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.

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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.

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 μ l/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 ($100,000\times g$ for 60 min) or nuclear–cytoskeletal-enriched (Nucl) ($1,000\times g$ for 10 min), mitochondria-enriched (Mito) ($8,000\times g$ for 10 min), microsomal (Micro) ($100,000\times g$ 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 $100,000\times g$ 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 μ m) 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™, 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™ 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™ co-localization tab.

Statistical analysis

The results are expressed as means $\pm$ 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*), *F1 α* 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 μ g for all subcellular fractions. Values are means $\pm$ 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).

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

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 μ g (homogenate), 15 μ g (cytosolic fraction), or 5 μ g (particulate fraction) for PKC- ϵ and 40 μ g (homogenate), 50 μ g (cytosolic fraction), or 40 μ g (particulate fraction) for p-PKC- ϵ (Ser729). Values are means $\pm$ SE from five hearts in each group. * $P < 0.05$ versus corresponding normoxic group

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

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

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

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 μ g (*homogenate*), 15 μ g (*Cyto*), 5 μ g (*Nucl*), 10 μ g (*Mito*), and 5 μ g (*Micro*). Values are means $\pm$ 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 μ m. (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 $\pm$ 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 $\pm$ 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⁺/Ca²⁺ 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% O₂, 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 F₁F₀ 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 F₁F₀ ATPase have suggested that this putative interaction mediates inhibition of F₁F₀ 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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