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Mol Cell Biochem
DOI 10.1007/s11010-016-2833-8

Chronic intermittent hypoxia affects the cytosolic phospholipase $A_2\alpha$/cyclooxygenase 2 pathway via β_2-adrenoceptor-mediated ERK/p38 stimulation

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Received: 3 June 2016 / Accepted: 23 September 2016

Abstract Cardiac resistance against acute ischemia/reperfusion (I/R) injury can be enhanced by adaptation to chronic intermittent hypoxia (CIH), but the changes at the molecular level associated with this adaptation are still not fully explored. Phospholipase A_2 (PLA_2) plays an important role in phospholipid metabolism and may contribute to membrane destruction under conditions of energy deprivation during I/R. The aim of this study was to determine the effect of CIH (7000 m, 8 h/day, 5 weeks) on the expression of cytosolic $PLA_2\alpha$ ($cPLA_2\alpha$) and its phosphorylated form (p-$cPLA_2\alpha$), as well as other related signaling proteins in the left ventricular myocardium of adult male Wistar rats. Adaptation to CIH increased the total content of $cPLA_2\alpha$ by 14 % in myocardial homogenate, and enhanced the association of p-$cPLA_2\alpha$ with the nuclear membrane by 85 %. The total number of β-adrenoceptors (β-ARs) did not change but the β_2/β_1 ratio markedly increased due to the elevation of β_2-ARs and drop in β_1-ARs. In parallel, the amount of adenylyl cyclase decreased by 49 % and $G_i\alpha$ proteins increased by about 50 %. Besides that, cyclooxygenase 2 (COX-2) and prostaglandin E_2 (PGE_2) increased by 36 and 84 %, respectively. In parallel, we detected increased phosphorylation of protein kinase Cα, ERK1/2 and p38 (by 12, 48 and 19 %, respectively). These data suggest that adaptive changes induced in the myocardium by CIH may include activation of $cPLA_2\alpha$ and COX-2 via β_2-AR/G_i-mediated stimulation of the ERK/p38 pathway.

Keywords Heart · Hypoxia · Ischemia/reperfusion · Phospholipase A_2 · Cyclooxygenase 2 · β-Adrenoceptor · MAPK

Introduction

Physiological adaptation to chronic intermittent hypoxia (CIH) has long-term cardioprotective effects against acute ischemia/reperfusion (I/R) injury, as manifested by reduced infarct size, attenuation of I/R-induced arrhythmias and improved recovery of contractility [1, 2]. We have shown that a number of signaling molecules including reactive oxygen species (ROS), protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K)/Akt are involved in the cardioprotective mechanism of CIH [3–5]. Nevertheless, the precise mechanism of this phenomenon is not yet known.

There are two main subtypes of β-adrenoceptors in the myocardium, β_1-AR and β_2-AR [6]. Unlike β_1-AR, β_2-AR are coupled dually to G_s and G_i proteins [7]. It has been suggested that β_2-AR/G_i signaling activates the PI3K/Akt cell survival pathway, which plays a crucial role in the protection of cardiomyocytes against apoptosis. Chesley et al. [8] observed that the β_2-AR stimulation prevented hypoxia or ROS-induced apoptosis in rat neonatal cardiomyocytes and it markedly increased MAPK/ERK and PI3K activity, as well as Akt phosphorylation. In addition, a selective inhibitor of PI3K blocked β_2-AR-mediated cardiomyocyte protection. As for our model of hypoxia,

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Published online: 30 September 2016

elevated abundance of p-Akt was detected in CIH-adapted myocardium [9]. Interestingly, it has been found that increased activation of cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) under stress conditions was connected with diminution in β_1-AR density, uncoupling of β_2-ARs from the G_s protein-regulated adenylyl cyclase (AC) pathway and increased coupling of β_2-ARs to the G_i protein/phospholipase C (PLC)/$cPLA_2\alpha$/COX-2 pathway [10, 11].

Phospholipases A_2 (PLA_2) are important enzymes that take part in the repairing and remodeling of the cell membranes. Moreover, PLA_2 are also involved in generation of lipid signaling molecules by hydrolysis of the *sn*-2 ester bound of glycerophospholipids to yield free long chain fatty acids (FA) and 2-lysophospholipids [12]. In myocardium the members of three PLA_2 classes are present differing in structure, cellular localization, and Ca^{2+} requirement for catalytic activity and function [13]. First, the most abundant heart PLA_2 is intracellular calcium-independent PLA_2 ($iPLA_2$). This group of PLA_2 do not require Ca^{2+} for the catalytic activity and main role of these enzymes is preferential hydrolysis of the peroxidized FA from phospholipids, thus they mainly participate in the membrane repair via the deacylation/reacylation cycle [14]. Second, secretory PLA_2 ($sPLA_2$) are low molecular weight enzymes requiring mM Ca^{2+} concentrations for the catalytic activity and they are believed to play an important role in inflammatory processes [12]. Third group comprises intracellular cytosolic PLA_2 ($cPLA_2$). Among six known $cPLA_2$ only $cPLA_2\alpha$ exhibits preference for hydrolysis of arachidonic acid (AA) from phospholipids. This FA can either function as an important signaling molecule or it can be oxidatively metabolized to various bioactive eicosanoids through cyclooxygenase (COX), lipooxygenase and epoxygenase pathways [12]. In fact, the catalytic activity of $cPLA_2\alpha$ is calcium-independent but low (μM) concentration of intracellular Ca^{2+} is necessary for the $cPLA_2\alpha$ translocation from the cytosol to the phospholipid membranes [15]. In addition, its activity is enhanced by phosphorylation of serine residues mediated by members of the MAPK family, Ca^{2+}/calmodulin-dependent protein kinases II and protein kinase C [16–18].

As outlines above, β_2-ARs may switch from G_s to $G_{i/o}$ coupling under certain conditions. Gβγ subunits released from $G_{i/o}$ heterotrimers are capable of stimulating the PI3K/Akt and PLC/PKC pathways. It is known that PI3K/Akt and PKCα may activate ERK1/2 and p38, and these MAPKs can stimulate $cPLA_2\alpha$ [16, 19, 20]. Besides that, activity of $cPLA_2\alpha$ can be also enhanced by the specific phosphorylation mediated by PKCα [18]. Importantly, the cAMP/protein kinase A (PKA) pathway has been found to oppose the activation of the MAPK cascades in many cell types [21]. Attenuation of AC activity and cAMP production may thus facilitate ERK1/2 and p38 activation, allowing these kinases to exert their stimulatory effect on $cPLA_2\alpha$. Free AA liberated by $cPLA_2\alpha$ may serve as substrate for COX-1 and COX-2, and their metabolic pathways lead to generation of prostaglandin E_2 (PGE_2). A schematic depiction of the putative interactions between β_2-AR signaling and the $cPLA_2\alpha$/COX/PGE_2 pathway is shown in Fig. 1. However, the possible role of β_2-AR signaling in regulation of $cPLA_2\alpha$-dependent PGE_2 synthesis in chronically hypoxic myocardium has not yet been explored. Therefore, in the present study, we used a standard rat model to investigate the effect of CIH on the expression of the main components of myocardial β-AR signaling system, PKCα and MAPKs that may affect the $cPLA_2\alpha$/COX/PGE_2 pathway.

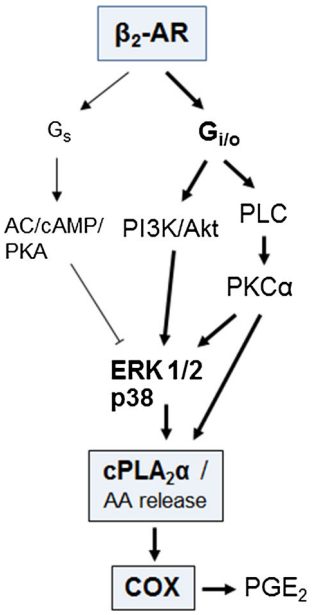

Fig. 1 A simplified scheme showing the putative interactions between β_2-AR signaling and the $cPLA_2\alpha$/COX/PGE_2 pathway in rat myocardium under chronically hypoxic conditions. Switching of β_2-AR from G_s to $G_{i/o}$ coupling results in activation of PI3K and/or PLC. The latter enzyme cleaves phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3). DG and IP_3-induced surges of internal Ca^{2+} stimulate PKCα. Activity of ERK1/2 and p38 MAPKs can be enhanced by phosphorylation mediated by both PI3K and PKCα. By contrast, cAMP/PKA signaling may diminish the activation of these MAPKs. ERK1/2 and p38 as well as PKCα stimulate $cPLA_2\alpha$ and release of AA, which serves as substrate for COX and generation of PGE_2. *AA* arachidonic acid; *AC* adenylyl cyclase; *β_2-AR* β_2-adrenergic receptor; *$cPLA_2\alpha$* cytosolic phospholipase $A_2\alpha$; *COX* cyclooxygenase; *MAPKs* mitogen-activated protein kinases; *PGE_2* prostaglandin E_2; *PKA* protein kinase A; *PKCα* protein kinase Cα; *PLC* phospholipase C

Materials and methods

Drugs and chemicals

Bovine serum albumin as the standard for analysis of protein concentration was obtained from Bio-Rad (Hercules, CA, USA). The antibodies against cPLA$_2\alpha$, p-cPLA$_2\alpha$, ERK1/2, p-ERK1/2, p38 and p-p38 were from Cell Signaling Technology (Beverly, MA, USA), the antibodies against COX-1, COX-2, p-PKCα, G$_i\alpha$3 and anti-goat IgG secondary antibody conjugated with horseradish peroxidase from Santa Cruz Biotechnology (Santa Cruz, CA, USA), the antibody against AC6 was from Acris Antibodies (Rockville, MD, USA), and the antibodies against AC5 and HPRT1 and MitoProfile Total OXPHOS Rodent Antibody Coctail were purchased from Abcam (Cambridge, UK). Preparation of G$_i\alpha$1,2 antibody was described previously [22]. PGE$_2$ EIA Kit and pyrrophenone (cPLA$_2\alpha$ inhibitor) were from Cayman Chemical Company (Ann Arbor, MI, USA). Anti-rabbit IgG 488 and anti-mouse A647 secondary antibodies, WGA and ProLong Gold Antifade Reagent were obtained from Life Technologies (Carlsbad, CA, USA). [^{3}H]CGP-12,177 was purchased from ARC (St. Louis, MO, USA) and scintillation cocktail Ecolite from MP Biomedicals (Santa Ana, CA, USA). Collagenase type 2 was from Worthington (Lakewood, NJ, USA), SYTOX Green nucleic acid stain (S7020) from Invitrogen-Molecular Probes (Eugene, OR, USA). All other chemicals and drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animal model

Adult male Wistar rats (320–350 g body wt; Velaz, Ltd., Czech Republic) were adapted to intermittent high-altitude hypoxia of 7000 m in hypobaric chamber for 8 h/day, 5 days/week, 5 weeks (25 exposures). Barometric pressure (P_B) was lowered stepwise, so that the final level equivalent to an altitude of 7000 m ($P_B = 40.9$ kPa, $P_{O2} = 8.5$ kPa) was reached after 13 exposures [3]. The control rats were kept for the same period of time at P_B and P_{O2} equivalent to an altitude of 200 m ($P_B = 99$ kPa, $P_{O2} = 20.7$ kPa). Rats had free access to water, were fed a standard laboratory diet and kept at the 12/12 h light/dark cycle. All rats were employed the day after the last hypoxic exposure and killed by decapitation. Hearts were rapidly excised, washed in cold saline (0 °C) and right, left ventricular walls and septum were dissected and weighed. Left ventricles (LV) were used for analyses (method see below). The study was conducted in accordance with the Animal Protection Law of the Czech Republic (311/1997). The experimental protocols were approved by the Ethics Committee of the Institute of Physiology, Czech Academy of Sciences.

Tissue homogenization and western blot analysis

Frozen LVs were pulverized to fine powder with liquid nitrogen and subsequently homogenized in eight volumes of ice-cold homogenization buffer consisting of 12.5 mM Tris (pH 7.4), 250 mM sucrose, 2.5 mM EGTA, 1 mM EDTA, 100 mM NaF, 0.3 mM phenylmethylsulfonyl fluoride, 6 mM β-mercaptoethanol, 10 mM glycerol-3-phosphate, 0.2 mM leupeptin, 0.02 mM aprotinin and 0.1 mM sodium orthovanadate. All steps were performed at 4 °C. The homogenate aliquots were stored at −80 °C until use. Nuclear fraction was isolated from LV myocardium as previously described [23]. The protein concentration of each preparation was determined by Bradford assay reagent using bovine serum albumin as the standard.

Samples were resolved by SDS-PAGE electrophoresis and transferred to nitrocellulose membranes (Amersham Biosciences, Freiburg, Germany). After blocking with 5 % dry low-fat milk in Tris-buffered saline with Tween 20 (TTBS) for 60 min at room temperature, membranes were washed and probed with primary antibodies either for 90 min (PKCα, p-PKCα and HPRT1) or overnight (cPLA$_2\alpha$, p-cPLA$_2\alpha$, COX-1, COX-2, ERK1/2, p-ERK1/2, p38, p-p38, G$_i\alpha$1,2, G$_i\alpha$3, AC5 and AC6), and subsequently incubated with the secondary anti-rabbit or anti-goat antibodies conjugated with horseradish peroxidase for 60 min. To ensure the specificity of immunoreactive proteins, prestained molecular mass protein standards and rat brain cortex homogenate as the positive control were used. The samples from each experimental group were run on the same gel and quantified on the same membrane. Bands were visualized by enhanced chemiluminescence on the autoradiographic film (Agfa HealthCare NV, Mortsel, Belgium). The analysis of each heart sample was repeated at least six times and HPRT1 was used for comparative quantification of the monitored protein amount in western blot analysis. The results were normalized to the total protein amount. ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA) was used for quantification of the relative abundance of proteins.

β-Adrenoceptor binding

Crude myocardial membranes for assessment of β-adrenoceptor binding were prepared from LV homogenates by centrifugation for 10 min at 600×g. Total amount of myocardial β-ARs was determined by radioligand binding assay with the β-AR antagonist [^{3}H]CGP-12177 as described previously [24]. Saturation isotherms were prepared by incubation of LV preparations with varying concentrations (0.06–4 nM) of the radioligand in the absence (total binding) or presence (nonspecific binding) of 1 μM propranolol. For competition binding experiments,

samples were incubated with 1 nM [^{3}H]CGP-12177 and increasing concentrations of the selective β_2-AR antagonist ICI 118.551 (10^{-4}–10^{-10} M). The reactions were stopped by rapid filtration using Brandel cell harvester over Whatman GF/C glass fiber filters that had been treated with 0.1 % polyethylenimine followed by washing with 3 ml cold wash buffer containing 25 mM Tris–HCl (pH 7.5) and 1 mM $MgCl_2$. The radioactivity remaining on the filter was counted by liquid scintillation counter.

Quantitative immunofluorescence microscopy

Preparation of hearts and cryosections was performed as described previously [25]. Briefly, five normoxic and five CIH hearts were perfused by Tyrode solution and then fixed by 4 % formaldehyde and cryoprotected in 20 % sucrose. LVs were then cut transversally and snapped into liquid nitrogen, stored in −80 °C till use. Longitudinal cryosections of the apex part from each heart were used. All cryosections (5–7 μM) were fixed in 4 % formaldehyde for 5 min and permeabilized in 1 % SDS for 5 min. Non-specific binding sites were blocked by an appropriate normal serum. Cryosections were incubated with primary antibody against p-cPLA$_2\alpha$ and further incubated with anti-rabbit IgG Alexa Fluor 488 secondary antibody. The mitochondrial compartment was stained with MitoProfile Total OXPHOS Rodent Antibody Cocktail and visualized with goat anti-mouse IgG Alexa Fluor 647 secondary antibody. Regarding the detection of sarcolemmal membranes, sections were incubated with wheat-germ agglutinin conjugated with tetramethylrhodamine (WGA). Cryosections were mounted in ProLong Gold Antifade Reagent containing DAPI nuclear marker. Cryosections were examined using the wide-field inverted fluorescence microscope (Olympus IX2-UCB) equipped with fully motorized stage (Corvus) and MT20 mercury arc illumination unit (Olympus). Each experimental sample was observed with 100 × 1.4NA Plan-Apochromat objective lens. At least five digital images from each sample were acquired using CCD camera (Orca C4742-80-12AG, Hamamatsu Photonics). NoN (No Neighbour) algorithm of Olympus Soft Imaging Solutions software was used for deconvolution of the scanning images. Images were quantitatively analyzed by using ICA plugin of Fiji Image J open source software [26]. The Mander's M2 correlation coefficient was used for calculation of the degree of co-localization between channels of multiple regions of interest from each sample [27].

Analysis of PGE_2 concentration

Prostaglandin E_2 (PGE_2) assay was conducted by non-competitive ELISA kit according to the manufactureŕs instructions (Cayman). This assay was performed on homogenized samples intended for western blot analysis from LVs of normoxic and CIH-adapted rats. The results are expressed per mg of total protein.

Isolation of cardiomyocytes and assessment of cell viability

Cardiomyocytes were isolated as previously described [28]. The rats were heparinized and killed by cervical dislocation. The hearts were perfused with Tyrode solution at 37 °C under constant flow (10 ml/min) for 5 min, followed by perfusion with nominally Ca^{2+}-free Tyrode for 8 min. Tissue digestion was initiated by adding 15,000 U collagenase, type 2 and 7 mg protease type XIV into 30 ml of Ca^{2+}-free Tyrode containing 50 mg BSA. All solutions were gassed with 100 % O_2. After 20 min, the collagenase-protease cocktail was washed out by 10-min perfusion with Ca^{2+}-free Tyrode. Myocytes isolated from the left ventricle (LVM) were dispersed mechanically and then filtered through a nylon mesh to remove non-dissociated tissue. LVM solutions were adjusted to the same cell density, transferred to culture medium (50 % Dulbecco's modified Eagle's medium and 50 % Nutrient Mixture F12HAM, containing 0.2 % BSA, 100 U/ml penicillin and 100 mg/ml streptomycin) and kept in a CO_2 incubator (95 % air, 5 % CO_2, 28 °C) for a 1-h stabilization period.

The dose–response of LVM viability to the cPLA$_2\alpha$ inhibitor pyrrophenone was determined. The concentrations of 0.1, 1, 5 and 10 μM pyrrophenone were tested. The percentage of living cells compared to the untreated control cells was assessed with SYTOX Green nucleic acid stain (S7020) at the beginning of the experiment (after stabilization) and after 2, 4 and 20 h. The fluorescence signal of SYTOX Green, which is proportional to the number of dead cells [29], was measured at an excitation wavelength of 490 nM and emission wavelength of 520 nM using a Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek, Winooski, VT, USA). The 1 μM concentration of pyrrophenone, which had no effect on the number of surviving cells during 20-h incubation, has been chosen for the following experiments. The 1 μM concentration of pyrrophenone was also previously used for experiments with H9c2 cells [30].

LVM isolated from hypoxic and normoxic rats were pre-treated for 20 min with 1 μM pyrrophenone or vehicle (0.01 % DMSO) and subjected to 25 min of metabolic inhibition (MI) followed by 30 min of re-energization (MI/R). LVM from each treatment group were split into two parts of equal volumes. Control cells were incubated in a normal Krebs solution and not exposed to MI/R. MI was induced by the modified Krebs solution (containing 1.5 mM NaCN and 20 mM 2-deoxyglucose instead of

glucose). The re-energization was achieved by replacing the MI solution with the normal cell culture medium (the same medium was applied to control cells).

Cell viability was analysed at the beginning of the experiments (after stabilization) and after re-energization as previously described [28]. The number of viable (unstained) myocytes was determined by Trypan blue exclusion. 50–100 myocytes were counted in duplicates from 5 to 8 independent experiments. Viable myocytes were divided according to the cell length-to-width ratio as follows: rod-shaped myocytes (ratio >3:1) and non-rod-shaped myocytes (ratio <3:1). Viability after MI/R was expressed as a percentage of rod-shaped cells that survived the MI/R insult and normalized to the appropriate control group not exposed to MI/R.

Statistical analysis

The results are expressed as means ± SEM from the indicated number of experiments. Statistical significance of comparing differences in normally distributed variables between the groups was determined by one-way ANOVA and subsequent Student–Newman–Keuls test. $P < 0.05$ were considered to be statistically significant.

Results

Weight parameters

The adaptation of rats to CIH led to the significant body growth retardation by 10 %. The heart weight of chronically hypoxic groups increased due to hypertrophy of both ventricles compared with normoxic ones. The right ventricular weight, normalized to body weight, increased by 61 % and that of LV by 22 % compared with normoxia (Table 1).

β-Adrenoceptors, G proteins and adenylyl cyclase

Myocardial β-ARs were characterized by saturation and competitive radioligand binding assays (Fig. 2). The total number of β-ARs (about 18 fmol/mg protein) and the dissociation constants (about 0.65 nM) of these receptors in crude membranes from LV myocardium were not affected by adaptation to CIH (Table 2). However, CIH markedly changed the proportion of β-AR subtypes. The proportion of β_2-ARs rose from 29 to 39 %, which corresponds to increase in β_2-ARs by 35 % and decrease in β_1-ARs by 14 % (Table 3). In other words, the β_2/β_1 ratio shifted from 0.40 to 0.64.

Western blot analysis of the dominant myocardial AC5 and AC6 isoforms and the inhibitory $G_i\alpha$ proteins revealed a significant effect of CIH on the expression of these main components of the myocardial β-adrenergic signaling system (Fig. 3). The decline in adenylyl cyclase was solely brought about by marked reduction in AC5 (by 56 %). AC6 remained apparently unaffected by CIH and the levels

Table 1 Weights parameters of normoxic and CIH-adapted rats

Parameter	Normoxia	CIH
BW, g	388 ± 4	351 ± 9*
HW, mg	919 ± 20	1048 ± 43*
HW/BW, mg/g	2.368 ± 0.056	2.987 ± 0.120*
LVW/BW, mg/g	1.289 ± 0.031	1.576 ± 0.076*
RVW/BW, mg/g	0.488 ± 0.014	0.788 ± 0.028*
SW/BW, mg/g	3.547 ± 0.015	3.742 ± 0.029

Values are mean ± SEM of six rats in each group

CIH chronic intermittent hypoxia; *BW* body weight; *HW* heart weight; *LVW* left ventricular weight; *LVW/BW* relative left ventricular weight; *RVW* right ventricular weight; *RVW/BW* relative right ventricular weight; *SW/BW* relative septum weight

* $P < 0.05$ hypoxic vs. corresponding normoxic group

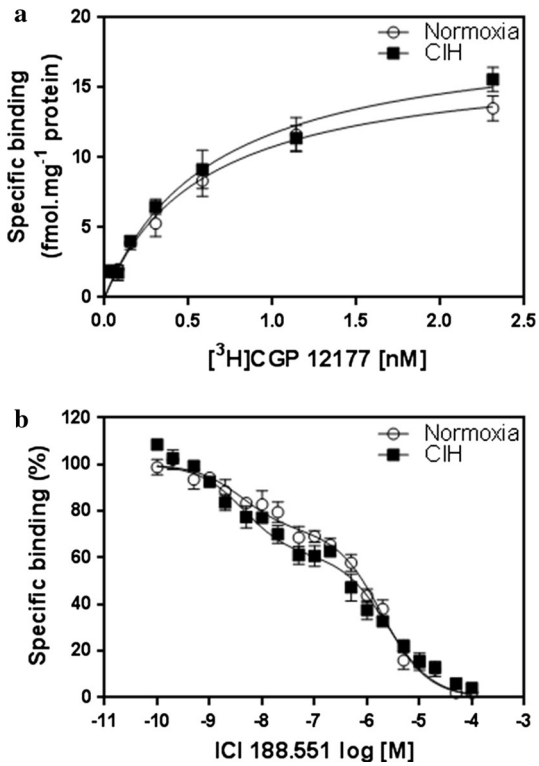

Fig. 2 Characterization of the β-adrenoceptors in LV preparations from normoxic (*open circles*) and CIH-adapted (*closed squares*) rats. There are displayed [^{3}H]CGP 12,177 saturation binding curves (**a**) and competitive binding curves (**b**) which were constructed using the β_2-AR antagonist ICI 188.551. Values are represented as mean ± SEM from four separate experiments performed in triplicates

Table 2 Binding characteristics of myocardial β-ARs in normoxic and CIH-adapted rats

	Normoxia	CIH
B_{max} [fmol·mg^{-1}]	17.23 ± 0.81	19.47 ± 0.70
K_D [nM]	0.62 ± 0.07	0.68 ± 0.09

Values are mean ± SEM of four left ventricles in each group

CIH chronic intermittent hypoxia; B_{max} maximal binding capacity; K_D dissociation constant

Table 3 Distribution and properties of myocardial β-AR subtypes in normoxic and CIH-adapted rats

	Normoxia	CIH
β2 (%)	28.86 ± 2.55	39.02 ± 1.42*
$K_i\beta_2$ [nM]	2.16 ± 0.67	2.03 ± 0.51
$K_i\beta_1$ [μM]	0.75 ± 0.14	1.03 ± 0.16

Values are mean ± SEM of four left ventricles in each group

CIH chronic intermittent hypoxia; K_i inhibition constant

* $P < 0.05$ hypoxic vs. corresponding normoxic group

of $G_i\alpha1,2$ and $G_i\alpha3$ increased by 53 and 49 %, respectively. There was no significant change in the expression of the stimulatory $G_s\alpha$ protein (data not shown).

cPLA$_2\alpha$ and its activating proteins

The amount of cPLA$_2\alpha$ and its phosphorylated form (p-cPLA$_2\alpha$) was increased in LV preparations of CIH-adapted rats compared with normoxic ones by 96 and 41 %, respectively (Fig. 4). Adaptation to CIH increased the immunoreactivity of total PKCα and p-PKCα by 14 and 12 %, respectively. As for ERK1/2 and p38, CIH did not affect the total protein levels but increased the level of phosphorylation (p-ERK1/2 by 48 % and p-p38 by 19 %) as compared with normoxic controls (Fig. 5).

Immunofluorescence analysis of p-cPLA$_2\alpha$

Immunofluorescence analysis revealed that nuclear localization of p-cPLA$_2\alpha$ in LV myocardium increased by 85 % after adaptation to CIH compared with normoxic controls. Co-localization of p-cPLA$_2\alpha$ with other membranes was not found (Fig. 6). In order to quantify CIH-induced p-cPLA$_2\alpha$ localization to cell nuclei, Mander's M2 correlation coefficient between the green (p-PLA$_2\alpha$) and the blue channels (DAPI) was calculated. Its mean value significantly increased from 0.35 ± 0.01 in normoxia to 0.65 ± 0.02 in tissue from CIH-adapted rats (Fig. 6c). Subsequent western blot analysis confirmed increased immunoreactivity of p-cPLA$_2\alpha$ (by 44 %) in the nuclear fraction isolated from LV myocardium of CIH-adapted rats compared to the normoxic group (Fig. 7).

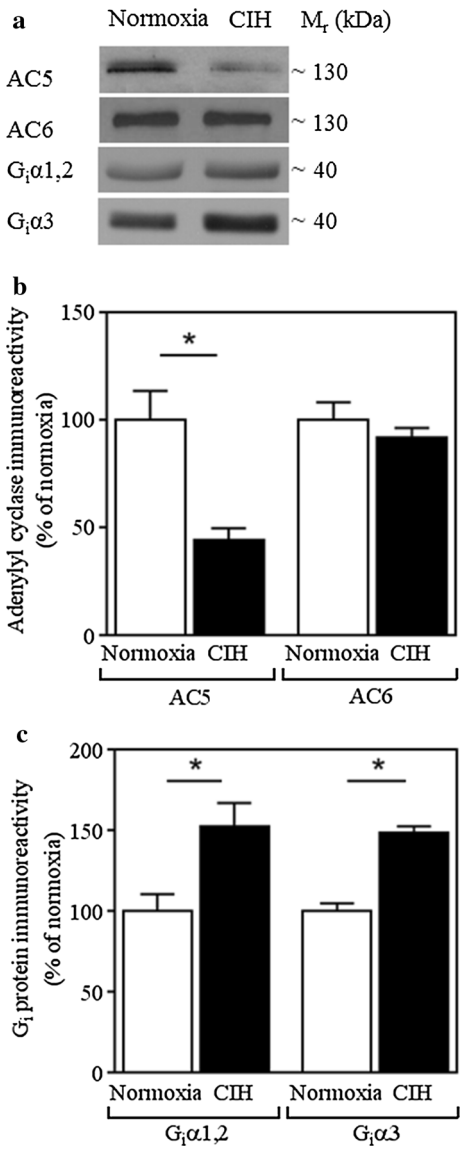

Fig. 3 Effect of chronic intermittent hypoxia on adenylyl cyclase and G_i proteins in rat myocardium. **a** Representative western blots of AC5, AC6, $G_i\alpha1,2$ and $G_i\alpha3$ proteins are shown. **b**, **c** The relative amount of individual proteins in LV preparations from normoxic (*empty columns*) and CIH-adapted (*solid columns*) rats is expressed as a percentage of normoxic values. Values are represented as mean ± SEM from four determinations. *$P < 0.05$ hypoxic vs. corresponding normoxic group

COX-1 and COX-2 expression and PGE$_2$ concentration

Figure 7 shows the protein abundance of COX-1 and COX-2 in LV preparations from rats after adaptation to CIH. Whereas there were no significant changes at the COX-1

protein level, the amount of COX-2 increased by 36 % compared to normoxic controls. Furthermore, the adaptation to CIH increased the total concentration of PGE_2 in LV myocardium by 84 % as compared with normoxia (Fig. 8).

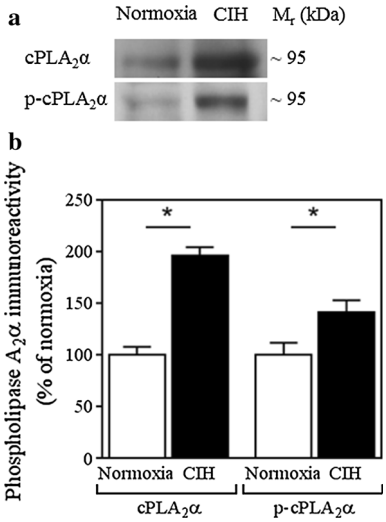

Fig. 4 Effect of chronic intermittent hypoxia on $cPLA_2\alpha$ and p-$cPLA_2\alpha$ in myocardial homogenates. **a** Representative western blots of $cPLA_2\alpha$ and p-$cPLA_2\alpha$ are shown. **b** The relative amount of these proteins in LV preparations from normoxic (*empty columns*) and CIH-adapted (*solid columns*) rats is expressed as a percentage of normoxic values. Values are represented as means ± SEM from six separate determinations. $^*P < 0.05$ hypoxic vs. corresponding normoxic group

Effect of CIH and acute administration of $cPLA_2\alpha$ inhibitor on the viability of isolated cardiomyocytes

Myocytes isolated from the left ventricles of CIH-adapted rats retained the improved resistance against injury caused by MI/R (Fig. 9). Treatment with vehicle/DMSO had no effect on survival of rod-shaped myocytes after the MI/R insult in either normoxic or CIH groups. The acute treatment of LVM with $cPLA_2\alpha$ inhibitor pyrrophenone did not affect the salutary effect of CIH.

Discussion

In the present study, we observed a significantly increased co-localization of activated $cPLA_2\alpha$ (p-$cPLA_2\alpha$) with the nuclear region of CIH-adapted LV myocardium. However, we did not find any co-localization of p-$cPLA_2\alpha$ with other membranes in cardiomyocytes. This observation corresponds well to a previous study where a relocation of $cPLA_2\alpha$ into the nuclear envelope and nuclear periphery but not into the endoplasmic reticulum (ER) or Golgi apparatus upon stimulation with the calcium mobilizing agonist in human endothelial cells was found [31]. These

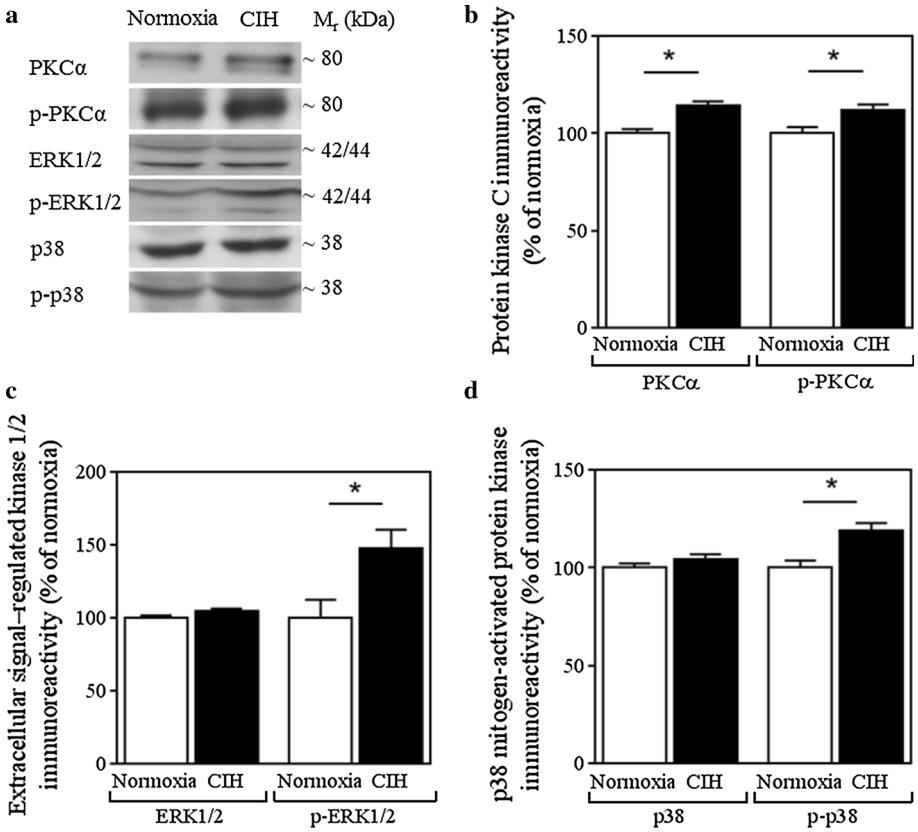

Fig. 5 Effect of chronic intermittent hypoxia on the PKCα and p-PKCα (**b**), ERK1/2 and p-ERK1/2 (**c**), p38 and p-p38 (**d**) protein levels in homogenate from left ventricular myocardium of rats adapted to CIH (*solid columns*) and of normoxic controls (*empty columns*) expressed as a percentage of normoxic values. Representative western blots are shown (**a**). Values are represented as mean ± SEM from six separate determinations. $^*P < 0.05$ hypoxic vs. corresponding normoxic group

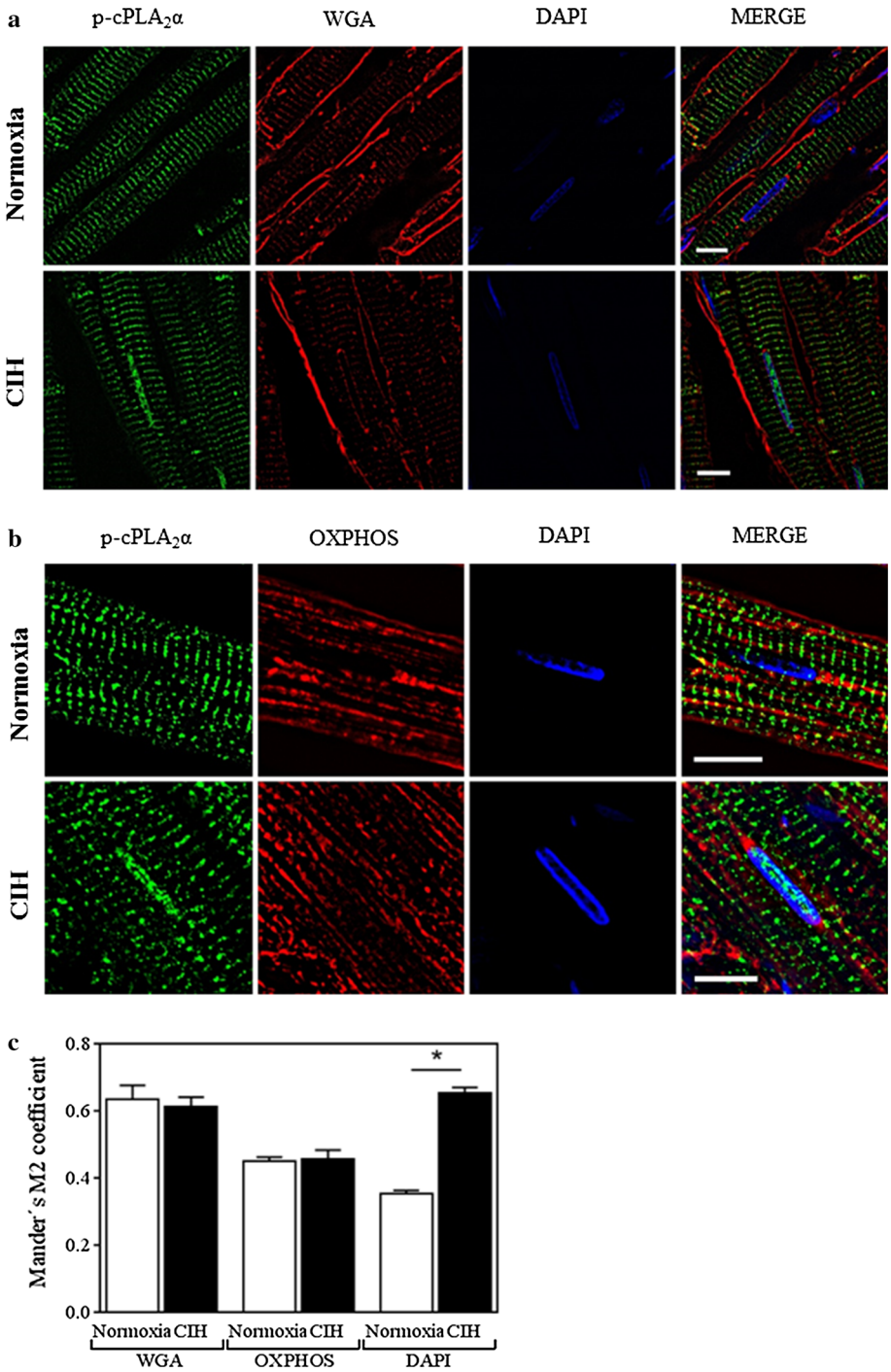

Fig. 6 Effect of chronic intermittent hypoxia on subcellular distribution of p-cPLA$_2\alpha$. Representative images show p-cPLA$_2\alpha$ distribution and co-localization with sarcolemma (**a**) and mitochondria (**b**) in longitudinal cryo-sections of the LV from normoxic and CIH-adapted rats. In all panels, *green* represents specific p-cPLA$_2\alpha$ staining and *blue* indicates the nuclear 4′,6-diamidino-2-phenylindole (DAPI) staining. In *panel* a, *red* represents the wheat-germ agglutinin (WGA) staining of the sarcolemmal membranes, and in *panel* b, *red* represents the OXPHOS complexes. *Scale bar* is 10 μm. **c** Quantification of the mean fluorescence intensity of p-cPLA$_2\alpha$ in sarcolemma (WGA staining), mitochondria (OXPHOS staining) and nuclei (DAPI staining) in cryosections from normoxic (*empty columns*) and CIH-adapted (*solid columns*) rats. The Mander's M2 coefficient was used for evaluation of the co-localization of p-cPLA$_2$ with sarcolemma, mitochondria and nuclei. (Color figure online)

data suggest that the nuclear envelope may serve as the primary site for the AA production in the myocardium after adaptation to CIH. This notion is supported by localization at the nuclear envelope of prostaglandin endoperoxide H synthase-1 and -2, i.e. enzymes catalyzing conversion of AA to its oxidative products [32].

To investigate the molecular mechanism of myocardial response to CIH conditions affecting cPLA$_2\alpha$ enzyme, we focused on the intracellular signaling cascade responsible for its activation. It has been previously shown that the activating phosphorylation of cPLA$_2\alpha$ is provided by MAPKs, notably ERK1/2 and p38 [16, 19]. Here we have found that adaptation to CIH was associated with increased p38 and ERK1/2 phosphorylation, although the total amount of these enzymes did not change. Our results are concordant with a study of Morel et al. [33] showing enhanced abundances of both p-ERK1/2 and p-p38 in cardiac tissue from chronically hypoxic rats. On the other

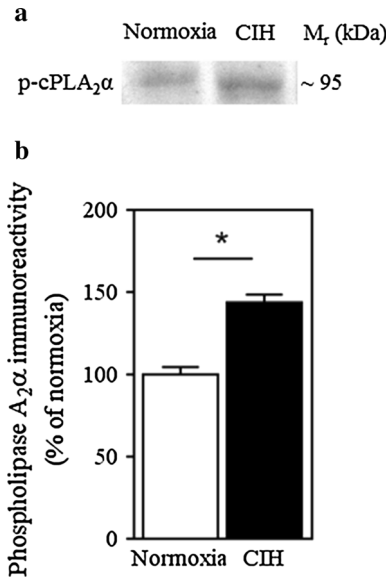

Fig. 7 Effect of chronic intermittent hypoxia on p-cPLA$_2$α association with the nuclear fraction. **a** Representative western blots of p-cPLA$_2$α are shown. **b** The relative amount of p-cPLA$_2$α in LV preparations from normoxic (*empty columns*) and CIH-adapted (*solid columns*) rats is expressed as a percentage of normoxic values. Values are means ± SEM from six determinations. *$P < 0.05$ hypoxic vs. corresponding normoxic group

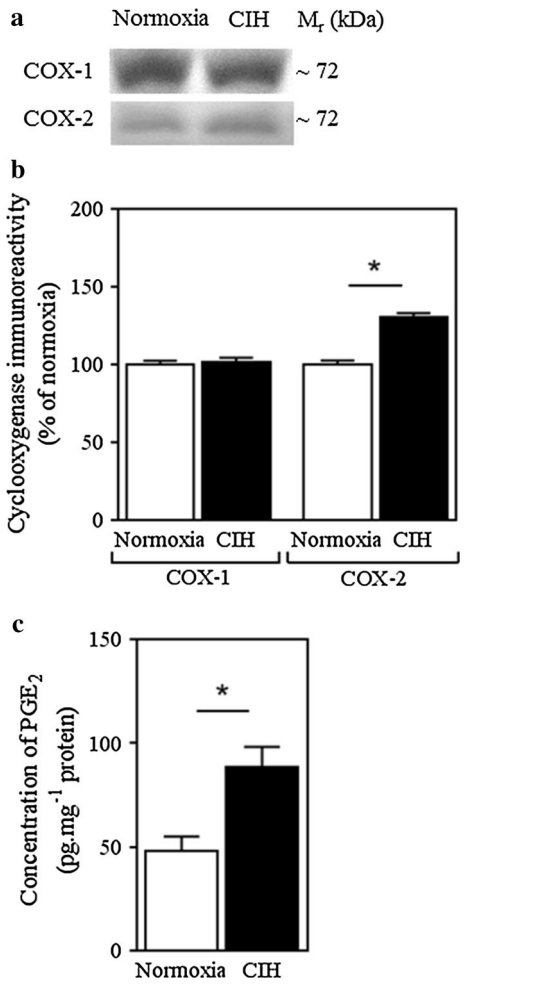

Fig. 8 Effect of chronic intermittent hypoxia on cyclooxygenase and PGE$_2$. **a** Representative western blots of COX-1 and COX-2 are shown. **b** The relative amount of these proteins in LV preparations from normoxic (*empty columns*) and CIH-adapted (*solid columns*) rats is expressed as a percentage of normoxic values. **c** Levels of PGE$_2$ were determined in LV preparations from normoxic (*empty columns*) and CIH-adapted (*solid columns*) rats. Values are means ± SEM from six separate determinations. *$P < 0.05$ hypoxic vs. corresponding normoxic group

hand, Rafiee et al. [34] demonstrated the activation of p38 and Jun kinases but not p-ERK1/2 in infant rabbit hearts adapted to chronic hypoxia. Moreover, the inhibition of these kinases abolished the cardioprotective effect of chronic hypoxia. Interestingly, Seko et al. [35] observed rapid activation of these stress kinases by hypoxia and hypoxia/reoxygenation in cardiac myocytes. MAPKs were strongly activated in the cells responding to increased oxidative stress [36], which had previously been observed under CIH conditions [3].

PKC enzymes were found to be also involved in the regulation of cPLA$_2$α activation and AA release. As for PKCε, Rafiee et al. [34] showed that the enzyme is upregulated and involved in the activation of p38 kinase in chronically hypoxic rabbit hearts. Moreover, inhibition of PKCε and p38 in that model abolished the cardioprotective effect of chronic hypoxia. Surprisingly, under CIH conditions PKCε was reduced whereas the amount of PKCδ was increased in LV myocardium [3, 4]. As regards PKCδ, You et al. [37] suggested that the PKCδ–ROS–NF-κB cascade plays a pivotal role in cPLA$_2$α induction in airways epithelium. In the present study, we observed that CIH was associated with PKCα induction. PKCα was demonstrated earlier as the crucial enzyme isoform participating in activation of cPLA$_2$α [18]. The activation of PKCα is mediated by DAG and IP$_3$/Ca^{2+}, i.e. second messengers generated by signaling pathways downstream of the G protein-coupled receptors and phospholipase C [38]. Interestingly, PKCα activity was also enhanced by increased protease activity induced by peroxynitrite treatment of endothelial cells, which was accompanied by phosphorylation of G$_i$α [39]. Pretreatment of the cells with PKCα inhibitor prevented this phosphorylation, cPLA$_2$α activity and AA release. Conversely, pretreatment with the inhibitor of G$_i$ proteins pertussis toxin inhibited only peroxynitrite-induced increase in cPLA$_2$α activity. Hence, there is a direct link between the inhibition of G$_i$ proteins by pertussis toxin and suppression of cPLA$_2$α activation and ROS generation in endothelial cells, which is regulated by PKCα-dependent phosphorylation [39].

There have been several studies reported in the literature dealing with myocardial β-adrenergic signaling during

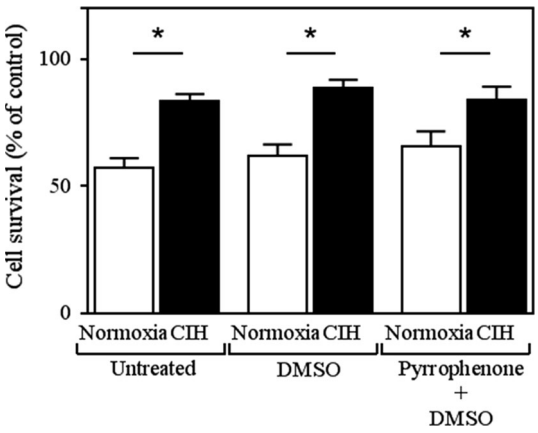

Fig. 9 Effect of the cPLA$_2\alpha$ inhibitor pyrrophenone on survival of left ventricular myocytes during acute metabolic inhibition and re-energization. Control cells were treated with 0.01 % dimethyl sulfoxide (DMSO) as a vehicle. The cells were isolated from rats kept in normoxic (*empty columns*) conditions or from rats adapted to CIH (*solid columns*). Values are means ± SEM from eight separate determinations. *$P < 0.05$ hypoxic vs. corresponding normoxic group

adaptation to hypoxia. However, rather discordant data have been published concerning the effect of hypoxia on myocardial β-ARs. Depending on the experimental conditions, either decrease or no change in the total amount of β-ARs was observed [40–42]. In parallel, the amount of $G_s\alpha$ was found to be unaffected or reduced, $G_i\alpha$ was usually unchanged or somewhat increased, and AC activity was diminished [43, 44]. In our experimental conditions, the adaptation to CIH did not change the total number and dissociation constants of β-ARs, but the proportion of β_2-AR subtype was increased at the expense of β_1-AR. This shift was accompanied by a significant decrease in AC5 and increase in the inhibitory $G_i\alpha$ proteins. These findings are in line with and extend our previous observation of decreased myocardial AC activity in CIH-adapted rats [41]. Interestingly, ablation of AC5 has been shown to be cardioprotective [45]. Moreover, Tong et al. [46] pointed to the significance of β_2-ARs in preconditioning-induced cardioprotection. Switching of β_2-AR coupling from G_s to G_i is apparently mediated by protein kinase A (PKA). It was previously found that PKA-mediated phosphorylation not only reduced β_2-AR coupling with G_s but also enhanced interaction of the receptor with G_i thus reducing cAMP production via G_i-mediated inhibition of AC activity [47].

Concerning cPLA$_2\alpha$, Magne et al. [48] demonstrated that β_2-AR agonists triggered AA release via p38- and ERK1/2-dependent activation of cPLA$_2\alpha$ in embryonic chick ventricular cardiomyocytes. In addition, AA as a hydrolytic product of cPLA$_2\alpha$ has been reported to directly modulate PKCδ and ε isoforms in myocardium [49]. Pavoine et al. [10] reported that regulation of cPLA$_2$ by β_2-ARs depends on the status of receptor coupling to AC in human myocardium and provided the first evidence of the recruitment of cPLA$_2$ by β_2-ARs. Interestingly, cPLA$_2$ as a member of cardiac β_2-AR pathway was found to limit β_2-AR/AC/PKA-induced Ca^{2+} signaling in rat cardiomyocytes through the constraint of phosphorylation of endothelial nitric oxide synthase and phospholamban [50]. Our present results suggest that CIH enhances β_2-AR/G_i signaling which can promote activation of the cPLA$_2\alpha$/COX-2 pathway via ERK/p38 MAPK cascade in the rat myocardium. The involvement of β_2-ARs in up-regulation and activation of ERK/p38 was previously observed under various experimental conditions [51, 52].

The role of cPLA$_2\alpha$ in the protective phenomenon of CIH has not yet been clearly elucidated. Generally, a number of studies on brain and lung tissues have demonstrated the damaging effects of cPLA$_2\alpha$ activation under I/R conditions [53]. However, mice with cPLA$_2\alpha$ gene deletion exhibited a significantly increased infarct size suggesting a protective role for cPLA$_2\alpha$ under I/R conditions [54]. Nevertheless, the acute inhibition of cPLA$_2\alpha$ before simulated ischemia in cardiomyocytes did not reveal any difference in the extent of hypoxic injury between cells isolated from control and cPLA$_2\alpha^{(-/-)}$ animals [54]. Likewise, another two earlier studies conducted on isolated cardiomyocytes failed to confirm the presumed protective role of cPLA$_2\alpha$ [30, 55]. This is in line with our current results on cardiomyocytes isolated from normoxic and CIH-adapted rats where acute administration of a specific inhibitor of cPLA$_2\alpha$ before simulated I/R did not abolish the cardioprotective effect of CIH. This likely indicates the importance of cPLA$_2\alpha$ activation during the adaptation period to chronic hypoxia but not during the acute I/R insult.

In connection with the function of cPLA$_2\alpha$, we studied the effect of CIH on the protein abundances of COXs, the rate-limiting enzymes in the eicosanoid synthesis. Two distinct isoforms have been characterized: constitutive COX-1 enzyme, which is present in most cells, and COX-2, which is induced in response to proinflammatory stimuli [56]. Under CIH conditions, we did not find any changes in COX-1, but the amount of COX-2 increased in LV myocardium. Interestingly, adaptation to chronic continuous hypoxia affected both myocardial COX isoforms in the same manner; there was no change in COX-1 and increase in COX-2 [57]. Similarly, hypoxic conditions increased expression of the COX-2 gene in human vascular endothelial cells mediated by hypoxia-induced binding of the NF-κB p65 protein to the COX-2 promoter region [58]. COX-2 is generally thought to be detrimental in cardiovascular homeostasis [59]. On the other hand, ischemic preconditioning was found to upregulate the expression and activity of COX-2 in the heart, which was necessary for the

protective effect of ischemia-induced late preconditioning against myocardial infarction [60]. As far as the formation of eicosanoids is concerned, adaptation to CIH increased PGE_2 concentration in rat heart. Kerkelä et al. [54] reported a significantly reduced PGE_2 level in mice with $cPLA_2\alpha$ gene deletion and suggested that $cPLA_2\alpha$-dependent production of PGE_2 is important for the infarct-reducing effect in rat heart.

In conclusion, our present study has demonstrated that adaptation of rats to CIH may lead to complex changes in signaling cascades downstream of β-ARs. The observed up-regulation of myocardial β_2-ARs and G_i proteins was accompanied by increased stimulation of ERK1/2 and p38 that are directly linked to activation of the $cPLA_2\alpha$/COX-2/PGE_2 pathway. These data corroborate the relevance of β_2-AR-initiated signaling mechanisms (depicted in Fig. 1) under hypoxic conditions and support the notion that $cPLA_2\alpha$ participates in the development of a cardioprotective phenotype during adaptation to CIH.

Acknowledgments This work was supported by the Charles University Grant Agency (392615), the Czech Science Foundation (13-10267S), the Ministry of Education, Youth and Sport of the Czech Republic (SVV-260313/2016) and the Operational Program OP VaVpI (CZ.1.05/4.1.00/16.0347).

Compliance with ethical standards

Conflict of interests There is no conflict of interest.

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PŘÍLOHA B

Antioxidant tempol supresses heart cytosolic phospholipase $A_2\alpha$ stimulated by chronic intermittent hypoxia

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Abstract

Adaptation to chronic intermittent hypoxia (CIH) is associated with reactive oxygen species (ROS) generation implicated in the improved cardiac tolerance against acute ischemia/reperfusion injury. Phospholipases A_2 (PLA_2s) play an important role in cardiomyocyte phospholipid metabolism influencing membrane homeostasis. Here we aimed to determine the effect of CIH (7000 m, 8h/day, 5 weeks) on the expression of cytosolic PLA_2 ($cPLA_2\alpha$), its phosphorylated form (p-$cPLA_2\alpha$), calcium-independent ($iPLA_2$) and secretory ($sPLA_2$IIA) at protein and mRNA levels as well fatty acids (FA) profile in left ventricular myocardium of adult male Wistar rats. Chronic administration of antioxidant tempol was used to verify the ROS involvement in CIH effect on PLA_2s expression and phospholipid FA remodeling. While CIH did not affect PLA_2s mRNA levels, it increased the total $cPLA_2\alpha$ protein in cytosol and membranes (by 191% and 38%, respectively) and p-$cPLA_2\alpha$ (by 23%) in membranes. On the other hand, both $iPLA_2$ and $sPLA_2$IIA were down-regulated by CIH. CIH further decreased phospholipid n-6 polyunsaturated FA (PUFA) and increased n-3 PUFA proportion. Tempol treatment prevented only CIH-induced $cPLA_2\alpha$ up-regulation and its phosphorylation on Ser^{505}. Our results show that CIH diversely affect myocardial PLA_2s and suggest that ROS are responsible for the activation of $cPLA_2\alpha$ under these conditions.

Key words: heart, chronic intermittent hypoxia, oxidative stress, phospholipases A_2, tempol

Introduction

Exposure to chronic intermittent hypoxia (CIH) leads to adaptive structural and functional changes of the heart that help in maintaining homeostasis with minimum energy expenditure (Ostadal and Kolar 2007). Using a rat model of CIH, a number of studies demonstrated an improved tolerance of the heart to all major end points of acute ischemia/reperfusion (I/R) injury including a reduced infarct size (Neckar et al. 2002*a*), decreased incidence and severity of arrhythmias (Asemu et al. 1999; Asemu et al. 2000) and better post-ischemic recovery of contractile function (Neckar et al. 2002*b*). We found that reactive oxygen species (ROS) generated during hypoxic adaptation play a key role in triggering endogenous protective pathways (Kolar et al. 2007). Although the precise mechanism of this phenomenon is unknown, a number of ROS-sensitive signaling molecules such as protein kinases C (Hlavackova et al. 2010; Neckar et al. 2005; Wang et al. 2011), mitochondrial ATP-sensitive potassium channels (Neckar et al. 2002*b*) or permeability transition pores (Zhu et al. 2006) have been identified as potential players in CIH-induced cardioprotection.

We observed that adaptation to CIH leads to the remodeling of membrane phospholipids, with increased heart n-3 polyunsaturated fatty acids (PUFA) proportion to the detriment of n-6 PUFA (Balkova et al. 2009; Jezkova et al. 2002). This effect can be attributed to deacylation-reacylation cycle in which phospholipases A_2 (PLA_2s) take part in maintaining cell membrane integrity as well as providing lipid mediators for the regulation of cell function under physiological and pathological conditions (Cedars et al. 2009; Jenkins et al. 2009). PLA_2s belong to ROS-dependent enzymes and recent studies have demonstrated their crucial role in mediating oxidative and inflammatory responses (Kim et al. 2008; Niessen et al. 2003; Swift et al. 2007). In myocardium, members of three major PLA_2 classes are present, differing in requirements for activating Ca^{2+}, cell localization and mode of activation: cytosolic PLA_2 ($cPLA_2$), secretory PLA_2 ($sPLA_2$) and calcium-independent PLA_2 ($iPLA_2$). All of them are involved in membrane lipid metabolism by splitting free FA from phospholipid *sn-2* position and producing

lysophospholipids (McHowat and Creer 2004). The most studied member of $cPLA_2$ family is $cPLA_2\alpha$ which preferentially hydrolyses phospholipids containing arachidonic acid (AA) (Clark et al. 1991; Grandits and Oostenbrink 2015) as substrate for enzymes providing biosynthesis of eicosanoids (Jenkins et al. 2009).

Our recent study on CIH-adapted rats demonstrated $cPLA_2\alpha$ translocation to the nuclear region and its activation by phosphorylation in heart left ventricle. The concomitant up-regulation of β_2-adrenergic receptors (β_2-AR) and G_i proteins was accompanied by increased phosphorylation of mitogen-activated protein kinases (ERK1/2, p38), cyclooxygenases 2 (COX-2) expression and prostaglandin E_2 (PGE_2) production (Micova et al. 2016). It is unclear whether the activation of the β_2-AR/G_i/$cPLA_2\alpha$/COX-2 pathway in CIH-adapted myocardium is ROS-dependent. Based on these results, the present study aimed to assess whether ROS play a role in the up-regulation and activation of $cPLA_2\alpha$ in the CIH-adapted heart using tempol as a ROS scavenger (Wilcox 2010; Wilcox and Pearlman 2008). In addition, we also examined the involvement of CIH-induced ROS production in myocardial expression of other members of phospholipase classes, $sPLA_2$IIA and $iPLA_2$.

Materials and methods

Drugs and chemicals

Bovine serum albumin was purchased from Bio-Rad (Hercules, CA, USA). The antibodies against $cPLA_2\alpha$ (2832) and p-$cPLA_2\alpha$ Ser^{505} (2831) were from Cell Signaling Technology (Beverly, MA, USA). The antibody against hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1; ab10479) was obtained from Abcam (Cambridge, UK). The antibodies against $sPLA_2$IIA (160502) and $iPLA_2$ (160507), and $sPLA_2$IIA Western Ready Control were from Cayman Chemical Company (Ann Arbor, MI, USA). For quantitative real-time PCR analysis, Trizol Reagent was from Life Technologies (Carlsbad, CA, USA), RevertAid™ H Minus First Strand cDNA Synthesis Kit from Fermentas (Burlington, Ontario, Canada), and Probe Master kit

from Roche Applied Science (Mannheim, Germany). Silica Gel H and magnon for phospholipid analysis were from Merck (Darmstadt, Germany). All other chemicals and drugs were supplied by Sigma-Aldrich (St. Louis, MO, USA).

Animal model

Adult male Wistar rats (320-350 g body wt; Velaz, Ltd., Czech Republic) were exposed to CIH corresponding to the altitude of 7,000 m in a hypobaric chamber for 8h/day, 5 days/week, 5 weeks (25 exposures). According to the experimental protocol described previously (Kolar et al. 2007), barometric pressure was lowered stepwise, so that the final level equivalent to the altitude of 7,000 m (P_B = 40.9 kPa, P_{O_2} = 8.5 kPa) was reached after 13 exposures. The control group of animals was kept under normoxic conditions equivalent to an altitude of 200 m (P_B = 99 kPa, P_{O_2} = 20.7 kPa) for the same period of time. All animals were housed in a controlled environment (free access to water, standard laboratory diet, 12/12 h light/dark cycle). One-half of the rats from each group received tempol (a cell membrane-permeable amphilite that dismutates superoxide, facilitates hydrogen peroxide metabolism and limits formation of hydroxyl radicals) dissolved in drinking water (1 mM) during the five-week adaptation period. The animals were killed 24 h after the last hypoxic exposure by cervical dislocation. Hearts were removed, washed in cold saline (0 °C), subsequently dissected into right and left ventricular walls (LV) and septum and weighed. LV myocardium was used for analyses (methods see below). The study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by US National Institutes of Health (NHI publication no. 85-23, revised 1996) and the Animal Protection Law of the Czech Republic (311/1997). The experimental protocols were approved by the Ethics Committee of the Institute of Physiology, Czech Academy of Sciences.

Homogenate preparation, fractionation and Western blot analysis

LV was chopped by scissors in eight volumes of ice-cold buffer consisting of 12.5 mM Tris (pH 7.4), 250 mM sucrose, 2.5 mM EGTA, 1 mM EDTA, 100 mM NaF, 0.3 mM phenylmethylsulfonyl fluoride, 6 mM β-mercaptoethanol, 10 mM glycerol-3-phosphate, 0.2 mM leupeptin, 0.02 mM aprotinin and 0.1 mM sodium orthovanadate, then homogenized (Ultra-Turax T8.01, IKA-Werke GmbH & Co. KG, Staufen, Germany) and spun at 200 × *g* for 10 min. The pellet consisting of unbroken cells and debris was discarded. The supernatant was further centrifuged at 105,000 x *g* for 90 min. The resulting supernatant was the cytosolic fraction and pellet represented the membrane fraction, which was re-suspended in homogenization buffer. All steps were performed at 4 °C. The sample aliquots were stored in -80 °C until use. The protein concentration was measured using Bradford dye binding assay using bovine serum albumin as the standard (Bradford 1976).

Individual samples were separated by SDS-PAGE electrophoresis. Resolved proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany). Membranes were blocked for 1 h at room temperature with 5% dry low-fat milk in Tris buffered saline with Tween 20 (TTBS), subsequently washed in TTBS and incubated with primary antibodies against $sPLA_2$IIA, $iPLA_2$ and HPRT1 (90 min, room temperature), and against $cPLA_2\alpha$ and p-$cPLA_2\alpha$ (overnight, 4 °C). After further washing in TTBS, anti-rabbit antibody conjugated with horseradish peroxidase was used as secondary antibody (1 h incubation, room temperature). In order to ensure the specificity of immunoreactive proteins, $sPLA_2$IIA Western Ready Control as the positive control and pre-stained molecular mass protein standards were used. The samples from each experimental group were run on the same gel. Proteins were detected by enhanced chemiluminescence and quantified on the same membrane using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). The analysis of each sample was repeated at least six times and HPRT1 was used for comparative quantification of the

monitored protein amount in Western blot analysis. The results were normalized to the total protein amount.

Quantitative real-time PCR

All procedures including isolation of total RNA from the LV of the normoxic and hypoxic rats (5 individual samples/each group), following reverse transcription as well as PCR quantification were described previously (Holzerova et al. 2015). Quantitative real-time PCR analyses were performed according to manufacture instructions by Light Cycler Roche using Light Cycler 480 Probes Master and following specific primers together with Mono-Color Hydrolysis Probes (#) designed by the Universal Probe Library Assay Design Center (UPL, Roche Applied Science). Sequences of the primers were:

cPLA$_2$α_F*: tctggctcacagaataaaggttc*,

cPLA$_2$α_R*: ctcacaatgtgctttgctgtaa* and #25,

iPLA$_2$_F*: ggagtgctgagaagtgctga*,

iPLA$_2$_R*: cgtccaaagaactgcatccta* and #123,

sPLA$_2$IIA_F*: gtgacctacaagttctcctaccg*,

sPLA$_2$IIA_R*: ttatcgcactggcacagc* and #15,

HPRT1_F*: gaccggttctgtcatgtcg*,

HPRT1_R*: acctggttcatcatcactaatcacc* and *#95*.

The level of analyzed transcripts was normalized to the level of the reference gene HPRT1 transcript (Bohuslavova et al. 2010) with regard to the specific PCR efficiency for each gene (Pfaffl 2001).

Phospholipid fatty acid analysis

Frozen LV samples were pulverized to fine powder with liquid nitrogen. Lipids were extracted from tissue samples according to the modified method of Folch et al. (1957) in three

consecutive steps. The first extraction was performed in three portions (0.25 ml each) of chloroform-methanol mixture (1:3, 2:1 and 2:1 v/v) in a chilled mortar. Subsequent extractions were performed in the chloroform-methanol mixture of 2:1 (0.6 ml each). Saline (20% of the extract volume) was added to the extract and vigorously shaken. The lower lipid layer was carefully separated and dried at 40 °C under a stream of nitrogen. Total phospholipids were separated from lipid extracts by one-dimensional thin-layer chromatography (0.5 mm Silica Gel H, Merck, Germany) using the solvent mixture hexane-ether-acetic acid (80:20:3 v/v/v) detected by 2,7-dichlorofluorescein (0.005% in methanol), scraped out, then stored in a nitrogen atmosphere at -20 °C. The following day, FA methyl esters were prepared and separated by gas chromatography (Tvrzicka et al. 2002). In detail, FA methyl esters were dissolved in 100 µl of hexane and 2 µl injected in split mode (split ratio 30:1) into the GC (FOCUS GC with auto-sampler AI 1310, Thermo Fisher Scientific, MA, USA). The injector and detector (FID) temperatures were both set to 240 °C. The flow of the carrier gas, hydrogen, was maintained through the column (CP WAX 52CB 25 m x 0.25 mm I.D., 0.2 µm, Agilent Technologies, CA, USA) under constant pressure mode (60 kPa). The oven temperature was programmed as follows: initial temperature 80 °C was kept for 1 min and then raised at 20 °C/min to 150 °C with the second ramp 3 °C/min to 230 °C; the final temperature was held for 3 min. The FA methyl esters were identified according to their retention time comparison with standard FA methyl ester mixture Nu-CHECK 566C (Nu-Check Prep., MN, USA).

Statistical analysis

All data are presented as means ± SEM. The one-way ANOVA or two-way ANOVA with Bonferroni post tests were used to examine the statistical significance between the experimental groups. P value of < 0.05 was considered as statistically significant. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., CA, USA).

Results

Weight parameters

The adaptation of rats to CIH led to significant body growth retardation. CIH increased right and left ventricular weight normalized to body weight compared with normoxic controls. The tempol treatment had no effect on body and heart weight parameters (Table 1).

Quantitative real-time PCR analysis of PLA_2

CIH did not influence $cPLA_2\alpha$, $iPLA_2$ and $sPLA_2IIA$ expression at mRNA level in LV tissue homogenate (Fig. 1).

Effect of CIH and tempol on PLA_2 protein levels

Adaptation to CIH increased the relative protein level of total $cPLA_2\alpha$ in the cytosol and membrane fraction (by 191% and 38%, respectively) compared to normoxic controls (Fig. 2a). Also, p-$cPLA_2\alpha$ was increased by 23% in the membrane fraction compared to normoxic controls in response to CIH but it was not detected in cytosol (Fig. 2b).

CIH increased the total $cPLA_2\alpha$ and p-$cPLA_2\alpha$ in homogenate by 14% and 16%, respectively. The chronic tempol treatment had no effect on $cPLA_2\alpha$ and p-$cPLA_2\alpha$ protein levels in normoxic LV. However, it completely prevented the $cPLA_2\alpha$ and p-$cPLA_2\alpha$ up-regulation induced by CIH (Fig. 2c, d).

Figure 3a shows that CIH induced a decrease of $iPLA_2$ protein amount in membrane fraction by 19%. The protein level of $sPLA_2IIA$ was also decreased in the cytosol and membrane fraction after CIH by 34% and 36%, respectively (Fig. 3b).

In homogenate of CIH-adapted rats, the protein amounts of $iPLA_2$ and $sPLA_2IIA$ were decreased by 12% and 15%, respectively, compared to normoxic controls. Chronic tempol treatment decreased $sPLA_2IIA$ protein level in normoxic hearts by 19%. However, no additional effect of tempol on $iPLA_2$ and $sPLA_2IIA$ protein levels was seen in CIH hearts (Fig. 3c, d).

Effect of CIH and tempol on distribution and proportion of main FA in heart phospholipids

CIH increased the total level of saturated FA (SFA) in heart phospholipids mainly due to the increase of palmitic acid (16:0). The proportion of monounsaturated FA (MUFA) was not influenced by CIH. A marked decrease in linoleic acid (18:2n-6) reduced the proportion of total n-6 PUFA in CIH hearts. Decrease of n-6 PUFA was compensated by an increase of n-3 PUFA, particularly docosahexaenoic acid (DHA, 22:6n-3). The tempol treatment significantly influenced FA composition neither in normoxic nor in CIH-adapted hearts (Fig. 4 and Table 2).

Discussion

The present study demonstrates that ROS play an important role in the up-regulation and activation (phosphorylation at Ser^{505}) of $cPLA_2\alpha$ in hearts of rats adapted to CIH. We have also shown that, contrary to the stimulating effect on $cPLA_2\alpha$, CIH decreased protein levels of other important PLA_2s in myocardium ($iPLA_2$ and $sPLA_2$IIA). Altered myocardial PLA_2 levels following CIH may contribute to the observed CIH-induced changes in FA composition of myocardial membrane phospholipids.

PLA_2s have a crucial role in mediating redox and inflammatory responses (Jenkins et al. 2009; Niessen et al. 2003; Swift et al. 2007), which are important for the adaptation to chronic hypoxia (Chytilova et al. 2015; Kolar et al. 2007). Recently, we have shown that CIH is associated with increased formation of ROS which are involved in the induction of an ischemia-resistant cardiac phenotype, since an antioxidant applied during the adaptation period eliminated the infarct size-limiting effect of CIH (Kolar et al. 2007). In this study, we demonstrate that a chronic treatment with the antioxidant tempol completely prevented the CIH-induced increase in myocardial protein levels of $cPLA_2\alpha$ and its active form p-$cPLA_2\alpha$ suggesting a ROS-dependent regulation. In line with these results, it was shown that hydrogen peroxide increased $cPLA_2$ activity in a concentration-dependent manner in Her14 fibroblasts (Van Rossum et al.

2004) and influenced $cPLA_2$-dependent AA release in H9c2 rat cardiac myoblast cells (Winstead et al. 2005). The increased $cPLA_2\alpha$ protein level observed after adaptation to CIH may further contribute to ROS production which is stimulated in CIH hearts (Hlavackova et al. 2010; Kolar et al. 2007). Firstly, the $cPLA_2\alpha$, as opposed to other PLA_2s, possesses a high specificity for AA (Clark et al. 1991; Grandits and Oostenbrink 2015). ROS may be generated as a by-product in further AA conversion by downstream metabolic enzymes (COX, lipoxygenases and cytochrome P450 pathways) (Kim et al. 2008). Moreover, the participation of $cPLA_2\alpha$ in modulating NADPH oxidase (NOX) activity and NOX-dependent ROS production was observed in heart tissue (Khan et al. 2015). Therefore, $cPLA_2\alpha$ up-regulation by CIH can significantly modulate ROS levels and related signaling events in chronically hypoxic hearts.

Results regarding the role of $cPLA_2\alpha$ in increased cardiac resistance to I/R injury are rather controversial. In our previous study, we observed that up-regulation of myocardial β_2-ARs and G_i proteins was accompanied by increased stimulation of ERK1/2 and p38 which are directly linked to the activation of the $cPLA_2\alpha$/COX-2/PGE_2 pathway in CIH hearts (Micova et al. 2016). It was shown that $cPLA_2\alpha$-dependent PGE_2 and possibly other prostaglandins (e.g. epoxyeicosatrienoic acids) production is critical for the infarct size-reducing effect seen in $cPLA_2\alpha$-deficient mice (Kerkela et al. 2011). On the other hand, another study on $cPLA_2\alpha$-deficient mice showed that $cPLA_2\alpha$ disruption attenuated myocardial I/R injury partly through TNFα-mediated signaling (Saito et al. 2012). Overall, the present data indicate that $cPLA_2\alpha$ activation as well as AA are crucially involved in both pathogenic and protective mechanisms and that their effects on the heart depend upon the redox and metabolic status of cardiomyocytes.

In this study, we confirmed that $sPLA_2$IIA mRNA is expressed in rat myocardium as published previously (De Windt et al. 1997; Nyman et al. 2000). The low molecular weight (14-19 kDa) $sPLA_2$ usually requires millimolar Ca^{2+} concentration for catalytic activity and lacks specificity for the FA in the *sn-2* position of phospholipid molecule (Murakami and Kudo 2004). CIH had decreasing trend on $sPLA_2$IIA mRNA and significantly decreased its protein amount.

This enzyme is considered as an inflammation-stimulated PLA_2 (Schwemmer et al. 2001). It was shown that pro-inflammatory cytokines such as TNFα elevate $sPLA_2$IIA levels in neonatal rat cardiomyocytes (De Windt et al. 1997). The explanation of decreased $sPLA_2$IIA protein level after adaptation to CIH is unclear. It seems that its activation and down-regulation is a time-dependent process as suggested by Kuwata et al. (2005). These authors observed that stimulation of fibroblasts by pro-inflammatory cytokines activate $cPLA_2$, thereafter leading to synthesis of 12-, 15-lipoxygenase products and subsequently to $sPLA_2$IIA mRNA up-regulation. The ensuing $sPLA_2$IIA-dependent PGE_2 increase leads to the anti-inflammatory cytokine receptors induction and subsequent signaling through these receptors down-regulates $sPLA_2$ mRNA expression (Kuwata et al. 2005). In line with this view, the increase of PGE_2 (Micova et al. 2016) and anti-inflammatory cytokine IL-10 levels (unpublished data) were found in CIH hearts. Studies dealing with a role of $sPLA_2$IIA in acute I/R injury show that its inhibition is connected with lesser myocardial tissue injury and better cardiomyocyte survival (Krijnen et al. 2006; Nijmeijer et al. 2003; Van Dijk et al. 2009) which is in agreement with decreased $sPLA_2$IIA protein level in hearts adapted to cardioprotective CIH.

We found that CIH decreased $iPLA_2$ protein amount in membrane fraction of rat myocardium and chronic administration of tempol did not reverse this effect. Although the observed down-regulation of $iPLA_2$ was relatively small, it may play an important role in the process of CIH-adaptation since $iPLA_2$ represents the majority of PLA_2s activity in the heart (McHowat and Creer 2004). The role of $iPLA_2$ is generally regarded as housekeeping enzyme in phospholipid remodeling (Balsinde and Dennis 1997). However, they also act as phospholipid repair enzymes preferentially splitting oxidatively damaged FA from phospholipids (Cummings et al. 2002). Among the members of $iPLA_2$ family that do not require Ca^{2+} for its activity (Wolf and Gross 1985), $iPLA_2\beta$ and $iPLA_2\gamma$ have been demonstrated to be the most important in regulating myocardial function (Cedars et al. 2009). Under control condition, $iPLA_2\beta$ is localized in the cytosol, although substantial amount of the enzyme is also present in heart mitochondria

(Williams and Gottlieb 2002). As for iPLA$_2\gamma$, this isoform localizes to either mitochondria or to peroxisomes (Mancuso et al. 2004). Mancuso et al. (2003) demonstrated that ischemia activates iPLA$_2\beta$ in intact myocardium and that iPLA$_2\beta$-mediated hydrolysis of membrane phospholipids can induce lethal malignant ventricular tachyarrhythmias during acute cardiac ischemia. Additionally, inhibition of iPLA$_2$ by bromoenol lactone was cardioprotective, as reflected by a reduction in infarct size after global I/R (Williams and Gottlieb 2002). The myocardial loss of iPLA$_2\gamma$ function substantially reduced infarct size and markedly decreased production of pro-inflammatory oxidized fatty acids after I/R *in vivo* (Moon et al. 2016). Function of iPLA$_2\gamma$ has also been demonstrated to play a role in the mitochondrial permeability transition that can facilitate the release of apoptotic mediators and cell death (Kinsey et al. 2007*a*). From this perspective, the reduced level of iPLA$_2$, observed in CIH-adapted heart could be beneficial. On the other hand, the dysregulation of iPLA$_2\gamma$ expression level and/or activity was shown to provoke mitochondrial dysfunction *via* alterations in cardiolipin content and species (Mancuso et al. 2007). Moreover, oxidant-induced mitochondrial lipid peroxidation and swelling were accelerated by pre-treatment with specific iPLA$_2\gamma$ inhibitor (Kinsey et al. 2007*b*).

In this study, we confirmed our previous results, showing the CIH-induced remodeling of FA in individual cardiac phospholipids leading to the increase in n-3 PUFA to the detriment of n-6 PUFA proportion. These changes were related also to mitochondrial cardiolipin where the proportion of linoleic acid (18:2n-6) decreased and DHA (22:6n-3) increased, accompanied by a reduction in cardiolipin concentration (Jezkova et al. 2002). We observed similar phospholipid remodeling when rat myocardium was exposed to pressure overload during postnatal development suggesting an adaptive response to stress conditions (Novak et al. 2012). The cumulative protective effect of CIH and dietary n-3 PUFA on the incidence of I/R arrhythmias in rat myocardium was demonstrated (Hlavackova et al. 2007). The involvement of ROS in the heart phospholipid remodeling seems unlikely because the administration of antioxidant tempol in the course of CIH exposure did not abolish phospholipid remodeling. Similarly, antioxidant L-

carnitine did not influence changes in phospholipid FA composition in chronically hypoxic hearts (Oka et al. 2008).

Our results show that CIH diversely affect myocardial PLA_2s and suggest that ROS are responsible for the activation of $cPLA_2\alpha$ under these conditions. On the other hand, we did not observe the ROS participation in the CIH-induced down-regulation of $sPLA_2IIA$ and $iPLA_2$, and in phospholipid remodeling. It remains to be elucidated whether the observed changes in individual PLA_2s are involved in CIH-induced cardiac ischemic tolerance.

Conflict of Interests

There is no conflict of interest.

Acknowledgments

This work was supported by the Czech Science Foundation (16-12420Y and 13-10267).

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Table 1

Body and heart weight parameters of normoxic and CIH vehicle- or tempol-treated rats

Parameter	Normoxia		CIH	
	Control	Tempol	Control	Tempol
BW, g	412 ± 7	437 ± 19	345 ± 15*	346 ± 9*
HW, mg	914 ± 28	1008 ± 62	1080 ± 78	1026 ± 51
HW/BW, mg/g	2.219 ± 0.060	2.294 ± 0.053	3.131 ± 0.164*	2.965 ± 0.118*
LVW/BW, mg/g	1.277 ± 0.037	1.281 ± 0.030	1.711 ± 0.102*	1.554 ± 0.080*
RVW/BW, mg/g	0.442 ± 0.016	0.464 ± 0.018	0.775 ± 0.038*	0.794 ± 0.027*

Values are means ± SEM of 8 rats in each group. CIH, chronic intermittent hypoxia; BW, body weight; HW, heart weight; HW/BW, relative heart weight; LVW/BW, relative left ventricular weight; RVW/BW, relative right ventricular weight; * $P < 0.05$ hypoxic vs. corresponding normoxic group.

Table 2

Fatty acid composition (mol %) of total phospholipids in left ventricles of normoxic and CIH vehicle- or tempol-treated rats

Fatty acids	Normoxia				CIH			
	Control		Tempol		Control		Tempol	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
16:0	12.25	0.29	11.94	0.27	13.16 *	0.09	13.39 *	0.12
16:1n-7	0.39	0.03	0.43	0.03	0.37	0.02	0.41	0.03
17:0	0.53	0.02	0.52	0.02	0.46	0.01	0.50	0.03
18:0	19.82	0.16	19.16	0.44	20.28	0.25	19.44	0.24
18:1n-9	2.46	0.17	2.70	0.17	2.58	0.14	2.88	0.17
18:1n-7	4.66	0.08	4.95	0.28	4.22	0.17	4.56	0.12
18:2n-6	28.72	0.81	32.18	1.76	23.42 *	0.71	25.56 *	0.83
18:3n-3	0.11	0.01	0.12	0.01	0.10	0.01	0.10 *	0.01
20:2n-6	0.15	0.01	0.16	0.01	0.15	0.02	0.18	0.02
20:3n-6	0.38	0.01	0.39	0.02	0.40	0.02	0.40	0.01
20:4n-6	14.39	0.45	13.89	0.81	14.54	0.37	13.64	0.56
20:5n-3	0.63	0.06	0.74	0.02	0.59	0.05	0.50 *	0.02
22:4n-6	0.24	0.02	0.22	0.01	0.29	0.02	0.28	0.02
22:5n-6	0.19	0.02	0.17	0.01	0.24	0.01	0.24 *	0.01
22:5n-3	2.73	0.03	2.57	0.05	2.79	0.13	2.99	0.10
22:6n-3	11.81	0.74	9.28	0.93	15.58 *	0.65	14.31 *	0.64

Values are means ± SEM of 6 rats in each group. CIH, chronic intermittent hypoxia. FA reaching at least 0.1% of the total are shown only. * $P < 0.05$ hypoxic vs. corresponding normoxic group.

Figure legends

Figure 1. Effect of CIH on myocardial PLA_2s mRNA

Myocardial $cPLA_2\alpha$, $iPLA_2$ and $sPLA_2IIA$ mRNA levels normalized to the reference gene HPRT1 in homogenate from left ventricular myocardium of normoxic controls *(empty columns)* and of rats adapted to CIH *(solid columns)* expressed as a percentage of normoxic values. Values are means ± SEM from 5 hearts in each group.

Figure 2. Effect of CIH and tempol on myocardial total $cPLA_2\alpha$ and p-$cPLA_2\alpha$ protein levels

Myocardial protein levels of $cPLA_2\alpha$ (a) and p-$cPLA_2\alpha$ (b) in cytosol and membrane fraction from left ventricular myocardium of normoxic controls *(empty columns)* and of rats adapted to CIH *(solid columns)*. The effect of tempol treatment on the protein levels of $cPLA_2\alpha$ (c) and p-$cPLA_2\alpha$ (d) in homogenate from the left ventricular myocardium of normoxic controls and of rats adapted to CIH. Representative Western blots (e) are shown. Values are means ± SEM from 5 hearts in each group. * $P < 0.05$ hypoxic vs. corresponding normoxic group; † $P < 0.05$ tempol-treated vs. corresponding untreated group.

Figure 3. Effect of CIH and tempol on myocardial $iPLA_2$ and $sPLA_2IIA$ protein levels

Myocardial protein levels of $iPLA_2$ (a) and $sPLA_2IIA$ (b) in cytosol and membrane fraction from left ventricular myocardium of normoxic controls *(empty columns)* and of rats adapted to CIH *(solid columns)*. The effect of tempol treatment on the protein levels of $iPLA_2$ (c) and $sPLA_2IIA$ (d) in homogenate from the left ventricular myocardium of normoxic controls and of rats adapted to CIH. Representative Western blots (e) are shown. Values are means ± SEM from 5 hearts in each group. * $P < 0.05$ hypoxic vs. corresponding normoxic group; † $P < 0.05$ tempol-treated vs. corresponding untreated group.

Figure 4. Effect of CIH and tempol on fatty acid composition (mol %) in total phospholipids

Distribution of main fatty acid classes in left ventricular myocardium of normoxic (N) and normoxic tempol-treated (NT) *(empty columns)*, and CIH (H) and CIH tempol-treated (HT) rats *(solid columns)*. Values are means ± SEM of 6 hearts in each group. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. * $P < 0.05$ hypoxic vs. corresponding normoxic group.

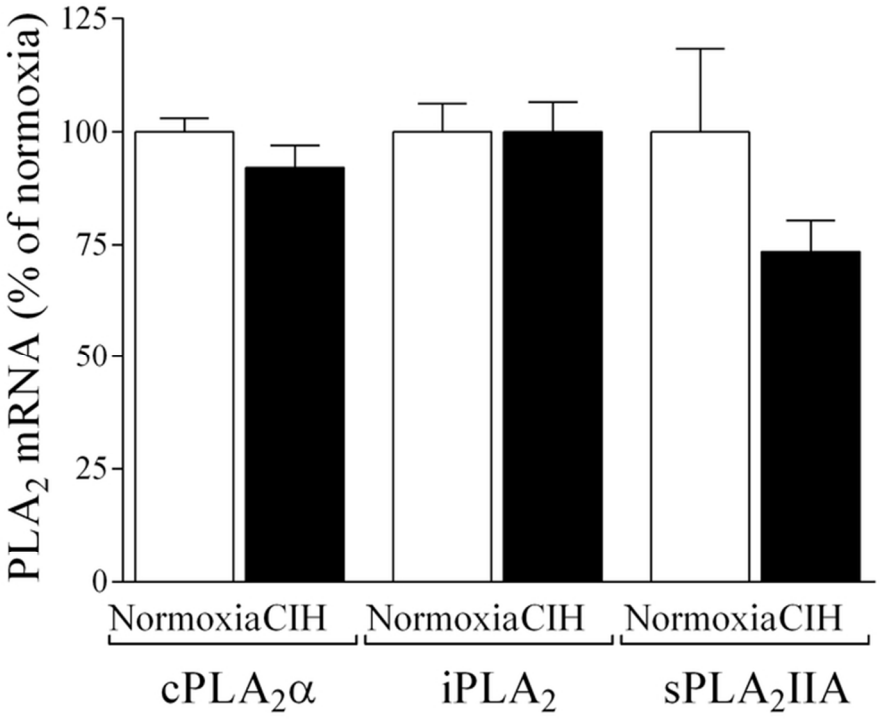

Figure 1

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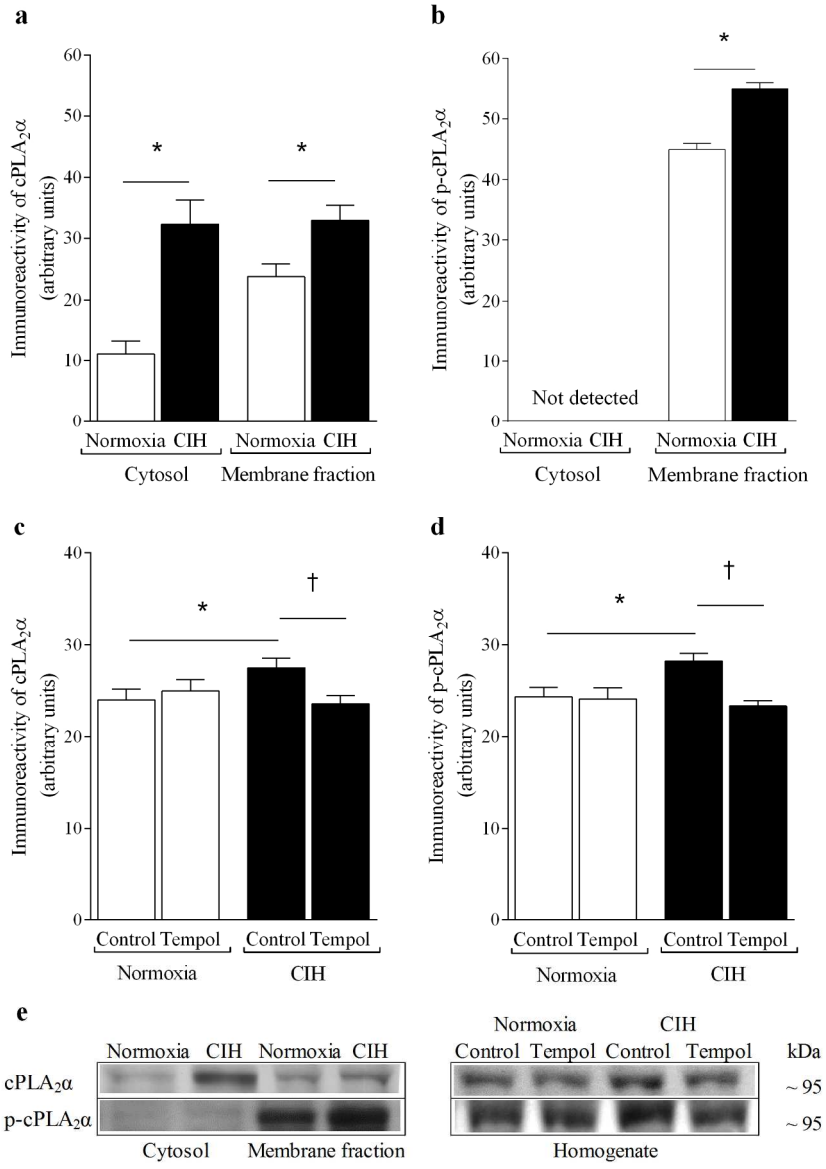

Figure 2

177x236mm (300 x 300 DPI)

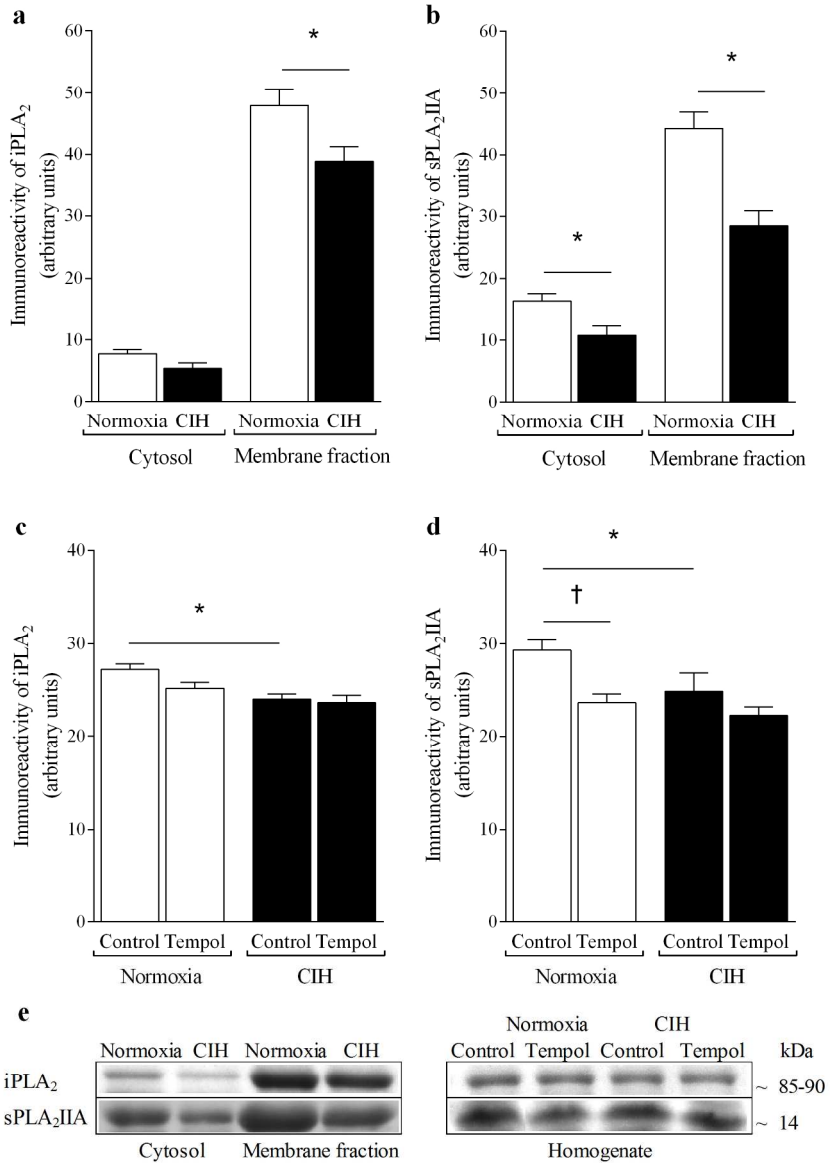

Figure 3

177x236mm (300 x 300 DPI)

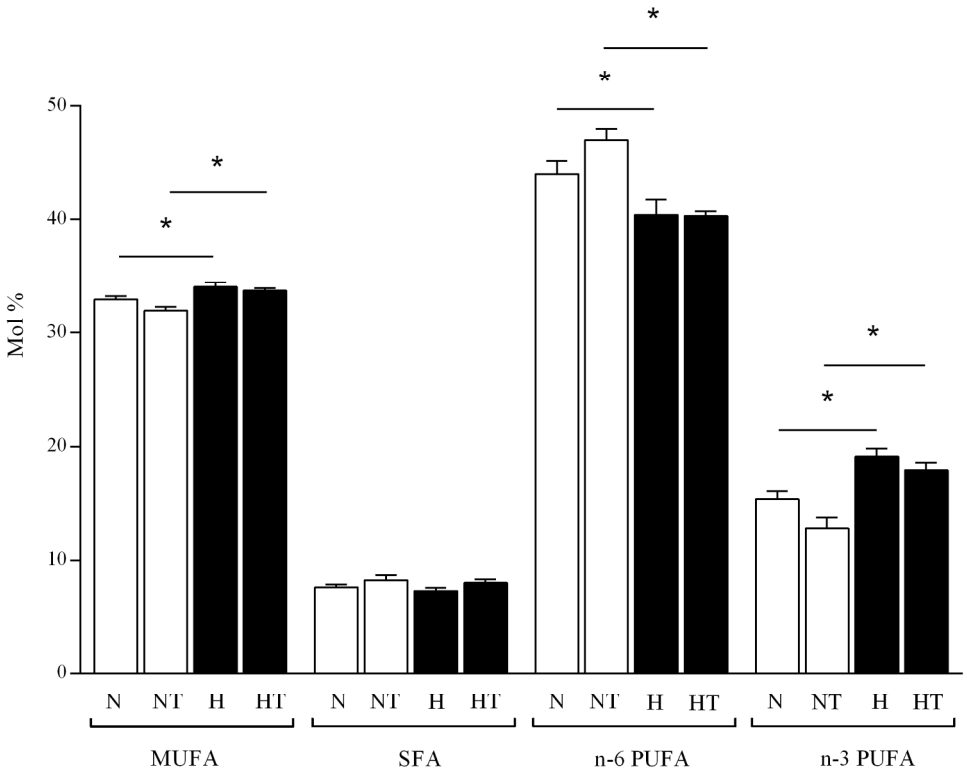

Figure 4

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6880 *Current Pharmaceutical Design*, 2013, *19*, 6880-6889

Brief Daily Episode of Normoxia Inhibits Cardioprotection Conferred by Chronic Continuous Hypoxia. Role of Oxidative Stress and BK_{Ca} Channels

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Abstract: The purpose of the present study was to assess the impact of brief daily reoxygenation during adaptation to chronic continuous hypoxia (CCH) on protective cardiac phenotype. Adult male Wistar rats were kept at CCH (10% oxygen) for 5, 15 or 30 days; a subgroup of animals was exposed to room air daily for a single 60-min period. While 5 days of CCH did not affect myocardial infarction induced by 20-min coronary artery occlusion and 3-h reperfusion, 15 days reduced infarct size from 62% of the area at risk in normoxic controls to 52%, and this protective effect was more pronounced after 30 days (41%). Susceptibility to ischemic ventricular arrhythmias exhibited reciprocal development. CCH increased myocardial abundance of mitochondrial superoxide dismutase (MnSOD) without affecting malondialdehyde concentration. Daily reoxygenation abolished both the infarct size-limiting effect of CCH and MnSOD upregulation, and increased malondialdehyde (by 53%). Ventricular cardiomyocytes isolated from CCH rats exhibited better survival and lower lactate dehydrogenase release caused by simulated ischemia/reperfusion than cells from normoxic and daily reoxygenated groups. The cytoprotective effects of CCH were attenuated by the large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel blocker paxilline, while the opener NS1619 reduced cell injury in the normoxic group but not in the CCH group. Daily reoxygenation restored the NS1619-induced protection, whereas paxilline had no effect, resembling the pattern observed in the normoxic group. The results suggest that CCH is cardioprotective and brief daily reoxygenation blunts its salutary effects, possibly by a mechanism involving oxidative stress and attenuation of the activation of mitochondrial BK_{Ca} channels.

Keywords: Chronic continuous hypoxia, reoxygenation, ischemia/reperfusion, myocardial infarction, potassium channels, oxidative stress.

INTRODUCTION

Adaptation to chronic intermittent hypoxia (CIH) has been recognized as an efficient protective phenomenon, which increases cardiac tolerance to acute ischemia/reperfusion (I/R) injury. The reduced size of myocardial infarction, improved recovery of contractility and lower incidence of life-threatening ventricular arrhythmias represent major protective endpoints of CIH [1]. Compared to the temporal character of preconditioning, these cardioprotective effects may persist weeks or even months after the cessation of hypoxic exposure [2,3] making this phenomenon interesting for potential therapeutic exploitation.

Critical evaluation of the available information on the cardioprotective effects of CIH implies that the potential influence of adaptation regimen in terms of the duration and periodicity of normoxic intervals (reoxygenation) between hypoxic bouts has not been sufficiently explored. Improved cardiac ischemic tolerance has been demonstrated in various models and regimens of CIH associated with periodic reoxygenation lasting minutes to hours. Thus, rats adapted for several weeks to a single daily hypoxic exposure of 6–8 h exhibited markedly reduced infarct size and decreased incidence and severity of ischemic arrhythmias [4-6]. Robust infarct size-limiting and antiarrhythmic effects have been shown in dogs conditioned for 3 wk by several daily cycles of hypoxia and normoxia lasting 5–10 min each [7]. On the other hand, exposures of animals to fast (seconds) repeated cycles of severe hypoxia and normoxia simulating obstructive sleep apnea (OSA) syndrome abrogated myocardial injury caused by the I/R insult [8,9].

Compared to cardioprotection afforded by adaptation to CIH, the experimental evidence concerning cardiac ischemic tolerance in animals adapted to chronic continuous hypoxia (CCH) is scarce and controversial. Milano *et al.* [10] reported that the hearts from rats maintained at CCH for 2 wk without any exposure to normoxia exhibited larger myocardial infarction and impaired postischemic recovery of contractile function than hearts of normoxic rats when subjected to the I/R insult either *in vivo* or *in vitro*. They further demonstrated that allowing the animals to recover at normoxia during the adaptation period for only 60 min per day prevented these harmful effects of CCH. This observation may suggest that brief daily reoxygenation episodes are needed to protect the chronically hypoxic hearts against I/R injury rather than hypoxia itself. According to this view, the protective influence of reoxygenation can be implicated in previous studies of "continuous" hypoxia using conventional hypoxic chambers that require regular opening for feeding and maintenance [11,12]. In contrast, we have recently shown that ventricular myocytes isolated from rats adapted for 3 wk to CCH without any reoxygenation episode exhibited markedly better survival and lower lactate dehydrogenase (LDH) release in response to a simulated I/R than cells from normoxic controls [13].

Considering these contrasting results, the primary goal of the present study was to compare cardiac ischemic tolerance in rats during their adaptation to CCH with and without brief daily normoxic episodes. An open-chest model of myocardial infarction and isolated ventricular myocytes subjected to a simulated I/R were used to quantify various endpoints of injury. It has been proposed that formation of reactive oxygen species (ROS) and increased capacity of antioxidant defense are linked to cardioprotection afforded by CIH [12,14-17]. Myocardial lipid peroxidation and expression of antioxidant enzymes were, therefore, studied in order to determine whether potential differences in cardiac ischemic tolerance between the two hypoxic regimens can be related to oxidative stress. Finally, we aimed at examining whether periodic reoxygenation affects the involvement of mitochondrial large-conduc-tance Ca^{2+}-activated K^+ (BK_{Ca}) channels in the cytoprotective mechanism of CCH.

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1873-4286/13 $58.00+.00

MATERIALS AND METHODS

Animals

Adult male Wistar rats (250–280 g body wt) were exposed to moderate chronic continuous hypoxia (10% oxygen) in a normobaric chamber equipped with hypoxic generators (Everest Summit, Hypoxico Inc., NY, USA) for 5 (H5), 15 (H15), or 30 (H30) days. No reoxygenation occurred during this period. A separate group of hypoxic animals was exposed to room air for 1 h/day during 30 days of adaptation (H30-R). The control rats were kept for the same period of time at room air (Fig. **1A**). All animals were housed in a controlled environment (23°C; 12:12-h light-dark cycle; light from 5:00 AM) with free access to water and a standard chow diet.

Hematocrit was measured in the tail blood. The animals assigned to biochemical analyses were killed by cervical dislocation, their hearts were rapidly excised, washed in cold (0°C) saline and dissected into the right (RV) and left (LV) free ventricular walls and the septum. All parts were weighed and the LV were frozen in liquid nitrogen and stored at –80°C until use.

The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (published by the National Academy of Science, National Academy Press, Washington, D.C.). Experimental protocols were approved by the Animal Care and Use Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic.

Myocardial Ischemia/Reperfusion

Animals were subjected to acute ischemia/reperfusion as described previously [5]. Anesthetized (sodium pentobarbital, 60 mg/kg i.p.) rats were ventilated (Ugo Basile, Italy) with room air at 68–70 strokes/min (tidal volume of 1.2 ml/100 g body wt). A single-lead electrocardiogram (ECG) and blood pressure in the carotid artery were continuously recorded (Gould P23Gb, USA) and subsequently analyzed by a custom-designed software. The rectal temperature was maintained between 36.5 and 37.5°C by a heated table throughout the experiment. Hypoxic rats were anesthetized in the hypoxic chamber and their exposure to normoxic air before the coronary artery occlusion was shorter than 40 min.

Left thoracotomy was performed and a silk braided suture 5/0 (Chirmax, Czech Republic) was placed around the left anterior descending (LAD) coronary artery about 1–2 mm distal to its origin. After 10-min stabilization, regional myocardial ischemia was induced by the tightening of the suture threaded through a polyethylene tube. After a 20-min occlusion period, the ligature was released and reperfusion of previously ischemic tissue continued. After 5 min of reperfusion, chest was closed, air from thorax was exhausted and spontaneously breathing animals were maintained in deep anesthesia following 3 h (Fig. **1B**).

Infarct Size Determination

Hearts were excised and washed with saline through the aorta. The area at risk was delineated by perfusion with 5% potassium permanganate as described earlier [5]. Frozen hearts were cut into slices 1 mm thick, stained with 1% 2,3,5-triphenyltetrazolium chloride (pH 7.4; 37°C) for 30 min and fixed in formaldehyde solution. Four days later, both sides of the slices were photographed. The infarct size (IS), the size of the area at risk (AR) and the size of the LV were determined by computerized planimetry using the software Ellipse (ViDiTo, Slovakia). The size of AR was normalized to the LV (AR/LV) and the IS was normalized to the LV (IS/LV) and to the AR (IS/AR).

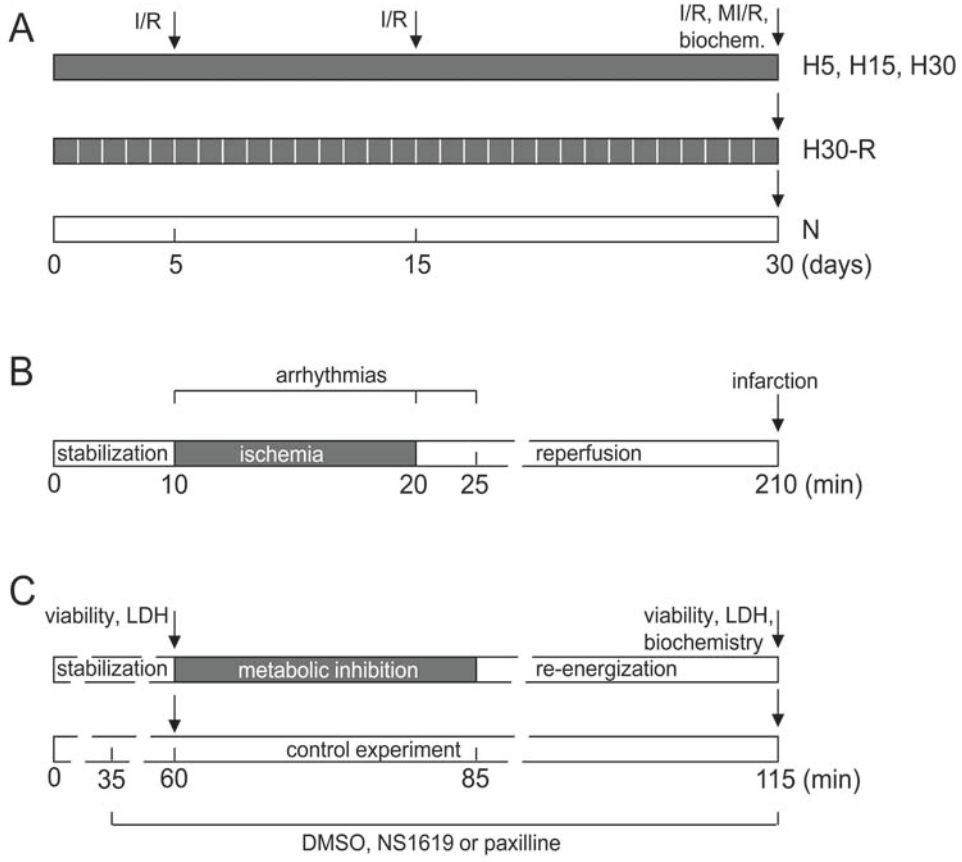

Fig. (1). Experimental schemes of adaptation to hypoxia (**A**), ischemia/reperfusion experiments in open-chest rats (**B**) and simulated ischemia/reperfusion experiments in isolated ventricular myocytes (**C**). Rats were exposed to hypoxia for 5 (H5), 15 (H15), or 30 (H30) days, or for 30 days with daily 60-min reoxygenation (H30-R) and compared with animals kept at normoxia (N). See Materials and Methods for the detailed description.

Analysis of Arrhythmias

The incidence and severity of ischemic arrhythmias during the 20-min ischemic insult and during the first 5 min of reperfusion were assessed according to the Lambeth Conventions [18]. Premature ventricular complexes (PVCs) occurring as singles, salvos or tachycardia (a run of 4 or more consecutive PVCs) were counted separately. The incidence of ventricular tachycardia (VT) and fibrillation (VF) was also evaluated. VF lasting more than two minutes was considered as sustained (VFs); hearts exhibiting VFs were excluded from further evaluation. The severity of arrhythmias in each individual heart was evaluated by means of a 5-point arrhythmia score as described elsewhere [19].

Isolation of Ventricular Myocytes

Animals were heparinized (2,500 IU i.p.) and killed by cervical dislocation. The heart was rapidly excised and perfused via the aorta with Tyrode solution under constant flow conditions (10 ml/min), followed by perfusion with nominally Ca^{2+}-free Tyrode for 8 min. Tissue digestion was initiated by adding 14,000 U collagenase and 7 mg proteinase type XIV into 30 ml of Ca^{2+}-free Tyrode containing 50 mg BSA. Digestion was stopped after 9–12 min by washing out the collagenase-protease cocktail by 10 min perfusion with Ca^{2+}free-Tyrode. The RV and the intraventricular septum were cut off and the LV was dispersed mechanically. LV myocyte solutions were adjusted to the same cell density, transferred to culture medium (50% Dulbecco's Modified Eagles Medium and 50% Nutrient Mixture F12HAM, containing 0.2% BSA, 100 U/ml penicillin, and 100 µg/ml streptomycin) and incubated for 1 h (stabilization) at 28˚C at 5% CO_2 and 95% air [13].

Simulated Ischemia/Reperfusion

In the first series of experiments, myocytes isolated from hypoxic and normoxic rats were subjected to 25 min of metabolic inhibition (MI) followed by 30 min of reenergization (MI/R). In the second set of experiments, cells were treated with BK_{Ca} modulators starting 25 min before MI and continuing during the whole experiment. Myocytes from each heart were divided into three groups treated with either 30 µM BK_{Ca} opener NS1619 (1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-trifuoromethyl-2*H*benzimidazol-one), 2 µM BK_{Ca} blocker paxilline or 0.1% dimethyl sulfoxide (DMSO; vehicle control).

Myocytes from each treatment group were split into two parts of equal volumes. Control cells were incubated in normal Krebs solution and not exposed to MI/R. Experimental cells were subjected to MI/R (Fig. **1C**). MI was induced by incubation the cells in modified Krebs solution containing 1.5 mM NaCN and 20 mM 2-deoxyglucose instead of glucose. The reenergization was achieved by removing the metabolic inhibitors and replacing the MI solution with the normal cell culture medium (the same medium was applied to control cells).

Cell Viability and LDH Release

Cell viability and LDH release were evaluated at the beginning of experiments (after stabilization) and after MI/R. The number of viable and dead (stained) myocytes was determined by Trypan blue exclusion [13]. Typically, 50–100 myocytes were counted in duplicates from 6–8 independent experiments. Viable myocytes were divided into two fractions: rod-shaped myocytes with the cell length-to-width ratio > 3:1 and non-rod-shaped myocytes with the ratio < 3:1. Viability after MI/R was expressed as a percentage of rod-shaped cells that survived the MI/R insult, normalized to the appropriate control group not exposed to MI/R.

LDH was determined spectrophotometrically using the LDH Liqui-UV kit (Stanbio, Boerne, TX, USA) according to manufacturer´s instructions. LDH released during MI/R was normalized to total LDH content in the cells and expressed as a percentage of appropriate control values.

Concentration of Malondialdehyde

Lipid peroxidation was quantified by measuring malondialdehyde (MDA) formation. Myocardial samples (100 mg) were pulverized to a fine powder and added to 500 µl ice-cold buffer (25 mM Tris and 0.10% Triton X 100). The homogenates were sonicated, centrifuged (1,000 *g,* 10 min, 4°C) and 100 µl aliquots of supernatant were hydrolyzed, derivatized and analyzed according to method described by Pilz *et al.* [20]. MDA concentration was normalized to protein, determined by the Bradford assay according to the manufacturer´s instructions.

Western Blot Analysis of Superoxide Dismutases

Detergent-treated extracts of LV homogenate were prepared as described earlier [14]. Proteins were separated by SDS-PAGE electrophoresis (15% gel) and transferred to nitrocellulose membranes (Amersham Biosciences, Freiburg, Germany). After blocking with 5% dry low-fat milk in Tris-buffered saline with Tween 20 (TTBS) for 60 min at room temperature, membranes were washed and probed with rabbit anti-MnSOD and Cu,ZnSOD polyclonal antibodies. The membranes were washed and incubated with anti-rabbit HRP-labeled secondary antibody (1:4,000 in TTBS) for 60 min at room temperature. Bands were visualized by enhanced chemiluminescence on the autoradiographic film, and ImageQuant software was used for quantification of the relative abundance of the enzymes. To ensure the specificity of immunoreactive proteins, prestained molecular weight protein and SOD standards were used. The samples from each experimental group were run on the same gel and quantified on the same membrane. The amounts of MnSOD and Cu,ZnSOD protein applied to the gel were 1 µg and 4 µg, respectively. Hypoxia did not affect the expression of GAPDH, which was used as a loading control.

Western Blot Analysis of BK_{Ca} Channel β_1-subunit

Myocytes were frozen in liquid nitrogen and stored at –80°C until use. Samples were prepared as described earlier [13]. The cells were pelleted at 500 *g* for 5 min, the isolation buffer was removed and 1 ml SDS-sample buffer (0.1 M Tris, 4% SDS, 20% glycerol) was added to achieve cells-to-buffer ratio 1:5 (v/v) and vortexed until the cell pellet was completely resolved. The samples were heated to 100°C for 10 min, and 100 µl was removed to measure protein concentration. This volume was replaced by 100 µl ß-mercaptoethanol, and 10 µl bromphenol blue was added. The samples were again exposed to 100°C for 10 min, aliquots of 50 µl were taken and stored at -80°C until use.

Samples of 20–30 µg per lane were loaded on a 10% acrylamide gel. The proteins were separated at constant voltage (100 V) for ~ 90 min, and transferred to polyvinylidene difluoride membrane at 0.35 A for 75 min. The transferred proteins were blocked overnight in 5% fat free milk and 1% BSA. The primary antibody against β_1 (diluted 1:200 in 5% milk and 1% BSA) was applied for 1 h at room temperature, followed by a 3-times washing with PBS. Then the blots were incubated with anti-rabbit HRP-labeled secondary antibody (1:30,000) for 1 h, followed by 3 more washes. The blots were exposed to the Amersham Hyperfilm ECL for 15 min. After developing the films, the blots were washed in PBS and the antibodies were removed using a common desorption protocol. The blots were then incubated with blocking solution (5% milk, 1% BSA) overnight, probed against GAPDH to verify the amount of protein loaded to the gel. Films were scanned and evaluated by using AIDA software.

Deglycosylation of BK_{Ca} Channel β_1-Subunit

Cells were centrifuged at 1,000 *g* for 5 min to a final pellet of 250 µl, added to the same volume of a solubilization buffer (50 mM Na-phosphate buffer, 150 mM NaCl, 10 mM KCl, 1.8% SDS, 17.5% glycerol, pH 7.2, and 3.3 µl/ml protease inhibitor cocktail P8340), vortexed and centrifuged at 12,000 *g* for 15 min. Deglyco-

sylation was performed using the GKE5006 kit (Prozyme, San Leandro, CA, USA) as described earlier [13] and Wester blotting of β_1-subunit was performed as described above.

Drugs and Chemicals

Collagenase was obtained from Yakult (Tokyo, Japan) and the PBS tablets from Gibco (Carlsbad, CA, USA). The antibody against BK_{Ca} channel subunit β_1 was from Alomone Labs (Jerusalem, Israel), the anti-GAPDH antibody from Applied Biosystems (Foster City, CA, USA), and the anti-rabbit secondary antibody from Bio-Rad (Hercules, CA, USA). The antibodies against MnSOD and Cu,ZnSOD, and both SOD standards were from Stressgen Bioreagents (Victoria, Canada), and the anti-rabbit secondary antibody from Sevapharma (Prague, Czech Republic). All other chemicals and drugs, including NS1619 and paxilline, were purchased from Sigma (Hamburg, Germany).

Statistics

The results are expressed as means ± SE from the indicated number of experiments. One-way ANOVA or ANOVA for repeated measurements and subsequent Student-Newman-Keuls test were used for comparison of differences in normally distributed variables between groups. Differences in the number of PVCs between the groups were compared by the Kruskal-Wallis non-parametric test. The incidence of tachycardia and fibrillation was examined by Fischer´s exact test. Differences were assumed statistically significant when $P < 0.05$.

RESULTS

Weight Parameters and Hematocrit

Adaptation of rats to CCH led to a gradual increase in hematocrit up to 58% after 30 days. The body mass was not significantly affected as compared with age-matched normoxic controls (Table **1**). CCH induced RV hypertrophy (RV/BW ratio increased to 148% of the normoxic value); on the other hand, CCH significantly reduced the relative LV mass (LV/BW decreased to 90% of the normoxic value). Daily brief reoxygenation did not affect the CCH-induced changes of these parameters (Table **1**).

Infarct Size

The mean normalized AR (AR/LV) was 32–37% and did not significantly differ among the groups (Table **1**). The IS reached 61.9 ± 4.0% of the AR in the normoxic group. 5 days of CCH had no effect on myocardial infarction (62.4 ± 3.6%), while 15-days induced a significant reduction (51.5 ± 2.4%) and the cardioprotective effect was more pronounced after 30 days (40.8 ± 3.1%; Fig. **2A**,**B**). Daily reoxygenation completely abolished the infarct size-limiting effect of CCH (69.0 ± 3.1%; Fig. **2A**,**B**). Similar results were obtained when IS was normalized to the size of the LV (Table **1**). Fig. **2C** shows the dependence of the IS on the AR in the normoxic, H30 and H30-R groups. CCH moderately decreased the slope of this linear relationship as compared with the normoxic group, while daily reoxygenation tended to reverse this effect.

Ventricular Arrhythmias

Compared with myocardial infarction, the incidence and severity of ischemic ventricular arrhythmias exhibited reciprocal development during adaptation to CCH. The strongest antiarrhythmic effect was observed already after 5-days of CCH, but it disappeared after prolongation of the hypoxic exposure (Fig. **2D**,**E**). 5 days of hypoxia markedly reduced all forms of arrhythmias and the incidence of VT (Table **2**); the total number of PVCs reached only 7.1 % of the normoxic value (25 ± 8 and 308 ± 66, respectively) and arrhythmia score was reduced to 1.67 ± 0.29 as compared with 2.89 ± 0.26 in normoxic controls (Fig. **2D**,**E**). In rats exposed to CCH for 15 and 30 days, the susceptibility to arrhythmias did not differ from normoxic controls. Daily reoxygenation markedly increased the total number of PVCs to 1069 ± 401 (Fig. **2D**), but the effect was not statistically significant due to extremely high variability in this group. The main form of arrhythmias in the H30-R group was VT, total duration of which was 6-fold and more than 2-fold longer compared with the normoxic and H30 groups, respectively (Table **2**).

The total number of PVCs occurring during reperfusion as well as arrhythmia severity were again significantly reduced in rats exposed to CCH for 5 days; the antiarrhythmic effect was not detected after a prolongation of the hypoxic exposure. Similarly to ischemic arrhythmias, daily reoxygenation tended to increase the number of PVCs in reperfusion, but this effect was not significant (Table **2**).

Cell Viability and LDH Release

Myocyte viability was about 51% after 1-h stabilization in both normoxic and H30 groups. Daily reoxygenation decreased the myocyte starting viability by about 15%, mainly due to a decreased number of viable non-rod-shaped cells (data not shown). The numbers of viable myocytes at the end of control experiments were similar to that counted after stabilization within each experimental group.

Exposure of myocytes to the MI/R insult decreased the numbers of viable cells in all groups (Fig. **3A**). About 62% of rod-shaped myocytes survived in the normoxic group when expressed as a percentage of corresponding control cell number in the absence of MI/R. Myocytes isolated from animals adapted to CCH for 30 days exhibited a significantly better survival after MI/R (79%) than the normoxic group of cells and daily reoxygenation completely abolished the protective effect of CCH on cell viability (60%).

Table 1. Body Weight, Relative Ventricular Weights, Hematocrit, Myocardial Area at Risk, and Infarct Size in Rats Exposed to Continuous Hypoxia with or Without Daily Reoxygenation and in Normoxic Controls.

Group	n	BW (g)	RV/BW (mg/g)	LV/BW (mg/g)	Hematocrit (%)	AR/LV (%)	IS/LV (%)
Normoxia (N)	9	376 ± 7	0.48 ± 0.02	1.25 ± 0.05	48.3 ± 0.5	35.2 ± 2.3	22.3 ± 2.7
Hypoxia							
5 days (H5)	9	284 ± 6	*N.D.*	*N.D.*	55.1 ± 0.7 *	36.5 ± 2.2	22.4 ± 1.3
15 days (H15)	9	304 ± 7	*N.D.*	*N.D.*	56.0 ± 0.3 *	33.5 ± 2.5	17.4 ± 1.6
30 days (H30)	9	350 ± 13	0.71 ± 0.02 *	1.12 ± 0.03 *	58.0 ± 0.7 *	32.2 ± 2.2	13.4 ± 1.8 *‡
30 days + Reox (H30-R)	8	343 ± 12	0.66 ± 0.01 *	1.15 ± 0.04	60.1 ± 2.6 *	37.0 ± 2.1	25.7 ± 2.1 †

n, number of rats; BW, body weight; RV/BW, relative right ventricular weight; LV/BW, relative left ventricular weight; AR/LV, area at risk normalized to the LV; IS/LV, infarct size normalized to the LV; *N.D.*, not determined. Values are means ± SE; *$p < 0.05$ vs. N; †$p < 0.05$ vs. H30; ‡$p < 0.05$ vs. H5.

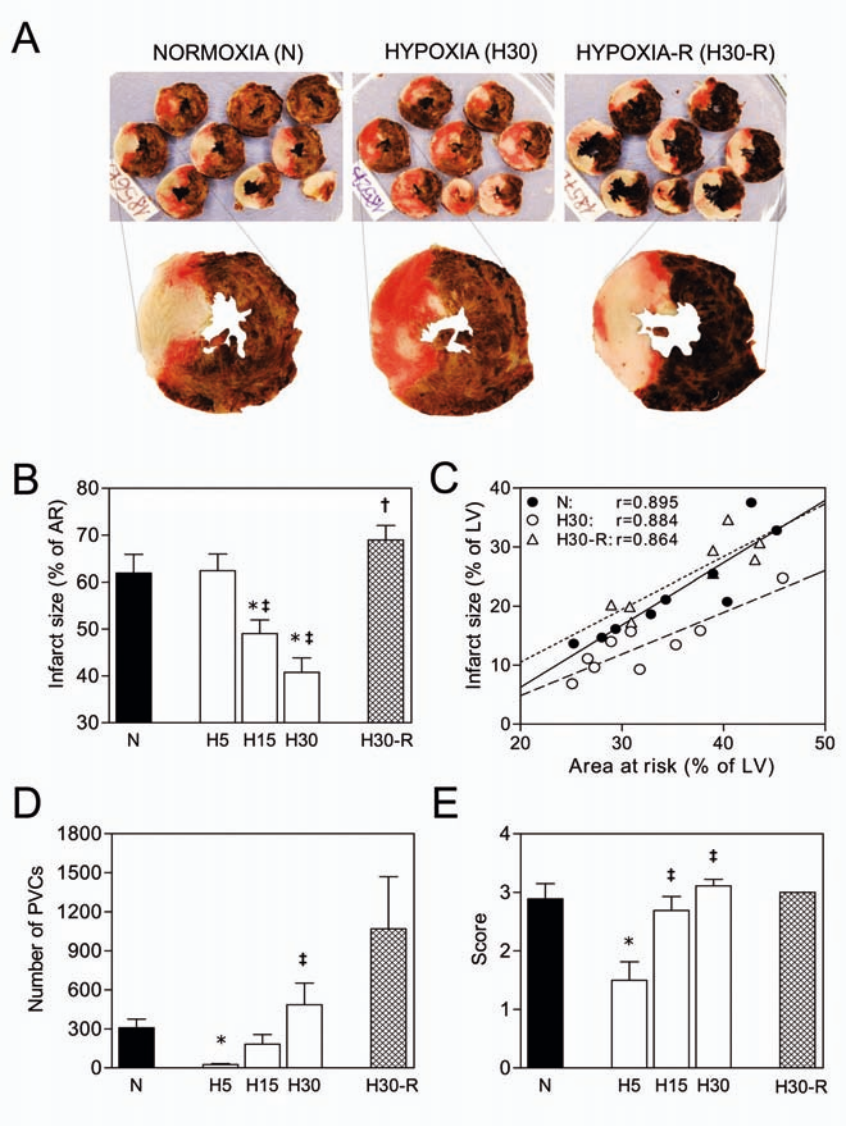

Fig. (2). Effects of continuous hypoxia with or without daily reoxygenation on infarct size and ischemic ventricular arrhythmias. Rats were exposed to hypoxia for 5 (H5), 15 (H15), or 30 (H30) days, or for 30 days with daily 60-min reoxygenation (H30-R) and compared with animals kept at normoxia (N). **A**: typical examples of myocardial infarction induced by 20-min coronary artery occlusion and 3-h reperfusion. Brown color represents normally perfused tissue stained by potassium permanganate. Red area, tetrazolium positive, represents tissue surviving the occlusion, and pale area, tetrazolium negative, is infarcted tissue. **B**: myocardial infarct size expressed as a percentage of the area at risk (AR). **C**: relationship between the area at risk and infarct size, both expressed as a percentage of the left ventricle (LV). **D**: total number of premature ventricular complexes (PVCs) during ischemia. **E**: score of ischemic arrhythmias. r, correlation coefficient. Values are means ± SE from 8–11 rats in each group; *$p < 0.05$ vs. N; †$p < 0.05$ vs. H30; ‡$p < 0.05$ vs. H5.

Neither total LDH content in cell preparations nor LDH release during control experiments differed among the groups (data not shown). LDH release from the normoxic group of cells increased after MI/R to 132% of the appropriate control value. 30-days of CCH significantly reduced MI/R-induced LDH release to only 112% of the control value in the absence of MI/R. In contrast, daily reoxygenation fully abolished the protective effect of CCH and significantly increased LDH release even above the value of the normoxic group to 171% of the control value (Fig. **3B**).

MDA Concentration and SOD Expression

Adaptation to CCH for 30 days significantly increased the myocardial protein level of mitochondrial MnSOD (by 23 %) but did not change the level of cytosolic Cu,ZnSOD and had no effect on the MDA concentration compared with the normoxic group (0.72 ± 0.05 kmol/g and 0.61 ± 0.05 kmol/g, respectively). Daily reoxygenation completely abolished the CCH-induced up-regulation of MnSOD without affecting Cu,ZnSOD, and significantly increased the myocardial MDA concentration to 0.94 ± 0.10 kmol/g (Fig. **4A**,**B**,**C**).

Effects of BK_{Ca} Channel Modulators

Figure **5**, **A** and **B**, respectively, illustrates the effects of BK_{Ca} channel modulators on myocyte viability and total LDH release during MI/R. We confirmed our recent observation [13] that the BK_{Ca} channel opener NS1619 significantly increased cell survival and decreased LDH release during the MI/R insult in the normoxic group. 30 days of CCH improved cell survival and reduced LDH release in the DMSO-treated myocytes, but NS1619 did not provide any additive protection. As expected, the BK_{Ca} channel blocker paxilline attenuated the protective effects of CCH without affecting cell injury in the normoxic group. The novel observation of the present study is that daily reoxygenation during adaptation to hypoxia restored the protective effects of NS1619 against MI/R-

Table 2. Characteristics of Ventricular Arrhythmias Occurring During 20-min Coronary Artery Occlusion and During the First 3 min of Reperfusion in Rats Exposed to Continuous Hypoxia with or without Daily Reoxygenation and in Normoxic Controls.

Group	Ischemic Arrhythmias							Reperfusion Arrhythmias	
	Number of PVCs			Incidence (%)		Duration (s)		Number of PVCs	Score
	Singles	Salvos	VT	VT	VFr	VT	VFr		
Normoxia (N)	117 ± 33	30 ± 8	161 ± 52	88.9	11.1	13.9 ± 4.0	0.4 ± 0.4	43.1 ± 12.8	2.78 ± 0.15
Hypoxia									
5 days (H5)	19 ± 7 *	6 ± 3	1 ± 1 *	11.1 *	0	0.1 ± 0.1 *	0	13.8 ± 4.7 *	1.78 ± 0.40 *
15 days (H15)	71 ± 27	21 ± 8	121 ± 71	77.7 ‡	11.1	7.0 ± 3.7	0.4 ± 0.4	30.4 ± 9.3	2.44 ± 0.24
30 days (H30)	88 ± 10 ‡	37 ± 8 ‡	360 ± 167 ‡	100.0 ‡	11.1	36.4 ± 18.3 ‡	0.1 ± 0.1	28.0 ± 9.0	2.56 ± 0.18
30 days + Reox (H30-R)	114 ± 25	33 ± 9	922 ± 383	100.0	0	83.9 ± 36.5	0	85.6 ± 39.4	2.75 ± 0.25

PVCs, premature ventricular complexes; VT, ventricular tachycardia; VFr, reversible ventricular fibrillation. Values are means ± SE from 8–9 rats in each group; *$p < 0.05$ vs. N; ‡$p < 0.05$ vs. H5.

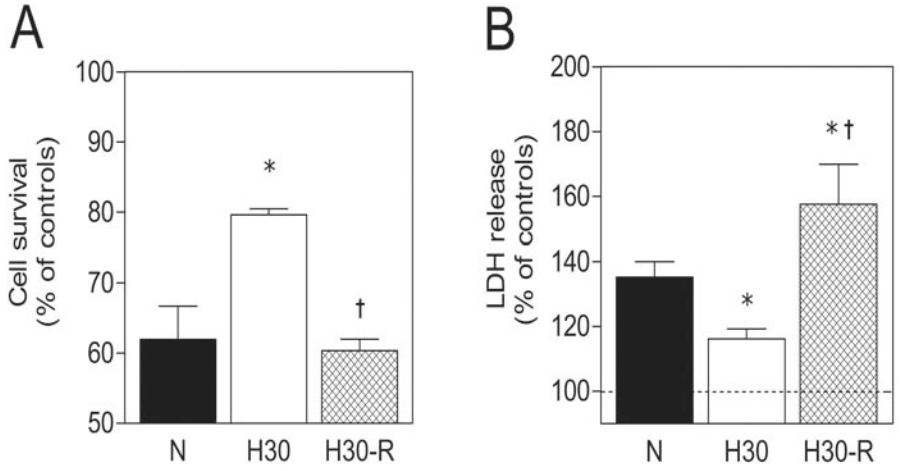

Fig. (3). Effects of chronic continuous hypoxia with or without daily reoxygenation on cell injury induced by acute metabolic inhibition and reenergization (MI/R). Cardiomyocytes were isolated from the left ventricles of rats adapted to hypoxia for 30 days without reoxygenation (H30) or with 60-min daily reoxygenation (H30-R) and compared with cells isolated from normoxic animals (N). **A**: survival of cardiomyocytes (rod-shaped), expressed as a percentage of control cells in the absence of MI/R. **B**: lactate dehydrogenase (LDH) release from cardiomyocytes, expressed as a percentage of LDH release from control cells not exposed to MI/R. Values are means ± SE from 6–8 hearts in each group; *$p < 0.05$ vs. N; †$p < 0.05$ vs. H30.

induced myocyte injury, whereas paxilline did affect neither cell survival nor LDH release, resembling the pattern observed in the normoxic group (Fig. **5A**,**B**).

Deglycosylation of BK_{Ca} Channel Regulatory Ķ-subunit

Western blot analysis showed two bands corresponding to the β_1-subunit (Fig. **6A**) that were previously detected as glycosylated (~40 kDa) and deglycosylated (~26 kDa) forms of the protein [13]. The total optical density of BK_{Ca} channel β_1-subunit (band 1 and band 2) did not differ among the experimental groups (Fig. **6B**). 30 days of CCH caused partial deglycosylation of the subunit (an increased proportion of the ~26 kDa band) and daily reoxygenation further stimulated the deglycosylation effect of chronic hypoxia (Fig. **6A**) as indicated by a sharp drop of the upper-to-lower band ratio (Fig. **6C**).

DISCUSSION

The major finding of the present work is that the tolerance to different end points of acute I/R injury of the heart developed in a reciprocal manner during the adaptation to CCH. While no reduction of myocardial infarct size occurred in rats exposed to CCH for 5 days compared with normoxic controls, the significant infarct size-limiting effect was observed after 15 days of hypoxia and the prolongation of hypoxic exposure to 30 days further reduced the extent of injury. In contrast, the marked suppression of ischemic ventricular arrhythmias was seen already after 5 days of CCH, vanishing with the prolongation of the hypoxic exposure. This observation reflects different mechanisms of ischemic arrhythmogenesis and lethal myocardial injury. Moreover, the considerably delayed appearance of the protective effect of CCH against infarction suggests that this phenomenon is not just another form of hypoxic preconditioning.

Powerful infarct size-limiting effects have been demonstrated in many previous studies using animals adapted to various models and regimens of CIH, whereby hypoxic exposure is interrupted regularly with normoxic periods [1]. It has been proposed that it is not hypoxic exposure itself but rather periodic reoxygenation, which is responsible for the CIH-induced cardioprotection [21]. This idea was based on the assumption that an interruption of the hypoxic exposure with normoxic period can be sensed as relative hyperoxia,

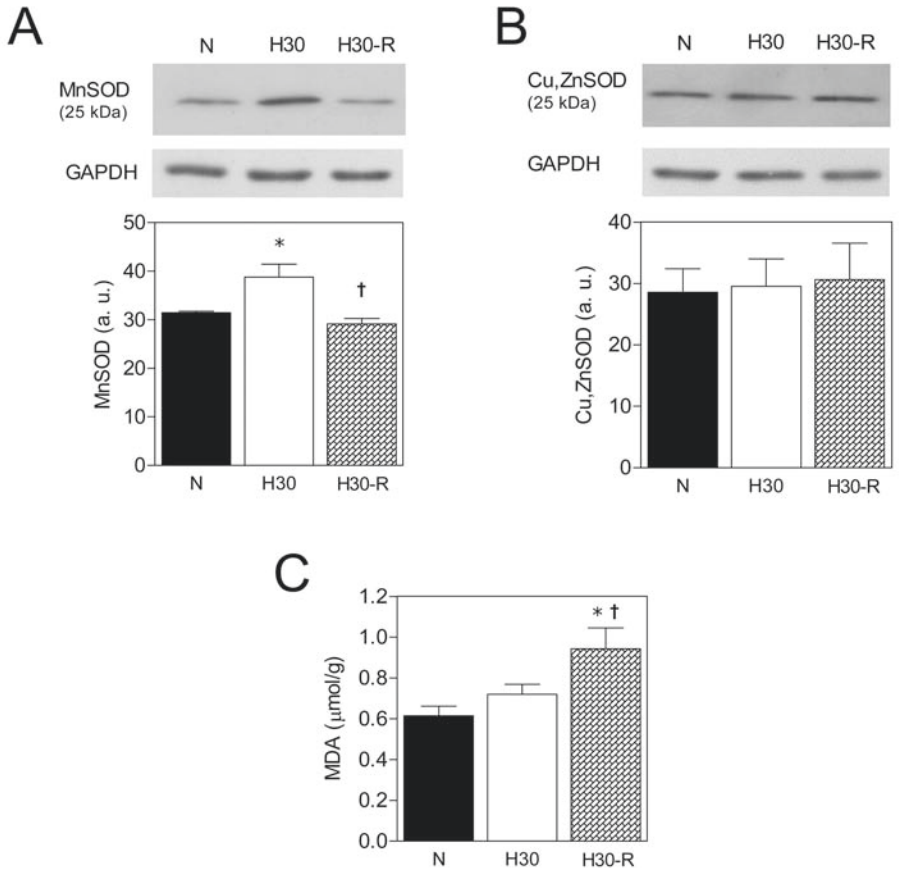

Fig. (4). Effects of chronic continuous hypoxia with or without daily reoxygenation on myocardial protein levels of (**A**) mitochondrial manganese superoxide dismutase (MnSOD) and (**B**) cooper-zinc superoxide dismutase (Cu,ZnSOD), and (**C**) myocardial concentration of malondialdehyde (MDA). Rats were adapted to hypoxia for 30 days without reoxygenation (H30) or with 60-min daily reoxygenation (H30-R) and compared with animals kept at normoxia (N). Representative Western blots of MnSOD and Cu,ZnSOD are shown; GAPDH was used as a loading control. Values are means ± SE from 6–7 hearts in each group; **p* < 0.05 vs. N; †*p* < 0.05 vs. H30.

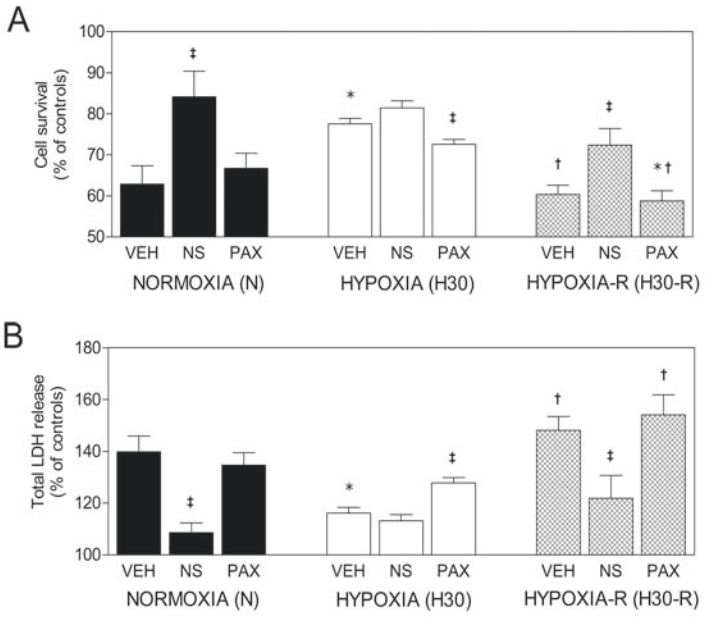

Fig. (5). Effects of NS1619 (NS) and paxilline (PAX) on cell injury induced by acute metabolic inhibition and reenergization (MI/R). Cardiomyocytes were isolated from the left ventricles of rats adapted to hypoxia for 30 days without reoxygenation or with 60-min daily reoxygenation (R) and compared with cells isolated from normoxic animals. **A**: survival of cardiomyocytes (rod-shaped), expressed as a percentage of control cells in the absence of MI/R. **B**: lactate dehydrogenase (LDH) release from cardiomyocytes, expressed as a percentage of LDH release from control cells not exposed to MI/R. VEH, vehicle-treated cells. Values are means ± SE from 6–8 hearts in each group; **p* < 0.05 vs. N; †*p* < 0.05 vs. H30; ‡*p* < 0.05 vs. VEH.

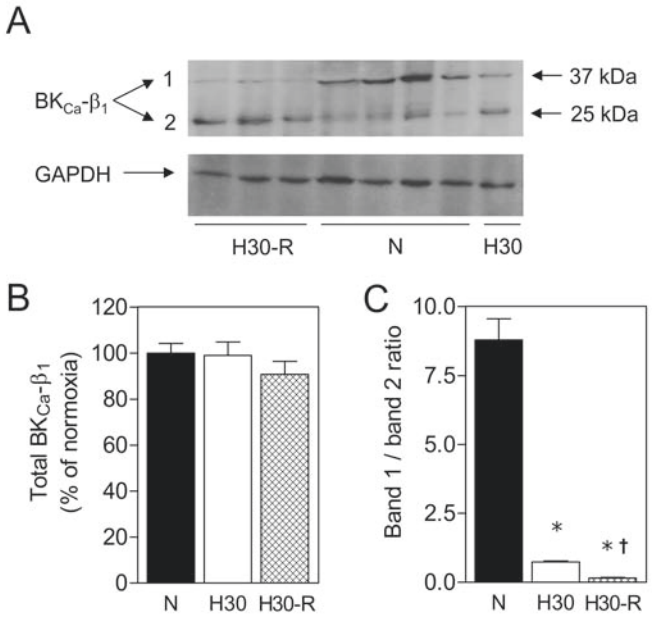

Fig. (6). Deglycosylation of the large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel regulatory β_1-subunit. Cardiomyocytes were isolated from the left ventricles of rats adapted to hypoxia for 30 days without reoxygenation (H30) or with 60-min daily reoxygenation (H30-R) and compared with cells isolated from normoxic animals (N). **A**: representative Western blots of the β_1-subunit show a dominant band at ~40 kDa band (*band 1*) in the normoxic group and an increased abundance of a ~26-kDa band (*band 2*) in the H30 and H30-R groups. GAPDH was used as a loading control. **B**: quantitative analysis of the total amount of the BK_{Ca} channel β_1-subunit expressed as a percentage of normoxic values. **C**: The ratio of glycosylated (*band 1*) to deglycosylated (*band 2*) β_1-subunits. Values are means ± SE from 6–8 hearts in each group; $^*p < 0.05$ vs. N; $^\dagger p < 0.05$ vs. H30.

which potentially can activate protective signaling pathways. Cardiac preconditioning by hyperoxic stimulus has been well documented [22]. Using regimens of hypoxic adaptation similar to those of the present study, Milano *et al.* [10] found an impaired tolerance to myocardial infarction induced by the I/R insult in rats exposed to CCH compared with normoxic animals; periodic reoxygenation prevented this effect, slightly decreasing infarct size below that of the normoxic group. This observation might support the crucial role of periodic reoxygenation in cardioprotection induced by CIH. However, our present study yielded quite opposite results: rats adapted to CCH exhibited significantly smaller infarct size than normoxic controls and this protective effect was absent in the group of daily reoxygenated animals. Although we cannot fully explain these apparently conflicting results, it should be noted that all animals in our study were ventilated with room air during the I/R experiment, while hypoxic rats in the study of Milano *et al.* [10] were still maintained at hypoxia even during the I/R insult in order to prevent any reoxygenation of animals after the cessation of adaptation to hypoxia. Our results clearly showed that this "terminal" reoxygenation cannot trigger any protective response as no infarct size reduction was seen after 5 days of CCH. Taken together, we showed that rats adapted to CCH are more resistant to lethal myocardial I/R injury, and hypoxia itself is responsible for the acquisition of the protective phenotype. It is conceivable that myocardial infarction induced in CCH animals under the hypoxic challenge [10] can cause more severe injury than the same insult in normoxic rats ventilated with room air.

However, the important question remains what is the mechanism associated with the brief daily reoxygenation that can be responsible for blunting the infarct size-limiting effect of CCH adaptation. The exact answer is unknown, yet several lines of evidence suggest that excess oxidative stress may play a role. It has been shown that chronic hypoxia leads to increased formation of ROS [16,23,24], which is required for the acquisition of protective cardiac phenotype [16]. ROS-dependent signaling can increase myocardial capacity of antioxidant defense systems, thereby preventing excess oxidative stress [15,17] and reducing tissue injury. In our recent study, the increased MnSOD expression and activity in myocardial mitochondria, induced by adaptation of rats to hypobaric CIH (PO_2 = 63.8 mm Hg, 8 h/day, 25 exposures), negatively correlated with infarct size [14]. On the other hand, CIH regimen mimicking OSA syndrome, which impaired cardiac ischemic tolerance, had no effect on MnSOD in mice [9]. Consistent with these observations, our present data demonstrated that adaptation to protective CCH increased the MnSOD expression without affecting the MDA level, while daily reoxygenation abolished the MnSOD upregulation and increased MDA, indicating lipid peroxidation. It appears, therefore, that 60-min periodic reoxygenation, similarly as CIH models of OSA syndrome, can result in perturbation of antioxidant defense and consequent oxidative stress, thereby promoting myocardial I/R injury. This view is supported by a recent study of Ramond *et al.* [25], showing that chronic treatment with SOD mimetic tempol prevented the impaired cardiac ischemic tolerance in rats with OSA syndrome. However, it remains unclear, why daily 60-min exposure to normoxia blunts the cardioprotective mechanisms of chronic hypoxia, while CIH regimens with longer normoxic periods per day are still associated with a resistant phenotype.

Our experiments performed on isolated ventricular myocytes subjected to MI/R support the aforementioned findings obtained using the open-chest model of myocardial infarction. The improved cell survival and reduced LDH release conferred by adaptation to CCH were completely abolished in myocytes isolated from daily reoxygenated rats. This finding suggests that the observed changes of ischemic tolerance reflect altered intrinsic properties of cells rather than any indirect systemic effects. Moreover, we confirmed our recent observation indicating that the activation of BK_{Ca} channels plays a role in the cytoprotective mechanism of CCH [13]. As ventricular myocytes most likely do not contain this type of channel in the sarcolemma [26], the observed effects of paxilline and NS1619 can be attributed to BK_{Ca} channels localized in the inner mitochondrial membrane [27]. The novel finding of the present

study is that these channels were not activated in unprotected cells from daily reoxygenated rats. While the BK_{Ca} opener NS1619 did not increase cell resistance against MI/R injury in addition to that conferred by CCH, reoxygenation restored the salutary effects of this agent. In opposite, the channel blocker paxilline attenuated protection in the CCH group of cells but had no effect on cells from reoxygenated animals. Thus, actions of these pharmacological modulators in myocytes isolated from animals subjected during hypoxic adaptation to daily reoxygenation resembled the pattern observed in the normoxic group, suggesting that this intervention prevented the activation of BK_{Ca} channels by CCH. It is possible to speculate that oxidative stress contributed to this effect because BK_{Ca} channels are sensitive to the inhibition by ROS [28].

It has been proposed that chronic hypoxia can affect the BK_{Ca} channel function via posttranscriptional mechanisms [29]. We have shown previously that CCH caused marked deglycosylation of the native regulatory β_1-subunit of the channel [13]. As enzymatic deglycosylation of the β_1-subunit in smooth muscle cells stimulated the channel activity by increasing its open probability and mean open time [30], it can be assumed that this mechanism is responsible for the activation of the channel and its involvement in the cytoprotection by CCH. However, our present observation of an even more reduced glycosylation level of the β_1-subunit in unprotected cells from daily reoxygenated rats compared to the protected CCH group of cells seems to rule out this possibility.

CONCLUSION

Our results show that long-term adaptation of rats to CCH protects against lethal myocardial injury caused by the acute I/R insult. This protective effect was demonstrated both in the open-chest model of myocardial infarction and in freshly isolated ventricular myocytes. While the infarct size-limiting effect of CCH needs several weeks to develop, the susceptibility to ventricular arrhythmias is most pronounced in the early phase of adaptation, vanishing with its prolongation. Daily interruption of the hypoxic exposure with 60-min normoxic episodes blunts the cardioprotective effect of CCH likely by a mechanism, which attenuates antioxidant defense, results in oxidative stress and interferes with the activation of mitochondrial BK_{Ca} channels independent of their glycosylation status.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

This work was supported by the Czech Science Foundation (305/07/0875 and 303/12/1162 to F.K.), the Grant Agency of the Academy of Sciences of the Czech Republic (IAAX01110901 to J.N.), the Ministry of Education, Youth and Sports of the Czech Republic (21620858 to O.N.), the Grant Agency of the Charles University in Prague (100809 to P.M.), and the institutional research project (AV0Z50110509). The authors thank J. Vašinová for excellent technical assistance.

J.N., G.H.B., and F.K. are responsible for the conception and design of the research; J.N., G.H.B., P.H., P.M., and M.H. performed the experiments; J.N., G.H.B., P.H., O.N., F.N., M.H., and F.P. analyzed the data; J.N., G.H.B., O.N., F.N., B.O., and F.K. interpreted the results of the experiments; J.N., and F.P. prepared the figures; J.N., G.H.B., and F.K. drafted the manuscript; F.K. edited and revised the manuscript; J.N., G.H.B., P.H., P.M., O.N., F.N., M.H., F.P., B.O., and F.K. approved the final version of the manuscript.

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Received: March 6, 2013 **Accepted: April 9, 2013**

PŘÍLOHA D

J Appl Physiol 122: 000–000, 2017.
First published February 16, 2017; doi:10.1152/japplphysiol.00671.2016.

RESEARCH ARTICLE

Myocardial ischemic tolerance in rats subjected to endurance exercise training during adaptation to chronic hypoxia

AQ: 1

AQ: au

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Submitted 27 July 2016; accepted in final form 13 February 2017

Alánová P, Chytilová A, Neckář J, Hrdlička J, Míčová P, Holzerová K, Hlaváčková M, Macháčková K, Papoušek F, Vašinová J, Benák D, Nováková O, Kolář F. Myocardial ischemic tolerance in rats subjected to endurance exercise training during adaptation to chronic hypoxia. *J Appl Physiol* 122: 000–000, 2017. First published February 16, 2017; doi:10.1152/japplphysiol.00671.2016.—Chronic hypoxia and exercise are natural stimuli that confer sustainable cardioprotection against ischemia-reperfusion (I/R) injury, but it is unknown whether they can act in synergy to enhance ischemic resistance. Inflammatory response mediated by tumor necrosis factor-α (TNF-α) plays a role in the infarct size limitation by continuous normobaric hypoxia (CNH), whereas exercise is associated with anti-inflammatory effects. This study was conducted to determine if exercise training performed under conditions of CNH (12% O_2) affects myocardial ischemic resistance with respect to inflammatory and redox status. Adult male Wistar rats were assigned to one of the following groups: normoxic sedentary, normoxic trained, hypoxic sedentary, and hypoxic trained. ELISA and Western blot analysis, respectively, were used to quantify myocardial cytokines and the expression of TNF-α receptors, nuclear factor-κB (NF-κB), and selected components of related signaling pathways. Infarct size and arrhythmias were assessed in open-chest rats subjected to I/R. CNH increased TNF-α and interleukin-6 levels and the expression of TNF-α type 2 receptor, NF-κB, inducible nitric oxide synthase (iNOS), cytosolic phospholipase A_2α, cyclooxygenase-2, manganese superoxide dismutase (MnSOD), and catalase. None of these effects occurred in the normoxic trained group, whereas exercise in hypoxia abolished or significantly attenuated CNH-induced responses, except for NF-κB, iNOS, and MnSOD. Both CNH and exercise reduced infarct size, but their combination provided the same degree of protection as CNH alone. In conclusion, exercise training does not amplify the cardioprotection conferred by CNH. High ischemic tolerance of the CNH hearts persists after exercise, possibly by maintaining the increased antioxidant capacity despite attenuating TNF-α-dependent protective signaling.

NEW & NOTEWORTHY Chronic hypoxia and regular exercise are natural stimuli that confer sustainable myocardial protection against acute ischemia-reperfusion injury. Signaling mediated by TNF-α via its type 2 receptor plays a role in the cardioprotective mechanism of chronic hypoxia. In the present study, we found that exercise training of rats during adaptation to hypoxia does not amplify the infarct size-limiting effect. Ischemia-resistant phenotype is maintained in the combined hypoxia-exercise setting despite exercise-induced attenuation of TNF-α-dependent protective signaling.

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AQ: 2

chronic hypoxia; exercise training; cardioprotection; cytokines; antioxidants

GIVEN THE WORLDWIDE epidemic prevalence of ischemic heart disease representing the leading cause of mortality, the search for effective approaches to improve myocardial ischemic tolerance and delay the onset of cell death is crucially important. It is well recognized that the heart has the ability to protect itself from lethal ischemia-reperfusion (I/R) injury if subjected to appropriate stimuli. Among them, chronic hypoxia and exercise training have received increasing attention as natural and clinically relevant cardioprotective stimuli that can induce prolonged or sustainable cardioprotective states.

In line with the results of human epidemiological surveys (2, 14, 25), the vast majority of animal studies demonstrate that chronic hypoxia confers the protective cardiac phenotype against major end points of acute I/R injury (18). Importantly, the notable infarct size-limiting effect of chronic hypoxia lasts at least for 5 wk after the cessation of hypoxia (30). Moreover, as demonstrated recently, rats exposed to chronic hypoxia several days after the induction of myocardial infarction exhibit better heart function and less progressive remodeling than infarcted normoxic animals (45). The benefits of regular exercise for healthy heart have also been well recognized and a strong association exists between physical activity and the rate of survival after myocardial infarction (24, 34). Similarly to chronic hypoxia, exercise has been shown to mitigate myocardial injury caused by acute I/R insult in various experimental settings (5, 9, 13, 21, 35), the protective effects being dependent on the type and intensity of exercise (10). Although chronic hypoxia and exercise obviously share several important mediators, limited evidence exists suggesting that there are some differences in detailed protective mechanisms. If these two protective measures involve discrete salutary pathways, it can be assumed that they act in synergy to improve myocardial survival on ischemic insult.

AQ: 4

It is now well established that reactive oxygen species (ROS) play a dual role in myocardial I/R injury: although excess ROS can trigger oxidative damage of biological structures, they also serve as important elements in protective signaling at physiologically relevant levels (4). We have shown recently that chronic hypoxia is associated with the increased formation of ROS, which plays an important role in the induction of the protective cardiac phenotype as various anti-

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oxidant treatments applied during the adaptation period eliminated its infarct size-limiting effect (19, 32). Regarding the involvement of ROS in cardioprotection induced by exercise, the available data are rather controversial. For example, although ROS have been implicated in the reduction of infarct size in exercising mice (1), exercise-induced late preconditioning of the rat heart was not affected by an antioxidant treatment (44).

ROS stimulate myocardial inflammatory reaction by promoting tumor necrosis factor-α (TNF-α) activity leading to pro-inflammatory cytokine cascade (12, 16). This is a self-amplifying process as TNF-α is involved in further production of ROS (26). Similar to ROS, TNF-α can contribute to both myocardial I/R injury (12, 46) and protective signaling (22, 23, 27). In our recent study, we observed the increased myocardial levels of TNF-α and its type 2 receptor (TNFR2) together with the increased antioxidant capacity in chronically hypoxic rats. Moreover, the treatment of animals during adaptation with an antibody against TNF-α suppressed not only these responses but also the infarct-sparing effect (7). In contrast, exercise can result in an anti-inflammatory phenotype, as indicated by the repression of related myocardial transcripts (6). Interestingly, regular exercise completely abolished the myocardial TNF-α increase induced by the chronic stimulation of β-adrenoceptors (40). This observation led us to hypothesize that exercise can also suppress TNF-α and the pro-inflammatory myocardial phenotype of chronically hypoxic rats. Therefore, we attempted to determine how regular exercise training performed under conditions of continuous chronic hypoxia affects myocardial ischemic tolerance with respect to the inflammatory response and redox status.

MATERIALS AND METHODS

Animals. Adult male Wistar rats (initial body wt 250–280 g, Charles River, Germany) were housed in a controlled environment (23°C, 12-h light-dark cycle, light from 5:00 AM) with free access to water and standard chow diet. Animals were randomly assigned to one of the following experimental groups: normoxic sedentary ($n = 20$), normoxic trained ($n = 28$), hypoxic sedentary ($n = 21$), and hypoxic trained ($n = 24$). The study was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Academy of Science, National Academy Press, Washington, D.C. The experimental protocols were approved by the Animal Care and Use Committee of the Institute of Physiology of the Czech Academy of Sciences.

Chronic hypoxia and exercise training. Rats were exposed to moderate continuous normobaric hypoxia (CNH) (inspired O_2 fraction 0.12) for 3 wk in a normobaric chamber (6 m^3) equipped with hypoxic generators (Everest Summit, Hypoxico, NY). No reoxygenation occurred during this period. The control normoxic rats were kept for the same period of time at room air.

Rats assigned to exercise groups were habituated to forced treadmill running by increasing the speed (from 25 to 30 m/min) and duration (from 10 to 50 min) of daily exercise session stepwise for 5 consecutive days. After 2 days of rest, the exercise protocol involved 5 days of running at 30 m/min for 60 min with a 0° inclination. Normoxic and hypoxic animals were trained either at room air or in the hypoxic chamber, respectively, during the light period. Corresponding sedentary and trained rats were housed in the same room. Habituation to running started at the beginning of the second week of hypoxic exposure. To check potential effects of hypoxia before starting exercise, an additional subgroup of animals ($n = 6$) exposed to CNH for only 1 week was examined. All hypoxic animals were used immediately after the cessation of hypoxic exposure, and all exercising animals were used the next day after the last exercise session.

The compliance of each rat with exercise training was evaluated during each session by a 5-point score: a score of 1 was given to well-compliant rats, whereas a score of 5 was given to totally noncompliant ones. Mean exercise compliance score of each rat during the whole training protocol was calculated. Two insufficiently compliant rats out of 15 in the combined CNH/exercise group were excluded from evaluations.

Myocardial ischemia-reperfusion. Acute I/R insult was performed as described previously (31) with minor modifications. Anesthetized (pentobarbital sodium, 60 mg/kg ip) rats were ventilated (Ugo Basile, Italy) with room air at 65–70 strokes/min (tidal volume of 12 ml/kg body weight). A single-lead electrocardiogram and blood pressure in the carotid artery were continuously recorded (Gould P23Gb) and subsequently analyzed by a custom-designed software. The rectal temperature was maintained between 36.5 and 37.5°C throughout the experiment. Hematocrit in the tail blood was measured by the capillary micromethod.

A left thoracotomy was performed, and, after 10-min stabilization, regional ischemia was induced by the tightening of a silk braided suture 5/0 (Chirmax, Czech Republic) placed around the left anterior descending coronary artery ~1–2 mm distal to its origin and threaded through a polyethylene tube. Characteristic changes in the configuration of the ECG and a transient decrease in blood pressure verified the complete coronary artery occlusion. After a 20-min occlusion period, the ligature was released and reperfusion of previously ischemic tissue started. Then the chest was closed, air from thorax was exhausted, and spontaneously breathing animals were maintained in deep anesthesia following 3 h.

Infarct size determination. Hearts were excised and washed with saline via aorta. The area at risk (AR) was delineated by perfusion with 5% potassium permanganate after the coronary artery reocclusion (31). Frozen hearts were cut into slices 1 mm thick, stained with 1% 2,3,5-triphenyltetrazolium chloride (pH 7.4, 37°C) for 30 min, and fixed in formaldehyde solution. The infarct size (IS), the size of AR, and the size of the left ventricle (LV) were determined by computerized planimetric method using Ellipse software (ViDiTo, Slovakia). The size of AR was normalized to LV (AR/LV) and the IS was normalized to the AR (IS/AR).

EQ:1 Table 1. *Body and heart weight parameters and hematocrit in chronically hypoxic and normoxic sedentary and exercise-trained rats*

Group	BW, g	LVW, mg	LVW/BW, mg/g	RVW, mg	RVW/BW, mg/g	Hematocrit, %
Normoxic sedentary, $n = 8$	422 ± 9	538 ± 20	1.275 ± 0.038	229 ± 6	0.542 ± 0.008	45.7 ± 0.8
Normoxic trained, $n = 9$	380 ± 7†	528 ± 20	1.391 ± 0.044	205 ± 5	0.541 ± 0.014	45.2 ± 0.8
Hypoxic sedentary, $n = 8$	397 ± 6	506 ± 22	1.271 ± 0.039	341 ± 15*	0.857 ± 0.033*	53.1 ± 1.3*
Hypoxic trained, $n = 8$	330 ± 3*†	474 ± 12	1.436 ± 0.031†	288 ± 7*†	0.875 ± 0.027*	55.9 ± 0.8*

Values are means ± SE. BW, body weight; LVW, left ventricle weight; LVW/BW, relative left ventricle weight; RVW, right ventricle weight; RVW/BW, relative right ventricle weight. *$P < 0.05$ vs. corresponding normoxic group; †$P < 0.05$ vs. corresponding sedentary group.

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Table 2. *Heart rate and mean arterial blood pressure after stabilization (Baseline), at the end of 20-min coronary artery occlusion (Ischemia), and at the end of 3-h reperfusion in chronically hypoxic and normoxic sedentary and exercise-trained rats*

	Baseline	Ischemia	Reperfusion
Heart rate, beats/min			
Normoxic sedentary, $n = 8$	392 ± 8	404 ± 11	416 ± 11
Normoxic trained, $n = 15$	372 ± 8	379 ± 12	378 ± 9†
Hypoxic sedentary, $n = 13$	371 ± 9	407 ± 5‡	393 ± 8‡
Hypoxic trained, $n = 13$	368 ± 8	374 ± 10	396 ± 8‡
Blood pressure, mmHg			
Normoxic sedentary	101 ± 4	87 ± 7	84 ± 4
Normoxic trained	107 ± 4	103 ± 5	95 ± 5
Hypoxic sedentary	122 ± 5*	121 ± 6*	105 ± 6*
Hypoxic trained	122 ± 4*	114 ± 6	108 ± 7

Values are means ± SE. *$P < 0.05$ vs. corresponding normoxic group; †$P < 0.05$ vs. corresponding sedentary group; ‡$P < 0.05$ vs. baseline.

Analysis of arrhythmias. The incidence and severity of ventricular arrhythmias during the 20-min ischemic insult and during the first 3 min of reperfusion were assessed according to the Lambeth Conventions (8) as previously described (3). Premature ventricular complexes (PVCs) occurring as singles, salvos, or tachycardia (VT, a run of 4 or more consecutive PVCs) were counted separately. The incidence and duration of life-threatening ventricular tachyarrhythmias, that is, VT and fibrillation (VF), were also evaluated. VF lasting more than 2 min was considered as sustained (VFs). Rats exhibiting VFs were excluded from further evaluations.

Tissue processing. The separate groups of animals (not subjected to myocardial I/R) assigned to biochemical analyses of the LV myocardium were killed by cervical dislocation; hearts were rapidly excised, washed in cold (0°C) saline, dissected into the right ventricle (RV), the LV, and the septum and weighed; the LV free wall was frozen in liquid nitrogen and stored at −80°C.

Samples for the cytokines assay and immunoblotting were pulverized into fine powder with liquid nitrogen and subsequently homogenized in eight volumes of ice-cold homogenization buffer containing 12.5 mM Tris (pH 7.4), 250 mM sucrose, 2.5 mM EGTA, 1 mM EDTA, 100 mM NaF, 0.3 mM phenylmethylsulfonyl fluoride, 6 mM β-mercaptoethanol, 10 mM glycerol-2-phosphate, 0.2 mM leupeptin, 0.02 mM aprotinin, and 0.1 mM activated sodium orthovanadate. All steps were performed at 4°C. The homogenate aliquots were stored at −80°C until use.

Inflammatory cytokines assay. For the measurement of TNF-α and interleukin-6 (IL-6) LV concentrations, ELISA kits (eBioscience, Vienna, Austria) were used. These assays were performed according to the standards described by the manufacturers. The results are expressed per milligrams of total protein.

Immunoblotting. Proteins were separated by SDS-PAGE electrophoresis (10 or 15% gels) and transferred to polyvinylidene difluoride membranes (Bio-Rad, Prague, Czech Republic). After blocking with 5% dry low-fat milk in Tween-20 Tris-base sodium for 1 h at room temperature, membranes were washed and probed at 4°C with the following primary antibodies against: catalase (1:2,000, ab16731, Abcam, Cambridge, MA), citrate synthase (CS) (1:2000, ab-96600, Abcam), cyclooxygenase-1 (COX-1) (1:1,000, TX sc-1752, Santa Cruz Biotechnology, Dallas, TX), cyclooxygenase-2 (COX-2) (1:1,000, sc-1747, Santa Cruz Biotechnology), cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) (1:2,000, 2832S, Cell Signaling, Danvers, MA), phosphorylated form of $cPLA_2\alpha$ ($p\text{-}cPLA_2\alpha$) (1:2,000, 2831S, Cell Signaling), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:500, sc-25778, Santa Cruz Biotechnology), inducible nitric oxide synthase (iNOS) (1:500, 610432, BD Biosciences, San Jose, CA), manganese superoxide dismutase (MnSOD) (1:1,000, S5069, Sigma Aldrich, Prague, Czech Republic), nuclear factor- κB (NF-κB) p65 (1:500, sc-372, Santa Cruz Biotechnology), TNF-α type 1 receptor (TNFR1) (1:1,000, sc-1070, Santa Cruz Biotechnology), and TNF-α type 2 receptor (TNFR2) (1:1,000, sc-7862, Santa Cruz Biotechnology). After overnight incubation, the membranes were washed and incubated for 1 h at room temperature with anti-rabbit (170-6515, Bio-Rad), anti-mouse (31432, Thermo Fisher Scientific, Prague, Czech Republic), and anti-goat (A8919, Sigma Aldrich), respectively,

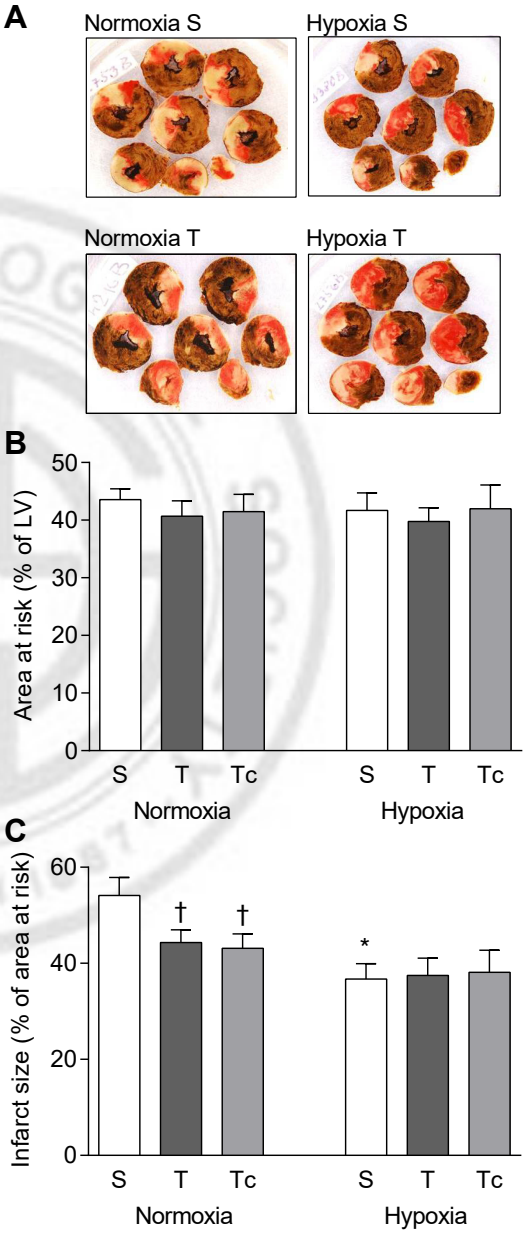

Fig. 1. Myocardial infarction induced by coronary artery occlusion and reperfusion in chronically hypoxic and normoxic sedentary (S) and exercise-trained (T) rats. Typical images (*A*), the size of area at risk expressed as a percentage of the left ventricle (*B*), and infarct size expressed as a percentage of the area at risk (*C*). Brown color represents normally perfused tissue stained by potassium permanganate. Red area, tetrazolium positive, represents tissue surviving the occlusion, and pale area, tetrazolium negative, is infarcted tissue. Tc denotes the subgroups of T rats well compliant to exercise training (see MATERIALS AND METHODS and RESULTS for details). Values are means ± SE from 7 to 15 hearts in each group; *$P < 0.05$ vs. corresponding normoxic group; †$P < 0.05$ vs. corresponding sedentary group.

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horseradish peroxidase-labeled secondary antibodies. Bands were visualized by enhanced chemiluminescence on the LAS system or on the medical X-ray films (Agfa, Berlin, Germany). ImageJ software (Java Technology, Cupertino, CA) was used for the quantification of the relative abundance of proteins. To ensure the specificity of immunoreactive proteins, prestained molecular weight protein standards (BioRad) were used. The samples from each experimental group were run on the same gel and quantified on the same membrane. Neither CNH nor exercise affected the expression of GAPDH, which was used as a loading control.

Malondialdehyde assay. LV samples for determination of lipid peroxidation marker malondialdehyde (MDA) were processed as described earlier (7) and analyzed by a high-performance liquid chromatography system (4.6 × 125 mm, flow 1.0 ml/min, sampling volume 30–100 μl, column EC Nucleosil 100-5 C18; Shimadzu, Japan) with the ultraviolet detection set on 310 nm. MDA concentration is expressed per milligrams of total protein.

Statistical analyses. Normally distributed variables are presented as means ± SE. Two-way ANOVA was used to determine effects of chronic hypoxia, exercise, and their interaction, followed by Bonferroni correction for multiple comparisons. Not normally distributed data (arrhythmias) are expressed as median ± interquartile range. Differences in the number of PVCs between the groups were compared by the Kruskal-Wallis nonparametric test. The incidence of ventricular tachycardia and fibrillation was examined by Fisher's exact test. Differences were assumed statistically significant when $P < 0.05$. Statistical analyses were performed with GraphPad Prism 5 software (La Jolla, CA).

RESULTS

Body and heart weight and hematocrit. Adaptation of rats to moderate continuous hypoxia did not significantly affect body weight, while exercise training caused growth retardation, which was more pronounced in animals trained under hypoxic conditions. No significant differences in LV weight were observed among the groups, except for the hypoxic exercised rats, which showed increased LV weight normalized to body weight. CNH led to RV hypertrophy and increased hematocrit. These variables were not affected by exercise training (Table 1).

Myocardial IS and arrhythmias. Baseline values of mean arterial pressure were slightly but significantly higher in both hypoxic groups compared with their normoxic counterparts. This difference persisted throughout ischemia and reperfusion in sedentary rats only. Neither CNH nor exercise training affected heart rate before ischemia. Both hypoxic groups exhibited higher heart rate at the end of reperfusion than at baseline and the rats trained at normoxia had lower heart rate than their sedentary controls (Table 2).

The mean normalized AR (AR/LV) was 39–43% and did not differ among the groups. The IS reached 54.1 ± 4.0% of the AR in the normoxic group, and exercise training decreased it to 44.3 ± 2.7%. CNH reduced IS to 36.7 ± 3.3%, but exer-

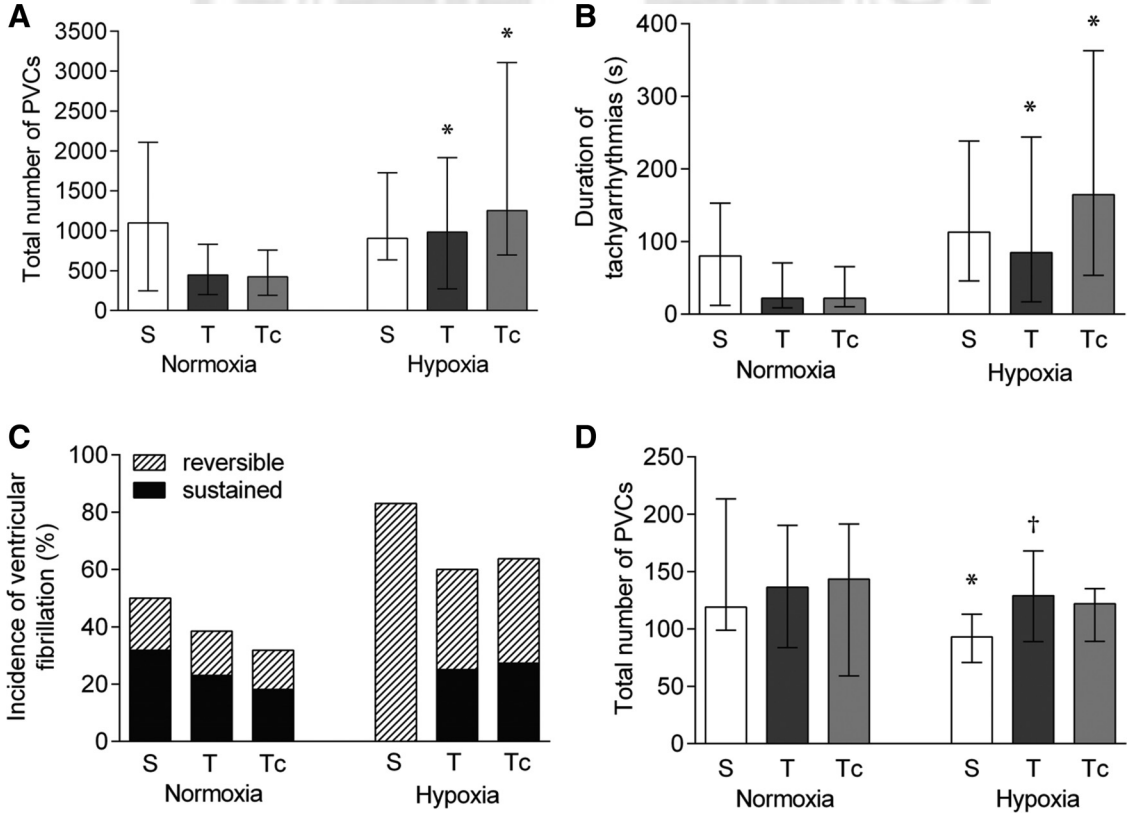

Fig. 2. Total number of premature ventricular complexes (PVCs) (*A*), total duration of tachyarrhythmias (*B*), the incidence of reversible/sustained ventricular fibrillation during coronary artery occlusion (*C*), and total number of PVCs during the first 3 min of reperfusion (*D*) in chronically hypoxic and normoxic sedentary (S) and exercise-trained (T) rats. Tc denotes the subgroups of T rats well compliant to exercise training (see MATERIALS AND METHODS and RESULTS for details). Values (graphs in *A*, *B*, and *D*) are shown as median with interquartil range from 7 to 15 hearts in each group; *$P < 0.05$ vs. corresponding normoxic group; †$P < 0.05$ vs. corresponding sedentary group.

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cise at hypoxia did not provide any additive protection (37.4 ± 3.7%) (Fig. 1).

Neither CNH nor exercise training significantly affected the total number of ischemic ventricular arrhythmias (Fig. 2*A*) and the total duration of tachyarrhythmias (VT and reversible VF; Fig. 2*B*). However, animals trained at hypoxia were significantly more susceptible to ischemic arrhythmias than their normoxic counterparts. Sustained VF (>2-min duration) occurred in 18–32% of rats, except for the sedentary hypoxic group which exhibited only reversible VF; the differences among groups did not reach statistical significance (Fig. 2*C*). CNH reduced the total number of arrhythmias occurring at the beginning of reperfusion but exercise abolished this effect (Fig. 2*D*).

The mean exercise compliance score of normoxic and chronically hypoxic animals was 1.29 and 2.06, respectively. To verify that the somewhat worse compliance of rats exercising at hypoxia compared with those trained at room air did not affect myocardial ischemic tolerance, we selected well-compliant (Tc) animals (a score of 1.0–1.5) from both trained (T) groups. The mean score was 1.23 and 1.24 in selected normoxic and chronically hypoxic subgroups, respectively. Figure 1 and Fig. 2 show that this selection did not significantly influence the effect of exercise on IS and susceptibility to arrhythmias.

IL-6, TNF-α, and their receptors. Adaptation to CNH for 3 wk increased myocardial levels of TNF-α and IL-6 by 53% and 88%, respectively, compared with the normoxic sedentary group. The effect was absent when TNF-α was measured after the first week of the hypoxic exposure (93% of normoxic level). Exercise training had no effect on TNF-α and IL-6 in the hearts of normoxic rats, but it significantly attenuated their increase induced by CNH. Nevertheless, both cytokines still remained significantly higher in rats exercising at hypoxia compared with their normoxic counterparts (Fig. 3, *A* and *B*). CNH had no effect on the myocardial protein level of TNFR1 while significantly increasing TNFR2 level (by 102%). Exercise training of normoxic rats affected neither TNFR1 nor TNFR2 but it prevented the increase in TNFR2 level in the group adapted to CNH (Fig. 3, *C* and *D*).

NF-κB and related signaling. The expression of transcription factor NF-κB increased CNH by 71% compared with the sedentary normoxic group. Exercise training only tended to attenuate this increase without affecting NF-κB in normoxic rats (Fig. 4*A*). Similarly, CNH increased the expression of iNOS (by 63%), which was not significantly affected by exercise (Fig. 4*B*). Both $cPLA_2\alpha$ and its phosphorylated form were upregulated by CNH by 13 and 26%, respectively. These increases were abolished by exercise training, which had no effect in the normoxic group (Fig. 4, *C* and *D*). Neither CNH nor exercise affected COX-1 level, whereas COX-2 level

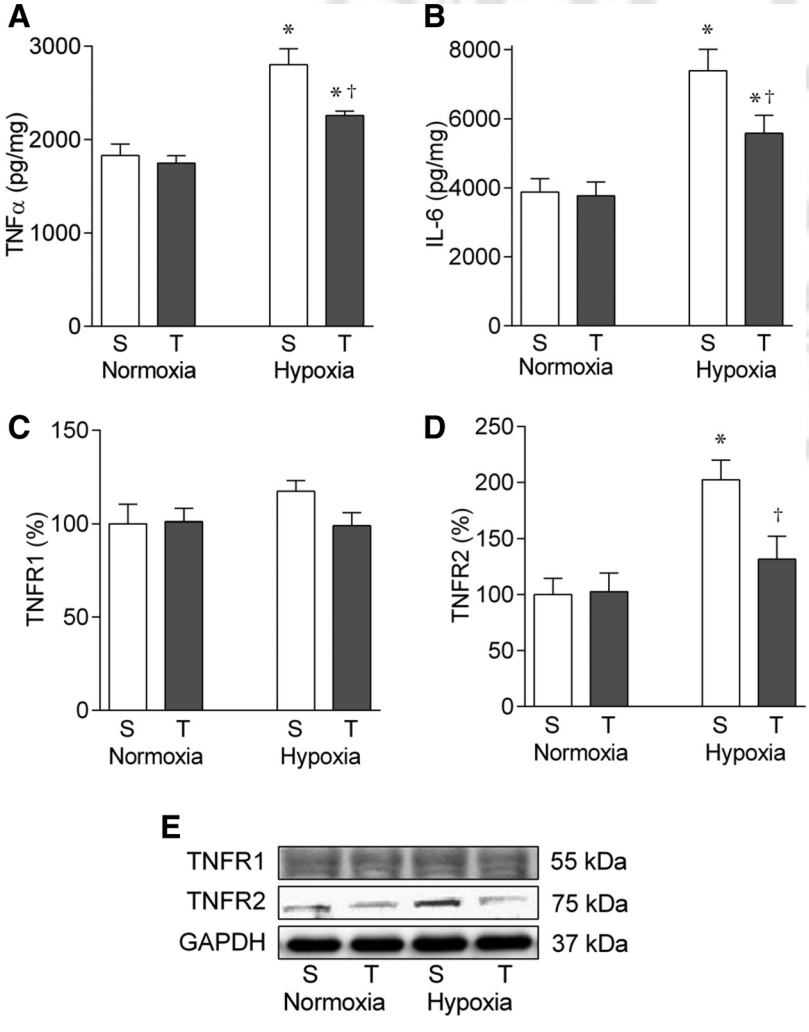

Fig. 3. Myocardial levels of tumor necrosis factor-α (TNF-α) (*A*), interleukin-6 (IL-6) (*B*), TNF-α type 1 receptor (TNFR1) (*C*) and TNF-α type 2 receptor (TNFR2) (*D*) in chronically hypoxic and normoxic sedentary (S) and exercise-trained (T) rats. Representative Western blots are shown (*E*); GAPDH was used as a loading control. Values are means ± SE from 8 hearts in each group; $*P < 0.05$ vs. corresponding normoxic group; $\dagger P < 0.05$ vs. corresponding sedentary group.

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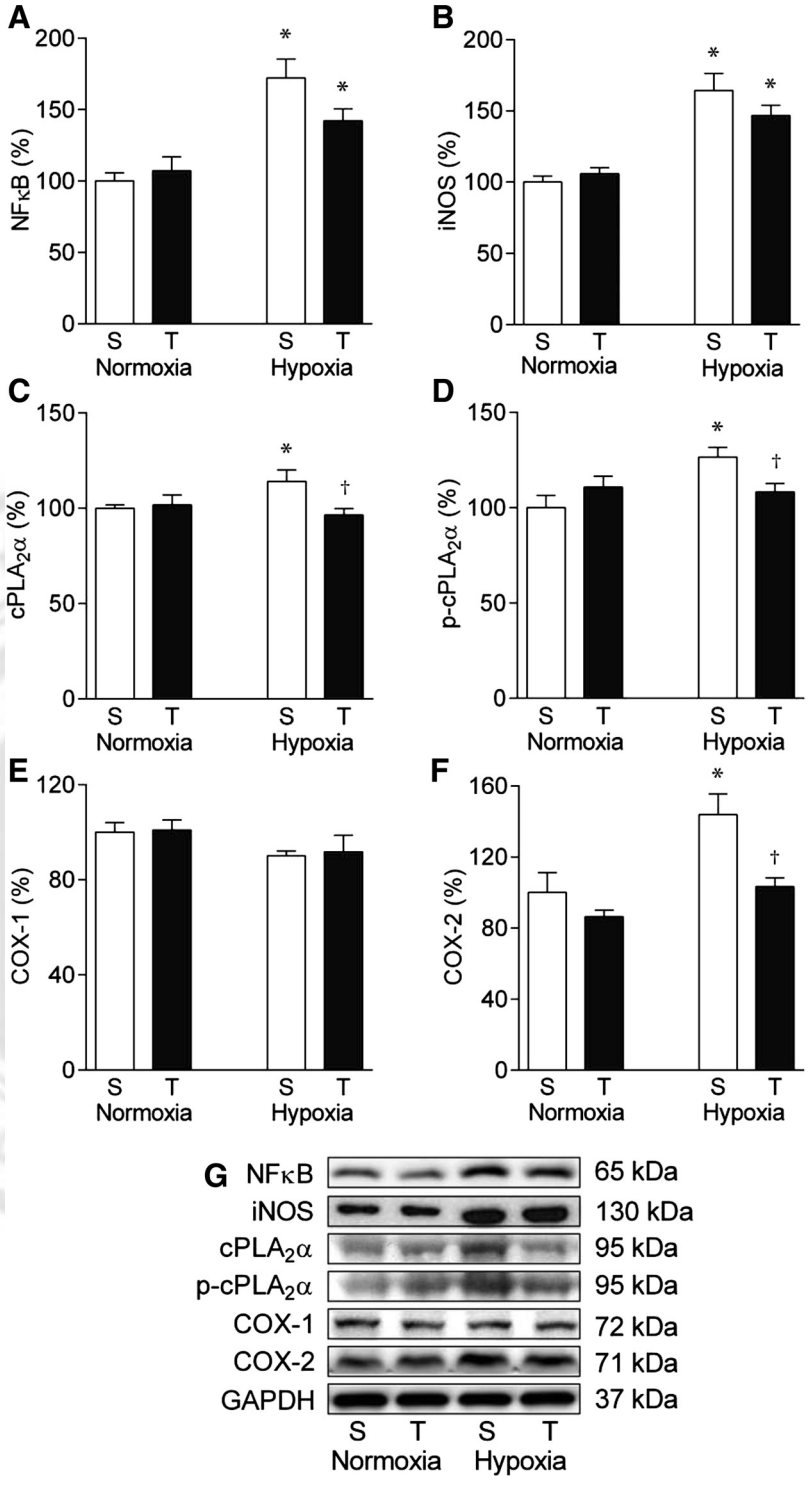

Fig. 4. Myocardial levels of nuclear factor-κB (NF-κB) (*A*), inducible nitric oxide synthase (iNOS) (*B*), cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) (*C*), phosphorylated form of $cPLA_2\alpha$ ($p\text{-}cPLA_2\alpha$) (*D*), cyclooxygenase-1 (COX-1) (*E*), and cyclooxygenase-2 (COX-2) (*F*) in chronically hypoxic and normoxic sedentary (S) and exercise-trained (T) rats. Representative Western blots are shown (*G*); GAPDH was used as a loading control. Values are means ± SE from 8 hearts in each group; $*P < 0.05$ vs. corresponding normoxic group; $\dagger P < 0.05$ vs. corresponding sedentary group.

increased by 43% in the CNH group, the effect being eliminated by exercise (Fig. 4, *E* and *F*).

MDA and antioxidant enzymes. Myocardial MDA concentration increased by 76%, and the expression of MnSOD and catalase rose by 75 and 24%, respectively, in the hearts of rats adapted to CNH for 3 wk. MnSOD measured after the first week of the hypoxic exposure remained unaffected, reaching 101% of normoxic value. Exercise training had no effect in the normoxic animals, and it only tended to attenuate the CNH-induced increases of MDA, MnSOD, and catalase without reaching statistical significance (Fig. 5, *A–C*). Neither CNH nor exercise affected the myocardial expression of CS, which is commonly used as a marker of mitochondrial mass (Fig. 5*D*). F5

DISCUSSION

The present study was designed to determine if a combination of two well-established forms of sustainable cardioprotec-

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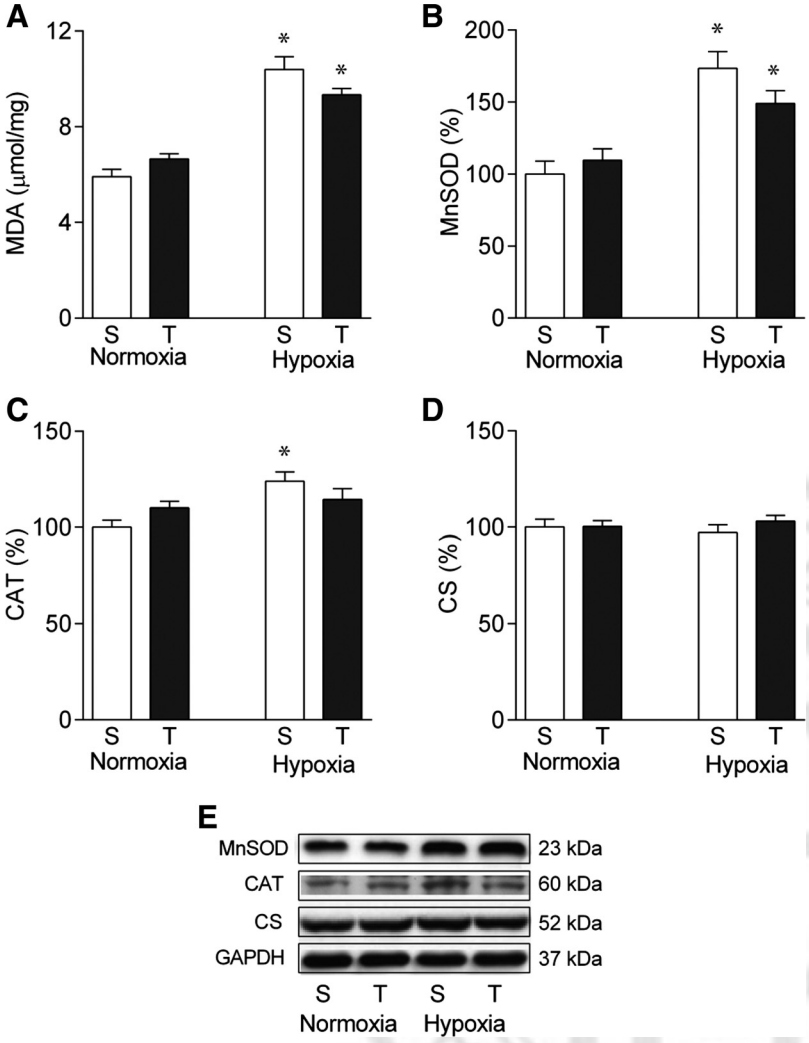

Fig. 5. Concentration of malondialdehyde (MDA) (*A*) and myocardial levels of manganese superoxide dismutase (Mn-SOD) (*B*), catalase (CAT) (*C*), and citrate synthase (CS) (*D*) in chronically hypoxic and normoxic sedentary (S) and exercise-trained (T) rats. Representative Western blots are shown (*E*); GAPDH was used as a loading control. Values are means ± SE from 8 hearts in each group; $*P < 0.05$ vs. corresponding normoxic group.

tion induced by chronic hypoxia and exercise training can result in the amplification of ischemia-resistant cardiac phenotype. Our data are in line with a number of earlier reports showing that these adaptive interventions acting separately reduced myocardial IS induced by acute I/R insult. The novel finding is that rats subjected to regular exercise during continuous exposure to hypoxic atmosphere exhibited the same infarct-sparing effect as their sedentary counterparts. CNH led to pro-inflammatory response, increased myocardial expression of several related potentially protective mediators, and antioxidant enzymes, whereas none of these effects were observed in the rats exercising at room air. On the other hand, exercise in hypoxia abolished or significantly attenuated most of the CNH-induced responses related to inflammation, including the increased TNF-α and IL-6 levels and the overexpression of TNFR2, cPLA$_2$α, and COX-2, without significantly affecting the upregulation of NF-κB, iNOS, and antioxidant enzyme MnSOD.

We have recently reported that the treatment of rats with antibodies against TNF-α during adaptation to CNH suppressed the infarct size-limiting effect and eliminated the CNH-induced increases in myocardial levels of TNF-α, its receptor TNFR2, NF-κB, and MnSOD. These results led us to conclude that TNF-α is involved in the protective mechanism of CNH, its effect being possibly mediated by TNFR2 and the NF-κB-dependent activation of redox signaling with increased antioxidant defense (7). TNF-α is a key cytokine that plays an essential role in the initiation of inflammatory response. Although excessive levels of TNF-α have detrimental actions on the heart mediated by TNFR1 (which was not affected in our study), the activation of TNFR2 by low levels of this cytokine is protective (39). Several studies have demonstrated that TNF-α can also induce various forms of conditioning (20, 33).

Regarding the involvement of cytokines in exercise-induced cardioprotection, the available data are scarce and conflicting. Serra et al. (40) did not observe any effect of regular exercise training itself on the myocardial levels of TNF-α and IL-6 in rats. On the other hand, TNF-α neutralization blunted the protection induced by a single exercise session, likely via the prevention of antioxidant response (47). Regarding IL-6, a recent report indicated that this myokine released from skeletal muscles mediated cardioprotective effects of exercise in mice. Exercise did not affect myocardial IL-6 level but it upregulated its receptor and activated IL-6 signaling pathways (23). Thus the absence of any effect of exercise alone on myocardial cytokines in our study does not necessarily mean that they are not involved in the induction of protective cardiac phenotype.

Exercise has been shown to reduce sympathetic activation and stimulation of myocardial adrenoceptors associated with the adaptation to chronic hypoxia (11), which plays an important role in the cardioprotection conferred by hypoxic conditioning (22). Interestingly, exercise training completely abolished the increase of myocardial TNF-α and IL-6 levels caused by the sustained pharmacological stimulation of β-adrenoceptors (40). Given our finding that TNF-α plays a role in the induction of the ischemia-resistant phenotype of CNH hearts (7), its blunted response to hypoxia in exercised rats may be expected to attenuate the protective effect. However, here we showed that exercise training abolished only the CNH-induced suppression of early reperfusion arrhythmias, whereas the infarct-sparing effect was unaffected. As the myocardial level of TNF-α remained still significantly higher in the combined CNH/exercise setting, we can suppose that it was sufficient to maintain the CNH-induced protective signaling and improved resistance to infarction. Another possibility is that TNF-α increase occurred already during the first week of CNH exposure, when the animals did not exercise, and this initial response could be able to induce the persisting cardioprotective state. Indeed, it has been shown that TNF-α can result in the long-lasting activation of NF-κB and its downstream targets (28). However, our observation of unchanged levels of TNF-α and MnSOD after 1 wk of hypoxia seems to rule out this possibility. Accordingly, we did not detect any reduction of IS during the first week of exposure to CNH in our previous study (29).

Alternatively, we cannot exclude that exercise activated distinct protective mechanisms not addressed in the present study that just compensated for the attenuated TNF-α signaling. The absence of any influence of exercise training alone on the TNF-α-related potentially protective molecules detected in our study seems to support this view. In line with our observation, previous studies reported unchanged levels of COX-2 (23, 37) and iNOS (43) in myocardium of exercising rats. On the other hand, iNOS has been suggested to mediate exercise-induced cardioprotection in mice (1). Unlike COX-2, iNOS upregulated by CNH remained significantly higher in exercised hearts compared with their normoxic counterparts. However, the major contribution of this enzyme to the maintenance of cardioprotection in the combined CNH/exercise group seems unlikely. We showed earlier that iNOS upregulation persisted despite the abolished infarct-sparing effect in CNH rats treated with TNF-α antibody (7).

Inflammation and oxidative stress are mutually related. Specifically, TNF-α stimulates ROS production, while ROS can promote the TNF-α-induced inflammatory cascade (16, 26, 38). It has been shown that mitochondria are the principal source of ROS formation in the TNF-α pathway (42). Signaling via ROS-dependent pathways appears to play a key role in cardioprotection against I/R insult conferred by various stimuli including chronic hypoxia (19) and long-term exercise training (1), as indicated by the elimination of their infarct-sparing effects by antioxidant treatments during hypoxic exposure and training sessions, respectively. Nevertheless, it has been shown that ROS are not required for late preconditioning of the heart induced by two exercise sessions (44). Numerous but not all studies demonstrated the increased myocardial capacity of antioxidant defense systems induced by chronic hypoxia or exercise as a prerequisite for their salutary effects against I/R injury. The enhanced expression of MnSOD and catalase in hearts of CNH rats in the present study is in line with these results. Excess formation of ROS without adequate activation of cellular antioxidants caused by a brief periodic interruption of hypoxic exposure may result in a disturbed redox balance and a loss of protection (15, 29). Regarding exercise training, we did not detect any major effect on MDA and antioxidant enzymes in ventricular homogenate, which is in agreement with a number of reports summarized in a recent review (36). On the other hand, it has been suggested that cardioprotection induced by a longer duration of exercise is mediated, at least in part, by MnSOD (13, 36), the primary mitochondrial antioxidant enzyme. Indeed, some studies detected the increased level and activity of this enzyme following exercise in myocardial mitochondrial fraction (17, 41). Although we cannot exclude that exercise led to the upregulation of MnSOD in mitochondria also in our present study, the unchanged level of CS reflecting mitochondrial mass makes this an unlikely possibility.

It is concluded that regular exercise training of rats during their adaptation to CNH conferred the same infarct size-limiting effect as CNH alone. The maintenance of ischemia-resistant cardiac phenotype in CNH combined with exercise can be likely attributed to the persisting increase in myocardial antioxidant defense capacity, despite attenuating the CNH-induced myocardial inflammatory response and TNF-α-dependent cardioprotective signaling. However, the possibility that another protective mechanism activated by exercise compensated for the downregulation of this pathway should be also taken into account.

GRANTS

Funding support was provided by the Czech Science Foundation Grants 302/12/1162 (to F. Kolář), 13-10267 (to J. Neckář), and 16-12420Y (to M. Hlaváčková), and the Grant Agency of the Charles University in Prague 798813 (to J. Hrdlička).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors. **AQ: 7**

AUTHOR CONTRIBUTIONS

P.A., A.C., J.N., J.H., P.M., K.H., M.H., K.M., F.P., J.V., and D.B. performed experiments; P.A., A.C., J.N., J.H., P.M., K.H., M.H., K.M., F.P., J.V., D.B., and O.N. analyzed data; P.A., A.C., J.N., J.H., P.M., K.H., M.H., K.M., F.P., O.N., and F.K. interpreted results of experiments; P.A., A.C., J.N., J.H., P.M., K.H., M.H., K.M., F.P., J.V., D.B., O.N., and F.K. approved final version of manuscript; F.K. conceived and designed research; F.K. prepared figures; F.K. drafted manuscript; F.K. edited and revised manuscript.

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