Ischemic preconditioning does not protect via blockade of electron transport

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Tanaka-Esposito C, Chen Q, Moghaddas S, Lesnefsky EJ. Ischemic preconditioning does not protect via blockade of electron transport. J Appl Physiol 103: 623–628, 2007. First published April 26, 2007; doi:10.1152/japplphysiol.00943.2006.—Ischemic preconditioning (IPC) before sustained ischemia decreases myocardial infarct size mediated in part via protection of cardiac mitochondria. Reversible blockade of electron transport at complex I immediately before sustained ischemia also preserves mitochondrial respiration and decreases infarct size. We proposed that IPC would attenuate electron transport from complex I as a potential effector mechanism of cardioprotection. Isolated, Langendorff-perfused rat hearts underwent IPC (3 cycles of 5-min 37°C global ischemia and 5-min reperfusion) or were perfused for 40 min without ischemia as controls. Subsarcolemmal (SSM) and interfibrillar (IFM) populations of mitochondria were isolated. IPC did not decrease ADP-stimulated respiration measured in intact mitochondria using substrates that donate reducing equivalents to complex I. Maximally expressed complex I activity measured as rotenone-sensitive NADH:ubiquinone oxidoreductase in detergent-solubilized mitochondria was also unaffected by IPC. Thus the protection of IPC does not occur as a consequence of a partial decrease in complex I activity leading to a decrease in integrated respiration through complex I. IPC and blockade of electron transport both converge on mitochondria as effectors of cardioprotection; however, each modulates mitochondrial metabolism during ischemia by different mechanisms to achieve cardioprotection.

NADH:ubiquinone oxidoreductase; oxidative phosphorylation; mitochondria

ISCHEMIC PRECONDITIONING (IPC) is an innate cytoprotective phenomenon evoked by transient episodes of brief, nonlethal ischemia interspersed with reperfusion (36). Although the mechanism of IPC has not been fully elucidated, IPC leads to the release of endogenous agonists that bind to cell surface receptors, in turn activating intracellular kinase cascades. These signal transduction networks converge on several end effectors, one of which is mitochondria (12, 35). Mitochondria isolated from hearts subjected to an IPC stimulus before sustained ischemia-reperfusion exhibit preserved respiratory function, compared with those from time-matched ischemia-reperfusion controls. The mechanism by which IPC impacts mitochondrial function that in turn leads to myocardial protection is unresolved.

IPC attenuates the release of cytotoxic reactive oxygen species (ROS) during the sustained index ischemia and subsequent reperfusion (30, 44). Mitochondria are major intracellular sources of cytotoxic ROS (2, 4, 24, 45, 46). Modulation of mitochondrial derived ROS production can be achieved either by uncoupling of respiration (18, 32, 38, 43) or blockade of electron transport (9, 10). In turn, pharmacological manipulation of mitochondrial metabolism via either mechanism leads to cardioprotection following ischemia and reperfusion (1, 6, 9, 18, 32, 38, 44).

Uncoupling of mitochondrial respiration during sustained ischemia, mediated via activation of either mitochondria ATP-sensitive potassium (KATP) channel or of uncoupling proteins located in the mitochondrial inner membrane, decreases ROS production and myocardial injury evident after the sustained ischemia and reperfusion. Mitochondria from ischemic-preconditioned hearts exhibit an augmented inducible proton leak that is sensitive to GDP, an inhibitor of uncoupling proteins (32, 37). Opening of the mitochondrial KATP channel, which has been implicated in IPC, also results in uncoupling of mitochondrial oxidation and phosphorylation (13, 31). Alternatively, blockade of electron transport into complex III of the electron transport chain (ETC) attenuates the net release of ROS (10) and immediately before ischemia attenuates ischemic mitochondrial damage (7, 28) and subsequent myocardial injury assessed after reperfusion (1, 9). In line with this observation, treatment of the isolated heart with a complex I inhibitor immediately before sustained ischemia preserves mitochondrial function and attenuates cardiomyocyte injury (9).

We propose that one of the effector mechanisms of IPC involves blockade of electron flux into complex III via a decrease in complex I activity. Complex III of the ETC is a major source of ROS release from intact mitochondria. Blockade of electron transport into complex III attenuates net ROS production during the oxidation of complex I substrates (10). A decrease in complex I activity at the onset of the index ischemia can result in a functional blockade of electron flux into complex III and thus attenuate cytotoxic ROS production. Periods of ischemia as brief as 10 min duration are sufficient to cause a 40% reversible decrease in complex I activity and integrated respiration through complex I (17, 40). Alternatively, posttranslational modification of complex I can also modulate its activity (5, 41). We studied whether the brief episodes of ischemia and reperfusion, which comprise the stimulus of IPC, lead to partial blockade of the proximal ETC via a decrease in complex I activity at the onset of the sustained index ischemia.

EXPERIMENTAL PROCEDURES

Preparation of rat hearts for perfusion. The Animal Care and Use Committees of the Louis Stokes Cleveland Veterans Affairs Medical Center and Case Western Reserve University approved the protocol. Male Fischer 344 rats [6–8 mo of age, 350–420 g] were anesthetized with pentobarbital sodium (100 mg/kg ip) and anticoagulated with heparin (1,000 IU/kg ip). Hearts were excised and retrograde perfused...
via the aorta at a constant pressure of 65 mmHg, with modified Krebs-Henseleit buffer (pH 7.35–7.45 at 37°C) containing 115 mM NaCl, 4 mM KCl, 1.2 mM MgSO₄, 0.9 mM KH₂PO₄, 22.5 mM NaHCO₃, 2.5 mM CaCl₂, and 5.5 mM glucose, and oxygenated with 95% O₂–5% CO₂. Left ventricular developed pressure and heart rate were measured with a balloon inserted into the left ventricle. Coronary flow rate was also recorded. To ascertain that IPC was effective under our conditions, we submitted one group of hearts to 10 min of equilibration followed by three successive cycles of 5-min duration of ischemia and then 5 min of reperfusion, in turn followed by 25-min stop-flow ischemia and 120 min of reperfusion. The second group was subjected to a time-matched 40-min perfusion, followed by 25-min ischemia and 120-min reperfusion. Subsequently, infarct size was measured using the triphenyltetrazolium chloride staining method (47). For the remaining experiments, hearts were subjected to the IPC stimulus or a time-matched continuous perfusion period of 40 min.

Isolation of subsarcolemmal and interfibrillar mitochondria. At the end of the perfusion period, hearts were decanulated and placed into Chappel-Perry buffer (pH 7.4) containing 100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA. In a separate set of experiments, SSM and IFM were isolated, from control and preconditioned hearts, in the presence of protein phosphatase inhibitors (100 mM KCl, 50 mM MOPS, and 0.5 mM EGTA). Mitochondria were incubated in buffer (pH 7.4) containing 80 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM MgSO₄, and 0.5 mM glucose, and oxygenated with 95% O₂–5% CO₂. Left ventricular developed pressure and heart rate were measured with a balloon inserted into the left ventricle. Coronary flow rate was also recorded. To ascertain that IPC was effective under our conditions, we submitted one group of hearts to 10 min of equilibration followed by three successive cycles of 5-min duration of ischemia and then 5 min of reperfusion, in turn followed by 25-min stop-flow ischemia and 120 min of reperfusion. The second group was subjected to a time-matched 40-min perfusion, followed by 25-min ischemia and 120-min reperfusion. Subsequently, infarct size was measured using the triphenyltetrazolium chloride staining method (47). For the remaining experiments, hearts were subjected to the IPC stimulus or a time-matched continuous perfusion period of 40 min.

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Mitochondrial oxidative phosphorylation. Oxygen consumption in mitochondria was measured using a Clark-type oxygen electrode at 30°C. Mitochondria were incubated in buffer (pH 7.4) containing 80 mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM KH₂PO₄, and 1 mg/ml defatted bovine serum albumin. Glutamate (complex I substrate), NADH, NAD⁺/NADH, NADPH, NAD₂/H₂NAD₂, and N,N,N,N′-tetramethyl-p-phenylenediamine-ascorbate (complex IV substrate) were used as electron donors to specific sites in the electron transport chain. State 3 (ADP stimulated), state 4 (ADP limited), maximally stimulated respiration, respiratory control ratios, and the ADP/O ratio were determined (29). Mitochondria were used within 2 h after isolation from tissue. Endogenous substrates were depleted by the addition of 0.1 mM ADP when complex I substrates were used.

ETC and citrate synthase enzyme activities. The following enzyme activities were measured in detergent-solubilized freshly isolated SSM and IFM at 37°C: rotenone-sensitive NADH:decyldubiquinone oxidoreductase (complex I), rotenone-sensitive NADH-cytochrome c oxidoreductase (NCR), and citrate synthase (CS) (20, 26, 27). Outer mitochondrial membrane integrity was assessed by measuring the rate of oxidation of exogenous reduced cytochrome c in the presence and absence of a detergent (9). The intact outer mitochondrial membrane is impermeable to exogenous cytochrome c. However, if the outer membrane is breached then added reduced cytochrome c can be oxidized by cytochrome oxidase. Detergent solubilization with dodecyl maltoside was used to measure the maximal rate of cytochrome oxidase activity. The percentage of cytochrome oxidase activity in the absence vs. presence of detergent reflects the permeability of the outer membrane.

Statistical analysis. Data are expressed as means ± SE. ETC data for the experiments where mitochondria were isolated in the presence of protein phosphatase inhibitors are reported as means ± SD. Differences among groups were compared by one-way analysis of variance. A difference of P < 0.05 was considered significant.

RESULTS

Cardiac function and infarct size study. Rate pressure product (RPP = heart rate × left ventricular developed pressure) was used as an index of functional capacity, because the hearts were not paced. Initial RPP was similar between the groups [IPC: 36,000 ± 3,400 mmHg × min, n = 5 vs. time control: 36,200 ± 2,100, n = 6; P = not significant (NS)]. At the end of the protocol period, there was a trend toward a decrease in the recovery of RPP as a percentage of that measured at 10 min of equilibration perfusion in the group subjected to the IPC stimulus (IPC: 92 ± 6%, n = 5 vs. time control: 97 ± 3%, n = 6; P = NS). There was no difference in the coronary flow rate at the end of the protocol period (IPC: 10.0 ± 0.4 ml/min, n = 5 vs. time control: 10.0 ± 1.5 ml/min, n = 6; P = NS).

To demonstrate that IPC was effective as previously described in the adult Fischer 344 rat (42) under our conditions, 6-mo-old Fischer 344 rat hearts were submitted to 25 min of stop-flow ischemia followed by 120 min of reperfusion with or without antecedent ischemic preconditioning as described. Infarct size was measured at the end of reperfusion. RPP was again similar in the two groups following 10 min of equilibration perfusion (IPC: 32,000 ± 1,400 mmHg × min, n = 4 vs. control: 37,500 ± 3,900, n = 3; P = NS). Immediately before the index ischemia, there again was a trend toward a decrease in RPP recovery in the group subjected to ischemic preconditioning stimulus (IPC: 81 ± 10%, n = 4 vs. control: 99 ± 6%, n = 3; P = NS). As expected, infarct size was significantly decreased in preconditioned hearts compared with untreated controls. Infarct size as a percentage of the left ventricle was reduced from 32 ± 4% (n = 3) to 16 ± 6% (n = 4; P < 0.05).

The recovery of RPP measured at 120 min of reperfusion was also improved by ischemic preconditioning (IPC: 60 ± 3%, n = 4 vs. control: 40 ± 8%, n = 3; P < 0.05).

Mitochondrial protein yield in preconditioned and control hearts. The protein yield of SSM and IFM (mg/g wet weight of heart) was similar in the preconditioned and control groups (Table 1). The specific activity of CS, a mitochondrial matrix enzyme, was measured and used as a marker of the relative purity of the mitochondrial fraction. There was no difference between the IPC and control groups (Table 1). The integrity of SSM and IFM, assessed by a measure of mitochondrial outer membrane intactness and reported as a percentage change of cytochrome oxidase activity without and with the added detergent dodecyl maltoside, was comparable between the two groups (Table 1).

Mitochondrial respiration. To address the question whether the stimulus of IPC attenuates complex I-dependent respiration in either population of cardiac mitochondria, the rate of oxidative phosphorylation was studied in SSM and IFM isolated from hearts subjected to the ischemic preconditioning stimulus and the time-matched control group. NADH-supported respi-
Table 1. Characterization of subsarcolemmal mitochondria and interfibrillar mitochondria isolated from hearts subjected to the ischemic preconditioning stimulus vs. time-matched controls

<table>
<thead>
<tr>
<th>Protein yield, mg/g tissue</th>
<th>Citrate synthase, mU/mg protein</th>
<th>Outer membrane permeability, %</th>
<th>Substate 4 respiration rate</th>
<th>Respiratory control ratio</th>
<th>ADP/O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TC</td>
<td>IPC</td>
<td>TC</td>
<td>IPC</td>
<td></td>
</tr>
<tr>
<td>SSM</td>
<td>9.3 ± 0.6</td>
<td>8.3 ± 0.7</td>
<td>10.2 ± 1.1</td>
<td>9.3 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>IFM</td>
<td>2.260 ± 160</td>
<td>2.160 ± 90</td>
<td>3.040 ± 200</td>
<td>2.960 ± 40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 ± 0.7</td>
<td>6 ± 1.4</td>
<td>2 ± 0.2</td>
<td>3 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. SSM, subsarcolemmal mitochondria; IFM, interfibrillar mitochondria; IPC, ischemic preconditioning; TC, time control. *P = not significant TC vs. IPC.

ration through complex I was measured with glutamate or pyruvate plus malate as substrates. The rate of oxidative phosphorylation was lower in SSM than IFM (39). SSM and IFM from both groups of hearts showed robust state 3 respiration (Table 2). Oxidative phosphorylation was tightly coupled in mitochondria from control hearts, reflected by normal state 4 respiration rates, respiratory control ratios, and ADP/O values (Table 2). Oxidative phosphorylation was mildly uncoupled in IFM from preconditioned hearts. SSM from preconditioned hearts also showed a trend toward decreased coupling of respiration. There was no difference between the groups in maximally stimulated complex I supported respiration in either population of mitochondria (Fig. 1A). Respiration with substrates that donate reducing equivalents distal to complex I was also unaltered by IPC (data not shown). These findings argue against a defect in complex I induced by the stimulus of IPC.

ETC activities. To address the potential effect of partial inhibition in complex I activity of a magnitude that did not alter the control of respiration by complex I, enzyme activity was measured. Rotenone-sensitive specific activity (mU/mg protein) of NADH:decyblubiquinone oxidoreductase (complex I) was measured in detergent solubilized mitochondria. Complex I activity was similar in IPC and control groups (Fig. 1B). Complex I is the rate-limiting enzyme in the NCR assay; thus a decrease in complex I activity would manifest as a decrease in activity. Again, there was no difference [IPC (SSM):

Table 2. Glutamate-stimulated respiration in SSM and IFM isolated from ischemic preconditioned hearts and time-matched control hearts

<table>
<thead>
<tr>
<th>State 3</th>
<th>State 4</th>
<th>RCR</th>
<th>ADP/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (n = 5)</td>
<td>296±24</td>
<td>38±6</td>
<td>9.0±1</td>
</tr>
<tr>
<td>IPC (n = 6)</td>
<td>298±20</td>
<td>46±7</td>
<td>7.5±1</td>
</tr>
<tr>
<td>IFM (n = 5)</td>
<td>405±20</td>
<td>37±4</td>
<td>11.2±1.2</td>
</tr>
<tr>
<td>IPC (n = 6)</td>
<td>432±24</td>
<td>58±8*</td>
<td>7.8±0.8</td>
</tr>
</tbody>
</table>

Values means ± SE; n, no. of animals. State 3 and state 4 mitochondrial respiration values are in nanoatoms O₂·min⁻¹·mg mitochondrial protein⁻¹, RCR, respiratory control ratio. *P < 0.05 compared with time-matched control.

Fig. 1. A: maximal ADP-stimulated respiration with complex I substrates is similar in subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria isolated from time control (TC) and ischemic preconditioned (IPC) hearts. Concentrations: glutamate, 20 mM; pyruvate, 10 mM + malate, 5 mM; ADP, 2 mM. P = not significant. B: maximal rotenone sensitive complex I activity (NADH:ubiquinone oxidoreductase) measured in SSM and IFM from TC and IPC hearts. nAO, nanoatoms of O. Activity is similar in both groups (P = not significant).

5,450 ± 620 mU/mg protein, n = 5 vs. control (SSM): 5,600 ± 860 mU/mg protein, n = 6; P = NS; IPC (IFM): 6,890 ± 890 mU/mg protein, n = 5 vs. control (IFM): 5,100 ± 750 mU/mg protein, n = 6; P = NS).

To address the question whether IPC led to a phosphorylation-mediated decrease of complex I activity that was negated by endogenous phosphatases during the process of mitochondrial isolation, additional hearts were subjected to either the same preconditioning stimulus or time-matched control protocol. Subsequently, the two populations of mitochondria were isolated using buffer containing protein phosphatase inhibitors as described in EXPERIMENTAL PROCEDURES. Mitochondrial isolation in the presence of phosphatase inhibitors did not alter complex I activity [IPC (SSM): 510 ± 30 mU/mg protein, n = 3 vs. control (SSM): 480 ± 60 mU/mg protein, n = 2; P = NS; IPC (IFM): 520 ± 20 mU/mg protein, n = 3 vs. control (IFM): 430 ± 80 mU/mg protein, n = 2; P = NS] or NCR activity [IPC (SSM): 6,520 ± 990 mU/mg protein, n = 3 vs. control (SSM): 5,610 ± 600 mU/mg protein, n = 2; P = NS; IPC
(IFM): 7,420 ± 590 mU/mg protein, n = 3 vs. control (IFM): 5,140 ± 780 mU/mg protein, n = 2; P = NS).

DISCUSSION

IPC protects in part by attenuating oxidant stress during the sustained ischemia and subsequent reperfusion (24, 45). Modulation of mitochondrial respiration, mediated either by kinase-mediated pathways or the opening of mitochondrial K\textsubscript{ATP} channel, appears to contribute to this protection (35). Ischemic preconditioning, adenosine, a known early mediator of IPC, and diazoxide, which opens the mitochondria K\textsubscript{ATP} channel, all decrease oxidant stress during the sustained ischemia and subsequent reperfusion. On the contrary, 5-hydroxydecanoate, which blocks the mitochondria K\textsubscript{ATP} channel and Go-6978, an inhibitor of protein kinase C, reversed this attenuation of oxidant stress (45). Stowe and colleagues (24) showed reduced ROS release from preconditioned intact hearts during the index ischemia and during reperfusion. Mitochondrial-derived cytotoxic ROS, produced during the sustained ischemia and reperfusion, are implicated in the mechanism of injury to both mitochondria and cardiomyocytes. In isolated mitochondria, net ROS production can be effectively reduced by inhibiting electron flux into complex III via pharmacologic blockade of the proximal ETC using an inhibitor of complex I (10). Mitochondrial respiratory function is protected following sustained ischemia by treatment of the isolated heart before ischemia with rotenone blockade of complex I (28). Moreover, treatment of the heart with amobarbital, which reversibly blocks complex I at the same site as rotenone, preserves mitochondrial function at the end of ischemia (7) that is carried forth into reperfusion with decreased infarct size and improved contractile recovery (1, 9).

We proposed that the stimulus of IPC induces an inhibition of complex I that results in partial blockade of electron transport as a potential mitochondria-dependent effector mechanism of IPC. Because complex I is upstream within the ETC, inhibition would result in blockade of electron flow into complex III, and in turn decrease the release of cytotoxic ROS from mitochondria. Regulation of complex I activity can occur via posttranslational modification. Despite the intramitochondrial location of the complexes of the ETC, Kadenbach and colleagues (23) demonstrated cAMP-dependent phosphorylation of cytochrome oxidase, which reversibly modulates its activity. IPC activates cytosolic protein kinases, in particular protein kinase A (21). Protein kinase A, a key mediator of the signaling cascade of IPC, is required for the phosphorylation of a regulatory subunit of complex I in vitro (41). S-nitrosation of cysteine residues in complex I resulting from S-nitrosglutathione treatment of mitochondria resulted in a 25% inhibition of complex I activity (5). However, despite an increase in total S-nitrosothiol content in mitochondria isolated from preconditioned hearts, neither nitrosation of complex I per se nor an effect on activity was demonstrated (5). S-nitrosation is one of the cellular reactions of nitric oxide, which has been proven to be a trigger of IPC (14, 16). Complex I activity is also inhibited by brief periods of ischemia. Ten minutes of ischemia resulted in 40% reversible inhibition of complex I, likely via loss of the flavin mononucleotide portion of NADH dehydrogenase (40). Thus it seemed plausible that the stimulus of IPC, consisting of brief episodes of ischemia and mediated by intracellular kinase signaling, could lead to partial inhibition of complex I.

We found that the stimulus of IPC does not induce a defect in complex I, manifested by a decrease in either integrated respiration or maximally expressed enzyme activity. The protein yields and CS activity of both populations of mitochondria were similar between IPC and control groups, indicating a similar recovery and purity of each population of mitochondria from time control and IPC hearts. Mitochondria were also isolated in the presence of the phosphatase inhibitors NaF and NaN\textsubscript{3}VO\textsubscript{4}; both have been independently shown to effectively prevent dephosphorylation of mitochondrial proteins, including proteins located in the mitochondrial matrix (11, 19). Complex I activity remained unaltered in these mitochondria, excluding the possibility that a phenotypic defect in complex I was reversed by endogenous phosphatases. Two previous investigations of mitochondrial respiratory function following an IPC stimulus showed conflicting results, with a modest decrease observed using a mitochondrial population likely consisting of a mixture of SSM and IFM (15) and no difference observed in a population likely consisting only of SSM (25). This present study, which systematically studied each population of cardiac mitochondria with clearly defined measures of mitochondrial yield, purity, and integrity found no effect of the IPC trigger on either integrated mitochondrial respiration or isolated complex I activity. Although the triggers of ischemic preconditioning increase the production of signaling ROS from complex I (3), the generation of these reactive oxygen species does not lead to a decrease in complex I activity as a subsequent effector of preconditioning-mediated cardioprotection.

In the present study, mitochondria isolated from preconditioned hearts did exhibit lower respiratory control ratios and higher state 4 rates, indicative of modest uncoupling of respiration (Table 2). This finding is in line with work by Sack and colleagues (33), who reported a “mitochondrial uncoupling” phenotype in a cellular model of IPC (33). Uncoupling of oxidative phosphorylation describes a process that decreases the stoichiometric efficiency of ATP production vs. oxygen consumption, based on dissipation of the proton motive force across the inner mitochondrial membrane (22). Uncoupling of respiration can also result from a decrease in the efficiency (“slip”) of the H\textsuperscript{+} pumping complexes of the ETC (22). The mechanism underlying the increased H\textsuperscript{+} flux induced by IPC was studied by Brookes and colleagues (37). They isolated mitochondria from Langendorff-perfused hearts subjected to an IPC stimulus and quantified H\textsuperscript{+} leak by simultaneously measuring membrane potential and state 4 respiration. IPC-treated mitochondria demonstrated a small but significantly increased proton leak compared with controls (37). This increased permeability across the inner mitochondrial membrane was completely attenuated by inhibitors of either uncoupling proteins or the adenine nucleotide translocase (37). This finding is in line with work by Sack and colleagues (32). Thus there appears to be a role for both uncoupling proteins and the translocase in the mechanism of extrinsic uncoupling induced by IPC (37). To date there is no data to support a decrease in efficiency of the H\textsuperscript{+} pumping within the ETC complexes as a mechanism of uncoupling in IPC. In conclusion, blockade of electron transport at complex I is not the mitochondria-dependent effector mechanism of IPC. Instead, pharmacological
blockade of electron transport remains an alternative mitochondrial-mediated mechanism of cardioprotection to that.

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