Warm ischemic preconditioning improves mitochondrial redox balance during and after mild hypothermic ischemia in guinea pig isolated hearts

Jianzhong An, Amadou K. S. Camara, Samhita S. Rhodes, Matthias L. Riess, and David F. Stowe. Warm ischemic preconditioning improves mitochondrial redox balance during and after mild hypothermic ischemia in guinea pig isolated hearts. Am J Physiol Heart Circ Physiol 288: H2620–H2627, 2005. First published January 14, 2005; doi:10.1152/ajpheart.01124.2004.—Ischemic preconditioning (IPC) induces distinctive changes in mitochondrial bioenergetics during warm (37°C) ischemia and improves function and tissue viability on reperfusion. We examined whether IPC before 2 h of hypothermic (27°C) ischemia affords additive cardioprotection and improves mitochondrial redox balance assessed by mitochondrial NADH and flavin adenine dinucleotide (FAD) autofluorescence in intact hearts. A mediating role of ATP-sensitive K⁺ (KATP) channel opening was investigated. NADH and FAD fluorescence were measured in the left ventricular wall of guinea pig isolated hearts assigned to five groups of eight animals each: hypothermia alone, hypothermia with ischemia, IPC with cold ischemia, 5-hydroxydecanoic acid (5-HD) alone, and 5-HD with IPC and cold ischemia. IPC consisted of two 5-min periods of warm global ischemia spaced 5 min apart and 15 min of reperfusion before 2 h of ischemia at 27°C and 2 h of warm reperfusion. The KATP channel inhibitor 5-HD was perfused from 5 min before until 5 min after IPC. IPC before 2 h of ischemia at 27°C led to better recovery of function and less tissue damage on reperfusion than did 27°C ischemia alone. These improvements were preceded by attenuated increases in NADH and decreases in FAD during cold ischemia and the reverse changes during warm reperfusion. 5-HD blocked each of these changes induced by IPC. This study indicates that IPC induces additive cardioprotection with mild hypothermic ischemia by improving mitochondrial bioenergetics during and after ischemia. Because effects of IPC on subsequent changes in NADH and FAD were inhibited by 5-HD, this suggests that mitochondrial KATP channel opening plays a substantial role in improving mitochondrial bioenergetics throughout mild hypothermic ischemia and reperfusion.

nicotinamide adenine dinucleotide; flavin adenine dinucleotide; ATP-sensitive potassium channels; 5-hydroxydecanoic acid

HEARTS SUBJECTED TO BRIEF periods of ischemia can withstand subsequent periods of longer ischemia with better functional recovery. This phenomenon, called ischemic preconditioning (IPC) and first described in 1986 (32), is assessed by reduced infarct size, decreased cytosolic Ca²⁺ loading during ischemia and reperfusion (I/R) (4), attenuated mechanical dysfunction, and limited ultrastructural abnormality on reperfusion after prolonged ischemia. The protection provided by myocardial preconditioning and the mechanisms that mediate its effects have been the focus of intense investigation.

Brief (5-min) cardiac ischemia causes a reversible increase in the reduced form of nicotinamide adenine dinucleotide (NADH), indicating altered mitochondrial energy balance (35). This imbalance likely has an important role in the triggering mechanisms of ischemic and pharmacological preconditioning (35). Many studies suggest that mitochondrial ATP-sensitive K⁺ (KATP) channels open during brief ischemia and also play a key protective role in normothermic I/R injury (5, 13, 17, 24, 30, 41). Opening of KATP channels is believed to be a common mechanism of IPC because KATP channel antagonists attenuate the protection of IPC and KATP agonists mimic the protection of IPC (18, 29). Although KATP channel opening may be an effective way to attenuate mitochondrial damage (18, 24), it remains unclear how this actually initiates or mediates cardioprotection. Mitochondrial membrane depolarization caused by K⁺ influx could lead to partial uncoupling of oxidative phosphorylation, thus improving the efficiency of oxidative phosphorylation and minimizing respiration inefficiency during I/R (1, 31, 39). Alternatively, KATP channel opening could reverse matrix contraction (18, 27) during ischemia and, thus, accelerate respiration on reperfusion.

Hypothermia decreases energy demand and, thus, is useful in protection of hearts metabolically in the course of ischemia during cardiac surgery or after cardiac arrest with cardiac injury. Hypothermia decreases energy utilization during ischemia and preserves essential mechanisms to rapidly regenerate ATP on reperfusion. We reported that IPC before 4 h of ischemia at 17°C added to the cardioprotective effects of hypothermia and that this was associated with decreased cytosolic Ca²⁺ loading and KATP channel opening (13). Recently, we showed that perfusion at 17°C before 30 min of ischemia at 17°C caused moderate and reversible changes in mitochondrial function; in addition, hypothermia preserved the energy balance supplied by NADH and prevented deleterious increases in mitochondrial Ca²⁺ concentration and reactive oxygen species (ROS) formation during cold ischemia (36).

The later study (36) suggested that a change in mitochondrial bioenergetics induced by perfusion at 17°C may underlie improved cardiac function after longer ischemia and warm reperfusion. In addition, prior IPC may further alter the mitochondrial redox state and lead to greater protection via improved redox state during I/R. In the present study, we tested

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the following hypotheses: 1) brief I/R before 2 h of ischemia at 27°C affords additive cardioprotection via a preconditioning mechanism; 2) protection elicited by hypothermia plus IPC is associated with improved mitochondrial bioenergetics, as evidenced by online recordings of reduced NADH and oxidized flavin adenine dinucleotide (FAD) fluorescence; and 3) the added protection by IPC before hypothermic ischemia is due to K\textsubscript{ATP} channel opening.

**METHODS**

**Langendorff heart preparation.** The investigation conformed to the Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996). Our methods have been described in detail previously (4, 13, 35, 36, 38, 43). Ketamine (30 mg) and heparin (1,000 units) were injected intraperitoneally into 40 guinea pigs (250–300 g) 15 min before the animals were decapitated when unresponsive to noxious stimulation. After thoracotomy, the aorta was cannulated (distal to the aortic valve) and the vena cavae were cut from the heart. Each heart was immediately perfused at 55 mmHg via the aortic root with a cold oxygenated modified Krebs-Ringer (KR) solution (equilibrated with 97% O\textsubscript{2}-3% CO\textsubscript{2}) and rapidly excised. The KR perfusate (pH 7.39 ± 0.1, 560 ± 10 mmHg PO\textsubscript{2}) was in-line filtered (5-μm pore size) and had the calculated composition of (nonionized, in mM) 137 Na\textsuperscript{+}, 5 K\textsuperscript{+}, 1.2 Mg\textsuperscript{2+}, 2.5 Ca\textsuperscript{2+}, 134 Cl\textsuperscript{−}, 15.5 HCO\textsubscript{3}−, 1.2 H\textsubscript{2}PO\textsubscript{4}−, 11.5 glucose, 2 pyruvate, 16 mannitol, 0.05 EDTA, and 0.1 probenecid, with 5 U/l insulin. Perfusion and bath temperatures were maintained at 37.2 ± 0.1°C before and after ischemia and at 27.1 ± 0.1°C during ischemia by two preset thermostatically controlled water circulators operated in parallel.

Left ventricular (LV) pressure (LVP) was measured isovolumetrically with a transducer connected to a thin, saline-filled latex balloon inserted into the LV through the mitral valve from a cut in the left atrium. Balloon volume was initially adjusted to a diastolic LVP of 0 mmHg, so that any subsequent increase in diastolic LVP reflected diastolic contracture. Bipolar electrodes were placed in the right atrial appendage and the right ventricular free wall to monitor spontaneous heart rate (HR). Coronary flow (CF; aortic inflow) was measured as systolic LVP × heart rate, and coronary outflow (coronary sinus PO\textsubscript{2}) was also measured continuously online with a Clark-type O\textsubscript{2} electrode. Myocardial O\textsubscript{2} consumption (MVO\textsubscript{2}) was calculated as (coronary flow/g heart weight) × (arterial PO\textsubscript{2} − venous PO\textsubscript{2}) × 24 μl O\textsubscript{2}/ml (37°C) or 27 μl O\textsubscript{2}/ml (27°C) at 760 mmHg, and cardiac work efficiency was calculated as systolic − diastolic LVP × HR/MVO\textsubscript{2}.

If ventricular fibrillation occurred on reperfusion, a 250-μg bolus of lidocaine was given within 20 s. All data were collected from hearts in sinus rhythm. At the end of 120 min of reperfusion, hearts were removed and the ventricles were cut into four horizontal sections. Infarct size was determined by the 2,3,5-triphenyltetrazolium chloride staining method (4, 13, 35, 36). After storage overnight in 10% formaldehyde, infarcted and noninfarcted tissues of whole hearts were carefully separated and weighed. Infarct size was expressed as a percentage of ventricular weight (4, 13, 35, 36).

**Measurement of NADH and FAD in intact hearts.** Tissue autofluorescence was used to measure relative changes in the redox state of flavoproteins contained in FADH\textsubscript{2}/FAD (12) and NADH/NAD\textsuperscript{+} (7, 34–36). To assess changes in NADH (reduced) and FAD (oxidized) fluorescence, each experiment was carried out in a light-blocking Faraday cage. The distal end of a trifurcated fiber-optic cable (6.8 mm\textsuperscript{2} per bundle) was placed against the LV anterior free wall. Netting was applied around the heart to optimize contact with the fiber-optic tip. This maneuver did not affect LVP. The three proximal ends of the fiber-optic cable were connected to a modified spectrophotofluorometer (Photon Technology International, London, Ontario, Canada). Autofluorescence was excited at the LV free wall with monochromatic light from a 75-W xenon arc lamp via one limb of the cable. Incident light was filtered through a 350-nm monochromator (Delta RAM, Photon Technology International) and focused onto the in-going fibers of the optic bundle. The arc lamp shutter was opened only for 2.5-s recording intervals to prevent photobleaching. Fluorescence emissions indicative of NADH were collected by fibers of a second limb of the optical bundle and filtered at 540 ± 15 nm; excitation wavelength was 480 nm. An electronic chopper switched between the excitation wavelengths for NADH and FAD, so that NADH and FAD fluorescence were each measured for 2.5 s at 200 Hz with a 1-s interval between measurements. All analog signals were digitized (PowerLab/16sp, ADInstruments, Castle Hill, Australia) and recorded (Chart & Scope version 3.6.3, ADInstruments) on Power Macintosh G4 computers (Apple, Cupertino, CA) for later analysis using MATLAB (MathWorks, Natick, MA) and Excel (Microsoft, Redmond, WA) software. All variables were averaged over the sampling period of 2.5 s.

**Protocol.** Each experiment lasted 305 min beginning after 30 min of equilibration. Hearts were randomly divided into five groups of eight hearts each: nontreated; hypothermic nonischemia time control (Time-C); hypothermic ischemia control (ISC); preconditioning (IPC), i.e., two 5-min periods of ischemia at 37°C, each 5 min apart; 5-hydroxydecanoic acid (5-HD, 200 μM) alone, i.e., perfusion for 25 min with a 20-min washout before ischemia; and 5-HD perfusion from 5 min before IPC until 20 min before ischemia (IPC + 5-HD). All hearts in the ISC, IPC, 5-HD, and IPC + 5-HD groups were subjected to 2 h of global ischemia at 27°C and 2 h of reperfusion at 37°C. Hearts were cooled from 37°C to 27°C over 15 min before ischemia and rewarmed from 27°C to 37°C over 10 min after ischemia. LVP and its maximal and minimal first derivatives (dLVP/dt\textsubscript{max} and dLVP/dt\textsubscript{min}), CF, and coronary sinus PO\textsubscript{2} as well as NADH and FAD were measured near continuously before and after hypothermia.

**Statistical analysis.** Values are means ± SE. Analysis of variance for repeated measures (Super Anova 1.11 software for Macintosh, Abacus Concepts, Berkeley, CA) was used to assess within-group differences over time. Among-group data were compared at discrete time points before cooling at 37°C (baseline), after 2 h of cold ischemia, and during rewarming and reperfusion. Two-way analysis of variance was used to assess among-group differences at these time points. If F values from the analyses of variance were significant, Student-Newman-Keuls multiple-comparison post hoc tests were used to differentiate within- or among-group differences. Differences among means were considered significant when P < 0.05.

**RESULTS**

Table 1 summarizes changes in indexes of CF, HR, MVO\textsubscript{2}, and cardiac efficiency in the Time-C, ISC, IPC, and IPC + 5-HD groups before cold ischemia and at 2, 10, 30, and 120 min of reperfusion. The 5-HD group did not show any significant differences from the ISC group for any measured variable (data not shown). CF was much lower than baseline throughout reperfusion in each group, but it was higher in the IPC group than in the ISC group throughout reperfusion. HR was higher in the IPC and IPC + 5-HD groups than in the ISC group at 2 and 10 min of reperfusion. MVO\textsubscript{2} was decreased throughout reperfusion in each group but was depressed less in the IPC group than in the ISC group during reperfusion. Cardiac efficiency was decreased in each group but was higher in the
Fig. 1. Time course of systolic − diastolic (s − d) left ventricular pressure (developed LVP) for each group. LVP decreased during transient global ischemia in the ischemia-preconditioned (IPC) group and was zero in each group during hypothermic ischemia. Developed pressure was depressed on rewarming and reperfusion in groups subjected to ischemia but increased more in IPC than in untreated ischemic control (ISC) hearts and IPC hearts treated with 5-hydroxydecanoic acid (IPC + 5-HD). *P < 0.05, time-control (Time-C) vs. other experimental groups; †P < 0.05, IPC vs. ISC or IPC + 5-HD group.

Table 1. Cardiac effects of 2 h of ischemia at 27°C and warm reperfusion in Time-C, ISC, IPC, and IPC+5-HD groups

<table>
<thead>
<tr>
<th>Coronary flow, ml·min⁻¹·g⁻¹</th>
<th>Baseline</th>
<th>2 min</th>
<th>10 min</th>
<th>30 min</th>
<th>2 h</th>
</tr>
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<tbody>
<tr>
<td>Time-C</td>
<td>12.4±0.4</td>
<td>8.2±0.6*</td>
<td>12.9±0.9</td>
<td>12.8±0.7</td>
<td>13.6±0.8</td>
</tr>
<tr>
<td>ISC</td>
<td>12.3±0.9</td>
<td>5.1±0.6*</td>
<td>5.9±0.3*</td>
<td>5.8±0.3*</td>
<td>6.3±0.4*</td>
</tr>
<tr>
<td>IPC</td>
<td>12.4±0.4</td>
<td>6.9±0.8*†</td>
<td>7.9±1.0*†</td>
<td>8.1±0.6*†</td>
<td>8.1±0.7†</td>
</tr>
<tr>
<td>IPC + 5-HD</td>
<td>12.4±0.5</td>
<td>5.9±0.6*</td>
<td>6.8±0.3*</td>
<td>7.7±0.5*</td>
<td>7.9±0.5*</td>
</tr>
</tbody>
</table>

HR, beats/min

| Time-C                     | 293±12 | 182±14*| 277±15 | 298±20 | 301±18 |
| ISC                        | 282±12 | 140±24*| 227±15 | 243±16 | 279±19 |
| IPC                        | 279±15 | 255±23*†| 285±20*| 254±20 | 288±18 |
| IPC + 5-HD                 | 279±16 | 210±13*†| 270±18*| 251±18 | 279±31 |

MVO₂, 0.1 μl·min⁻¹·g⁻¹

| Time-C                     | 123±10 | 72±6*| 115±6 | 120±10 | 122±10 |
| ISC                        | 119±12 | 42±5*| 66±5*| 69±7*| 72±8*|
| IPC                        | 120±10 | 62±9*†| 89±9*†| 88±9*†| 93±9†|
| IPC + 5-HD                 | 121±10 | 52±7*| 77±8*| 73±6*| 82±7*|

Cardiac efficiency, mmHg·beat·0.1 μl O₂·g⁻¹

| Time-C                     | 16.8±1.2| 14.3±1.3| 15.0±1.7| 15.8±1.5| 15.4±1.2|
| ISC                        | 15.9±1.8| 0.7±0.1*| 3.5±0.2*| 6.1±0.7*| 9.4±1.1*|
| IPC                        | 16.3±2.0| 1.0±0.1*†| 5.9±0.6*†| 8.8±0.7*†| 10.4±0.8*†|
| IPC + 5-HD                 | 16.1±1.5| 1.1±0.1*| 4.8±0.5*†| 6.9±0.5*†| 9.7±1.3*†|

Values are means ± SE; n = 8 for each group. Temperature was 23.8 ± 1°C at 2 min of reperfusion and 37°C at all other reperfusion time points. Time-C, time control; ISC, ischemia; IPC, ischemic preconditioning; 5-HD, 5-hydroxydecanoate acid; MVO₂, myocardial O₂ consumption; HR, heart rate. Cardiac efficiency = LVDP·HR/MVO₂, where LVDP is left ventricular developed pressure. *P < 0.05 vs. baseline within each group. †P < 0.05 vs. ISC. ‡P < 0.05 vs. ISC.

IPC group than in the ISC group at 30 min, but not after 2 h of reperfusion, at which time it remained lower than baseline in each group.

Figures 1 and 2 display systolic − diastolic LVP (developed pressure), dLVP/dt_max (contractility), and dLVP/dt_min (relaxation) for each group before, during, and after hypothermic

Fig. 2. Time course of maximal and minimal 1st derivatives of LVP [dLVP/dt_max (contractility) and dLVP/dt_min (relaxation)] for each of the 4 groups. dLVP/dt_max and dLVP/dt_min were reversibly depressed during IPC. During reperfusion, contractility indexes were improved only in IPC hearts compared with untreated ISC control and IPC + 5-HD hearts. *P < 0.05, Time-C vs. other experimental groups; †P < 0.05, IPC vs. ISC or IPC + 5-HD group.
ischemia. Hypothermia to 27°C without ischemia did not alter developed LVP, although HR was slower (240 ± 10 beats/min at 37°C vs. 102 ± 8 beats/min at 27°C). However, diastolic and systolic LVP at 27°C were each increased by ~12 mmHg. Developed LVP was decreased after compared with before ischemia in each group but was higher in the IPC group than in the ISC group on reperfusion; this improvement by IPC was inhibited by 5-HD. dLVP/dt_max and dLVP/dt_min were depressed during hypothermia in the Time-C group and approached zero with ischemia in all other groups. On reperfusion, these values increased but remained lower than before ischemia throughout reperfusion in each group. The improvements in contractility and relaxation were abolished by 5-HD.

Figure 3 shows changes in NADH fluorescence before, during, and after cold ischemia. NADH was not altered significantly by 27°C perfusion in the absence of ischemia (Time-C group). In the ISC group, NADH initially increased and peaked at 5 min of ischemia and gradually declined below baseline levels later during ischemia and during rewarming and reperfusion. IPC caused a completely reversible increase in NADH during the preconditioning stimuli. In IPC hearts, the initial increase in NADH with ischemia was not evident, and the later decline during ischemia was significantly less than that in the ISC group. 5-HD reversed the attenuating effect of IPC on NADH during ischemia and on reperfusion.

Figure 4 shows changes in FAD fluorescence in each study group. The FAD (oxidized) signals were generally the inverse of the NADH (reduced) signals for each group. The two preconditioning stimuli caused a completely reversible decrease in FAD. FAD fluorescence was not significantly altered at 27°C in the nonischemia Time-C group. During cold ischemia, FAD gradually decreased to lower levels in nonprecon-

ditioned hearts than in IPC hearts. During rewarming, FAD returned toward baseline and gradually increased throughout reperfusion to above the baseline preischemia levels. The decrease of FAD during cold ischemia in IPC hearts and the later increase during reperfusion were significantly attenuated compared with the ISC hearts. 5-HD reversed the attenuating effects of IPC on FAD during I/R. In each group, throughout cold ischemia, FAD remained lower than NADH, which increased early during cold ischemia and then declined during the course of ischemia.

Figure 5 shows that ventricular infarct size was significantly reduced in the IPC group compared with the ISC group. Infarct size was not different between the IPC + 5-HD and ISC groups. Wet heart weight was not different among groups.

**DISCUSSION**

We found that two brief periods of global IPC applied before a sustained 2-h period of global ischemia at 27°C improved contractility, CF, and cardiac efficiency and significantly reduced infarct size. Moreover, IPC before cold ischemia led to smaller changes in mitochondrial redox state during cold ischemia, as evidenced by the smaller increase in NADH and the smaller decrease in FAD than during cold ischemia without IPC. During reperfusion, the added improvement in mitochondrial bioenergetics induced by IPC was accompanied by a smaller decrease in NADH and no increase in FAD. Each of these beneficial changes induced by IPC was reversed by 5-HD, a putative mitochondrial K_{ATP} channel blocker.

Our study indicates that 1) IPC adds to the cardioprotection of mild hypothermia during prolonged ischemia, 2) IPC and hypothermia-induced protection resulted from a more normal-
Thus it is important to determine whether IPC protects against ischemia at the intermediate temperature range examined in this study. We selected a 2-h ischemic period at 27°C because functional return in control hearts was <40% of baseline, indicating that cardioprotective effects of hypothermia diminished over time. Because a high-K⁺ solution protects against ischemia and adds to the protection of hypothermia (3, 10), we perfused hearts only with a normal-K⁺ solution to produce a significant degree of injury in control hearts.

Mitochondrial respiration and IPC. Mitochondrial alterations promoted by IPC could be responsible for the protective properties against tissue damage induced by I/R. IPC helps preserve the capacity to undergo state 3 respiration at the end of reperfusion compared with non-IPC controls in dogs (19). In IPC of rat hearts, a normal rate of oxygen consumption (state 3 respiration) and adenosine nucleotide translocator content were maintained during index ischemia and reperfusion (45). Another study showed that IPC preserved mitochondrial respiratory function and conserved the functional coupling between creatine kinase and adenosine nucleotide translocase compared with diminished functioning in nonpreconditioned controls after 15 min of reperfusion (28). Our previous studies also showed that IPC maintains mitochondrial respiration to enhance survival after I/R injury (35, 43).

Our recent study (36) suggested that hypothermia, similar to IPC (35), affords cardiac protection by a mitochondrial redox mechanism. In our previous study (35) of 30 min of ischemia at 37°C, NADH exhibited a similar initial increase but a slower decline in the IPC group than in the ISC group. In the present study, NADH remained lower in the IPC group than in the non-IPC control group during 2 ho f ischemia at 27°C. In another study (36), we found that NADH increased during perfusion at 17°C before ischemia; this rise could result from increased production or, more likely, decreased consumption of NADH; either effect would change ΔHm. In contrast, Zuwier et al. (46) reported that NADH, assessed by videofluorometry, was not significantly higher during index ischemia or reperfusion in preconditioned hearts than in nonpreconditioned hearts. They concluded that IPC attenuates cardiac reperfusion injury without altering mitochondrial redox state. Differences between their study, our previous normothermic (35) and hypothermic ischemia studies (36), and the present study may be due to different methods to measure NADH, rat vs. guinea pig heart models, and different IPC protocols.

Mitochondrial respiration during and after hypothermic ischemia. During normothermic ischemia, as the supply of O₂ to accept electrons from NADH and FADH₂ diminishes, electron flux through the electron transport chain falters, NADH (7, 35, 43) and FADH₂ accumulate, and oxidative phosphorylation is impaired (8, 16, 43). FAD- or NADH-linked autofluorescence can be used to assess mitochondrial redox balance. In our previous study (35) of 30 min of ischemia at 37°C, NADH exhibited a similar initial increase but a slower decline in the IPC group than in the ISC group. In the present study, NADH remained lower in the IPC group than in the non-IPC control group during 2 ho f ischemia at 27°C. In another study (36), we found that NADH increased during perfusion at 17°C before ischemia; this rise could result from increased production or, more likely, decreased consumption of NADH; either effect would change ΔHm. In contrast, Zuwier et al. (46) reported that NADH, assessed by videofluorometry, was not significantly higher during index ischemia or reperfusion in preconditioned hearts than in nonpreconditioned hearts. They concluded that IPC attenuates cardiac reperfusion injury without altering mitochondrial redox state. Differences between their study, our previous normothermic (35) and hypothermic ischemia studies (36), and the present study may be due to different methods to measure NADH, rat vs. guinea pig heart models, and different IPC protocols.
regulates mitochondrial function through Ca\textsuperscript{2+}-sensitive dehydrogenases. Both processes are driven by the IMM potential (\(\Psi_m\)), generated mostly by electron transport. Energy stored by \(\Psi_m\) drives ATP synthesis by the F_0F_1-ATP synthetic complex. Electron transfer from NADH and/or FADH\textsubscript{2} through mitochondrial complexes I–III to O\textsubscript{2} via cytochrome c oxidase (complex IV) generates the proton-motive force to drive H\textsuperscript{+} through complex V to power ATP synthesis (8). Complex I, NADH ubiquinone oxidoreductase, accepts electrons from NADH and transfers them to complex III, ubiquinol cytochrome c oxidoreductase. Complex II, succinate ubiquinone oxidoreductase, oxidizes succinate and, similar to complex I, transfers electrons from FADH\textsubscript{2} to ubiquinone, also known as coenzyme Q, and on to complex IV, where cytochrome c transfers the electrons to O\textsubscript{2}, the terminal electron acceptor. Thus, in the mitochondrial matrix, there is equilibrium between the redox potentials of the NADH-NAD\textsuperscript{+} and FADH\textsubscript{2}-FAD pairs (44).

During 2 h of ischemia at 27°C and 2 h of reperfusion, we found that NADH and FAD remained stable for 5 h in the hypothermic nonischemic control group. Ischemia at 27°C reversibly increased NADH and decreased FAD compared with before cold ischemia. The redox state during I/R was more normalized in the IPC group than in the ISC group. This is consistent with higher mitochondrial ATP activity during reperfusion after IPC (33). NADH and FAD fluorescence signals measured in our study are a product of average cell NADH or FAD and the number of viable cells. The marked and irreversible decline in NADH and increase in FAD fluorescence on reperfusion in preconditioned hearts could represent greater cell death on reperfusion (14) or increased volume of irreversibly oxidized, i.e., energy-depleted, mitochondria. In contrast, the relative normalization of NADH and FAD on reperfusion in IPC hearts may represent less oxidized mitochondria or greater cell salvage. We reported earlier that tissue damage during reperfusion correlates with the rate of NADH decline during ischemia (35).

Reduced ATP synthesis at complex V, due to decreased metabolic demands by hypothermia, would be expected to decrease oxidation of NADH to NAD\textsuperscript{+}. In contrast, FAD fluorescence remained low throughout ischemia, signifying a relative increase in FADH\textsubscript{2} with the assumption of a constant pool of reduced and oxidized forms, whereas NADH declined during the course of ischemia. During later ischemia, these differences could arise from 1) decreased matrix reduction of NAD\textsuperscript{+} to NADH by mitochondrial substrate dehydrogenases, 2) accelerated oxidation of matrix NADH at complexes I and III relative to oxidation of matrix FADH\textsubscript{2} at complex II, or 3) increased oxidation of cytosolic NADH coupled to lactic acid production by lactate dehydrogenase. The return to baseline values of FAD, but not NADH, in the IPC group during reperfusion also suggests a preferential flow of electrons through complex II relative to complexes I and III after ischemia.

A possible mechanism of protection is suggested by our finding that IPC led to a lower initial accumulation of NADH and depletion of FAD during ischemia. We showed that the same IPC protocol led not only to a rapid increase in NADH, but also to a rapid and reversible increase in ROS (26). The more oxidized redox state during IPC pulses causes increased release of ROS and, along with other downstream factors, may mediate K\textsubscript{ATP} channel opening. This, in turn, may induce matrix swelling and/or alter \(\Psi_m\) to modify respiration and redox balance before cold perfusion, as shown by smaller changes in NADH and FAD in the IPC group during ischemia as well as during reperfusion.

Putative role of K\textsubscript{ATP} channels: Evidence for participation of mitochondria in IPC is based on findings that pharmacological inhibition of mitochondrial K\textsubscript{ATP} channels can prevent IPC (5, 13, 17, 18, 24, 33, 41). Also IPC can be mimicked by mitochondrial K\textsubscript{ATP} channel agonists (18). The mechanism by which mitochondrial K\textsubscript{ATP} channels are activated during IPC is unknown. This study demonstrated that 5-HD, presumed to be a selective mitochondrial K\textsubscript{ATP} channel blocker, not only prevented functional metabolic and tissue cardioprotection by IPC after ischemia for 2 h at 27°C followed by reperfusion, but also reversed IPC-induced changes in NADH, mostly mitochondrial, and changes in mitochondrial FAD. Because 5-HD abrogated changes in NADH and FAD induced by IPC, our results suggest that mitochondrial K\textsubscript{ATP} channel opening is, at least in part, responsible for providing the added improvement in mitochondrial bioenergetics.

Although opening mitochondrial K\textsubscript{ATP} channels may be an effective way to attenuate mitochondria damage, it remains unclear how mitochondrial K\textsubscript{ATP} channel opening might protect mitochondrial function (18, 20). Opening of mitochondrial K\textsubscript{ATP} channels and subsequent mitochondrial K\textsuperscript{+} uptake have been reported to cause a significant matrix swelling and a small decrease in \(\Psi_m\) (18, 27). Other data suggest that K\textsuperscript{+} influx only partially dissipates the \(\Psi_m\) and, if compensated by accelerated electron transfer, leads to a net oxidation of mitochondrial substrates (1). This was demonstrated by an increase in flavoprotein fluorescence by diazoxide in rabbit ventricular myocytes having an oxidized energy state (29).

Comparison with other hypothermic ischemia studies: In one isolated rat heart study, IPC added to the protection of ischemia at 20°C (11); in another study, IPC did not improve postischemic function after 3.5 h of ischemia at 6°C (42). In other studies using isolated rat hearts, IPC improved cardiac function after 75 min of ischemia at 23°C, but not after 5 h of ischemia at 6°C (40), and improved function after 1 h of ischemia at 34°C, but not after 1 h of ischemia at 10°C (25). Our studies offer a partial answer for these different results. Without IPC, recovery of developed LVP was 57% after 4 h of ischemia at 17°C (13) and, in the present study, 34% after 2 h of ischemia at 27°C. However, contractility on reperfusion induced by IPC before 2 h of ischemia at 27°C was greater (35%) than that observed after 4 h of ischemia at 17°C (16%) (13). Although hypothermia itself is cardioprotective, it appears that IPC adds to the protection after 2 h of ischemia, when protective effects at 27°C alone wane, compared with after 4 h of ischemia, when protective effects at 17°C alone are greater (13). Moreover, our studies provide a possible mitochondrial mechanism for the added protective effects of hypothermia and IPC.

In summary, IPC before 2 h of ischemia at 27°C not only resulted in better recovery of cardiac function, CF, MV\textsubscript{O2}, and reduced infarct size but also in improved mitochondrial function. This was evidenced by the markedly improved mitochondrial redox state induced by IPC, which was found not only during reperfusion, but also very early during the 2-h hypothermic ischemia period. Because effects of IPC on changes in
NADH and FAD were inhibited by bracketing with 5-HD, this suggests that mitochondrial KATP channel opening exerts a substantial role in improving mitochondrial bioenergetics during hypothermia protection. A limitation of this and other studies is that putative mitochondrial KATP channel openers and blockers may have other unrelated effects (21, 22).

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Portions of this work have appeared in abstract form (3a).

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