Intermittent hypobaric hypoxia improves postischemic recovery of myocardial contractile function via redox signaling during early reperfusion

Zhi-Hua Wang,1 Yi-Xiong Chen,1 Cai-Mei Zhang,1 Lan Wu,1 Zhuo Yu,1 Xiao-Long Cai,1 Yi Guan,1 Zhao-Nian Zhou,3 and Huang-Tian Yang1,2
1Key Laboratory of Stem Cell Biology and Laboratory of Molecular Cardiology, Institute of Health Sciences, Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences (CAS), and Shanghai Jiao Tong University School of Medicine (SJTUSM), Shanghai; 2Shanghai Key Laboratory of Vascular Biology, Ruijin Hospital, SJTUSM, Shanghai; and 3Laboratory of Hypoxic Cardiovascular Physiology, SIBS, CAS, Shanghai, China
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EARLY REPERFUSION during evolving myocardial infarction is essential for saving the myocardium, but lethal reperfusion injury can occur and limit the beneficial effects (49). A number of cardioprotective strategies have been developed to ameliorate or retard the irreversible injury. However, the clinical translation of these strategies has failed to achieve the anticipated results (13, 34). Intermittent hypobaric hypoxia (IHH) has been shown to protect the heart against ischemia-reperfusion (I/R) injury by improving the manifestations including contractile dysfunction (3, 33), arrhythmias (31, 52), and cell death (8, 27). Recently, we (48) revealed a therapeutic effect of IHH on permanent coronary artery ligation-induced myocardial infarction by attenuating infarct size, myocardial fibrosis, and apoptosis and improving cardiac performance. Because IHH is a relatively simple intervention with a longer protection duration and fewer adverse effects and may offer profound benefit to patients with acute myocardial infarction (3, 37), elucidating the mechanistic insights underlying the cardioprotective effects of IHH is critical to potential clinical applications.

Excessive generation of ROS during the early phase of reperfusion after myocardial ischemia has been proposed to contribute to reperfusion injury (2, 45, 55). Paradoxically, ROS generated at the same phase also act as signaling molecules, triggering the cardioprotection induced by ischemic or pharmacological conditioning (4, 16, 18, 36, 43). Mitochondrial permeability transition pore (MPTP) opening is a crucial event in lethal reperfusion injury and is regulated by glycogen synthase kinase (GSK)-3β activity (20, 21, 23). Activation of reactive oxygen species; ischemia-reperfusion injury

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Fig. 1. Effects of intermittent hypobaric hypoxia (IHH) on body weight (A) and heart weight (B) of rats. Left ventricle (LV)-to-body weight and right ventricle (RV)-to-body weight ratios are shown. n = 46.
prosurvival signaling pathways [in particular, PKB (Akt) and PKC-ε] relies on the generation of ROS in the protected myocardium (14, 15, 41) and converge on the inhibition of GSK-3β activity by phosphorylation of Ser9 (23). It has been suggested that the concentration of ROS determines their different roles, i.e., ROS are cardioprotective at low levels but detrimental at high levels (6, 39). However, this theory is still clouded by the failure of a number of animal studies and most clinical studies with antioxidants to convey cardioprotection (24, 29, 42). An obvious increase of ROS in cardiomyocytes during early reperfusion after ischemia has been observed by several groups (24, 46, 55), but whether such an increase is already excess to cardiomyocytes remains uncertain. It also remains a debate whether the increase of ROS directly relates to cell injury (24, 42).

IHH increases myocardial oxidative stress during the intervals of hypoxia and reoxygenation. Treatment with the antioxidant N-acetylcysteine during exposure to IHH partially reduced the infarct size-limiting effect of IHH in the rat heart (26). However, it is unknown how IHH influences ROS production during myocardial I/R and whether ROS generated at this phase play a role in IHH-induced cardioprotection. To address these questions, we examined the effects of IHH on ROS generation during I/R in isolated perfused rat hearts and isolated cardiomyocytes and determined the roles of ROS as well as downstream signaling pathways in IHH-induced cardioprotection. Our results demonstrate that elevated ROS generation during early reperfusion is critical for triggering the cardioprotection induced by IHH. Our findings provide new insights into the

Fig. 2. Role of ROS generated during early reperfusion in the IHH-improved postischemic recovery of myocardial contractile function. A: representative traces of LV pressure (LVP) during ischemia-reperfusion (I/R) in isolated rat hearts from normoxic and IHH groups. H2O2 (20 μmol/l) and the ROS scavengers N-(2-mercaptopropionyl)glycine (MPG; 100 μmol/l) and manganese (III) tetrakis (1-methyl-4-pyridyl)porphyrin (MnTMPyP; 10 μmol/l) were added at the beginning of reperfusion for 5 min followed by washout, as indicated by the solid and open arrowheads, respectively. B–E: summarized effects of H2O2, MPG, and MnTMPyP on postischemic recovery of LV developed pressure (LVDP), LV end-diastolic pressure (LVEDP), and maximum rates of pressure development or decay over time (+dP/dt max and −dP/dt max) in normoxic and IHH groups. Experimental numbers are indicated in each bar. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the corresponding normoxic group; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. the corresponding I/R control.
explanation of the controversial roles of ROS in myocardial I/R injury and cardioprotection.

**MATERIALS AND METHODS**

**Animal care.** The animals used in this study were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23), and all procedures were approved by the Institutional Review Board of the Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and School of Medicine, Shanghai Jiao Tong University (Shanghai, China).

**IHH-adapted rat model.** To establish animal models adapted to IHH, male Sprague-Dawley rats (Shanghai Slac Laboratory Animal, Shanghai, China) were intermittently exposed to hypobaric hypoxia (equivalent to an altitude of 5,000 m, barometric pressure: 404 mmHg, PO2: 84 mmHg) in a hypobaric chamber for one 4-h period each day for 4 wk as previously described (1, 51). During this period, their body weights rose from 100–120 to 310–360 g. Age-matched normoxic animals were maintained in a normoxic environment for a corresponding period. All animals had free access to water and standard laboratory diet.

**I/R injury model in Langendorff-perfused rat hearts.** After rats have been anesthetized with pentobarbital sodium (45 mg/kg ip), hearts were rapidly excised and perfused with Krebs-Henseleit (K-H) solution at 37°C using a Langendorff apparatus at a constant pressure of 80 mmHg as previously described (54). A water-filled latex balloon connected to a pressure transducer (Gould P23Db, AD Instruments, Sydney, NSW, Australia) was inserted into the left ventricular (LV) cavity to achieve a stable LV end-diastolic pressure (LVEDP) of 5–10 mmHg during initial equilibration. After equilibration perfusion, the heart was subjected to 30 min of global noflow ischemia followed by 45 min of reperfusion. LV developed pressure (LVDP) and maximum rates of pressure development or decay over time (+dP/dt max and −dP/dt max) were evaluated with the PowerLab system (AD Instruments).

**Experimental protocols.** H2O2 (20 μmol/l) was perfused at the beginning of reperfusion for 5 min to examine the effects of exogenous ROS on the posts ischemic recovery of myocardial performance in normoxic and IHH groups. The ROS scavengers N-(2-mercaptoptooyl)glycine (MPG; 100 μmol/l, Sigma-Aldrich, St. Louis, MO) and manganese (III) tetrakis (1-methyl-4-pyridyl)porphyrin (MnT-MPyP; 10 μmol/l, Merck, Darmstadt, Germany) were perfused at the beginning of reperfusion for 5 min. The phosphoinositide 3-kinase (PI3K) inhibitor wortmannin (300 nmol/l, Millipore) or the PKC-ε inhibitor εV1-2 (10 μmol/l, Anaspec) was perfused for 5 min with a 5-min washout before ischemia. The mitochondrial ATP-sensitive K+ (KATP) channel inhibitor 5-hydroxydecanoate (5-HD; 200 μmol/l) was perfused during the last 5 min of ischemia and the first 5 min of reperfusion.

**ROS production in isolated cardiomyocytes during simulated I/R.** ROS detection in isolated cardiomyocytes during simulated I/R was detected using 5(−and 6)−carboxy-2′,7′−dichlorodihydrofluorescein diacetate (DCF; Invitrogen) as previously described (17, 46). Briefly, cardiomyocytes from normoxic or IHH groups were loaded with DCF (20 μmol/l) for 10 min before being plated on the dish. The acetate groups of the probe can be removed by intracellular esterases, which allow it to be retained by the cells. Because DCF is nonfluorescent until it is oxidized by ROS within the cell, the intracellular generation of ROS can be reflected by monitoring the increase in DCF fluorescence.

**Infarct size estimation.** To determine whether IHH and MPG affect the irreversible cell injury after I/R, isolated rat hearts subjected to 30 min of ischemia followed by 2 h of reperfusion were frozen, and the LV was cut into 2-mm-thick slices. Sections were then incubated in 1% (wt/vol) triphenyltetrazolium chloride (phosphate buffer, pH 7.4) for 15 min for staining (54). Slices were fixed in 10% formaldehyde to enhance the contrast between stained viable and unstained necrotic tissue. Infarct size was calculated using Image Pro Plus software (MediaCybernetics), and the infarct area was expressed as a percentage of the LV area at risk.

**Fig. 3. Effects of IHH and MPG on cell injury during myocardial I/R.** A: lactate dehydrogenase (LDH) activity was measured with coronary effluent collected at baseline, at 5 min of reperfusion (R5), and at 45 min of reperfusion (R45). n = 3. B: infarct size determined with triphenyltetrazolium chloride was expressed as a percentage of the LV area at risk from isolated hearts subjected to 30 min of ischemia followed by 2 h of reperfusion. n = 5. *P < 0.05 and **P < 0.01 vs. the corresponding normoxic group; #P < 0.05 and ##P < 0.01 vs. the corresponding IHH group.
Fluorescence was detected using an inverted microscope (Nikon, Tokyo, Japan) and recorded (excitation/emission: 495/525 nm) every 5 min during I/R with a fixed field of view including 30–50 rod-shaped cardiomyocytes. The excitation light source was set at low power to avoid inducing oxidation and opened only when it was needed to get a photo. Fluorescence intensity was analyzed with Image Pro Plus software.

**Western blot analysis.** Proteins were prepared as previously described (8). Briefly, freeze-clamped LV tissue (200–300 mg) was homogenized in 10 volumes of lysis buffer containing 20 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl, 2.5 mmol/l EDTA, 50 mmol/l NaF, 0.1 mmol/l Na2HPO4, 1 mmol/l Na3VO4, 1 mmol/l PMSF, 1 mmol/l DTT, 0.2% (vol/vol) protease inhibitor cocktail (Sigma-Aldrich), 1% (vol/vol) Triton X-100, and 10% (vol/vol) glycerol. Homogenates were centrifuged twice at 20,000 g for 15 min, and supernatants were saved as total proteins. To separate membrane and cytosolic fractions, LV tissue was homogenized in lysis buffer containing (in mmol/l) 20 Tris-HCl (pH 7.4), 250 sucrose, 1 EDTA, 1 EGTA, 1 NaF, 1 Na3VO4, 1 PMSF, and 1 DTT with 0.2% (vol/vol) protease inhibitor cocktail and centrifuged at 1,000 g for 10 min at 4°C. The supernatant was centrifuged at 10,000 g for 30 min at 4°C, and the pellet was washed once with lysis buffer by centrifugation, resuspended with 0.5% Triton X-100 in lysis buffer, sonicated on ice, and then centrifuged at 20,000 g for 10 min at 4°C. The resultant supernatant was defined as the membrane fraction. The supernatant after the membrane fraction had been pelleted was centrifuged at 100,000 g for 1 h at 4°C, and the resultant supernatant was defined as the cytosolic fraction. Triton X-100 was added at a final concentration of 0.5%. Protein concentrations were determined by the BCA method.

Equal amounts of proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Western blot analysis was performed under standard conditions with specific antibodies including anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-PKC-ε (Ser202), anti-PKC-ε, anti-phospho-GSK-3β (Ser9), and anti-GSK-3β. Antibodies were purchased from Cell Signaling. The immunoreaction was visualized using an enhanced chemiluminescent detection kit (Amersham, London, UK), exposed to X-ray film, and quantified by densitometry with a video documentation system (Gel Doc 2000, Bio-Rad).

**Protein oxidation and lipid peroxidation.** To evaluate the effect of IHH on oxidative stress during I/R, we measured protein carbonyls and malondialdehyde (MDA), respective products of protein oxidation and lipid peroxidation (22, 24), in LV tissue homogenated in protein lysis buffer without DTT. Protein carbonyls were measured using an immunoblot kit to detect the 2, 4-dinitrophenylhydrazine (DNPH) derivatization of protein carbonyls following the manufacturer’s instruction (Cell Biolabs). MDA content was determined by the thiobarbituric acid reaction as previously described (24).

**Statistical analysis.** Data are expressed as means ± SE. Significant differences between two mean values were estimated using Student’s t-test. For multiple comparisons, ANOVA or repeated ANOVA followed by a least-significant-difference post hoc test was used. All statistics were done using SPSS software (version 13.0, SPSS, Chicago, IL). P values of <0.05 were considered statistically significant.

**RESULTS**

**IHH improves the postischemic recovery of myocardial contractile function and cell survival.** IHH-adapted rats showed comparable body weights compared with normoxic controls (Fig. 1A). LV-to-body weight and right ventricle-to-body weight ratios were slightly increased after exposure to IHH for 4 wk but did not reach statistic significance (Fig. 1B). In Langendorff-perfused rat hearts, LV contractile function was markedly suppressed after 30 min of no-flow ischemia followed by 45 min of reperfusion (Fig. 2A). IHH did not affect baseline LV contractile function but significantly improved the postischemic recovery of LVDP, LVEDP, +dP/dt max, and –dP/dt max compared with the normoxic group (Fig. 2, B–E). Consistently, IHH significantly inhibited the I/R-induced robust increase of LDH activity in coronary perfusate at 5 and 45

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**Fig. 4. Effects of IHH and 5-hydroxydecanoate (5-HD) on myocardial protein oxidation and lipid peroxidation during I/R.** A: representative immunoblot of 2,4-dinitrophenylhydrazine (DNPH) derivatization of protein carbonyls, a product of protein oxidation, in the LV during I/R in normoxic and IHH groups. Parallel gels were stained with Coomassie brilliant blue R250 dye as a control for protein loading. B: averaged data of protein oxidation shown as the DNPH signal over Coomassie blue stain. C: lipid peroxidation as assessed by melondialdehye (MDA) content normalized against the protein concentration in the LV during I/R. n = 3. The mitochondrial ATP-sensitive K+ (KATP) channel inhibitor 5-HD (200 μmol/l) was added during the last 5 min of baseline perfusion and first 5 min of reperfusion. *P < 0.05 and **P < 0.01 vs. the corresponding normoxic group; #P < 0.05 and ###P < 0.01 vs. the corresponding IHH group; †P < 0.05, ††P < 0.01, and †††P < 0.001 vs. the corresponding baseline.
min of reperfusion (Fig. 3A) and attenuated the I/R-induced myocardial infarct size after 2 h of reperfusion (20.5 ± 5.3% in the IHH group vs. 42.1 ± 3.8% in the normoxic group, *P < 0.01; Fig. 3B), suggesting that IHH protects the heart against I/R injury.

**IHH increases ROS production during early reperfusion.** To determine the effect of IHH on ROS, we examined myocardial protein oxidation and lipid peroxidation by measuring protein carbonyls and MDA contents during I/R. DNPH derivatization of myocardial protein carbonyls and MDA content were increased after reperfusion (Fig. 4). Compared with the normoxic group, IHH significantly increased the baseline level of protein carbonyls and further enhanced the I/R-induced increase of DNPH at the first 5 min of reperfusion (Fig. 4, A and B). MDA content was also significantly enhanced by IHH at the first 5 min of reperfusion (Fig. 4C).

To directly confirm the influence of IHH on ROS, we then monitored ROS production in isolated cardiomyocytes subjected to simulated I/R (20/30 min) using DCF fluorescence. Fluorescence intensity was significantly enhanced during reperfusion in cardiomyocytes isolated from normoxic rat hearts (Fig. 5A). DCF fluorescence intensity during baseline perfusion and the ischemic phase was similar between IHH and normoxic groups, but IHH markedly augmented the fluorescence intensity during reperfusion, especially during the first 5 min (Fig. 5A). Such increases were abolished by the ROS scavenger MPG (100 μmol/l; Fig. 5A). These data suggest that IHH enhanced ROS production during early reperfusion in the ischemic heart.

**Mitochondrial K<sub>ATP</sub> channels contribute to IHH-increased ROS production.** To determine whether the increased ROS production during early reperfusion by IHH is derived from the mitochondria through the opening of mitochondrial K<sub>ATP</sub> channels, we examined the effects of the mitochondrial K<sub>ATP</sub> inhibitor 5-HD (200 μmol/l) on ROS production and IHH-induced cardioprotection. 5-HD did not alter I/R-increased protein carbonyls and MDA content in the normoxic group, but it attenuated IHH-increased protein carbonyls and MDA content at the first 5 min of reperfusion (Fig. 4). In isolated cardiomyocytes, 5-HD attenuated ROS production during reperfusion and blocked the IHH-enhanced increase of ROS production (Fig. 5B). These data support that the opening of the mitochondrial K<sub>ATP</sub> channel contributes to IHH-increased ROS production during early reperfusion.

**IHH-induced cardioprotection depends on ROS generation during early reperfusion.** Although it has been suggested that the ROS generated during early reperfusion participate in cardioprotection induced by ischemic or pharmacological conditioning (4, 16, 36, 43), little is known about the effect of introducing exogenous ROS into the heart during early reperfusion. To further determine the role of increased ROS during early reperfusion in IHH-induced cardioprotection, we treated hearts with H<sub>2</sub>O<sub>2</sub> and ROS scavengers during the first 5 min of reperfusion and examined their effects on the posts ischemic recovery of contractile function. Postconditioning with H<sub>2</sub>O<sub>2</sub> (20 μmol/l) significantly improved the posts ischemic recovery of LVDP, LVEDP, +dP/dt max, and −dP/dt max in the normoxic group but did not affect those functional parameters in the IHH group (Fig. 2). However, scavenging ROS with MPG (100 μmol/l) and MnTMPyP (10 μmol/l) completely blocked the IHH-improved posts ischemic recovery of LV contractile function without changes in the normoxic group (Fig. 2). Moreover, IHH-reduced LDH activity and myocardial infarct size were significantly reversed by MPG (Fig. 3). Consistently, inhibition of mitochondrial K<sub>ATP</sub> channels with 5-HD also abolished IHH-improved LV performance after I/R (Fig. 6). These data reveal that the increased ROS during early reperfusion are critical for IHH-induced cardioprotection.

**ROS mediates the IHH-enhanced activation of protective signaling after I/R.** To understand the underlying signaling pathways, we next examined the phosphorylation of Akt (Ser<sup>473</sup>), PKC-ε (Ser<sup>206</sup>), and GSK-3β (Ser<sup>9</sup>), the putative last step of the cytoplasmic cardioprotective signaling cascade (23). Compared with the normoxic group, IHH significantly increased the phosphorylation levels of Akt (Fig. 7A), PKC-ε (Fig. 7B), and GSK-3β (Fig. 7C) after I/R. Such effects were not additive to the enhancement induced by H<sub>2</sub>O<sub>2</sub> (20 μmol/l) but were completely blocked by the ROS scavengers MPG and MnTMPyP (Fig. 7, A–C). To further confirm the activation of PKC-ε by IHH, we then measured the translocation of PKC-ε by Western blot analysis. IHH significantly promoted the
translocation of PKC-ε from the cytosolic fraction to the membrane fraction in the LV after I/R. Such an effect was not additive to that of H₂O₂ but was blocked by MPG and MnT-MPyP (Fig. 7D). These results suggest that IHH activates protective signaling pathways via enhancement of ROS generation during early reperfusion.

**Activation of Akt and PKC-ε contributes to IHH-induced cardioprotection.** To confirm the contribution of Akt and PKC-ε pathways in IHH-induced cardioprotection, we then examined the effects of wortmannin and εV1-2, respective inhibitors of PI3K (the upstream activator of Akt) and PKC-ε, on the posts ischemic recovery of LV contractile function. Treatment with wortmannin (300 μmol/l) or εV1-2 (10 μmol/l) did not affect the posts ischemic recovery of LVDP, LVEDP, +dP/dt max, and −dP/dt max in normoxic and IHH groups. n = 5. **P < 0.01 vs. the corresponding normoxic group; #P < 0.05 and ##P < 0.01 vs. the corresponding I/R control.

Interestingly, the IHH-increased Akt phosphorylation after I/R was inhibited by the PKC-ε inhibitor, and vice versa (Fig. 9, A and B). Consistently, the IHH-promoted translocation of PKC-ε to the membrane fraction was blocked by both εV1-2 and wortmannin (Fig. 9D). These data suggest that Akt and PKC-ε pathways may form a positive feedback loop and mediate IHH-induced ROS-dependent cardioprotection.

**DISCUSSION**

In this study, we demonstrated, for the first time, that adaptation of rats to IHH confers protection of the heart against I/R injury through elevation of ROS production during early reperfusion. A moderate and sufficient increase of ROS during early reperfusion is required to efficiently activate the downstream protective signaling pathways, whereas the endogenous ROS generated during early reperfusion in I/R rat hearts appear to be insufficient to trigger efficient cardioprotection.
Enhancement of ROS generation during early reperfusion is critical to IHH-induced cardioprotection. ROS have been shown to participate in cardioprotection induced by ischemic or pharmacological conditioning (4, 16, 18, 36, 43). However, how ROS perform the protective function at the reperfusion phase rather than inducing injury is controversial. Our data showed that IHH increases ROS generation during early reperfusion (Figs. 4 and 5), and this was accompanied with an antioxidant-sensitive improvement of myocardial contractile function (Fig. 2) and a reduction of cell damage (Fig. 3). The fact that postconditioning with H2O2 (20 μmol/l) during the first 5 min of reperfusion improved the postischemic recovery of myocardial contractile function (Fig. 2) confirms that the endogenous ROS generated during early reperfusion in I/R hearts are insufficient to trigger cardioprotection. Otherwise, an aggravated injury should be observed if ROS are already excessively produced during this phase. This is further supported by the observations of Ytrehus et al. (50) showing that treatment with H2O2 during the first 30 min of reperfusion reduces myocardial infarct size after I/R injury in a coronary artery occlusion model. Although a lower concentration (1 μmol/l) of H2O2 was used in their study, the much longer treatment time (30 min) makes it explicable as the activation of protective signaling pathways by ROS accumulates with time (44).

Guo et al. (12) found that IHH upregulated the expression of antioxidant enzymes after myocardial I/R injury in guinea pigs, whereas we did not observed a reduction of oxidative stress in rat hearts. Besides the difference in species, this may be due to a more significant generation of ROS in IHH-protected hearts. Ischemic preconditioning and postconditioning have been reported to attenuate oxidative stress during reperfusion (46, 53). However, the repetitive episodes in those procedures lead to cyclic release of ROS (46) that may alter the quantitative threshold for ROS to trigger cardioprotection. In the present model, we provided direct evidence showing that a moderate increase of ROS generation during early reperfusion in cardiomyocytes from IHH-adapted rats is sufficient to activate protective signaling and trigger cardioprotection. This is supported by observations showing that scavenging ROS generation during early reperfusion abolishes the cardioprotection induced by ischemic postconditioning (4, 36) as well as by ischemic preconditioning (16), indicating that the early phase of reperfusion is crucial for ROS to convey cardioprotection.

**Fig. 7.** ROS mediate the IHH-increased activation of protective signaling pathways. A–C: Western blot analysis of total and phosphorylation levels of PKB (Akt), PKC-ε, and glycogen synthase kinase (GSK)-3β during I/R in LVs from normoxic and IHH groups. D: Western blot analysis of PKC-ε expression in membrane and cytosolic fractions of the LV from normoxic and IHH groups. PKC-ε translocation is presented as the ratio of the particulate fraction to the cytosolic fraction. H2O2 (20 μmol/l), MPG (100 μmol/l), and MnTMPyP (10 μmol/l) were added at the beginning of reperfusion for 5 min. n = 4 each. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. the corresponding normoxic group; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. the corresponding I/R control; †P < 0.05, ††P < 0.01, and †††P < 0.001 vs. the corresponding balance (Bal) group.
Mitochondria as the main source of ROS in IHH-induced cardioprotection. Mitochondria represent a main source of ROS generation in cardiomyocytes (45). It has been shown that more ROS are generated in isolated mitochondria during re-oxygenation after hypoxia than after anoxia, suggesting that the mitochondria-derived ROS rely to a certain extent on mitochondrial function (28). We (47, 54) have reported previously that IHH effectively protects mitochondrial structure and function after myocardial I/R injury. Moreover, the abolishment of the IHH-induced increase in ROS production and cardioprotection by the mitochondrial KATP channel inhibitor 5-HD (Figs. 4, 5B, and 6) further demonstrates that mitochondria-derived ROS contribute to IHH-induced cardioprotection. This is consistent with observations showing that the activation of mitochondrial K\textsubscript{ATP} channels increases ROS generation, which subsequently activates prosurvival signaling pathways and inhibits MPTP opening during myocardial I/R (6, 18, 35, 38). However, mitochondrial K\textsubscript{ATP} channels might not participate in the cardioprotection of right ventricular function induced by chronic hypoxia in rats (9). Other ROS-producing enzymes, e.g., NADPH oxidase and xanthine oxidase, have also been proposed to participate in cardioprotection (10, 25). Whether this mechanism contributes to IHH-induced cardioprotection needs to be investigated.

ROS trigger the cardioprotection of IHH via the activation of Akt and PKC-ε pathways. Of note, we found that the increased ROS generation during early reperfusion is essential for IHH-induced activation of Akt and PKC-ε pathways, suggesting a quantitative threshold of ROS in the efficient activation of these protective signaling pathways. Myocardial I/R has been shown to induce a noticeable activation of Akt and PKC-ε, which could be further enhanced in the protected myocardium (23, 30, 32). We also observed that adaptation of rats to IHH enhanced the I/R-increased phosphorylation of Akt...
and PKC-ε in LVs (Fig. 7, A and B). Considering their sensitivity to antioxidants, as confirmed by our data (Fig. 7A and 7B) and those of others (14, 15, 41), it is persuasive that ROS production during early reperfusion in I/R cardiomyocytes is lower than the threshold to effectively activate the signaling pathways responsible for cardioprotection. Moreover, as downstream signaling pathways of ROS, Akt and PKC-ε form a positive feedback loop in the IHH-protected myocardium (Fig. 9, A, B, and D). This may explain the quantitative threshold of ROS to confer cardioprotection.

Three programs of protein kinases have been suggested to be involved in the cardioprotection induced by ischemic preconditioning and postconditioning by Heusch et al. (19). In the first program, nitrogen oxide is a central step with the upstream activation of PI3K/Akt and downstream activation of mitochondrial KATP channels and inhibition of MPTP. The second program involves the activation of PI3K/Akt and the ERK system with the downstream inhibition of GSK-3β/H9252, although this pathway varies with species, e.g., it seems not to participate in ischemic preconditioning-induced protection of pig hearts (40). Activation of signal transducer and activator of transcription 3 (STAT3) constitutes the third program. A positive feedback loop between Akt and STAT3 has been shown to mediate opioid-induced cardioprotection (11). Whether STAT3 contributes to IHH-induced cardioprotection needs to be determined.

**Potential clinical significance.** Although a number of cardioprotective strategies have been discovered, their clinical translation is disappointing, probably because of the inadequacy of animal I/R models to simulate what happens at the bedside (13, 34). IHH appears to be a promising therapeutic strategy for coronary heart disease due to its easier manipulation, longer protection duration, and fewer adverse effects (3, 37, 48). Moreover, we (48) have shown that IHH has a significant therapeutic effect after 1 wk of coronary artery occlusion. IHH has also been demonstrated to improve myocardial perfusion in patients with severe coronary heart disease (7). As a relatively simple intervention, IHH may offer profound benefits to patients with acute myocardial infarction upon the elucidation of protective mechanisms (37). Considering the failure of clinical usage of antioxidants (29), understanding the precise regulation of ROS generation and subsequent signaling pathways in IHH is of clinical significance.

Taken together, our data demonstrate that IHH confers cardioprotection against I/R injury by enhancing the production of ROS during early reperfusion, which subsequently...
activate downstream protective signaling pathways, Akt and PKC-ε. These results also suggest that the endogenous ROS generated during early reperfusion in I/R hearts seem not reach the threshold to trigger efficient cardioprotection. Our findings provide a new angle to interpret the controversial roles of ROS.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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