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Determination of PKC isoform-specific protein expression in pulmonary arteries of rats with chronic hypoxia-induced pulmonary hypertension

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Evidence indicates that protein kinase C (PKC) plays a pivotal role in hypoxia-induced pulmonary hypertension (PH), but PKC isoform-specific protein expression in pulmonary arteries and their involvement in hypoxia-induced PH are unclear.

Material/Methods: Male SD rats (200–250 g) were exposed to normobaric hypoxia (10% oxygen) for 1, 3, 7, 14 and 21 d (days) to induce PH. PKC isoform-specific membrane translocation and protein expression in pulmonary arteries were determined by using Western blot and immunostaining.

Results: We found that only 6 isoforms of conventional PKC (cPKC) α, βI and βII, and novel PKC (nPKC) δ, ε and η were detected in pulmonary arteries of rats by Western blot. Hypoxic exposure (1–21 d) could induce rat PH with right ventricle (RV) hypertrophy and vascular remodeling. The cPKCβII membrane translocation at 3–7 d and protein levels of cPKCα at 3-14 d, βI and βII at 1-21 d decreased, while the nPKCδ membrane translocation at 3–21 d and protein levels at 3–14 d after hypoxic exposure in pulmonary arteries increased significantly when compared with that of the normoxia control group ($p<0.05$ *vs.* 0 d, n=6 per group). In addition, the down-regulation of cPKCα, βI and βII, and up-regulation of nPKCδ protein expressions at 14 d after hypoxia were further confirmed by immunostaining.

Conclusions: This study is the first systematic analysis of PKC isoform-specific membrane translocation and protein expression in pulmonary arteries, suggesting that the changes in membrane translocation and protein expression of cPKCα, βI, βII and nPKCδ are involved in the development of hypoxia-induced rat PH.

key words: **protein kinase C (PKC) • chronic hypoxia • pulmonary hypertension (PH) • membrane translocation • protein expression**

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Background

Pulmonary hypertension (PH) is characterized by high pulmonary arterial pressure, increased pulmonary vascular resistance, right ventricular (RV) hypertrophy, and RV failure [1–3]. The pathogenesis of PH remains unclear, but is probably mediated by hypoxia. Hypoxic exposure induces a selective pulmonary arterial vasoconstriction and an increase in pulmonary arterial pressure [4]. When hypoxic pulmonary vasoconstriction (HPV) is persistent, pro-proliferative signals are activated in vascular cells, which leads to vascular remodeling and chronic hypoxic PH [5]. Pulmonary vascular remodeling is characterized by intimal thickening, medial smooth muscle cell (SMC) hypertrophy and hyperplasia, adventitial fibroblast proliferation, and matrix protein synthesis [6,7]. The magnitude of the fixed structural changes depends on many factors, including developmental stage, genetic factors and type of insult. Protein kinase C (PKC) is an important signaling, intermediate in many of the vascular responses such as contraction and growth, and may play a key role in chronic hypoxic PH [8].

The PKC family of serine/threonine protein kinases is involved in multiple biochemical processes, including cell growth, differentiation and transformation [9,10]. So far, at least 10 PKC isoforms have been identified in mammals and classified into 3 subgroups based on their molecular structures and sensitivity to activators: conventional PKC (cPKCα, βI, βII and γ), requiring both Ca^{2+} and diacylglycerol for activation; novel PKC (nPKCδ, ε, η and θ); and atypical PKC (aPKCι/λ and ζ) [11]. Numerous PKC isozymes are expressed in pulmonary vascular media smooth muscle and adventitial fibroblasts [12,13], and their activations are generally marked by its translocation from the cytosolic fraction to the particulate or membrane-related fraction [14,15].

PH is a major cause of disability in patients with chronic lung, heart and sleep disorders, and supplemental oxygen and supportive care are the only treatments currently available. PKC-based therapy might be a complementary therapeutic tool, but the role of individual PKC isoforms in the regulation of chronic hypoxic PH remains unclear. Previous studies have suggested that PKC mediates hypoxia-induced proliferation and growth of pulmonary artery adventitial fibroblasts and smooth muscle cells [16–19]. Ca^{2+}-dependent cPKCα and β expressed in pulmonary artery smooth muscle cells (PASMCs) are particularly important in their hypoxic growth and, ultimately, the development of chronic hypoxic PH [17,20,21]. Das et al. found that bovine pulmonary artery adventitial fibroblasts expressed cPKCα, βI and βII, nPKCδ and ε, and aPKCζ, but only cPKCβI and aPKCζ activations were associated with the exaggerated growth responses of pulmonary artery adventitial fibroblasts under chronic hypoxia condition [16,22]. However, Short et al. found that aPKCζ attenuates hypoxia-induced proliferation of fibroblasts by regulating MAP kinase phosphatase-1 (MKP-1) expression [23].

Unfortunately, most of the experiments were carried out *in vitro* and some results are contradictory. The systematic investigation of PKC isoform-specific protein expression and activation in pulmonary vascular regions were rare in whole animal models with chronic hypoxia. Therefore, the primary aim of this study was to determine the PKC isoform-specific involvement in the development of hypoxic PH by observing their protein expression and membrane translocation in pulmonary arteries of hypoxia-induced PH rats.

Material and Methods

The following materials were obtained from the indicated sources: proteinase inhibitors (leupeptin, aprotinin, pepstatin A and chymostatin); phosphatase inhibitors (okadaic acid, sodium pyrophosphate and potassium fluoride); and monoclonal anti-β-actin antibody, as well as other reagents, such as dithiothreitol (DTT), Nonidet P-40, ethylene diamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), and sodium dodecyl sulfate (SDS), were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Protein assay reagent, horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG were purchased from Bio-Rad Company (Hercules, CA, USA).

Animals and hypoxic exposure

All procedures conducted in this study were approved by the Animal Care and Use Committee of Capital Medical University and were consistent with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). Specific pathogen-free adult male Sprague-Dawley (SD) rats, weighing 200–250 g, were exposed for the specified time periods (1, 3, 7, 14 and 21 d [days]) to normobaric hypoxia (10% oxygen) in a ventilated Plexiglas chamber while age- and weight-matched control rats were maintained in a 21% oxygen environment (n=12 per group).

To establish the hypoxic conditions as previously reported [24], the chamber was flushed with a mixture of oxygen and nitrogen from high-pressure cylinders, and an oxygen analyzer was used to monitor the chamber environment. CO_2 was removed with soda lime, excess humidity was prevented by Drierite granules, and boric acid was used to keep ammonia levels within the chamber to a minimum. The chamber was opened every other day for 30 min (minutes) to clean the cages and replenish food and water.

Hemodynamics and estimation of RV hypertrophy

At the end of the hypoxic exposure, the animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and a 1.4F microtip pressure transducer (SPR-671; Millar Instruments; Houston, TX, USA) was inserted into the right ventricle (RV) through the jugular vein for hemodynamic measurements. RV systolic pressure (RVSP, an indirect index of pulmonary artery pressure) was measured with a polygraph system (PowerLab; AD Instruments, Australia). The RV was dissected from the left ventricle (LV) and the septum (S), and weighed separately to determine the ratio of RV to LV plus S, RV/(LV+ S) as RV hypertrophy [25].

Subcellular fractionation and whole tissue homogenate preparations

The animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.), exsanguinated in the hypoxic chamber, and the lungs were removed from the thoracic cavity. Under a microscope, pulmonary arteries, including the main trunk plus the right and left branches, were isolated in ice-cold

phosphate-buffered saline (PBS) containing 120 mM NaCl, 2.7 mM KCl, and 10 mM phosphate buffer, and were quickly frozen in liquid nitrogen for later analysis.

As in our previous reports [15,26], the frozen samples were placed into 100 ìl freshly prepared homogenization buffer A (50 mM Tris-Cl, pH 7.5, 2 mM DTT, 2 mM EDTA, 2 mM EGTA, 5 mg/ml each of leupeptin, aprotinin, pepstatin A and chymostatin, 50 mM potassium fluoride, 50 μM okadaic acid, 5 mM sodium pyrophosphate) and homogenized. The homogenate was centrifuged at 30 000 g for 30 min, and the supernatant was collected as cytosolic fraction. The pellet was further solubilized in 100 μl homogenization buffer B (buffer A containing 0.5% Nonidet P-40) before being sonicated and centrifuged at 30 000 g for 30 min again, and the resulting supernatants were taken as particulate fractions. Both fractions were prepared for PKC isoform-specific membrane translocation analysis. To determine PKC isoform-specific protein expression levels, frozen pulmonary arteries were homogenized in 250 μl homogenization buffer C (buffer A containing 2% SDS) and sonicated to dissolve completely as the whole tissue homogenate. The protein concentration was determined by the BCA protein assay (Pierce Company, Rockford, IL, USA).

Western blot analysis

As described previously [26,27], equal amounts of vascular protein (50 μg from either cytosolic or particulate fractions, and 20 μg from whole tissue homogenate) per lane were loaded on 10% SDS-PAGE gels. After separation, the proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Buckinghamshire, UK) at 4°C. After several rinses with TTBS (20 mM Tris-Cl, pH7.5, 0.15 M NaCl and 0.05% Tween-20), the transferred PVDF membrane was blocked with 10% non-fat milk in TTBS for 1 h (hour) and incubated with the primary rabbit polyclonal antibodies against cPKCα, βI, βII and γ, nPKCδ, ε, η and θ, and aPKCι/λ and ζ (Santa Cruz Biotechnology Inc., CA, USA) at 1:5000 dilution for 3 h. To verify equal loading of protein, the blots were stripped by incubating the membranes for 45 min in stripping buffer containing 62.5 mM Tris-Cl (pH 6.7), 2% SDS and 100 mM 2-mercaptoethanol at 55°C and reprobed with primary mouse monoclonal antibody against β-actin (Sigma-Aldrich Company, St. Louis, MO, USA). The horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Stressgen Biotechnologies Corporation, Victoria, BC, Canada) were used as second antibodies at a 1:3000 dilution for 1 h incubation. Following incubation with the primary and secondary antibodies, the Enhanced Chemiluminescence (ECL) kit (GE Healthcare, Buckinghamshire, UK) was employed to detect the signals.

To quantify membrane translocation, the ratio of PKC isoform (band density in particulate/band densities in both particulate and cytosolic fractions) of the control group was normalized to 100%, and hypoxia groups were expressed as percentages of the control group. For the quantitative analysis of PKC isoform-specific protein expressions, the optical density of each band corresponding to PKC isoforms (from whole tissue homogenate) was normalized to that of β-actin. The protein expression ratio in the control group was regarded as 100%, and data from the hypoxia groups were also expressed as percentages of the control group.

Hematoxylin and eosin staining and immunohistochemistry

The animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and lungs were removed from the thoracic cavity. Right lower lungs were fixed in 10% formaldehyde for at least 24 h. Paraffin slices (6 μm thickness) were stained with hematoxylin and eosin (HE) solution. HE-stained images of the slices were examined under a microscope to look for the structural changes of pulmonary artery walls in response to hypoxic exposure.

To determine the changes of PKC isoform-specific expressions in pulmonary arteries of rats after chronic hypoxia-induced pulmonary hypertension, immunostaining with cPKCα, βI, βII, γ and nPKCδ (Santa Cruz Biotechnology Inc., CA, USA) were performed as reported [27]. Lungs were infused by the fixative (3% formaldehyde in PBS) via the trachea, and then immersed in the fixative at 4°C for 24 h. The tissues were dehydrated, embedded in paraffin, and sectioned at a thickness of 5 μm. The slices were incubated with 0.3% H_2O_2 for 10 min to exhaust the endogenous peroxidase and washed in 0.1 M phosphate-buffered saline (PBS, pH7.4). Next, they were blocked with 10% bovine serum for 1 h. The blocked slices were incubated with primary rabbit polyclonal antibodies against cPKCα, βI, βII and γ (Santa Cruz Biotechnology Inc., CA, USA) at a dilution of 1:200 for 12 h at 4°C. The specimens were incubated in secondary antibodies of horseradish peroxidase-conjugated goat anti-rabbit IgG for 2 h. Finally, the slices were thoroughly washed in PBS, and a solution containing H_2O_2 (0.03%) and 3,3'-diaminobenzidine (DAB, 60%) was added to visualize the slices. Control sections were conducted the same way, but primary antibodies were omitted. The images were captured by a Leica microscope imaging system (Leica DM4000B, Wetzlar, Germany).

Statistical analysis

Quantitative analysis for immunoblotting was done after scanning of the X-ray film with Quantitative-one software (GelDoc 2000 imaging system, Bio-Rad, Hercules, CA, USA). The presented values were expressed as mean ±SE from at least 6 independent experiments. Statistical analysis was conducted by one-way analysis of variance followed by all pairwise multiple comparison procedures using the Bonferroni test, and the significance was regarded as $p<0.05$.

Results

Physiologic analysis and pathologic changes in pulmonary arteries of hypoxia – induced PH rats

To confirm the establishment of hypoxia-induced PH in the rats, RVSP (a marker of systolic pulmonary arterial pressure) and the RV/(LV+S) ratio (a marker of RV hypertrophy) were measured. RVSP (mmHg) increased significantly ($p<0.05$ *vs.* 0 day 23.8±0.8, n=12) with the time of hypoxic exposure (1 d: 34.6±1.4; 3 d: 40.4±1.7; 7 d: 48.3±1.1; 14 d: 46.2±2.4; 21 d: 50.1±1.3, n=12 per group). There was also a significant increase in the RV/(LV+S) ratio ($p<0.05$ *vs.* 0 day 100%, n=12) of rats following hypoxic exposure (1 d: 111.7±8.4; 3 d: 127.8±9.3; 7 d: 133.9±10.1; 14 d: 148.7±9.8; 21 d: 156.5±8.6, n=12 per group). In addition, we also observed the pathologic changes at days 3, 7 and 21 in pulmonary

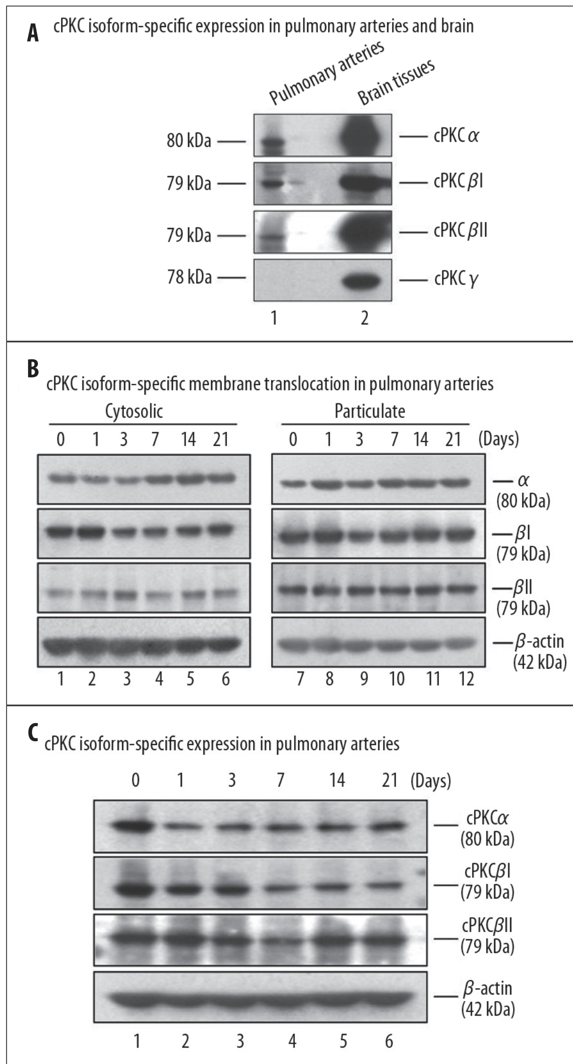

Figure 1. Determination of cPKC isoform-specific protein expression and membrane translocation in pulmonary arteries of PH rats. (**A**) Typical Western blot results showed cPKC isoform-specific protein expression in rat pulmonary arteries (lane 1) and brain (lane 2). (**B**) Typical Western blot results showed the changes in cPKCα (80 kDa), βI (79 kDa) and βII (79 kDa) membrane translocation in pulmonary arteries of rats following hypoxic exposures. (**C**) Typical Western blot results showed the changes in cPKCα, βI and βII protein expressions in pulmonary arteries of rats following hypoxic exposures for 0, 1, 3, 7, 14 and 21 days. n=6 per group.

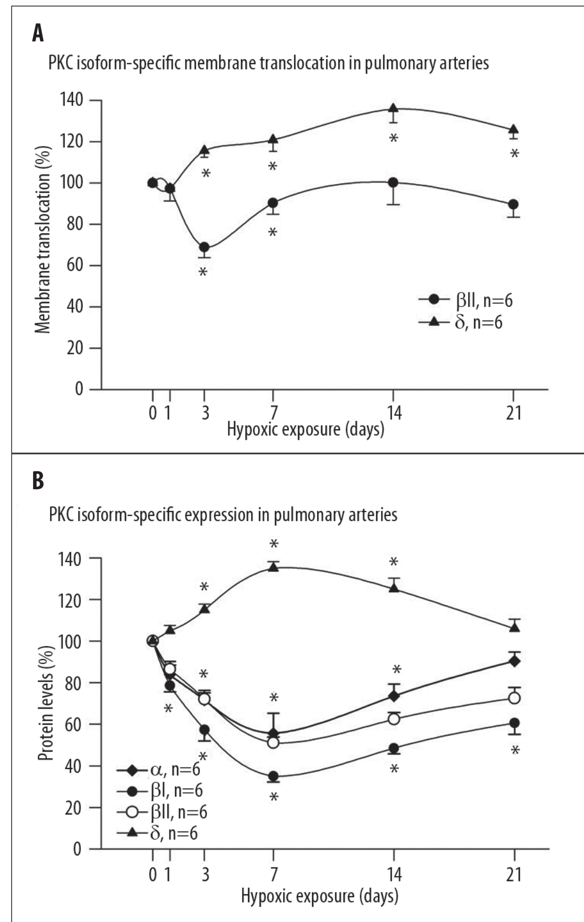

Figure 2. Changes of PKC isoform-specific membrane translocation and protein expressions in pulmonary arteries of PH rats. (**A**) Quantitative analysis indicated that the membrane translocation levels of nPKCδ increased significantly in response to hypoxic exposure 3–21 days, while cPKCβII membrane translocation decreased in pulmonary arteries of rats after hypoxic exposure 3–7 days when compared with that of the normoxia control group (0 day, 100%). * $p<0.05$, n=6 per group. (**B**) Quantitative analysis demonstrated that the protein expression levels of nPKCδ increased significantly in response to hypoxic exposure 3–14 days, while cPKCα, βI and βII protein expression levels decreased significantly at day 1, 3, 7, 14 and 21 (except cPKCα) after hypoxic exposures when compared with that of normoxia control group (*vs.* 0 day, 100%). * $p<0.05$, n=6 per group.

arteries of rats after hypoxic exposure. There was marked vascular remodeling, including medial hypertrophy and adventitial proliferation, in both the distal and the proximal pulmonary arteries of the PH rats when compared with that of control rats. These results suggest that prolonged exposure to hypoxia can induce PH with RV hypertrophy of rats.

PKC isoform-specific expression and membrane translocation in pulmonary arteries of hypoxia-induced PH rats

The typical Western blot results in Figure 1A showed that cPKCα (80 kDa), βI (79 kDa) and βII (79 kDa), not γ (78 kDa), presented in pulmonary arteries of rats. In addition, we found a decrease in membrane translocation of cPKCβII, but not α and βI, in pulmonary arteries of PH rats (Figures 1B and 2A). The translocation of cPKCβII from cytosolic to particulate fraction significantly decreased at 3 d (68.9±5.1%) and 7 d (90.3±5.5%) after hypoxia when compared with that of the normoxic control group (0 day 100%).

As shown in Figures 1C and 2B, cPKC isoform-specific protein levels decreased significantly during 1–14d (83.9±6.2%, 71.6±4.7%, 55.6±9.7% and 73.6±5.7%, $p<0.05$) for cPKCα, 1–21d (68.6±2.9%, 47.4±7.4%, 25.0±2.7%, 38.5±2.7% and 40.6±5.5%, $p<0.05$) for cPKCβI and 1–21d (72.1±3.1%,

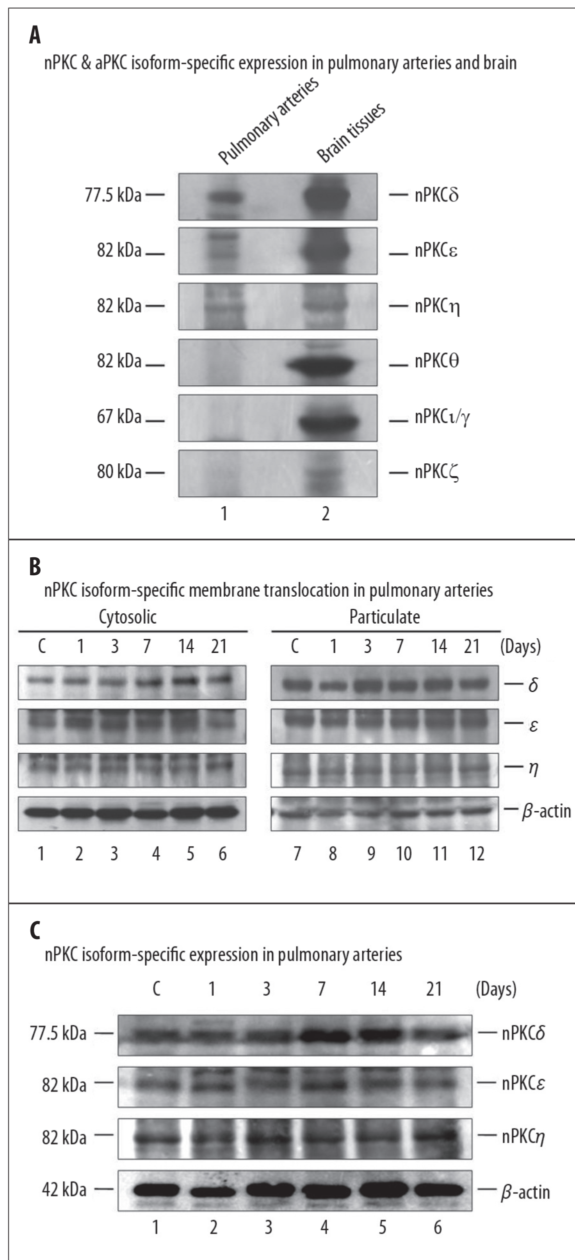

Figure 3. Identification of nPKC and aPKC isoform-specific membrane translocation and protein expression in pulmonary arteries of PH rats. (**A**) 20 µg of whole tissue homogenates from rat pulmonary arteries (lane 1) and brain (lane 2) were separated by 10% SDS-PAGE gel, electrophoretically transferred onto PVDF membrane and then probed with nPKC and aPKC isoform-specific primary antibodies. (**B**) Typical Western blot results showed the changes in nPKCδ (77.5 kDa), ε (82 kDa) and η (82 kDa) membrane translocation in pulmonary arteries of rats following hypoxic exposures. (**C**) Typical Western blot results showed the changes in nPKCδ, ε and η protein expressions in pulmonary arteries of rats under hypoxic exposures for 0, 1, 3, 7, 14 and 21 days. n=6 per group.

41.1±2.8%, 62.4±3.2% and 72.5±5.1%, $p<0.05$) for cPKCβII after hypoxia when compared with that of the normoxic control group (0 day 100%).

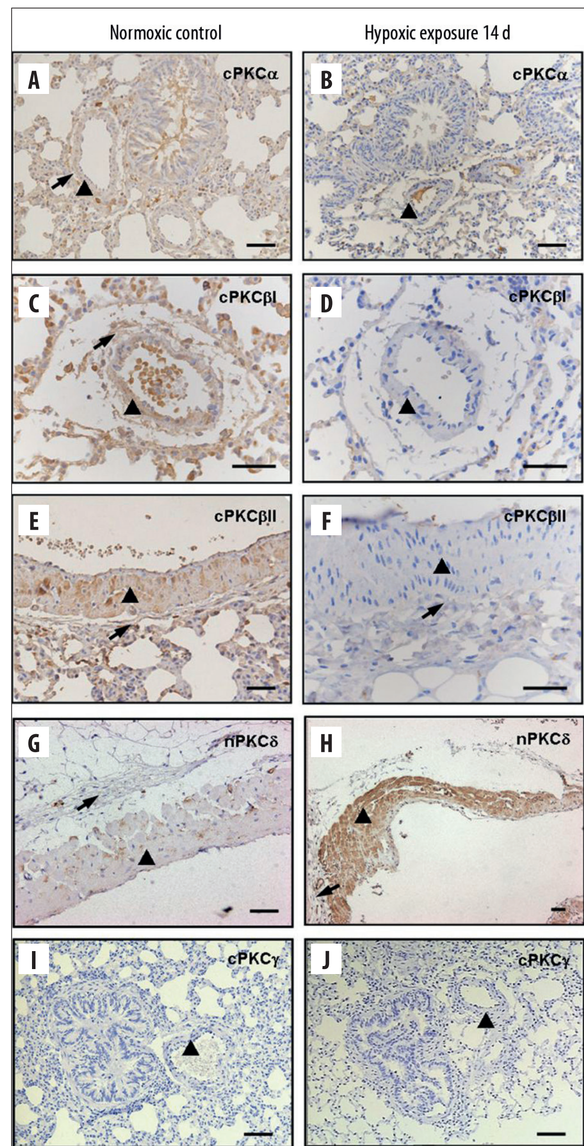

Figure 4. Immunostaining of cPKCα, βI, βII and nPKCδ in pulmonary arteries of rats following hypoxic exposure 14 days. Lung sections of rats under normoxia (**A, C, E, G and I**) and hypoxia exposure 14 days (**B, D, F, H and J**) were stained with polyclonal antibodies against cPKCα (**A and B**), βI (**C and D**), βII (**E and F**), nPKCδ (**G and H**) and cPKCγ (**I and J**, negative control). Triangle and black arrow indicated medial layer and adventitial layer respectively, bars =50 µm, n=6 per group.

However, we could only detect the protein expressions of nPKCδ (77.5kDa), ε (82kDa) and η (82kDa), but not nPKCθ and aPKCι/λ and ζ, in pulmonary arteries of rats by Western blot (Figure 3A). Unlike cPKCβII, significant increases of membrane translocations (Figures 2A and 3B) and protein expressions (Figures 2B and 3C) of nPKCδ, not ε and η, were observed in pulmonary arteries of rats following hypoxia exposures.

cPKC isoform-specific cellular localization in pulmonary arteries of chronic hypoxia-induced HP rats

To further characterize the cellular localization of cPKCα, βI, βII and nPKCδ in the pulmonary arteries of hypoxia-induced

PH rats, we performed immunostaining and found that the immunolabeled cPKCα, βI, βII and nPKCδ proteins were diffusely located throughout the cytoplasmic areas of pulmonary artery smooth muscle cells and fibroblasts (Figure 4A, C, E, G, n=6 per group). Furthermore, the decreased protein expressions of cPKCα, βI and βII, and the increased expression of nPKCδ could be observed at 14 d after hypoxia (Figure 4B, D, F, H, n=6 per group).

Discussion

This study reports the first description of the pattern of PKC isoform-specific membrane translocation and protein expression in pulmonary arteries of rats with hypoxia-induced PH. Assender et al. analyzed the expression of PKC isoforms in smooth muscle cells (SMC) by using Western blots and thermocycle amplification of mRNA, and demonstrated the presence of cPKCα, nPKCδ, ε and aPKCζ in SMC in various states of differentiation [28]. Liao et al. showed the presence of cPKCα, βI, nPKCδ, ε, aPKCζ in rat aortic vascular SMC using Western blot analysis [29]. cPKCα, βI and βII, nPKCδ and ε, and aPKCζ were reported in neonatal bovine pulmonary artery adventitial fibroblasts and pulmonary artery SMCs from chronically hypoxic neonatal calves [16,19]. In addition, the expressions of cPKCα, βII and γ, nPKCδ and ε, and aPKCζ were observed in ventricles of chronically hypoxic rats [30,31]. Similarly, our *in vivo* experiment demonstrated that 6 isoforms of cPKCα, βI and βII, nPKCδ, ε and η expressed in SMC and fibroblasts of rat pulmonary arteries by using Western blot and immunohistochemistry methods.

Pulmonary artery SMC and fibroblast proliferation occurs with hypoxic PH *in vivo* [6]. PKC is a key protein kinase cascade leading to cell proliferation in response to growth factors, hormones and antigens. However, it is not known whether PKC is involved in the signaling of hypoxia-induced pulmonary remodeling in an *in vivo* model. Dempsey et al. reported that the Ca^{2+}-dependent PKC, especially cPKCα, is particularly important in hypoxia-induced pulmonary artery SMC growth and ultimately the development of chronic PH by observing the cultured pulmonary artery SMC *in vitro* [17,19–21]. Das et al. found that cPKCα and βII contribute to the augmented proliferative response of immature bovine pulmonary artery adventitial fibroblasts [16]. Zhu et al. reported that specific PKC isozymes mediate forskolin-induced activation of large-conductance, calcium- and voltage-activated potassium (BKCa) channels in pulmonary vascular smooth muscle (PVSM) [32]. Although investigators have reported cPKC activation in hypoxic condition, we found in this study that the decreases of cPKCβII membrane translocation and cPKCα, βI and βII protein expressions, as well as the increases of nPKCδ membrane translocation and protein expression, were involved in the development of hypoxia-induced PH of rats. These results are consistent with previous observations from *in vivo* cerebral ischemia and primary neuronal cultures damaged by glutamate [33,34], which suggest that decreased cPKCβII activation probably provides a negative feedback mechanism in cell hypoxic growth, and the down-regulation of cPKC isoform-specific protein expression might be linked to the augmented growth capacity of pulmonary artery SMC. Mayr et al. also reported that nPKCζ is crucial in maintaining SMC differentiation [35]. The decrease of cPKCα, βI and βII protein expressions may be the result of enhanced proteolytic degradation by Ca^{2+}-activated proteinases during chronic hypoxia [36]. The discrepancy of studies shows the role of PKC isoform in proliferation might be cell type- and species-specific.

In this study, we also found that the development of hypoxia-induced rat PH was not accompanied by exaggerated pulmonary vascular remodeling at the early stage, suggesting that vasoconstriction is a major contributor to the increased pulmonary artery pressure. PKC activity was reported to be involved in the transduction pathway forwarding pulmonary vasoconstriction in response to alveolar hypoxia [37], but which PKC isoform is responsible for vasoconstriction is unclear. Dempsey et al. demonstrated that nPKCε-null mice have decreased hypoxic pulmonary vasoconstriction (HPV) [38]; the Ca^{2+}-independent vascular contraction appears to be associated with plasmalemmal translocation of nPKCε [39]. Our finding of increased nPKCδ membrane translocation and protein expression in pulmonary arteries of hypoxia-induced PH rats is consistent with the observation that nPKCδ was involved in modulating contraction via actions on the myofilaments [13]. These data show that the different PKC isoforms appear to have distinct functions regarding phosphorylation of specific substrate proteins such as cell proliferation and vasoconstriction.

Conclusions

In conclusion, our study is the first to demonstrate the PKC isoform-specific membrane translocation and protein expression systematically in a preparation of a whole animal model of hypoxia-induced rat PH. The present results indicate that the changes in membrane translocation and protein expression of cPKCα, βI, βII and nPKCδ are involved in the development of hypoxia-induced rat PH. Future experiments will focus on evaluating the PKC isoform-specific role in pulmonary artery constriction and proliferation.

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