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Ischemic Preconditioning Preserves Mitochondrial Membrane Potential and Limits Reactive Oxygen Species Production

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Abstract

Background—Mitochondrial superoxide radical ($O_2^{\bullet-}$) production increases after cardiac ischemia-reperfusion (IR). Ischemic preconditioning (IPC) preserves mitochondrial function and attenuates $O_2^{\bullet-}$ production, but the mechanism is unknown. Mitochondrial membrane potential ($m\Delta\Psi$) is known to affect $O_2^{\bullet-}$ production; mitochondrial depolarization decreases $O_2^{\bullet-}$ formation. We examined the relationship between $O_2^{\bullet-}$ production and $m\Delta\Psi$ during IR and IPC.

Materials/Methods—Rat hearts were subjected to Control or IPC. Mitochondria were isolated at end-equilibration (End EQ), end-ischemia (End I) and end-reperfusion (End RP). $m\Delta\Psi$ was measured using a tetraphenylphosphonium electrode. Mitochondrial $O_2^{\bullet-}$ production was measured by electron paramagnetic resonance (EPR) using DMPO spin trap. Cytochrome *c* levels were measured using high pressure liquid chromatography.

Results—IPC preserved $m\Delta\Psi$ at End I (-156 ± 5 vs. -131 ± 6 mV, $p<0.001$) and End RP (-168 ± 2 vs. -155 ± 2 mV, $p<0.05$). At End RP, IPC attenuated $O_2^{\bullet-}$ production (2527 ± 221 vs. 3523 ± 250 AU/mg protein, $p<0.05$). IPC preserved cytochrome *c* levels (351 ± 14 vs. 269 ± 16 picomoles/mg protein, $p<0.05$) at End RP, and decreased mitochondrial cristae disruption (10 ± 4 vs. $33\pm 7\%$, $p<0.05$) and amorphous density formation (18 ± 4 vs. $28\pm 1\%$, $p<0.05$).

Conclusion—We conclude that IPC preserves $m\Delta\Psi$, possibly by limiting disruption of mitochondrial inner membrane. IPC also decreases mitochondrial $O_2^{\bullet-}$ production and preserves mitochondrial ultrastructure after IR. While it was previously held that slight decreases in $m\Delta\Psi$ decrease $O_2^{\bullet-}$ production, our results indicate that preservation of $m\Delta\Psi$ is associated with decreased $O_2^{\bullet-}$ and preservation of cardiac function in IPC. These findings indicate that the mechanism of IPC may not involve $m\Delta\Psi$ depolarization, but rather preservation of mitochondrial electrochemical potential.

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Keywords

Ischemia-reperfusion; cardiac; mitochondria; reactive oxygen species; membrane potential; ischemic preconditioning; heart

Introduction

Understanding of the function of mitochondria has evolved from that of the cellular powerhouse to one where mitochondria are involved in the regulation of multiple cellular processes.¹ Reactive oxygen species (ROS) such as superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are known to play an important role in cellular signaling including the induction of apoptosis.^{1, 2, 3} Heart mitochondria produce large amounts of ROS during ischemia-reperfusion (IR).^{4, 5} Increased ROS production during IR is responsible for cell death and cardiac dysfunction.⁴ Mitochondrial membrane potential ($m\Delta\Psi$) directly affects the production of ROS through its effects on the mitochondrial electron transport chain (ETC). At high $m\Delta\Psi$ the ETC is extremely reduced leading to increased ROS production.^{6, 7} In isolated mitochondria, decreasing $m\Delta\Psi$ decreases ROS production through its effects on the redox status of the ETC. ROS production is also increased when the mitochondria is in a highly oxidized state, therefore extreme reduction of $m\Delta\Psi$ would also increase ROS formation.^{6, 7, 8} Increased ROS production is a necessary part of the signal pathway that leads to cell death after IR.^{1, 5} Therefore, changes in $m\Delta\Psi$ may be important in the regulation of cell death due to its effects on ROS production in the setting of IR. Complex I and III of the ETC are the main sources of ROS production after IR, with complex III being the major producer.^{9, 10} During IR, damage to mitochondrial respiratory complexes leads to inefficient transfer of electrons; these electrons then leak mainly from complex I and III and bind to oxygen to produce $O_2^{\bullet-}$, the main free radical ROS produced by the mitochondria.^{7, 11, 12}

Ischemic preconditioning (IPC) is the phenomenon where brief episodes of ischemia followed by reperfusion protect the heart from the damaging effects of subsequent episodes of IR.^{11, 12} It is well established that IPC preserves mechanical function, decreases myocyte death, preserves mitochondrial respiratory function, and decreases ROS formation after IR.^{11–14} Though many signaling pathways are activated during IPC, the exact mechanism of cardioprotection remains unknown.¹²

It is unclear how IPC affects $m\Delta\Psi$ and how this correlates with changes in $O_2^{\bullet-}$ production from complex I and III of the ETC. Studies have shown that mild mitochondrial uncoupling through increased proton (H^+) leak, the activation of uncoupling proteins or other mechanisms that decrease $m\Delta\Psi$, lead to decreased production of ROS (for review see 15). It has been proposed that mild uncoupling may be part of the protective mechanism of IPC. Mitochondrial depolarization may limit cell damage by decreasing mitochondrial ROS formation leading to improved cell function and survival.^{15, 16} The purpose of these experiments was to examine the changes in $m\Delta\Psi$ and ROS production during ischemia-reperfusion in IPC and control mitochondria. We demonstrated that IPC preserves $m\Delta\Psi$ as well as cytochrome *c* levels, decreases $O_2^{\bullet-}$ production and preserves mitochondrial structural integrity at end reperfusion.

Materials/Methods

Isolated heart preparation

Male Sprague–Dawley rats (275–300 g) were anesthetized with sodium pentobarbital (60 mg/kg intraperitoneally, ip) and heparinized (heparin sodium, 500 U ip). Hearts were

excised quickly and arrested in cold Krebs–Henseleit solution. Hearts were then perfused in a non-recirculating Langendorff apparatus at 37°C with Krebs–Henseleit buffer consisting of [in mM] NaCl [118]; KCl [4.6]; KH₂PO₄ [1.17]; MgSO₄ [1.17]; CaCl₂ [1.16]; NaHCO₃ [23]; and glucose [5.3]; pH: 7.4 and equilibrated with 95% O₂ and 5% CO₂ gas. Left ventricular rate pressure product (RPP, peak systolic pressure minus end diastolic pressure multiplied by heart rate) was recorded using an intraventricular latex balloon connected to a pressure transducer.¹⁴ Data were continuously recorded using a PowerLab Chart v4.2 (AD Instruments Inc., Milford, MA).

Prior to the experiments, rats were acclimated in a quiet environment and fed a standard diet. They were treated in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources of the National Research Council.¹⁷

Ischemic preconditioning protocol

Hearts were assigned to Control and Ischemic preconditioning (IPC) groups, figure 1. The Control group (n=8) was subjected to 30 minutes of equilibration (EQ), 30 minutes of global normothermic ischemia, and 30 minutes of reperfusion (RP). The IPC group (n=8) was subjected to 10 minutes of EQ, then ischemic preconditioning was induced by two 5-minute episodes of ischemia (I5) each followed by 5 minutes of reperfusion (R5), then 30 minutes of global normothermic ischemia, and 30 minutes of reperfusion. Ischemia was achieved by using a stopcock located directly above the aorta. Hearts were immersed at all times in a water-jacketed, non-gassed perfusate bath to maintain normothermia (37°C).

Isolation of subsarcolemmal (SS) mitochondria

SS mitochondria were isolated at end equilibration (end EQ), end Ischemia (End I), and end reperfusion (End RP) by differential centrifugation according to Palmer et al.¹⁸ Briefly, hearts were minced in the isolation buffer (MSH): [in mmol/L] mannitol [230]; sucrose [70]; HEPES [3]; pH: 7.4 at 4°C, with the addition of 0.2% bovine serum albumin (BSA) and 1 mM EGTA, and homogenized with a polytron tissue processor (Brinkman Instruments, Westbury, NY) for 3 seconds at a rheostat setting of 6.5. Further homogenization was performed with a glass homogenizer (Kontes Glass Co., Vineland, NJ). The polytron homogenate was centrifuged at 500 × g for 10 minutes. The supernatant was centrifuged at 5000 × g for 10 minutes for isolation of SS mitochondria. Subsequent pellets were washed twice at 5000 × g for 10 minutes each with MSH buffer and lastly, suspended in 0.5 mL of MSH buffer with 0.2% BSA and EGTA 1 mM. Protein concentration was determined by the Lowry method.¹⁹

EPR Measurements

Experiments were carried out on a Bruker EMX (Bruker Instruments, Billerica, MA) spectrometer operating at 9.849 GHz with 100 kHz modulation frequency at room temperature.²⁰ Mitochondria (approximately 0.3 mg protein) were suspended in 225 mM sucrose, 5 mM MgCl₂ • 6 H₂O, 10 mM KH₂PO₄, 20 mM KCl, 10 mM Tris, and 5 mM HEPES, pH 7.4; 40U catalase; 200 mM 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO); 7.5 mM β-cyclodextrin. To study O₂^{•-} production by complex I, 16 mM glutamate, 8 mM malate, were used as substrates and 2 μM rotenone to inhibit forward flow of electrons. To measure complex III O₂^{•-} production, 24 mM succinate was used as substrate, 2 μM rotenone to inhibit backflow into complex I, and 1.37 mM antimycin A to prevent forward electron flow. To measure combined ROS production from complex I and III, 16 mM glutamate, 8 mM malate, were used as substrates, and 1.37 mM antimycin A to inhibit forward electron flow from complex III. 50 μL samples were then transferred to a capillary tube and scanned using the following parameters: center field, 3510 G; sweep width, 80 G;

power, 2 milliwatt; receiver gain, 5.02×10^5 ; modulation amplitude, 1 G; time of conversion, 20.48 ms; time constant, 81.92 ms; signal average, 12 scans. For negative control studies 1.5 U/mL of superoxide dismutase (SOD) was added to samples. The spectral simulations were performed using the WinSim program.²¹

Mitochondrial membrane potential—Mitochondrial membrane potential was measured using a tetraphenylphosphonium (TPP⁺) electrode as described by Kamo et al.²² The electrode was calibrated prior to the start of each experiment using a total of 2.8 μ M TPPCl. Membrane potential was calculated from the Nernst equation using the TPP binding correction factor.^{22–25} The Nernst constant at 37°C = 61.540, TPP external is estimated from a standard curve of [TPP⁺] using 0.33 μ M TPPCl additions to a total concentration of 4 μ M. The TPP⁺ binding correction factor used was 0.16. The incubation media contained [in mmol/L]: KCl [100]; KH₂PO₄ [1]; HEPES [50], pH: 7.4, at 37°C. Succinate [5 mM] was used as substrate for complex II. Rotenone [4.15 μ M] was used to inhibit backflow of electrons to complex I.

Electron microscopy

At End RP, Control and IPC hearts (n=6/group) were infused with 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 at 1 ml/min for 5 minutes. The tissue was cut into thin slices and then into strips less than 1 mm in diameter. Tissue was fixed for 1 hour at room temperature and rinsed 3 times in 0.1 M phosphate buffer. Tissue was then post-fixed for 1 hour in 1% osmium tetroxide in phosphate buffer. Tissue was rinsed again 3 times in 0.1 M phosphate buffer for 5 min for each rinse. Further tissue preparations for observation under electron microscope were carried out according to Joshi et al.²⁶ The tissue was embedded in eponate 12 resin, polymerized at 60°C for 24 hours, and sectioned at 80 nm on a Leica EM U6 ultramicrotome. Grids were stained using 2% aqueous uranyl acetate followed by Reynolds lead citrate. Tissue was observed in a FEI Tecnai Spirit TEM at 80 kV and magnification of 18,500 $\times$. A total of 10 slices were obtained for each heart sample and a total of 100 mitochondria were measured from each heart. Mitochondria were observed for the presence of cristae disruption, amorphous densities, and outer membrane disruption. The area, roundness and perimeter of the mitochondria were also measured.

Cytochrome c measurement

Cytochrome *c* was measured by reverse-phase high-performance liquid chromatography (HPLC) as described by Crouser et al.²⁷ Briefly, 100 μ L samples of isolated mitochondria were injected into a Shimadzu HPLC and Photodiode Array Detector (Shimadzu Scientific Instruments, Columbia, MD), equipped with Shimadzu software. A Vydac C4 reverse-phase analytical column (Grace & Co., Deerfield, IL) preceded by a guard column was used. A linear gradient from 20 to 60% acetonitrile in water was employed, each containing 100 mM KCl and 0.1% trifluoroacetic acid (v/v). The gradient was developed over a 15 minute period at a flow rate of 1.0 mL/min and was followed by a 5 minute wash with the 60% solution and subsequent 5 minute wash with the 20% solution before injection of the next sample. The eluted cytochrome was detected at 393 nm. Quantification was determined from standard curves obtained using known stock solutions of cytochrome *c*.

Statistical analysis

Data are expressed as means $\pm$ SEM. Student's T-test and Mann-Whitney Rank Sum Test were used to compare differences between pairs; two-way ANOVA and the Holm-Sidak post hoc analysis was used to compare multiple groups. A *p* value < 0.05 was considered to be statistically significant.

Materials

DMPO was obtained from Dojindo Molecular Technologies Inc., Rockville, Maryland. All other chemicals were obtained from Sigma-Aldrich Inc., St. Louis, Missouri. Rats were obtained from Harlan Laboratories, Indianapolis, Indiana.

Results

Mechanical Function

IPC improved the recovery of left ventricular function at End RP. IPC hearts recovered $63\pm4\%$ of their End EQ RPP compared to $21\pm2\%$ in the Control group ($p<0.05$, figure 2).

Membrane potential

There was no difference in $m\Delta\Psi$ between IPC and Control mitochondria at End EQ (-178 ± 3 vs. -178 ± 2 mV, figure 3). At End I, IPC preserved $m\Delta\Psi$ (-156 ± 5 vs. -131 ± 6 mV, $p<0.05$) compared to Control. At End RP, Control mitochondria had a lower $m\Delta\Psi$ compared to End EQ (-155 ± 2 vs. -178 ± 2 mV, $p<0.001$); while $m\Delta\Psi$ for IPC at End RP was not significantly different than at End EQ (-168 ± 2 vs. -178 ± 3 mV, $p>0.05$).

Superoxide Radical Production at End-equilibration by EPR

Figure 4 shows representative EPR spectra of $O_2^{\bullet-}$ measured at End RP using DMPO spin trap. We see that Control mitochondria had the higher peak signals compared to IPC; while mitochondria treated with superoxide dismutase (SOD) as a negative control had no change in signal amplitude. This demonstrates the effectiveness of using EPR and DMPO spin trap to measure $O_2^{\bullet-}$ production.

At End EQ, combined $O_2^{\bullet-}$ production by complex I and III was greater for IPC compared to Control though this did not reach statistical significance (1047 ± 136 vs. 543 ± 76 arbitrary units (AU)/mg protein, $p=0.16$; figure 5). This increased $O_2^{\bullet-}$ at End EQ for IPC is due to the induction of preconditioning by the brief episodes of IR (figure 1). There was no significant difference in $O_2^{\bullet-}$ production by individual complexes (complex I or III) between IPC and Control. $O_2^{\bullet-}$ production for complex I was 237 ± 43 for IPC and 263 ± 15 AU/mg protein for Control. $O_2^{\bullet-}$ production for complex III was 484 ± 54 for IPC and 386 ± 47 AU/mg protein for Control.

Superoxide Radical Production at End-ischemia by EPR

Ischemia increased combined $O_2^{\bullet-}$ production from complex I and III in both IPC and Control mitochondria when compared to End EQ (1878 ± 296 and 2220 ± 340 AU/mg protein, $p<0.05$ vs. End EQ values; figure 5). $O_2^{\bullet-}$ production by complex I was 334 ± 9 for IPC vs. 325 ± 38 AU for Control, $p<0.05$ vs. End EQ values. Complex III $O_2^{\bullet-}$ production was 1000 ± 68 for IPC vs. 1236 ± 133 AU for Control, $p<0.05$ vs. End EQ values.

Superoxide Radical Production at End-reperfusion by EPR

Reperfusion caused the greatest increase in $O_2^{\bullet-}$ production in both IPC and Control mitochondria (figure 5). Complex III $O_2^{\bullet-}$ was 2132 ± 127 for IPC vs. 3273 ± 244 AU/mg protein for Control ($p<0.05$). Combined $O_2^{\bullet-}$ production by complex I and III was 2527 ± 221 for IPC vs. 3523 ± 250 AU/mg protein for Control ($p<0.05$). There was no significant difference in $O_2^{\bullet-}$ production by complex I in IPC vs. Control (330 ± 35 vs. 459 ± 57 AU/mg protein). Also, combined $O_2^{\bullet-}$ production from complex I and III increased significantly at End RP compared to End I for Control (3523 ± 250 vs. 2220 ± 227 AU/mg protein, $p<0.010$), while IPC mitochondria demonstrated no significant change in $O_2^{\bullet-}$ production (2527 ± 221 vs. 1878 ± 175 AU/mg protein).

Electron microscopy at End RP in IPC and Control mitochondria

A total of 100 mitochondria were measured from each heart sample. IPC preserved mitochondrial ultrastructure at End RP (figure 6A and B). IPC mitochondria had fewer cristae disruption (10 ± 4 vs. $33 \pm 7\%$ of total mitochondria, $p < 0.05$) and decreased amounts of total number of amorphous density formation (18 ± 4 vs. $28 \pm 1\%$, $p < 0.05$) when compared to Control (figure 6A and B). Outer mitochondrial membrane disruption (1.83 ± 1.17 vs. $2.31 \pm 1.38\%$), area (0.21 ± 0.01 vs. $0.24 \pm 0.01 \mu\text{m}^2$), roundness (1.36 ± 0.06 vs. $1.39 \pm 0.08 \mu\text{m}$) and perimeter (1.82 ± 0.03 vs. $1.96 \pm 0.09 \mu\text{m}$) were similar between IPC and Control at End RP.

Cytochrome c

IPC preserved Cytochrome *c* levels at End RP when compared to Control (351 ± 14 vs. 269 ± 16 picomoles/mg protein, $p < 0.05$, figure 7).

Discussion

The main findings of the current experiments are that: 1) IPC decreased mitochondrial $\text{O}_2^{\bullet-}$ production from complex I and III after IR; 2) IPC preserved $\text{m}\Delta\Psi$ after ischemia and leads to greater restoration of $\text{m}\Delta\Psi$ at End RP; 3) IPC decreased mitochondrial cristae disruption and amorphous density formation after IR; and 4) IPC preserved mitochondrial cytochrome *c* levels at End RP.

Reactive oxygen species production

EPR is an important tool used in the detection and quantitation of reactive oxygen species.^{28–31} Free radicals such as $\text{O}_2^{\bullet-}$ and hydroxyl radical $[\text{OH}\cdot]$ are highly reactive and spin traps are used to stabilize these molecules for detection. Spin traps react with free radicals to produce adducts which are paramagnetic and therefore EPR visible.^{28, 29} DMPO reacts with $\text{O}_2^{\bullet-}$ to form DMPO-OOH, the superoxide adduct which exhibits a unique EPR spectrum.²⁹ There are some complexities in using DMPO as a spin trap. The superoxide adduct DMPO-OOH spontaneously decomposes to form DMPO-OH, which is also formed by the trapping of OH radicals ($\cdot\text{OH}$).^{28, 29} Quenching of the observed signals by SOD can be used to confirm that they are derived from $\text{O}_2^{\bullet-}$. In the current experiments, SOD was used as a negative control (figure 4). This demonstrates that the measured adducts are $\text{O}_2^{\bullet-}$ derived.

Ischemia leads to inefficient transfer of electrons along the ETC.⁷ This inefficient transfer causes electron leak, mainly from respiratory complex I and III, leading to the incomplete reduction of oxygen to produce $\text{O}_2^{\bullet-}$.^{4, 7, 9, 10} $\text{O}_2^{\bullet-}$ is highly reactive and can cause protein modifications through oxidation, disruption of ionic homeostasis, lipid peroxidation, DNA damage, and inhibition of mitochondrial respiratory activity (for a review see 33 and 34). Our results indicate that combined $\text{O}_2^{\bullet-}$ production from complex I and III is slightly increased in IPC mitochondria after the induction of preconditioning (figure 3). It is proposed that low levels of ROS including $\text{O}_2^{\bullet-}$ are produced by the brief episodes of preconditioning, and act as mediators for the cardioprotective effects of IPC.^{35–38} The administration of scavengers of ROS prior to the preconditioning episode abolishes the cardioprotective effects of IPC, further supporting the role of low concentrations of ROS as mediators of IPC.^{35–37}

$\text{O}_2^{\bullet-}$ production was increased at End I for both groups; however there was no significant difference between IPC and Control when looking at $\text{O}_2^{\bullet-}$ production from complex I and III individually or combined. The increase in $\text{O}_2^{\bullet-}$ production at End I would seem to be paradoxical given the low O_2 concentration $[\text{O}_2]$ at End I and the fact that $\text{O}_2^{\bullet-}$ formation is

directly dependent on $[O_2]$.^{7, 34} The exact mechanism of increased $O_2^{\bullet-}$ at End I is unknown but it appears that it is not simply the low $[O_2]$ that increases $O_2^{\bullet-}$ production, but other potential contributing factors such as decreased activity of matrix and intermembrane space peroxidases and changes in the response of cytochrome *c* oxidase to low $[O_2]$.^{7, 34}

At End RP, $O_2^{\bullet-}$ production increased in both IPC and Control mitochondria when compared to End EQ values, however the increase in IPC ($237 \pm 17\%$) was far less than that seen in Control ($649 \pm 42\%$). The greatest increase in $O_2^{\bullet-}$ occurred during reperfusion (figure 5C), which would have significant impact in the clinical setting. Previous studies have shown that the addition of superoxide dismutase during reperfusion significantly improved cardiac contractile function and decreased the formation of superoxide adducts.^{31, 32} Therefore, low levels of $O_2^{\bullet-}$ as seen at the end of the preconditioning cycle, may play a role in the protective effects of IPC; but high levels of $O_2^{\bullet-}$ as seen at end-reperfusion in Control mitochondria may contribute to reperfusion injury.

$O_2^{\bullet-}$ production after IR is partly a function of mitochondrial respiration by the ETC.^{4, 6, 8, 9} Ischemia damages the respiratory complexes of the ETC leading to increased production of $O_2^{\bullet-}$ by complex I and III.⁹ Continued respiration by damaged ETC complexes leads to increased electron leak and $O_2^{\bullet-}$ production and further myocardial damage.^{9, 10} Agents that act as inhibitors of specific ETC complexes such as rotenone and amobarbital (complex I) and malonate (complex II) inhibit further ROS production by inhibiting respiration in a damaged system.^{9, 10, 39, 40} Complex I and III are the sources of ROS production by the ETC, with complex III being the major producer in heart mitochondria (figure 5).^{10, 34, 40} Inhibition of respiration at the proximal ETC (complex I or II) of ischemic/damaged mitochondria provides cardioprotection, partly by inhibiting ROS production by both the proximal and distal ETC complexes.^{4, 10, 34, 39-40}

Superoxide radical production and membrane potential

High $m\Delta\Psi$ increases $O_2^{\bullet-}$ production due to the highly reduced state of the ETC when mitochondria are respiring on either complex I or complex II substrates.^{6, 34, 41} Ischemia decreases mitochondria state 3 respiration (ADP consumption with ATP production) and increases state 4 respiration (respiration without ATP production).⁹ This leads to an increase in electron leak into the matrix and increased $O_2^{\bullet-}$ production.³⁴ At high levels of $m\Delta\Psi$, the ROS production is significantly increased but can be attenuated by depolarization.^{6, 34, 41} Starkov and Fiskum have demonstrated that depolarization in rat brain mitochondria resulted in up to 70% reduction of ROS production when respiring on complex I substrates.⁴¹ Korshunov et al. found a 90% reduction in ROS production after depolarization when respiring on complex II substrates.⁶ Changes in $m\Delta\Psi$ affect the utilization of redox substrates such as NADH.⁴² NADH is important in the antioxidant defense of cardiac cells, therefore changes in $m\Delta\Psi$ can affect the ability of cells to resist ROS damage.⁴²

Previous studies have demonstrated that ROS production increases during hypoxia as occurs during acute cardiac ischemia.^{4, 7, 9, 10, 23, 34} The increased ROS production observed in mitochondria during hypoxia can be relieved by partial oxidation of redox couples.⁴ This can occur, for example, through the overexpression of uncoupling proteins. Our results indicate that preconditioned mitochondria exhibit less membrane depolarization which would indicate uncoupling may not be a part of the protective mechanism of IPC.

Our results indicate that $m\Delta\Psi$ changes throughout an episode of IR (figure 3). The higher ROS production seen in IPC at End EQ is associated with the induction of preconditioning (figure 3). $m\Delta\Psi$ is equal in both groups at End EQ, which shows that the preconditioning protocol used in this experiment does not cause a net change in the $m\Delta\Psi$. The increased ROS production observed in IPC mitochondria at End EQ is triggered by the brief episodes

of IR used to induce preconditioning; this alters the mitochondrial respiratory complexes inducing increased $O_2^{\bullet-}$ production. At End I we observed that IPC leads to preservation of the electrical integrity of the mitochondrial inner membrane, which is demonstrated as preservation of $m\Delta\Psi$ when compared to Control (-156 ± 5 vs. -131 ± 6 mV, $p<0.001$). It can be postulated that this preservation of $m\Delta\Psi$ could be beneficial to mitochondria since loss of $m\Delta\Psi$ leads to disruption of oxidative phosphorylation, metabolic changes, caspase activation and eventual cell death.⁴³ The preservation of $m\Delta\Psi$ seen in IPC at End I is carried over into End RP where IPC had recovery of $m\Delta\Psi$ to levels similar to that observed at End EQ (-168 ± 2 vs. -178 ± 3 mV, $p>0.05$). There was less recovery of $m\Delta\Psi$ in Control mitochondria at End RP when compared to End EQ (155 ± 2 vs. -178 ± 2 mV, $p<0.001$).

It is known that minor fluctuations in $m\Delta\Psi$ can occur due to transient opening of the mitochondrial permeability transition pore (MPTP).⁴⁴⁻⁴⁶ This opening can be triggered by Ca^{2+} influx caused by oxidative stress.⁴⁴ High oxidative stress leads to non-reversible opening of the MPTP, collapse of $m\Delta\Psi$, and necrotic cell death.⁴⁴ More recent experiments have shown that other ion channels may be involved in depolarization of mitochondrial inner membrane.^{43, 47, 48} The inner membrane anion channel (IMAC) is a reversible channel that is activated under moderate oxidative stress leading to a decrease in $m\Delta\Psi$.^{43, 48} It is currently proposed that moderate levels of oxidative stress lead to activation of reversible ion channels such as IMAC accompanied by slight oscillations in $m\Delta\Psi$ (for review see 43). Greater levels of oxidative stress lead to activation of the MPTP and collapse of $m\Delta\Psi$; therefore prevention of severe $m\Delta\Psi$ depolarization leads to beneficial effects in mitochondria and cardiac cells.^{43, 47} Previous studies have shown that IPC inhibits opening of the MPTP.⁴⁹ In the current experiment, we measured changes in $m\Delta\Psi$ throughout an episode of ischemia reperfusion. Our results indicate that IPC leads to greater preservation of $m\Delta\Psi$ when compared to Control. The mechanism by which IPC leads to preservation of $m\Delta\Psi$ in mitochondria may therefore involve direct inhibition of IMAC and/or the MPTP or by limiting factors that promote the opening of these channels.⁴⁹ We propose that the attenuated $O_2^{\bullet-}$ production seen in IPC prevents activation of the MPTP and preserves $m\Delta\Psi$, while the higher levels of $O_2^{\bullet-}$ seen in Control mitochondria lead to greater loss of $m\Delta\Psi$ due to activation of irreversible channels such as the MPTP.^{43, 47-49}

The recovery of membrane potential observed at End RP cannot be entirely explained by this experiment, but it is possible that it may be attributed to closure of the MPTP during reperfusion. Javadov et al. have shown that IPC reduces MPTP opening during early reperfusion⁴⁹, and also increases subsequent pore closure as reperfusion continues (for review see 50). The recovery of $m\Delta\Psi$ at End RP to levels that are similar to End EQ in IPC mitochondria may be due to enhanced MPTP closure as a result of preconditioning.

Mitochondrial structure as assessed by electron microscopy

It has been demonstrated that oxidative stress alters mitochondrial permeability as well as mitochondrial structure.⁵¹ Cole et al. demonstrated that isolated mitochondria exposed to both exogenous and endogenous oxidative stress developed increased permeability with the translocation of cytoplasmic proteins into the matrix.⁵¹ They also demonstrated changes in mitochondrial structure such as loss of cristae density, narrower cristae with decrease in cristae number and mis-orientation; there was also an increase in intra-mitochondrial amorphous densities which likely represent matrix precipitates and degenerating cristae.⁵¹

In the current experiment, electron microscopic examination of IPC and Control mitochondria at End RP revealed that IPC mitochondria had decreased amounts of amorphous densities (figure 6A and B). IPC mitochondria also had less cristae disruption when compared to Control. There was no apparent difference in membrane disruption, perimeter, roundness or mitochondrial area between the two groups. The decreased cristae

disruption and formation of amorphous densities observed in preconditioned mitochondria is significant because it demonstrates that IPC preserves the structural integrity of mitochondria after ischemia and the increased oxidative stress of reperfusion. Lesnefsky et al. looked at mitochondrial structure after 45 minutes of ischemia and concluded that only minimal structural alterations (several organelles with expanded cristae) were present and morphologic evidence of irreversible structural damage was absent.⁵² The difference in structural changes observed in the current study could possibly be attributed to the fact that our samples were prepared at end reperfusion, which is what occurs in the clinical setting, while Lesnefsky and colleagues examined samples after 45 minutes of ischemia.

The results of the current study and that of Cole et al. reiterate the importance of maintaining mitochondrial structural integrity. Cristae disruption is associated with the release of cytochrome *c* which is a marker of mitochondrial membrane disruption and is associated with activation of apoptosis.^{53, 54} To confirm these findings in our experimental protocol, we evaluated cytochrome *c* levels at End RP (see below). The central role of mitochondria in energy production, maintaining homeostasis, and initiating apoptosis makes preservation of mitochondrial integrity vital for cardioprotection.⁴⁷

The Role of Cytochrome *c*

The results of our electron microscopy studies showed a preservation of mitochondrial cristae structure in IPC. We therefore sought to find if this was associated with preservation of cytochrome *c*. Cytochrome *c* is an electron carrier that transports electrons from complex III to complex IV of the ETC. In the setting of IR injury, cytochrome *c* is released by damaged mitochondria into the cytosol, most likely via the MPTP (for review see 53). Cytochrome *c* release leads to activation of apoptosis and is a marker of mitochondrial injury. *SS* mitochondria have been shown to be more susceptible to calcium-overload mediated release of cytochrome *c* compared to interfibrillar (IF) mitochondria.⁴⁰

In the current experiments, we demonstrate that IPC leads to greater preservation of *SS* mitochondria cytochrome *c* levels (figure 7). Cytochrome *c* is located in the intermembrane space and is localized to the surface of the mitochondrial inner membrane due to interactions with cardiolipin.^{40, 53, 55} The release of cytochrome *c* after IR injury is due to increased permeability of the mitochondrial outer membrane.^{40, 53, 54} Previous experiments have shown that increased oxidative stress as well as loss of $m\Delta\Psi$ lead to reversible increases in mitochondrial permeability.^{51, 53} Lundberg and Szweda demonstrated that IPC abolished the insertion of Bax, a pro-apoptotic, pore-forming member of the Bcl-2 family of proteins that is inserted into the mitochondrial outer membrane in response to IR.⁵⁵ This led to a reduction in the release of cytochrome *c* after IR.⁵⁵ The probability of MPTP opening increases with increased oxidative stress and decreasing $m\Delta\Psi$; both of these conditions are decreased by IPC as demonstrated by the data from our current experiments.

We propose that IPC attenuates ROS production at End RP and preserves mitochondrial membrane potential. This then leads to decreased probability of MPTP activation. Decreased MPTP activation means less loss of cytochrome *c* and decreased activation of apoptotic pathways and less myocyte loss during IR. The current study reemphasizes the important role of mitochondria in IR injury. Therapies that target mitochondria, specifically by limiting ROS production during reperfusion, stabilizing membrane structure and protecting $m\Delta\Psi$, offer opportunities for points of intervention in patients with ischemic heart disease.

Conclusion

Ischemic preconditioning decreases reactive oxygen species production leading to preservation of mitochondrial membrane integrity as well as membrane potential after an

episode of ischemia-reperfusion. While the current findings do not answer the question as to the mechanism of decreased ROS production by IPC, it was previously held that minor depolarization of mitochondrial membrane potential may be a part of the mechanism of decreased ROS production in IPC. However, the results of the current studies would indicate that IPC leads to decreased loss of mitochondrial membrane potential during ischemia and greater restoration of membrane potential during reperfusion. This preservation of mitochondrial function and structure is associated with improved outcomes after ischemia-reperfusion and may be part of the mechanism of IPC. Further studies looking at how specifically targeting mitochondrial function prior to ischemia-reperfusion affects outcomes are currently being undertaken.

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Figure 1.

Experimental protocol: Hearts were perfused on a non-recirculating Langendorff apparatus. Control hearts underwent 30 minutes of continuous perfusion (equilibration), then 30 minutes of ischemia, and 30 minutes of reperfusion. IPC hearts underwent 10 minutes of equilibration, followed by the induction of preconditioning by two 5 minute episodes of ischemia (I5), each followed by 5 minutes of reperfusion (R5). This was followed by 30 minutes of ischemia, then 30 minutes of reperfusion. Mitochondria were isolated at end equilibration (End EQ), end ischemia (End I) and at end reperfusion (End RP).

Figure 2.

Mechanical function during reperfusion: Mechanical function during reperfusion was measured as the recovery of rate pressure product (RPP, systolic pressure minus diastolic pressure multiplied by heart rate) expressed as percentage of End EQ values. After 30 minutes of reperfusion, IPC hearts recovered a greater percentage of RPP compared to Control; * $p < 0.05$ at each time point.

Figure 3.

Mitochondrial membrane potential ($m\Delta\Psi$) during ischemia-reperfusion. $m\Delta\Psi$ was measured using a tetraphenylphosphonium (TPP) electrode as described in Methods section. Mitochondria were isolated at end equilibration (End EQ), end ischemia (End I) and at end reperfusion (End RP). $m\Delta\Psi$ was equal in IPC and Control mitochondria at End EQ, indicating that the induction of preconditioning did not significantly affect $m\Delta\Psi$. At End I, IPC had greater preservation of $m\Delta\Psi$ compared to Control. At End RP, $m\Delta\Psi$ for IPC was not significantly different compared to End EQ values, while $m\Delta\Psi$ for Control mitochondria remained significantly lower compared to End EQ values. * $p<0.05$ vs. Control; ‡ $p<0.05$ vs. Control at End EQ; ¥ $p<0.05$ vs. IPC at End EQ.

Figure 4. Representative EPR spectra of $O_2^{\bullet-}$ measurements. Mitochondrial $O_2^{\bullet-}$ production was measured by EPR using DMPO spin trap as described in Methods section. A) Control mitochondria at End RP. B) IPC mitochondria at End RP. C) Negative control experiments using SOD added to Control mitochondria at End RP. The addition of SOD abolished the EPR signal, demonstrating that changes in EPR spectra are due to the production of $O_2^{\bullet-}$.

Figure 5.

ROS production at each endpoint: $O_2^{\bullet-}$ production by EPR using DMPO spin trap. To measure $O_2^{\bullet-}$ production from complex I, glutamate and malate were used as substrates, while rotenone was used to inhibit forward flow past complex I. To measure complex III $O_2^{\bullet-}$ production, succinate was used as a substrate while rotenone was used to inhibit reverse electron flow to complex II and antimycin-A was used to inhibit forward flow past complex III. To measure combined $O_2^{\bullet-}$ from complex I and III, glutamate and malate were used as substrates and antimycin-A to inhibit electron flow past complex III. ‡ p<0.05 vs. Control at End EQ; * p<0.05 vs. Control; ¶ p<0.05 vs. Control at End I; § p<0.05 vs. IPC at End I; ¥ p<0.05 vs. IPC at End EQ.

Figure 6.

A) Mitochondrial structure examined by electron microscopy. Black arrow-amorphous density; white arrow-normal cristae; black arrowhead-cristae disruption. IPC decreased cristae disruption and amorphous density formation. B) Graphic representation of data from

electron microscopy; * $p < 0.05$ vs. Control. Other measured parameters were not significantly different between the two groups.

Figure 7.

Mitochondrial cytochrome *c* content was measured by HPLC at End RP using a C4 reverse phase analytic column. IPC led to greater preservation of cytochrome *c* compared to Control mitochondria; * $p < 0.05$ vs. Control.