Controlled reperfusion after hypothermic heart preservation inhibits mitochondrial permeability transition-pore opening and enhances functional recovery

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Bopassa, J. C., David Vandroux, M. Ovize, and R. Ferrera. Controlled reperfusion after hypothermic heart preservation inhibits mitochondrial permeability transition-pore opening and enhances functional recovery. Am J Physiol Heart Circ Physiol 291: H2265–H2271, 2006—We investigated whether low-pressure reperfusion may attenuate postischemic contractile dysfunction, limits necrosis and apoptosis after a prolonged hypothermic ischemia, and inhibits mitochondrial permeability transition-pore (MPTP) opening. Isolated rat hearts (n = 72) were exposed to 8 h of cold ischemia and assigned to the following groups: 1) reperfusion with low pressure (LP = 70 cmH2O) and 2) reperfusion with normal pressure (NP = 100 cmH2O). Cardiac function was assessed during reperfusion using the Langendorff model. Mitochondria were isolated, and the Ca2+ resistance capacity (CRC) of the MPTP was determined. Malondialdehyde (MDA) production, caspase-3 activity, and cytochrome c were also assessed. We found that functional recovery was significantly improved in LP hearts with rate-pressure product averaging 30,380 ± 1,757 vs. 18,000 ± 1,599 mmHg/min in NP hearts (P < 0.01). Necrosis, measured by triphenyltetrazolium chloride staining and creatine kinase leakage, was significantly reduced in LP hearts (P = 0.01). This study demonstrated that low-pressure reperfusion after hypothermic heart ischemia improves postischemic contractile dysfunction and attenuates necrosis and apoptosis. This protection could be related to an inhibition of mitochondrial permeability transition.

mitochondria; apoptosis

RECENT EVIDENCE INDICATES that lethal reperfusion injury occurs at the time of reperfusion after normothermic ischemia. Mitochondrial permeability transition appears to be a pivotal event in cell death, after ischemia-reperfusion (43). Mitochondrial permeability transition results in the opening of a large pore in the inner mitochondrial membrane, whose structure remains incompletely known. Mitochondrial permeability transition-pore (MPTP) opening is related with uncoupling of the respiratory chain, efflux of Ca2+ and small proteins such as the proapoptotic cytochrome c, and matrix swelling (4, 28). MPTP opening is mostly induced by matrix Ca2+ accumulation, adenine nucleotide depletion, increased inorganic phosphate concentration, and oxidative stress, all features of ischemia-reperfusion. Griffiths and Halestrap (16) showed that the MPTP is closed during ischemia but opens in the early minutes of reperfusion. Hausenloy et al. (20) proposed that pharmacological and ischemic preconditioning could act at the time of reperfusion by inhibiting MPTP opening. These authors also proposed that the inhibition of MPTP opening during the reperfusion could exert a myocardial protection (19). Our group also confirmed the crucial role played by MPTP during pre- (1) and postconditioning (2).

Many studies performed using animal preparations or clinical models attempted to modify the conditions of reperfusion to attenuate its deleterious effects. Controlled reperfusion after an ischemic insult has been proposed to protect the reperfused myocardium (21, 26, 32, 38).

Our group recently demonstrated that a low-pressure reperfusion might protect the heart at the time of reperfusion after an irreversible normothermic ischemic insult (6). Furthermore, the protection afforded with a low-pressure reperfusion seems to act by the same route (phosphatidylinositol 3-kinase-Akt pathway) as postconditioning (5).

However, the protective effect of controlled reperfusion after a hypothermic cardioplegic arrest remains debated (13, 34–36). Whether a similar mechanism applies during reperfusion after a hypothermic ischemia is currently unknown. During cold preservation, damage to isolated hearts depends on numerous factors, including the temperature, the pH, the composition of the cardioplegia, and/or the preservative solutions.

Therefore, the objective of this study was to determine whether a controlled low-pressure reperfusion could protect against cellular necrosis and apoptosis by acting on MPTP opening and improve postischemic contractile dysfunction after a prolonged hypothermic ischemia.

METHODS

Surgical Procedure

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (National Institutes of Health Publication No. 85-23, Revised 1996).

Male Wistar rats, weighing 350–450 g, were anesthetized with pentobarbital sodium (50 mg/kg). Heparin (200 IU/kg) was injected in the femoral vein. Hearts were quickly removed, the aorta was cannulated, and cold Celsior solution was perfused during 1 min to arrest hearts.

Experimental Design

Hearts were then immersed in the same hypothermic solution during 8 h at 4°C (ischemic period). Two different protocols were performed.

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Protocol I was applied to evaluate functional recovery and tissue necrosis after 8 h of global cold ischemia at 4°C and 60 min of reperfusion. Reperfusion was performed by using a Langendorff system with Krebs-Henseleit bicarbonate buffer [containing (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. The left ventricle (LV) was paced at a constant rate of 300 beats/min.

Protocol II was used to assess the Ca²⁺ resistance capacity (CRC) of the MPTP and to measure cytochrome c release, caspase-3 activity (indicators of apoptosis), and myocardial malondialdehyde (MDA) production (an index of lipid peroxidation by oxygen-derived free radicals).

Protocol I. One group of hearts underwent no intervention for the whole duration of the experiment (control, n = 6). All other hearts underwent 8 h of global hypothermic (4°C) ischemia followed by reperfusion. Animals were randomly assigned to one of the two following groups (n = 6–8/group): NP group (normal pressure): myocardium was reperfused at normal pressure (i.e., 100 cmH₂O) after the cold ischemic storage and LP group (low pressure): myocardium was reperfused at a pressure of 70 cmH₂O.

These two perfusion pressures were obtained by adjusting the higher of the perfusion column. It can be noted that 100 cmH₂O is considered as a normal perfusion pressure for a rat heart under physiological conditions.

After 60 min reperfusion using the two perfusion pressures, hearts were reperfused an additional 15 min. These last 15 min corresponded to the return to normal pressure in LP hearts. Functional recovery was analyzed throughout the reperfusion, whereas myocardial necrosis [TTC staining, creatine kinase (CK) and lactate dehydrogenase (LDH) release] was assessed at the end of reperfusion (75 min). We chose this short period of reperfusion because we found on previous experiments that extending the duration of reperfusion to 120 min in our model did not change either the absolute values of infarct size or the difference between the two groups. It also did not change enzymatic release (CK, LDH) and functional recovery (unpublished data).

Protocol II. All hearts underwent 8 h of global hypothermic (4°C) ischemia followed by 10 min of reperfusion. Animals were randomly assigned to one of the two previously defined groups (n = 6/group). At the end of the 10-min reperfusion, hearts were excised for measurement of CRC, MDA content, cytochrome c release, and caspase-3 activity.

Analysis

Functional recovery. The LV systolic pressure (LVSP) and the LV end-diastolic pressure (LVEDP) were measured using a latex balloon introduced in the LV and expanded to exert a physiological end-diastolic pressure of 5 mmHg. The rate-pressure product [RPP = (LVSP – LVEDP) × heart rate], the rate of systolic contraction (dP/dtmax), and diastolic relaxation (–dP/dtmax) were calculated. Coronary flow was measured by timed collections of the coronary effluent flow.

Myocardial necrosis. Cellular injury was evaluated by both measurements of CK and LDH release in the coronary effluent at the end of the reperfusion period (Coulter kit; Beckman, Galway, Ireland). Enzyme leakage measurements were corrected by taking into account the coronary flow rate and the weight of the heart. Myocardial necrosis was also assessed using triphenyltetrazolium chloride (TTC) staining, as previously described (41). Briefly, the heart was cut into five transverse slices, parallel to the atrioventricular groove. After the right ventricular tissue was removed, 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. The extent of the area of necrosis was quantified by computerized planimetry. Total area of necrosis was then calculated and expressed as a percentage of total LV area.

Ca²⁺-Induced Mitochondrial Permeability Transition

Preparation of isolated mitochondria. Preparation of mitochondria was adapted from a previously described procedure (14). All operations were carried out in the cold. Myocardial sections (~1 g) were placed in isolation buffer A containing (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, successively, a Kontes tissue grinder and a Potter Elvejem homogenizer. The homogenate was centrifuged at 1,300 g for 3 min. The supernatant was poured through cheesecloth and centrifuged at 10,000 g for 10 min. The supernatant was collected and stored at −80°C for subsequent cytochrome c and caspase-3 activity measurements. The mitochondrial pellet was resuspended in isolation buffer B containing (in mM) 70 sucrose, 210 mannitol, and 0.1 EDTA in 50 Tris-HCl, pH 7.4. After aliquots were removed for protein measurements, the mitochondria (by aliquots of 5–mg proteins) were washed in isolation buffer B, centrifuged at 6,800 g for 10 min, and stored as pellets on ice before Ca²⁺-induced MPTP opening experiments. Protein content was routinely assayed according to Lowry’s procedure using BSA as a standard (15).

Ca²⁺-induced MPTP opening. CRC of the MPTP was assessed after in vitro Ca²⁺ overload. Isolated mitochondria (5 mg proteins) were suspended in 100 μl buffer B and added to 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. Modifications of the medium (i.e., extramitochondrial) Ca²⁺ concentration were continuously recorded using custom-made Synchronie software. Mitochondria were stirred gently for 1.5 min. At the end of the preincubation period, 20 μM CaCl₂ were added every 60 s. Each 20 μM CaCl₂ administration is recorded as a peak of extramitochondrial Ca²⁺ concentration. Ca²⁺ is then rapidly taken up by the mitochondria, resulting in a return of extramitochondrial Ca²⁺ concentration to near baseline level. After sufficient Ca²⁺ loading, extramitochondrial Ca²⁺ concentration abruptly increases, indicating a massive release of Ca²⁺ by mitochondria because of MPTP opening, as previously shown (1, 2). The amount of Ca²⁺ necessary to trigger this massive Ca²⁺ release is used here as an indicator of the CRC of the mitochondrial permeability transition.

MDA production. After 10 min of reperfusion, biopsies were harvested from the LV and quickly frozen. The MDA level was determined by HPLC using the thiobarbituric acid test and expressed as picomoles per milligram of protein, as previously described (6). Caspase-3 activity. The supernatant obtained from mitochondria centrifugation was collected. Aliquots containing 80 μg of supernatant proteins were incubated in 50 μl of buffer A [25 mmol/l HEPES, 5 mmol/l MgCl₂, 1 mmol/l EGTA, 10 μg/ml leupeptin, and 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), pH 7.5]. The previous mixture was diluted with 225 μl of freshly prepared buffer B [25 mmol/l HEPES, 0.1% (wt/vol) 3-(cholamidopropyl dimethylammonio)-1-propane sulfonate, 10 mmol/l dithiothreitol, 100 U/ml aprotinin, and 1 mmol/l PMSF, pH 7.5] containing 167 μmol/l substrate caspase-3 inhibitor DEVD and incubated for 60 min at 37°C. Fluorescence was measured using an excitation wavelength of 342 nm and emission wavelength of 441 nm. Standards containing 0–3,000 pmol of aminopeptidase (AP) activity of the heart were done according to the manufacturer’s instructions (Bio-Rad, Hercules, CA). Membranes were blocked for 60 min at room temperature with 5% nonfat milk in Tris-buffered saline (in mM: 25 Tris, 137 NaCl, and 2.7 KCl) containing (in mM) 0.1% Tween 20 and incubated with anti-cytochrome c antibody (Pharmingen, San Diego, CA) at 1:1,000 dilution for 120 min in the same buffer with gentle agitation. After
three washes, the blots were incubated for 60 min with 1:10,000
dilution of peroxidase-conjugated donkey anti-mouse IgG. After
being further washed, the immunocomplexes were visualized using
an enhanced chemiluminescence system (Amersham, Little Chalfont,
UK).

Statistics

Statistical comparisons were performed using the ANOVA and
Fischer protected least significant difference test. All results are
expressed as means ± SE. A P value <0.05 was considered as
indicative of a statistically significant difference.

RESULTS

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

Functional recovery. In the NP group, baseline RPP averaged
38,000 ± 2,000 mmHg/min. During the reperfusion period,
recovery of RPP was impaired, ranging from 11,375 ±
1,016 mmHg/min at 10 min to 18,000 ± 1,599 mmHg/min at
60 min (P < 0.001 vs. baseline and control; Fig. 1). After 60
min of reperfusion, mean LV dP/dt max and LV dP/dt min
were significantly decreased, averaging 1,270 ± 69 and 8,43 ± 90
mmHg/s, respectively (P < 0.001 vs. control). Mean CF
was unchanged in NP hearts compared with control values
(Table 1).

In the LP group, baseline RPP was comparable to that of the
NP group. During the reperfusion period, RPP was signifi-
cantly higher than in the NP group at all times, being, e.g.: 22,316 ±
1,825 mmHg/min at 10 min and 24,316 ± 1,758
mmHg/min at 60 min (P < 0.05 vs. NP; Fig. 1). Both mean LV
dP/dt max and LV dP/dt min of hearts in the LP groups were
significantly higher than in NP hearts (P < 0.01) despite a
reduced mean coronary flow (Table 1). Return to normal
perfusion pressure at the end of the experiment slightly in-
creased RPP in the LP group to reach 30,380
perfusion pressure at the end of the experiment slightly in-
significantly higher than in the NP group (P < 0.01; Fig. 1). TTC staining revealed that
infarct size averaged 6.5 ± 2% of LV weight in LP vs. 15 ±
3% in NP (P < 0.01; Fig. 3).

Myocardial necrosis. CK release was significantly reduced
in the LP vs. NP group, averaging 21 ± 12 vs. 90 ± 16 IU/l
at 60 min of reperfusion (P < 0.01; Fig. 2). LDH release
was similarly reduced in the LP vs. NP group, averaging
80 ± 21 and 137 ± 44 IU/l at 60 min of reperfusion,
respectively (P < 0.05; Fig. 2). TTC staining revealed that
infarct size averaged 6.5 ± 2% of LV weight in LP vs. 15 ±
3% in NP (P < 0.01; Fig. 3).

Protocol II: Ca2+-Induced Mitochondrial Permeability
Transition and Apoptosis

CRC. The effect of 10 min of controlled reperfusion after
8 h of cold ischemia on MPTP opening is depicted in Fig. 4.
In the control group, the amount of Ca2+ required to open
the MPTP averaged 372 ± 14 μmol. The CRC was signif-
icantly reduced in the NP group to 156 ± 38 μmol/5 μmol
proteins (P < 0.001 vs. control). In the LP group, the Ca2+
load required to open MPTP was significantly higher than in
NP hearts, averaging 272 ± 19 μmol/5 μmol proteins (P <
0.01 vs. NP group).

MDA production. Myocardial MDA content assessed after
8 h of cold ischemia and 10 min of reperfusion are reported in
Fig. 5. Myocardial MDA content was significantly increased in
the NP group (69 ± 12 pmol/mg protein) compared with

Table 1. Hemodynamics and functional recovery
at 60 min of reperfusion

<table>
<thead>
<tr>
<th>Groups</th>
<th>CF, ml·min⁻¹·g⁻¹</th>
<th>RPP, mmHg/min</th>
<th>dP/dt max, mmHg/s</th>
<th>dP/dt min, mmHg/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.5±1.1</td>
<td>38,000±2,000</td>
<td>3,377±183</td>
<td>2,323±143</td>
</tr>
<tr>
<td>NP</td>
<td>14±1.7</td>
<td>15,535±1,416</td>
<td>1,270±69</td>
<td>843±90†</td>
</tr>
<tr>
<td>LP</td>
<td>9.2±0.6†</td>
<td>23,953±2,049*†</td>
<td>2,025±235*†</td>
<td>1,812±93†</td>
</tr>
</tbody>
</table>

Values shown are means of 6 values ± SE. CF, coronary flow; RPP,
rate-pressure product; dP/dt max, recovery of contractility; −dP/dt min,
the maximum of isovolumetric rate of relaxation. Comparison of the postischemic
function (CF, RPP, dP/dt max, and −dP/dt min) of hearts reperfused at normal
(NP) and low (LP) pressure. *P < 0.01 and †P < 0.01, different from control value.
‡P < 0.01, different from LP group value.

![Image 1](http://ajpheart.physiology.org/March30.2017/fig1.jpg)

Fig. 1. Postischemic functional recovery. Effects of low pressure (LP, 70
cmH2O) and normal pressure (NP, 100 cmH2O) during reperfusion of cold
ischemic hearts on the rate-pressure product (RPP). Values shown are means
of 6 values ± SE. †P < 0.05 and ††P < 0.01, different from LP group value.

![Image 2](http://ajpheart.physiology.org/March30.2017/fig2.jpg)

Fig. 2. Creatine kinase (CPK) and lactate dehydrogenase (LDH) release.
Enzymatic release during reperfusion in the coronary effluent during of hearts
reperfused at NP (100 cmH2O) and LP (70 cmH2O). Values shown are means
of 6 values ± SE. ***P < 0.001, different from control value. †P < 0.05 and
††P < 0.01, different from LP group value.
shams. LP hearts displayed a reduced MDA content that averaged $45 \pm 3$ pmol/mg protein ($P < 0.05$ vs. NP, $P = \text{not significant vs. control}$).

**DISCUSSION**

Lethal reperfusion injury after a prolonged ischemic insult has been a matter of debate over the past two decades. Recent evidence clearly indicated that irreversible myocardial damage was absent in control hearts (Fig. 6). LP significantly reduced cytochrome release compared with NP.

**Cytochrome c release.** Extramitochondrial cytochrome $c$ was absent in control hearts (Fig. 6). LP significantly reduced cytochrome release compared with NP.

**Caspase-3 activity.** Caspase-3 activity was dramatically increased in NP, averaging $2,360 \pm 259$ pmol AMC·h$^{-1}$·mg$^{-1}$ at 10 min of reperfusion ($P < 0.001$ vs. control; Fig. 7). LP hearts displayed a reduced caspase-3 activity that averaged $606 \pm 71$ AMC·h$^{-1}$·mg$^{-1}$ ($P < 0.001$ vs. NP, $P < 0.05$ vs. control).

**Fig. 3. Infarct size evaluated by triphenyltetrazolium chloride staining.** LP significantly reduced infarct size vs. NP group. Values shown are means of 6 values $\pm$ SE. ***$P < 0.001$, different from control value. LV, left ventricular.

**Fig. 4. Ca$^{2+}$-induced mitochondrial permeability transition-pore (MPTP) opening.** Final quantity of extramitochondrial Ca$^{2+}$ addition [$\mu$mol/5 mg protein (prot)] from hearts reperfused 10 min at LP (70 cmH$2O$) and NP (100 cmH$2O$) pressure after 8 h of global cold ischemia. Values shown are means of 6 values $\pm$ SE. *$P < 0.05$ and ***$P < 0.001$, different from control value. $\dagger\dagger$ $P < 0.01$, different from LP group value. Inset: example of typical recording of MPTP opening in isolated mitochondria obtained from one NP and one LP heart. In the NP mitochondria, a Ca$^{2+}$ overload of 156 $\mu$M (mean of 8 pulses of 20 $\mu$M) was required to induce MPTP opening. In LP mitochondria, a significantly higher Ca$^{2+}$ overload (272 $\mu$M, mean of 14 pulses) was required to induce MPTP opening. Vertical arrows indicate the first administration of 20 $\mu$M of Ca$^{2+}$ in the NP or LP mitochondria suspension. Each spike of Ca$^{2+}$ concentration results from a repeated administration of 20 $\mu$M of Ca$^{2+}$.

**Fig. 5. Myocardial malondialdehyde (MDA).** MDA levels of hearts reperfused 10 min at LP (70 cmH$2O$) and NP (100 cmH$2O$). Values shown are means of 6 values $\pm$ SE. **$P < 0.01$, different from control and LP values.

**Fig. 6. Cytochrome c (Cyt C) release.** Cytosolic cytochrome $c$ release of hearts reperfused 10 min at LP (70 cmH$2O$) and NP (100 cmH$2O$). Values shown are means of 6 values $\pm$ SE. ***$P < 0.001$, different from control values. $\dagger\dagger\dagger$ $P < 0.001$, different from LP group value. Top: example of Western blotting analysis of cytochrome $c$ for 2 hearts in each group.
occurs during reperfusion, as demonstrated by the existence of a postconditioning-induced cardioprotection (42). This implies that the conditions of reperfusion are determinant for myocardial viability after a sustained ischemia. Previous studies conducted by Buckberg (9) and by Vinten-Johansen et al. (39) investigated the role of several factors modifying the conditions of reperfusion and the composition of the reperfusate. Kaneda et al. (23) demonstrated, in the rat model, that reducing the conditions of reperfusion and the composition of the reperfusate.

Fig. 7. Caspase-3 activity. Activity of caspase-3 of hearts reperfused 10 min at LP (70 cmH2O) and NP (100 cmH2O). AMC, aminomethylcoumarin. Values shown are means of 6 values ± SE. *P < 0.05 and ***P < 0.001, different from control values. †††P < 0.001, different from LP group value.

We might suppose that, submitted to high-potassium cardioplegia, endothelial cells would be impaired and produce inappropriate nitric oxide (40). Nitric oxide at physiological levels is known to protect the cell by preserving ATP, preventing MPTP opening and limiting apoptosis (22, 24, 30). Conversely, inadequate nitric oxide production would induce the negative inotrope effect, as previously shown (27, 33) and 2 activation of MPTP opening (nitric oxide) is a potent activator of MPTP (8).

In the present study, we showed that, after cold cardioplegia induced with Celsior solution and 8 h of global hypothermic ischemia, significant reperfusion injury occurred with uncontrolled reperfusion. Applying a low pressure in the early period of reperfusion blunted this irreversible damage. A notable point is that this protection was efficient as soon as 10 min after reflow and persisted when normal pressure was set back 1 h later, strongly suggesting that low pressure prevents irreversible myocardial injury by acting during the early minutes of reperfusion (Fig. 1).

Low-pressure reperfusion also results in a reduced coronary flow (Table 1). Kin et al. (25) recently proposed an interesting hypothesis. They suggested that the protection induced by postconditioning could be because of a delayed washout of intravascular adenosine (and increased retention time of adenosine in the extracellular space), associated with a reduced coronary flow during the first 2 min of reperfusion. In the present study, the 35% reduction of coronary flow induced by controlled reperfusion is in accordance with this hypothesis. Moreover, initial low-flow reperfusion may reduce oxygen influx and consequently limit free radical production. Indeed, ischemic hearts reperfused at low pressure exhibited a significantly reduced MDA content, suggesting a reduced lipid peroxidation via reactive oxygen species (Fig. 5). The reduction of coronary flow may also prevent Ca2+ overload after normothermic ischemia in the pig heart, as we previously showed (12). Free radical and Ca2+ overload are two major regulators of MPTP. We thus investigated whether MPTP opening might be associated with controlled reperfusion after cold ischemia. MPTP opening is indeed a crucial event in cardiomyocyte death after ischemia-reperfusion (16, 20, 28). MPTP opening at the onset of reperfusion after a prolonged ischemia is controlled by many factors, especially matrix Ca2+ accumulation and/or a production of free radicals. MPTP opening results in cell death. Recent papers point out the crucial role of MPTP in necrosis of the heart and brain rather than apoptosis (17). Moreover, cyclophilin D knockout mice are protected against necrosis but not against apoptosis after an ischemia-reperfusion sequence (3). Other reports showed the role of MPTP in apoptosis induced by myocardial ischemia. It was shown that opening the transition pore of the inner mitochondrial membrane releases proapoptotic proteins such as cytochrome c, apoptosis-inducing factor, and others, that could activate caspases enzymes, which are the executors of the apoptotic process (7). In the present study, we observed that LP reperfusion prevented both MPTP opening, necrosis, and apoptosis processes. Mitochondria isolated from low-pressure
hearts were more resistant to Ca\(^{2+}\) loading (Fig. 4). CK and LDH release as well as TTC staining (Figs. 2 and 3) showed that low pressure was also associated with less necrosis compared with NP hearts. With regard to apoptosis, our results showed an increased leakage of cytochrome c from hearts reperfused at normal pressure (Fig. 6) and also an increased activity of caspase-3 (Fig. 7), suggesting that apoptosis was significantly reduced in hearts reperfused at low pressure after prolonged cold ischemic arrest.

In conclusion, this study demonstrates that low-pressure reperfusion protects the myocardium after a prolonged hypothermic ischemia. Our results suggest that this protection may involve a reduction of oxygen free radicals production and consecutive inhibition of MPT openning. Low-pressure (or low-flow) reperfusion, like postconditioning, proceeds after ischemia at the initiation of reperfusion. This maneuver offers a reliable alternative strategy to protect the ischemic heart, simple to apply in clinical settings.

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REFERENCES


