Příloha A

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Involvement of PKCε in Cardioprotection Induced by Adaptation to Chronic Continuous Hypoxia

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Summary

Continuous normobaric hypoxia (CNH) renders the heart more tolerant to acute ischemia/reperfusion injury. Protein kinase C (PKC) is an important component of the protective signaling pathway, but the contribution of individual PKC isoforms under different hypoxic conditions is poorly understood. The aim of this study was to analyze the expression of PKCε after the adaptation to CNH and to clarify its role in increased cardiac ischemic tolerance with the use of PKCε inhibitory peptide KP-1633. Adult male Wistar rats were exposed to CNH (10 % O_2, 3 weeks) or kept under normoxic conditions. The protein level of PKCε and its phosphorylated form was analyzed by Western blot in homogenate, cytosolic and particulate fractions; the expression of PKCε mRNA was measured by RT-PCR. The effect of KP-1633 on cell viability and lactate dehydrogenase (LDH) release was analyzed after 25-min metabolic inhibition followed by 30-min re-energization in freshly isolated left ventricular myocytes. Adaptation to CNH increased myocardial PKCε at protein and mRNA levels. The application of KP-1633 blunted the hypoxia-induced salutary effects on cell viability and LDH release, while control peptide KP-1723 had no effect. This study indicates that PKCε is involved in the cardioprotective mechanism induced by CNH.

Key words

Chronic hypoxia • Cardioprotection • Ventricular myocytes • Protein kinase C • PKCε inhibitory peptide KP-1633

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Introduction

The resistance of the heart to ischemia/reperfusion (I/R) injury can be increased by many acute and chronic stimuli such as various forms of preconditioning (Yellon and Downey 2003), postconditioning (Ovize *et al.* 2010), exercise training (Powers *et al.* 2008), caloric restriction (Shinmura *et al*. 2005) or exposure to chronic hypoxia (Kolar and Ostadal 2004). A number of studies have shown repeatedly that chronic hypoxia renders the heart more tolerant to deleterious ischemia followed by reperfusion that was manifested by decreased infarct size (Neckar *et al*. 2002a, b), lower incidence of ventricular arrhythmias (Asemu *et al*. 2000, Neckar *et al*. 2002a) and better recovery of cardiac contractile function (Neckar *et al*. 2002b, Wang *et al*. 2011, Xie *et al*. 2005). Despite the fact that hypoxia-induced cardioprotection has been known for many decades and its elucidation may have potential therapeutic repercussions, the complex mechanism underlying this form of a sustained protective phenotype is still a matter of debate. Among many components of protective signaling cascades stimulated by chronic hypoxia, various protein kinases such as protein kinase A

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(Xie *et al*. 2005, Yeung *et al*. 2007), phosphatidylinositol 3-kinase/Akt (Milano *et al*. 2013, Ravingerova *et al*. 2007, Wang *et al*. 2011), glycogen synthase kinase-3β (McCarthy *et al*. 2011), Ca^{2+}/calmodulin-dependent protein kinase II (Xie *et al*. 2004, Yu *et al*. 2009), p38-mitogen-activated protein kinase and c-Jun NH_2-terminal kinase (Rafiee *et al*. 2002) and last but not least protein kinase C (PKC) (Ding *et al*. 2004, Li *et al*. 2007, Neckar *et al*. 2005, Rafiee *et al*. 2002, Wang *et al*. 2011, Yeung *et al*. 2007) have been shown to play a role.

PKC is a family of serine/threonine kinases that are important components in processes of cellular signaling. PKC includes several isoforms usually divided according to structure and requirement for second messengers. The three groups are as follows: a) classical (isoforms α, ßI, ßII and γ), b) novel (isoforms δ, ε, η and θ) and c) atypical (isoforms ζ and ι/λ) (Steinberg 2008). The discovery of general PKC inhibitors (chelerythrine, calphostin C) helped to reveal the essential role of PKC in the mechanism of hypoxia-induced cardioprotection as administration of these inhibitors abolished the protective phenotype (Ding *et al*. 2004, Neckar *et al*. 2005, Rafiee *et al*. 2002). The most frequently mentioned is the novel PKC isoform PKCε, but its involvement in cardioprotection induced by chronic hypoxia still remains to be clarified (Ding *et al*. 2004, Hlavackova *et al*. 2007, Rafiee *et al*. 2002). However, the specificity of various inhibitors for individual PKC isoforms has been often questioned (Soltoff 2007). This was the case until a PKCε-specific inhibitory peptide was synthesized by the Mochly-Rosen group which provided a powerful tool to elucidate the role of this isoform in ischemic preconditioning (Gray *et al*. 1997, Johnson *et al*. 1996).

It needs to be mentioned that the expression and activation of PKC isoforms associated with increased myocardial I/R resistance depends on the concrete model and regimen of chronic hypoxia. This complicates the interpretation of diverse results gained in individual studies (Ding *et al*. 2004, Neckar *et al*. 2005, Uenoyama *et al*. 2010). Recently, we have shown that adaptation of rats to continuous normobaric hypoxia (CNH) reduces the size of myocardial infarction induced by acute I/R (Maslov *et al*. 2013, Neckar *et al*. 2013). The aim of this study was to analyze the effect of CNH on myocardial expression of PKCε and to examine its involvement in the protective mechanism using PKCε inhibitory peptide KP-1633 and its inactive (scrambled) form KP-1723 (Mochly-Rosen 1995, Souroujon and Mochly-Rosen 1998).

Methods

Animals

Adult male Wistar rats (322±11 g body weight) were exposed to CNH (inspired O_2 fraction: 0.1) in a normobaric chamber equipped with hypoxic generators (Everest Summit, Hypoxico Inc., NY, USA) for 3 weeks. The control group of animals was kept under normoxic conditions (inspired O_2 fraction: 0.21). All animals had free access to water and a standard laboratory diet and were housed with 12 hours light/12 hours dark cycle. They were killed by cervical dislocation 24 h after the hypoxic exposure, the hearts were removed and either used for cell isolation (method see below) or washed in cold saline (0 °C) and dissected into right and left free ventricular walls and septum. All samples were frozen in liquid nitrogen until use. The experiments were performed in accordance with 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) and were approved by the Ethics Committee of the Institute of Physiology, Czech Academy of Sciences.

Gene expression determined by Real-Time PCR

Total cellular RNA was extracted from each left ventricle (LV) sample using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was converted to cDNA using the RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas UAB, Vilnius, Lithuania) with oligo(dT) primers. Real-Time PCR was performed on a Light Cycler 480 (Roche Applied Science, Penzberg, Germany) using Light Cycler 480 Probes Master according to the manufacturer's protocol. Following specific primers together with Mono-Color Hydrolysis Probes were designed by the Universal Probe Library Assay Design Center:
PKCε (F): *aaacacccttatctaacccaactct*,
PKCε (R): *catattccatgacgaagaagagc*, *#38,*
HPRT1 (F): *gaccggttctgtcatgtcg*,
HPRT1 (R): *acctggttcatcatcactaatcac, #95*.

The level of analyzed transcripts was normalized to the level of the reference gene hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT1) gene transcript (Bohuslavova *et al*. 2010) according to Pfaffl (2001). For more details see Waskova-Arnostova *et al*. (2013).

Tissue fractionation and Western blot analysis

LV samples were pulverized to fine powder with liquid nitrogen, dissolved in ice-cold homogenization

buffer (12.5 mM Tris-HCl (pH 7.4), 250 mM sucrose, 2.5 mM EGTA, 1 mM EDTA, 100 mM NaF, 0.3 mM phenylmethylsulfonyl fluoride, 6 mM ß-mercaptoethanol, 10 mM glycerol-3-phosphate, 0.2 mM leupeptin, 0.02 mM aprotinin and 0.1 mM sodium orthovanadate) and homogenized by Potter-Elvehjem homogenizer at 4 °C. The part of the homogenate was centrifuged at 100,000 × g for 90 min to obtain the pellet of particulate fraction and cytosolic fraction (Kolar *et al*. 2007). The other part of the homogenate and the pellet of the particulate fraction were resuspended in homogenization buffer containing 1 % Triton X-100, held on ice for 60 min, with occasionally mixing, and then centrifuged at 100,000 × g for 90 min. Resulting supernatants were used for Western blot analyses. The protein concentration of individual samples was determined using the Bradford method (Bradford 1976).

Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis using 10 % bis-acrylamide gel. Resolved proteins were transferred to a nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany). Membranes were incubated with primary antibodies against PKCε (Sigma-Aldrich, St. Louis, MO, USA), phosphorylated PKCε (Upstate, Billerica, MA, USA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and actin (Santa Cruz Biotechnology, Inc.). Horseradish peroxidase-conjugated anti-rabbit (Sigma-Aldrich) and anti-goat IgGs (Santa Cruz Biotechnology, Inc.) were used as secondary antibodies. Bands were visualized by enhanced chemiluminescence and quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). In order to ensure the specificity of immunoreactive proteins, blocking was performed with immunizing peptides and rat brain homogenate was used as a positive control. GAPDH and actin were used as internal loading controls. The results were normalized to total protein amount.

Isolation of cardiomyocytes

Cardiomyocytes were isolated as previously described (Borchert *et al*. 2011). 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 Ca^{2+}-free Tyrode for 8 min. Tissue digestion was initiated by adding 14000 U collagenase (Yakult, Tokyo, Japan) and 7 mg protease type XIV (Sigma-Aldrich) into 30 ml of Ca^{2+}-free Tyrode containing 50 mg BSA. All solutions were gassed with 100 % O_2. After 12-15 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.

Assessment of cell viability with SYTOX Green

The dose-response of LVM viability to the TAT-conjugated PKCε inhibitory peptide KP-1633 and control peptide KP-1723 (scrambled amino acid sequence) obtained from KAI Pharmaceuticals, Inc. (South San Francisco, CA, USA) (Mochly-Rosen 1995, Souroujon and Mochly-Rosen 1998) was determined. Having considered the effective concentrations of the KP-1633 resembling peptide εV1-2 used in other studies (Chen *et al*. 1999), the concentrations of 0.1, 1, 5, 10 and 50 μM KP-1633 and KP-1723 were tested. The percentage of living cells compared to the untreated control cells was assessed with SYTOX Green nucleic acid stain (S7020) (Invitrogen-Molecular Probes, Eugene, OR, USA) 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 (Hofgaard *et al*. 2006), was measured at an excitation wavelength of 490 nm and emission wavelength of 520 nm using a Synergy™ HT Multi-Detection Microplate Reader (BioTek, Winooski, VT, USA). Decreasing viability of LVM was already observed after 4-h incubation with 10 μM KP-1633 and after 2, 4 and 20-h incubation with 50 μM KP-1723 (data not shown). Therefore, the 5 μM concentration of peptides, which had no effect on the number of surviving cells during 20-h incubation, has been chosen for the following experiments.

Simulated ischemia/reperfusion

LVM isolated from hypoxic and normoxic rats were pre-treated for 15 min with KP-1633 or KP-1723 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

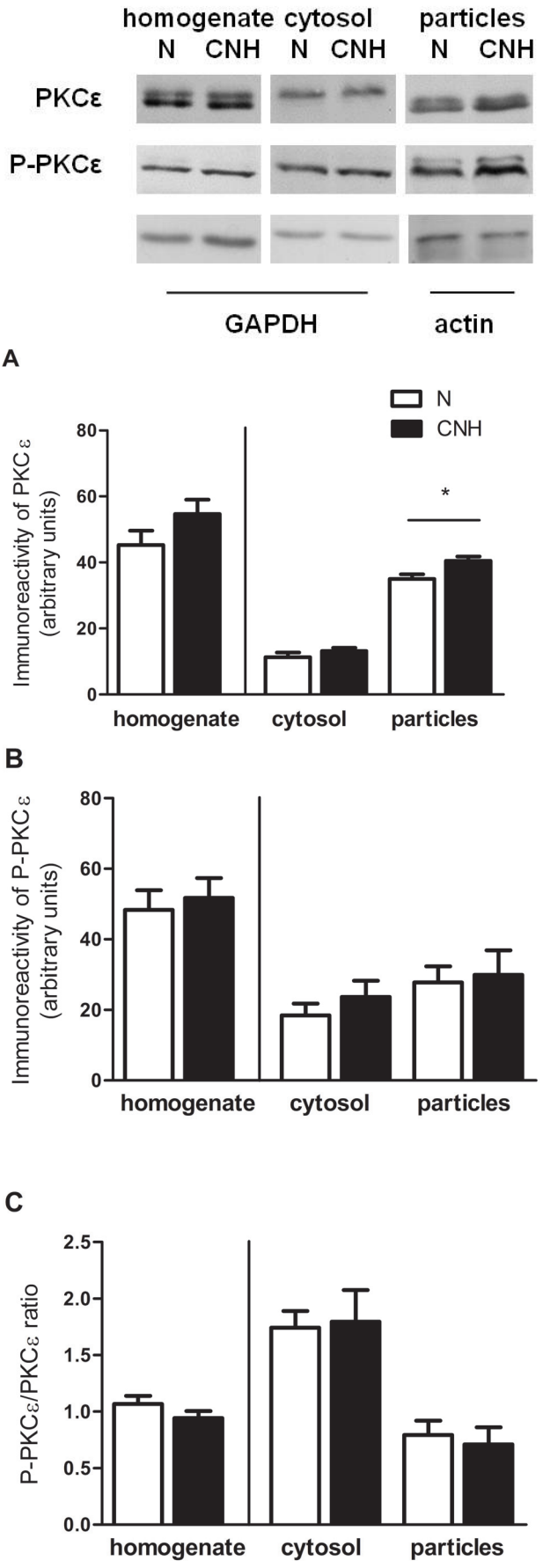

Fig. 1. Effect of continuous normobaric hypoxia (CNH) on the protein levels of PKCε (**A**), P-PKCε (Ser 729) (**B**) and the ratio P-PKCε/PKCε (**C**) in the left ventricular myocardium. Representative Western blots of PKCε and P-PKCε (Ser 729) are shown. The rats were adapted to CNH or kept under normoxic (N) conditions. The amount of protein applied to the gel was 10 μg (homogenate), 15 μg (cytosolic fraction) and 5 μg (particulate fraction) for PKCε and 40 μg (homogenate), 50 μg (cytosolic fraction) and 40 μg (particulate fraction) for P-PKCε. GAPDH and actin were used as loading controls. Values are presented as mean ± SE (n=5/group); * $P<0.05$.

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 and lactate dehydrogenase release

Cell viability and lactate dehydrogenase (LDH) release were analyzed at the beginning of the experiments (after stabilization), after MI (LDH release only) and after re-energization as previously described (Borchert *et al.* 2011). The number of viable (unstained) myocytes was determined by Trypan blue exclusion (Wu *et al.* 1999). 50-100 myocytes were counted in duplicates from 6-10 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. LDH release was measured spectrophotometrically (Buhl and Jackson 1978) using the LDH Liqui-UV kit (Stanbio, Boerne, TX, USA). LDH released during MI and during re-energization was normalized to total LDH content in the cells and expressed as a percentage of appropriate control group not exposed to MI/R.

Statistical analysis

All values are presented as means ± SE. The results were compared using t-test or One-way ANOVA with Bonferroni post hoc test when appropriate. A p-value < 0.05 was considered as statistically significant.

Results

The analysis of PKCε and its phosphorylated form (P-PKCε) after the adaptation to CNH was

performed in homogenate, cytosolic and particulate fractions. Figure 1A shows the hypoxia-induced increase of PKCε protein level in the particulate fraction (by 15 %) compared to the normoxic group. The level of P-PKCε as well as the ratio P-PKCε/PKCε were not affected significantly (Fig. 1B and 1C, respectively). The PKCε mRNA level increased after the adaptation to CNH by 48 % compared to normoxic controls (Fig. 2).

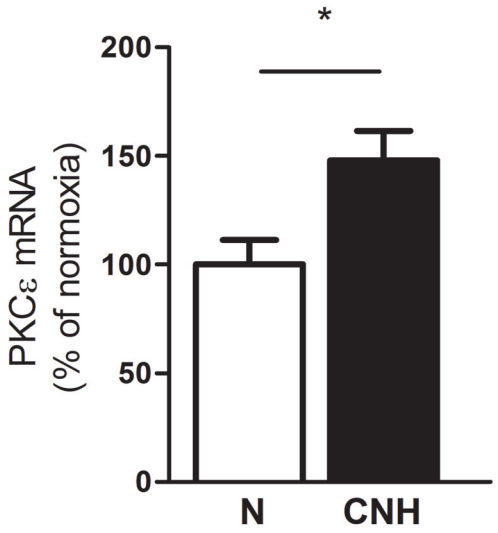

Fig. 2. Effect of continuous normobaric hypoxia (CNH) on myocardial expression of PKCε mRNA. Total mRNA was extracted from left ventricles of rats adapted to CNH or kept in normoxic (N) conditions. The values of mRNA were normalized to the reference gene HPRT1. Values are presented as mean ± SE (n=5/group); * P<0.05.

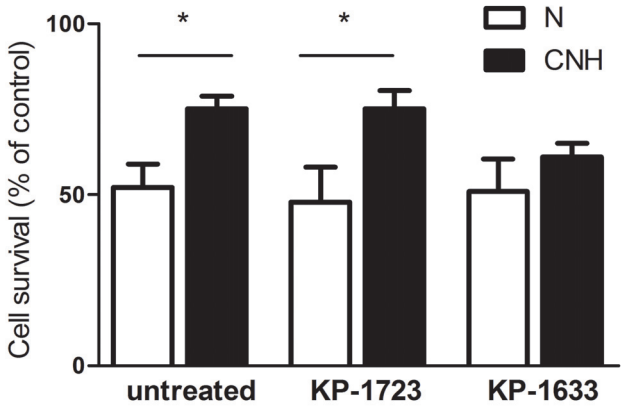

Fig. 3. Effect of the control peptide KP-1723 and the PKCε inhibitory peptide KP-1633 on survival of left ventricular myocytes during acute metabolic inhibition and re-energization, expressed as a percentage of control values. The cells were isolated from rats adapted to continuous normobaric hypoxia (CNH) or from rats kept in normoxic (N) conditions. Values are presented as mean ± SE (n=6-10/group); * P<0.05.

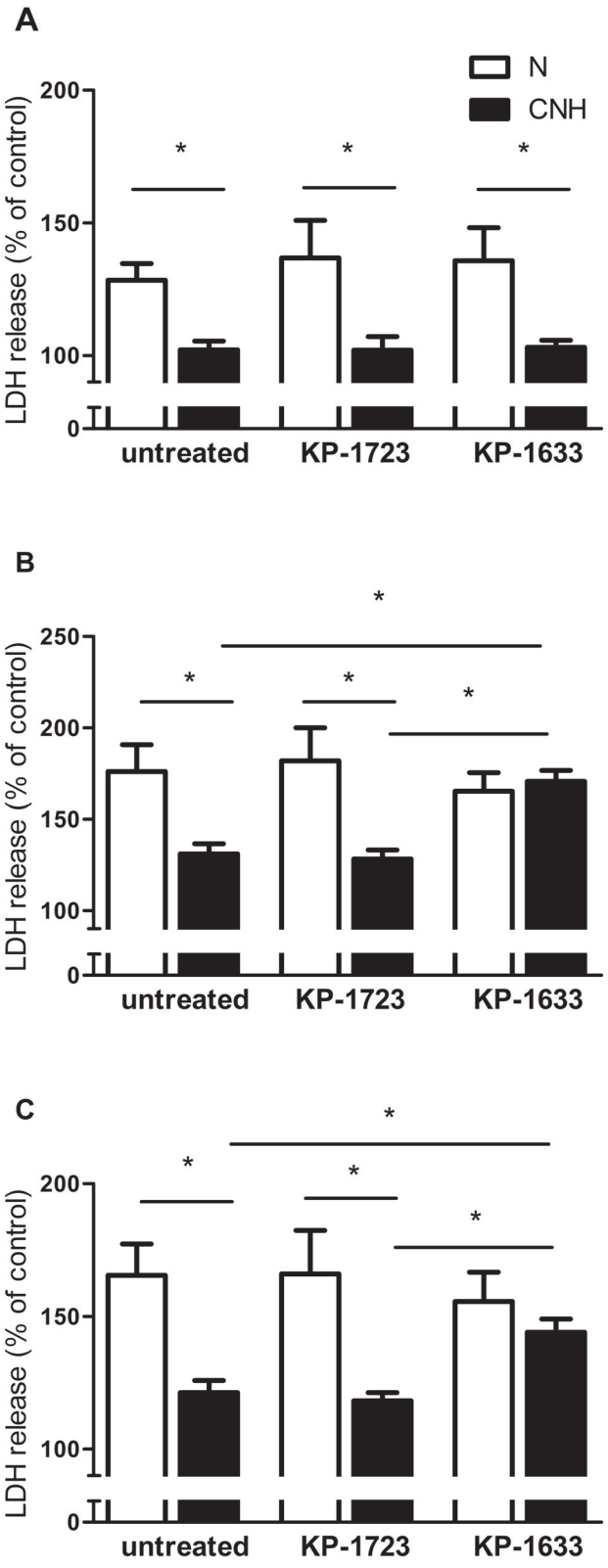

Fig. 4. Effect of the control peptide KP-1723 and the PKCε inhibitory peptide KP-1633 on lactate dehydrogenase (LDH) release from left ventricular myocytes during metabolic inhibition (**A**), during re-energization (**B**), and total release (**C**), expressed as a percentage of corresponding LDH release from control cells. The cells were isolated from rats adapted to continuous normobaric hypoxia (CNH) or from rats kept in normoxic (N) conditions. Values are presented as mean ± SE (n=6-10/group); * P<0.05.

Figure 3 shows the improved viability of LVM from the hypoxic group after MI/R. The pre-treatment of LVM with KP-1723 did not affect the salutary effect of CNH. However, the pre-treatment of LVM with PKCε inhibitory peptide KP-1633 blunted the hypoxia-induced increase in the cell survival.

Figure 4, A-C, respectively, show the effect of KP-1723 and KP-1633 on LDH release from LVM during MI, during re-energization and total LDH release during MI/R, expressed as a percentage of appropriate control values. In the untreated hypoxic group, LDH release was attenuated during MI, during re-energization and during MI/R. The pre-treatment of hypoxic as well as normoxic LVM with KP-1723 did not affect the LDH release and the salutary effect of CNH was preserved. In contrast, the pre-treatment of LVM with KP-1633 abolished the hypoxia-induced attenuation of LDH release in the re-energization phase.

Discussion

Recently, we demonstrated that the uninterrupted exposure of rats to CNH for 3 weeks improved myocardial resistance to acute ischemic injury. This was evidenced by reduced size of myocardial infarction induced by coronary artery occlusion/reperfusion in open-chest animals (Neckar *et al*. 2013) as well as by decreased LDH release and improved survival of isolated LVM subjected to simulated I/R (Borchert *et al*. 2011, Neckar *et al*. 2013). The present study shows that CNH increases PKCε mRNA expression and protein level in the particulate fraction of LV myocardium. To study the involvement of PKCε in CNH-induced cardioprotective mechanism, we used the PKCε-specific inhibitory peptide KP-1633, which inhibits the association of activated PKCε with its anchoring protein, receptor for activated C kinase 2 (RACK2 or β-COP) (Mackay and Mochly-Rosen 2001, Mochly-Rosen 1995, Souroujon and Mochly-Rosen 1998). The pre-treatment of LVM with KP-1633 completely abolished the CNH-induced salutary effects on cell survival and LDH release during re-energization without affecting cells isolated from the hearts of normoxic animals. This indicates that PKCε is critically involved in the CNH-induced cardioprotective mechanism.

Our study corresponds with other reports emphasizing the involvement of PKCε in chronic hypoxia-induced cardioprotection (Rafiee *et al*. 2002, Wang *et al*. 2011). However, these studies used various hypoxic stimuli/regimens and the PKCε involvement was determined in different ways (analysis of translocation, phosphorylation or the loss of cardioprotective phenotype after the PKCε inhibition). Wang *et al.* (2011) perfused isolated rat hearts with PKCε-specific inhibitory peptide εV1-2, which abolished both PKCε translocation (activation) from cytosolic to particulate fractions and the improvement of postischemic recovery of LV contractile function induced by moderate intermittent hypobaric hypoxia (PO_2=11.2 kPa, 4 h/day, 4 weeks). Similarly, the general PKC inhibitor chelerythrine suppressed PKCε activation and eliminated the infarct size-limiting effect in the hearts of infant rabbits adapted to CNH (10 % O_2, 10 days) (Rafiee *et al*. 2002). Interestingly, the prenatal exposure to chronic hypoxia had an adverse effect on myocardial resistance to I/R injury that was associated with PKCε downregulation. Adult offspring of rats exposed to CNH (10.5 % O_2) during the last trimester of gestation exhibited decreased myocardial levels of PKCε and its phosphorylated form together with impaired postischemic recovery of LV function and increased infarct size compared with controls (Xue and Zhang 2009). The same regimen of prenatal CNH led to PKCε downregulation and abolished heat stress-mediated cardioprotection in the later adulthood (Li *et al*. 2004). In contrast, decreased myocardial PKCε expression was observed in our previous experiments on adult rats adapted to severe intermittent hypobaric hypoxia (PO_2=8.5 kPa, 8 h/day, 5 weeks), which is cardioprotective (Hlavackova *et al*. 2010, Kolar *et al*. 2007). However, a beneficial role of another novel PKC isoform, PKCδ, was identified using this hypoxic regimen as indicated by a negative correlation of infarct size with PKCδ protein level (Hlavackova *et al*. 2007) and by an attenuation of infarct size-limiting effect using the PKCδ-selective inhibitor rottlerin (Neckar *et al*. 2005). Therefore, the involvement of the various PKC isoforms in hypoxia-induced cardioprotection is likely dependent on the hypoxic regimen used.

Although the available data mostly support the involvement of PKCε in chronic hypoxia-induced cardioprotection, the comparison of individual studies is difficult and does not allow an unequivocal conclusion. Apart from differences among normobaric, hypobaric, continuous and intermittent hypoxia regimens, the intensity and total duration of hypoxic stimulus as well as the frequency and duration of individual hypoxic bouts are highly variable among models used (Asemu *et al*.

2000, Kolar *et al*. 2007, Milano *et al*. 2013, Neckar *et al*. 2013, Zong *et al*. 2004) and are likely to significantly influence the impact on myocardial ischemic resistance and the role of individual PKC isoforms. It is still unclear which of these factors plays a decisive role in terms of cardioprotection. On the other hand, the investigation of different modes of chronic hypoxic exposure has its importance, because the human heart also can be exposed to the various hypoxic conditions. This may occur either naturally (e.g. during prenatal period or living at high altitude) or under disease states (cyanotic congenital heart defects, chronic obstructive lung disease, ischemic heart disease, sleep apnea etc.) (Ostadal and Kolar 2007). Apart from different hypoxic modes, other factors need to be considered, such as gender differences (Ostadal *et al*. 1984, Xue and Zhang 2009), age of animals (La Padula and Costa 2005, Ostadalova *et al*. 2002), nutrition (Hlavackova *et al*. 2007) or animal species used (Manukhina *et al*. 2013, Wauthy *et al*. 2004, Zong *et al*. 2004). It is also important to take into account which part of the heart is analyzed, as marked differences exist in the effect of chronic hypoxia on PKC expression between right and left ventricles (Uenoyama *et al*. 2010).

The precise mechanism by which PKCε activation exerts its protective effect is not fully understood. To date, several studies, mostly on preconditioning, identified many PKCε target proteins that may play a role in cardioprotection. It has been demonstrated that PKCε-mediated cardioprotection is linked to phosphorylation of connexin 43 (Doble *et al*. 2000, Jeyaraman *et al*. 2012), which among the other effects influences the gap junctional intercellular communication and thereby may prevent the spreading of injury during I/R. PKCε also activates aldehyde dehydrogenase-2, which metabolizes toxic aldehydes formed during I/R (Budas *et al*. 2010). In addition, PKCε may play an anti-apoptotic role by inhibition of pro-apoptotic Bcl-2 associated death domain protein (BAD) *via* its phosphorylation (Baines *et al*. 2002). It has been shown that an interaction of PKCε with cytochrome *c* oxidase subunit IV improved cytochrome *c* oxidase activity in preconditioned rat myocardium (Guo *et al*. 2007). Interestingly, PKCε, or more precisely yin/yang effect of both PKCε and PKCδ was also shown to inhibit and stimulate pyruvate dehydrogenase complex, respectively, and may thus play an important role in the maintenance of energy homeostasis in mitochondria (Gong *et al*. 2012). Another molecule which should not be omitted in connection with the mechanism of cardioprotection is nitric oxide (Ding *et al*. 2005), a direct activator of PKCε (Balafanova *et al*. 2002). PKCε-Akt-eNOS signaling modules were identified as critical signaling elements during PKCε-induced cardiac protection (Zhang *et al*. 2005). The association of PKCε and eNOS might thus represent a positive-feedback loop by which PKCε activity can be modulated. PKCε also phosphorylates glycogen synthase kinase-3β (Terashima *et al*. 2010) resulting in decreased mitochondrial permeability transition pore opening and improved resistance to myocardial infarction (Juhaszova *et al*. 2004, 2009). The involvement of reactive oxygen species, PKCε and glycogen synthase kinase-3β phosphorylation was observed also in cardioprotection induced by adaptation to moderate intermittent hypobaric hypoxia (PO_2=11.2 kPa, 4 h/day, 4 weeks) (Wang *et al*. 2011).

In conclusion, adaptation of rats to CNH increased myocardial expression of PKCε and protected isolated ventricular myocytes against injury caused by simulated I/R. The salutary effects of CNH were abolished by PKCε-specific inhibitory peptide KP-1633, indicating the involvement of this PKC isoform in the cardioprotective mechanism.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Příloha B

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Up-regulation and redistribution of protein kinase C-δ in chronically hypoxic heart

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Abstract The adaptation to chronic hypoxia confers long-lasting cardiac protection against acute ischemia–reperfusion injury. Protein kinase C (PKC) appears to play a role in the cardioprotective mechanism but the involvement of individual PKC isoforms remains unclear. The aim of this study was to examine the effects of chronic intermittent hypoxia (CIH; 7,000 m, 8 h/day) and acute administration of PKC-δ inhibitor (rottlerin, 0.3 mg/kg) on the expression and subcellular distribution of PKC-δ and PKC-ε in the left ventricular myocardium of adult male Wistar rats by Western blot and quantitative immunofluorescence microscopy. CIH decreased the total level of PKC-ε in homogenate without affecting the level of phosphorylated PKC-ε (Ser729). In contrast, CIH up-regulated the total level of PKC-δ as well as the level of phosphorylated PKC-δ (Ser643) in homogenate. Rottlerin partially reversed the hypoxia-induced increase in PKC-δ in the mitochondrial fraction. Immunofluorescent staining of ventricular cryo-sections revealed increased co-localization of PKC-δ with mitochondrial and sarcolemmal membranes in CIH hearts that was suppressed by rottlerin. The formation of nitrotyrosine as a marker of oxidative stress was enhanced in CIH myocardium, particularly in mitochondria. The expression of total oxidative phosphorylation complexes was slightly decreased by CIH mainly due to complex II decline. In conclusion, up-regulated PKC-δ in CIH hearts is mainly localized to mitochondrial and sarcolemmal membranes. The inhibitory effects of rottlerin on PKC-δ subcellular redistribution and cardioprotection (as shown previously) support the view that this isoform plays a role in the mechanism of CIH-induced ischemic tolerance.

Keywords Chronic hypoxia · Heart · Protein kinase C · Mitochondria · Cardioprotection · PKC delta up-regulation

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Introduction

It has been shown that adaptation of rats to chronic intermittent hypoxia (CIH) protects their hearts against acute ischemia–reperfusion injury. This long-lasting protective phenomenon manifests itself as a reduction in infarct size, limitation of ventricular arrhythmias, and improved recovery of contractile function [1]. Although the cardioprotective effects of CIH have been known for half a century, and several signaling pathways have been proposed to play a role [1], the detailed mechanism of improved cardiac ischemic tolerance induced by CIH still remains to be elucidated.

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In analogy to short-lived protection induced by preconditioning, we and others have shown that, for example, protein kinase C (PKC) [2, 3], phosphatidylinositol 3-kinase/Akt pathway [4], mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channels [5, 6], and reactive oxygen species (ROS) [7] are involved in the cardioprotective mechanism of CIH, although the precise interactions among these components are unclear. A link among PKC, especially the PKC-ε isoform, ROS, and mitoK_{ATP} channels has been postulated in protection induced by preconditioning [8, 9].

Whereas numerous studies have implicated individual PKC isoforms in cardioprotection induced by preconditioning, data concerning their potential role in the cardioprotective mechanism of CIH are rather limited and controversial probably due to different animal and hypoxia models [10]. In line with other reports [3, 11], the involvement of PKC in cardioprotection by chronic hypoxia is supported by the observation that chelerythrine, a general inhibitor of PKC, completely abolished the infarct size-limiting effect of CIH [2]. Several studies reported enhanced expression and/or redistribution of PKC-ε to particulate fraction under conditions of chronic hypoxia [3, 11] but our recent experiments have shown that redox-sensitive PKC-δ was up-regulated, and PKC-ε was either not affected or even moderately down-regulated by CIH in rat myocardium [2, 7, 12]. Moreover, the infarct size of rats adapted to CIH exhibited close negative correlation with PKC-δ up-regulation in the myocardial particulate fraction [12]. Chronic antioxidant treatment during the hypoxic adaptation period abolished both PKC-δ up-regulation and cardioprotective effect, indicating that these events are critically dependent on increased production of ROS associated with adaptation to CIH [7]. The involvement of PKC-δ in cardioprotection induced by CIH is further supported by our observation that rottlerin, a PKC-δ inhibitor, administered before the ischemic period, significantly attenuated the infarct size-limiting effect of CIH [2]. While these results have brought indirect evidence for the role of PKC, especially PKC-δ, in the cardioprotective mechanism of CIH, the precise localization of PKC isoforms in cellular compartments of CIH myocardium and its relevance to the improved ischemic tolerance remains to be elucidated.

The aim of this study, therefore, was to further investigate the potential involvement of PKC-δ and PKC-ε in the cardiac adaptation to CIH. We analyzed the effect of CIH on the myocardial level of total and phosphorylated forms of PKC-δ and PKC-ε. Furthermore, we studied effect of rottlerin on myocardial PKC-δ level and its detailed subcellular distribution after adaptation of rats to CIH by means of both Western blot analysis and quantitative immunofluorescence microscopy. We used the same experimental protocol under which the inhibitory effect of rottlerin on myocardial ischemic tolerance was observed [2]. Our results suggest that mitochondrial and sarcolemmal membranes are the major targets of PKC-δ in CIH-adapted hearts and support the involvement of this isoform in the cardioprotective mechanism.

Materials and methods

Animals

Adult male Wistar rats (250–280 g) were exposed to intermittent high-altitude hypoxia of 7,000 m in a hypobaric chamber for 8 h/day, 5 days a week (24–32 exposures). Barometric pressure (P_B) was lowered stepwise, so that the level equivalent to an altitude of 7,000 m ($P_B = 40.9$ kPa; $P_{O_2} = 8.5$ kPa) was reached after 13 exposures [7]. The animals were employed the day after the last hypoxic exposure. The control group of rats was kept for the same period of time at P_B and P_{O_2} equivalent to an altitude of 200 m ($P_B = 99$ kPa; $P_{O_2} = 20.7$ kPa). All animals had free access to water and a standard laboratory diet.

Animals were anesthetised with sodium pentobarbital (60 mg/kg ip, Sanofi, Montpellier, France). Rottlerin (Biomol, Plymouth Meeting, PA) was dissolved in DMSO and then diluted with saline and administered into the jugular vein in a dose of 0.3 mg/kg as a single bolus (1 ml/kg) 15 min before the excision of the heart; the final dose of DMSO was 6 µl/kg [2]. Controls were given saline (1 ml/kg) with DMSO. The animals were killed by decapitation, and their hearts were rapidly excised and perfused with Krebs–Henseleit solution containing 2,3-butanedione monoxime (20 mmol/l). Apex and the remaining part of the left ventricular (LV) free wall were dissected, weighed, snap-frozen in liquid nitrogen and stored at liquid nitrogen until use. The study was conducted in accordance with 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). Experimental protocols were approved by the Animal Care and Use Committee of the Institute of Physiology, Academy of Sciences of the Czech Republic. All the chemical compounds were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

Tissue fractionation and Western blot analysis

Frozen LV myocardium was pulverized at the temperature of liquid nitrogen, followed by Potter–Elvehjem homogenization as described previously [7]. The homogenization buffer for analyses of phosphorylated PKC isoforms contained phosphatases inhibitor sodium orthovanadate

(0.1 mM) and glycerol-3-phosphate (10 mM) in addition. The homogenate was centrifuged to obtain particulate (all membranes) fraction (100,000×*g* for 60 min) or nuclear–cytoskeletal-enriched (Nucl) (1,000×*g* for 10 min), mitochondria-enriched (Mito) (8,000×*g* for 10 min), microsomal (Micro) (100,000×*g* for 60 min) and cytosolic (Cyto) fractions [2, 7]. The homogenate and pellets of all fractions were re-suspended either in a homogenization buffer (non-extracted fractions; oxidative phosphorylation (OXPHOS) complex analysis) or in a homogenization buffer containing 1% Triton X-100, held on ice for 60 min and then centrifuged at 100,000×*g* for further 60 min (TX-extracted fractions; PKC analysis). Triton X-100 was also added to the cytosolic fraction to reach the final concentration of 1%.

Proteins from both TX-extracted and non-extracted fractions were separated by electrophoresis (10 or 15% SDS-PAGE) and transferred to nitrocellulose membranes (Amersham Biosciences, Freiburg, Germany). Equal protein transfer efficiency was verified by staining of membranes with Ponceau S. 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 (90 min at room temperature) with polyclonal antibodies against PKC-δ (662–673) and PKC-ε (728–737) (Research & Diagnostic Antibodies, Benicia, CA) or (overnight at 4°C) phosphorylated form of PKC-δ (p-PKC-δ) (Ser643) (Cell Signaling Technology, Beverly, MA) and p-PKC-ε (Ser729) (Upstate, Billerica, MA). After the membranes were washed with TTBS, they were incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase for 60 min at room temperature. Bands were visualized by enhanced chemiluminescence on the autoradiographic film (Amersham Biosciences), and ImageQuant software was used for quantification of the relative abundance of PKC isoforms and OXPHOS complexes. In order to ensure the specificity of PKC-δ and PKC-ε-immunoreactive proteins, prestained molar-mass protein standards, recombinant human PKC-δ and PKC-ε standards, rat brain extract, and the blocking immunizing peptides were used. In order to ensure the specificity of OXPHOS complexes-immunoreactive protein, mouse brain mitochondria (MitoSciences, Eugene, OR) were used as a positive control. From each group, one sample was run on the same gel and quantified on the same membrane. Sources for other antisera were as follows: lamin, GAPDH and Na,K-ATPase (Abcam, Cambridge, MA), the kit of monoclonal antibodies against five OXPHOS complexes from rodent mitochondria (MitoSciences).

Quantitative immunofluorescence microscopy

The subcellular redistribution of PKC-δ induced by CIH, the effect of rottlerin and the effect of CIH on myocardial nitrotyrosine content were investigated by immunofluorescent staining followed by digital imaging fluorescence microscopy. LV apex cross cryo-sections (5 μm) were incubated with a primary antibody raised against rat PKC-δ (662–673) or nitrotyrosine (Molecular Probes, Eugene, OR) and counterstained for nuclei with 4′,6-diamidino-2-phenylindole (DAPI)-containing mounting medium (Vectashield; Vector Laboratories, Burlingame, CA) and either for the sarcolemma with 10% (vol/vol) wheat-germ agglutinin (WGA; Molecular Probes) or for mitochondria with anti-OXPHOS complexes. They were then incubated with the appropriate Alexa Fluor secondary antibody conjugate (Molecular Probes) and visualized by immunofluorescence microscopy, as described by Bouwman et al. [13]. Sections were qualitatively and quantitatively analyzed with the use of imaging and analysis software (SlideBook^{TM}, version 4.1). Regions of interest from the digital images of the LV cross cryo-sections were selected in a process termed masking, a mask being a binary overlay on a digital image. Masks of segments of cross cryo-sections were created by automatically selecting standardized fluorescence thresholds of the counterstains (either sarcolemmal glycocalyx or mitochondrial OXPHOS complexes). Next, the mean intensities of fluorescence of PKC-δ or nitrotyrosine within these masks were calculated using SlideBook^{TM} imaging software. Pearson's correlation factors between channels of multiple regions of interest from at least three digital images per experimental group were derived using the SlightBook^{TM} co-localization tab.

Statistical analysis

The results are expressed as means ± SE. A one-way ANOVA and subsequent Student–Newman–Keuls test were used for comparison of differences in normally distributed variables between groups. Differences were considered as statistically significant when $P < 0.05$.

Results

Purity of subcellular fractions, distribution of OXPHOS complexes

Before PKC analysis in TX-extracted fractions, we attempted to determine the level of non-extracted subcellular fractions purity (Fig. 1). GAPDH as cytosolic and lamin as nuclear markers were distinctly detected in the corresponding fractions. Na,K-ATPase as a sarcolemmal membrane marker was detected in all particulate fractions and OXPHOS complexes as a mitochondria marker were present across all fractions analyzed. As for the effect of

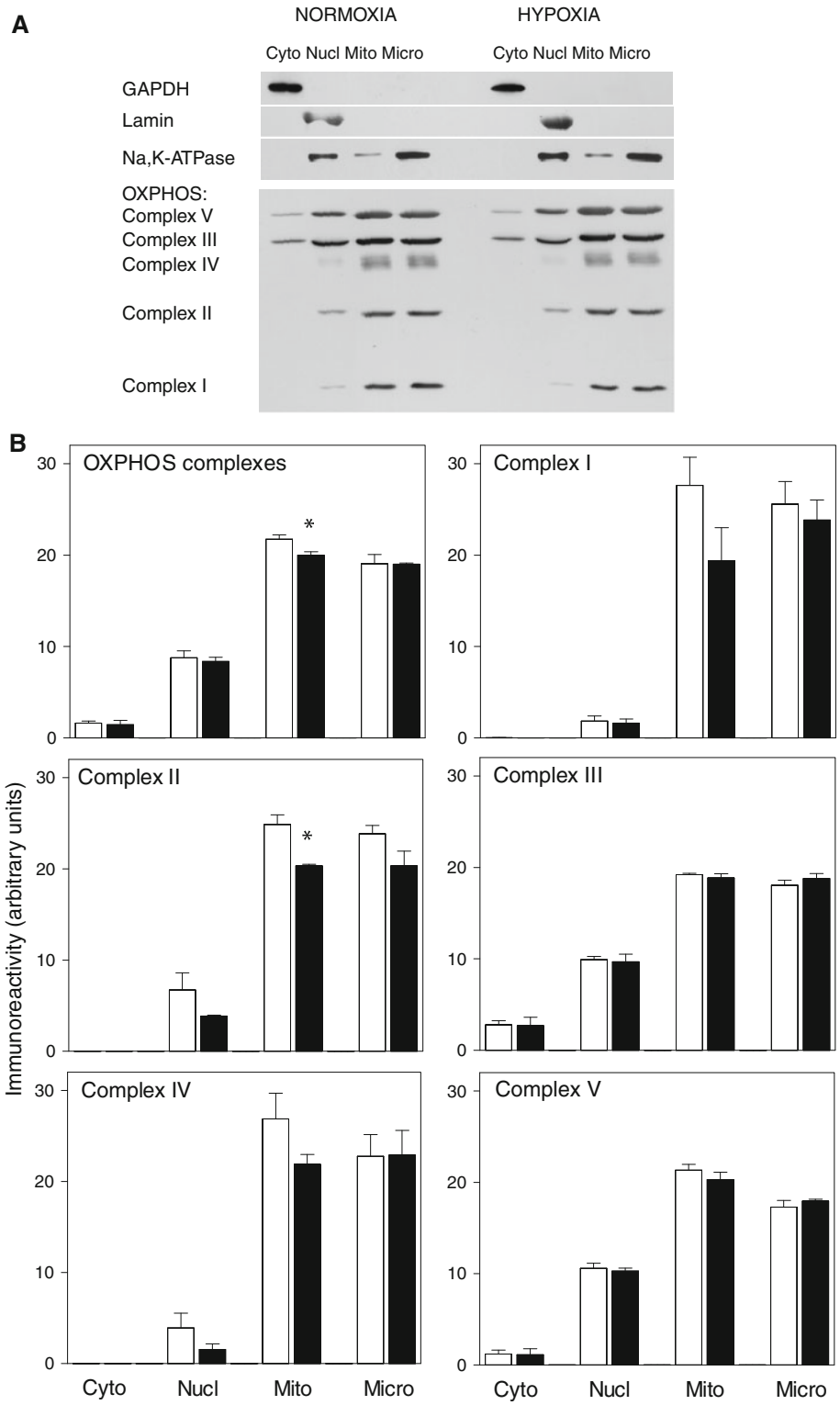

Fig. 1 Western blot analysis of fraction contamination (**a**) and mitochondrial OXPHOS complex distribution (**b**) in individual subcellular fractions. Purity of fractions was analyzed using antisera directed against marker proteins localized predominantly in the cytosol (*GAPDH*), nucleus (*lamin*), sarcolemma (*Na,K-ATPase*), and mitochondria [OXPHOS complexes: NDUFB8 (*Complex I*), SDHB (*Complex II*), core protein 2 (*Complex III*), COX I (*Complex IV*), F1α ATP synthase (*Complex V*)]. Cytosolic (*Cyto*), nuclear–cytoskeletal (*Nucl*), mitochondrial (*Mito*), and microsomal (*Micro*) non-extracted fractions from left ventricles of normoxic (*open columns*) and chronically hypoxic (*black columns*) rats were used. The amount of protein applied to the gel was 5 μg for all subcellular fractions. Values are means ± SE from three hearts in each group. * $P < 0.05$ versus the corresponding normoxic group

CIH on OXPHOS complexes, a slight decrease of the total OXPHOS complexes level in the Mito fraction was observed (Fig. 1b, upper left panel); Complexes I, II, and IV contributed to this effect (Fig. 1b, relevant panels).

Western blot analysis of PKC-ε

We have shown previously that CIH either did not influence [2] or moderately down-regulated PKC-ε in

particulate fractions [7, 12]. The possible explanation for this discrepancy is that different homogenization protocol was used and part of tissue was discarded as a debris in the study where no change of the PKC-ε level in CIH hearts was detected [2]. It was shown that, in addition to the choice of detergent and its concentration, the way of homogenization and centrifugation protocol could affect myocardial PKC isoforms distribution [14]. In this study, we confirmed that CIH decreased total level of PKC-ε in LV myocardium. We verified our Western blot results with two different antibodies, the antibody from Sigma and the antibody from Research & Diagnostic (used also for immunofluorescence microscopy analysis) and obtained comparable results. The adaptation to CIH decreased the total level of PKC-ε in homogenate and particulate fraction (by 40 and 37%, respectively) without affecting the level of p-PKC-ε (Ser729) (Fig. 2a, b). The ratio of p-PKC-ε (Ser729) to total PKC-ε was increased by CIH in homogenate and cytosolic fraction; the increase in particulate fraction did not reach statistical significance due to higher variability (Fig. 2c). The more detailed analysis of CIH effect on subcellular fractions revealed the decreased abundance of PKC-ε in the Mito and Micro fractions (Fig. 3).

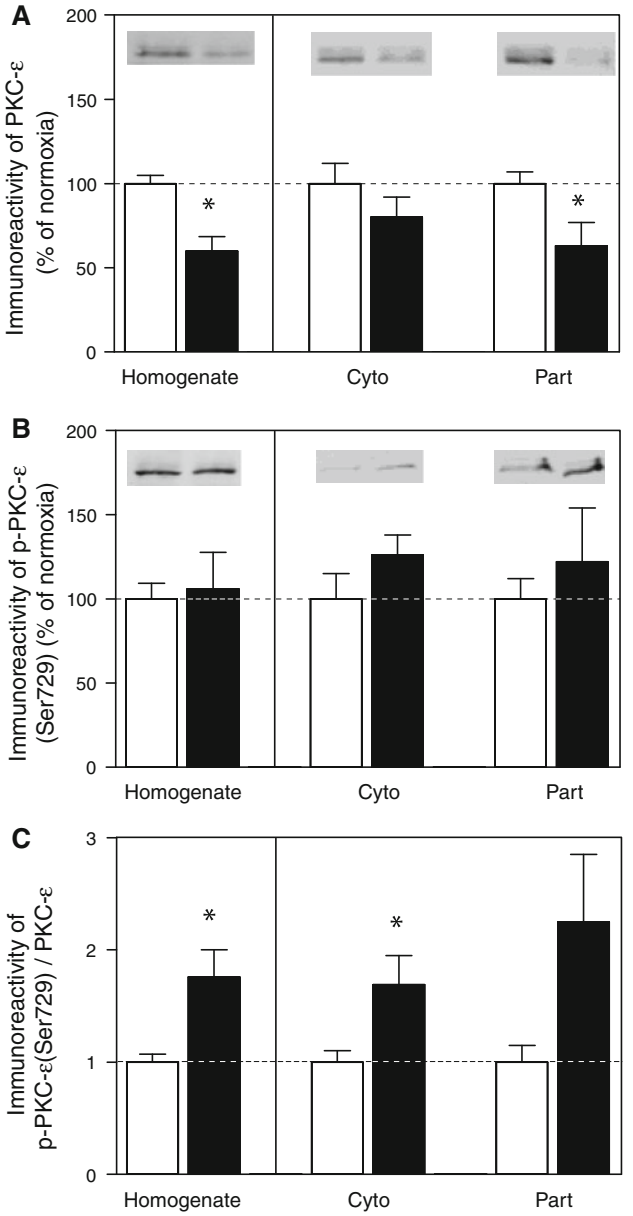

Fig. 2 Effect of CIH on the level of total PKC-ε (**a**), p-PKC-ε (Ser729) (**b**), and p-PKC-ε (Ser729) to total PKC-ε ratio (**c**) in *homogenate*, cytosolic (*Cyto*), and total particulate (*Part*) fractions from left ventricles of normoxic (*open columns*) and chronically hypoxic (*black columns*) rats, including representative Western blots. The amount of protein applied to the gel was 10 μg (*homogenate*), 15 μg (*cytosolic fraction*), or 5 μg (*particulate fraction*) for PKC-ε and 40 μg (*homogenate*), 50 μg (*cytosolic fraction*), or 40 μg (*particulate fraction*) for p-PKC-ε (Ser729). Values are means ± SE from five hearts in each group. * $P < 0.05$ versus corresponding normoxic group

Western blot analysis of PKC-δ

Chronic intermittent hypoxia increased the relative protein content of total PKC-δ as well as the level of p-PKC-δ (Ser643) in homogenate by 190 and 204%, respectively; similar changes were observed in cytosolic and particulate fractions (Fig. 4a, b). The ratio of p-PKC-δ (Ser643) to total PKC-δ was not influenced by CIH (Fig. 4c). Rottlerin had no effect on this isoform content in homogenate from either normoxic or hypoxic hearts (Fig. 5a). The detailed analysis showed that CIH up-regulated PKC-δ protein amount in all subcellular fractions with the highest increase in Mito and Micro fractions (Fig. 5b, c). Interestingly, the acute rottlerin treatment significantly decreased the relative PKC-δ protein content in the Mito fraction in favor of cytosolic and Nucl fractions of CIH-adapted hearts (Fig. 5d).

Immunofluorescence microscopy analysis

Figure 6 shows the PKC-δ redistribution pattern in response to CIH and rottlerin in LV cross cryo-sections. In the normoxic group, a diffuse staining of PKC-δ can be observed. PKC-δ was co-localized with nuclei in normoxic as well as in hypoxic hearts. CIH induced PKC-δ redistribution and increased PKC-δ co-localization with the sarcolemma (Fig. 6a, red WGA sarcolemmal glycocalyx counterstain) as well as with the mitochondria (Fig. 6b, red OXPHOS counterstain) as indicated by the increase in the yellow-orange color.

Figure 6a, c (left panel) show that the CIH-induced redistribution of PKC-δ to the sarcolemma (the mean intensity of fluorescence of PKC-δ in the sarcolemmal

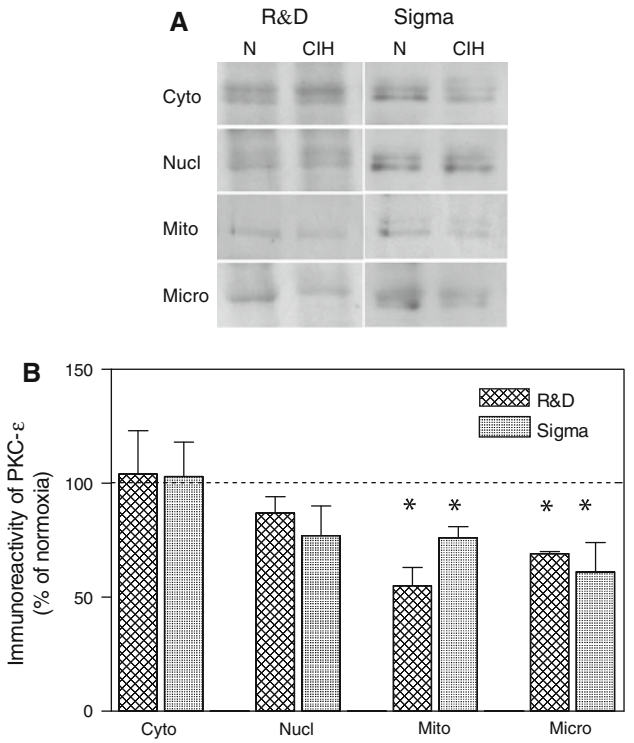

Fig. 3 Representative Western blots (**a**) showing effect of CIH on PKC-ε level in cytosolic (*Cyto*), nuclear–cytoskeletal (*Nucl*), mitochondrial (*Mito*), and microsomal (*Micro*) myocardial fractions analyzed by Research & Diagnostic (R&D) and Sigma antibodies. Quantified data (**b**) showing effect of CIH on PKC-ε level in subcellular fractions expressed as percentage of normoxic values. The amount of protein applied to the gel was 15 μg (*Cyto*), 5 μg (*Nucl*), 10 μg (*Mito*), and 5 μg (*Micro*). Values are means ± SE from four hearts in each group. * $P < 0.05$ versus corresponding normoxic group

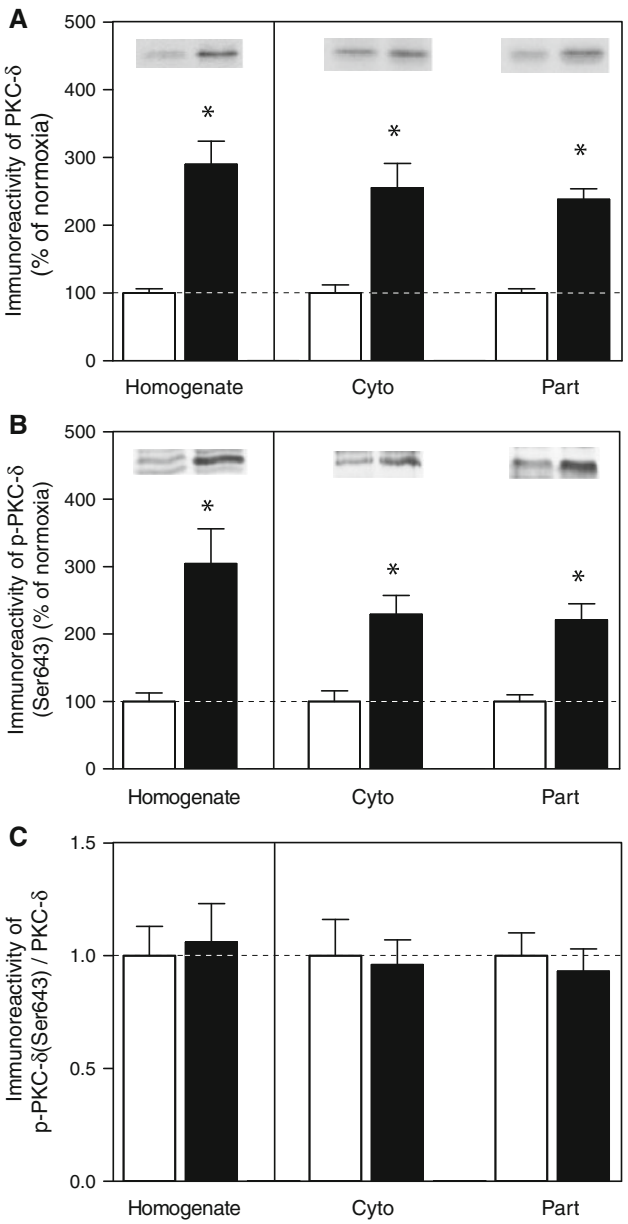

Fig. 4 Effect of CIH on the level of total PKC-δ (**a**), p-PKC-δ (Ser643) (**b**), and p-PKC-δ (Ser643) to total PKC-δ ratio (**c**) in *homogenate*, cytosolic (*Cyto*), and total particulate (*Part*) fractions from left ventricles of normoxic (*open columns*) and chronically hypoxic (*black columns*) rats, including representative Western blot. The amount of protein applied to the gel was 10 μg (*homogenate*), 15 μg (*cytosolic fraction*), or 5 μg (*particulate fraction*) for PKC-δ and 40 μg (*homogenate*), 50 μg (*cytosolic fraction*), or 40 μg (*particulate fraction*) for p-PKC-δ (Ser643). Values are means ± SE from six hearts in each group. * $P < 0.05$ versus corresponding normoxic group

mask was increased by up to 25%) was inhibited by rottlerin (the mean intensity of fluorescence of PKC-δ decreased to the normoxic value). Pearson's correlation factor between the green (PKC-δ) and the red channels (sarcolemmal WGA) was calculated to further quantify CIH-induced PKC-δ co-localization with the sarcolemmal membrane: its mean value in normoxia was 0.31 ± 0.04 and significantly increased in CIH to 0.42 ± 0.03. Rottlerin decreased its value in hypoxic tissue down to 0.27 ± 0.05. ($P < 0.05$; maximum correlation factor is 1).

In Fig. 6b, the PKC-δ co-localization with mitochondria (the red OXPHOS counterstain) is shown. In sections of hearts adapted to CIH, PKC-δ was present as a dotted-like distribution pattern with a yellow-orange color (arrows), which indicates the co-localization with the mitochondria. CIH increased the mean intensity of fluorescence of PKC-δ in the mitochondrial OXPHOS complexes mask by 19% (Fig. 6c, right panel), as well as the Pearson's correlation factor between the green (PKC-δ) and the red (OXPHOS complexes) channels. Its mean value significantly increased from 0.42 ± 0.04 in normoxic to 0.58 ± 0.06 in hypoxic tissue. Rottlerin reduced PKC-δ localization in hypoxic mitochondria, decreasing the mean intensity of PKC-δ fluorescence in the mitochondrial OXPHOS complexes mask to the normoxic control value. Rottlerin did

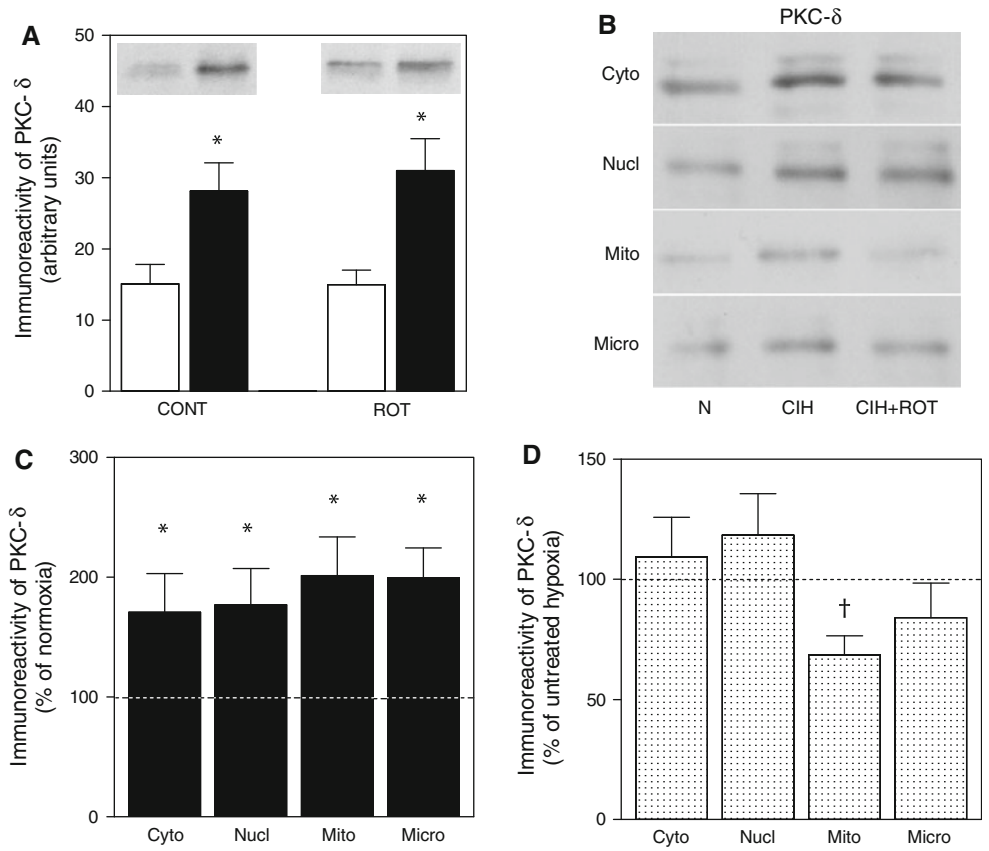

Fig. 5 Effect of CIH and rottlerin on the level of PKC-δ in homogenate from left ventricles of normoxic (*N*; *open columns*) and chronically hypoxic (*CIH*; *black columns*) rats treated by vehicle (*CONT*) or rottlerin (*ROT*), including representative Western blots (**a**). Representative Western blots (**b**) showing effect of CIH and rottlerin (*ROT*) on PKC-δ abundance in cytosolic (*Cyto*), nuclear–cytoskeletal (*Nucl*), mitochondrial (*Mito*), and microsomal (*Micro*) fractions. Quantified data (**c**) show effect of CIH on PKC-δ level in subcellular fractions expressed as percentage of normoxic values. (**d**) Effect of rottlerin on PKC-δ level in subcellular fractions from chronically hypoxic rats expressed as percentage of untreated hypoxic values. The amount of protein applied to the gel was 10 μg (*homogenate*), 15 μg (*Cyto*), 5 μg (*Nucl*), 10 μg (*Mito*), and 5 μg (*Micro*). Values are means ± SE from five hearts in each group. * $P < 0.05$ versus the corresponding normoxic group; † $P < 0.05$ versus the untreated hypoxic group

not significantly affect the PKC-δ distribution in normoxic tissue.

Unlike the PKC-δ staining in normoxic tissue, PKC-ε displayed a dotted-like distribution pattern. Partial PKC-ε co-localization with OXPHOS complexes suggests its presence in the mitochondria. Neither CIH nor rottlerin affected the PKC-ε distribution (data not shown).

Effect of CIH on nitrotyrosine formation

Figure 7a demonstrates CIH-induced formation of nitrotyrosine. CIH led to an increase in protein nitrosylation (the overall intensity of fluorescence of nitrotyrosine was increased by 24% compared to normoxia) and induced protein nitrosylation in mitochondria, nuclei, and sarcolemma (Fig. 7b). The mean Pearson's correlation factor between green (nitrotyrosine) and red channels (OXPHOS complexes) significantly increased from 0.32 ± 0.02 in normoxic tissue to 0.40 ± 0.03 in hypoxic tissue.

Discussion

The major novel finding of this study is that the up-regulation of PKC-δ in LV myocardium of CIH-adapted rats was associated with its enhanced phosphorylation on Ser643 and increased co-localization with markers of mitochondrial and sarcolemmal membranes. Rottlerin, PKC-δ inhibitor, attenuated CIH-induced effects on the expression and subcellular redistribution of PKC-δ. Concerning PKC-ε, the adaptation to CIH decreased its total abundance in myocardium without affecting its distribution and the level of p-PKC-ε (Ser729). The level of total oxidative phosphorylation complexes was slightly decreased and the formation of nitrotyrosine as a marker of oxidative stress was enhanced in CIH myocardium, particularly in mitochondria.

The studies dealing with effects of chronic hypoxia on PKC-ε expression, activity and subcellular distribution are rather controversial, probably due to the diversity of the

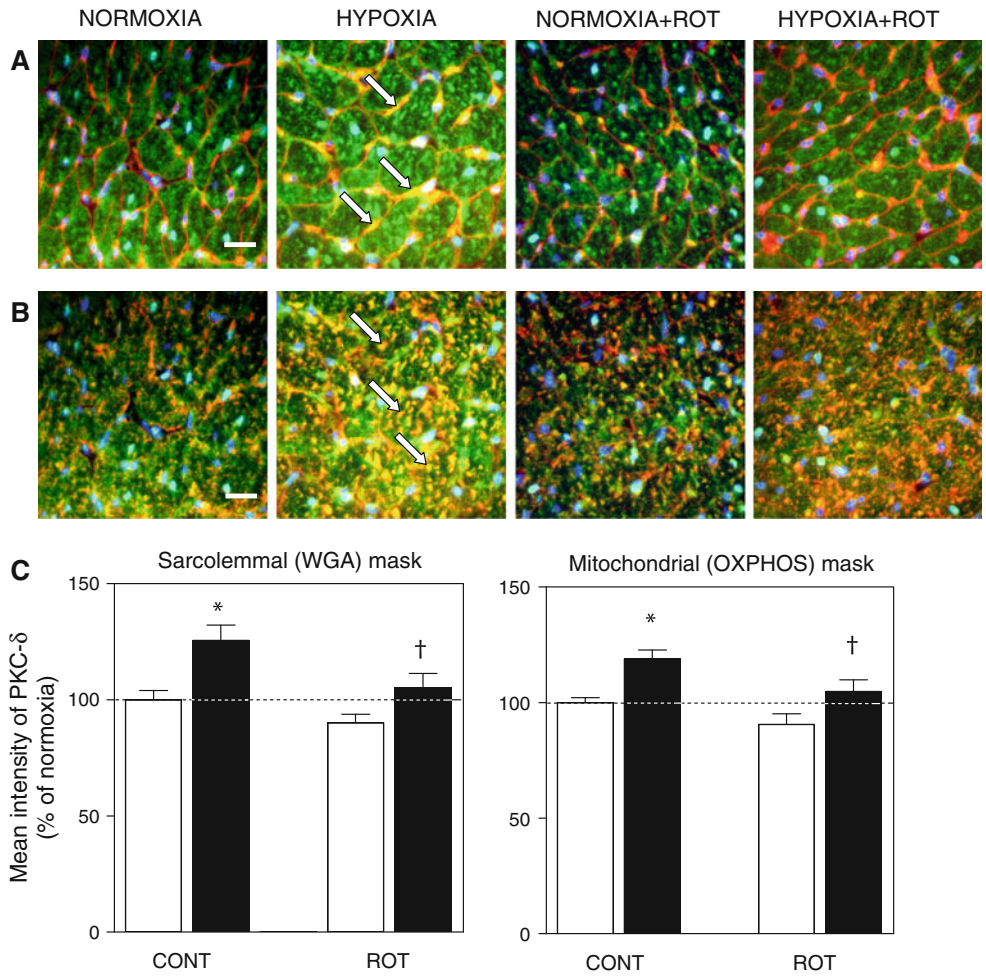

Fig. 6 PKC-δ distribution and co-localization with sarcolemma (**a**) and mitochondria (**b**) in normoxic and chronically hypoxic cross cryo-sections of left ventricles, and the effect of rottlerin (*ROT*). In all panels, *green* represents specific PKC-δ staining and *blue* indicates the nuclear 4′,6-diamidino-2-phenylindole (DAPI) staining. In panels **a**, *red* represents the wheat-germ agglutinin (WGA) staining of the sarcolemmal glycocalyx, and in panels **b**, *red* represents the OXPHOS complexes. Note the increase in the yellow-orange color in both panels, indicating increased CIH-induced co-localization of PKC-δ with sarcolemma and mitochondria (*arrows*). *Bar* represents 20 μm. (**c**) Quantification of the mean intensity of fluorescence of PKC-δ in sarcolemma (WGA staining, *left panel*) and mitochondria (OXPHOS complexes staining, *right panel*) in cryo-sections from normoxic (*open columns*) and chronically hypoxic (*black columns*) rats treated by vehicle (*CONT*) or rottlerin (*ROT*) expressed as percentage of normoxic values. Values are means ± SE from three hearts in each group. * $P < 0.05$ versus the normoxic group; † $P < 0.05$ versus the untreated hypoxic group (See online version for color pictures)

hypoxic models used [3, 10, 15, 16]. In our previous experiments, adaptation to CIH decreased PKC-ε protein amount [7] and similar effect was observed in adult rat myocardium prenatally exposed to hypoxic conditions [17]. It has been reported that PKC-ε down-regulation could be caused by PKC-δ-dependent hydrophobic motif phosphorylation of PKC-ε on Ser729 [18]. Despite the decrease of total PKC-ε content in this study, the level of phosphorylated form of PKC-ε (Ser729) was not influenced by CIH, leading to the increase in p-PKC-ε (Ser729) to total PKC-ε ratio. It suggests that the maintenance of phosphorylated (active) PKC-ε level is important for myocardial adaptation to CIH. An increased phosphorylation and translocation of PKC-ε was detected in hearts of neonatal rabbits under conditions of chronic continuous hypoxia [11]. It is necessary to emphasize that PKC-ε is the key component of signal transduction of various forms of preconditioning [19] and cross-regulation of PKC-ε and PKC-δ function in cardiomyocytes has been well documented [18, 20]. The work of Inagaki and Mochly-Rosen [21] showed that the activation of PKC-δ before ischemic insult led to the activation of PKC-ε and consequently to cardioprotection. Despite the absence of PKC-ε up-regulation or subcellular redistribution by CIH in this study, the fact that the phosphorylated PKC-ε level was preserved does not allow to unequivocally exclude the potential involvement of this isoform in the protective mechanism.

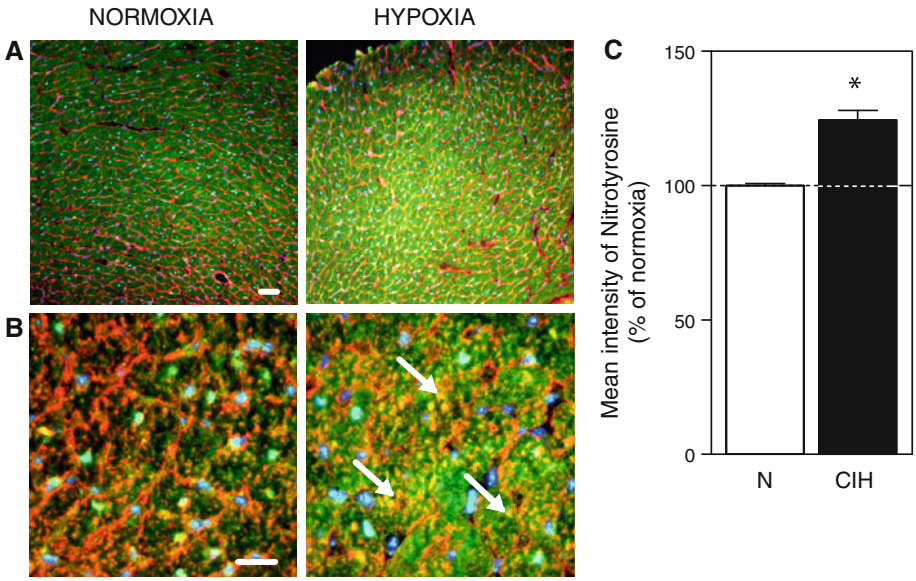

Fig. 7 Nitrotyrosine staining in left ventricular myocardium of normoxic and chronically hypoxic rats. In all panels, *green* indicates specific nitrotyrosine staining, and *blue* represents the nuclear 4′,6-diamidino-2-phenylindole (DAPI) staining. In panels **a**, *red* represents the wheat-germ agglutinin staining of the sarcolemmal glycocalyx, and in panels **b**, *red* represents the OXPHOS complexes. Note the increase in mitochondrial protein nitrosylation in hypoxia compared to normoxia (*arrows*). *Bar* represents 20 μm. (**c**) Quantification of the mean intensity of fluorescence of nitrotyrosine in cryo-sections from normoxic (*N*; *open columns*) and chronically hypoxic (*CIH*; *black columns*) rats expressed as percentage of normoxic values. Values are means ± SE from three hearts in each group. * $P < 0.05$ versus the normoxic group (See online version for color pictures)

Concerning PKC-δ, our observation of its significant up-regulation due to adaptation of rats to CIH corresponds to previous reports using the same [2, 7, 12] or similar [3] experimental models. Furthermore, we showed that CIH increased the phosphorylation of PKC-δ on Ser643 and promoted its increased co-localization with mitochondrial and sarcolemmal membranes. Phosphorylation of PKC-δ on Ser643 and its translocation to mitochondria was also observed in cardioprotection induced by pharmacological preconditioning [22]. The beneficial role of PKC-δ associated with its translocation to mitochondria and/or sarcolemma was confirmed in a variety of experiments on ischemic and pharmacological preconditioning [13, 22–25]. These protective effects of PKC-δ were ROS-dependent and related to either mitoK_{ATP} or sarcolemmal K_{ATP} (sarcK_{ATP}) channels activation. Similarly to preconditioning, the participation of sarcK_{ATP} and/or mitoK_{ATP} channels in increased ischemic tolerance of chronically hypoxic hearts was reported [5, 6, 26]. In addition, it was shown that PKC-δ translocation to sarcolemmal membrane is connected with the Na^+/Ca^{2+} exchanger-dependent cardioprotection [25]. Therefore, it can be speculated that increased co-localization of PKC-δ with sarcolemmal membrane could also play a role in CIH-induced myocardial protection.

Konishi et al. [27] reported that PKC-δ is a redox-sensitive enzyme, and ROS can modulate its function *via* tyrosine phosphorylation. This study demonstrated increased oxidative/nitrosative stress in CIH hearts by enhanced nitrotyrosine formation. Tyrosine nitration leads to the generation of antigenic epitopes on proteins, the changes in catalytic activity of enzymes [28] and could also influence their translocation, as shown, e.g., for PKC-ε [29]. Concerning the role of PKC-δ and ROS in CIH-induced cardioprotection, our recent study showed that chronic antioxidant treatment during the adaptation period eliminated both PKC-δ up-regulation and infarct size reduction, suggesting ROS-dependence of both events [7].

The observation that PKC-δ up-regulation in CIH hearts was co-localized with mitochondria prompted us to analyze OXPHOS complexes in detail. OXPHOS complexes were distributed across all subcellular fractions, though with different abundance. The OXPHOS distribution in fractions is in line with the study of Guo et al. [30], who measured immunoreactivity and activity of cytochrome *c* oxidase in the rat heart using similar homogenization and fractionation protocol. Mito and Micro fractions exhibited the highest OXPHOS enrichment. The later fraction is considered to contain mitochondrial fragments in addition to other membranes, such as sarcolemma and sarcoplasmic reticulum. The relatively high abundance of OXPHOS complexes in the Nucl fraction, which contains mainly broken cell debris, nuclei, cytoskeletal, and myofibrillar proteins, probably reflects the presence of interfibrillar mitochondria [31]. Our observation of the decrease in OXPHOS complexes is in agreement with our previous

study where CIH reduced the concentration of myocardial cardiolipin [32], a mitochondrial inner membrane lipid marker. Less severe chronic continuous hypoxia (5,000 m, 21 days) caused a global decrease in all OXPHOS complex activities [33]. Furthermore, decreased LV mitochondrial respiratory capacity in rats adapted to chronic continuous hypoxia (11% O_2, 4 weeks) was observed [34]. On the other hand, an increase in the activity of OXPHOS complexes I and III was found in the hearts of rats adapted to long-lasting high-altitude hypoxia (4,340 m, 84 days) [35]. As for the relation between PKC-ε and OXPHOS complexes, an interaction of PKC-ε with cytochrome *c* oxidase subunit IV associated with improved cytochrome *c* oxidase activity was documented in preconditioned myocardium [30, 36]. In agreement with these results, we observed a tendency to CIH-induced attenuation of OXPHOS complex IV and the down-regulation of PKC-ε in fractions with the highest OXPHOS enrichment. Concerning PKC-δ, the d-subunit of F_1F_0 ATPase was shown as its possible target in mitochondria. Enhanced PKC-δ expression in cardiac mitochondria and its co-immunoprecipitation with the d-subunit of F_1F_0 ATPase have suggested that this putative interaction mediates inhibition of F_1F_0 ATPase or ATP synthase activities during prolonged hypoxia [37]. In addition, Mayr et al. [38] postulated the necessity of PKC-δ presence for a protective shift from aerobic to anaerobic metabolism induced by ischemic preconditioning. In accordance, it has been shown that PKC-δ inhibits the pyruvate dehydrogenase complex [39], thereby likely contributing to the shift from aerobic to anaerobic metabolism observed in CIH-adapted hearts [40]. Furthermore, a link among PKC-δ, autophagy, and cardioprotection was documented. Autophagy and cardioprotection were abolished in rat hearts perfused with recombinant inhibitor of autophagy Tat-Atg5 (K130R) [41]. Autophagy may be the way for removing damaged mitochondria under CIH conditions, when the decrease in OXPHOS complexes occurs.

The function of PKC-δ in myocardial ischemia–reperfusion injury and its precise involvement in the mechanism of protection is still a matter of debate. One of the reasons of this ambiguity could be the greatest flexibility of PKC-δ among all PKC isoforms to affect various cellular functions because its subcellular localization is finely regulated by phosphorylation at multiple serine/threonine and tyrosine residues [42]. The timing and subcellular location of PKC-δ activation appears to be a critical factor in the manifestation of either protective or detrimental functions of this isoform in the heart. Whereas the activation of PKC-δ during reperfusion leads to a stimulation of pro-apoptotic pathways [43] and exacerbation of myocardial injury [44], the activation of the enzyme well before an ischemic insult is cardioprotective [21]. The advanced up-regulation of PKC-δ in cellular membranes, as an important prerequisite of its protective action, is well fulfilled under our CIH conditions. Mayr et al. [45] evidenced PKC-δ involvement in the preconditioning-induced cardioprotection using PKC-δ-null mice. In another study, they also showed that inhibition of PKC-ε resulted in compensatory phosphorylation and mitochondrial translocation of PKC-δ, providing a possible explanation for the synergy of PKC-δ and PKC-ε in cardioprotection [46]. This phenomenon can play a role under CIH conditions where PKC-δ was up-regulated and redistributed to mitochondria while PKC-ε was either not affected or even moderately down-regulated in rat myocardium [2, 7, 12].

In this study, we observed that rottlerin, a PKC-δ inhibitor, reversed CIH-induced PKC-δ redistribution to the Mito fraction without affecting the total protein amount of PKC-δ in homogenate. It was shown that acute rottlerin administration before preconditioning inhibited PKC-δ translocation to mitochondria [47] and nuclei [22]. Moreover, it was documented that ATP-competitive inhibitors, where rottlerin belongs to [48], affect redistribution of DAG-sensitive PKC isoforms (e.g., PKC-δ) by altering their DAG sensitivity perhaps by disrupting their closed conformation [49]. Although PKC-δ is inhibited by rottlerin more effectively than other PKC isoforms [48], it is necessary to consider possible non-specific effects of this compound [50]. Nevertheless, our immunofluorescence microscopy analysis revealed that rottlerin treatment partially reversed CIH-induced PKC-δ co-localization with the sarcolemma and mitochondria. These results are in line with the inhibitory effect of rottlerin on the improved ischemic tolerance of CIH-adapted rat hearts [2] and support the view that PKC-δ plays a role in the cardioprotective mechanism of CIH. However, we are aware that these results need to be verified by using more selective PKC inhibitors and searching for possible PKC-δ targets in sarcolemmal and mitochondrial membranes. The involvement of other PKC isoforms in cardioprotective effect of CIH cannot be excluded in view of the complete inhibition of cardioprotection by chelerythrine [2].

We conclude that the increase of PKC-δ expression in CIH-adapted rat hearts is localized mainly to mitochondrial and sarcolemmal membranes. CIH-induced PKC-δ subcellular redistribution was reversed by acute treatment with its inhibitor, rottlerin. Our results are in line with the view that PKC-δ mediates the cardioprotective effect of chronic hypoxia, probably via its mitochondrial and sarcolemmal target(s).

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Příloha C

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 (7,000 m, 8 h/day, 5 weeks) on the expression of cytosolic $PLA_2\alpha$ ($cPLA_2\alpha$) and its phosphorylated form ($p\text{-}cPLA_2\alpha$), 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 $p\text{-}cPLA_2\alpha$ 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 Giα 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/Gi-mediated stimulation of the ERK/p38 pathway.

Key words: chronic intermittent hypoxia, ischemia/reperfusion, phospholipase A_2, cyclooxygenase 2, β-adrenoceptor, adenylyl cyclase, 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) [3], PKC [4, 5] and phosphatidylinositol 3-kinase (PI3K)/Akt [6] are involved in the cardioprotective mechanism of CIH. 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 [7]. Unlike β_1-AR, β_2-AR are coupled dually to Gs and Gi proteins [8]. It has been suggested that β_2-AR/Gi signaling activates the PI3K/Akt cell survival pathway which plays a crucial role in protection of cardiomyocytes against apoptosis. Chesley et al. [9] observed that the β_2-AR stimulation prevented hypoxia or ROS-induced apoptosis in rat neonatal cardiomyocytes and it also 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, elevated abundance of p-Akt was detected in CIH-adapted myocardium [10]. 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 Gs protein-regulated adenylyl cyclase (AC) pathway and increased coupling of β_2-ARs to the Gi protein/phospholipase C/$cPLA_2\alpha$/COX-2 pathway [11, 12].

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-lysophopholipids [13]. In myocardium the members of three PLA_2 classes are present [14] differing in structure, cellular localization, and Ca^{2+} requirement for catalytic activity and function. 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 [15, 16]. Second, secretory PLA_2 ($sPLA_2$) are low molecular weight enzymes requiring mM Ca^{2+} concentrations for the catalytic activity [17]. They are believed to play an important role in inflammatory processes [18]. Third group comprises intracellular cytosolic PLA_2 ($cPLA_2$). Among six known $cPLA_2$ only $cPLA_2\alpha$ exhibits preference for arachidonic acid (AA) hydrolysis from phospholipids. This FA can either function as an important signaling molecule or be

oxidatively metabolized to various bioactive eicosanoids through cyclooxygenase, lipooxygenase and epoxygenase pathways. In fact, the catalytic activity of $cPLA_2\alpha$ is calcium-independent but low (μM) concentration of intracellular Ca^{2+} [19], which binds to the C2 domain of the enzyme [20], is necessary for the $cPLA_2\alpha$ translocation from the cytosol to the phospholipid membranes [21]. In addition, its activity is enhanced by phosphorylation of serine residues mediated by members of the MAPK family [22], Ca^{2+}/calmodulin-dependent protein kinases II [23], MAPK-interacting kinase Mnk1 [24] and protein kinase C [25].

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 that may affect the $cPLA_2\alpha$/COX-2 pathway.

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$_2$α, p-cPLA$_2$α, 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, Giα(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 Giα(1,2) antibody was described previously [26]. PGE$_2$ EIA Kit and pyrrophenone (cPLA$_2$α 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-12177 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 7,000 m in hypobaric chamber for 8 hours/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 7,000 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 hours 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 [27]. The protein concentration of each preparation was determined by Bradford assay reagent using bovine serum albumin as the standard [28].

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$_2$α, p-cPLA$_2$α, COX-1, COX-2, ERK1/2, p-ERK1/2, p38, p-p38, Giα(1,2), Giα(3), AC5 and AC6), and subsequently incubated with the secondary anti-rabbit or anti-goat antibodies conjugated with horseradish peroxidase for 60 min. In order 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 [29]. 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 [30]. 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 to 7 μm) were fixed in 4% formaldehyde for 5 min and permeabilized in 1% SDS for 5 min. Nonspecific binding sites were blocked by an appropriate normal serum. Cryosections were incubated with primary antibody against p-cPLA$_2$α 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 [31]. 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 [32].

Analysis of PGE_2 concentration

Prostaglandin E_2 (PGE_2) assay was conducted by noncompetitive 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 [33]. 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 15000 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 [34], was measured at an excitation wavelength of 490 nm and emission wavelength of 520 nm using a Synergy™ HT Multi-Detection Microplate Reader (BioTek, 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 [35].

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 [33]. The number of viable (unstained) myocytes was determined by Trypan blue exclusion [36]. 50-100 myocytes were counted in duplicates from 5-8 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 ± S. E. M. 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* values of < 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. 1). 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 Giα proteins revealed a significant effect of CIH on the expression of these main components of the myocardial β-adrenergic signaling system (Fig. 2). 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 of Giα(1,2) and Giα(3) increased by 53% and 49%, respectively. There was no significant change in the expression of the stimulatory Gsα protein (data not shown).

$cPLA_2\alpha$ and its activating proteins

The amount of $cPLA_2\alpha$ and its phosphorylated form ($p\text{-}cPLA_2\alpha$) was increased in LV preparations of CIH-adapted rats compared with normoxic ones by 96% and 41%, respectively (Fig. 3). 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. 4).

Immunofluorescence analysis of $p\text{-}cPLA_2\alpha$

Immunofluorescence analysis revealed that nuclear localization of $p\text{-}cPLA_2\alpha$ in LV myocardium increased by 85% after adaptation to CIH compared with normoxic controls. Co-localization of $p\text{-}cPLA_2\alpha$ with other membranes was not found (Fig. 5). In order to quantify CIH-induced p-

cPLA$_2$α localization to cell nuclei, Mander´s M2 correlation coefficient between the green (p-PLA$_2$α) 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. 5c). Subsequent Western blot analysis confirmed increased immunoreactivity of p-cPLA$_2$α (by 44%) in the nuclear fraction isolated from LV myocardium of CIH-adapted rats compared to the normoxic group (Fig. 6).

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

Fig. 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. 7).

Effect of CIH and acute administration of cPLA$_2$α 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. 8). 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$_2$α 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$_2$α (p-cPLA$_2$α) with the nuclear region of CIH-adapted LV myocardium. However, we did not find any co-localization of p-cPLA$_2$α with other membranes in cardiomyocytes. This observation corresponds well to a previous study where a relocation of cPLA$_2$α 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 [37]. These 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 [38].

To investigate the molecular mechanism of myocardial response to CIH conditions affecting cPLA$_2$α enzyme, we focused on the intracellular signaling cascade responsible for its activation. It has been previously shown that the activating phosphorylation of cPLA$_2$α is provided by MAPKs, notably ERK1/2 and p38 [22, 25, 39]. 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. [40] showing enhanced abundances of both p-ERK1/2 and p-p38 in cardiac tissue from chronically hypoxic rats. On the other hand, Rafiee et al. [41] 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. [42] 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 [43, 44], which had previously been observed under CIH conditions [3].

PKC enzymes were found to be also involved in the regulation of cPLA$_2$α activation and AA release [25] (Nemenoff et al., 1993). As for PKCε, Rafiee et al. [41] 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, 5]. As regards PKCδ, You et al. [45] suggested that the PKCδ–ROS–NF-κB cascade plays a pivotal role in cPLA$_2$α induction in airways epithelium. In the present study, we observed that CIH was associated with PKCα induction. PKCα was demonstrated earlier as the crucial enzyme isoform participating in activation of cPLA$_2$α [46, 47]. 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 [48]. Interestingly, PKCα activity was also enhanced by increased protease activity induced by peroxynitrite treatment of endothelial cells, which was accompanied by phosphorylation of Giα [49]. Pretreatment of the cells with PKCα inhibitor prevented this phosphorylation, $cPLA_2α$ activity and AA release. Conversely, pretreatment with the inhibitor of Gi proteins pertussis toxin inhibited only peroxynitrite-induced increase in $cPLA_2α$ activity. Hence, there is a direct link between the inhibition of Gi proteins by pertussis toxin and suppression of $cPLA_2α$ activation and ROS generation in endothelial cells, which is regulated by PKCα-dependent phosphorylation [49].

There have been several studies reported in the literature dealing with myocardial β-adrenergic signaling during 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 [50, 51, 52, 53, 54, 55]. In parallel, the amount of Gsα was found to be unaffected or reduced, Giα was usually unchanged or somewhat increased, and AC activity was diminished [56, 57, 58]. 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 Giα proteins. These findings are in line with and extend our previous observation of decreased myocardial AC activity in CIH-adapted rats [54]. Interestingly, ablation of AC5 has been shown to be cardioprotective [59]. Moreover, Tong et al. [60] pointed to the significance of $β_2$-ARs in preconditioning-induced cardioprotection. Switching of $β_2$-AR coupling from Gs to Gi is apparently mediated by protein kinase A (PKA). It was previously found that PKA-mediated phosphorylation not only reduced $β_2$-AR coupling with Gs [61] but also enhanced interaction of the receptor with Gi thus reducing cAMP production *via* Gi-mediated inhibition of AC activity [62].

Concerning $cPLA_2α$, Magne et al. [63] demonstrated that $β_2$-AR agonists triggered AA release *via* p38- and ERK1/2-dependent activation of $cPLA_2α$ in embryonic chick ventricular cardiomyocytes. In addition, AA as a hydrolytic product of $cPLA_2α$ has been reported to directly modulate PKCδ and ε isoforms in myocardium [64]. Pavoine et al. [11] 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 [65]. Our present results suggest that CIH enhances β_2-AR/Gi 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 [66, 67].

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 [68, 69, 70]. 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 [71]. 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 [71]. Likewise, another two earlier studies conducted on isolated cardiomyocytes failed to confirm the presumed protective role of cPLA$_2\alpha$ [35, 72]. 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 [73]. 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 [74]. 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 [75]. COX-2 is generally thought to be detrimental in cardiovascular homeostasis [76, 77]. 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 [78]. As far as the formation of eicosanoids is concerned, adaptation to CIH increased PGE_2 concentration in rat heart. Kerkelä et al. [71] 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 [71].

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 Gi 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 support the notion that $cPLA_2\alpha$ participates in the development of a cardioprotective phenotype during adaptation to CIH.

Conflict of Interests

There is no conflict of interest.

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Table 1. Weights parameters of normoxic and CIH adapted rats

Parameter	Normoxia	CIH
BW, g	388 ± 4	351 ± 9*
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 ± S. E. M. of 6 rats in each group. CIH, chronic intermittent hypoxia; BW, body weight; RVW, right ventricular weight; LVW, relative left ventricular weight; RVW/BW, relative right ventricular weight; LVW/BW, relative left ventricular weight; SW/BW, relative septum weight; * $P < 0.05$ hypoxic vs. corresponding normoxic group.

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 ± S. E. M. of 4 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
β_2 (%)	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 ± S. E. M. of 4 left ventricles in each group. CIH, chronic intermittent hypoxia; Ki, inhibition constant; * $P < 0.05$ hypoxic vs. corresponding normoxic group.

Figure legends

Fig. 1

Characterization of the β-adrenoceptors in LV preparations from normoxic *(open circles)* and CIH-adapted *(closed squares)* rats. There are displayed [^{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. Values are represented as mean ± S. E. M. from 3 separate experiments performed in triplicates.

Fig. 2

Effect of chronic intermittent hypoxia on adenylyl cyclase and Gi proteins in rat myocardium. (a) Representative Western blots of AC5, AC6, Gi(1,2)α and Gi3α 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 ± S. E. M. from 4 determinations. * $P < 0.05$ hypoxic vs. corresponding normoxic group.

Fig. 3

Effect of chronic intermittent hypoxia on cPLA$_2$α and p-cPLA$_2$α. (a) Representative Western blots of cPLA$_2$α and p-cPLA$_2$α 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 ± S. E. M. from 6 separate determinations. * $P < 0.05$ hypoxic vs. corresponding normoxic group.

Fig. 4

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 ± S. E. M. from 6 separate determinations. * $P < 0.05$ hypoxic vs. corresponding normoxic group.

Fig. 5

Effect of chronic intermittent hypoxia on subcellular distribution of p-cPLA$_2$α. Representative images show p-cPLA$_2$α 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$_2$α 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$_2$α 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.

Fig. 6

Effect of chronic intermittent hypoxia on p-cPLA$_2$α association with the nuclear fraction. (a) Representative Western blots of p-cPLA$_2$α are shown. (b) The relative amount of p-cPLA$_2$α in LV preparations from normoxic (*empty columns*) and CIH-adapted (*solid columns*) rats is expressed as a percentage of normoxic values. Values are means ± S. E. M. from 6 determinations. * $P < 0.05$ CIH vs. corresponding normoxic group.

Fig. 7

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 ± S. E. M. from 6 separate determinations. * $P < 0.05$ hypoxic vs. corresponding normoxic group.

Fig. 8

Effect of the cPLA$_2$α 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 ± S. E. M. from 8 separate determinations. * $P < 0.05$ hypoxic vs. corresponding normoxic group.

Fig. 1

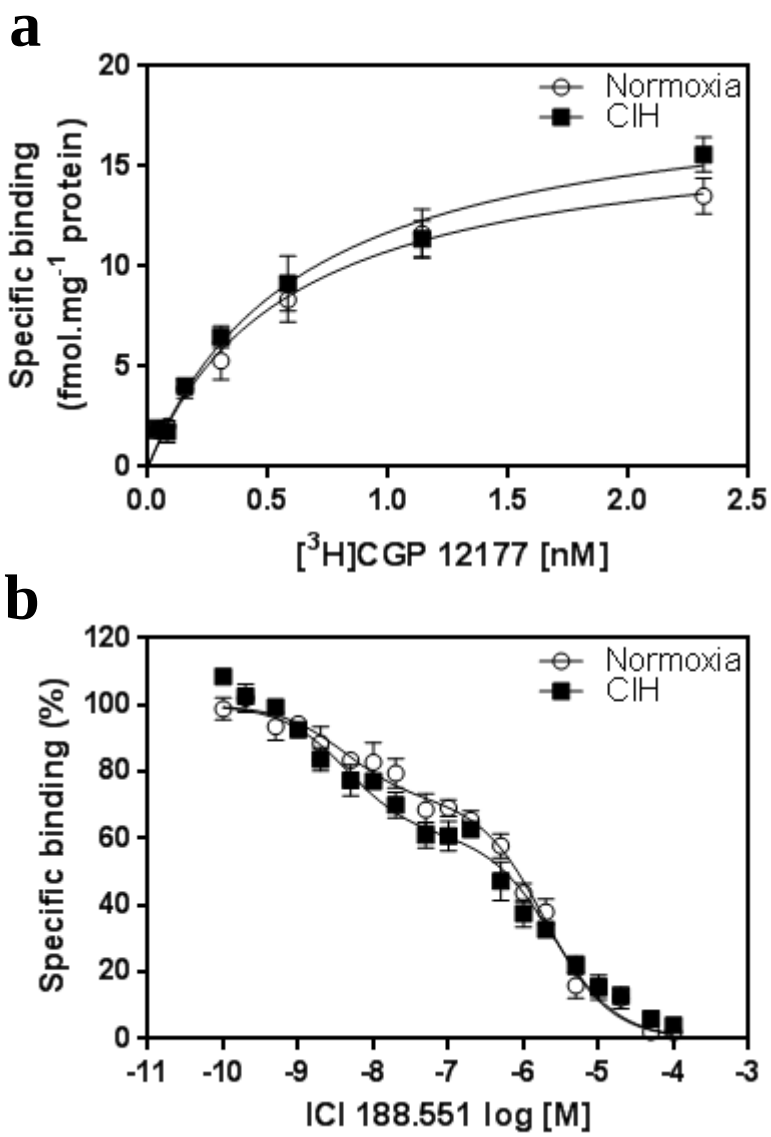
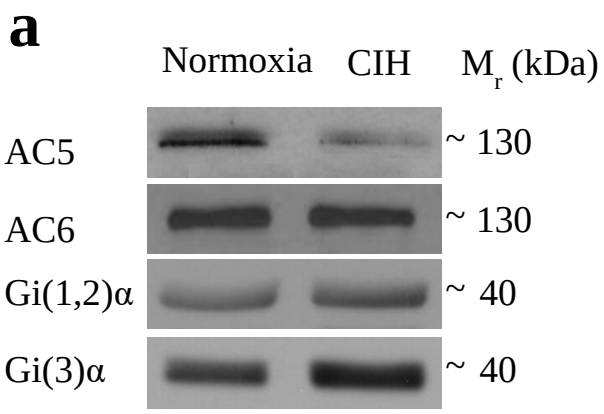
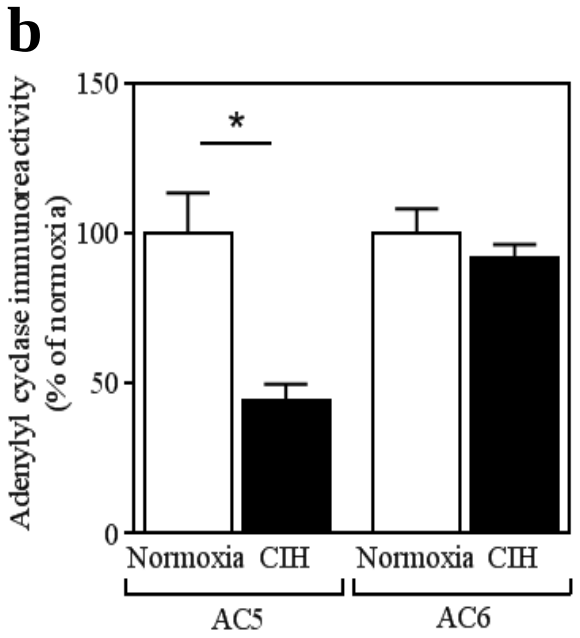

a
Normoxia
CIH
Specific binding (fmol.mg-1 protein)
[3H]CGP 12177 [nM]
b
Normoxia
CIH
Specific binding (%)
ICI 188.551 log [M]

Fig. 2


a
Normoxia
CIH
M r (kDa)
AC5
~ 130
AC6
~ 130
Gi(1,2)α
~ 40
Gi(3)α
~ 40


b
Adenylyl cyclase immunoreactivity (% of normoxia)
150
100
50
0
*
Normoxia CIH
Normoxia CIH
AC5
AC6

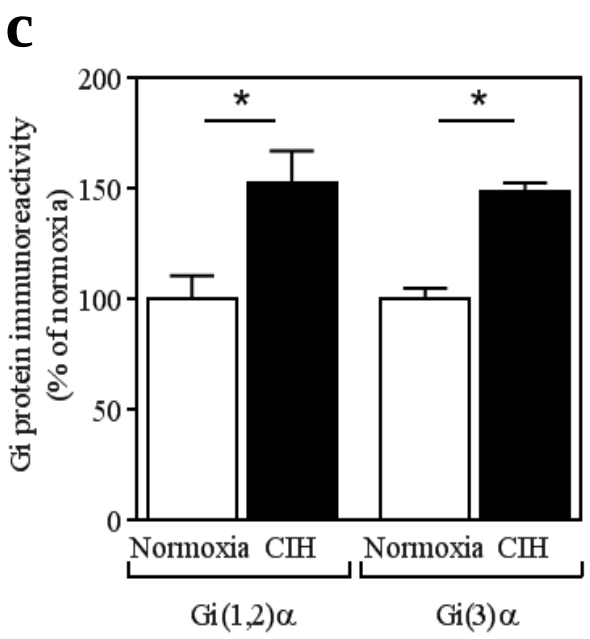

c
Gi protein immunoreactivity (% of normoxia)
200
150
100
50
0
*
*
Normoxia CIH
Normoxia CIH
Gi(1,2)α
Gi(3)α

Fig. 3

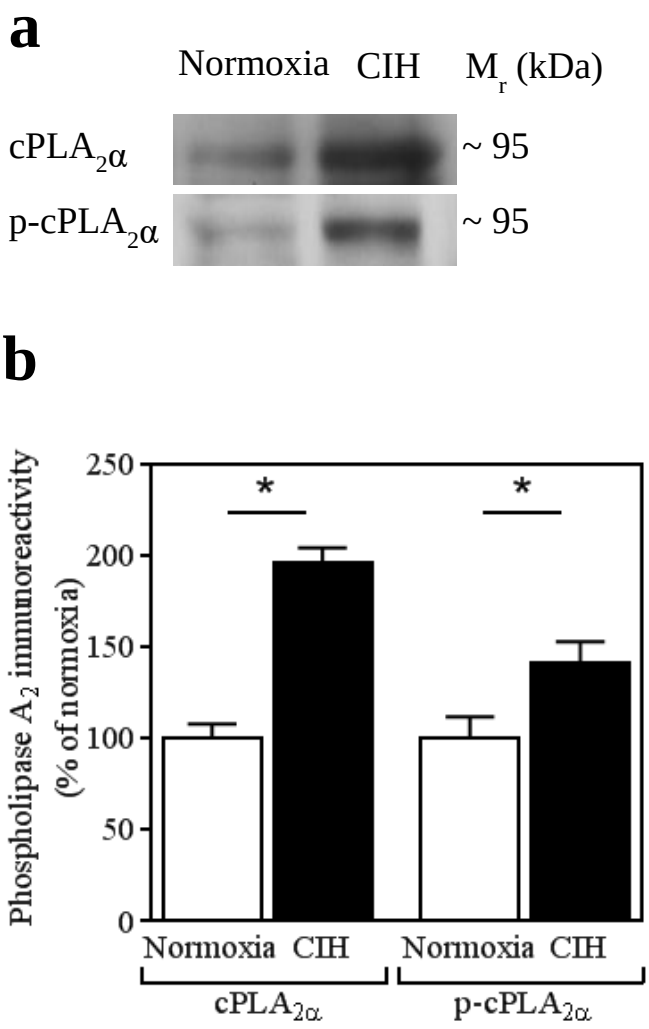

a
Normoxia
CIH
M_r (kDa)
$cPLA_{2}\alpha$
~ 95
$p\text{-}cPLA_{2}\alpha$
~ 95
b
Phospholipase A_2 immunoreactivity (% of normoxia)
250
200
150
100
50
0
*
*
Normoxia
CIH
Normoxia
CIH
$cPLA_{2\alpha}$
$p\text{-}cPLA_{2\alpha}$

Fig. 4

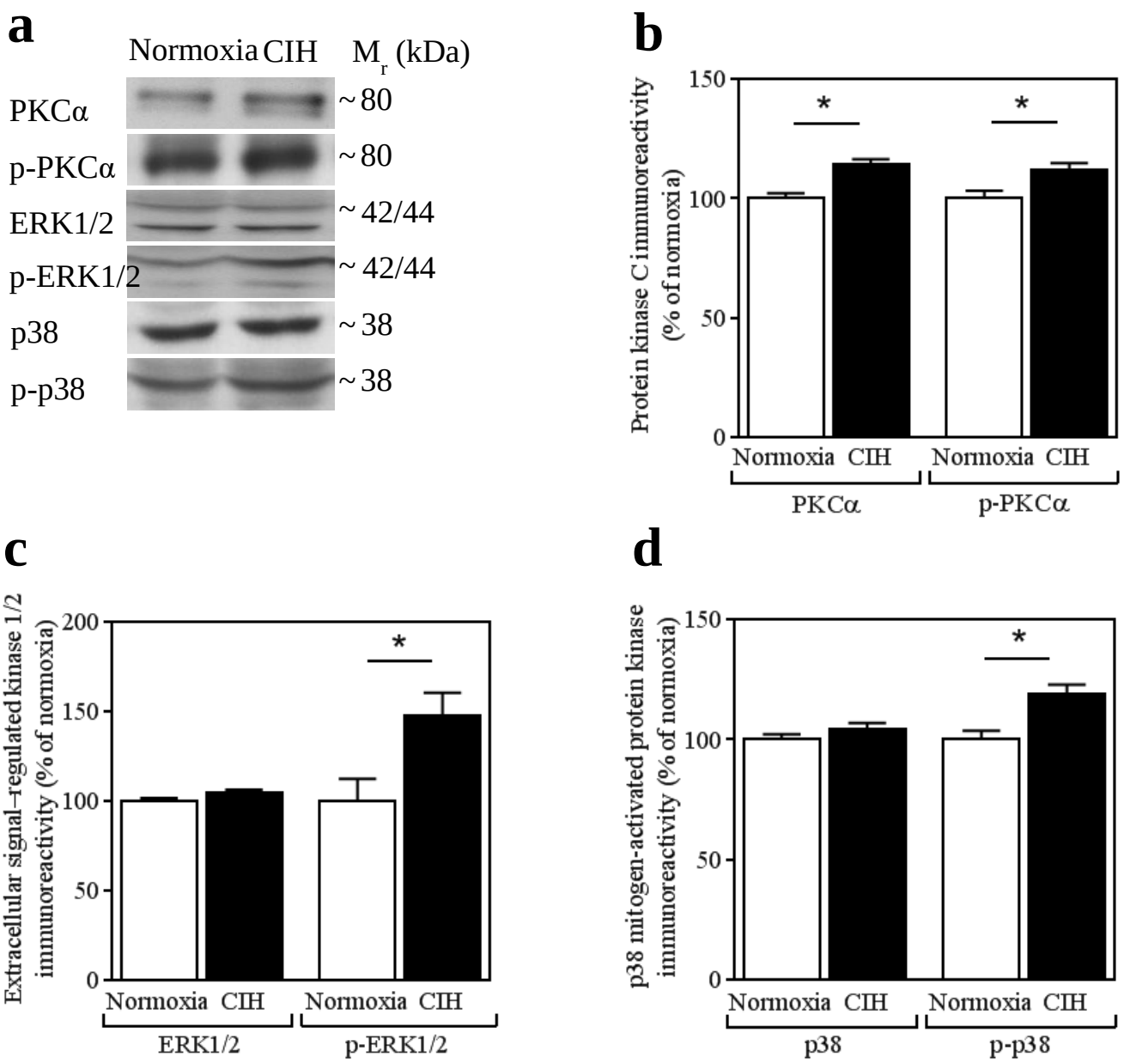
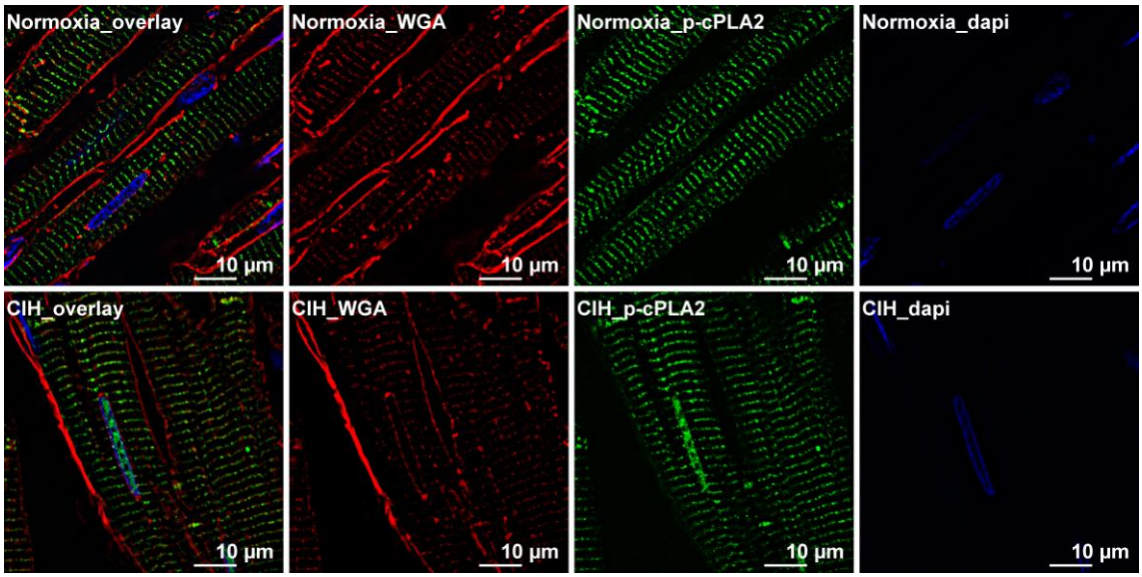
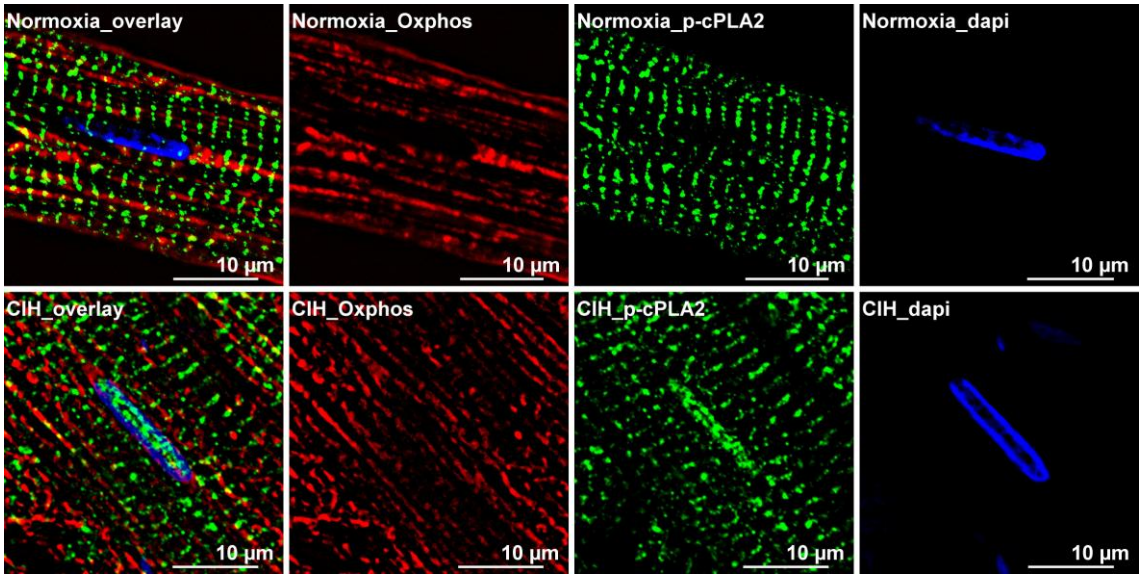

a
Normoxia CIH
M r (kDa)
PKCα
p-PKCα
ERK1/2
p-ERK1/2
p38
p-p38
~ 80
~ 80
~ 42/44
~ 42/44
~ 38
~ 38
b
Protein kinase C immunoreactivity (% of normoxia)
150
100
50
0
*
*
Normoxia CIH
Normoxia CIH
PKCα
p-PKCα
c
Extracellular signal–regulated kinase 1/2 immunoreactivity (% of normoxia)
200
150
100
50
0
*
Normoxia CIH
Normoxia CIH
ERK1/2
p-ERK1/2
d
p38 mitogen-activated protein kinase immunoreactivity (% of normoxia)
150
100
50
0
*
Normoxia CIH
Normoxia CIH
p38
p-p38

Fig. 5

a


Normoxia_overlay
Normoxia_WGA
Normoxia_p-cPLA2
Normoxia_dapi
CIH_overlay
CIH_WGA
CIH_p-cPLA2
CIH_dapi
10 μm

b


Normoxia_overlay
Normoxia_Oxphos
Normoxia_p-cPLA2
Normoxia_dapi
CIH_overlay
CIH_Oxphos
CIH_p-cPLA2
CIH_dapi
10 μm

C

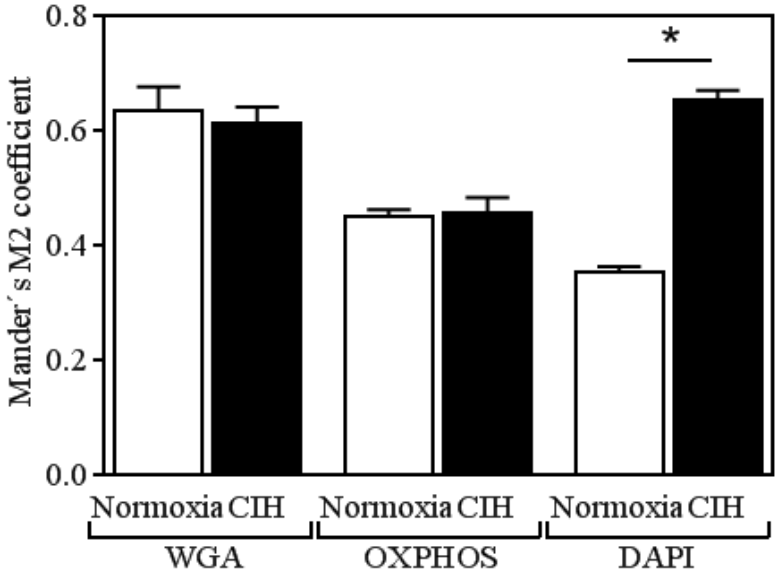

0.8
0.6
0.4
0.2
0.0
Mander´s M2 coefficient
*
Normoxia CIH
Normoxia CIH
Normoxia CIH
WGA
OXPHOS
DAPI

Fig. 6

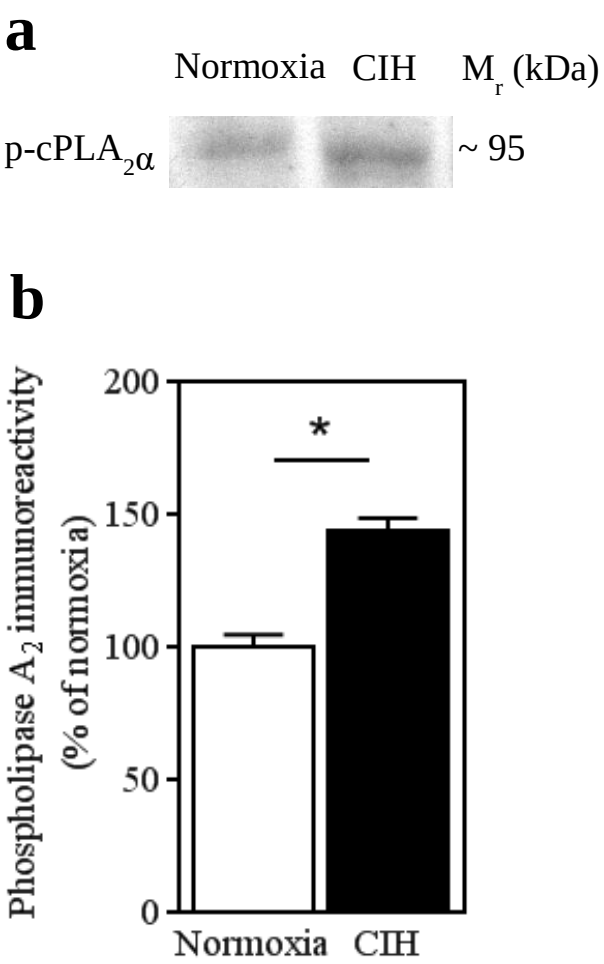

a
Normoxia
CIH
M_r (kDa)
p-cPLA$_2\alpha$
~ 95
b
Phospholipase A_2 immunoreactivity (% of normoxia)
200
150
100
50
0
*
Normoxia
CIH

Fig. 7

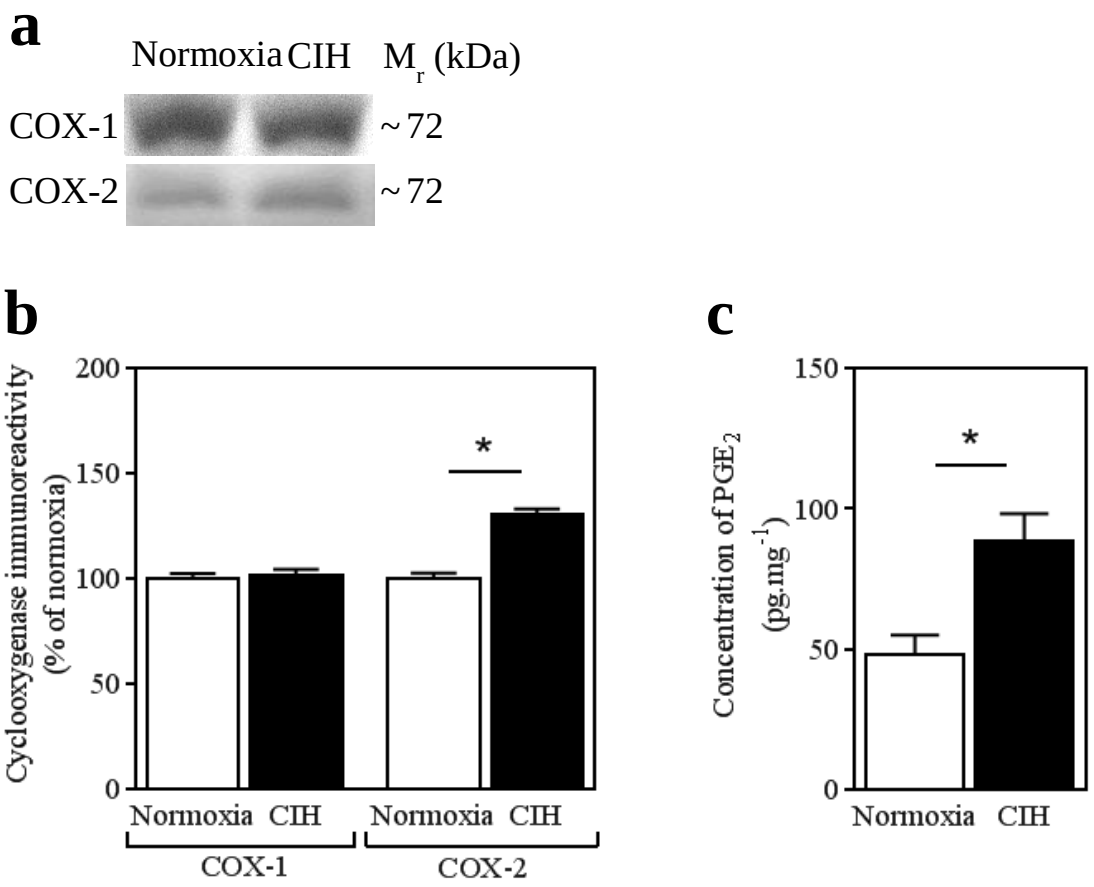
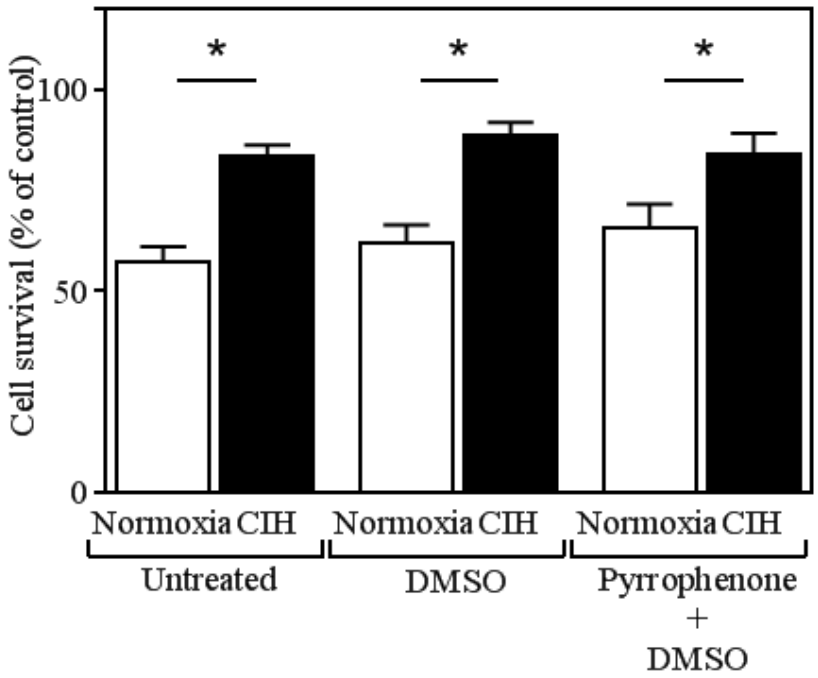

a
Normoxia
CIH
M r (kDa)
COX-1
~ 72
COX-2
~ 72
b
Cyclooxygenase immunoreactivity (% of normoxia)
200
150
100
50
0
*
Normoxia
CIH
Normoxia
CIH
COX-1
COX-2
c
Concentration of PGE2 (pg.mg-1)
150
100
50
0
*
Normoxia
CIH

Fig. 8


Cell survival (% of control)
100
50
0
*
*
*
Normoxia CIH
Normoxia CIH
Normoxia CIH
Untreated
DMSO
Pyrrophenone
+
DMSO