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Calcium/calmodulin-dependent protein kinase II mediates cardioprotection of intermittent hypoxia against ischemic-reperfusion-induced cardiac dysfunction

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Yu Z, Wang Z, Yang H. Calcium/calmodulin-dependent protein kinase II mediates cardioprotection of intermittent hypoxia against ischemic-reperfusion-induced cardiac dysfunction. *Am J Physiol Heart Circ Physiol* 297: H735–H742, 2009. First published June 12, 2009; doi:10.1152/ajpheart.01164.2008.—Intermittent high-altitude (IHA) hypoxia-induced cardioprotection against ischemia-reperfusion (I/R) injury is associated with the preservation of sarcoplasmic reticulum (SR) function. Although Ca²⁺/calmodulin (CaM)-dependent protein kinase II (CaMKII) and phosphatase are known to modulate the function of cardiac SR under physiological conditions, the status of SR CaMKII and phosphatase during I/R in the hearts from IHA hypoxic rats is unknown. In the present study, we determined SR and cytosolic CaMKII activity during preischemia and I/R (30 min/30 min) in perfused hearts from normoxic and IHA hypoxic rats. The left ventricular contractile recovery, SR CaMKII activity as well as phosphorylation of phospholamban at Thr¹⁷, and Ca²⁺/CaM-dependent SR Ca²⁺-uptake activity were depressed in the I/R hearts from normoxic rats, whereas these changes were prevented in the hearts from IHA hypoxic rats. Such beneficial effects of IHA hypoxia were lost by treating the hearts with a specific CaMKII inhibitor, KN-93. I/R also depressed cytosolic CaMKII and SR phosphatase activity, but these alterations remained unchanged in IHA hypoxic group. Furthermore, we found that the autophosphorylation at Thr²⁸⁷, which confers Ca²⁺/CaM-independent activity, was not altered by I/R in both groups. These findings indicate that preservation of SR CaMKII activity plays an important role in the IHA hypoxia-induced cardioprotection against I/R injury via maintaining SR Ca²⁺-uptake activity.

cardiac sarcoplasmic reticulum; phospholamban phosphorylation residue; sarcoplasmic reticulum calcium pump adenosinetriphosphatase; phosphatase; cardiac contractile function

ISCHEMIC HEART DISEASE IS ONE of the most important causes of cardiovascular mortality in advanced countries. This reflects the lack of effective strategies targeted to the molecular basis of ischemia-reperfusion (I/R) injury. Therefore, there is an urgent need for exploring new approaches to reduce postischemic injury based on the understanding of intrinsic cardioprotective mechanisms. Cumulated evidence has shown that adaptation to intermittent high-altitude (IHA) hypoxia increases myocardial tolerance to the subsequent severe hypoxic, Ca²⁺ overload, or ischemic injury (3, 6, 22, 34, 40, 43). Attractively, this form of protection is noninvasive, persists longer than ischemic preconditioning (IP), and has fewer side effects, such as right ventricular hypertrophy, compared with chronic hypoxia (3, 39). However, much less is known about the protective mechanism of IHA hypoxia compared with the extensive studies in IP (41).

Sarcoplasmic reticulum (SR) is known to play a central role in the regulation of intracellular concentration of Ca²⁺ ([Ca²⁺]_i) and subsequent cardiac contraction and relaxation processes. Dysfunction of cardiac sarco(endo)plasmic reticulum Ca²⁺-pump ATPase (SERCA2) during I/R appears to be involved in the Ca²⁺ overload and thereby affects the extent of contractile dysfunction that follows ischemia (21, 31), while improvement of Ca²⁺ handling by SR has been indicated to contribute to the beneficial effects of IHA hypoxia in the I/R cardiomyocytes (6). We have demonstrated recently that IHA hypoxia mitigates I/R-induced depression in the SERCA2 activity by improving dual-site phosphorylation of phospholamban (PLB), especially phosphorylation at threonine 17 (PThr¹⁷-PLB) during reperfusion, and these effects are associated with the improved recovery of contractile relaxation (40). The phosphorylation state of the protein in cells is regulated by a balance between activities of protein kinases and protein phosphatases (10, 15). Although we found that calcium/calmodulin (CaM)-dependent protein kinase II (CaMKII) inhibitor blocks IHA hypoxia-induced improvement in PLB PThr¹⁷, as well as in Ca²⁺-pump ATPase activity (40), it is unknown whether protein phosphatases or the status of SR CaMKII are altered in the hearts from IHA hypoxic animals.

CaMKII, one of the major regulators of Ca²⁺ homeostasis in the heart, phosphorylates cardiac contractile regulatory proteins, such as troponin T (17), and SR proteins, such as ryanodine receptors (RyRs), SERCA2, and PLB (28). CaMKII can be autophosphorylated, i.e., CaMKII is a substrate for itself, at Thr²⁸⁷ (PThr²⁸⁷), which confers Ca²⁺/CaM-independent activity, and, once activated, sustains the activity of CaMKII even in the absence of elevated Ca²⁺/CaM and thus extends CaM-dependent regulation over time (1). Several pieces of evidence have shown that IP may prevent I/R-induced alterations in SR Ca²⁺ handling abilities by preserving SR CaMKII activity (30). On the other hand, chronic hypoxia significantly decreases CaMKII activity in PC12 cells (4). However, the status of CaMKII in IHA hypoxia is unclear.

To address these issues, in this study, we investigated 1) the changes of total CaMKII activity (Ca²⁺/CaM dependent plus Ca²⁺/CaM independent) and autophosphorylation of CaMKII-PThr²⁸⁷ (as Ca²⁺/CaM-independent CaMKII activity) in the hearts from normoxic and IHA hypoxic rats, with or without I/R; 2) the importance of CaMKII in protecting SR function in the IHA hypoxia-protected hearts; and 3) the status of cardiac SR phosphatase activity during I/R, with or without adaptation

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to IHA hypoxia. Our data provide a new insight into the understanding of the intrinsic defensive mechanism and demonstrate the importance of SR CaMKII pathway in preserving SR function in the cardiac protection afforded by IHA hypoxia.

MATERIALS AND METHODS

Animals used in this study were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* (publication no. 85-23, revised 1996; National Institutes of Health, Bethesda, MD), 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 Shanghai Jiao Tong University School of Medicine (Shanghai, China).

IHA hypoxic animals. Male Sprague-Dawley rats were exposed to IHA hypoxia in a hyperbaric chamber (equivalent to an altitude of 5,000 m; barometric pressure = 404 mmHg, P_{O_2} = 84 Torr) for one 6-h period each day for 42 days, as previously described (6, 40). Their body weights during this period rose from 100–130 g to 310–340 g. Age-matched normoxic animals were maintained in the normoxic environment for a corresponding period. Their body weights and weight gains were identical to those of rats exposed to IHA hypoxia. All animals had free access to water and to a standard laboratory diet.

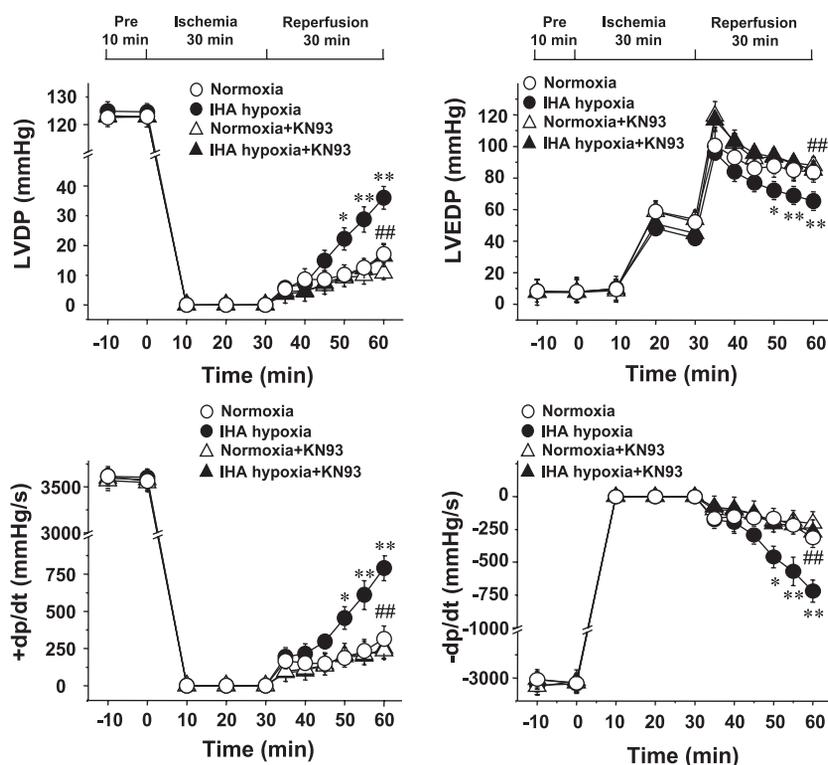
Heart perfusions and experimental protocols. Rats were anesthetized with pentobarbital sodium (60 mg/kg ip). The hearts were rapidly excised and perfused with Krebs-Henseleit solution at 37°C by using the Langendorff technique at a constant pressure of 80 mmHg, as previously described (40, 43). The perfusion solution containing (in mM) 118 NaCl, 4.7 KCl, 1.2 $MgSO_4 \cdot 7H_2O$, 2.5 CaCl₂, 1.2 KH_2PO_4 , 25 $NaHCO_3$, 0.026 Na_2-EDTA , and 11.1 glucose was gassed with 95% O_2 -5% CO_2 (pH 7.4). A water-filled latex balloon connected to a pressure transducer (model Gould P23 Db, AD Instrument, Castle Hill, Australia) was inserted into the left ventricular (LV) cavity to achieve a stable LV end-diastolic pressure (LVEDP) of ~8 mmHg. Contractile performance of the LV was evaluated on the basis of its developed pressure (LVDP), LVEDP, rate of pressure development

over time (+dP/dt), and rate of pressure decay over time (-dP/dt) with PowerLab system (AD Instrument).

After stabilization, hearts were perfused for 10 additional min (preischemia) and then subjected to 30-min no-flow global ischemia followed by 30 min of reperfusion, as shown in Fig. 1. To test whether CaMKII is involved in the beneficial effects of IHA hypoxia against I/R, CaMKII-specific inhibitor KN-93 (Sigma, St. Louis, MO) was delivered at 1 ml/min, with the various concentrations in different preparations adjusted according to the different coronary flow (Supplemental Fig. 1; the online version of this article contains supplemental data) to reach a final concentration of 1 μM by an infusion pump into the perfusion stream above the aortic cannula at 10 min before ischemia and ended after reperfusion (30, 40). At the end of the experiments, the LVs were freeze-clamped and stored at -80°C until being used in assays later.

Preparation of SR vesicles and cytosolic fraction. Frozen LV tissue was homogenized in an extraction buffer containing (in mM) 15 Tris·HCl, 10 $NaHCO_3$, 5 NaN_3 , 250 sucrose, and 1 EDTA (2°C, pH 7.0) using a homogenizer (Polytron PT 3100; Kinematica, Littau-Lucerne, Switzerland). The homogenate was centrifuged for 5 min at 3,000 g to remove cellular debris. For preparation of the SR vesicles (6, 33), the 3,000 g supernatant was further centrifuged at 48,000 g for 75 min. The resulting supernatant was processed for the isolation of the cytosolic fraction, and the pellet was suspended in a mixture of 0.6 mM KCl and 20 mM Tris·HCl (pH 7.0) and centrifuged at 48,000 g for 60 min. The final pellet was rehomogenized in 250 mM sucrose and 40 mM imidazole-HCl as SR fraction. For isolation of the cytosolic fraction (26), the supernatant obtained after the first spin (48,000 g) was centrifuged at 100,000 g for 1 h, and the resulting supernatant was employed. The resulting SR and cytosolic suspensions were stored at -80°C and used in assays later. All solutions contained three protease inhibitors: soybean trypsin inhibitor (40 $\mu g/ml$), 0.1% PMSF, and leupeptin (0.5 $\mu g/ml$). SR vesicles for Western blot of total CaMKII or CaMKII-PThr²⁸⁷ were isolated in the presence of a phosphatase inhibitor, sodium pyrophosphate (1 mM), to prevent any dephosphorylation occurring during the isolation pro-

Fig. 1. Left ventricular (LV) performance during ischemia-reperfusion (I/R) in isolated, perfused hearts from normoxic and intermittent high-altitude (IHA) hypoxic rats. KN-93 (1 $\mu mol/l$) was infused into the heart for 10 min before ischemia and ended after reperfusion. Pre, preischemia; LVDP, LV developed pressures; LVEDP, LV end-diastolic pressure; +dP/dt, maximal rate of LV pressure development; -dP/dt, maximal rate of LV pressure decline. Values are means \pm SE; $n = 6$ hearts in normoxic or IHA hypoxic group, and 5 hearts in KN93-treated group. * $P < 0.05$, ** $P < 0.01$ vs. corresponding normoxic values. ## $P < 0.01$ vs. respective IHA hypoxia values.



cedure (7). The concentration of the proteins was determined using the method of Bradford. To assess whether the contamination in each group is to an equal extent, the activities of NADPH cytochrome *c* reductase (SR marker), ouabain-sensitive $\text{Na}^+\text{-K}^+\text{-ATPase}$ (sarcolemmal marker), and cytochrome-*c* oxidase (mitochondrial marker) in the cytosolic and SR preparations were measured as described previously (28, 31) in each group by using cytochrome *c* reductase (NADPH) assay kit (Sigma, St. Louis, MO), ouabain-sensitive $\text{Na}^+\text{-K}^+\text{-ATPase}$ assay kit (Jiancheng Bioengineering Institute, Nanjing, China), and cytochrome-*c* oxidase assay kit (Sigma). In the cytosolic preparations, there was no detection of NADPH cytochrome *c* reductase, ouabain-sensitive $\text{Na}^+\text{-K}^+\text{-ATPase}$, and cytochrome-*c* oxidase (data not shown). In the SR preparations, no difference in the sarcolemmal and mitochondrial markers was detected between normoxic and IHA hypoxic groups (Supplemental Table 1). Thus the SR preparations from both groups were equally contaminated with other subcellular organelles.

Measurement of CaMKII activity. The CaMKII activities of SR and cytosolic preparations were determined according to the procedure described previously (30) by using CaMKII assay kit (Upstate Biotechnology, Lake Placid, NY). The assay is based on the phosphorylation of specific synthetic peptide autocalmitide-3 (KKALRRQETVDAL), as indicated by the transfer of $[\gamma\text{-}^{32}\text{P}]$ from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by CaMKII. The phosphorylated substrate was then separated from the residual $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ using P81 phosphocellulose paper and quantitated by using a liquid scintillation counter (Micro Beta 1450–024, USA). The experiments were performed in the presence and absence of the exogenous substrate, autocalmitide-3. The CaMKII activity was calculated by subtracting the values in the absence from those in the presence of the exogenous substrate (30).

Western blot analysis. The expression of PLB, CaMKII, and CaMKII-PThr²⁸⁷ proteins in the SR was detected by Western blot analysis, as previously described (28, 40, 44). Protein samples for PLB or CaMKII/CaMKII-PThr²⁸⁷ were subjected to 7.5% (wt/vol) urea SDS-PAGE and 10% SDS-PAGE, respectively. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes and probed with the phosphorylation site-specific PS-17 PLB antibody (1:10,000 dilution, Phosphoprotein Research, Leeds, UK), the total PLB antibody anti-PLB A1 (1:2,000 dilution; Upstate Biotechnology, Lake Placid, NY), anti-CaMKII antibody (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-phospho-CaMKII (Thr²⁸⁶, 1:1,000 dilution; ABR, Golden, Thr²⁸⁶ in the alpha-isoform is equivalent to Thr²⁸⁷ in the delta-isoform of the heart, and the peptide used to raise this antibody is conserved 100% in delta isoform), respectively. Appropriate secondary antibodies were used, and the antibody-antigen complexes in the membranes were visualized using an ECL detection kit (Amersham Life Science, Oakville, Ontario, Canada), exposed to X-ray film, and quantified using a video documentation system (Gel Doc 2000; Bio-Rad Laboratories, Hercules, CA). Equal protein loading was checked in experiments by staining the membrane with Coomassie Brilliant Blue at the end of the experiment.

Measurement of $\text{Ca}^{2+}/\text{CaM}$ -dependent Ca^{2+} -uptake activity. Ca^{2+} -uptake activity of SR phosphorylated in the presence or absence of $\text{Ca}^{2+}/\text{CaM}$ was determined by employing $^{45}\text{Ca}^{2+}$ and Millipore filtration technique, as described previously (18, 30). The incubation medium for $\text{Ca}^{2+}/\text{CaM}$ -mediated phosphorylation contained 50 mM HEPES (pH 7.4), 10 mM MgCl_2 , 100 μM CaCl_2 , 100 μM EGTA, 1 μM CaM, 0.8 mM ATP, 10 nM microcystin-LR (ALEXIS Biochemical, Germany), and 1 mM sodium pyrophosphate (both to inhibit any endogenous phosphatase activity), and 10 μg SR protein. Phosphorylation reaction was initiated by the addition of ATP following preincubation of the rest of the assay components for 3 min at 37°C. The incubation medium for the phosphorylation of SR vesicles in the absence of $\text{Ca}^{2+}/\text{CaM}$ contained 10 μM W-7 (Sigma; to inhibit endogenous CaM activity) and 1 mM EGTA (to chelate Ca^{2+}). The phosphorylated SR vesicles were transferred into the Ca^{2+} -uptake

assay medium after 1 min of the reaction. The Ca^{2+} -uptake assay medium contained (in mM) 50 Tris-maleate (pH 6.8), 5 NaN_3 , 5 ATP, 5 MgCl_2 , 120 KCl, 5 potassium-oxalate, 0.1 EGTA, 0.1 $^{45}\text{CaCl}_2$, 25 $\mu\text{mol/l}$ ruthenium red (to inhibit activity of Ca^{2+} -release channels), and 6 μg SR proteins. The reaction was terminated after 1 min by filtering the incubation mixture through the Millipore filter (Millipore, Billerica, MA). The radioactivity was counted by using the standard liquid scintillation counting technique. $\text{Ca}^{2+}/\text{CaM}$ -dependent Ca^{2+} -uptake activities were calculated as the difference in Ca^{2+} -uptake activities of the phosphorylated SR vesicles in the presence and absence of $\text{Ca}^{2+}/\text{CaM}$.

Measurement of protein phosphatase activity. The activity of SR protein phosphatase was determined by using Ser/Thr phosphatase assay kit (Upstate Biotechnology, Lake Placid, NY), as described previously (30). This assay is based on the dephosphorylation of synthetic phosphopeptide (KRpTIRR). Inorganic phosphate released during the reaction was detected by the addition of Malachite Green Solution, and the absorbance was measured at 650 nm. The experiments were performed in the presence and absence of the exogenous substrate, and the phosphatase activity was calculated by subtracting the values in the absence from those in the presence of the exogenous substrates. Because type 1 (PP1) and 2A phosphatases (PP2A) are the main phosphatases of the myocardium, and both phosphatases dephosphorylate PLB (16, 20), PP1 and PP2A of SR were measured as previously described (9, 36). PP1 and PP2A were differentiated in the presence of okadaic acid (OA; Sigma), which is a broad range inhibitor of serine/threonine phosphatases causing complete inhibition of PP2A and PP4 at ~ 1 nM, whereas the IC_{50} of OA for PP1 is 10–15 nM (13). PP1 activity was defined as the activity measured in the presence of 2.5 nM OA minus the residual activity in the presence of 2.5 μM OA, and PP2A activity was defined as the component of total phosphatase activity that was inhibited by 2.5 nM OA.

Statistical analysis. Data are expressed as means \pm SE. Statistical significance was determined by ANOVA or repeated ANOVA for multiple comparisons or repeated measurements. Significant differences between the two mean values were estimated using Student's *t*-test (SPSS 11.5). $P < 0.05$ was considered statistically significant.

RESULTS

Improvement in postischemic recovery of LV function. To evaluate whether IHA hypoxia is capable of enhancing contractile functional recovery after ischemia, we examined postischemic hemodynamic changes of normoxic and IHA hypoxic groups in isolated, perfused hearts with global no-flow I/R (30 min/30 min). No significant differences were observed in LVDP, LVEDP, and $\pm\text{dP}/\text{dt}$ between normoxic and IHA hypoxic groups during the preischemic phase, whereas, during the reperfusion phase, IHA hypoxic group exhibited significantly better functional recovery than the normoxic group (Fig. 1). After 30 min of reperfusion, the recovery of LVDP, $+\text{dP}/\text{dt}$, and $-\text{dP}/\text{dt}$ were higher in the IHA hypoxic group than that in the normoxic group. Concomitantly, this was associated with a marked decrease of LVEDP in the IHA hypoxic group compared with that in the normoxic group ($P < 0.01$). To confirm the involvement of CaMKII pathway in the beneficial effect of IHA hypoxia, a CaMKII-specific inhibitor, KN-93 (1 μM), was added before I/R. KN-93 did not affect preischemic contractile and relaxation in normoxic and IHA hypoxic groups, but it completely abolished the improved recovery of postischemic contractile function afforded by IHA hypoxia, with no effect in the hearts from the normoxic group.

Preservation of postischemic activity of SR CaMKII but not cytosolic CaMKII. The observation that KN-93 abolished the improved recovery of cardiac performance by IHA hypoxia,

but not in the normoxic group, suggests that IHA hypoxia may alter the activity of CaMKII during the postischemic phase. We thus compared activities of SR and cytosolic CaMKII from both groups, with and without I/R. The cytosolic CaMKII activity in the normoxic group decreased by 76.8 ± 2.2 and $63.6 \pm 4.0\%$ at 30 min of ischemia and reperfusion, respectively, and it decreased to a similar level in IHA hypoxic group (Fig. 2A). The SR CaMKII activity in the normoxic group decreased after I/R, and such suppression was attenuated by IHA hypoxia at 30 min of reperfusion, but not at preischemia and 30 min of ischemia (Fig. 2B). CaMKII inhibitor KN-93 decreased SR CaMKII activity at the preischemia phase in both groups and completely abolished IHA hypoxia-improved activity of SR CaMKII at 30 min of reperfusion, but not in the normoxic group.

SR total CaMKII protein abundance and autophosphorylation of CaMKII-PThr²⁸⁷ during I/R. To gain further insight into the mechanisms underlying IHA hypoxia-improved SR CaMKII activity during reperfusion, we next examined SR total CaMKII protein abundance and autophosphorylation of SR CaMKII at Thr²⁸⁷ [an important autophosphorylation site in the CaM binding domain and representing Ca²⁺/CaM-independent activity (1, 12)], with or without I/R in both groups. SR total CaMKII protein expression and autophosphorylation of CaMKII at Thr²⁸⁷ remained unchanged during I/R compared with that at preischemia in both groups (Fig. 3). These results indicate that IHA hypoxia-protected SR CaMKII activity is not related to the alteration of SR total CaMKII

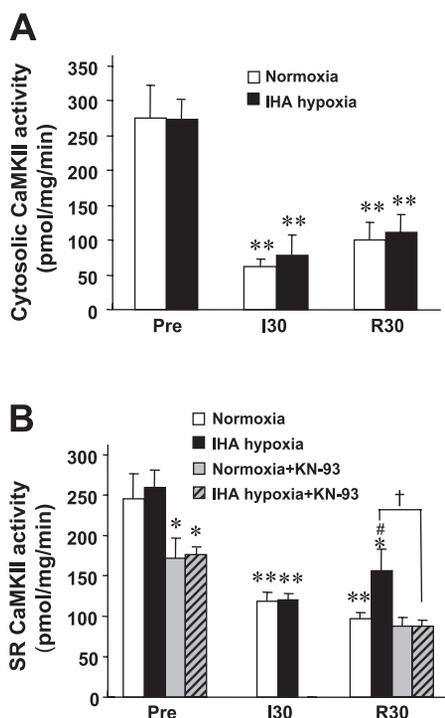


Fig. 2. Ca²⁺/calmodulin (CaM)-dependent protein kinase II (CaMKII) activities of LVs during I/R in normoxic and IHA hypoxic group. A: cytosolic CaMKII activities at Pre, 30 min of ischemia (I30), and 30 min of reperfusion (R30) in both groups. B: sarcoplasmic reticulum (SR) CaMKII activities at Pre, I30, and R30 in both groups, with or without KN-93 treatment. Values are means \pm SE; $n = 5$ each. * $P < 0.05$, ** $P < 0.01$ vs. corresponding Pre values. # $P < 0.05$ vs. the corresponding normoxic values. † $P < 0.05$ compared with the group indicated.

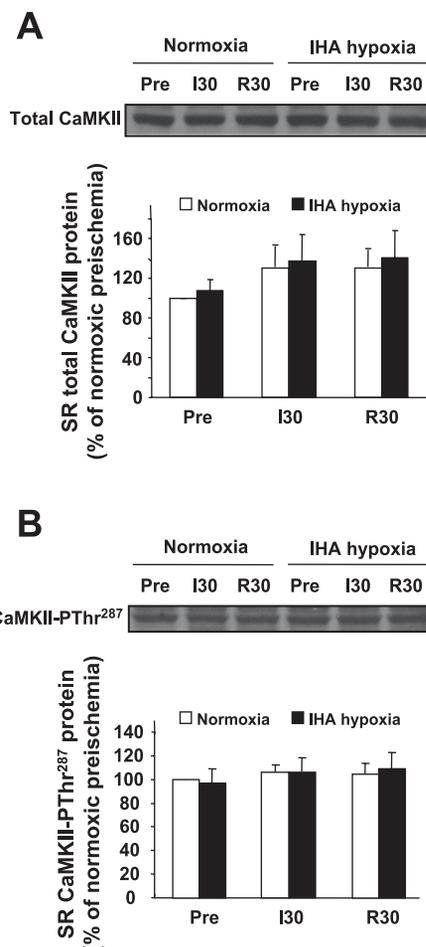


Fig. 3. Total CaMKII expression and Thr²⁸⁷ autophosphorylation (CaMKII-PThr²⁸⁷) in LV SR during I/R from normoxic and IHA hypoxic group. A: representative immunoblots of SR total CaMKII (top) and averaged densitometric results of immunoblot analysis (bottom) at Pre, I30, and R30. B: representative immunoblots of SR CaMKII-PThr²⁸⁷ (top) and average densitometric results of immunoblot analysis (bottom) at Pre, I30, and R30. Values are means \pm SE; $n = 5$ each.

expression level or Ca²⁺/CaM-independent activity of SR CaMKII.

Improvement of postischemic Thr¹⁷ PLB phosphorylation. Site-specific phosphorylation of PLB at Thr¹⁷ is mediated by CaMKII pathway (19). To evaluate the contribution of this pathway to inducement of the beneficial effects of IHA hypoxia, we determined phosphorylation of PLB at Thr¹⁷ in I/R hearts, with or without KN93 treatment. In the normoxic group, phosphorylation of PLB at Thr¹⁷ significantly decreased at 30 min of reperfusion by $74.9 \pm 9.0\%$ ($P < 0.01$, Fig. 4), while total PLB expression remained unchanged. IHA hypoxia did not affect PThr¹⁷ at preischemic and ischemic phases, but it markedly attenuated I/R-induced decrease in PThr¹⁷ at 30 min of reperfusion, without affecting the total PLB expression. KN-93 partly depressed preischemic PThr¹⁷ of PLB in both groups, and it completely abolished the protection induced by IHA hypoxia, but had no effect in the normoxic group (Fig. 4). These results suggest that the improvement of PLB PThr¹⁷ by CaMKII may contribute to the cardioprotection of IHA hypoxia against I/R injury.

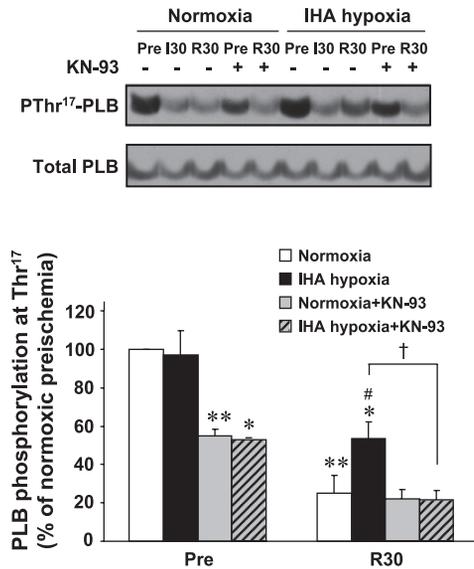


Fig. 4. Effects of CaMKII inhibitor KN-93 on phosphorylation (P) of Thr¹⁷ phospholamban (PLB) of LV during I/R from normoxic and IHA hypoxic group. *Top*: representative original immunoblots of PLB PThr¹⁷ and total PLB expression. *Bottom*: average densitometric results of immunoblot analysis of PLB PThr¹⁷. Values are means \pm SE; $n = 4-6$ hearts at different time points in each group. * $P < 0.05$, ** $P < 0.01$ vs. corresponding Pre values. # $P < 0.05$ vs. the corresponding normoxic value. † $P < 0.05$ compared with the group indicated.

Preservation of postischemic Ca²⁺/CaM-dependent Ca²⁺-uptake activity. Because CaMKII phosphorylation has been shown to regulate the activity of cardiac SR Ca²⁺-uptake (14), we next examined the effect of CaMKII phosphorylation on SR Ca²⁺-uptake in the absence or presence of Ca²⁺/CaM in both groups, with or without I/R. The Ca²⁺-uptake activity of the phosphorylated SR vesicles in the presence of Ca²⁺/CaM in normoxic group decreased at 30 min of ischemia and reperfusion, but the suppression was partially reversed by IHA hypoxia (Fig. 5A). In contrast, the Ca²⁺-uptake activity in the absence of Ca²⁺/CaM, which resulted from the unphosphorylated SR vesicles, remained unchanged in both normoxic and IHA hypoxic groups (Fig. 5B). Ca²⁺/CaM-dependent Ca²⁺-uptake activity, calculated as differences between the Ca²⁺-uptake activity of SR preparations in the presence and absence of Ca²⁺/CaM (30), decreased during I/R in the normoxic group (Fig. 5C). IHA hypoxia did not significantly affect preischemic and ischemic Ca²⁺/CaM-dependent Ca²⁺-uptake activity, but it markedly attenuated the I/R-induced decrease in Ca²⁺/CaM-dependent Ca²⁺-uptake activity at 30 min of reperfusion. KN-93 decreased Ca²⁺/CaM-dependent Ca²⁺-uptake activity at preischemia phase in both groups and completely abolished IHA hypoxia-improved Ca²⁺/CaM-dependent Ca²⁺-uptake activity at 30 min of reperfusion, but not in the normoxic group (Fig. 5C).

Effect of IHA hypoxia on SR protein phosphatase activity. The phosphorylation state of SR proteins is regulated by a balance between activities of protein kinases and phosphatase (10, 15). To determine whether IHA hypoxia-improved postischemic phosphorylation of PLB is also a result of the alteration of protein phosphatase activity, we then examined SR phosphatase activity during I/R. In the normoxic group, SR total phosphatase, PP1, and PP2A activities decreased at 30

min of ischemia and 30 min of reperfusion compared with the preischemic level, respectively. These alterations due to I/R remained unchanged in IHA hypoxic group (Fig. 6). Thus the SR phosphatase appears not to contribute to the preserved Thr¹⁷ phosphorylation of PLB by IHA hypoxia during reperfusion.

DISCUSSION

In the present study, we evaluated the contribution of CaMKII and protein phosphatase to the cardioprotective effect of IHA hypoxia on the cardiac performance, as well as the SR Ca²⁺-uptake activity during I/R. We found that IHA hypoxia does not alter CaMKII activity during the ischemic phase, while it preserves postischemic SR total CaMKII activity by showing an increase in the Ca²⁺-dependent CaMKII activity, but not in the Ca²⁺/CaM-independent CaMKII activity at 30 min of reperfusion. This beneficial effect is verified to be associated with the improvement of phosphorylation of PLB at Thr¹⁷, as we have shown previously (40), and is demonstrated to regulate SR Ca²⁺/CaM-dependent Ca²⁺-uptake activity, as well as cardiac performance during reperfusion by using CaMKII-specific inhibitor KN-93. Furthermore, we showed that decreased total SR protein phosphatase, as well as PP1 and PP2A activities due to I/R, remain unchanged in the IHA hypoxic group. These results extend previous findings, indicating that improved SR Ca²⁺-dependent CaMKII activity, but not the SR-CaMKII-PThr²⁸⁷ and SR phosphatase activity,

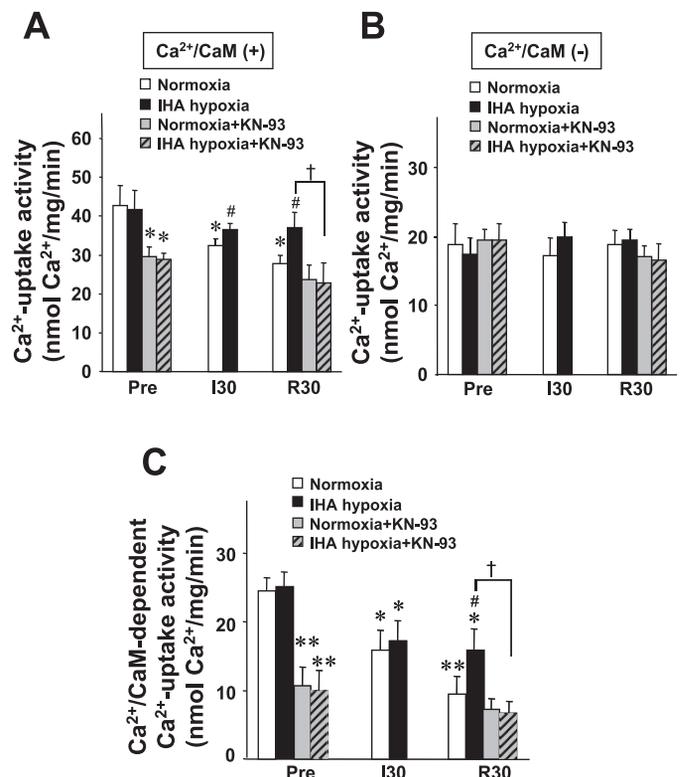


Fig. 5. SR Ca²⁺-uptake activities in LV during I/R in normoxic and IHA hypoxic group, with or without treatment of KN93. Ca²⁺/CaM-dependent SR Ca²⁺-uptake activities (C) are shown as difference between Ca²⁺-uptake activities in the presence (A) and absence (B) of Ca²⁺/CaM. Values are means \pm SE; $n = 5$ at different time points in each group. * $P < 0.05$, ** $P < 0.01$ vs. corresponding Pre values. # $P < 0.05$ vs. the corresponding normoxic value. † $P < 0.05$ compared with the group indicated.

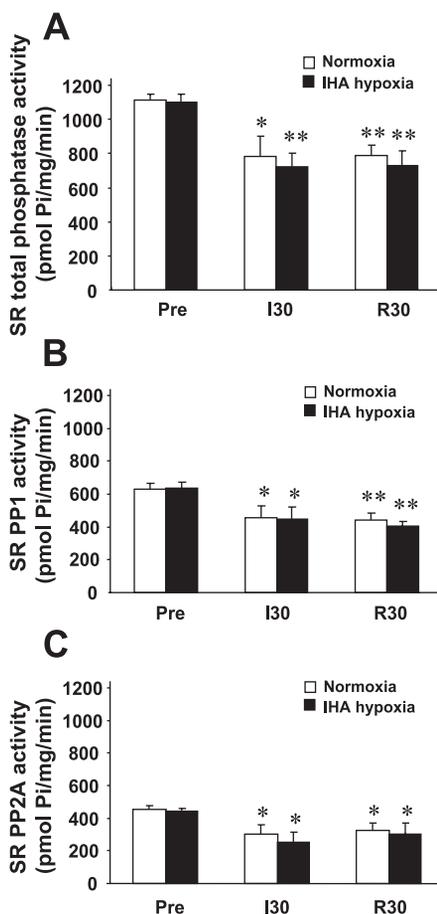


Fig. 6. Activities of SR phosphatase in LV during I/R in normoxic and IHA hypoxic group. A: SR total phosphatase activity. B: SR type 1 phosphatase (PP1) activity. C: SR type 2A phosphatase (PP2A) activity. Values are means \pm SE; $n = 5$ at different time points in each group. * $P < 0.05$, ** $P < 0.01$ vs. corresponding Pre values.

serves as a major player in protecting the SR function in the hearts adapted to IHA hypoxia.

CaMKII is a most abundant CaMK in the heart. Its established significance for the myocardium lies in the fact that it phosphorylates not only SR Ca^{2+} handling proteins, e.g., RyRs, SERCA2, and PLB, but also cardiac contractile proteins, such as troponin T (17). We have recently demonstrated that IHA hypoxia improves PLB PThr¹⁷ and relaxation during perfusion, and the beneficial effects can be abolished by CaMKII inhibitor KN-93 (40). These observations indirectly suggest that CaMKII may be an important mediator in the cardioprotection of IHA hypoxia. In the present study, we confirm that I/R decreases SR and cytosolic CaMKII activity, a consistent result with the observation from similar I/R model (30, 37). Interestingly, IHA hypoxia significantly preserves SR CaMKII activity during reperfusion, but it does not affect CaMKII protein abundance and cytosolic CaMKII activity. Moreover, the blockage of CaMKII inhibitor KN-93 on the improvement of postischemic SR CaMKII activity is accompanied by the loss of protection on the cardiac performance by IHA hypoxia. These results suggest that SR CaMKII plays more important roles than cytosolic CaMKII in the cardioprotection of IHA hypoxia. Furthermore, IHA hypoxia does not affect preischemic and ischemic Ca^{2+} /CaM-dependent Ca^{2+} -uptake activ-

ity, but it attenuates I/R-induced depression of SR Ca^{2+} /CaM-dependent Ca^{2+} -uptake activities significantly. This can be interpreted by the stimulation of SR Ca^{2+} pump activity via PLB phosphorylation, as well as direct phosphorylation of the Ca^{2+} pump by CaMK (27). This is supported by our observation of consistent alternations of PLB PThr¹⁷ in the absence or presence of KN-93 with the changes of SR CaMKII activity, as well as the SR Ca^{2+} /CaM-dependent Ca^{2+} -uptake during I/R. Therefore, SR CaMKII appears to be an important player in the cardioprotection of IHA hypoxia.

In addition, the improvement of SR CaMKII activity by IHA hypoxia verifies that SR CaMKII activity is dependent on Ca^{2+} homeostasis. Because CaMKII is responsive to changes in the intracellular Ca^{2+} (i.e., increased amplitude, prolonged pulse duration, and increased stimulation frequency) (1), which is achieved by grading Ca^{2+} influx through the plasma membrane, modulating Ca^{2+} release and re-uptake into the SR, Ca^{2+} efflux through Ca^{2+} pumps and exchangers, and Ca^{2+} buffering by a multitude of Ca^{2+} binding proteins (5), alterations of the precise regulation of local and global $[\text{Ca}^{2+}]_i$ during I/R may be an explanation of decreased SR CaMKII activity that can occur without a change in the SR CaMKII protein abundance. Indeed, intracellular Ca^{2+} overload has been confirmed to inhibit the cardiac SR CaMKII activity (29). Our laboratory previously observed that I/R-induced significant Ca^{2+} overload in cardiomyocytes is associated with a decrease in the amplitude of the Ca^{2+} transients, and these changes can be improved by IHA hypoxia (6). Such protection is related to maintaining activity of $\text{Na}^+/\text{Ca}^{2+}$ exchange, SR RyRs, and SERCA2, as well as mitochondrial function (6, 45). Thus the improved I/R-induced alterations of resting $[\text{Ca}^{2+}]_i$ and Ca^{2+} transients by IHA hypoxia may interpret the improvement of postischemic SR CaMKII activity in the heart from IHA hypoxic rats.

CaMKII is known to have an autophosphorylation site in the CaM binding domain (Thr²⁸⁷), which, once activated, allows conversion of the kinase to a Ca^{2+} /CaM-independent or autonomous enzyme (1). This phosphorylation may markedly increase the affinity of the subunit for Ca^{2+} /CaM (23) and maintain the kinase activity even after the Ca^{2+} level has declined (25, 35). The latter property endows CaMKII with a memory function (35). However, we did not detect the changes of autophosphorylation of CaMKII at Thr²⁸⁷ during I/R in normoxic and IHA hypoxic group, indicating that IHA hypoxia-preserved SR total CaMKII activity during reperfusion is a result of the improvement of Ca^{2+} -dependent CaMKII activity, but not of the Ca^{2+} /CaM-independent CaMKII activity.

It has been noticed that 6 wk of IHA hypoxia did not alter preischemic SR and cytosolic CaMKII activity, although CaMKII activity is decreased significantly by chronic hypoxia (4). As CaMKII is involved in the dynamic modulation of cellular Ca^{2+} homeostasis and has been implicated in the development of cardiac hypertrophy and heart failure (21), our findings suggest that the cardiac protection afforded by IHA hypoxia is associated with less adverse effects, such as ventricular hypertrophy, than those associated with chronic hypoxia (3, 33, 39, 42).

We have recently demonstrated that IHA hypoxia mitigates I/R-induced injury by upregulating dual-site PLB phosphorylation (40). In the present study, we also confirmed that IHA hypoxia-upregulated PLB PThr¹⁷ is mediated by the improved

SR CaMKII activity during I/R. The phosphorylation state of proteins in the cells is regulated by a balance between the activities of protein kinases and protein phosphatases (10, 15). It has been shown that phosphatase activity is decreased by hypoxia (38). In addition, IP attenuates the suppression of phosphatase activity due to I/R, and inhibition of phosphatase activity enhances preconditioning and protects against I/R injury in adult and aged rat heart (2, 10, 30). Thus IHA hypoxia might alter SR phosphatase activity during I/R, which, in turn, contributes to the IHA hypoxia-mediated protection. Here, we confirmed that I/R decreases SR phosphatase activity in the normoxic group. This is consistent with the previous observation obtained in the similar I/R model (30). However, IHA hypoxia does not affect SR phosphatase activity, with or without I/R. Therefore, the improved PLB phosphorylation during I/R appears mainly regulated by the SR CaMKII activity. These results also indicate that the protective mechanisms of IHA hypoxia and IP may differ in the subtle regulations, although both of them share similar mechanisms, such as inhibition of Ca^{2+} overload, activation of PKC, and mitochondrial ATP-sensitive potassium (mito- K_{ATP}) channels (6, 8, 24, 32, 39, 45). Because PP1/PP2A comprise the majority of serine-threonine phosphatase activity and have a widely subcellular location in the myocardium, whether IHA hypoxia affects the status of PP1/PP2A that regulate other components of the excitation-contraction coupling cascade, such as ion channels on the plasma membrane and the myofilaments (9), needs to be explored.

Besides the restoration of SR function and the improvement of CaMKII activation during I/R, several other mechanisms may also contribute to the cardioprotective effects of IHA hypoxia. We previously observed that Ca^{2+} paradox (5 min of Ca^{2+} depletion followed by 30 min of Ca^{2+} repletion) incapacitated contractility of the hearts from normoxic rats, whereas the IHA hearts significantly preserved contractile activity. Such protective effect is abolished by 5-hydroxydecanoate, a selective mito- K_{ATP} channel blocker, and significantly attenuated by KN-93, indicating an important contribution of mitochondria in the cardiac protection of IHA hypoxia (39). This has been confirmed by our observations that IHA hypoxia inhibits I/R-induced Ca^{2+} overload and improved myocyte contraction during I/R, via activating the mito- K_{ATP} channels (43), and inhibiting the opening of mitochondrial permeability transition pores (45). In addition, a bigger decrease in cell contraction at reperfusion than that in the Ca^{2+} transients observed in the normoxic group is not detected in the IHA hypoxic group, suggesting an improvement of Ca^{2+} sensitivity of myofilaments during I/R by IHA hypoxia (6). Moreover, IHA hypoxia-activated cAMP-dependent protein kinase A pathway appears to contribute to the functional recovery during reperfusion by upregulating Ser¹⁶ phosphorylation of PLB at the end of ischemia and onset of reperfusion (40). Furthermore, IHA hypoxia activates ϵ -isoform of protein kinase C (PKC- ϵ) at the preischemic (8) and I/R hearts (our unpublished data). We showed recently that activation of PKC- ϵ maintains mitochondrial function during I/R, which subsequently improves mitochondrial and cytosolic Ca^{2+} homeostasis and contraction in the cardiomyocyte (11). Whether such mechanism is involved in the cardioprotection of IHA hypoxia needs to be determined.

In conclusion, the present study verifies that IHA hypoxia attenuates I/R-induced suppression on the PLB PThr¹⁷ through the preservation of CaMKII activity, but not SR phosphatases. Therefore, CaMKII appears to play an important role in the form of long-lasting cardioprotection.

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