Activation of $\alpha_{1B}$-adrenoceptors contributes to intermittent hypobaric hypoxia-improved postischemic myocardial performance via inhibiting MMP-2 activation

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1Key Laboratory of Stem Cell Biology and Laboratory of Molecular Cardiology, Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and Shanghai Jiao Tong University School of Medicine Shanghai, China; and 2Institute of Vascular Medicine, Peking University Third Hospital and Key Laboratory of Molecular Cardiovascular Sciences Ministry of Education, Beijing, China

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Gao L, Chen L, Lu ZZ, Gao H, Wu L, Chen YX, Zhang CM, Jiang YK, Jing Q, Zhang YY, Yang HT. Activation of $\alpha_{1B}$-adrenoceptors contributes to intermittent hypobaric hypoxia-improved postischemic myocardial performance via inhibiting MMP-2 activation. Am J Physiol Heart Circ Physiol 306: H1569–H1581, 2014. First published April 4, 2014; doi:10.1152/ajpheart.00772.2013.—Inhibition of matrix metalloproteinases-2 (MMP-2) activation renders cardioprotection from ischemia/reperfusion (I/R) injury; however, the signaling pathways involved have not been fully understood. Intermittent hypobaric hypoxia (IHH) has been shown to enhance myocardial tolerance to I/R injury via triggering intrinsic adaptive responses. Here we investigated whether IHH protects the heart against I/R injury via the regulation of MMP-2 and how the MMP-2 is regulated. IHH (PO$_2$ = 84 mmHg, 4-h/day, 4 wk) improved postischemic myocardial contractile performance, lactate dehydrogenase (LDH) release, and infarct size in isolated perfused rat hearts. Moreover, IHH reversed I/R-induced MMP-2 activation and release, disorders in the levels of MMP-2 regulators, peroxynitrite (ONOO$^-$) and tissue inhibitor of metalloproteinase-4 (TIMP-4), and loss of the MMP-2 targets α-actin and troponin I. This protection was mimicked, but not augmented, by a MMP inhibitor doxycycline and lost by the $\alpha_1$-adrenoceptor (AR) antagonist prazosin. Furthermore, IHH increased myocardial $\alpha_{1A}$-AR and $\alpha_{1B}$-AR density but not $\alpha_{1D}$-AR after I/R. Concomitantly, IHH further enhanced the translocation of PKC epsilon (PKCe) and decreased the release of mitochondrial cytochrome $c$ due to I/R via the activation of $\alpha_{1B}$-AR but not $\alpha_{1A}$-AR or $\alpha_{1D}$-AR. IHH-conferred cardioprotection in the postischemic contractile function, LDH release, MMP-2 activation, and nitrotyrosine as well as TIMP-4 contents were mimicked but not additive by $\alpha_1$-AR stimulation with phenylephrine and were abolished by an $\alpha_{1B}$-AR antagonist chloroethylclonidine and a PKCe inhibitor PKCε V1–2. These findings demonstrate that IHH exerts cardioprotection through attenuating excess ONOO$^-$ biosynthesis and TIMP-4 loss and sequential MMP-2 activation via the activation of $\alpha_{1B}$-AR/PKCe pathway.

intermittent hypobaric hypoxia; ischemia/reperfusion injury; matrix metalloenzymes-2; $\alpha_1$-adrenoceptors; mitochondria
by IHH. Moreover, cardiac sympathetic stimulation and α1-AR activation have been shown to play an important role in the cardioprotective effect of IHH (35, 56), while it is unclear whether IHH exerts cardioprotection against MMP-2 activation via α1-ARs. Considering a critical role of MMP-2 in the proteolysis of myofilament, such as troponin I (TnI) and α-actinin (2, 4, 47, 54), we hypothesized that the inhibiting of MMP-2 activation might contribute to the chronic IHH-confirmed cardioprotection against I/R injury through the activation of α1-AR.

To address those questions, the present study was designed 1) to determine whether IHH protects the heart via the regulation of MMP-2 activation; 2) to clarify whether α1-ARs regulate MMP-2 and its regulators during cardioprotection against I/R injury; and 3) to identify the signaling pathway implicated in the regulation of MMP-2 during 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 were exposed to hypoxia in a hypobaric chamber (equivalent to an altitude of 5,000 m; barometric pressure = 404 mmHg, PO2 = 84 mmHg) for one 4-h period each day for 4 wk as previously described (58). During this period, their body weight rose from 100–130 g to 310–360 g. Age-matched normoxic animals were maintained in the normoxic environment for a corresponding period. All animals had free access to water and a standard laboratory diet and were raised at room temperature (23–26°C) with a barometric chamber (equivalent to an altitude of 5,000 m; barometric pressure = 404 mmHg, PO2 = 84 mmHg) for one 4-h period each day for 4 wk as previously described (58).

Heart perfusion. The rats were anesthetized with sodium pentobarbital (45 mg/kg ip). The hearts were promptly excised and perfused with Krebs-Henseleit buffer containing the following (in mM): 118 NaCl, 4.7 KCl, 1.2 MgSO4·7H2O, 2.5 CaCl2, 1.2 KH2PO4, 25 NaHCO3, 0.026 Na2EDTA, and 11.1 glucose, gassed with 95% O2–5% CO2 (pH 7.4) at 37°C by using the Langendorff technique under a constant pressure of 80 mmHg as previously described (58, 66). Then, proteins of concentrated samples were measured by the BCA method and balanced to an equal protein concentration by adding Krebs-Henseleit buffer. Twenty-microliter balanced samples (5 μg total proteins) were added to an 80-μl reaction mixture containing MMP fluorescence resonance energy transfer (FRET) peptide substrate [Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2, where Mca is (7-methoxy-coumarin-4-yl) acetyl and Dpa is N-3-(2,4-dinitrophenyl)-L-α,β-diaminopropionyl]. The fluorescence intensity was measured at 2-min intervals for 1 h at 37°C using a SPECTRAMax Gemini XPS fluorescence microplate reader (λex: 328 nm; λem: 393 nm; Molecular Devices). The specific activity of MMP-2/μg protein in the coronary effluent was calculated as the OA-Hy-nonsensitive enzyme activity subtracted from the total activity. One unit of MMP-2 activity was defined as the amount of OA-Hy-sensitive enzyme that hydrolyzes 1 nM of peptide substrate per min at 37°C (pH 7.5).

Western blotting. The total protein fraction was prepared as described previously (58). Briefly, LV tissues were homogenized in lysis buffer containing the following (in mM): 20 Tris, 150 NaCl, 2.5 EDTA, 50 NaF, 0.1 Na3P2O7, 1 Na2VO4, 1 PMSF, 1 DTT, 1 μg/ml leupeptin, and 1% Triton X-100 (pH 7.4, 4°C) using a Polytron PT 1000 homogenizer (Kinetica, Littauer-Lucerne, Switzerland). The homogenate was kept on ice for 30 min and then centrifuged at 10,000 g for 20 min at 4°C, and the supernatant was saved as total protein fraction. For examining PKCε translocation, the membrane and cytosol fractions were separated as previously described (58). LV tissues were homogenized in lysis buffer containing the following (in mM): 20 Tris-HCl (pH 7.4), 250 sucrose, 1 EDTA, 1 EGTA, 1 NaF, 1 Na2VO4, 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 and 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%. For analysis of cytochrome c release, the mitochondrial and cytoplasmic compartments were fractionated as previously described (18). Briefly, frozen LV tissues were homogenized in lysis buffer containing the following (in mM): 20 HEPEs (pH 7.5), 10 KCl, 1.5 MgCl2, 1 EDTA, 1 EGTA, 1 DTT, 0.1 PMSF, 1 leupeptin, and 250 sucrose. The homogenate was centrifuged at 750 g at 4°C. The supernatant was aspirated and centrifuged at 10,000 g at 4°C. The pellet containing the mitochondrial fraction was resuspended in lysis buffer. The superna-
tant was centrifuged at 100,000 g at 4°C to remove any mitochondrial contamination and saved as cytoplasmic compartments. The protein concentrations were determined by BCA method. The specific protein marker and contamination control for each fractions were assayed by immunoblotting.

The standard Western blot was performed as previously described (58). Equal protein fractions were separated by electrophoresis on SDS-PAGE gels. After being transferred to PVDF, proteins were probed with primary specific antibodies [anti-MMP-2, 72 kDa, 1:500, Chemicon, CA; anti-TnI, anti-voltage-dependent anion-selective channel proteins-1 (VDAC-1) and anti-cytochrome c, 1:1,000, Santa Cruz, TX; anti-α-actinin and anti-PKCε, 1:1,000, Sigma; anti-Na+/Ca2+ exchanger-1 (NCX-1), 1:1,000, Abcam, Cambridge, UK; and GAPDH, 1:10,000; KangChen, Shanghai, China] overnight at 4°C. 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, Hercules, CA).

RT-PCR. Total RNA was extracted from LV tissue with Trizol Reagent (Invitrogen, Carlsbad, CA). cDNA was prepared by reverse transcription of 1 μg total RNA using the oligo (dT) primer and ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). PCR was

Fig. 1. Effects of intermittent hypobaric hypoxia (IHH) on the left ventricular (LV) functional performance in isolated rat hearts subjected to 30 min of no-flow global ischemia followed by 30 min of reperfusion with or without the matrix metalloproteinase (MMPs) inhibitor doxycycline (100 μM) and α1-adrenoceptor (α1-AR) antagonist prazosin (1 μM) treatment. A: LV developed pressure (LVDP). B: LV end-diastolic pressure (LVEDP). C: maximal rate of LV pressure development (+dP/dt_{max}). D: maximal rate of LV pressure decline (−dP/dt_{max}). E: heart rate. F: rate-pressure product. Pre, preischemia; R30, 30 min of reperfusion. The number of individual rat hearts per group is indicated in parentheses. **P < 0.01 vs. corresponding preischemic values; #P < 0.05, ##P < 0.01 vs. corresponding normoxic values; †P < 0.05 vs. corresponding I/R control values.
carried out for 30 cycles using Taq DNA Polymerase (Takara, Shiga, Japan) as previously described (67). The following primers were used: MMP-2 sense, 5'-CCCCTATCTACCTACCCAAGACG-3'; MMP-2 antisense, 5'-CATCAGAGGCTCTCCGATGACG-3'; GAPDH sense, 5'-ACGACCCCTTCATTGCACC-3'; and GAPDH antisense, 5'-TGCTTCAACCCTCTCCTG-3'.

Cardiac nitrotyrosine measurement. As a marker of the cardiac ONOO− formation, the nitrotyrosine level of LV tissue extracts at 30 min of reperfusion was measured using an enzyme-linked immunosorbent assay (Cell Biolabs) as previously described (20, 50). Nitrotyrosine content was normalized to protein content of myocardium extracts and expressed as picogram per milligram of protein.

Radioligand binding assay. The preparation of crude LV membrane fraction and radioligand binding assay were performed as described previously (67). The α1-AR antagonist 2-b(4-hydroxyphenyl)-ethylaminomethyl-tetralone (BE2254; Beiersdorf, Hamburg, Germany) was radioiodinated with 125I-Na1+ (Beijing Institute of Atomic Energy, Beijing, China) using the chloramine T method. Then, the membrane preparation (1 mg/ml) was incubated with 125I-BE2254 (2,200 Ci/mM) for 20 min at 37°C in the presence or absence of competing drugs, and the incubation was terminated by 10 mM Tris·HCl. The mixture was filtered, washed, dried, and measured for its radioactivity and protein concentration. Nonspecific binding was determined in the presence of 10 μM of phentolamine (Sigma). To determine the affinity (Kd) and the maximal binding capacity (Bmax) of 125I-BE2254 to cardiac α1-ARs, saturation curves were determined by incubating membrane preparations with increasing concentrations of 125I-BE2254 (35–640 pM, 2.8–52.1 MBq) and the data were analyzed by the method of Scatchard. About 50 pM 125I-BE2254 (4 MBq) was incubated with 100-μl membrane preparations in the presence of 16 concentrations of 5-MU and BMY7378 to gain the competitive binding curve. The Bmax of α1A- and α1D-subtypes were calculated from the percentage of high affinity site of the 5-MU and BMY7378 using Prism software, and the rest of the total Bmax was evaluated as of α1HR-AR subtype.

Statistical analysis. Data are presented as means ± SE. Statistical significance was determined using ANOVA or repeated ANOVA for multiple comparisons or repeated measurements. P < 0.05 was regarded as statistically significant.

RESULTS

IHH improves the postischemic recovery of myocardial performance and cell survival via inhibiting MMP-2 activation. To determine whether IHH protects the heart against I/R injury via the regulation of MMPs, we first examined hemodynamic changes during I/R in Langendorff-perfused rat hearts with or without a MMP inhibitor doxycycline (10). The LV functional performance, characterized by LVDP,
Fig. 3. Effects of IHH on the release, protein, and mRNA levels and sensitive substrates of MMP-2 during I/R with or without doxycycline and prazosin treatment. A: MMP-2 activity in coronary effluent. The number of preparations from individual rat hearts per group is indicated in parentheses. B: MMP-2 protein content in LV tissue. C: MMP-2 mRNA content in LV tissue. D: α-Actinin and troponin I (TnI) protein contents in LV tissue; n = 5 hearts from individual rat per group. **P < 0.01 vs. corresponding preischemic (Pre) values; #P < 0.05, ##P < 0.01 vs. corresponding normoxic values; †P < 0.05 vs. corresponding I/R control values.
LVEDP, ±dP/dt\text{max}, and RPP, was suppressed by I/R (30 min/30 min), although the heart rate remained unchanged (Fig. 1). These suppressions were significantly improved by IHH, while the preischemic values and heart rate were comparable between the two groups (Fig. 1). Consistently, IHH significantly attenuated I/R-induced increases of LDH release in coronary perfusate (an indicator of cell damage) at 3 and 30 min of reperfusion and LVDP and RPP suppression and myocardial infarct size after 2 h of reperfusion without affecting the heart rate (Fig. 2). Doxycycline (100 μM) mimicked the cardioprotective effects of IHH on the postischemic myocardial performance, LDH release, and infarct size, but it had no additive protective effects to the IHH and did not affect the preischemic values and heart rate during I/R (Figs. 1 and 2). Besides, the weight gain of normoxic and IHH rats was comparable (IHH: from 120.5 ± 1.4 to 323.5 ± 2.4 g vs. normoxia: from 120.9 ± 1.3 to 325.3 ± 2.6 g, n = 30 each) as observed previously (58).

Because MMP-2 has been shown to play an important role in early myocardial I/R injury (29), we next analyzed whether IHH affects the MMP-2 activity and expression during I/R. The MMP-2 activity in coronary effluent was significantly increased at 3 min of reperfusion in the normoxic control group (Fig. 3A). Concomitantly, the protein content of 72-kDa MMP-2 in LV tissue from normoxic group was decreased (Fig. 3B) because of its activation and release from myocytes during reperfusion for the purpose of cell self-rescue (10), while the mRNA level of MMP-2 remained unchanged after I/R (Fig. 3C). In contrast, IHH attenuated I/R-induced MMP-2 activation and release and preserved the myocardial MMP-2 protein content during reperfusion without affection the MMP-2 mRNA level (Fig. 3, A–C). Moreover, α-actinin and TnI, two sensitive proteolysis substrates of MMP-2 (47, 54), were decreased after I/R. The lower molecular mass fragment of TnI (~22 kDa) after I/R was also detected (Fig. 3D). These changes were reversed by IHH or doxycycline, but both of them had no additive protective effects (Fig. 3, A, B, and D), indicating that the cardioprotection of IHH is mediated through the inhibition of MMP-2 activation during I/R.

**IHH inhibits MMP-2 activation via reversing I/R-induced ONOO− overgeneration and TIMP-4 loss.** ONOO− overgeneration and TIMP-4 loss have been reported to undertake the responsibility for the activation of MMP-2 (29, 44, 53). We thus examined the effects of IHH on the alternations of ONOO− formation and TIMP-4 level due to I/R. I/R induced a marked increase of nitrotyrosine content (Fig. 4A), reflecting the ONOO− formation (50), and a decrease of TIMP-4 protein level in the LV (Fig. 4B), while these disorders were significantly attenuated by IHH (Fig. 4). Thus IHH reduced MMP-2 activation against I/R injury through the inhibition of overgeneration of ONOO− and loss of TIMP-4.

**α1D-AR mediates the IHH-conferred cardioprotection on the contractile function, cell survival, and MMP-2 activation.** Because the activation of α1-ARs mimics IPC against I/R injury by activating endogenous adaptive responses and contributes to IHH-avoided cardioprotection (5, 18, 37, 52, 56), we then investigated a potential involvement of α1-ARs in the IHH-regulated MMP-2 activation. The α1-ARs antagonist prazosin at 1 μM abolished IHH-conferred improvement of postischemic myocardial performance, LDH release, and myocardial infarction, but it did not affect I/R-induced disorders (Figs. 1 and 2). Consistently, IHH-attenuated I/R-induced activation and release of α-actinin and TnI protein contents, increase of nitrotyrosine content, and decrease of TIMP-4 protein content were also abolished by prazosin (Fig. 3 and 4).

To determine if there were changes in the α1-AR subtype, we then performed competition ligand binding experiment as reported previously (67). The total density and affinity of α1-ARs at preischemia were comparable between the LV from normoxic and IHH rats, while IHH not only reversed I/R-decreased density of α1-ARs but also enhanced it further at 30 min of reperfusion, although it did not alter the affinity of α1-ARs before or after I/R (Fig. 5, A and B). The densities of α1A-AR and α1D-AR at 30 min of reperfusion were lower than the values at preischemia, whereas the density of α1B-AR remained unchanged (Fig. 5C). However, IHH significantly increased the densities of α1A-AR and α1B-AR at both preischemia and reperfusion, but it decreased the density of α1D-AR only at preischemia (Fig. 5C). Therefore, IHH changed the...
ratio of three α1-AR subtypes by increases of α1A- and α1B-AR ratios but the decrease of α1D-AR ratio in LV myocardium before and after I/R.

Next, the contribution of three α1-AR subtypes to the IHH- afforded cardioprotection was investigated by the α1-AR stimulation with 10 μM of phenylephrine or by the specific inhibition of α1A-, α1B-, or α1D-AR subtype with the antagonist 5-MU (1 μM), CEC (10 μM), or BMY7378 (1 μM), respectively. The cardioprotective effects of IHH on the postischemic myocardial performance, LDH release, activation and release of MMP-2 (Fig. 6), as well as nitrotyrosine and TIMP-4 protein contents (Fig. 7) were mimicked but not augmented by phenylephrine, while they were totally inhibited by the α1B-AR antagonist CEC but not the α1A- or α1D-AR antagonist 5-MU or BMY7378 (Figs. 6 and 7). These results reveal a crucial role of α1B-AR in the cardioprotection of IHH against I/R-induced overgeneration of ONOO⁻, loss of TIMP-4 protein, and the subsequent activation of MMP-2, as well as resultant myocardial performance and cell survival.

IHH-conferred cardioprotection on the contractile function, cell survival, and MMP-2 activation through α1B-AR-mediated PKCe activation and subsequent mitochondrial protection. We previously demonstrated that stimulation of α1B-AR activates PKCe, resulting in the cardioprotection on the mitochondrial function (18). To further determine whether α1B-AR activates PKCe pathway is involved in the IHH-conferred cardioprotection on the regulation of MMP-2, we analyzed the effect of PKCe-specific inhibitor PKCe V1–2 on the cardioprotection of IHH, the translocation of PKCe (an indirect index of PKCe activation), and mitochondrial cytochrome c release (a marker of mitochondrial damage) (18, 58). PKCe V1–2 (10 μM) did not affect the corresponding normoxic values, but it totally reversed the IHH-improved postischemic myocardial contractile function, inhibited LDH release and MMP-2 activation and release (Fig. 6), and attenuated nitrotyrosine content and TIMP-4 loss (Fig. 7). In addition, IHH further enhanced I/R-promoted translocation of PKCe from cytosol to membrane and decreased I/R-induced release of cytochrome c from mitochondria without affecting the preischemic values (Fig. 8). The effects of IHH were mimicked but not further augmented by phenylephrine, while it was inhibited by CEC rather than 5-MU or BMY7378 (Fig. 8). Further, IHH-decreased mitochondrial cytochrome c release due to I/R was reversed by PKCe V1–2 (Fig. 8B). No significant contamination between the membrane and cytosolic fraction was observed as NCX-1 (membrane marker) and GAPDH (cytosolic marker) were only detected in the membrane and cytosolic fractions, respectively (Fig. 8A, top). Similarly, no significant contamination between the mitochondrial and cytosolic fractions was observed as VDAC-1 (mitochondrial marker) and GAPDH (cytosolic marker) were only detected in the mitochondrial and cytosolic fractions, respectively (Fig. 8B, top). These results indicate that the α1B-AR plays an important role in the IHH-conferred cardioprotection via the regulation of MMP-2 activation through the activation of PKCe and the protection of the mitochondria integrity under I/R insult.

DISCUSSION

In this study, we confirmed the cardioprotection of IHH on the improvement of the postischemic myocardial performance and cell survival and revealed the new mechanisms responsible for its cardioprotective effects. The main findings are: 1) IHH improves the postischemic myocardial performance and cell survival via inhibiting I/R-induced activation and release of MMP-2 and subsequently reduce the decrease of α-actinin and...
TnI; 2) the inhibitory effect of IHH on the MMP-2 activation is dependent on the reversal of I/R-induced ONOO⁻ overgeneration and TIMP-4 loss; and 3) IHH inhibits MMP-2 activation and release through the attenuation of I/R-induced ONOO⁻ overgeneration and TIMP-4 loss via α₁B-AR and subsequently activated PKCε pathway. A schematic representation for the IHH-stimulated protective pathway in the regulation of MMP-2 activation during I/R is shown in Fig. 9. These findings extend previous knowledge and provide a new insight into the importance of α₁B-AR/PKCε pathway on the regulation of MMP-2 activation in the cardioprotection against I/R injury.

**Inhibiting MMP-2 activation plays a crucial role in the cardioprotection afforded by IHH.** Cumulated evidence shows that MMP-2 is involved in the acute myocardial I/R injury (1, 10, 29, 54). MMP-2 widely locates in the sarcomere, cytosol, mitochondrion, nucleus, and other organelles of cardiomyocyte (12, 26, 29). Upon first reperfusion, MMP-2 is largely activated and cleaves a series of proteins, such as TnI, myosin light chain-1 (MLC-1), and titin, resulting in severe contractile

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**Fig. 6. Effects of IHH on I/R-induced alternations of LV functional performance, LDH release and MMP-2 activation with or without the α₁A-AR antagonist 5-methylurapidil (5-MU; 1 μM), α₁B-AR antagonist chloroethylclonidine (CEC; 10 μM), α₁D-AR antagonist BMY7378 (BMY; 1 μM), PKCε inhibitor PKCε V1–2 (V1–2, 10 μM), or α₁-AR agonist phenylephrine (Phe; 10 μM) treatment. A: LVDP. B: heart rate. C: rate-pressure product. D: LDH activity in coronary effluent. E: MMP-2 activity in coronary effluent. n = 10 in normoxic group; 7 in IHH group; 4 each in normoxia + 5-MU, IHH + 5-MU, normoxia + BMY7378, IHH + BMY7378, normoxia + V1–2 group, and IHH + V1–2 groups; 5 each in normoxia + CEC, normoxia + Phe, and IHH + Phe groups, and 6 in IHH + CEC group from individual rat hearts in each group. *P < 0.05, **P < 0.01 vs. corresponding preischemic (Pre) values; #P < 0.05, ##P < 0.01 vs. corresponding normoxic values; †P < 0.05, ††P < 0.01 vs. corresponding I/R control values.
dysfunction and cell death (1, 29, 47, 54), while the MMP-2 inhibition decreases the intracellular protein degradation, improves the recovery of myocardial contractile dysfunction, and reduces infarct size in I/R hearts (10, 13, 17). Therefore, inhibiting the activation of MMP-2 appears to be a common downstream mediator in various forms of cardioprotection-triggered intrinsic adaptive responses, such as IPC, postischemia conditioning, noninvasive limb IPC, and pharmacological conditioning (rosuvastatin and high-density lipoproteins) (6, 13, 15, 31, 32). This view is supported by our findings here by using of the chronic IHH adaptive model.

Since the first report for the cardiac beneficial effects of altitude anoxia in 1958 (30), more and more evidence has shown that intermittent hypoxia, characterized by repeated episodes of hypoxia/reoxygenation, has profound effects on the susceptibility of the myocardium to I/R injury (3, 34, 58, 61, 62, 68). Moreover, IHH improves myocardial perfusion in patients with severe coronary heart diseases (14) and has a therapeutic effect on the permanent coronary artery ligation-induced animal myocardial infarction by attenuating infarct size and myocardial fibrosis and improving cardiac performance (61). Therefore, IHH may offer beneficial effects to patients with acute myocardial infarction if the mechanistic insights underlying the cardioprotective effects of IHH are achieved (14, 39). We previously showed that the activation of protein kinase A during the end of ischemia and early reperfusion, phosphoinositide 3-kinase/protein kinase B (PI3K/Akt), calcium/calmodulin-dependent protein kinase II, reactive oxygen species during the reperfusion, the preservation of Ca\(^{2+}\)/H\(_{1001}\) handling proteins abundance/activity at sarcollemma and sarcoplasmic reticulum, and the mitochondrial structure and function contribute to the IHH-conferred cardioprotection (9, 58, 60, 62). These findings together with the observation of the regulation of MMP-2 activation by IHH here suggest that the protective mechanisms in IHH are similar as the classic IPC (64), but the chronic IHH has more significant regulations in the levels of protein expressions (57).

Fig. 7. Effects of IHH on I/R-induced disorders of nitrotyrosine content and TIMP-4 protein level with or without 5-MU, CEC, BMY7378, PKCε V1–2, or phenylephrine treatment. A: nitrotyrosine content; n = 10 in normoxic group; 7 in IHH group; 4 each in normoxia + 5-MU, IHH + 5-MU, normoxia + BMY7378, IHH + BMY7378, normoxia + V1–2 group, and IHH + V1–2 groups; 5 each in normoxia + CEC, normoxia + Phe, and IHH + Phe groups; and 6 in IHH + CEC group from individual rat hearts in each group. B: TIMP-4 protein level; n = 4 hearts from individual rat per group. *P < 0.05, **P < 0.01 vs. corresponding preischemic (Pre) values; #P < 0.05, ##P < 0.01 vs. corresponding normoxic values; †P < 0.05, ††P < 0.01 vs. corresponding I/R control values.
logical conditions have not yet been fully clarified. Chronic hypoxia (48 h) was reported to inhibit MMP-2 activation, but it does not affect the MMP-2 protein expression in human cardiac myofibroblasts (41). Similarly, chronic IHH alone does not alter LV MMP-2 protein level (46). This is supported by our data here showing that the chronic IHH does not alter the preischemic MMP-2 mRNA and protein level but reduces the postischemic MMP-2 activation in LV.

MMP-2 is synthesized as a proenzyme and can be activated by proteolytic cleavage of its inhibitory pro-peptide domain (29). A large body of experimental evidence supports that the early reperfusion leads to an increase in oxidative stress when oxygen is reintroduced to ischemic tissue. Therefore, simultaneous production of NO and $O_2^-$ will react to generate ONOO$^-$, which activates the endogenic MMP-2 by oxidizing the sulphhydryl bond between a cysteine residue of the prodomain and the Zn$^{2+}$ catalytic center and produces myocardial dysfunction (29, 53). Endogenous inhibitors, such as TIMPs, tightly control MMP-2 activity. Four TIMPs have been identified to date, of which TIMP-4 is expressed predominantly in the heart compared with others (29, 44). I/R injury-induced rapid loss of myocardial TIMP-4 also results in an increase of net myocardial MMP-2 activity (33, 44). Moreover, the over-generation of ONOO$^-$ inhibits TIMP-4 activity, which turns to indirectly activate MMP-2 (16). Interestingly, we found here that the IHH can reverse I/R-induced activation of MMP-2.

Fig. 8. Translocation of PKCε from the cytosol to the membrane fraction and release of mitochondrial cytochrome c (Cyto-c) from the mitochondrian to the cytoplasmic fraction during preischemia (Pre) and I/R with or without 5-MU, CEC, BMY7378, PKCε V1–2, or phenylephrine treatment. A: representative immunoblots of the membrane and cytosolic fractions and purity validated with the cytosolic marker GAPDH and the membrane marker NCX-1 (top), and representative immunoblots (middle) and analysis (bottom) of PKCε translocation. B: representative immunoblots of the cytosolic and mitochondria fractions and purity validated with the cytosolic marker GAPDH and the mitochondrial marker VDAC-1 (top), and representative immunoblot (middle) and analysis (bottom) of mitochondrial cytochrome c release; n = 4 hearts from individual rat per group. *P < 0.05, **P < 0.01 vs. corresponding preischemic values; #P < 0.05, ##P < 0.01 vs. corresponding normoxic values; ††P < 0.01 vs. corresponding I/R control values.
through the posttranslational regulation by modulating the amount of two critical MMP-2 regulators, i.e., reducing the ONOO\(^-\) formation and TIMP-4 loss, and thus subsequently decreases I/R-induced MMP-2 activation. Whether other mechanisms are involved in the IHH-regulated MMP-2 activation, such as MMP-2 phosphorylation and endogenous proteases (MT-MMPs) (29), needs to be further investigated.

The \(\alpha_{1B}\)-AR/PKC\(\varepsilon\) pathway plays an important role in the regulation of MMP-2 activation. The PI3K/Akt and JAK2-ERK pathways have been shown to be involved in the modulation of MMP-2 activation under cardioprotection against I/R injury (8, 45). Here we showed another signaling pathway mediated by \(\alpha_{1B}\)-AR/PKC\(\varepsilon\) being involved in the regulation of MMP-2 activation in the cardioprotection against I/R injury. It has been well documented that \(\alpha_{1}\)-AR activation induces the cardioprotection against I/R injury in animal experiments (5, 18, 37, 52), while the role of \(\alpha_{1}\)-AR subtypes (\(\alpha_{1A}\)-AR, \(\alpha_{1B}\)-AR, and \(\alpha_{1D}\)-AR) in the cardioprotection remains controversial (18, 19, 25, 43). IHH has been shown to increase the activity of \(\alpha_{1}\)-ARs and thus improve the contraction of papillary muscle during simulated ischemia (56). Our data here further reveal that IHH exerts cardioprotection via the increases of postischemic \(\alpha_{1B}\)-AR density and activity and the subsequent activation of PKC\(\varepsilon\) but not other \(\alpha_{1}\)-AR subtypes in rat hearts. This is consistent with the reports from other and our laboratories showing that the activation of \(\alpha_{1B}\)-AR/PKC\(\varepsilon\) pathway plays an important role in the IPC and \(\alpha_{1}\)-AR stimulation-conferred cardioprotection (18, 25). Consistent with our results of the increase in \(\alpha_{1B}\)-AR density by the chronic IHH, an 8-wk treatment of testosterone has been shown to confer cardioprotection against I/R injury by upregulating the cardiac \(\alpha_{1}\)-AR and enhancing the effects of \(\alpha_{1}\)-AR stimulation (51). However, we could not find reports related to the increase of \(\alpha_{1B}\)-AR density in classic IPC. Therefore, the activation of \(\alpha_{1B}\)-AR/PKC\(\varepsilon\) pathway appears to be a common mechanism in cardioprotection, while the increase of \(\alpha_{1B}\)-AR density seems to be mainly involved in the chronic conditioning. This mechanism may help the cell to deal with the stress more effectively and needs to be further investigated for the correlation between the extent and duration of IHH with the magnitude of increases in the \(\alpha_{1B}\)-AR density.

PKC\(\varepsilon\) is a most abundant novel PKC isozyme found in adult rat cardiac myocytes and plays an essential role in the development of cardioprotection (18, 24, 58). Its activation, accompanying with the translocation from cytosolic to particulate compartments, has been showed to arouse the intrinsic cardioprotective mechanisms, such as opening mitochondrial ATP-sensitive potassium (mitoK\(_{ATP}\)) channels and maintaining mitochondrial integrity and function against I/R injury (18, 24, 58). The protection on mitochondria attenuates oxidative stress and sequential \(O_2^-\) and ONOO\(^-\) overgeneration (7, 48). Moreover, mitochondria protection-mediated decreases of oxidative stress and intracellular \(Ca^{2+}\) overloading alter the activities of proteases and reduce intracellular protein lysis and release (18, 24, 36). Because IHH inhibits the opening of mitochondrial permeability transition pores and opens mitoK\(_{ATP}\) channels to enhance the mitochondria tolerance to reperfusion injury (3, 38, 59, 68), the mitochondrial protection mediated by the IHH-stimulated \(\alpha_{1B}\)-AR/PKC\(\varepsilon\) pathway may subsequently attenuate \(O_2^-\) and sequential ONOO\(^-\) overgeneration and inhibit protease activation and sequential TIMP-4 loss to reduce MMP-2 activation against I/R injury.

One limitation of the present study is that all of the data were collected from the use of inhibitors or activator. To exclude potential nonspecific and side effects of those reagents, genetic manipulation of \(\alpha_{1}\)-AR subtypes by overexpression or specific knockdown in the hearts from normoxic and IHH-adapted rats needs to be carried out to further confirm those findings.

In conclusion, our results demonstrate that IHH improves the postischemic recovery of myocardial dysfunction and cell death via the suppressing of I/R-induced overgeneration of ONOO\(^-\) and loss of TIMP-4 through the activation of the \(\alpha_{1B}\)-AR/PKC\(\varepsilon\) pathway and the subsequent inhibition of MMP-2 activation.

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AUTHOR CONTRIBUTIONS
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