Přílohy

Příloha A

Hahnova K., Kasparova D., Zurmanova J., Neckar J., Kolar F., Novotny J. (2016) β-Adrenergic signaling in rat heart is similarly affected by continuous and intermittent normobaric hypoxia. *Gen Physiol Biophys*, 35(2):165-73

Příloha B

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Příloha C

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Příloha A

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β-Adrenergic signaling in rat heart is similarly affected by continuous and intermittent normobaric hypoxia

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Abstract. Chronic hypoxia may produce a cardioprotective phenotype characterized by increased resistance to ischemia-reperfusion injury. Nevertheless, the molecular basis of cardioprotective effects of hypoxia is still not quite clear. The present study investigated the consequences of a 3-week adaptation to cardioprotective (CNH, continuous normobaric hypoxia) and nonprotective (INH, intermittent normobaric hypoxia; 23 h/day hypoxia followed by 1 h/day reoxygenation) regimen of hypoxia on β-adrenergic signaling in the rat myocardium. Both regimens of hypoxia lowered body weight and led to marked right ventricular (RV) hypertrophy, which was accompanied by 25% loss of β1-adrenergic receptors (β1-ARs) in the RV. No significant changes were found in β-ARs in left ventricular (LV) preparations from animals adapted to hypoxia. Although adenylyl cyclase (AC) activity stimulated through the G proteins was decreased in the RV and increased in the LV after exposure to hypoxia, there were no significant changes in the expression of the dominant myocardial AC 5/6 isoforms and the stimulatory G proteins. These data suggest that chronic normobaric hypoxia may strongly affect myocardial β-adrenergic signaling but adaptation to cardioprotective and nonprotective regimens of hypoxia does not cause notably diverse changes.

Key words: Rat myocardium — Chronic hypoxia — β-adrenergic receptors — Adenylyl cyclase

Abbreviations: AC, adenylyl cyclase; β-ARs, β-adrenergic receptors; BSA, bovine serum albumin; CNH, continuous normobaric hypoxia; EDTA, ethylenediaminetetraacetic acid; INH, intermittent normobaric hypoxia; LV, left ventricle; RV, right ventricle.

Introduction

Hypoxia can evoke different effects, either adaptive or pathological, depending on the severity, pattern, and duration of exposure. Chronic hypoxia has been found to induce a wide range of adaptive changes in the heart, which could be considered as cardioprotective. Adaptation of myocardium to certain regimens of chronic hypoxia can contribute to the improvement of ischemic tolerance and enhancement of left ventricular contractility in heart failure (Zhuang and Zhou 1999; Ostadal and Kolar 2007; Naghshin et al. 2012). Chronic hypoxia leads to increased activity of the sympathetic nervous system, thus increasing catecholamine levels in the body. The increased level of catecholamines and their effect on β-adrenergic signaling could contribute to the development of a cardioprotective phenotype (Mallet et al. 2006). Nevertheless, the exact molecular mechanisms underlying hypoxia-induced cardioprotection are still unclear.

Chronic hypoxia leads to pulmonary hypertension and subsequently to the right ventricular (RV) hypertrophy. The left ventricle (LV) usually does not hypertrophy unless at rather severe and prolonged intermittent hypoxia (Pelouch et al. 1997). Besides increased tolerance to an acute ischemic injury, animals adapted to chronic hypoxia exhibit the impaired chronotropic and inotropic responsiveness to β-adrenergic stimulation (Pei et al. 2000). We have previously shown that severe chronic intermittent

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high-altitude hypoxia deranges myocardial adenylyl cyclase (AC) signaling in both ventricles (Hrbasova et al. 2003). A number of earlier studies reported down-regulation of β-adrenoceptors (β-ARs) and desensitization of AC in the hypoxic heart (Mader et al. 1991; Mardon et al. 1998; Leon-Velarde et al. 2001). All these changes may reflect increased sympathetic activity under hypoxic conditions. However, the concequencess of chronic hypoxia may differ in different experimental settings as illustrated by increased expression of β-ARs and unchanged AC activity in isolated cardiac myocytes (Li et al. 1996).

There are some indications that β-adrenergic signaling may play a role in cardioprotection. The engagement of β-ARs in the induction of a cardioprotective phenotyp has been demonstrated by administration of metoprolol to dogs throughout their exposure to intermittent hypoxia. This β_1-AR antagonist markedly blunted hypoxia-evoked cardioprotection (Mallet et al. 2006). Okruhlicova et al. (1999) reported the involvement of AC in mechanisms underlying ischemic preconditioning in the rat heart. We and others have previously observed certain changes in the stimulatory G proteins (Gs) of animals exposed to chronic hypoxia. Whereas Pei et al. (2000) reported oposite changes in the short and long Gsα isoforms in ventricular myocytes isolated from hypoxia-exposed rats, we noticed a sligh increase in cytosolic but not membrane-bound Gsα (Hrbasova et al. 2003). There were no significant changes in the amount of the inhibitory G proteins (Gi) after adaptation to hypoxia. The discordant data on gene expression, protein levels, and their functional activity do not provide a clear evidence for the role of trimeric G proteins in the cardioprotective mechanisms. Anyway, the stimulatory and inhibitory G proteins are key regulatory elements of the β-adrenergic signaling pathway which modulate the enzyme activity of AC under variable physiological conditions (El-Armouche et al. 2003). Besides Gα subunits, also Gβγ subunits may regulate isoform-dependent AC activity. The dominant cardiac AC isoforms (5 and 6) are known to be inhibited by Gβγ (Beazely and Watts 2006).

Many investigators tested different regimens of chronic hypoxia for their cardioprotective effects in the past. Apparently, both continuous and intermittent hypoxia can enhance cardiac ischemic tolerance under certain conditions (Neckar et al. 2002; Guo et al. 2009; Maslov et al. 2013). However, the outcome obviously depends not only on the degree and duration of hypoxia, but also on the number, duration and periodicity of daily normoxic episodes. Interestingly, it was recently demonstrated that a brief daily episode of reoxygenation can abolish cardioprotection conferred by adaptation to chronic normobaric hypoxia. Daily reoxygenation eliminated both the infarct size-limiting effect of continuous hypoxia in open-chest rats subjected to coronary artery occlusion and cytoprotective effects of hypoxic adaptation in isolated ventricular myocytes exposed to acute anoxic insult (Neckar et al. 2013). So far, there is no information about the possible difference between the effect of protective and nonprotective regimens of hypoxia on the myocardial β-adrenergic signaling system that might potentially contribute to diverse ischemic tolerance. Therefore, the present study was aimed to evaluate the presumed impact of protective continuous (CNH) and nonprotective intermittent (INH) normobaric hypoxia on β-adrenergic signaling in the RV and LV myocardium of adult rats. We have assessed the distribution of β-ARs receptors, G proteins and AC, as well as functional status of this crucial myocardial signaling system.

Materials and Methods

Materials

TRIzol Reagent was from Invitrogen (Carlsbad, CA, USA), [α-^{32}P]ATP, [^{3}H]cAMP and [^{3}H]CGP 12177 were purchased from Amersham Biosciences (Buckinghamshire, UK) and scintillation cocktail CytoScint from ICN Biomedicals (Irvine, CA, USA). Acrylamide and bis-acrylamide were from SERVA (Heidelberg, Germany), aluminum oxide 90 (neutral, activity I) was from Merck (Darmstadt, Germany) and Protran nitrocellulose transfer membranes were from Schleicher & Schuell BioScience (Dassel, Germany). All other chemicals were from Sigma (St. Louis, MI, USA) and they were of the highest purity available.

Animal model

Adult male Wistar rats (Velaz, Ltd., Czech Republic) with initial body weight (BW) about 280 ± 15 g were used throughout the study. Animals were fed an *ad libitum* standard chow diet and kept 3 *per* cage in a controlled environment (23°C, 12 h:12 h light-dark cycle). One group of rats was exposed to continuous normobaric hypoxia (CNH, 24 h/day, 10% O_2) for 3 weeks in a chamber equipped with hypoxic generators (Everest Summit, Hypoxico Inc., NY, USA). The chamber construction allowed for regular animal maintenance without any reoxygenation during this period. Another group of rats was exposed to hypoxia intermittently (INH, 23 h/day) with single 1 h/day episod of normoxia (room air) during 3 weeks of adaptation. A control group (N) was kept at room air for the same period of time. All animal experiments were approved by the Institutional Animal Use and Care Committee of the Institute of Physiology, Czech Academy of Sciences (No. 140/2011). Rats were maintained according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Processing of heart tissue for biochemical analyses

After sacrifying the rats by decapitation, hearts were rapidly excised and washed in ice-cold saline solution. The LV and RV free walls were dissected from the septum, immediately frozen in liquid nitrogen and weighed. The pieces of frozen tissue were homogenized either in TRIzol Reagent for isolation of mRNA or in homogenization buffer H (20 mM Tris, 3 mM $MgCl_2$, 0.25 M sucrose, 1 mM EDTA and protease inhibitor cocktail (Complete, Roche Diagnostics); pH 7.4) for radioligand binding assay, Western blotting and enzyme activity analysis. In the latter case, the rat ventricles were homogenized for 1 min on ice using a motor-driven homogenizer (Teflon-glass). The homogenates were subsequently clarified by centrifugation at 600 × *g* for 10 min (4°C) in order to remove nuclei and particulate cellular debris. Thereafter, the resulting postnuclear supernatant was centrifuged at 50 000 × *g* for 30 min (4°C) in order to isolate crude membranes. The pellet containing crude membranes was resuspended in TME buffer (20 mM Tris, 3 mM $MgCl_2$ and 1 mM EDTA; pH 7.4), aliquoted and stored at –80°C until use.

Real-time PCR analysis

Total cellular RNA was extracted from samples of the individual ventricles using TRIzol Reagent. One microgram of total RNA was converted to cDNA using oligo(dT) primers and RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas UAB, Vilnius, Lithuania) according to manufacturer's protocol. Real-time PCR protocol was performed on a LightCycler 480 (Roche Applied Sciences, Penzberg, Germany) using a MESA GREEN qPCR MasterMix Plus for SYBR Assay No ROX (Eurogentec, Belgium) according to the manufacturer's protocol. Gene-specific primer pairs for β-adrenergic receptors were designed using the Universal Probe Library Assay Design Center (UPL, Roche Applied Science) and the sequences of forward and reverse primers were: 5'-AGAGCAGAAGGCGCTCAAG-3' and 5'-AGCCAGCAGAGCGTGAAC-3' for *AdrB1*, and 5'-ACGAGCTCAGTGTGCAGGA-3' and 5'-TCCTGGAAGCTTCATTCAGAG-3' for *AdrB2*. The levels of analyzed transcripts were quantified after normalization to the level of hypoxanthine-guanine phosphoribosyltransferase 1 (*Hprt1*) reference gene transcript (Waskova-Arnostova et al. 2013). All measurements were performed in triplicates.

β-Adrenergic receptor binding

Myocardial β-ARs were determined by radioligand binding assay with the β-antagonist [^{3}H]CGP 12177 as described previously (Klevstig et al. 2013). Briefly, samples of myocardial membranes (100 μg protein) were incubated in a buffer B (50 mM Tris-Cl, 10 mM $MgCl_2$ and 1 mM ascorbic acid; pH 7.4) containing 4 nM [^{3}H]CGP 12177 at 37°C for 1 h (total volume of 0.5 ml). The binding reaction was terminated by adding 3 ml of ice-cold buffer C (50 mM Tris-Cl and 10 mM $MgCl_2$; pH 7.4) and subsequent filtration through GF/C filters presoaked for 1 h with polyethylenimine. The filters were then washed 2 times with 3 ml of ice-cold buffer C. After addition of 4 ml scintillation cocktail CytoScint, radioactivity retained on the filters was measured by counting for 5 min. Nonspecific binding was defined as that not displaceable by 10 μM L-propranolol and it represented about 30% of total binding. For competition 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 characteristics of β-adrenergic binding sites and the β_1- and β_2-AR proportions in myocardial membranes were calculated using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA).

Electrophoresis and Western blotting

Samples of myocardial membranes were solubilized (3:1) in Laemmli buffer and loaded (30 μg *per* lane) on standard (10% acrylamide/0.26% *bis*-acrylamide) polyacrylamide gels (Novotny et al. 2001). SDS-PAGE was carried out at 200 V for 60 min on a Mini-Protean II apparatus (BIO-RAD, Hercules, CA, USA). After electrophoresis, the resolved proteins were transferred to nitrocellulose membrane (Schleicher & Schuell), blocked with 5% non-fat dry milk in TBS buffer (10 mM Tris, 150 mM NaCl; pH 8.0) for 1 h and then incubated with relevant primary antisera (all antibodies were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at room temperature. After washing in TBS containing 0.3% Tween 20, the membranes were incubated with secondary anti-rabbit IgG labeled with horseradish peroxidase for 1 h. Immunoreactive proteins on the blots were visualized by enhanced chemiluminiscence technique according to the manufacture´s instructions (Pierce Biotechnology, Rockford, IL, USA) and quantitatively analyzed by the ImageQuant program (Molecular Dynamics, Sunnyvale, CA, USA). To correct for errors associated with sample loading and gel transfer, β-actin was used as a housekeeping protein for reliable quantification of Western blot data.

Determination of adenylyl cyclase activity

Activity of AC was determined as described previously (Ihnatovych et al. 2001). Briefly, the reaction mixture (in a total volume of 0.1 ml) contained 20 μg of protein, 48 mM Tris-HCl buffer (pH 7.4), 100 mM NaCl, 2 mM $MgCl_2$, 1 mM EDTA, 3.2 U/ml pyruvate kinase, 5 mM potassium phosphoenolpyruvate, 0.8 g/ml BSA, 40 μM 3-isobutyl-1-methylxanthine, 20 μM GTP, 0.1 mM cAMP, 15 000 cpm

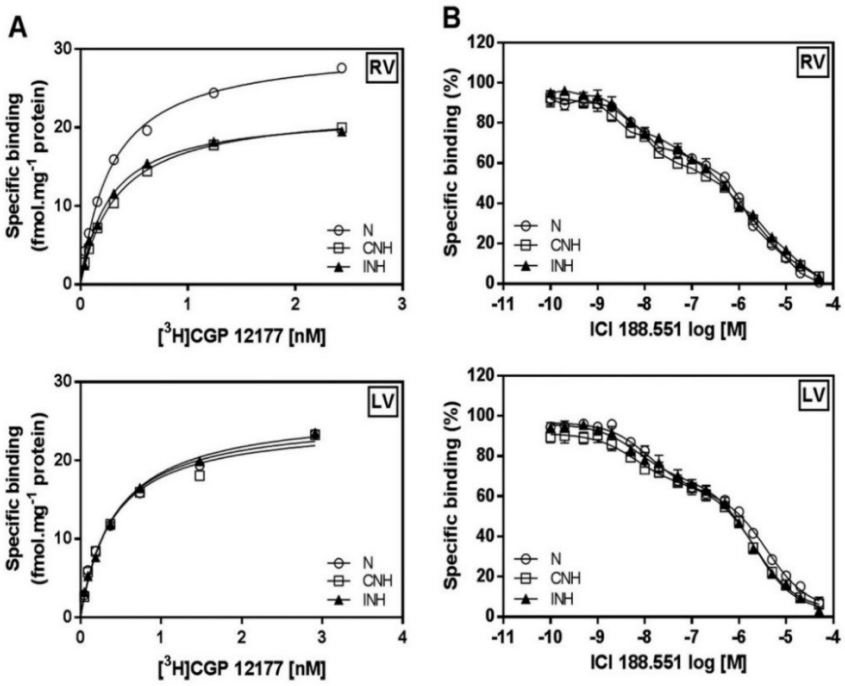

Figure 1. Characterization of β-adrenoceptors in right (RV) and left (LV) ventricular preparations from rats exposed to CNH (open squares) or INH (closed triangles) and in corresponding normoxic controls (open circles). Shown are representative [^{3}H]CGP12177 saturation binding curves (**A**) and competitive binding curves which were constructed using the β_2-AR antagonist ICI 188.551 (**B**). Data represent means (± S.E.M.) of three separate experiments performed in triplicates. N, normoxia; CNH, continuous normobaric hypoxia; INH, intermittent normobaric hypoxia.

per sample of [^{3}H]cAMP and 0.4 mM ATP with [α-^{32}P]ATP (about 1×10^6 cpm *per* sample). For stimulation of AC, the following stimulators were used in separate experiments: 10 μM isoprenaline, 10 μM forskolin, 100 μM GTPγS, 10 mM $MnCl_2$ and 10 mM NaF. After 1 min preincubation 0.4 mM ATP was added along with 200,000 cpm [α-^{32}P]ATP and incubation proceeded for 20 min at 30°C. The reaction was terminated by adding 0.2 ml of 0.5 M HCl and heating for 5 min at 100°C. The cyclic AMP formed was separated by alumina columns and the detected amount of [^{32}P]cAMP corrected for recovery with [^{3}H]cAMP.

Table 1. Weight parametres

	Group		
	N (n = 10)	CNH (n = 10)	INH (n = 10)
Body weight (g)	359.9 ± 8.3	305.3 ± 7.7*	309.9 ± 6.9*
Heart weight (mg)	836.4 ± 20.9	936.4 ± 43.3*	971.8 ± 56.5*
RV weight (mg)	185.0 ± 5.9	314.4 ± 15.5*	343.4 ± 27.6*
LV weight (mg)	452.9 ± 15.1	442.5 ± 22.4	441.7 ± 26.2
RV/BW ($\times 10^{-3}$)	0.52 ± 0.01	1.03 ± 0.04*	1.10 ± 0.07*
LV/BW ($\times 10^{-3}$)	1.26 ± 0.04	1.44 ± 0.05	1.43 ± 0.08

Data are mean ± S.E.M., * $p < 0.05$ *vs.* N group. N, normoxia; CNH, continuous hypoxia; INH, intermittent hypoxia for 23 h/day; BW, body weight; RV, right ventricle; LV, left ventricle; RV/BW, relative weight of the RV; LV/BW, relative weight of the LV.

Data analysis

The results are expressed as means ± S.E.M. One-way analysis of variance (ANOVA) and subsequent Student-Newman-Keuls test were used for comparison of differences in normaly distributed variables between the groups. Differences between appropriate groups were considered to be statistically significant when the p-value was smaler than 0.05 ($p < 0.05$).

Results

The effect of hypoxia on body and heart weight

Body and heart weight parameters of rats kept under normoxia and those adapted for 3 weeks to CNH or INH are summarized in Table 1. Majority of weight parameters were affected by exposure of animals to chronic hypoxia. Hypoxia led to a significant retardation of body growth, which was accompanied by an increase of the heart weight due to hypertrophy of the right ventricles. The RV weight increased by about 80% and the ratio RV/BW doubled in both CNH and INH groups of rats.

The effect of hypoxia on β-adrenoceptors

Saturation binding experiments (Fig. 1A) performed on crude myocardial membranes indicated that total number

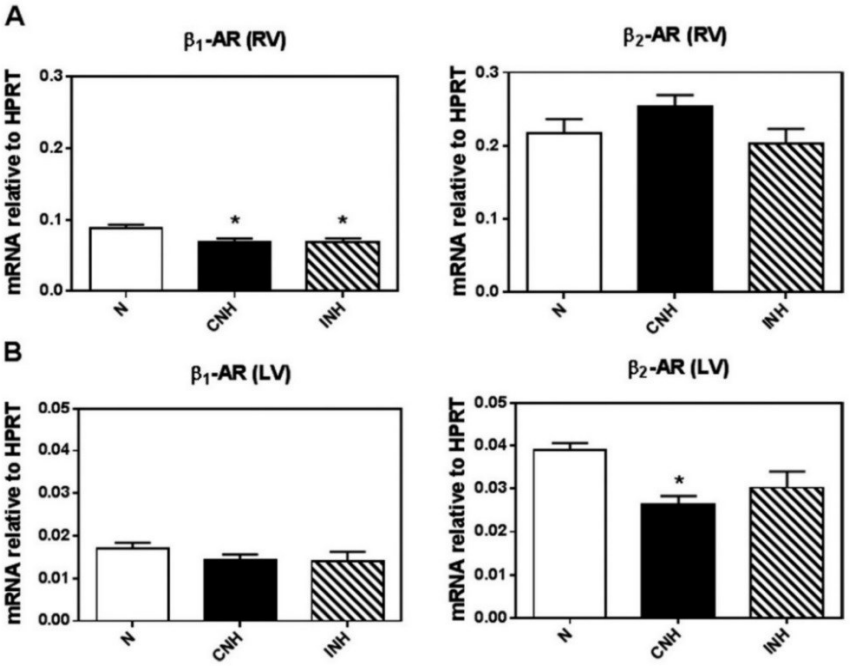

Figure 2. Determination of mRNA levels of β_1- and β_2-adrenoceptors in the RV (**A**) and LV (**B**) from rats exposed to CNH (solid bars) or INH (hatched bars) and in corresponding normoxic control (empty bars). Values were expressed relative to expression of the housekeeping gene HPRT. Data represent means (± S.E.M.) of five experiments performed in triplicates. Statistically significant differences ($p < 0.05$) between samples from chronically hypoxic rats (CNH or INH) and corresponding age-matched controls (N) are indicated by the asterisk. For more abbreviations see Fig. 1.

of β-ARs (B_{max}) and dissociation constant (K_D) of these receptors in the LV was not affected by any regimen of chronic hypoxia. By contrast, CNH and INH reduced the number of β-ARs in the RV by about 25% (Table 2). Subsequently, competition binding experiments were conducted to assess the distribution of β-AR subtypes (Fig. 1B). As indicated in Table 3, the proportion β_2-AR was increased in RV preparations from rats exposed to hypoxia, but this increase was statistically significant only in the case of CNH. Interestingly, our real-time PCR analyses revealed a significant decrease (by about 20%) in the levels of β_1-AR transcripts in RV preparations after exposure to hypoxia (Fig. 2). Interestingly, similar drop was found in β_2-AR mRNA in LV preparations from animals affected by CNH.

The effect of hypoxia on the expression of G proteins and adenylyl cyclase

To assess the possible effect of adaptation to hypoxia on myocardial AC signaling, first we investigated the distribu-

Table 2. Binding characteristics of β-ARs

	Group		
	N	CNH	INH
RV			
B_{max} (fmol/mg)	29.47 ± 1.24	21.68 ± 1.45*	21.17 ± 0.65*
K_D (nM)	0.30 ± 0.01	0.35 ± 0.03	0.30 ± 0.02
LV			
B_{max} (fmol/mg)	24.64 ± 0.73	25.62 ± 0.54	25.78 ± 1.00
K_D (nM)	0.41 ± 0.03	0.38 ± 0.05	0.37 ± 0.06

Data are mean ± S.E.M., * $p < 0.05$ *vs.* N group. N, normoxia; CNH, continuous hypoxia; INH, intermittent hypoxia for 23 h/day; RV, right ventricle; LV, left ventricle; B_{max}, maximal binding; K_D, dissociation constant.

Table 3. Distribution and properties of β-AR subtypes

	Group		
	N	CNH	INH
RV			
β_2 (%)	32.30 ± 1.41	40.43 ± 0.29*	37.80 ± 3.08
$K_i\beta_2$ (nM)	2.24 ± 0.67	2.11 ± 0.73	1.95 ± 0.56
$K_i\beta_1$ (μM)	0.51 ± 0.06	0.68 ± 0.10	0.52 ± 0.17
LV			
β_2 (%)	34.93 ± 2.13	29.67 ± 1.56	30.33 ± 1.59
$K_i\beta_2$ (nM)	3.58 ± 0.71	2.04 ± 0.52	2.19 ± 0.53
$K_i\beta_1$ (μM)	1.26 ± 0.21	0.62 ± 0.07*	0.56 ± 0.02*

Data are mean ± S.E.M., * $p < 0.05$ *vs.* N group. N, normoxia; CNH, continuous hypoxia; INH, intermittent hypoxia for 23 h/day; RV, right ventricle; LV, left ventricle; K_i, inhibition constant.

tion of the stimulatory G protein and the dominant isoforms (5 and 6) of cardiac AC in membrane preparations from both the right and left ventricles of rats exposed to chronic hypoxia. Our Western blot analyses (Fig. 3) revealed a pronounced decrease (by about 40–50%) in Gsα protein expresion in the RV from animals affected by CNH or INH, and content of the other tested proteins was not significantly changed by hypoxia.

The effect of hypoxia on adenylyl cyclase activity

Besides determination of basal AC activity, the enzyme activity was modulated by different stimulatory agents to assess

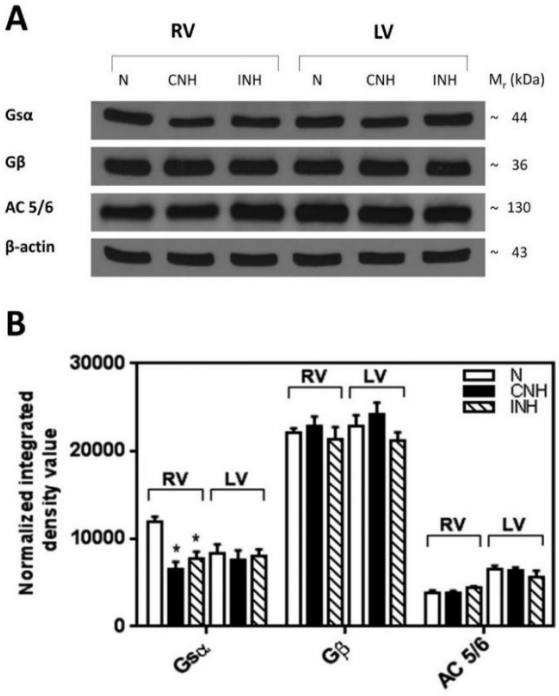

Figure 3. Immunoblot analysis of selected G protein subunits and adenylyl cyclase in right (RV) and left (LV) ventricular preparations from rats exposed to hypoxia (CNH and INH) and in corresponding normoxic controls (N). Samples were resolved by SDS-PAGE, transferred onto nitrocelulose membranes, and probed with specific antibodies for Gsα and Gβ subunits of G proteins and AC isoforms 5/6. After stripping, the blots were reprobed with anti-β-actin antibody. Representative Western blots are shown (**A**). The relative protein expression levels of Gsα, Gβ and AC 5/6 were quantified by computer analysis and normalized to the internal standard β-actin (**B**). Bar graphs showing normalized integrated optical density values represent means (± S.E.M.) of three separate experiments. Statistically significant differences ($p < 0.05$) between samples from chronically hypoxic rats (CNH or INH) and corresponding age-matched controls (N) are indicated by the asterisk. For more abbreviations see Fig. 1.

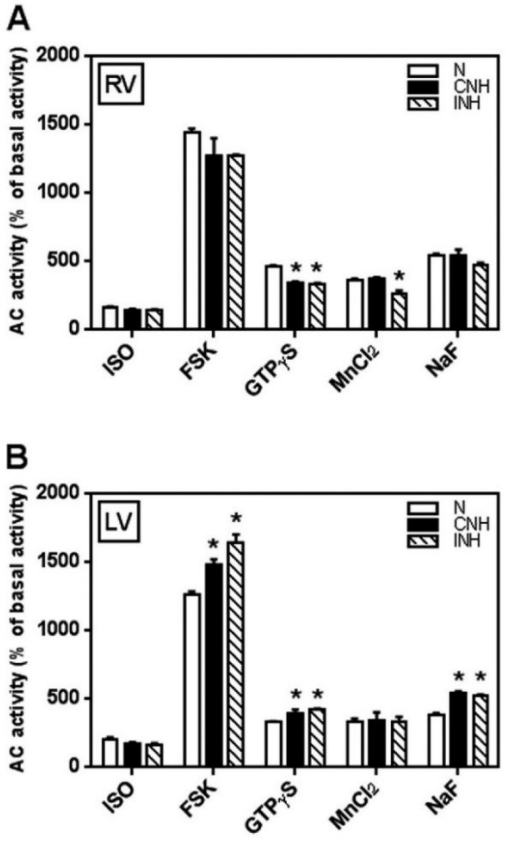

Figure 4. Effect of adaptation to chronic normobaric hypoxia on myocardial adenylyl cyclase activity. AC activity was determined in samples from normoxie rats (N; empty bars) and those adapted to CNH (solid bars) or INH (hatched bars) using the following stimulators: isoprenaline (ISO), forskolin (FSK), GTPγS, $MnCl_2$ and NaF. Data are expressed as a percentage of corresponding basal AC activity (100%). Basal AC activity (9.98 ± 0.90 pmol cAMP/mg/min in the RV and 9.90 ± 1.05 pmol cAMP/mg/min in the LV) was not affected by exposure to hypoxia. Values represent the mean (± S.E.M.) of five independent measurements performed in duplicates. Statistically significant differences ($p < 0.05$) between samples from chronically hypoxic rats (CNH or INH) and corresponding age-matched controls (N) are indicated by the asterisk. For more abbreviations see Fig. 1.

functional status of the individual components of the signaling pathway. AC was activated either directly by its cofactor Mn^{2+} or through stimulation of both the enzyme and Gs protein by forskolin or through stimulation of Gs protein by GTPγS and by NaF, or through stimulation of β-ARs by isoprenaline. Although basal AC activity did not significantly differ between different samples, chronic hypoxia apparently affected the ability of Gs protein to regulate the enzyme activity (Fig. 4). Whereas adaptation to hypoxia lowered (by about

25%) AC activity stimulated by GTPγS in RV preparations, thusly modulated activity was increased by about 20% in LV preparations from CNH- or INH-adapted rats, compared to the corresponding normoxic controls. Similar enhancement of AC activity in the LV was observed after stimulation by NaF. Forskolin, a potent activator of both the Gs protein and AC, did not significantly reduce the enzyme activity in RV preparations and increased by about 30% its activity in LV preparations from rats exposed to hypoxia.

Discussion

The results of our current study indicate that adaptation of adult Wistar rats to different regimens of chronic normobaric hypoxia leads to a significant retardation of body growth, which is accompanied by a pronounced RV hypertrophy. This is in line with many previous observations on similar models (Tual et al. 2006; Laursen et al. 2008; Baandrup et al. 2011; Neckar et al. 2013).

However, the core of the present work is represented by our analysis of β-adrenergic signaling in ventricular myocardium of rats adapted to cardioprotective (CNH) and nonprotective (INH) regimens of chronic normobaric hypoxia. We first noted a significant reduction in the total number of β-ARs in RV preparations from both groups of chronically hypoxic rats, which can be attributed to the loss of β_1-ARs because the β_1/β_2 proportion appreciably decreased. It is known that the total number of myocardial β-ARs is closely related to catecholamine levels but a selective decrease in β_1-ARs has usually been observed during cardiac hypertrophy. The vast majority of studies focused on this issue were done in the LV (Galinier et al. 1992; Communal et al. 1998; Sethi et al. 2007). For RV hypertrophy, a significant decrease in the total number of β-ARs was also reported but receptor subtypes were not discriminated in these early studies (Ishikawa et al. 1991; Yoshie et al. 1994; Mardon et al. 1998). Our present data show that RV hypertrophy elicited by exposure to chronic hypoxia exhibits similar changes in the expression of β-AR subtypes as those found in different types of LV hypertrophy. Moreover, the observed diminution of β_1-ARs at the protein level in the RV after hypoxia was well matched by significantly lower β_1-AR mRNA levels. We have also detected a noticeable drop in β_2-AR mRNA in the LV from rats exposed to CNH, but it was not followed by altered expression of this receptor subtype at the protein level.

Our next experiments revealed that some myocardial G proteins and AC activity were also affected by chronic hypoxia. The observed decreased content of Gsα in the RV after adaptation to CNH or INH is analogical to lower expression of this protein in the RV affected by hypobaric hypoxia (Guan et al. 2010). Interestingly, differently stimulated AC activity changed in opposite manner in RV and LV preparations. Although chronic exposure to CNH and INH did not cause any appreciable changes in basal AC activity, both these hypoxic regimens reduced the enzyme activity stimulated through Gs protein (by GTPγS) in the RV and increased this activity in the LV. The reduction of AC activity in the RV may be at least partly explained by the drop in Gs protein level and corresponds well to previously observed derangement of AC in samples of hypertrophied heart (Bohm et al. 1997; Tse et al. 2000; Novotny et al. 2003). Intriguingly, some previous studies exploring the effect of chronic hypoxia on myocardial AC signaling reported similar changes, namely suppression, of this system in both ventricles (Kacimi et al. 1992; Mardon et al. 1998; Leon-Velarde et al. 2001; Hrbasova et al. 2003). It is important to note, hovewer, that severe intermittent hypoxia usually leads to biventricular hypertrophy, the LV being struck to a lesser extent than the RV. On the contrary, no detectable LV hypertrophy was developed by exposure to moderate chronic normobaric hypoxia in our experiments. Under these conditions, it is quite concievable that different changes may occur in both ventricles. Hence, the partially discordant modulation of AC activity by different stimulatory agents in RV and LV from rats exposed to hypoxia may be ascribed to RV hypertrophy leading to derangement of this signaling system. On the other hand, the observed increase in AC activity stimulated by forskolin, GTPγS and NaF in the LV after adaptation to CNH or INH indicates more efficient coupling between Gs protein and AC, which may perhaps somehow participate in the development of a cardioprotective phenotype. Interestingly, the abilility of the β-AR agonist isoprenaline to stimulate AC in both ventricles was only slightly reduced which can be ascribed to lesser amount of β-ARs in the RV and to lower coupling efficiency in the LV. Attenuated β-adrenergic signaling has been frequently found in cardiac hypertrophy, as well as in other stressful conditions (Bohm et al. 1997; Vatner et al. 1999; Nishizawa et al. 2004).

In conclusion, our present study demonstrates that adaptation to chronic normobaric hypoxia is accompanied by discordant alterations in the myocardial β-adrenergic signaling system in the right and left ventricles. Exposure to both continuous and intermittent regimen of hypoxia invariably impaired this signaling in the RV but not in the LV, and there was no significant difference between the effects of protective CNH and nonprotective INH. Although these data do not allow to identify a specific role of β-ARs and AC signaling in the adaptive process to chronic hypoxia, participation of this signaling system in the development of a cardioprotective phenotype cannot be excluded. Further research is needed to better understand the possible role of β-adrenergic signaling in cardioprotection.

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ORIGINAL PAPER

β-Adrenergic signaling, monoamine oxidase A and antioxidant defence in the myocardium of SHR and SHR-mtBN conplastic rat strains: the effect of chronic hypoxia

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Abstract The β-adrenergic signaling pathways and antioxidant defence mechanisms play important roles in maintaining proper heart function. Here, we examined the effect of chronic normobaric hypoxia (CNH, 10% O_2, 3 weeks) on myocardial β-adrenergic signaling and selected components of the antioxidant system in spontaneously hypertensive rats (SHR) and in a conplastic SHR-mtBN strain characterized by the selective replacement of the mitochondrial genome of SHR with that of the more ischemia–resistant Brown Norway strain. Our investigations revealed some intriguing differences between the two strains at the level of β-adrenergic receptors (β-ARs), activity of adenylyl cyclase (AC) and monoamine oxidase A (MAO-A), as well as distinct changes after CNH exposure. The β_2-AR/β_1-AR ratio was significantly higher in SHR-mtBN than in SHR, apparently due to increased expression of β_2-ARs. Adaptation to hypoxia elevated β_2-ARs in SHR and decreased the total number of β-ARs in SHR-mtBN. In parallel, the ability of isoprenaline to stimulate AC activity was found to be higher in SHR-mtBN than that in SHR. Interestingly, the activity of MAO-A was notably lower in SHR-mtBN than in SHR, and it was markedly elevated in both strains after exposure to hypoxia. In addition to that, CNH markedly enhanced the expression of catalase and aldehyde dehydrogenase-2 in both strains, and decreased the expression of Cu/Zn superoxide dismutase in SHR. Adaptation to CNH intensified oxidative stress to a similar extent in both strains and elevated the IL-10/TNF-α ratio in SHR-mtBN only. These data indicate that alterations in the mitochondrial genome can result in peculiar changes in myocardial β-adrenergic signaling, MAO-A activity and antioxidant defence and may, thus, affect the adaptive responses to hypoxia.

Keywords SHR · Mitochondrial genome · Myocardium · β-adrenergic receptors · Adenylyl cyclase · Monoamine oxidase A · Antioxidant defence · Chronic hypoxia

Introduction

The spontaneously hypertensive rat (SHR) is one of the most commonly used animal models in cardiovascular research. This strain, which harbors a deletion variant of the Cd36 gene, is predisposed to the development of hypertension and cardiac hypertrophy in adulthood [1]. The hearts of SHR exhibit higher vulnerability to ischemia/reperfusion (I/R) injury and susceptibility to ventricular arrhythmias when compared to normotensive rats [2–4]. Interestingly, despite their reduced ischemic tolerance, ischemic preconditioning was found to provide beneficial antiarrhythmic effects in these animals [5]. We have previously documented that transgenic rescue of defective Cd36 in SHR rat leads to smaller infarct size induced by coronary artery occlusion [6]. In addition, transgenic SHR-Cd36 rats were shown to express a higher number of myocardial β-adrenergic receptors (β-ARs) and displayed

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increased adenylyl cyclase (AC) activity [7]. These data imply that transgenic expression of an apparently unrelated gene may strongly affect β-adrenergic signaling and myocardial resistance to IR injury.

β-ARs and their signaling machinery are known to play a key role in regulating myocardial function [8]. There are numerous indications that β-AR-mediated signaling may also participate in the development of preconditioning-induced ischemic tolerance [9–11]. Adaptation to chronic hypoxia, which may provide potent cardioprotection, can also be associated with changes in cardiac β-ARs and AC signaling. Down-regulation of β-ARs and desensitization of AC were occasionally observed in hearts from hypoxia-adapted animals [12–16]. It is well known that hypoxia promotes the activity of the sympathetic adrenergic system [17] and that stimulation of β-ARs may enhance mitochondrial reactive oxygen species (ROS) generation in cardiomyocytes [18]. β-AR activation seems to play an essential role in the development of powerful myocardial ischemic resistance conferred by chronic hypoxic exposure [19]. However, the adaptive changes induced by different conditioning regimens or hypoxia are not limited just to β-ARs or other membrane-bound receptors and their signaling systems, but they are also tightly linked to changes in ROS production and their detoxification.

ROS, among other factors, are well-known mediators of the beneficial effects of hypoxic conditioning [20]. Whereas the appropriate rise in ROS is important for achieving suitable protective outcomes of hypoxic adaptation [21, 22], high levels of ROS can cause excessive oxidative stress in cardiomyocytes. In this respect, mitochondria have drawn a great deal of attention as a major site of ROS production and control of redox-sensitive transcription factors. At the same time, these organelles are also the major targets of the detrimental effects of ROS overproduction [23]. Interestingly, the functional properties and vulnerability of mitochondria to oxidative stress may vary somewhat between different tissues and species [24, 25]. On the other hand, increasing evidence indicates that mitochondrial DNA (mtDNA) is essential for the cell phenotype and, thus, may contribute to stress and environmental adaptability [26]. It is known that mtDNA modulates cellular bioenergetics and mitochondrial ROS generation and mtDNA sequence variation may, thus, contribute to disease susceptibility [27]. The role of mtDNA in modulating physiological plasticity and stress responses at the cellular, tissue and whole organism level can be investigated using mitochondrial replacement technology. We have shown recently that the conplastic SHR-mtBN strain characterized by the selective replacement of the mitochondrial genome with that of the more ischemia–resistant Brown Norway strain exhibited the same myocardial infarct size caused by I/R insult as the progenitor SHR. Although adaptation to chronic hypoxia improved cardiac ischemic resistance in both strains, the infarct size-limiting effect was stronger in SHR-mtBN than in SHR, and correlated with reduced sensitivity of mitochondrial permeability transition to Ca^{2+}-induced opening [28].

To further define the potential role of mitochondrial genome in the modulation of cardiac function and resistance to I/R injury, here we focused on exploring β-AR-mediated signaling and selected components of the antioxidant defence system in LV preparations from SHR and SHR-mtBN. We monitored the levels of selected signaling molecules, antioxidant enzymes and markers of oxidative stress and inflammation in samples obtained from both normoxic and hypoxia-adapted animals. In addition, we also determined the expression and activity of monoamine oxidase A (MAO-A). MAO-A belongs among the main ROS producers in cardiac cells [29], but as yet there is a lack of information about behavior of this mitochondrial enzyme in the chronically hypoxic heart. Interestingly, replacement of the mitochondrial genome resulted in distinct changes in myocardial β-ARs and MAO-A, as well as in some components of the antioxidant system under both normal and hypoxic conditions.

Materials and methods

Materials

[^{3}H]CGP-12177 was purchased from PerkinElmer, Inc. (Boston, MA, USA), [^{3}H]cAMP was from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA), [α-^{32}P]ATP was from Hartmann Analytic, GmbH (Braunschweig, Germany) and EcoLite liquid scintillation cocktail was from MP Biomedicals (Santa Ana, CA, USA). Acrylamide and bis-acrylamide were from SERVA (Heidelberg, Germany), aluminum oxide 90 (neutral, activity I) was from Merck (Darmstadt, Germany) and cOmplete protease inhibitor cocktail was from Roche Life Science (Indianapolis, IN, USA). Anti-MAO-A and anti-ALDH-2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-catalase antibody was from Abcam (Cambridge, UK) and anti-Cu/ZnSOD and anti-MnSOD antibodies were from Cayman Chemical Company (Ann Arbor, MI, USA). SuperSignal West Dura chemiluminescent substrate was from Pierce Biotechnology (Rockford, IL, USA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Animal model

The SHR-mtBN conplastic strain harboring the mitochondrial genome of a highly inbred strain BN on the nuclear genetic background of SHR was created by selective

replacement of a mitochondrial genome of SHR with the mitochondrial genome of BN rats as described earlier [28]. Adult male SHR and SHR-mtBN rats (280–300 g body wt) were exposed to continuous normobaric hypoxia (CNH; inspired O_2 fraction 0.1) in a normobaric chamber (6 m^2) equipped with hypoxic generators (Everest Summit, Hypoxico Inc., NY, USA) for 3 weeks. No reoxygenation occurred during this period. Animals were used immediately after the cessation of hypoxic exposure. The control rats were kept for the same period of time at room air. All animals were housed in a controled environment (22 ± 2 °C; 12:12 h light–dark cycle; light from 5:00 a.m. with free access to water and standard chow diet. 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, DC). Experimental protocols were approved by the Animal Care and Use Committee of the Institute of Physiology, Czech Academy of Sciences.

Processing of heart tissue for biochemical analyses

Immediately after sacrifice, the hearts were rapidly excised, washed in ice-cold saline solution and the left ventricles (LV) were dissected from the right ventricles and the septum. The pieces of frozen tissue were either pulverized in liquid nitrogen and subsequently homogenized in RNAzol® RT (Molecular Research Center, Inc.) for isolation of mRNA or homogenized in TMES buffer (20 mM Tris, 3 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose; pH 7.4) supplemented with protease inhibitors for radioligand binding assay, Western blotting, enzyme activity determination or other biochemical analyses. In the latter case, the ventricles were homogenized on ice by Ultra-Turrax blender for 30 s and subsequently by Potter–Elvehjem glass-Teflon homogenizer for 1 min. The homogenates were clarified by centrifugation at 600×*g* for 10 min (4 °C) in order to remove nuclei and particulate cellular debris. The resulting postnuclear supernatant was centrifuged at 50,000×*g* for 30 min (4 °C) to separate the membrane and cytosolic fractions. The pellet containing crude membranes was resuspended in TME buffer (20 mM Tris, 3 mM $MgCl_2$ and 1 mM EDTA; pH 7.4). Both membrane and cytosolic fractions were aliquoted and stored at −80 °C until use.

Real time RT-PCR

RNA isolation and real time RT-PCR were performed as described previously [30] with a slight modification as follows. Briefly, tissue homogenization and total RNA isolation was performed according manufacturer's instruction using RNAzol Reagent (Molecular Research Center, Inc.). One microgram of total RNA was loaded to reverse transcription using RevertAidTM H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) with oligo(dT) primers according to the manufacturer's instructions. Real Time PCR analyses were, performed on Light Cycler LC 480 (Roche Applied Science, Branford, CT, USA) using Syber green Master Mix (Eurogentec SA, Seraing, Belgium). Gene-specific primers were designed to be compatible with the Roche Universal Probe Library (UPL) and are listed in Table 1. The relative levels of analyzed gene transcripts were calculated according to Pfaffl [31] using the 18S rRNA gene as a suitable reference gene (18S_F: *tctagacaacaagctgcgtga*; 18S_R: *cctctatgggctcggatttt*). This reference gene was selected from six candidates using GenEx software (MultiD Analyses AB, Göteborg, Sweden).

β-Adrenergic receptor binding

Myocardial β-ARs were determined by radioligand binding assay with a nonselective β-adrenergic antagonist [^{3}H]CGP 12177 as described previously [32]. Samples of crude membranes (100 μg protein) were incubated in incubation buffer (50 mM Tris, 10 mM $MgCl_2$ and 1 mM ascorbic acid; pH 7.4) containing increasing concentrations of [^{3}H]CGP 12177 (0.06–4 nM) for 1 h at 37 °C in a total volume of 0.5 ml; at this time the specific binding of radioligand had reached an equilibrium. The binding reaction was terminated by addition of 3 ml of ice-cold washing buffer (50 mM Tris, 10 mM $MgCl_2$; pH 7.4) and subsequent filtration through Whatman GF/C filters, which

Table 1 PCR primers for the real time PCR

Gene	Forward primer	Reverse primer
AC5	*gggagaaccagcaacagg*	*catctccatggcaacatgac*
AC6	*atgagatcatcgcggacttt*	*gccatgtaagtgctaccgatg*
ACO1	*ttgctgtgtctgagattgaaaag*	*cttgaaaacctttaaatccttgct*
ACO2	*cgccttacagcctactggtc*	*ggcagaggccacatggta*
ALDH2	*agacgtcaaagatggcatga*	*ttgaggatctgcatcactgg*
CAT	*cagcgaccagatgaagca*	*ggtcaggacatcgggtttc*
CuZnSOD	*taagaaacatggcggtcca*	*tggacacattggccacac*
GSTO1	*aagcttgccagaagatgacc*	*ctcttcgccctaataaaactcg*
MAOA	*tggtatcatgacccagtatgga*	*tgtgcctgcaaagtaaatcct*
MnSOD	*tggacaaacctgagccctaa*	*gacccaaagtcacgcttgata*
NRF1	*atagtcctgtctggggaaacc*	*tccatgcatgaactccatct*
NRF2	*agcatgatggacttggaattg*	*cctccaaaggatgtcaatcaa*
PRX3	*agaagaacctgcttgacagaca*	*caggggtgtggaatgaaga*
PRX5	*gactatggccccgatcaa*	*aaaacacctttcttgtccttgaa*
TXN2	*cacacagaccttgccattga*	*acgtccccgttcttgatg*
TXNRD2	*gcacatggtgaagctacctaga*	*gctccatccacatcttctcag*

were presoaked with 0.3% polyethyleneimine for 1 h. The filters were then washed twice with ice-cold washing buffer and placed into scintillation vials. After addition of 4 ml EcoLite scintillation cocktail, radioactivity retained on the filters was measured by counting for 5 min. Nonspecific binding was assessed by incubating the samples with radioligand in the presence of 10 μM L-propranolol, and it represented less than 30% of the total binding. For competition experiments, samples of crude membranes 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 characteristics of β-adrenergic binding sites and the proportions of β_1- and β_2-ARs in myocardial crude membranes were calculated using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA).

Assessment of adenylyl cyclase activity

Activity of AC was determined by measuring the conversion of [α-^{32}P]ATP to [^{32}P]cAMP as described previously [33]. Samples of crude myocardial membranes (20 μg protein) were incubated in the reaction mixture (in a total volume 100 μl) containing 48 mM Tris buffer (pH 8), 2 mM $MgCl_2$, 20 μM GTP, 0.8 mg/ml BSA, 40 μM 3-isobutyl-1-methylxanthine, 5 mM potassium phosphoenolpyruvate, 3.2 U of pyruvate kinase, 100 mM NaCl, 0.1 mM cAMP and about 15,000 cpm [^{3}H]cAMP as a tracer. For stimulation of AC, the following stimulators were used in separate experiments: 10 μM isoprenaline, 10 μM forskolin, 10 mM $MnCl_2$ and 10 mM NaF. After 1 min preincubation, 0.4 mM ATP was added along with 2,000,000 cpm [α-^{32}P]ATP and incubation proceeded for 20 min at 30 °C. The reaction was terminated by addition of 200 μl 0.5 M HCl and heating for 5 min at 100 °C. Samples were neutralized by 200 μl 1.5 M imidazole. Separation of cAMP produced by stimulated membrane preparations from other nucleotides was performed by filtration through alumina columns, and the detected amount of [^{32}P]cAMP in each vial was corrected for recovery with [^{3}H]cAMP as the internal standard.

Assessment of monoamine oxidase A activity

MAO-A activity in the LV was determined using kynuramine dihydrobromide as substrate in the presence of deprenyl (inhibitor MAO-B) as described previously [34] with a slight modification. Myocardial crude membranes (100 μg protein) were lysed by treatment with 2% Triton X-100. Resulting lysate samples (200 μl) were mixed with 0.2 μl of 1 mM deprenyl and 2.5 ml of 50 mM phosphate buffer (pH 7.4) and incubated for 60 min at 37 °C. After incubation, 30 μl of 2.19 mM kynuramine dihydrobromide were added to each reaction mixture as substrate and incubation was continued for another 30 min at 37 °C. The reaction was terminated by addition of 200 μl of 5 M perchloric acid. Samples were centrifuged at 1500×*g* for 10 min and 500 μl aliquots of supernatant were transferred into test tubes containing 2.5 ml of 1 M NaOH. The fluorescence of the reaction product 4-quinolinol was measured at Ex 310-nm/Em 380-nm using a Biotek Synergy HT plate reader. A standard curve of 4-quinolinol (in the range of 0.03–0.5 mM) was used to calculate MAO-A enzyme activity.

Electrophoresis and Western blotting

Individual samples of myocardial preparations were solubilized in Laemmli buffer and loaded (10–30 μg per lane) on 10 or 15% acrylamide gels for SDS-PAGE as described previously [35]. After electrophoresis, the resolved proteins were transferred to nitrocellulose membranes (GE Healthcare Life Sciences, Buckinghamshire, UK), blocked with 5% non-fat dry milk in TBS buffer (10 mM Tris, 150 mM NaCl; pH 8.0) for 1 h and then incubated for 1.5 h at room temperature or overnight at 4 °C with relevant primary antibodies. After three 10-min washes in TBS containing 0.3% Tween 20, the membranes were incubated with secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. Immunoreactive proteins on the blots were visualized by enhanced chemiluminiscence technique according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA) and quantitatively analyzed by ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). To correct for errors associated with sample loading and gel transfer, β-actin was used as a housekeeping protein for reliable quantification of Western blot data.

Determination of malondialdehyde

Lipid peroxidation was quantified by measuring malondialdehyde (MDA) formation. Myocardial samples (100 mg) were pulverized to a fine powder and dissolved in 500 μl of ice-cold buffer (25 mM Tris and 0.10% Triton X 100; pH). The homogenates were sonicated, centrifuged (1000×*g*, 10 min, 4 °C) and 100 μl samples of supernatant were taken and analyzed as described by Pilz et al. [36] with a slight modification. Briefly, 20 μl of 6 M NaOH was added to each sample, vortexed and incubated for 30 min at 60 °C. The solution was then cooled on ice and 50 μl of 35% perchloric acid was added. After centrifugation (10,000×*g*, 5 min, 4 °C), 100 μl of supernatant was taken and derivatization was performed using 10 μl of 5 mM 2,4-dinitrophenylhydrazine. After 10 min in the dark, the solution was analyzed using a HPLC system

(Shimadzu, Kyoto, Japan) with UV detection at 310 nm (column: EC Nucleosil 100-5 C18, 4.6 mm × 125 mm heated to 30 °C; mobile phase: acetonitrile–water–acetic acid 380:620:2 (v/v/v); flow rate: 1.0 ml/min). MDA concentration was normalized to total protein content.

Determination of TNF-α, IL-6 and IL-10

Levels of TNF-α (tumor necrosis factor-α), IL-6 (interleukin-6) and IL-10 (interleukin-10) in myocardial homogenates from different experimental groups were measured using DuoSet ELISA kits (eBioscience, Vienna, Austria) according to the standard protocols described by the manufacturer. The content of cytokines is given in picograms per milligram of total protein [37].

Data analysis

Biochemical data were determined in at least three independent preparations. All results were expressed as the mean ± SEM. The Kolmogorov–Smirnov test was used to assess the normality and all parameters were distributed normally. One-way analysis of variance (ANOVA) and subsequent Student–Newman–Keuls tests were used for comparison of differences in normally distributed variables between the groups. Differences between appropriate groups were considered to be statistically significant when the p value was smaller than 0.05 ($p < 0.05$).

Results

All measurements were done on LV myocardial preparations obtained from normoxic rats (SHR and SHR-mtBN) as well as from those exposed to continuous normobaric hypoxia (CNH) for 3 weeks (SHR/H and SHR-mtBN/H).

β-Adrenergic receptors

The total number of β-ARs and dissociation constants of these receptors in myocardial membrane preparations from LVs were determined by using saturation binding experiments (Fig. 1a). We found that the total number of β-ARs, expressed as B_{max}, tended to be higher (by about 15%) in SHR-mtBN than SHR, but this difference was not statistically significant. The values of B_{max} were also affected by adaptation of rats to hypoxia. Whereas CNH induced a significant increase (by 16%) in the total number of β-ARs in SHR/H, the expression of these receptors was markedly diminished (by 26%) in SHR-mtBN/H (Table 2). Dissociation constants (K_d) did not differ significantly between the strains and their values were not affected by adaptation to hypoxia. Subsequently, competition binding experiments were conducted to assess the distribution of β-AR subtypes in LV preparations (Fig. 1b). As indicated in Table 3, the proportion of β_2-ARs was significantly increased (by 37%) in SHR-mtBN, compared to SHR. Exposure to hypoxia increased the proportion of β_2-AR in SHR/H by 30% but did not affect the relative proportion of β-AR subtypes in SHR-mtBN/H.

Adenylyl cyclase

Activity of AC in myocardial membrane preparations was determined by measuring cAMP production under different experimental conditions. Besides determining basal AC activity, the enzyme activity was modulated by the following stimulatory agents: isoprenaline, forskolin, $MnCl_2$ and NaF. Isoprenaline is a β-AR agonist, forskolin or $MnCl_2$ can stimulate the AC catalytic subunit directly and NaF elicits the enzymatic response through activation of the stimulatory G proteins [38]. Results of experiments in which AC activity was tested under different conditions are

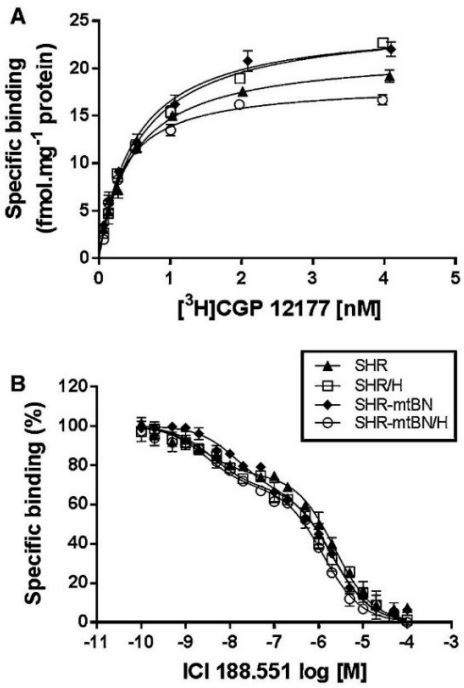

Fig. 1 Effect of hypoxia on myocardial β-adrenergic receptors. β-ARs in LV preparations from SHR (*closed triangles*), SHR/H (*open squares*), SHR-mtBN (*closed diamonds*) and SHR-mtBN/H (*open circles*) were characterized by radioligand binding experiments. Shown are [^{3}H]CGP 12177 saturation binding *curves* (**a**) and competitive binding *curves* (**b**) which were constructed using the β_2-AR antagonist ICI 188.551. Data represent means (±SEM) of three separate experiments performed in triplicate

Table 2 Binding characteristics of β-ARs

	SHR	SHR/H	SHR-mtBN	SHR-mtBN/H
B_{max} (fmol mg^{-1})	21.71 ± 0.88	25.20 ± 0.38^{+}	24.87 ± 0.73	18.37 ± 0.46$^{\S\#}$
K_D (nM)	0.49 ± 0.10	0.58 ± 0.07	0.51 ± 0.03	0.32 ± 0.01

Data are means (±SEM) of three separate experiments performed in triplicates

B_{max} maximal binding capacity, K_D equilibrium dissociation constant of radioligand ([^{3}H]CGP 12177)

$^{+}$ $p < 0.05$ SHR vs. SHR/H; § $p < 0.05$ SHR/H vs. SHR-mtBN/H rats; $^{\#}$ $p < 0.05$ SHR-mtBN vs. SHR-mtBN/H

Table 3 Distribution and properties of β-AR subtypes

	SHR	SHR/H	SHR-mtBN	SHR-mtBN/H
β_2 (%)	24.03 ± 0.30	31.12 ± 1.42^{+}	32.97 ± 2.01*	31.15 ± 0.42
$K_i\beta_2$ (nM)	0.80 ± 0.53	1.43 ± 0.17	4.80 ± 1.25	0.75 ± 0.21
$K_i\beta_1$ (μM)	0.73 ± 0.06	0.77 ± 0.25	0.70 ± 0.07	0.31 ± 0.03

Data are means (±SEM) of three separate experiments performed in triplicates

β_2 (%) percentage of β_2-ARs of total myocardial β-ARs, $K_i\beta_2$ (β_1) apparent dissociation constant representing the affinity of ICI 118.551 to β_2-ARs (β_1-ARs)

* $p < 0.05$ SHR vs. SHR-mtBN; $^{+}$ $p < 0.05$ SHR vs. SHR/H

summarized in Fig. 2. Basal AC activity did not significantly differ between both strains or even after adaptation to hypoxia, but the enzyme activity was diversely influenced by different stimulators. Whereas there was no significant difference between SHR and SHR-mtBN in AC activity stimulated by forskolin or $MnCl_2$, the enzyme activity stimulated by isoprenaline and NaF was markedly increased in SHR-mtBN (by about 35%). Adaptation of rats to hypoxia also led to specific changes in variably stimulated AC activity. Whereas CNH increased AC activity stimulated by forskolin or NaF by about 30% in preparations from SHR, there was no change in forskolin-stimulated AC activity and decrease (by 17%) in NaF-stimulated AC activity in hypoxia-adapted SHR-mtBN/H, compared to the corresponding normoxic controls. The stimulatory effects on AC activity of forskolin and NaF were lower by 10 and 14%, respectively, in SHR-mtBN/H than in SHR/H. Hypoxia did not significantly affect the ability of isoprenaline to stimulate AC activity in SHR but decreased its stimulatory effect by 13% in SHR-mtBN. Interestingly, determination of transcript levels of the dominant myocardial isoforms of AC (AC5 and AC6) revealed a decrease (by about 35%) in mRNA levels of both these isoforms in SHR-mtBN adapted to hypoxia (Suppl. Fig. S1).

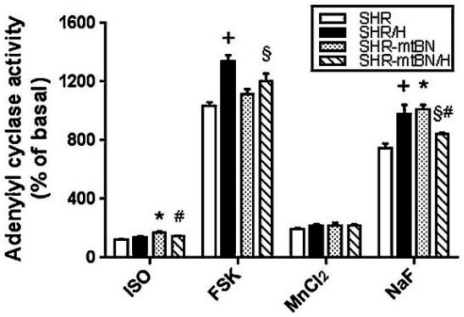

Fig. 2 Effect of hypoxia on activity of myocardial adenylyl cyclase. AC activity in LV preparations from SHR (*empty bars*), SHR/H (*closed bars*), SHR-mtBN (*dotted bars*) and SHR-mtBN/H (*hatched bars*) was determined in the presence of the following stimulators: 10 μM isoprenaline (ISO), 10 μM forskolin (FSK), 10 mM $MnCl_2$ and 10 mM NaF. Differently stimulated AC activity is expressed as a percentage of the corresponding basal AC activity, which did not significantly differ between different samples and was in the range of 7.9–9.2 pmol cAMP/min per mg protein in all measurements. Data represent means (±SEM) of five independent measurements performed in triplicate. $^{*}p < 0.05$ SHR vs. SHR-mtBN rats; $^{+}p < 0.05$ SHR vs. SHR/H rats; $^{\S}p < 0.05$ SHR/H vs. SHR-mtBN/H rats; $^{\#}p < 0.05$ SHR-mtBN vs. SHR-mtBN/H rats

Monoamine oxidase A

Gene expression and enzyme activity of MAO-A were assessed in myocardial preparations from both normoxic and hypoxia-exposed SHR and SHR-mtBN. The mRNA level of MAO-A was significantly lower (by 30%) in SHR-mtBN than in SHR (Fig. 3a). Adaptation to hypoxia resulted in marked increase of MAO-A mRNA in both strains (by 97 and 132% in SHR and SHR-mtBN, respectively). The same trend was detected at the protein level and in the enzyme activity of MAO-A. The expression of MAO-A protein was lower by 28% in SHR-mtBN than in SHR, and CNH elevated the amount of MAO-A protein by 66 and 92% in SHR and SHR-mtBN, respectively (Fig. 3b). The enzyme activity of MAO-A in SHR-mtBN

was lower by 21% than in SHR, and CNH increased the enzyme activity by 33 and 22% in SHR and SHR-mtBN, respectively (Fig. 3c).

Antioxidant defence enzymes

In the next set of experiments, we tested whether adaptation to hypoxia affects expression of selected antioxidant defence enzymes in the LV of both rat strains. CNH did not change the mRNA level of catalase (CAT) in SHR, but markedly increased (by 51%) the amount of this transcript in SHR-mtBN (Fig. 4, panel a). The expression of CAT protein was increased by 105 and 68% in hypoxia-exposed SHR/H and SHR-mtBN/H, respectively (Fig. 4, panel b). Adaptation to hypoxia elevated the level of aldehyde dehydrogenase 2 (ALDH-2) mRNA in both strains by about 40–50% (Fig. 4, panel a). The expression of ALDH-2 protein was increased by 25 and 32% in SHR/H and SHR-mtBN/H, respectively, when compared to corresponding normoxic controls (Fig. 4, panel b). In contrast to CAT and ALDH-2, mRNA and protein levels of superoxide dismutases (SODs) were decreased (Cu/ZnSOD) or remained unchanged (MnSOD) after adaptation to hypoxia (Fig. 5). The mRNA level of cytosolic Cu/ZnSOD was reduced by 12 and 16% in SHR/H and SHR-mtBN/H, respectively, but the expression of Cu/ZnSOD protein was significantly changed (a drop by 31%) just in SHR/H.

A similar downward trend was also observed in the expression of myocardial glutathione S-transferase ω1 (GTSO1), thioredoxin 2 (TXN2) and thioredoxin reductase 2 (TXNRD2) and peroxiredoxin 5 (PRX5) after adaptation to hypoxia (Suppl. Fig. S2a–c). The mRNA levels of these enzymes dropped by 19% (GSTO1 and TXN2), 18% (TXNRD2) and 16% (PRX5) in SHR/H and by 32% (TXN2), 27% (TXNRD2) and 25% (PRX5) in SHR-mtBN/H. Although hypoxia exposure did not markedly affect comparable levels of PRX3 mRNA in both rat strains, the amount of this transcript was higher (by 48%) in SHR-mtBN/H than in SHR/H (Suppl. Fig. S2c). The mRNA levels of ACO1 and ACO2 remained without significant changes (Suppl. Fig. S2d). In addition, we detected increased expression (by 31%) of nuclear respiratory factor 1 (NRF1) mRNA in SHR-mtBN/H and no change in the mRNA level of nuclear factor (erythroid-derived 2)-like 2 (NFE2L2, NRF2) (Suppl. Fig. S2e).

Markers of lipid peroxidation and inflammation

Malondialdehyde (MDA) was determined as a marker of oxidative stress-induced lipid peroxidation. Myocardial preparations from both rat strains kept under normoxic conditions did not exhibit any differences between MDA levels. Adaptation to hypoxia increased the MDA concentration by 104 and 52% in SHR/H and SHR-mtBN/H, respectively (Fig. 6a). Levels of anti-inflammatory

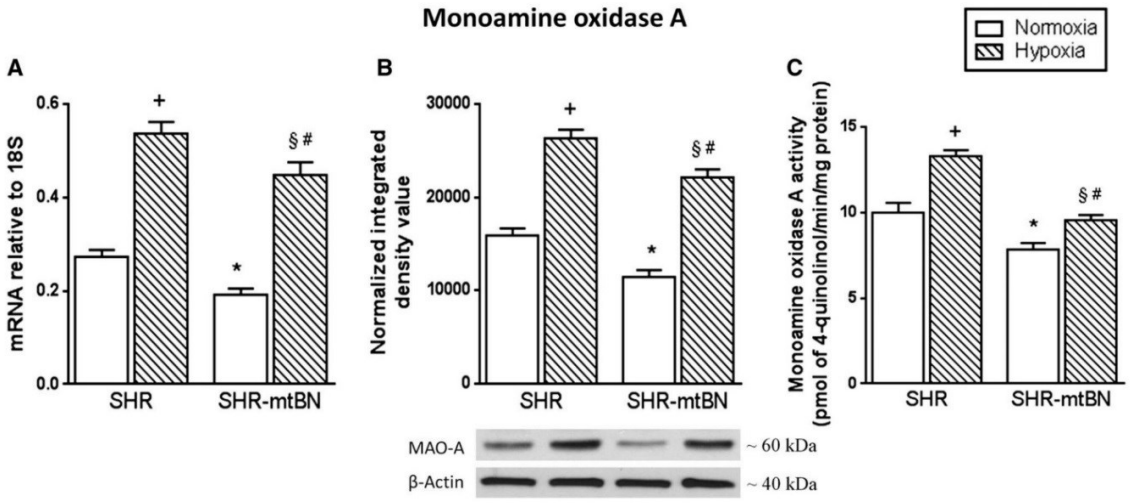

Fig. 3 Effect of hypoxia on myocardial monoamine oxidase. Expression levels of mRNA and protein of MAO-A in LV preparations from SHR and SHR-mtBN rats kept in normoxic (*empty bars*) and hypoxic (*hatched bars*) conditions were assessed by RT-PCR and Western blotting, respectively. The amounts of specific mRNA species were normalized with 18S levels (**a**). The 18S levels were not significantly affected by hypoxic exposure. Representative Western blots are shown below the corresponding *bar* graphs depicting the relative protein expression levels (**b**). Specific enzyme activity of MAO-A (**c**) was determined spectrophotometrically by using kynuramine as substrate. Data represent means (±SEM) of at least three separate experiments. $^{*}p < 0.05$ SHR vs. SHR-mtBN rats; $^{+}p < 0.05$ SHR vs. SHR/H rats; $^{\S}p < 0.05$ SHR/H vs. SHR-mtBN/H rats; $^{\#}p < 0.05$ SHR-mtBN vs. SHR-mtBN/H rats

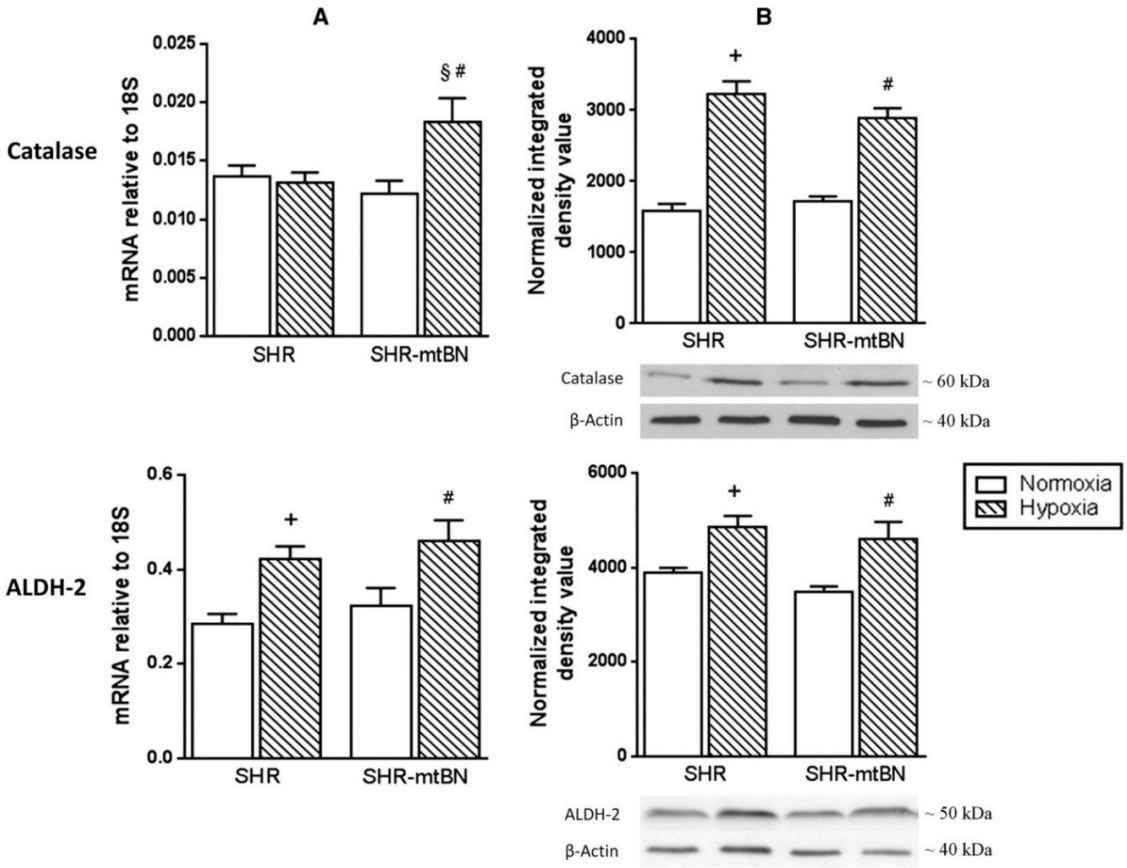

Fig. 4 Effect of hypoxia on myocardial catalase and aldehyde dehydrogenase-2. Expression levels of mRNA and protein of CAT and ALDH-2 in LV preparations from SHR and SHR-mtBN rats kept in normoxic (*empty bars*) and hypoxic (*hatched bars*) conditions were assessed by RT-PCR and Western blotting, respectively. The amounts of specific mRNA species were normalized with 18S levels (column **a**). The 18S levels were not significantly affected by hypoxic exposure. Representative Western blots are shown below the corresponding *bar* graphs depicting the relative protein expression levels (column **b**). Data represent means (±SEM) of at least three separate experiments. $^{+}p < 0.05$ SHR vs. SHR/H rats; $^{\S}p < 0.05$ SHR/H vs. SHR-mtBN/H rats; $^{\#}p < 0.05$ SHR-mtBN vs. SHR-mtBN/H rats

interleukin-10 (IL-10) and pro-inflammatory tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) tended to be lower in SHR-mtBN than in SHR, and these levels tended to increase in both strains after hypoxic exposure (Suppl. Fig. 3). CNH did not significantly change the IL-10/TNF-α ratio in SHR/H and increased this ratio (by 17%) in SHR-mtBN/H (Fig. 6b).

Discussion

In the present study, we sought to explore the impact of mitochondria replacement on myocardial β-adrenergic signaling and antioxidant defence in the spontaneously hypertensive rat (SHR) under normoxic and hypoxic conditions. The experiments were conducted in parallel on SHR and SHR-mtBN, i.e., the SHR-based conplastic strain carrying the mitochondrial genome from Brown Norway rats [28]. Our investigation revealed a number of distinct changes at the level of β-ARs, AC and MAO-A, as well as in some components of the antioxidant defence system.

We observed that the proportion of myocardial β_2-ARs was significantly higher in SHR-mtBN than in SHR and that adaptation to hypoxia diversely affected the distribution of β-ARs in both rat strains. Whereas CNH elevated β-AR density in SHR due to increased expression of β_2-AR, the total number of β-ARs in SHR-mtBN declined without a change in the proportion of β-AR subtypes. The higher β-AR density in SHR-mtBN was reflected by increased ability of isoprenaline to stimulate AC activity. However,

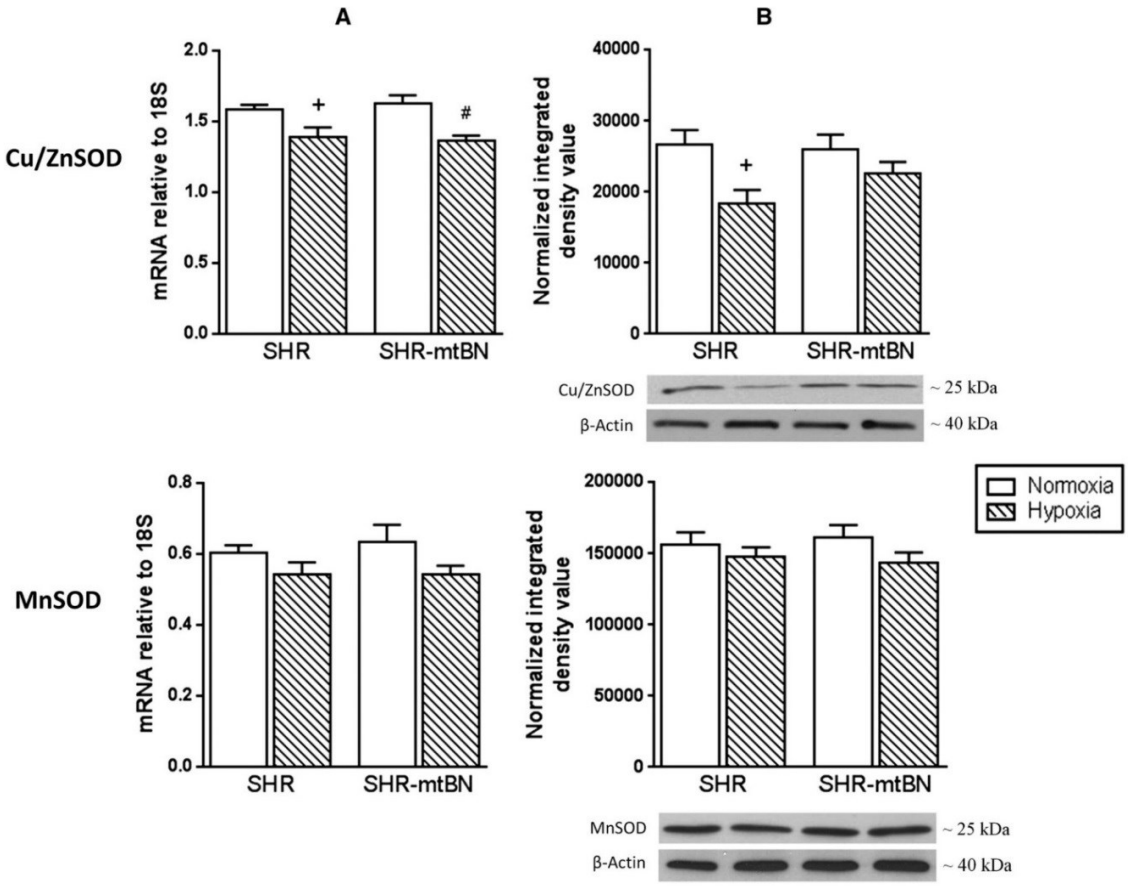

Fig. 5 Effect of hypoxia on myocardial cytosolic copper/zinc superoxide dismutase and mitochondrial manganese superoxide dismutase. Expression levels of mRNA and protein of Cu/ZnSOD and MnSOD in LV preparations from SHR and SHR-mtBN rats kept in normoxic (*empty bars*) and hypoxic (*hatched bars*) conditions were assessed by RT-PCR and Western blotting, respectively. The amounts of specific mRNA species were normalized with 18S levels (column **a**). The 18S levels were not significantly affected by hypoxic exposure. Representative Western blots are shown below the corresponding *bar* graphs depicting the relative protein expression levels (column **b**). Data represent means (±SEM) of at least five separate experiments. $^{+}p < 0.05$ SHR vs. SHR/H rats; $^{\#}p < 0.05$ SHR-mtBN vs. SHR-mtBN/H rats

isoprenaline-stimulated AC activity was reduced in preparations from SHR-mtBN exposed to hypoxia, which is in line with the lower expression of β-ARs and AC determined in these animals. The enzyme activity stimulated by forskolin and NaF (the agents capable of stimulating AC both directly and through G proteins or only through G proteins, respectively) was enhanced in SHR after their exposure to CNH. Interestingly, no such elevation was found in CNH-adapted SHR-mtBN. By contrast, NaF-stimulated AC activity in these rats was already increased under normoxic conditions, and it was diminished after exposure to CNH. These strain-specific differences may presumably be explained by altered ability of G proteins to regulate AC activity. Both forskolin and NaF enhanced AC activity much more strongly than isoprenaline, which is a well-known and commonly reported phenomenon [14]. The observed decline in the number β-ARs in SHR-mtBN after CNH exposure is consistent with previous findings of lower β-AR expression in rat heart following hypoxia [12, 13, 15]. As a rule, β-AR subtypes were not discerned in these studies. The only exception is a study of Mardon et al. [14], which demonstrated a selective decrease in β_1-ARs caused by 5-day hypoxia. These investigators, like others [15, 16], found reduced AC activity in myocardial preparations from hypoxia-exposed rats. The reduction in NaF-stimulated AC activity observed in CNH-exposed SHR-mtBN, thus, conforms well to the previously published data. On the other hand, the CNH-

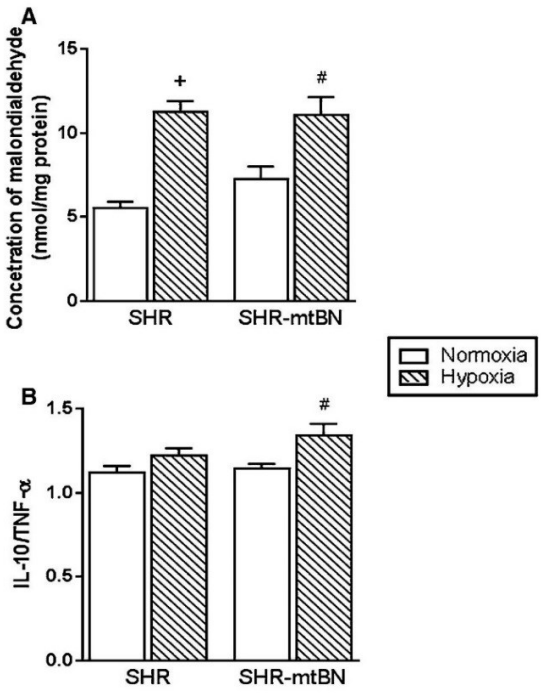

Fig. 6 Effect of hypoxia on myocardial malondialdehyde levels and the ratio of interleukin-10/tumor necrosis factor-α. Concentrations of malondialdehyde (**a**) and the IL-10/TNF-α ratio (**b**) were assessed in LV preparations from SHR a SHR-mtBN rats kept in normoxic (*empty bars*) and hypoxic (*hatched bars*) conditions. Data represent means (±SEM) of at least five separate experiments. $^{+}p < 0.05$ SHR vs. SHR/H rats; $^{\#}p < 0.05$ SHR-mtBN vs. SHR-mtBN/H rats

induced increase in the ability of G proteins to stimulate myocardial AC activity in SHR has not been previously noted and may represent a specific feature of this rat strain. It is worth mentioning that especially β_2-AR appears to be important for generating the salutary effects of preconditioning [11, 39]. Accordingly, β_2-ARs were found to activate pro-survival kinases and attenuate mitochondrial dysfunction during oxidative stress [40]. The increased proportion of β_2-AR in SHR-mtBN, compared to SHR, and reduction in the total number of β-ARs after adaptation to CNH may, thus, contribute to better protection of this rat strain against acute I/R injury [28].

It is well known that hypoxic stress is associated with increased production and release of catecholamines, and these compounds may be subjected to oxidative deamination catalyzed by monoamine oxidases [41]. These enzymes have recently emerged as important mitochondrial sources of oxidative stress in the cardiovascular system and MAO inhibition may apparently have a therapeutic value for treating cardiac affections of ischemic and non-ischemic origin [42]. Interestingly, the presence of MAO inhibitors during ischemic preconditioning (IPC) was shown to potentiate the IPC-induced post-ischemic functional recovery of a rat heart [43]. In the present study, we found that the expression and activity of MAO-A (the predominant cardiac isoform) was lower in SHR-mtBN than in SHR and that CNH exposure increased similarly the expression and activity of this enzyme in both strains. Nevertheless, MAO-A activity remained still significantly lower in SHR-mtBN under these conditions, which can be considered as a strain-specific feature. The observed elevation of myocardial MAO-A after hypoxic exposure may suggest the importance of MAO-A in adaptive responses to chronic hypoxia. It should be mentioned here that our finding of a relatively large increase in MAO-A activity after CNH is in contrast to the work of Maher et al. [44]. These authors did not find any change in MAO activity in a goat heart exposed to chronic hypoxia. However, this discrepancy can be explained by using different models and experimental conditions. There are some indications that the possible changes in MAO activity may depend on the duration of hypoxia. Whereas 5-day exposure to hypoxia led to decrease of MAO activity in rat liver, 21-day exposure increased the enzyme activity [45].

The harmful effects of ROS generated from different sources, including mitochondria and MAO activity, are regularly combated by a number of endogenous antioxidant defence mechanisms. The enzyme antioxidant defence system consists of different types of enzymes with different functions [46]. In the present study, we focused on selected ROS-degrading enzymes (SODs, CAT, PRXs, GSTO1) and enzymes participating in maintenance of cellular redox homeostasis (TXN2, TXNRD2, ALDH-2, ACO1 and 2). Assessment of expression levels of all these enzymes in myocardial preparations from SHR and SHR-mtBN did not reveal any significant differences between both these strains under normoxic conditions. Moreover, adaptation to hypoxia elicited very similar changes in the levels of most of these enzymes in both progenitor and conplastic SHR strains. CNH appreciably elevated the protein levels of CAT and ALDH-2, and did not change MnSOD in myocardial preparations from both strains. In contrast, CNH markedly reduced the amount of Cu/ZnSOD protein in SHR but not in SHR-mtBN. The transcript levels of mitochondrial antioxidants TXN2, TXNRD2 and PRX5 were always significantly lower in CNH-exposed animals than in corresponding normoxic controls. On the other hand, CNH did not change the mRNA levels of PRX3, ACO1 and ACO2, and decreased GSTO1 in SHR only. Interestingly, most changes associated with CNH in the expression of antioxidant enzymes in SHR and SHR-mtBN markedly differ from those reported in previous studies. In contrast to our current findings, the expression levels of Cu/ZnSOD, MnSOD, TXN2, TXNRD2, PRX5 and ACO2 were found to increase, CAT to decrease and GSTO1 not to

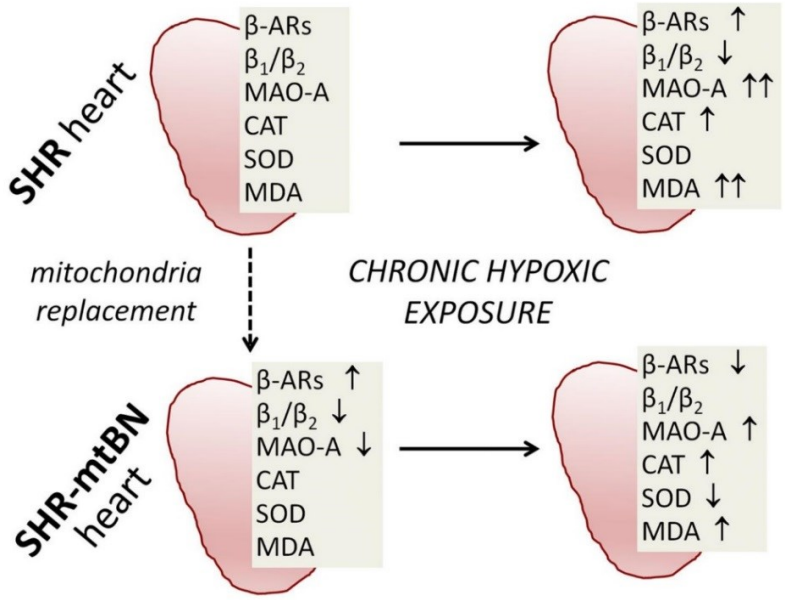

Fig. 7 Schematic summarizing the main findings. Replacement of the mitochondrial genome of SHR with that of the more ischemia–resistant Brown Norway strain (mtBN) distinctly affects myocardial expression of β-ARs and MAO-A. Adaptation to chronic hypoxia is associated with partially dissimilar changes in β-ARs, MAO-A and some components of the antioxidant defence in the myocardium of SHR and SHR-mtBN. These differences may contribute to more efficient cardioprotection conferred by chronic hypoxia in SHR-mtBN, compared to progenitor SHR. The *arrows* indicate changes relative to SHR or to SHR-mtBN in the case of SHR-mtBN/H. β-ARs, β-adrenergic receptors; β_1/β_2, the β_1-AR/β_2-AR ratio; *MAO-A* monoamine oxidase A, *CAT* catalase, *SOD* superoxide dismutase, *MDA* malondialdehyde, *up arrow* (*double up arrow*) and *down arrow*, increase (higher increase) and decrease, respectively

change after adaptation of Wistar rats to the same cardioprotective regimen of hypoxia [22]. A significant increase in myocardial Cu/ZnSOD and MnSOD was also detected in Wistar rats adapted to different hypoxic conditions [47–49]. Hence, the observed discordant changes in antioxidant enzymes induced by hypoxic exposure appear to be strain-specific. The unique downregulation of Cu/ZnSOD and GSTO1 in SHR following hypoxia may partly explain the lower cardioprotective effect of CNH against acute I/R injury in these rats when compared to SHR-mtBN [28]. The effect of hypoxia on ALDH-2 expression has not yet been investigated and our finding of increased levels of ALDH-2 in both rat strains exposed to CNH supports the notion that this enzyme is highly important for reducing ischemic heart damage [50] and may follow activation of MAO-A. NRF2 is a transcription factor crucially involved in the regulation of antioxidant enzyme expression, and they can modulate the capacity of the antioxidant system at the protein level [51]. The observed increase in NRF1 in CNH-exposed SHR-mtBN might contribute to upregulation of some nuclear-encoded mitochondrial respiratory genes (such as cytochrome c oxidase (subunit VIc) and reductase, the γ subunit of ATP synthase) as NRF1 is one of the important transcription factors for structural components of the mammalian electron transport chain [52, 53]. Moreover, NRF1 has been shown to play a role in the mitochondrial DNA replication machinery and other biosynthetic and degradative pathways [54]. The elevated NRF1 expression after CNH exposure may promote mitochondrial biogenesis and, thus, increase cardiac resistance to I/R injury of SHR-mtBN/H.

Chronic hypoxia is usually accompanied by increased ROS formation [55, 56]. Therefore, we decided to assess the extent of lipid peroxidation by monitoring MDA content in myocardial preparations from rats under normoxic and hypoxic conditions. Interestingly, the MDA concentration was markedly increased in both rat strains after CNH exposure, but this increase was much more pronounced in hearts of SHR, which may reflect lower antioxidant capacity of these animals when compared to SHR-mtBN. Similar increase in myocardial MDA concentration was previously found in Wistar rats or mice after exposure to different regimens of hypoxia [37, 57–59]. These data indicate that, irrespective of animal model, ROS formation accompanied by increased lipid peroxidation is a typical sign of chronic hypoxia.

It is known that the adaptive response to hypoxia of the heart tissue can be modulated by inflammatory cytokines [60]. In the present study, we found that the amount of the

principal anti-inflammatory (IL-10) and pro-inflammatory (TNF-α and IL-6) cytokines tended to be increased under hypoxic conditions. In parallel, these animals exhibited a significant increase in the IL-10/TNF-α ratio. Interestingly, these results appear rather contradictory with respect to the previously observed decrease in IL-10 and lower IL-10/TNF-α ratio in Wistar rats adapted to hypoxia [37]. We think that the disproportionate changes in the content of myocardial cytokines under hypoxic conditions can be ascribed to specific differences between individual rat strains. Moreover, the increased IL-10/TNF-α ratio in SHR-mtBN exposed to CNH can likely contribute to the lesser extent of myocardial damage caused by I/R.

The main findings obtained in the present study are schematically shown in Fig. 7. Taken together, our results indicate that the selective replacement of mitochondria of SHR with those of more ischemia–resistant Brown Norway strain can modify the functioning of myocardial AC signaling regulated by β-ARs and G proteins, as well as the expression and activity of MAO-A and some components of the antioxidant defence system. This is the first study to demonstrate relatively far-reaching consequences arising from the manipulation of the mitochondrial genome for β-adrenergic signaling and redox balance in rat heart. Concurrently, these data provide new evidence that mitochondria function not only as key ATP-producing organelles, but they may influence other aspects of normal cell functioning. Importantly, manipulating the mitochondrial genome may apparently lead to complex alterations within target cells, and this should be taken into account when planning application of such manipulations for research or therapeutic purposes.

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Compliance with ethical standards

Conflict of interests The authors have no conflict of interest to declare.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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Příloha C

Chronic intermittent hypoxia affects the cytosolic phospholipase A2α/cyclooxygenase 2 pathway via β_2-adrenoceptor-mediated ERK/p38 stimulation

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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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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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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.

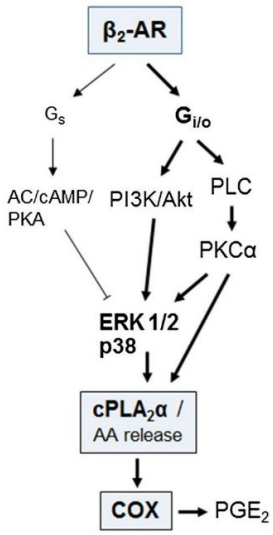

Fig. 1 A simplified scheme showing the putative interactions between β_2-AR signaling and the $cPLA_2\alpha$/COX/PGE_2 pathway in rat myocardium under chronically hypoxic conditions. Switching of β_2-AR from G_s to $G_{i/o}$ coupling results in activation of PI3K and/or PLC. The latter enzyme cleaves 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 manufacturer's 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

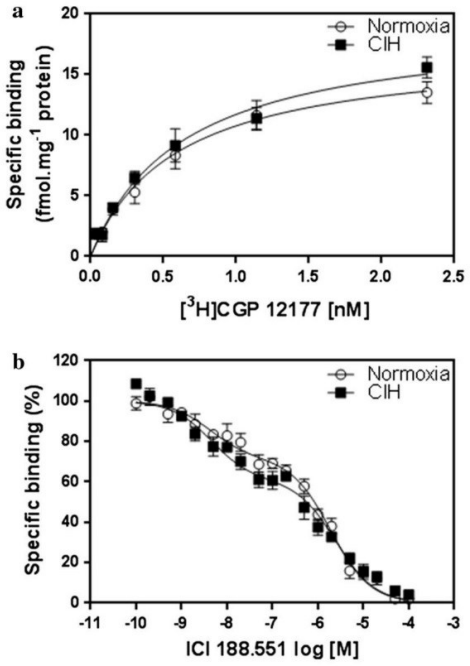

Fig. 2 Characterization of the β-adrenoceptors in LV preparations from normoxic (*open circles*) and CIH-adapted (*closed squares*) rats. There are displayed [^{3}H]CGP 12,177 saturation binding curves (**a**) and competitive binding curves (**b**) which were constructed using the β_2-AR antagonist ICI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

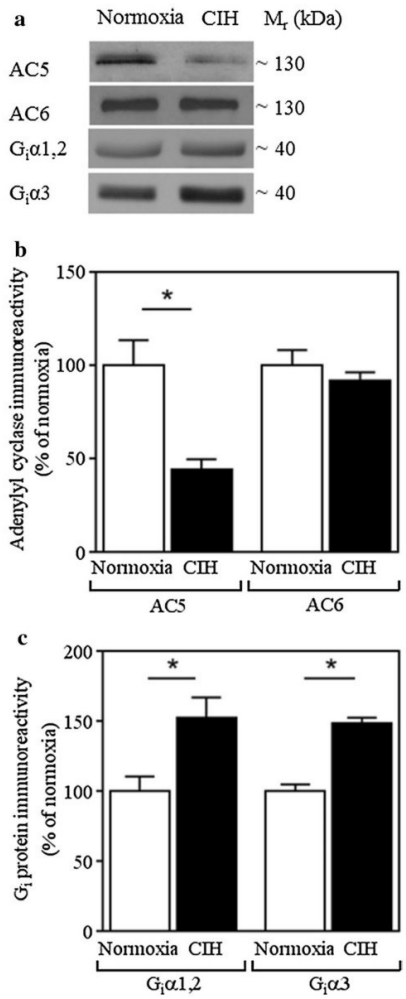

Fig. 3 Effect of chronic intermittent hypoxia on adenylyl cyclase and G_i proteins in rat myocardium. **a** Representative western blots of AC5, AC6, $G_i\alpha1,2$ and $G_i\alpha3$ proteins are shown. **b**, **c** The relative amount of individual proteins in LV preparations from normoxic (*empty columns*) 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).

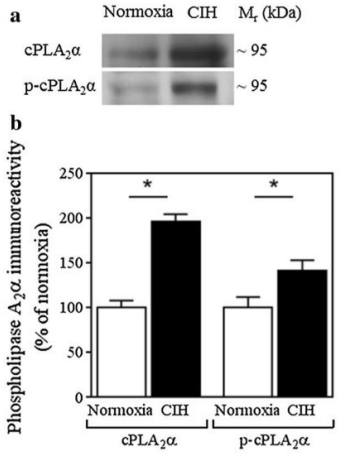

Fig. 4 Effect of chronic intermittent hypoxia on $cPLA_2\alpha$ and p-$cPLA_2\alpha$ in myocardial homogenates. **a** Representative western blots of $cPLA_2\alpha$ and p-$cPLA_2\alpha$ are shown. **b** The relative amount of these proteins in LV preparations from normoxic (*empty columns*) and CIH-adapted (*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

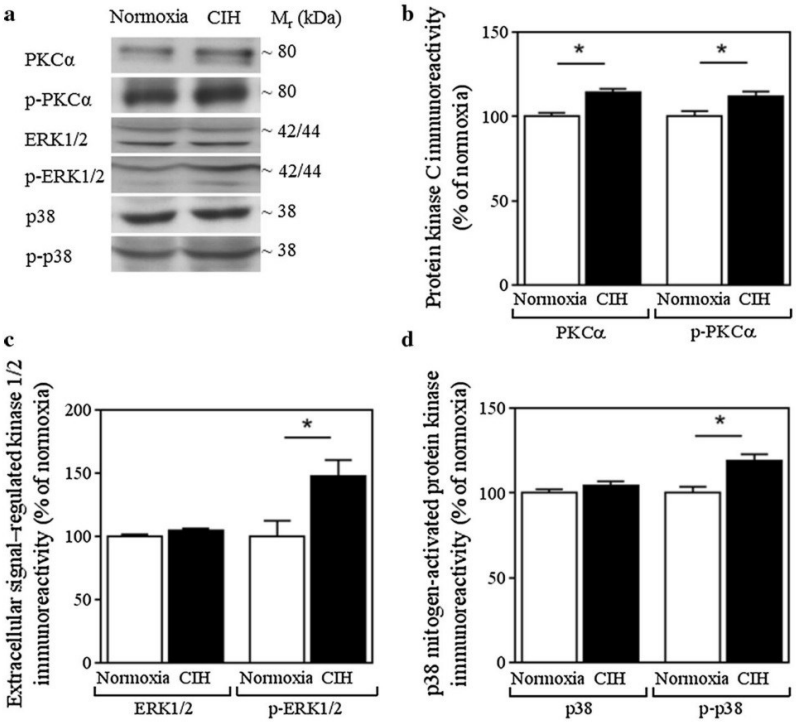

Fig. 5 Effect of chronic intermittent hypoxia on the PKCα and p-PKCα (**b**), ERK1/2 and p-ERK1/2 (**c**), p38 and p-p38 (**d**) protein levels in homogenate from left ventricular myocardium of rats adapted to CIH (*solid columns*) and of normoxic controls (*empty columns*) expressed as a percentage 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

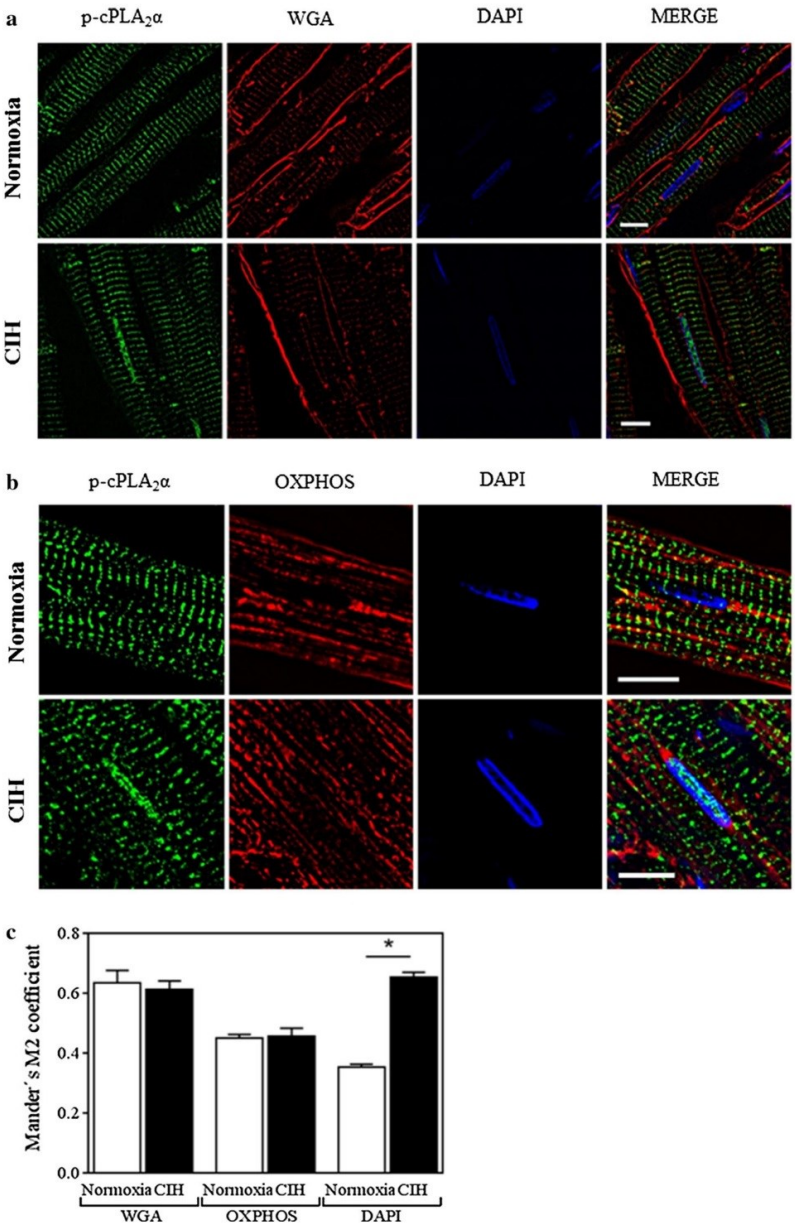

Fig. 6 Effect of chronic intermittent hypoxia on subcellular distribution of p-cPLA$_2\alpha$. Representative images show p-cPLA$_2\alpha$ distribution and co-localization with sarcolemma (**a**) and mitochondria (**b**) in longitudinal cryo-sections of the LV from normoxic and CIH-adapted rats. In all 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

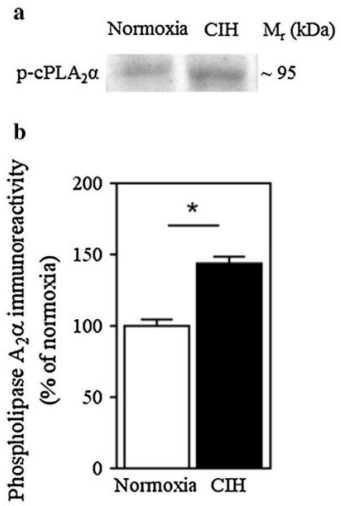

Fig. 7 Effect of chronic intermittent hypoxia on p-cPLA$_2\alpha$ association with the nuclear fraction. **a** Representative western blots of p-cPLA$_2\alpha$ are shown. **b** The relative amount of p-cPLA$_2\alpha$ in LV preparations from normoxic (*empty columns*) and CIH-adapted (*solid columns*)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

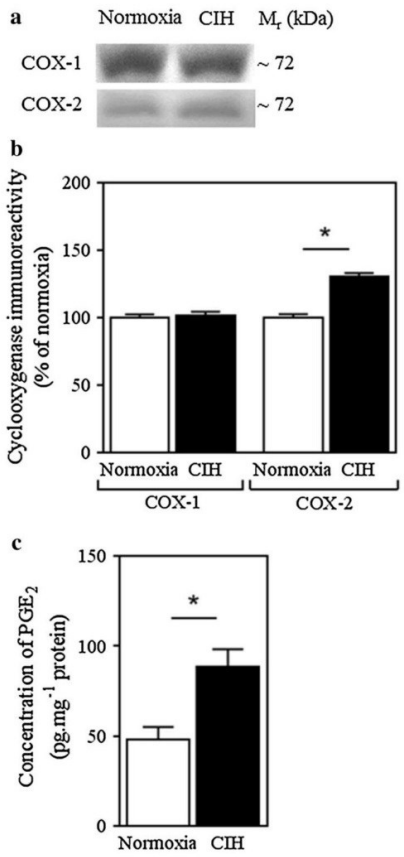

Fig. 8 Effect of chronic intermittent hypoxia on cyclooxygenase and PGE$_2$. **a** Representative western blots of COX-1 and COX-2 are shown. **b** The relative amount of these proteins in LV preparations from normoxic (*empty columns*) and CIH-adapted (*solid columns*) rats is expressed as a percentage 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$_2\alpha$ 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$_2\alpha$ 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$_2\alpha$ [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$_i\alpha$ [39]. Pretreatment of the cells with PKCα inhibitor prevented this phosphorylation, cPLA$_2\alpha$ activity and AA release. Conversely, pretreatment with the inhibitor of G$_i$ proteins pertussis toxin inhibited only peroxynitrite-induced increase in cPLA$_2\alpha$ activity. Hence, there is a direct link between the inhibition of G$_i$ proteins by pertussis toxin and suppression of cPLA$_2\alpha$ 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

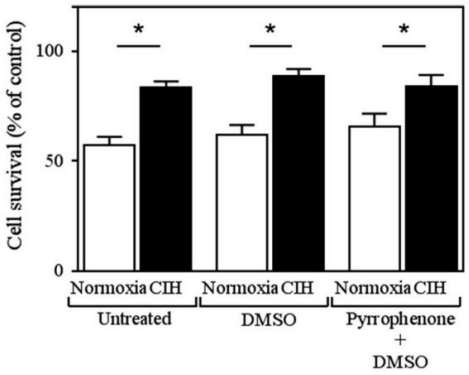

Fig. 9 Effect of the cPLA$_2\alpha$ inhibitor pyrrophenone on survival of left ventricular myocytes during acute metabolic inhibition and re-energization. Control cells were treated with 0.01 % dimethyl sulfoxide (DMSO) as a vehicle. The cells were isolated from rats kept in normoxic (*empty columns*) 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.

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Compliance with ethical standards

Conflict of interests There is no conflict of interest.

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