Low-pressure reperfusion alters mitochondrial permeability transition

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Low-pressure reperfusion alters mitochondrial permeability transition. Am J Physiol Heart Circ Physiol 288: H2750–H2755, 2005. First published January 14, 2005; doi:10.1152/ajpheart.01081.2004.—We hypothesized that low-pressure reperfusion may limit myocardial necrosis and attenuate postischemic contractile dysfunction by inhibiting mitochondrial permeability transition pore (mPTP) opening. Male Wistar rat hearts (n = 36) were perfused according to the Langendorff technique, exposed to 40 min of ischemia, and assigned to one of the following groups: 1) reperfusion with normal pressure (NP = 100 cmH2O) or 2) reperfusion with low pressure (LP = 70 cmH2O). Creatine kinase release and tetraphenyltetrazolium chloride staining were used to evaluate infarct size. Modifications of cardiac function were assessed by changes in coronary flow, heart rate (HR), left ventricular developed pressure (LVDP), the first derive of the pressure curve (dP/dt), and the rate-pressure product (RPP = LVDP × HR). Mitochondria were isolated from the reperfused myocardium, and the Ca2+-induced mPTP opening was measured using a potentiometric approach. Lipid peroxidation was assessed by measuring malondialdehyde production. Infarct size was significantly reduced in the LP group, averaging 17 ± 3 vs. 33 ± 3% of the left ventricular weight in NP hearts. At the end of reperfusion, functional recovery was significantly improved in LP hearts, with RPP averaging 10,392 ± 876 vs. 3,969 ± 534 mmHg/min in NP hearts (P < 0.001). The Ca2+-load required to induce mPTP opening averaged 232 ± 10 and 128 ± 16 μM in LP and NP hearts, respectively (P < 0.001). Myocardial malondialdehyde was significantly lower in LP than in NP hearts (P < 0.05). These results suggest that the protection afforded by low-pressure reperfusion involves an inhibition of the opening of the mPTP, possibly via reduction of reactive oxygen species production.

Mitochondrial permeability transition pore (mPTP) opening is triggered by matrix Ca2+ accumulation, adenine nucleotide depletion, increased inorganic phosphate concentration, and oxidative stress, all features of ischemia-reperfusion (16). Griffths and Halestrap (15) demonstrated that the mPTP is closed during ischemia but opens in the early minutes of reperfusion. Hausenloy et al. (20) proposed that ischemic and pharmacological preconditioning may exert their protective effect by inhibiting mPTP opening at reperfusion. Several groups including ours recently proposed that pharmacological inhibition of mPTP opening at reperfusion is protective and might explain the beneficial effect of ischemic preconditioning (2, 19, 22). The aim of the present study was to determine whether a controlled, low-pressure reperfusion might limit lethal reperfusion injury by inhibiting mPTP opening.

METHODS

Surgical Preparation

The investigation conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996) and was approved by Institutional Animal Care and Use Committee (IACUC) of the Claude Bernard University. Male Wistar rats, weighing 350–450 g, were anesthetized with pentobarbital sodium (50 mg/kg), and heparin (200 IU/kg) was injected into the femoral vein. Hearts were removed and immediately arrested in ice-cold St. Thomas solution. The aorta was rapidly cannulated and perfused for 10 min in the Langendorff mode using Krebs-Henseleit bicarbonate buffer (in mmol/L: 11.0 glucose, 118.5 NaCl, 4.75 KCl, 1.19 MgSO4, 1.18 KH2PO4, 25.0 NaHCO3, and 1.4 CaCl2) at pH 7.4. The buffer was bubbled with 95% O2-5% CO2 at 37°C, and perfusion was performed under a hydrostatic pressure of 100 cmH2O. The left ventricle (LV) was paced at a constant rate of 300 beats/min.

Experimental Design

Global normothermic ischemia was induced by clamping the aorta. The temperature was maintained by immersion of the heart in the perfusion medium at 37°C. Two different protocols were performed. The aim of protocol I was to evaluate functional recovery and tissue injury after 40 min of global ischemia and 60 or 120 min of reperfusion. Protocol II was used to assess Ca2+-induced mitochondrial permeability transition and measurement of malondialdehyde (MDA) production, an index of lipid peroxidation by oxygen-derived free radicals.

Protocol I. One group of hearts underwent no intervention throughout the experiment (control, n = 12). All other hearts underwent 40 min of global ischemia followed by 60 min (protocol IA) or 120 min (protocol IB) of reperfusion. In protocols IA and IB, all animals were randomly assigned to one of the two following groups (n = 6/group): 1) the normal-pressure (NP) group, in which the myocardium was

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reperfused at normal pressure (i.e., 100 cm H2O) after the ischemic insult, and 2) the low-pressure (LP) group, in which the myocardium was reperfused at 70 cm H2O.

In protocol IB, reperfusion in the LP group was set at 70 cm H2O for the first 60 min; then normal perfusion pressure (i.e., 100 cm H2O) was established for the remaining 60 min. These two levels of perfusion pressure were obtained by adjusting the perfusion column to the adequate height. Perfusion pressure of 100 cm H2O is considered normal for a rat heart under physiological conditions.

Protocol II. All hearts underwent 40 min of global ischemia followed by 10 min of reperfusion. Animals were randomly assigned to one of the two previously defined groups, and cyclosporin A (CsA, 0.2 μmol/l) or its vehicle was administered before the onset of ischemia and throughout reperfusion (n = 6/group). At the end of the 10-min reperfusion, hearts were excised for Ca2+-induced mPTP assessment and MDA measurements.

Analysis

Functional recovery. LV systolic pressure (LVSP) and LV end-diastolic pressure (LVEDP) were measured by introducing a latex balloon into the LV and expanding it to exert a physiological end-diastolic pressure of 5 mmHg. The rate-pressure product [RPP = (LVSP – LVEDP) × HR, where HR is heart rate], the maximum rate of rise of LV pressure (dP/dt max), and the maximum isovolumetric rate of relaxation (–dP/dt min) were calculated. Coronary flow (CF) was measured by timed collections of the pulmonary effluent.

Myocardial necrosis. Myocardial necrosis was evaluated by measurement of creatine kinase (CK) release in the coronary effluent during the reperfusion period (Beckman Coulter kit, Galway, Ireland) and triphenyltetrazolium chloride (TTC) staining, as previously described (35). The heart was cut into four to five transverse slices, parallel to the atrioventricular groove. After removal of right ventricular tissue, heart slices were weighed and incubated for 20 min in a 1% solution of TTC at 37°C to differentiate infarcted (pale) from viable (brick-red) myocardial area. The slices were then photographed, and enlarged projections of these slices were traced for determination of the boundaries of the area of necrosis. Extent of the area of necrosis was quantified by computerized planimetry. Total area of necrosis was then calculated and expressed as percentage of total LV.

Ca2+-induced mitochondrial permeability transition. PREPARATION OF ISOLATED MITOCHONDRIA. Preparation of mitochondria was adapted from a previously described procedure (13). All operations were carried out in the cold. Myocardial sections (~1 g) were placed in isolation buffer A (in mM: 70 sucrose, 210 mannitol, and 1 EDTA in 50 Tris-HCl, pH 7.4). The tissue was finely minced with scissors and then homogenized in the same buffer (1 ml buffer/g tissue) using a Kontes tissue grinder and then a Potter-Elvejem homogenizer. The homogenate was centrifuged at 1,300 g for 10 min, and stored as pellets on ice before mPTP opening experiments. Protein content was routinely assayed according to Gornall’s procedure, with bovine serum albumin as a standard (14).

CA2+-INDUCED MPTP OPENING. mPTP opening was assessed after in vitro Ca2+ overload. Isolated mitochondria (6 mg of protein) were suspended in 100 μl of buffer B and added in 900 μl of buffer C (in mM: 150 sucrose, 50 KCl, 2 KH2PO4, and 5 succinic acid in 20 Tris-HCl, pH 7.4) within a Teflon chamber equipped with a Ca2+-specific microelectrode, in conjunction with a reference electrode (12). Modifications of the medium (i.e., extramitochondrial) Ca2+ concentration were continuously recorded using custom-modified synchronich software. Mitochondria were gently stirred for 1.5 min. At the end of the preincubation period, 20 μM CaCl2 was administered every 60 s. Each administration of 20 μM CaCl2 induces an abrupt rise in extramitochondrial Ca2+ concentration (Fig. 1). Ca2+ is then rapidly taken up by the mitochondria, resulting in a return of extramitochondrial Ca2+ concentration to near baseline. After sufficient Ca2+ loading, extramitochondrial Ca2+ concentration abruptly increases, indicating a massive release of Ca2+ by mitochondria due to mPTP opening (Fig. 1). The amount of Ca2+ required to trigger this massive Ca2+ release is used here as an indicator of susceptibility of the mPTP to Ca2+ overload.

MDA production. After 10 min of reperfusion, biopsies were performed in the LV and quickly frozen. MDA level was determined by high-performance liquid chromatography using the thiobarbituric acid test and expressed as picomoles per milligram of protein.

Statistics

Statistical comparisons were performed using the analysis of variance and Fisher’s protected least significant difference test. Values are means ± SE. P < 0.05 was considered as indicative of a statistically significant difference.

RESULTS

Protocol I: Myocardial Damage and Functional Recovery After Ischemia and Reperfusion

Protocol IA: 60 min of reperfusion. In the NP group, baseline RPP averaged 30,002 ± 3,100 mmHg/min. During the reperfusion period, recovery of RPP was poor, ranging from 1,117 ± 140 mmHg/min at 10 min to 3,969 ± 534 mmHg/min at 60 min (P < 0.001 vs. baseline and control; Fig. 2). At 60 min of reperfusion, LV dP/dt max and LV dP/dt min were significantly decreased, averaging 300 ± 26 and 248 ± 26 mmHg/s, respectively (P < 0.001 vs. control). CF was significantly reduced, averaging 9.1 ± 0.7 ml·min⁻¹·g⁻¹ vs. 13.8 ± 0.3 ml·min⁻¹·g⁻¹ in control (P < 0.05).

In the LP group, baseline RPP was comparable to that in the NP group. In contrast, during the reperfusion period, RPP was significantly higher than in NP group, ranging from 7,545 ± 401 mmHg/min at 10 min to 10,392 ± 876 mmHg/min at 60 min (P < 0.001 vs. NP). Return to normal perfusion pressure at the end of the experiment did not significantly alter RPP in the NP or LP group [P = not significant (NS) vs. 60 min]. LV systolic pressure (LVSP) and LV end-diastolic pressure (LVEDP) are shown in Fig. 2B. LVSP in the LP group was lower than in the NP group throughout the experiment (P < 0.01 vs. NP).

The protocol IB: 60 min of reperfusion. In the NP group, baseline RPP averaged 30,002 ± 3,100 mmHg/min. During the reperfusion period, recovery of RPP was poor, ranging from 1,117 ± 140 mmHg/min at 10 min to 3,969 ± 534 mmHg/min at 60 min (P < 0.001 vs. baseline and control; Fig. 2). At 60 min of reperfusion, LV dP/dt max and LV dP/dt min were significantly decreased, averaging 300 ± 26 and 248 ± 26 mmHg/s, respectively (P < 0.001 vs. control). CF was significantly reduced, averaging 9.1 ± 0.7 ml·min⁻¹·g⁻¹ vs. 13.8 ± 0.3 ml·min⁻¹·g⁻¹ in control (P < 0.05).

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![Fig. 1. Ca2+-induced mitochondrial permeability transition pore (mPTP) opening. Typical recording of mPTP opening in isolated mitochondria from 1 normal-pressure (NP = 100 cm H2O) and one low-pressure (LP = 70 cm H2O) heart. In the NP mitochondria, a Ca2+ overload of 120 μM (6 pulses of 20 μM) was required to induce mPTP opening vs. 200 μM Ca2+ (10 pulses of 20 μM Ca2+) in the LP heart. Vertical arrows indicate administration of 20 μM Ca2+ into the NP mitochondrial suspension.](http://ajpheart.physiology.org/)

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dP/dt\text{max} and LV dP/dt\text{min} were significantly higher in LP than in NP hearts \((P < 0.001)\), despite a reduced mean CF (Table 1).

CK release was significantly reduced in the LP vs. the NP group, averaging 95 \pm 7 and 177 \pm 24 IU/l at 60 min of reperfusion \((P < 0.05; \text{Fig. 3A})\). This was confirmed by TTC staining, with infarct size averaging 17 \pm 3 and 33 \pm 3% in LP and NP hearts, respectively \((P < 0.01; \text{Fig. 3B})\).

**Protocol IB: 120 min of perfusion.** At 60 min of reperfusion, CF averaged 9.7 \pm 1.0 and 6.8 \pm 0.4 ml/min-g\text{^{-1}} in NP and LP hearts, respectively \((P < 0.05)\). Restoration of normal perfusion pressure in the LP group abolished this difference, with CF averaging 8.4 \pm 1.3 and 7.4 \pm 0.6 ml/min-g\text{^{-1}} in the NL and LP groups, respectively, at 120 min of reperfusion \((P = \text{NS})\).

At 120 min of reperfusion, RPP averaged 25,790 \pm 1,440, 2,330 \pm 1,800, and 5,240 \pm 3,350 mmHg/min in control (sham), NP, and LP hearts, respectively \((P < 0.05,\text{ LP vs. NP})\).

Infarct size averaged 34 \pm 5 and 17 \pm 1% of the LV weight in the NP and LP groups, respectively \((P < 0.05)\). At 120 min of reflow, mean CK release averaged 150 \pm 20 and 80 \pm 6 IU/l in NP and LP groups, respectively, whereas lactate dehydrogenase release averaged 65 \pm 8 and 41 \pm 2 IU/l in NP and LP groups, respectively \((P < 0.05\text{ for both})\). In other words, the difference between NP and LP groups at 60 min was maintained when the reperfusion duration was extended to 120 min.

**Protocol II: \text{Ca}^{2+}\text{-Induced Mitochondrial Pore Transition and MDA Production}**

In the control group, the amount of \text{Ca}^{2+} required to open the mPTP averaged 280 \pm 10 \mu M (Fig. 4). This \text{Ca}^{2+} load was significantly reduced in the NP group, averaging 128 \pm 16 \mu M \((P < 0.001\text{ vs. control})\). The \text{Ca}^{2+} load required to open the mPTP was significantly higher in the LP than in the NP hearts, averaging 232 \pm 10 \mu M. CsA (0.2 \mu mol/l) significantly inhibited mPTP opening in NP hearts; the \text{Ca}^{2+} overload required to open the mPTP averaged 233 \pm 7 \mu M in CsA-treated NP hearts \((P < 0.05\text{ vs. NP, } P = \text{NS vs. LP})\). CsA did not modify mPTP opening in LP hearts (216 \pm 5 \mu M; Fig. 4).

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**Table 1. Hemodynamics**

<table>
<thead>
<tr>
<th>Groups</th>
<th>CF, ml/min-g\text{^{-1}}</th>
<th>RPP, mmHg/min</th>
<th>dP/dt\text{max}, mmHg/s</th>
<th>(\text{dP/dt}\text{min}, \text{mmHg/s})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.8 \pm 0.3*</td>
<td>30,000 \pm 3,100 *</td>
<td>2,630 \pm 110</td>
<td>1,860 \pm 70 *</td>
</tr>
<tr>
<td>NP</td>
<td>9.1 \pm 0.7*</td>
<td>2,572 \pm 214*</td>
<td>300 \pm 26*</td>
<td>248 \pm 26*</td>
</tr>
<tr>
<td>LP</td>
<td>6.4 \pm 0.3*†</td>
<td>9,415 \pm 790*†</td>
<td>981 \pm 64†</td>
<td>641 \pm 35†</td>
</tr>
</tbody>
</table>

Values are means \pm SE \((n = 6)\). NP, normal pressure (100 cmH2O); LP, Low pressure (70 cmH2O); CF, coronary flow; RPP, rate-pressure product; dP/dt\text{max}, maximum rate of rise of left ventricular pressure; dP/dt\text{min}, maximum isovolumetric rate of relaxation. \*Significantly different from control \((P < 0.001)\). Significantly different from NP: \*\*P < 0.05; \*\*\*P < 0.001.
Myocardial MDA levels were significantly increased in the NP group compared with control: 150 ± 5 vs. 87 ± 13 pmol/mg of protein (P < 0.05; Fig. 5). MDA production was significantly lower in LP than in NP hearts: 119 ± 15 pmol/mg of protein.

**DISCUSSION**

In the present study, we report that low reperfusion pressure inhibits opening of the mPTP at the time of reperfusion and prevents irreversible myocardial injury after a prolonged ischemic insult. Although there is no doubt that reperfusion salvages a significant amount of jeopardized tissue, it has also been recognized as a “double-edged sword,” because it can trigger arrhythmias, myocardial stunning, and no reflow (7). Among various interventions aimed at reducing the deleterious effects of reperfusion, several authors used modifications of the conditions of reperfusion (33). Limiting Ca\(^{2+}\) overload, scavenging oxygen-derived free radicals, reducing arterial PO\(_2\), or applying a transient acidosis in the early minutes of reperfusion can improve functional recovery after an ischemic insult (8, 10, 24, 27). In the present study, we reported that low-pressure reperfusion reduced infarct size and improved functional recovery after a prolonged global normothermic ischemia in the rat heart. Here, we confirmed a recent study from our group in which we observed the beneficial effect of low-pressure reperfusion after warm ischemia, but not after a cardioplegic arrest or cold ischemic preservation in the rat model (28). The present results are in agreement with studies demonstrating that controlled reperfusion (reduced coronary perfusion pressure or flow) may improve functional recovery after brief or sustained ischemic episodes (5, 21, 25, 30, 34), although low PO\(_2\) or a reperfusion pressure <50 mmHg may be detrimental, especially after a cold cardioplegic arrest (23). Low-pressure reperfusion has also been successfully used after cold preservation of rat and pig lungs (1, 6, 18) and helped our group transplant human cardiac grafts subjected to a prolonged cold ischemia lasting up to 10–13 h (29).

How low-perfusion pressure can protect the reperfused heart remains unknown. Takeo et al. (34) attributed this protective effect to a limitation of the cytosolic accumulation of Na\(^{+}\) and Ca\(^{2+}\) after 35 min of global ischemia in the isolated rat heart. Hori et al. (21) demonstrated that staged reperfusion after a 15-min coronary artery occlusion in the dog heart attenuates myocardial stunning via a delayed correction of acidosis during the first minutes of reperfusion. We observed here that the protection was efficient as soon as 10 min after reflow and persisted when normal pressure was briefly resumed 1 h later. The protective effect was maintained when reperfusion was extended up to 120 min, suggesting that irreversible injury was actually limited, rather than simply delayed. This strongly suggests that low pressure prevents irreversible myocardial injury by acting during the early minutes of reperfusion.

Because mPTP opening occurs in the early minutes of reperfusion and plays an important role in lethal injury, we investigated whether low pressure may inhibit mitochondrial permeability transition (17, 19). We demonstrated that mitochondria isolated from myocardium reperfused at a low pressure displayed a reduced susceptibility to in vitro mPTP opening. The mitochondrial permeability transition inhibitor CsA increased the resistance of the transition pore to in vitro Ca\(^{2+}\) overload, whereas it did not affect that of mitochondria from hearts that had been reperfused with a low pressure. In our experimental conditions, LP mitochondria behaved as if they had been pretreated with CsA. One might question whether Ca\(^{2+}\)-induced mPTP opening might be a consequence, rather than a cause, of the improved myocardial viability observed in LP reperfused hearts. We, however, recently demonstrated in the rabbit heart model that in vivo inhibition of mitochondrial permeability transition by CsA or preconditioning modifies Ca\(^{2+}\)-induced mPTP opening in a way very similar to low-pressure reperfusion; importantly, this was observed after a fully reversible (i.e., 10 min of ischemia followed by 5 min of reperfusion) ischemia-reperfusion (2). This observation demonstrates that a modification of Ca\(^{2+}\)-induced mPTP opening is not a consequence of reduced cardiomyocyte death. Although we have not demonstrated a causal relation, this strongly suggests that altered Ca\(^{2+}\)-induced mPTP opening in myocardium excised from hearts with low-pressure reperfusion is not a consequence (but possibly a cause) of improved myocardial viability. Specific pharmacological inhibition of the transition pore by the nonimmunosuppressive cyclosporin derivative NIM-811 also provided comparable protection (2).

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**Fig. 4.** Ca\(^{2+}\)-induced mPTP opening. Ca\(^{2+}\) load required to induce mPTP opening in hearts reperfused for 10 min at LP or NP after 40 min of global ischemia in the presence or absence of cyclosporin A (CsA, 0.2 μmol/l). Values are means ± SE (n = 6). *P < 0.05 vs. sham. **P < 0.05 vs. NP.

**Fig. 5.** Malondialdehyde (MDA) levels of hearts reperfused for 10 min at LP and NP. Values are means ± SE (n = 6). *P < 0.05; ***P < 0.001 vs. control. †P < 0.05 vs. NP.
A decrease in matrix Ca\textsuperscript{2+} accumulation and/or a limited production of free radicals are the two main explanations for this enhanced resistance of LP mitochondria to transition pore opening. We found that myocardial MDA content at 10 min of reflow was reduced in these hearts, indirectly suggesting that low-pressure reperfusion limited the production of oxygen-derived free radicals. Yet we cannot exclude that a diminished mitochondrial Ca\textsuperscript{2+} overload also played a role, because we previously reported that a reduced CF subsequent to low-pressure reperfusion, as observed in the present study, limits cytosolic Ca\textsuperscript{2+} overload after normothermic ischemia in the pig heart (11). Peng et al. (31a) also reported that controlled flow during reperfusion limits cytosolic accumulation of Ca\textsuperscript{2+}, increases the rate of mitochondrial oxidative phosphorylation, and preserves myocardial ATP content.

Protection afforded by low-pressure reperfusion shares similarities with the recently described “postconditioning” phenomenon (36). Zhao et al. (36) reported that repeated brief episodes of ischemia-reperfusion in the early minutes of reflow after a sustained ischemia can dramatically reduce infarct size. They attributed this protection to a limitation of reactive oxygen species production. We recently demonstrated that postconditioning modulates mPTP opening (3). Further investigations are required to determine whether postconditioning might represent a form of controlled reperfusion.

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