mitochondrial protein isolated from control and hypoperfused hearts. Furthermore, the MDHm activity in the isolated mitochondrial fractions was equivalent in the hypoperfused and control groups. These findings indicate that similar amounts of mitochondria were isolated from control and hypoperfused hearts and that the purity of these fractions was equivalent. In contrast to these findings, mitochondria of hearts isolated from control and hypoperfused hearts actually represent an alteration in mitochondrial function and is not an artifact resulting from differences of purity of the control and hypoperfused mitochondrial fractions.

In ischemic hearts, in contrast, normalization for MDHm activity revealed a mild decrease in state 3 respiration that was not statistically significant with glutamate and malate as respiratory substrates (10.2 ± 2.5 ng atoms O/min/IU MDHm, p versus control = 0.11). With succinate as substrate, ischemic heart mitochondrial state 3 respiration was reduced to 12.5 ± 4.2 ng O/min/IU MDHm (p versus control < 0.01).

Discussion

These experiments demonstrate an unexpected augmentation of the kinetics of mitochondrial oxidative phosphorylation in hypoperfused hearts when glutamate and malate are used as tricarboxylic acid cycle substrates. These alterations in mitochondrial function were not observed when succinate was utilized as the respiratory substrate. The alterations in ADP-stimulated oxidative phosphorylation were similar to those measured after DNP uncoupling in each group of hearts with a given tricarboxylic acid cycle substrate. In contrast to these findings, mitochondria of hearts subjected to 30 minutes ischemia, with complete cessation of coronary perfusion, had reduced rates of state 3 respiration with both glutamate/malate and succinate as respiratory substrates. These data are consistent with a hypoperfusion-induced enhancement in the activity of the reduced nicotinamide adenine dinucleotide (NADH)-coenzyme Q reductase segment of the electron transport chain (electron transfer complex I) and/or in the rate of mitochondrial glutamate and malate uptake.

The results for the hypoperfused hearts in these experiments differ from those in previous studies that have shown a reduction in mitochondrial function caused by ischemia. In dogs, 1 hour of complete

Table 2. Effect of Hypoperfusion on Mitochondrial Function Using Glutamate (10 mM) and Malate (10 mM) as Substrates

<table>
<thead>
<tr>
<th>Mitochondrial function</th>
<th>Control</th>
<th>Hypoperfused</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of hearts</td>
<td>17</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>State 2*</td>
<td>42.4 ± 2.5</td>
<td>48.7 ± 2.1</td>
<td>NS</td>
</tr>
<tr>
<td>State 3*</td>
<td>290.7 ± 13.4</td>
<td>448.8 ± 14.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>State 4*</td>
<td>71.6 ± 2.8</td>
<td>97.4 ± 3.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Acceptor control ratio</td>
<td>7.2 ± 0.5</td>
<td>9.4 ± 0.3</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Respiratory control ratio</td>
<td>4.1 ± 0.2</td>
<td>4.7 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>ADP/O ratio</td>
<td>2.38 ± 0.04</td>
<td>2.32 ± 0.04</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Nanograms atoms oxygen per minute per milligram mitochondrial protein.

Table 3. Effect of Hypoperfusion on Mitochondrial Function Using Succinate (5 mM) as Substrate

<table>
<thead>
<tr>
<th>Mitochondrial function</th>
<th>Control</th>
<th>Hypoperfused</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of hearts</td>
<td>11</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>State 2*</td>
<td>129.2 ± 5.4</td>
<td>134.9 ± 6.9</td>
<td>NS</td>
</tr>
<tr>
<td>State 3*</td>
<td>536.1 ± 20.5</td>
<td>556.1 ± 31.9</td>
<td>NS</td>
</tr>
<tr>
<td>State 4*</td>
<td>129.2 ± 5.4</td>
<td>134.9 ± 6.9</td>
<td>NS</td>
</tr>
<tr>
<td>Acceptor control ratio</td>
<td>4.2 ± 0.2</td>
<td>4.1 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Respiratory control ratio</td>
<td>3.1 ± 0.1</td>
<td>3.0 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>ADP/O ratio</td>
<td>1.47 ± 0.03</td>
<td>1.46 ± 0.03</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Nanograms atoms oxygen per minute per milligram mitochondrial protein.

Table 4. Kinetics of Oxidative Phosphorylation

<table>
<thead>
<tr>
<th>Control</th>
<th>Hypoperfused</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate/malate as substrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Vmax</td>
<td>582.5 ± 30.8</td>
<td>568.3 ± 62.7</td>
</tr>
<tr>
<td>Apparent K_m</td>
<td>8.3 ± 0.7</td>
<td>7.3 ± 1.1</td>
</tr>
</tbody>
</table>

* Nanograms atoms oxygen per minute per milligram mitochondrial protein. ᵃ(ADP) μM.
and flow. Under these conditions, there is an increase in state 3 respiration with glutamate and malate as the tricarboxylic acid cycle substrates.

There are other experimental models in which increases in mitochondrial function have been observed. Although not specifically addressed in each study, it is possible that acute or chronic myocardial oxygen supply/demand imbalance may have occurred in each of these models. Increases in cardiac work were studied both in vivo and in isolated perfused hearts. Starczewski et al.\textsuperscript{26} achieved prolonged exercise training in rats, possibly inducing repeated episodes of supply/demand imbalance. After chronic exercise, this study measured cardiac performance in an ejecting, and therefore working, isolated perfused heart apparatus. In the hearts of exercised animals, cardiac output, and therefore myocardial demand, was higher than in nonexercised controls, while coronary flow was similar in the two groups. This relative shift in the relation between myocardial supply and demand was associated with increased state 3 respiration with both glutamate/malate and succinate as substrates. Acute alterations in mitochondrial function related to coronary flow were not examined. Increases in the work of isolated perfused rat hearts, achieved by increasing aortic afterload, also have been shown to increase the activity of the key mitochondrial enzyme pyruvate dehydrogenase, possibly via a decrease in the NADH/NAD ratio.\textsuperscript{27} Although the relation between cardiac work and coronary flow was not addressed in this study, myocardial supply/demand imbalance may have occurred.

Other studies may have induced supply/demand imbalance via reduced myocardial oxygen delivery, with resultant increases in mitochondrial function. Perfusion of isolated rat hearts with doxorubicin has been shown to cause an increase in coronary resistance with systolic and diastolic left ventricular dysfunction and reduction in intracellular high-energy phosphate levels similar to those observed during ischemia.\textsuperscript{28} Although coronary flow rate was not reduced, it was thought that altered coronary resistance and elevated left ventricular diastolic pressure may have caused uneven distribution of coronary flow with resultant areas of hypoperfusion. Mitochondria isolated from such doxorubicin-perfused hearts demonstrated en

\begin{table}[h]
\begin{center}
\begin{tabular}{|c|c|c|}
\hline
Mitochondrial function & Control & Hypoperfused \\
\hline
Glutamate/malate as substrate & & \\
Number of hearts & 6 & 6 \\
State 3* & 298.3±22.3 & 418.0±20.4 \\
DNP rate† & 275.9±26.6 & 442.4±31.0 \\
Succinate as substrate & & \\
Number of hearts & 6 & 6 \\
State 3* & 523.2±25.0 & 530.0±53.3 \\
DNP rate† & 549.0±39.8 & 562.6±56.5 \\
\hline
\end{tabular}
\end{center}
\caption{Comparison of Rates of Oxidative Phosphorylation and Uncoupled Respiration}
\end{table}

* Nanograms atoms oxygen per minute per milligram mitochondrial protein (after addition of ADP, final concentration 195.9 μM).
† Nanograms atoms oxygen per minute per International Unit of the key mitochondrial enzyme pyruvate dehydrogenase, possibly via a decrease in the NADH/NAD ratio.27 Although the relation between cardiac work and coronary flow was not addressed in this study, myocardial supply/demand imbalance may have occurred.

Other studies may have induced supply/demand imbalance via reduced myocardial oxygen delivery, with resultant increases in mitochondrial function. Perfusion of isolated rat hearts with doxorubicin has been shown to cause an increase in coronary resistance with systolic and diastolic left ventricular dysfunction and reduction in intracellular high-energy phosphate levels similar to those observed during ischemia.28 Although coronary flow rate was not reduced, it was thought that altered coronary resistance and elevated left ventricular diastolic pressure may have caused uneven distribution of coronary flow with resultant areas of hypoperfusion. Mitochondria isolated from such doxorubicin-perfused hearts demonstrated en

\begin{table}[h]
\begin{center}
\begin{tabular}{|c|c|c|c|}
\hline
Mitochondrial yield & MDHm activity & No. heart pairs & Control & Hypoperfused \\
\hline
No. heart pairs & 5 & 10.6±0.7 & 9.7±0.9 & NS \\
& 5 & 8.3±1.2 & 20.9±1.7 & NS \\
MGDH activity (IU/mg mito protein) & 5 & 15.5±1.6 & 24.1±2.1 & <0.02 \\
State 3, glut/malate* & 5 & 31.0±3.4 & 29.8±1.5 & NS \\
State 3, succinate* & 5 & & & \\
\hline
\end{tabular}
\end{center}
\caption{Mitochondrial Yield, MDHm Activity, and Rates of Oxidative Phosphorylation Expressed Relative to MDHm Activity}
\end{table}

* Nanograms atoms oxygen per minute per milligram mitochondrial protein (after addition of ADP, 195.9 μM).
enhanced state 3 respiration with the respiratory substrates glutamate and malate but not succinate. Increases in state 3 respiration also have been observed when oxygen delivery to the myocardium is reduced by hypoxia in the isolated perfused rat heart. In these studies, hypoxia caused an increase in coronary flow rate, a decrease in heart rate, and decreased MVO₂. Cardiac work or developed pressure was not assessed. State 3 respiration was increased in the hypoxic hearts with either glutamate or succinate as substrates. Other models of decreased oxygen delivery include cyanosis induced by surgical creation of right to left shunts in dogs and in vivo hypoxia in dogs and rats. These models also demonstrated increases in state 3 respiration. Since oxygen availability was reduced, supply/demand imbalance may again have been responsible for the alterations in mitochondrial function.

The increase in state 3 respiration with glutamate/malate as substrates, which occurs during hypoperfusion, may represent an adaptive mechanism to maximize ATP synthesis during or following myocardial supply/demand imbalance (e.g., during recovery from hypoxia or extremes of exercise). The enhanced utilization of glutamate and malate during ischemia may also provide a rational basis for using these agents to improve myocardial function during reperfusion. In this light, it is interesting that previous studies have shown an improvement in left ventricular function and increase in myocardial ATP content when anoxic isolated perfused rat hearts were perfused with the tricarboxylic acid cycle intermediates fumarate, malate, and glutamate. Isolated perfused rabbit heart septae also benefit from perfusion with amino acids during and after ischemia and anoxia. Only those amino acids that accumulate as glutamate and malate were effective. In dogs, reperfusion after ischemia resulted in enhanced myocardial oxygen consumption, ventricular function, and ATP content when the reperfusion was enriched with glutamate and aspartate.

In summary, these results indicate that hypoperfusion is associated with an enhancement of the kinetics of mitochondrial oxidative phosphorylation with the NAD-linked substrates glutamate and malate. Similar alterations may occur in human beings during myocardial infarction or cyanosis and may be important in the development of, or protection from, myocardial damage.

Acknowledgments

The authors wish to thank Dr. Glenn A. Langer for his critical review of this manuscript, Mr. Russel Stokes for assistance with statistical computation, and Dr. Paul Fu for kindly performing the creatine kinase assays.

References

11. Reimer KA, Jennings RB: The wavefront phenomenon of myocardial ischemic cell death: 2. Transmural progression of necrosis within the framework of ischemic bed size (myocardium at risk) and collateral flow. Lab Invest 1979;40:633–644


Key Words • hypoperfusion • myocardial ischemia • mitochondrial oxidative phosphorylation • isolated perfused rat heart
Enhancement of mitochondrial oxidative phosphorylation capability by hypoperfusion in isolated perfused rat heart.

P C Pelikan, J T Niemann, G Z Xia, G Jagels and J M Criley

*Circ Res*. 1987;61:880-888
doi: 10.1161/01.RES.61.6.880

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1987 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/61/6/880

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the *Permissions and Rights Question and Answer* document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org/subscriptions/