Ischemic preconditioning prevents in vivo hyperoxygenation in postischemic myocardium with preservation of mitochondrial oxygen consumption

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1The Center for Biomedical Electron Paramagnetic Resonance Spectroscopy and Imaging, Davis Heart and Lung Research Institute, and the Division of Cardiovascular Medicine, Department of Internal Medicine, The Ohio State University College of Medicine, Columbus, Ohio; and 2Key Laboratory of Organ Transplantation, Ministry of Education Institute of Organ Transplantation, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

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Zhu X, Liu B, Zhou S, Chen Y-R, Deng Y, Zweier JL, He G. Ischemic preconditioning prevents in vivo hyperoxygenation in postischemic myocardium with preservation of mitochondrial oxygen consumption. Am J Physiol Heart Circ Physiol 293: H1442–H1450, 2007. First published May 18, 2007; doi:10.1152/ajpheart.00256.2007.—Ischemic preconditioning (IPC) strongly protects against ischemia-reperfusion injury; however, its effect on subsequent myocardial oxygenation is unknown. Therefore, we determined in an in vivo mouse model of regional ischemia and reperfusion (I/R) if IPC attenuates postischemic myocardial hyperoxygenation and decreases formation of reactive oxygen/nitrogen species (ROS/RNS), with preservation of mitochondrial function. The following five groups of mice were studied: sham, control (I/R), ischemic preconditioning (IPC), I/R and IPC, and IPC/eNOS knockout mice. I/R and IPC + I/R mice were subjected to 30 min regional ischemia followed by 60 min reperfusion. Myocardial Po2 and redox state were measured by electron paramagnetic resonance spectroscopy. In the IPC + I/R, but not the I/R group, regional blood flow was increased after reperfusion. Po2 upon reperfusion increased significantly above preischemic values in I/R but not in IPC + I/R mice. Tissue redox state was measured from the reduction rate of a spin probe, and this rate was 60% higher in IPC than in non-IPC hearts. Activites of NADH dehydrogenase (NADH-DH) and cytochrome oxidase (CcO) were reduced in I/R mice after 60 min reperfusion but conserved in IPC + I/R mice compared with sham. There were no differences in NADH-DH and CcO expression in I/R and IPC + I/R groups compared with sham. After 60 min reperfusion, strong nitrotyrosine formation was observed in I/R mice, but only weak staining was observed in IPC + I/R mice. Thus IPC markedly attenuates postischemic myocardial hyperoxygenation with less ROS/RNS generation and preservation of mitochondrial O2 metabolism because of conserved NADH-DH and CcO activities.

ischemia reperfusion; reactive oxygen species; free radicals; nitric oxide; peroxynitrite; redox; mitochondria

ISCHEMIC HEART DISEASE has been a leading cause of morbidity and mortality. Ischemia is characterized by insufficient oxygen and nutrient supply to the area at risk and leads to tissue infarction. Prompt return of blood to the area at risk reduces mortality by 50% (15). Never the less, reperfusion injury introduces further complications to the reperfused myocardium, including cardiac arrhythmias and diminished contractile function (8). Rapid entry of calcium ions in cardiac myocytes produces contraction bands, mitochondrial granules, and a loss of vascular integrity, resulting in hemorrhage in the infarct. In addition, reactive oxygen/nitrogen species (ROS/RNS) generated upon reperfusion play an important role in the process of cellular damage with oxidation and denaturation of a number of critical proteins and peroxidation of membrane lipids. Nitration of functional proteins occurs, and this further contributes to cellular injury (7, 28). Reperfusion injury is associated with loss of contractile function, cell death, and reperfusion arrhythmias, including ventricular tachycardia and fibrillation, which also has been reported to induce ROS formation (14).

In 1986, Murry et al. (32) reported a profound myocardial protective mechanism induced by mild ischemic stress that conferred resistance to a subsequent ischemic stress, namely ischemic preconditioning (IPC). Since then, IPC has been shown to reduce infarction in pigs, dogs, rabbits, rats, and mice (16, 24, 26, 47, 53). Several mechanisms have been identified to result in IPC. These include G protein-coupled receptor-mediated processes, including those agonized by adenosine (11), bradykinin (49), and opioids (38), and free radical-mediated processes, including those initiated by nitric oxide (NO), superoxide (O2•−), and their reaction product peroxynitrite (ONOO−; see Refs. 13, 51, and 60). So far, the downstream signal transduction pathways have included a key role for activation of protein kinase C (30, 34), with subsequent activation of tyrosine kinases (3, 20), mitogen-activated protein kinases (29, 35), and the mitochondrial ATP-sensitive K+ channel (mitoKATP; see Refs. 17 and 48). To date, the exact cellular mechanism(s) responsible for IPC are still debated; however, the production of free radicals, including NO, O2•−, and ONOO−, are believed to play an important role in ischemia-induced reperfusion injury, and IPC is thought to diminish their subsequent formation (4, 6, 39, 58, 59).

NO and ONOO− have been reported to interact with NADH dehydrogenase (NADH-DH) and cytochrome c oxidase (CcO) on the mitochondrial respiratory chain, thereby regulating mitochondrial oxygen consumption (27). Recently, we and others have demonstrated that NO, O2•−, and their derivative ONOO− suppress myocardial tissue oxygen consumption by regulating mitochondrial respiration on NADH-DH and CcO after ischemia-reperfusion (I/R; see Refs. 9, 40, 52, 54, 55). We have also demonstrated in an in vivo mouse heart model that there is a hyperoxygenation status after regional I/R because of

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the suppression of mitochondrial oxygen consumption (55). IPC was reported to reduce the generation of ROS/RNS after both the acute and late phase of postischemic reperfusion (12, 23). Therefore, we hypothesize that IPC attenuates in vivo postischemic myocardial hyperoxygenation through attenuating ROS/RNS formation, thereby upregulating tissue oxygen consumption and therefore preserving mitochondrial respiration that in turn contributes to postischemic myocardial protection.

However, in vivo assessment of myocardial tissue oxygenation and redox status has been challenging. The standard oxygen electrode techniques suffer from motion artifacts and nonrepeatability if the electrode is inserted in the myocardium of a beating heart, although this technique can be applied to measure tissue oxygen consumption by monitoring coronary oxygenation in the effluent/affluent in large animals (2, 43). The development of electron paramagnetic resonance (EPR) techniques using oxygen-sensitive probes such as lithium phthalocyanine (LiPc) or redox-sensitive probes such as 2,2,5,5-tetramethyl-3-carboxylpyrrolidine-N-oxyl (PCA) has provided fast and accurate methods for monitoring tissue PO2 or redox status in various organs and tissues in vivo (19, 41, 56). Doppler blood flow measurement, in conjunction with in vivo EPR measurement of tissue oxygenation, has enabled in vivo assessment of myocardial oxygen consumption and mitochondrial function.

There are two phases of protection afforded by IPC, e.g., the early phase and the late phase (5). Both share some common signaling pathways and some distinct mechanisms. The current study focuses on the early phase of IPC. In untreated or preconditioned mice, EPR spectroscopy was applied to monitor cardiac tissue PO2 and along with laser Doppler flow measurements of tissue perfusion. The effects of early phase IPC on oxygen consumption and redox status were determined. We show that, in the early phase, IPC attenuates postischemic myocardial hyperoxygenation and prevents oxidative stress and preserves tissue oxygen consumption and mitochondrial function through protection of the mitochondrial respiratory chain, and this is associated with infarct size reduction in the postischemic heart.

**MATERIALS AND METHODS**

**Animals and materials.** Male wild-type C57BL/6 and eNOS knockout (eNOS−/−) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Wild-type mice were randomly selected to be administered 1 mg/ml X2-nitro-L-arginine methyl ester (L-NAME; Sigma Chemical) in drinking water for 3 days before experiment (L-NAME; Sigma). NADH-DH and CcO. Activities of NADH-DH and CcO. Frozen myocardial tissue obtained from the risk region, was homogenized in ice-cold HEPES buffer (3 mmol/l, pH 7.2) containing sucrose (0.25 mol/l), EGTA (0.5 mmol/l), and protease-inhibitor cocktail (1:40; Roche). NADH-DH activity was measured in the presence of Tris-HCl buffer (20 mmol/l, pH 8.0), NADH (150 mol/l; Sigma), and coenzyme Q1 (100 mol/l; Sigma). CcO activity was measured in the presence of phosphate buffer (50 mmol/l, pH 7.4) and reduced cytochrome c (60 mol/l; Sigma). The extinction coefficients, ε340 nm = 6.22 mmol/l·cm for NADH and ε550 nm = 18.5 mmol/l·cm for cytochrome c were used for

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activity calculation (10). Protein concentration of the tissue homogenate was measured by the Lowry method, and the activities were normalized to protein concentrations as micromoles per minute per milligram protein.

Western blot analysis of protein expression of NADH-DH and CcO. Proteins of the homogenate were subjected to electrophoresis on 4–20% Tris-glycine polyacrylamide gradient gels. Anti-OxPhos Complex I subunit 39 kDa and Anti-OxPhos Complex IV subunit VIb (Invitrogen, Carlsbad, CA) and anti-GAPDH were used to confirm equal loading (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibodies were conjugated with horseradish peroxidase, and the protein was detected by use of enhanced chemiluminescence secondary antibodies were conjugated with horseradish peroxidase, and the protein was detected by use of enhanced chemiluminescence (Vector, Burlingame, CA) was used as the secondary antibody at a dilution of 1:200 and incubated for 30 min at room temperature. Rabbit polyclonal nitrotyrosine (Upstate, Charlottesville, VA) was used as the primary antibody at a dilution of 1:50 and incubated for 1 h at room temperature. Slides were next biotin blocked for endogenous biotin. Biotinylated goat anti-rabbit antibody (Vector, Burlingame, CA) was used as the secondary antibody at a dilution of 1:200 and incubated for 30 min at room temperature. Vectastain Alkaline Phosphatase (Vector) was used as the detection system for 30 min. Permanent Red (Dako) was used as the substrate chromogen. The slides were then counterstained with hematoxylin, dehydrated through graded ethanol solutions, and cover slipped.

Infarct size. Hearts were excised and cannulated through the ascending aorta after 30 min ischemia and 24 h reperfusion with a 23-gauge needle for perfusion with 3–4 ml of 1.0% triphenyl tetrazolium chloride (TTC) in phosphate buffer (pH 7.4, 37°C). The LAD was reoccluded by tightening the suture left in the myocardium after I/R and TTC staining. The hearts were then perfused with 2–3 ml of 10% Phthalo Blue (Heubach) to delineate nonischemic myocardium. The hearts were weighed, frozen, and cut into five transverse slices, each with ~1 mm thickness. Each slice was photographed from both sides with a high-resolution digital camera on a dissecting microscope. The sections were photographed and contoured with a planimeter (Adobe PhotoShop 5.0) to delineate the borders of the entire heart, the nonischemic area, and the infarct area. The sizes of the nonischemic area, AAR, and infarct area (INF) were calculated as percentages of the total left ventricle (LV) area multiplied by the total weight of that slice. The infarct size was measured by an independent, blinded observer using the above-mentioned computer planimetry.

Statistical analysis. A two-way ANOVA was used for data analysis of PO2, arterial pressure, mean arterial pressure, and RPP. A 1-way ANOVA was used for analysis of enzyme activity; these were followed by Newman-Keuls multiple-comparison test among the groups. A t-test was used for data analysis of AAR and INF size. Data were represented as means ± SE. A value of P < 0.05 was considered significant.
RESULTS

Regional blood flow measurement. Regional blood flow was measured before ischemia in both I/R and IPC/I/R mice with basal values defined as 100% (Fig. 2). During the IPC episodes, regional coronary blood flow measured within the risk region decreased by just over 60% during the 5-min period of ischemia and rebounded to just over 100% of basal values after the 5-min reperfusion period. In both groups, when hearts were subjected to 30 min ischemia with coronary ligation, blood flow decreased rapidly with values of 15.0 ± 6.9 and 14.0 ± 6.8% in the AAR after 30 min ischemia in I/R and IPC + I/R mice, respectively. Upon reperfusion, blood flow was rapidly restored to 95.9 ± 6.0 or 102.6 ± 8.9% of basal values in the first 5 min in I/R and IPC + I/R mice, respectively. In I/R mice, reperfusion blood flow remained constant, with 93.8 ± 7.9% of the basal value seen at 60 min reperfusion. However, in IPC + I/R mice, reperfusion blood flow increased significantly above basal values (P < 0.05) and was higher than the values seen in I/R mice (P < 0.05).

Fig. 3. In vivo measurement of myocardial tissue Po2 with EPR oximetry on wild-type control and preconditioned mice. Wild-type C57BL/6 mice were subjected to 30 min left anterior descending coronary artery (LAD) occlusion followed by 60 min reperfusion (I/R) or 3 cycles of 5 min LAD occlusion followed by 5 min reperfusion (the last reperfusion was prolonged to 15 min), and 30 min ischemia and 60 min reperfusion (IPC + I/R). Tissue Po2 overshoot the baseline values in the I/R group, but this was attenuated in the IPC + I/R group. *I/R vs. IPC + I/R at the end of 60 min reperfusion; n = 7/group.

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Fig. 4. In vivo measurement of myocardial tissue Po2 with EPR oximetry on preconditioned eNOS knockout (eNOS−/−) and Nω-nitro-L-arginine methyl ester (L-NAME)-treated mice. eNOS−/− and L-NAME-treated mice were subjected to 30 min LAD occlusion followed by 60 min reperfusion (I/R) or 3 cycles of 5 min LAD occlusion followed by 5 min reperfusion (the last reperfusion was prolonged to 15 min), and 30 min ischemia and 60 min reperfusion (IPC + I/R). There was no overshoot of tissue Po2 in both groups in the postischemic hearts compared with that in the wild-type control group as shown in Fig. 3; n = 7/group.

Fig. 5. In vivo EPR measurement of tissue redox status using the 2,2,5,5-tetramethyl-3-carboxylpyrrolidine-N-oxyl (PCA) redox probe. After thoracotomy and exposure of the heart, 5 μl of 10 mM PCA in PBS was injected in the area at risk, and EPR spectroscopy was performed. A: representative decay profiles of the EPR signal intensity after reperfusion, shown as open circles for the I/R group and triangles for the IPC + I/R group. Fitting curves are shown as the lines, with solid line for I/R group and dashed line for IPC + I/R group. *I/R-ischemia vs. normal, P < 0.01. **I/R-reperfusion vs. normal, P < 0.01. **I/R-ischemia vs. normal, P < 0.01. + + IPC + IR-reperfusion vs. normal, P = not significant; n = 7/group.
Regional myocardial tissue \( \text{PO}_2 \) in the AAR. The baseline values of tissue \( \text{PO}_2 \) in the preischemic state were 16.3 ± 0.7 and 15.1 ± 0.8 mmHg in wild-type I/R and IPC I/R mice, respectively (Fig. 3). Decreases of tissue \( \text{PO}_2 \) were clearly observed during each 5-min ischemic period followed by increases with each reperfusion phase. In both groups, tissue \( \text{PO}_2 \) values dropped rapidly following the onset of ischemia to <2 mmHg within 10 min and then gradually decreased to values of 0.7 ± 0.2 and 1.2 ± 0.3 mmHg in I/R and IPC + I/R mice, respectively. Upon reperfusion, the values of \( \text{PO}_2 \) increased rapidly in both groups. In wild-type I/R mice, tissue \( \text{PO}_2 \) increased and overshot basal values, with 26.2 ± 0.8 mmHg seen at the end of the 60 min reperfusion period. In wild-type IPC + I/R mice, the \( \text{PO}_2 \) increased back to preischemic values, reaching 15.6 ± 1.5 mmHg at 60 min reperfusion. In contrast to the I/R mice, no overshoot in myocardial oxygenation was seen. In eNOS\(^{-/-}\) and L-NAME-treated preconditioned mice, there was no overshoot of tissue \( \text{PO}_2 \) after reperfusion in both groups, as shown in Fig. 4.

In vivo myocardial tissue redox status. After injection of PCA in the AAR, EPR spectra before, during, and after ischemia were collected every 60 s for up to 1 h in both the preconditioned and nonpreconditioned mice. Representative time-dependent profiles of the EPR signal intensity in the reperfused hearts are shown in Fig. 5A. Figure 5B shows the reduction rate constants of PCA in wild-type I/R and IPC + I/R mice in the preischemic state (0.042 ± 0.003 vs. 0.041 ± 0.003/min), ischemic state (0.084 ± 0.015 vs. 0.079 ± 0.005/min), and postischemic state (0.028 ± 0.004 vs. 0.044 ± 0.003/min). A 60% faster decay of the EPR signal was observed upon reperfusion in the preconditioned than in the nonpreconditioned hearts. Potassium ferricyanide, a known...
standard oxidant that oxidizes reduced PCA back to its paramagnetic form, was injected to the same spot 10 min after the injection of PCA, and the signal was restored, confirming that PCA was reduced in the tissue as previously described (56).

**RPP.** Basal values of RPP in I/R and IPC + I/R mice were measured as 20.0 ± 2.3 × 10³ and 20.9 ± 2.7 × 10³ mmHg/min, respectively (Fig. 6). RPP values were 16.2 ± 2.1 and 18.5 ± 1.5 × 10³ mmHg/min at the end of 60 min reperfusion in I/R and IPC + I/R mice. The RPP values at the end of reperfusion were slightly higher in IPC mice than that in I/R mice; however, the difference was not significant.

**Mitochondrial NADH-DH and CcO activity.** To investigate the mechanism(s) by which ROS/RNS regulate mitochondrial O₂ consumption, the activities of NADH-DH and CcO were measured. Baseline values of the activities of NADH-DH and CcO in the sham group were measured as 0.23 ± 0.01 and 0.44 ± 0.03 μmol min⁻¹ mg protein⁻¹, respectively (Fig. 7). The activities of NADH-DH and CcO at the end of 60 min reperfusion were 0.17 ± 0.01 and 0.35 ± 0.03 μmol min⁻¹ mg protein⁻¹ in the I/R group, respectively, and 0.21 ± 0.01 and 0.41 ± 0.02 μmol min⁻¹ mg protein⁻¹ in IPC + I/R mice, respectively. Compared with the sham group, the activities of NADH-DH and CcO were significantly lower in the I/R group (P < 0.05) but not in the IPC + I/R group.

**Protein expression of NADH-DH and CcO.** Protein expressions of NADH-DH and CcO were measured by Western blotting to determine if the expression of these key mitochondrial electron transport proteins is altered by I/R and IPC. The NADH-DH and CcO protein levels after I/R were 95.5 ± 6.3 and 103.8 ± 11.8%, respectively, of basal preischemic values, in I/R mice and 94.0 ± 11.7 and 97.8 ± 10.5% in IPC + I/R mice (Fig. 8). There was no significant difference among the groups.

**Formation of nitrotyrosine.** Nitrotyrosine staining showed very prominent red coloration in the vascular beds in the positive control group that was infused with ONOO⁻ (see Fig. 9). There was no observable nitrotyrosine staining in the negative control group. Only very weak red staining for nitro-

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**Fig. 9.** Immunohistochemical staining of nitrotyrosine, a biomarker of peroxynitrite formation. Sections were fixed and prepared as described in MATERIALS AND METHODS, and photomicrographs are obtained at ×400 magnification. A: positive control with direct injection of peroxynitrite; strong diffuse nitrotyrosine staining in the vascular beds was present (red color, arrows). B: negative control; no nitrotyrosine staining (red) was present. Only weak staining in hearts of sham group was seen (C), whereas strong red staining was present in the I/R heart (D; arrows). E: in the IPC + I/R heart only weak staining was seen.
tyrosine was seen in the sham group. In contrast, strong nitrotyrosine staining was observed with a homogeneous distribution throughout the myocytes in the I/R group, however, staining was much weaker in the IPC + I/R group.

**AAR and INF.** Data of AAR as a percentage of LV area in I/R and IPC + I/R mice (Fig. 10, A and B) were 61.3 ± 3.2 and 55.0 ± 4.1%, respectively, and with no significant difference between the groups. Infarct size (INF) as a percentage of AAR in I/R and IPC + I/R mice were 32.2 ± 3.4% and 12.8 ± 1.9%, respectively, and the difference was significant (*P < 0.01).

**DISCUSSION**

It has been clearly established that IPC imparts profound myocardial protection against subsequent ischemia (5). A number of mechanisms have been shown to mediate IPC, and these include alterations in oxygen radical and NO production. Of note, it has been reported that IPC-associated protection is diminished in the disease states with preexisting oxidant stress such as diabetes or hypercholesterolemia (22, 45, 46). Oxygen is critical for myocardial function and aerobic metabolism, with oxygen reduction by the mitochondria providing 90% of the energy in the heart (36). During global myocardial ischemia, tissue oxygen levels fall to near zero and on reperfusion a rapid rise occurs (57, 58). Al-Obaidi et al. (1) measured myocardial tissue oxygen supply and utilization during coronary artery bypass surgery using an electrode and observed an overshoot of tissue oxygenation. However, there were no data showing the mechanisms leading to this hyperoxygenation status. Tanoue et al. (43) measured the unloaded myocardial oxygen consumption using an electrode in an in vivo sheep model and observed an increase in tissue oxygen consumption; however, there were no data showing the myocardial tissue oxygenation. We have previously measured in vivo myocardial tissue PO2 using EPR oximetry and observed a hyperoxygenation status after I/R. The hyperoxygenation status was caused by endothelium-induced NO and its derivatives (55). However, questions still remain regarding the alterations in myocardial oxygenation during IPC and the effects of IPC on in vivo oxygenation during subsequent regional I/R.

Recently, we have developed methods suitable for noninvasive measurement of myocardial oxygenation and redox status in living mice utilizing in vivo EPR spectroscopy with oxygen-sensitive microcrystals and redox probes such as PCA (55, 56). The EPR oximetry technique has been applied to measure tissue oxygenation in a broad range of disease applications. In the current work, EPR oximetry was performed using a highly characterized oxygen-sensitive spin probe (LiPc) that has been demonstrated to be a very powerful in vivo probe for measuring tissue PO2 (19, 25, 41, 55). Once LiPc is implanted, the technique is noninvasive, and tissue PO2 can be measured repeatedly for hours or even days. This technique in conjunction with Doppler determination of blood flow can be used to assess alterations in tissue oxygenation and oxygen consumption in vivo (55).

In the current study, we investigated the effects of IPC on myocardial oxygenation, perfusion, redox status, and mitochondrial function in the postischemic heart. After I/R in IPC hearts, regional blood flow was increased compared with that in non-IPC hearts. The increased blood flow after reperfusion in the preconditioned hearts could be because of adenosine-mediated vasodilation, since it is known that IPC stimulates adenosine formation (5). The enhanced flow could also be because of decreased cellular swelling with prevention of microvascular occlusion and no-reflow in IPC hearts. Measurements of the in vivo redox status in the preconditioned and nonpreconditioned hearts demonstrated that IPC maintains the redox status of the reperfused heart at preischemic levels. This prevention of the shift to a more oxidized state confirms that IPC attenuates oxidant stress. Tissue PO2 overshot preischemic baseline values in non-IPC hearts, but this overshoot was not seen with IPC. This overshoot with higher levels of myocardial oxygenation in postischemic myocardium suggested that oxygen consumption is decreased by the process of I/R injury. These observations are consistent with prior reports that ROS/RNS formed upon postischemic reperfusion inhibit mitochondrial oxygen consumption and electron transport (40, 55). When NO formation was diminished as in the eNOS−/− or 1-NAME-treated preconditioned groups, the overshoot of tissue PO2 diminished after reperfusion as reported previously (55). These data confirmed that the postischemic hyperoxygenation in the wild-type control heart was induced by endothelium-derived NO and its derivatives. Furthermore, we observe that inhibition of NO formation by eNOS−/− or inhibition of the enzyme did not further alter myocardial oxygenation in IPC.
hearts, and similar levels of reoxygenation were observed. This is consistent with prior reports that IPC results in inhibition of NO formation from NOS (15, 51).

In our current study, we have observed for the first time in the regional I/R mouse heart model that IPC attenuates in vivo myocardial hyperoxygenation status in the postischemic myocardium. The attenuation of the hyperoxygenation after regional I/R may have profound effect on postischemic cardiac remodeling, since oxygenation is a key mediator of the development and maturation of cardiac myocytes (37). Furthermore, the higher blood flow and lower PO2 with IPC indicates that IPC attenuates postischemic myocardial hyperoxygenation and preserves higher levels of O2 consumption in postischemic myocardium. We also observed that IPC prevented the loss of the activities of the critical mitochondrial electron transport enzymes NADH-DH and CcO.

It has been previously demonstrated that ONOO⁻ is formed in postischemic myocardium from the reaction of NO and O2⁻ and results in tyrosine nitration (44, 50). We observed in our studies with the regional I/R mouse heart model that nitrotyrosine formation in postischemic myocardium is increased and that this is greatly diminished in ischemic preconditioned hearts after I/R. Thus our data suggest that IPC attenuates ROS/RNS formation, and this may contribute to the preservation of myocardial oxygen consumption and mitochondrial function leading to the attenuation of hyperoxygenation after I/R. This regional improvement of tissue oxygen consumption in IPC hearts was seen on histopathology to be associated with markedly decreased myocardial infarction.

It was observed that, while the levels of expression of NADH-DH and CcO were unchanged after I/R, their activities were significantly decreased. This I/R-induced downregulation of the function of these mitochondrial enzymes is consistent with a prior report and the fact that these have been shown to be sensitive to oxidant-induced modification and denaturation (42, 55). IPC abolished this loss of mitochondrial function. Therefore, our results suggest that IPC decreased formation of NO and O2⁻ during subsequent postischemic reperfusion, and this protected the myocardium at risk and preserved mitochondrial function.

There are redundant signaling pathways for the protection afforded by IPC; some are linear and some are branching and converging (31). The mechanisms demonstrated in our study, possibly together with other aforementioned receptor-mediated protective mechanism(s), led to a decrease in the infarct size after I/R as demonstrated by the decreased infarct size seen on TTC staining in IPC + I/R vs. I/R mice. The attenuation of pathological ROS/RNS production with preconditioning after I/R is one of the mechanisms by which mitoKATP channel openers seem to be acting to protect against myocardial injury (31, 33). Thus there may be one or more interconnections between the processes of altered ROS/RNS or oxygen metabolism and other critical molecular pathways of IPC-induced protection.

Conclusions. In conclusion, our study suggests that IPC attenuates in vivo postischemic myocardial hyperoxygenation by improving myocardial oxygen consumption and decreasing ROS/RNS generation after regional I/R, which in turn preserves mitochondrial function and electron transport enzyme activity. This preservation of mitochondrial function and oxygen consumption contributes to myocardial protection. Further studies of the detailed mechanisms by which IPC induces suppression of pathological ROS/RNS formation and how this process relates to other pathways of protection are warranted.

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