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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

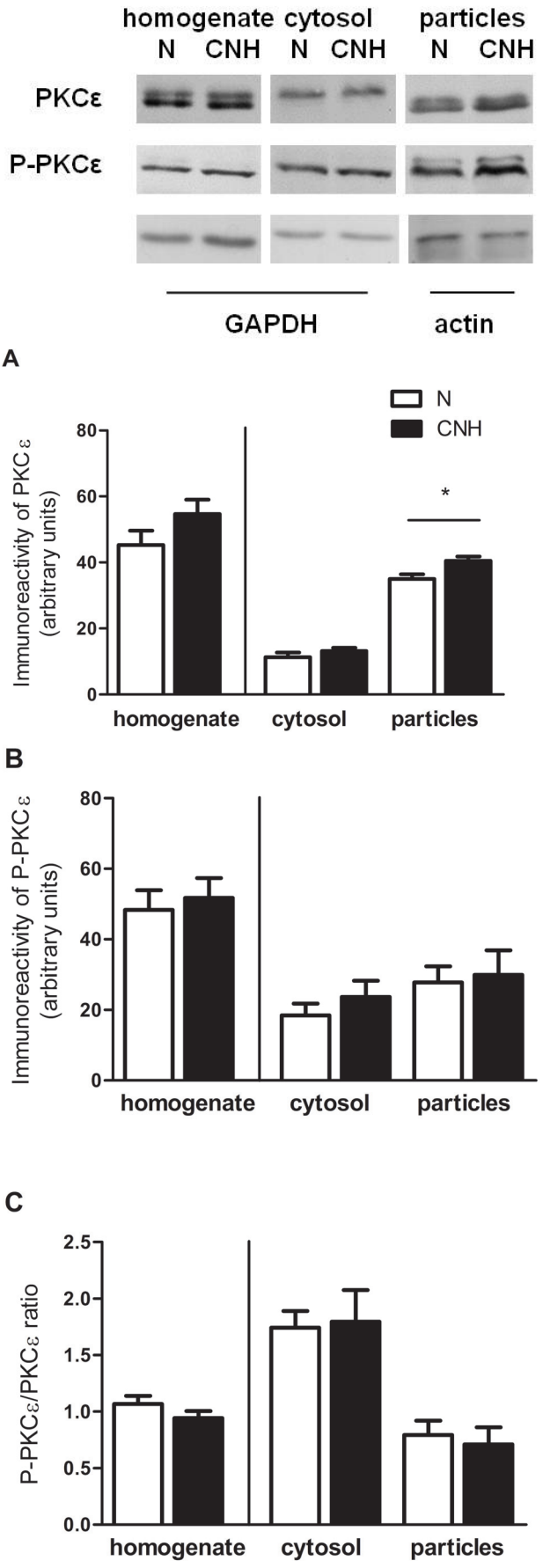

Fig. 1. Effect of continuous normobaric hypoxia (CNH) on the protein levels of PKCε (**A**), P-PKCε (Ser 729) (**B**) and the ratio P-PKCε/PKCε (**C**) in the left ventricular myocardium. Representative Western blots of PKCε and P-PKCε (Ser 729) are shown. The rats were adapted to CNH or kept under normoxic (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).

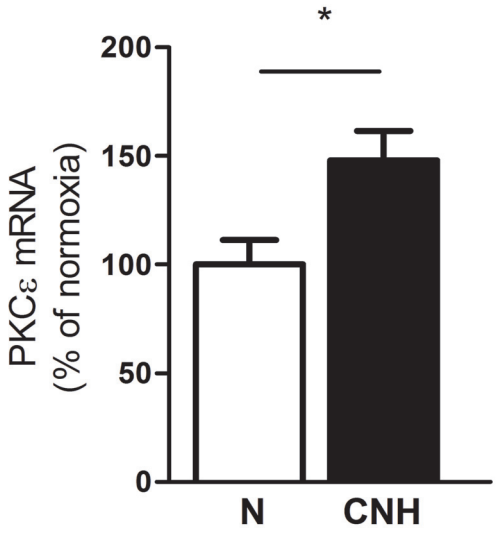

Fig. 2. Effect of continuous normobaric hypoxia (CNH) on myocardial expression of PKCε mRNA. Total mRNA was extracted from left ventricles of rats adapted to CNH or kept in normoxic (N) conditions. The values of mRNA were normalized to the reference gene HPRT1. Values are presented as mean ± SE (n=5/group);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

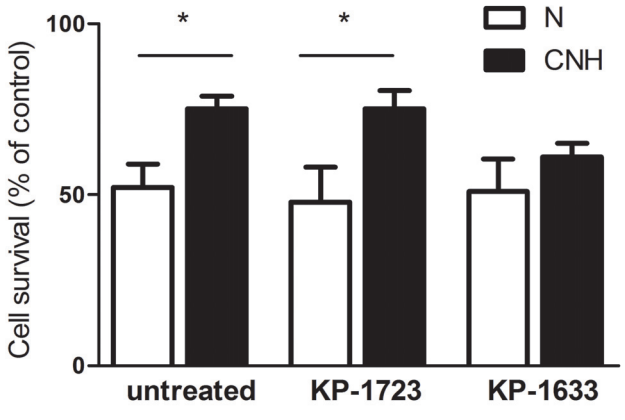

Fig. 3. Effect of the control peptide KP-1723 and the PKCε inhibitory peptide KP-1633 on survival of left ventricular myocytes during acute metabolic inhibition and re-energization, expressed as a percentage of control values. The cells were isolated from rats adapted to continuous normobaric hypoxia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

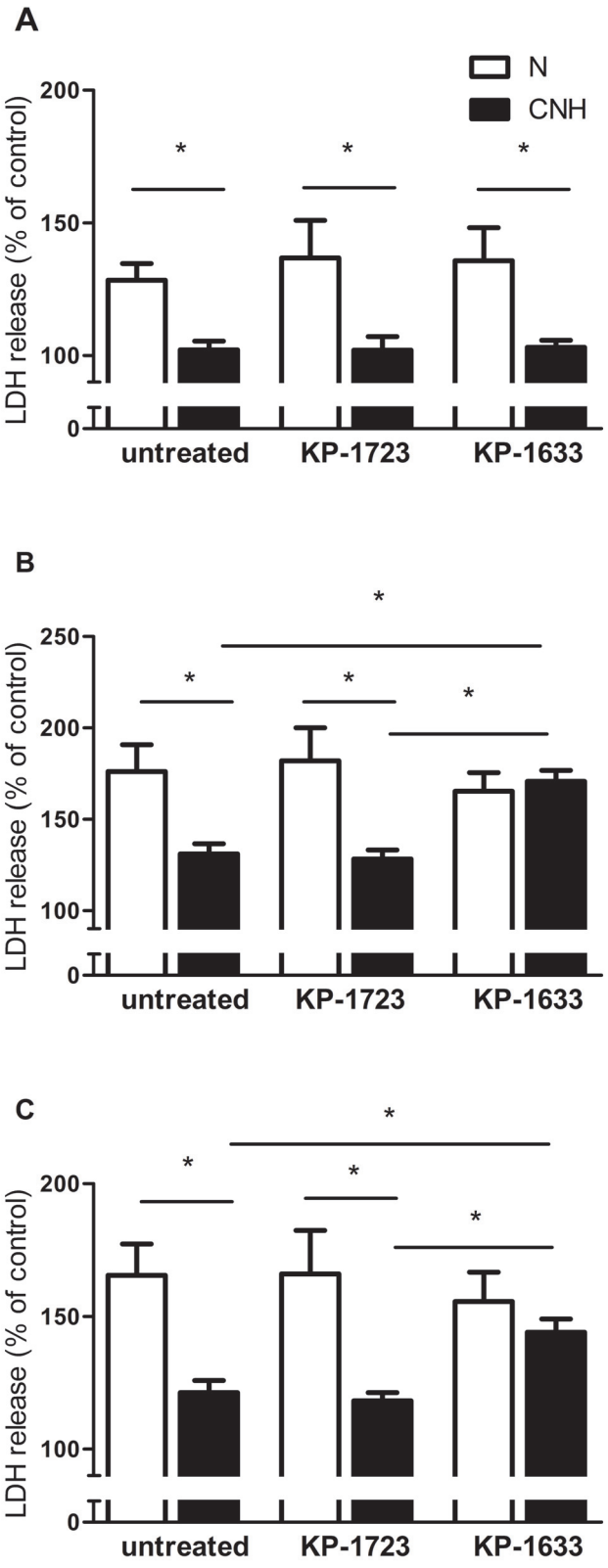

Fig. 4. Effect of the control peptide KP-1723 and the PKCε inhibitory peptide KP-1633 on lactate dehydrogenase (LDH) release from left ventricular myocytes during metabolic inhibition (**A**), during re-energization (**B**), and total release (**C**), expressed as a percentage of corresponding LDH release 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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