Up-regulation of nPKC Contributes to Proliferation of Pulmonary Artery Smooth Muscle Cells in Hypoxia-induced Pulmonary Hypertension

Yiwei Shi
Department of respiratory and critacal care medicine, Shanxi Mecical University Affiliated First Hospital
Rui Jiang
Shanxi Provincial Peoples Hospital
Xiaojiang Qin (sph@sxmu.edu.cn)
Shanxi Medical University https://orcid.org/0000-0001-6393-9355
Anqi Gao
School of Public Health, Shanxi Medical University
Xiaomin Hou
Department of Pharmacology, Shanxi Medical University
Liangjing Chen
Shanxi Medical University
Xinrong Xu
Shanxi Medical University School of Public Health
Lina Chai
School of Public Health, Shanxi Medical University
Yunting Guo
School of Public Health, Shanxi Medical University
Liangyuan Zhao
Department of Exercise Rehabilitation, Shanxi Medical University
Furong Wu
Shanghai Clinical Research Center for Tuberculosis, Shanghai Pulmonary Hospital, Tongji University School of Medicine

Research

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Abstract

Background It has been indicated that protein kinase C (PKC) plays a vital role in the pathogenesis of hypoxia-induced pulmonary hypertension (PH). The functions or the pathogenic roles of PKCs vary from different types, and their related downstream pathways may also be distinct. Therefore, the specific role of different types of PKC deserves to be elucidated. Discussions regarding conventional PKC (cPKC) have dominated research in recent years, however, the relationship between novel PKC (nPKC) and the development of PH remain unclear. In addition, it is less known whether nPKC has a direct effect on the proliferation of pulmonary artery smooth muscle cells (PASMCs). This study is designed to investigate the role of nPKC in mediating PASMCs proliferation in PH and the underlying mechanisms.

Methods Mouse PASMCs was isolated using magnetic separation technology. The PASMCs were divided into 24 h group, 48 h group and 72 h group according to different hypoxia treatment time, then detected cell proliferation rate and nPKC expression level in each group. We treated PASMCs with agonists or inhibitors of PKCδ and PKCε and exposed them to hypoxia or normoxia for 72 h, then measured the proliferation of PASMCs. We also constructed a lentiviral vector containing siRNA fragments for inhibiting PKCδ and PKCε to transfected PASMCs, then examined their proliferation.

Results PASMCs isolated successfully by magnetic separation method and were in good condition. Hypoxia promoted the proliferation of PASMCs, and the treatment for 72 h had the most significant effect. Hypoxia upregulated the expression of PKCδ and PKCε in mouse PASMCs, leading to PASMCs proliferation. Moreover, Our study demonstrated that hypoxia induced upregulation of PKCδ and PKCε expression resulting to the proliferation of PASMCs via up-regulating the phosphorylation of AKT and ERK.

Conclusions Our study provides clear evidence that increased nPKC expression contributes to PASMCs proliferation and uncovers the correlation between AKT and ERK pathways and nPKC-mediated proliferation of PASMCs. These findings may provide novel targets for molecular therapy of pulmonary hypertension.

Background

PH refers to a pathological state in which the mean pulmonary artery pressure ≥ 25 mmHg measured by cardiac catheterization in a resting state[1]. It is a lethal syndrome that results in the progressive increase of right ventricular load, right ventricular failure, and ultimately whole heart failure[2]. With early age of onset, numerous causes, great risks and difficulties in cure, PH has attracted much attention in the world, and was currently divided into five categories, including: pulmonary arterial hypertension (PAH), pulmonary hypertension due to left heart disease, hypoxic pulmonary hypertension, chronic thromboembolic pulmonary hypertension, and PH with unclear and/or multifactorial mechanisms, of which HPH is the most common[3].

HPH was first proposed by von Euler and Liljestrand in 1946. When they provided the anesthetized cats with 10% O_2, they found a notably increase in pulmonary artery pressure but only a slight increase in left atrial pressure, then named this phenomenon as the von Euler-Liljestrand mechanism[4]. With further researches, pulmonary vasoconstriction and excessive proliferation of PASMCs caused by hypoxia have been recognized as important contributors to HPH[5]. HPH is characterized by remodeling of pulmonary arterioles, rarely affecting pulmonary arteries[6], and the significant increase in PASMCs proliferation is one of the main reasons that lead to pulmonary arterioles hyperplasia and remodeling[7]. Although abnormal proliferation of PASMCs plays a critical role in the pathogenesis of HPH, the specific mechanisms are still not clearly understood.

Recent studies suggest that PKC is closely correlated with the development of HPH. PKC belongs to the Ser/Thr protein kinase family and has been detected to contain about 12 types divided into three categories: classical PKC, novel PKC and atypical PKC[8]. nPKC includes four types: PKCδ, PKCε, PKCη and PKCθ. As shown in researches, PKC activated RhoA/Rock to promote contraction of PASMCs by inhibiting MLCP, which disappeared when PKC inhibitors were used[9]. It has also been confirmed that hypoxia induced up-regulation of PKCα expression resulting to the proliferation of PASMCs[10]. Moreover, other studies have suggested that PKC expression changes do not directly affect cell proliferation but may alter a series of downstream proliferation-related pathways. To illustrate, PKC can inhibit cell proliferation by regulating the NF-κB signaling pathway to reduce the expression of proliferation-related genes[11]. In addition to PASMCs proliferation, there have also been reports that PKC promotes cell proliferation in endothelial cells[12]. As described above, the pathogenic roles of PKCs vary from different types, and their related downstream pathways may also be distinct. Therefore, the specific role of different types of PKC deserves to be further elucidated.

PKC has been implicated in hypoxic-induced proliferation of PASMCs and discussions regarding cPKC have dominated research in recent years, however, the relationship between nPKC and the development of HPH has rarely been studied directly. Thus, in this study, mice PASMCs were used to explore the relationship between 4 types of nPKC and cell proliferation under hypoxia-induced condition and to investigate their underlying mechanisms.

Our results demonstrated that hypoxia induced upregulation of PKCδ and PKCε expression resulting to the proliferation of PASMCs via up-regulating the phosphorylation of AKT and ERK. Proliferation of PASMCs has been recognized as a major factor in PH, nPKC may therefore represent a potential target for the treatment of PH. These findings may provide novel targets for molecular therapy of pulmonary hypertension.

Methods

Isolation of mouse PASMCs by magnetic separation

Healthy adult C57BL/6J mice weighing about 20 g were obtained from the Laboratory Animal Center, Shanxi Medical University. After anesthesia by intraperitoneal injection of 4% chloral hydrate (0.01 ml/g), the mice were planned to expose the kidneys, and their renal arteries were severed. After the thorax was exposed, 3-5 ml of sterilized PBS solution was slowly infused from the right ventricle to the pulmonary artery until the lungs turned white. Slowly injected 3-5 ml PA agarose into the right lung until the lungs turned gray. Removed heart and lungs and placed in ice-cold PBS for about 5 min to allow the gel to coagulate. The lung tissue was shredded and then transferred to a 50 ml sterile centrifuge tube. The magnet was attached, and the ferrous tissue moved to the wall of the centrifuge tube near the magnet. The PBS solution was then aspirated, and the lung tissue was washed 3 times with 5 ml sterile PBS solution. 6 ml of collagenase solution was added to the centrifuge tube, poured into a petri dish and digested for 1 hour at 37℃. One hour later, lung tissue and collagenase were repeatedly aspirated and transferred to a sterilized centrifuge tube. The magnet was connected, and the supernatant was aspirated and washed with complete medium to inactivate the collagenase. Finally, added complete medium and then transferred the cell suspension into Petri dish and incubate overnight (5% CO_2, 37℃). On the second day, the tissue pieces in the culture dish were poured into a 50 ml centrifuge tube. Connected the magnet, used a complete culture medium to clean the centrifuge tube for 3 times, and then transferred into a new sterile Petri dish, placed in 5% CO_2, 37 ℃ incubator to continue culturing. 3-5 days to replace the medium. During the culturing process, magnetic separation techniques may again be used to separate the tissue fragments in the culture vessel according to cell growth. About 50% of the cells that first crawled out of the iron-containing vessels were smooth muscle cells and the other 50% were fibroblasts. Because the remarkable growth superiority of fibroblast, the culture dish was discarded to promise the better growth of smooth muscle cells. When the cells were fused to about 80%, 500 μL trypsin was added into T25 culture bottle, and the cells were left for 3-5 min at room temperature. When the cells became round, complete culture medium were added to neutralize the pancreatin, and then a pipette was used to suck the cells to promote shedding. In the first passage, after the cell suspension was made and the magnets were connected, the iron particles were seen to gather on the wall of the centrifuge tube near the magnet. The cells were collected into the T25 culture bottle with the discard of the centrifuge tube, and undergo repeated magnetic separation, the iron particles were eventually all be discarded. The cells were cultured to the desired number and state through several passages.

Immunofluorescence staining

PASMCs were fixed with 4% paraformaldehyde for 10 min, and were incubated with 0.2% permeable Triton X-100 for 15 min. Being washed three times with PBS, the cells were blocked with goat serum for 1 h, and then incubated with anti-smooth muscle (1:100) and anti-smooth muscle heavy chain (1:80) primary antibodies overnight at 4°C. Cells were then washed three times with PBS, followed by incubation with fluorescein isothiocyanate-conjugated secondary antibody and tetramethylrhodamine-conjugated secondary antibody for 1 h in the dark. After three washes with PBS, the cell nuclei were stained with DAPI for 10 min and observed by confocal laser scanning microscope. All the above procedures were carried out at room temperature.

Hypoxia treatment of mouse PASMCs

According to different hypoxia treatment times, the cells were divided into 24 h group, 48 h group and 72 h group, and each group was set up with a normoxia control group. The cells of the normoxia group were cultured in a CO_2 incubator with the following culture conditions: 37℃, 5% CO_2, 21% O_2; the cells of the hypoxia group were cultured in a tri-gas incubator with the following culture conditions: 37℃, 5% CO_2, 3% O_2, 92% N_2. Cell proliferation in each group was detected by using CCK-8 and Brdu method. Similarly, PASMCs with good growth status were selected and randomly divided into 0 h group, 24 h group, 48 h group and 72 h group according to different hypoxia treatment time. Western-blot and flow cytometry were used to determine the expression of PKCδ, PKCε, PKCη and PKCθ under different hypoxic induction time. In order to investigate the relationship between hypoxia-induced up-regulation of nPKCs and the proliferation of PASMCs and its possible mechanism, PASMCs were divided into the following groups according to different interventions. PKCδ: Normoxia group, Normoxia+PMA group, Hypoxia group, Hypoxia+Rottlerin group. PKCε: Normoxia group, Normoxia+PMA group, Hypoxia group, Hypoxia+PKCε inhibitor peptide group.

Small interfering RNA treatment of mouse PASMCs

For siRNA treatment, PASMCs with a growth density of approximately 70-80% of the culture flask bottom were selected. A lentiviral vector containing siRNA fragments for inhibiting PKCδ and PKCε, respectively, was constructed and the control group was set up with no-load lentiviral vector. The volume of lentiviral vector required was calculated based on MOI=40 and the concentration of the different vectors. After calculation, the lentiviral vectors were then mixed with incomplete medium and incubated in a hypoxic incubator for 72h. Group by different interventions: Normal group, Scramble group, PKCδ knockdown group, PKCε knockdown group. After treatments, western blot was used to detect the expression of PKCδ, PKCε, P-AKT, AKT, P-ERK and ERK in each group. Cell proliferation was detected by using Brdu method.

Brdu incorporation

Cells were washed three times with PBS and Brdu solution was diluted 2000 times. 5 μl of Brdu solution was then mixed with 10 mL of FBS-DMEM solution at a concentration of 10%, avoiding light throughout the process, the total volume of the mixture depends on the number of samples. Then 5 mL of the mixture was added to each flask of cells, followed by incubation in a normoxia incubator for 72 h. After the treatment, discarded the mixture and washed with PBS three times. Cells were then fixed at room temperature with 4% paraformaldehyde for 20 min. After fixation, cells were washed three times with PBS and 2 M HCl was added, followed by a 37°C water bath for 10 min. 0.1% TritonX-100 was used to break the cell membrane structure for 5 min. Being washed three times with PBS, the cells were blocked with goat serum for 1 h, and then incubated with Brdu primary antibody overnight at 4°C. Cells were then washed with PBS, followed by incubation with fluorescein isothiocyanate-conjugated secondary antibody at 37°C for 3 h. After three washes with PBS, the cells were stained with DAPI for 10 min and observed by confocal laser scanning microscope.

CCK-8 assay

CCK-8 assay was used to detect PASMCs proliferation. PASMCs were seeded in 96-wells plates at the concentration of 4×10^3 cells/well, then the 96-wells plates were put into normoxic and hypoxic incubators respectively to detect cell proliferation in the normoxic group for 24 h, 48 h and 72 h and the hypoxic group for 24 h, 48 h and 72 h. Each well was added with 10 μl of CCK-8 solution and continued to incubate for 2 h. Finally, the optical density value of absorbance at 450 nm was measured.

Western blotting

After treatments, cells were washed in cold PBS for three times, collected and scraped with cell lysis buffer and then kept in a 4°C refrigerator for 2 h. The lysates were then centrifuged at 12000 r/min for 15 min, the supernatants were collected, and the total protein concentrations were calculated. Proteins with equal amounts (20 μg) were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking with 5% BSA at room temperature for 1 h, the membranes were then incubated with antibodies specific for rabbit anti-PKCδ (1:5,000), rabbit anti- PKCε (1:1,500), rabbit anti-PKCη (1:100), rabbit anti-PKCθ (1:5,000) and rabbit anti-β-actin (1:5,000) overnight at 4°C. Subsequently, the membranes were incubated with a secondary antibody for 2 h at 4°C.

Flow cytometry

Cells in good condition were digested with trypsin free of EDTA, then washed with PBS, centrifuged at 1000 r/min for 5 min, and the cell sediment was collected. Cells were then fixed with 1% paraformaldehyde for 10 min and 0.05% Triton was used to break the cell membrane structure for 1 min. After three washes with PBS, the cells were blocked with goat serum for 1 h. Being washed three times with PBS, the cells incubated with primary antibody at room temperature for 1 h. Cells were then washed with PBS, followed by incubation with secondary antibody at room temperature for 30 min. After the treatment, washed with PBS three times and detected the result.

Statistical analysis

All statistical analyses were performed using SPSS16.0, and the results were presented as Mean ± SD. The one sample *t* test was used for comparison between groups. $P<0.05$ was considered statistically significant. The statistical graphs were drawn using Prism Graph Pad 8.01.

Results

PASMCs isolated by magnetic separation were in good condition

To improve the success rate of cell separation and lay a good experimental foundation for the construction of hypoxia models, we isolated mouse PASMCs by magnetic separation. As shown in Fig. 1A, after the first isolation of PASMCs, small pieces of microvascular tissue that were black and contained iron particles were visible by ordinary light microscopy. It was shown that gel and iron powder

were successfully injected into the right lung and the iron powder bound to pulmonary artery tissue through the gel. After 3 days, cells were seen to crawl out of the small iron-containing vessels (Fig. 1B). The morphology of the cells appeared long fusiform radial. After passage, vascular smooth muscle cells showed "peak-valley" growth after 7–10 days (Fig. 1C). Thus, initially identified from the morphology of pulmonary artery smooth muscle cells. Therefore, it could be preliminarily identified as PASMCs from morphology. We further observed the immunofluorescence staining of mouse PASMCs under a laser scanning confocal microscope. The blue fluorescence was the nucleus, the green fluorescence was positive for smooth muscle protein expression, and the red fluorescence was positive for smooth muscle myosin heavy chain (Fig. 1D-G). The positive expression of two specific proteins related to smooth muscle cells in immunofluorescence indicated that the cells isolated by magnetic separation method were PASMCs. These results show that the magnetic separation of mouse PASMCs is feasible, and the isolated PASMCs are in good condition for the following experiments.

Hypoxia induced proliferation of PASMCs in mice

To address the effects of hypoxia on PASMCs proliferation, we divided the cells into 24 h group, 48 h group and 72 h group according to different hypoxia treatment. The results of CCK-8 showed that there was no significant difference in cell viability between Hypoxia 24 h group and Normoxia 24 h group ($P > 0.05$), and there was no significant difference between Hypoxia 48 h group and Normoxia 48 h group ($P > 0.05$). However, the cell viability of 72 h Hypoxia group was remarkably higher than that of Normoxia 72 h group ($P < 0.05$) (Fig. 2A). Likewise, the results of cell proliferation detected by Brdu suggested that there was no obvious difference between Hypoxia 24 h group and Normoxia 24 h group ($P > 0.05$). The survival rate of 48 h Hypoxia group was higher than that of Normoxia 48 h group ($P < 0.05$), and notably, the survival rate of 72 h Hypoxia group was higher than that of Normoxia 72 h group ($P < 0.05$) (Fig. 2B-C). These experiments demonstrated that hypoxia promote the proliferation of PASMCs, and the effect can be significantly increased when hypoxia is induced for 72 h. Therefore, we select hypoxia induction 72 h as the follow-up experimental conditions.

PKCδ and PKC□ were up-regulated in hypoxia-exposed PASMCs

Furthermore, we investigated the expression levels of nPKC in hypoxia-exposed PASMCs. The results of flow cytometry suggested that the expressions of PKCδ and PKCε in hypoxia 72 h were outstandingly increased ($P < 0.05$) compared with those in normoxia group, while PKCη and PKCθ were not significantly different at 72 h ($P > 0.05$) (Fig. 3A-B). In parallel with flow cytometry, western blot showed that the expression of PKCδ and PKCε increased notably at 72 h ($P < 0.05$), while PKCη and PKCθ had no clear difference (Fig. 3C-D). Taken together, the above results confirm that in normal PASMCs, hypoxia can induce the upregulation of PKCδ and PKCε expression but has no obvious effect on the expression of PKCη and PKCθ.

Hypoxia induced up-regulation of PKCδ and PKC□ resulting to PASMCs proliferation

To address the roles of PKCδ and PKC in PASMCs proliferation, we divided PASMCs into several groups according to different interventions. According to the results of Brdu test (Fig. 4A-B), compared with Hypoxia group, there was no significant difference in cell proliferation of Normoxia + PKCδ group and Normoxia + PKCε group ($P > 0.05$); equally, compared with Normoxia group, there was no significant difference in cell proliferation of Hypoxia + Rottlerin group and Hypoxia + PKCε inhibitor peptide group ($P > 0.05$). These results indicate that the inhibitory and agonistic effects of inhibitors and agonists of PKCδ and PKCε were in accordance with the experimental requirements. Compared with Normoxia group, cell proliferation of Normoxia + PMA group was remarkably increased ($P < 0.05$), proving that simply up-regulating PKCδ and PKCε can induce PASMCs proliferation. Moreover, compared with Hypoxia group, cell proliferation of Hypoxia + Rottlerin group and Hypoxia + PKCε inhibitor peptide group was reduced. This suggests that the inhibition of PKCδ and PKC can reverse hypoxia-induced PASMCs proliferation.

Hypoxia induced AKT and ERK phosphorylation by upregulating PKCδ and PKCε

To further explore the possible mechanism of PKCδ and PKCε up-regulation promoting PASMCs proliferation, we measured the phosphorylation levels of AKT and ERK, which play an important role in cell proliferation. Western blot results revealed that compared with Normoxia group, the expressions of PKCδ and PKCε in Normoxia + PMA group were significantly increased ($P < 0.05$), and compared with hypoxia group, the expressions of PKCδ and PKCε in Hypoxia + Rottlerin group and Hypoxia + PKCε inhibitor peptide group were notably decreased ($P < 0.05$), which indicated that the agonistic and inhibitory effects of PKCδ and PKCε were in line with experimental requirements (Fig. 5A-B). Compared with Normoxia group, the phosphorylation of AKT and ERK in Normoxia + PMA group was increased significantly ($P < 0.05$) (Fig. 5C-D). Compared with the Hypoxia group, the phosphorylation of AKT and ERK in Hypoxia + Rottlerin group and Hypoxia + PKCε inhibitor peptide group was surprisingly inhibited ($P < 0.05$) (Fig. 5C-D). These results indicate that hypoxia can increase the phosphorylation of AKT and ERK by upregulating PKCδ and PKCε. In order to reinforce the role of nPKC in mediating hypoxia-induced migration of PASMCs, the cells were transfected with lentiviruses containing siRNA fragments for inhibiting PKCδ and PKCε. After lentiviral transfection, PASMCs were cultured under hypoxia for 72 h. The transfection efficiency was confirmed by Western blot analysis (Fig. 5E-F). Compared with the Normal hypoxia group, the phosphorylation of AKT and ERK in the PKCδ and PKCε knockdown group was significantly inhibited ($P < 0.05$) (Fig. 5G-H). Similarly, compared with the Normal hypoxia group, cell proliferation of PKCδ and PKCε knockdown group was remarkably inhibited (Fig. 5I-J). These results suggest that knockdown of PKCδ and PKCε resulting to the inhibition of PASMCs proliferation via down-regulating the phosphorylation of AKT and ERK.

Discussion

Our study demonstrated that hypoxia induced upregulation of PKCδ and PKCε expression resulting to the proliferation of PASMCs via up-regulating the phosphorylation of AKT and ERK.

It has been documented that PKC plays an important role in the development of PH. The functions or the pathogenic roles of PKCs vary from different types, and their related downstream pathways can also be distinct. Discussions regarding cPKC have dominated research in recent years, for instance, it has shown that hypoxia induced up-regulation of PKCα expression resulting to the proliferation of PASMCs[13]. Based on the similarities between cPKC and nPKC, and the structural characteristics of nPKC, we therefore speculate that nPKC may also participate in hypoxia-induced PASMCs proliferation in PH. Thus, in this study, we applied mice PASMCs to investigate the relationship between nPKC and cell proliferation under hypoxia-induced condition and to explore their possible downstream pathways.

PASMCs culture in vitro has been widely used in studies of pulmonary vascular diseases, which is a vital method to study the pathogenesis of PH. Presently, the main methods for isolation and culture of PASMCs are enzyme digestion and tissue explant method. The outstanding advantage of enzyme digestion is high specificity of the isolated PASMCs. However, its disadvantages are also obvious, such as complicated operation and low success rate of separation. Compared with enzyme digestion, tissue explant method is simple in operation, and the isolated cells are in better state with less probability of cell contamination. On the other hand, the isolated cells have poor specificity and need to be purified. We have observed the deficiencies of above two methods in the previous experiments, so we determine to find a more efficient method to improve the success rate of PASMCs separation. According to previous study, much attention has been drawn to magnetic separation method[14]. For instance, Lee KJ[15] has successfully isolated the pulmonary artery of newborn mice using a method similar to magnetic separation. Thus, on the fundamental of summarizing the above methods, we have innovated the magnetic separation method, which can accurately locate and successfully separate a great deal of pulmonary arterioles in mice. Moreover, magnetic separation does not require long-time isolation by laboratory staff, which significantly reduces the chance of tissue damage and cell contamination. This method provides a more effective way for the separation of pulmonary arteries and pulmonary arterioles in mice and has great methodological significance.

It has well been demonstrated that hypoxia induce the proliferation of PASMCs. For instance, in isolated PASMCs, hypoxia leads to the rise of reactive oxygen species through tissue thioredoxin 2, which promotes cell proliferation[16]. Hypoxia also causes abnormal secretion of NO via upregulating hypoxic inducers such as HIF-1α, which inhibits vasodilation and accelerates PASMCs proliferation[17], resulting in HPH. Many studies have focused on the pathogenic mechanism of HPH. In those experiments, the hypoxia model was created by cultivating PASMCs in hypoxia incubators, applying O_2 concentrations ranging from 1–10%. We determined a 3% O_2 concentration based on preliminary experiments[18]. In line with previous studies, we also observed the effect of hypoxia on the proliferation of PASMCs. The results of CCK-8 showed that PASMCs proliferation rate was notably increased after 72 h with hypoxia. In parallel with CCK-8 assay, we gained the same conclusion from the Brdu assay. These experiments demonstrated that hypoxia promoted the proliferation of PASMCs, and the effect can be significantly increased when hypoxia is induced for 72 h. Therefore, we selected hypoxia induction 72 h as the follow-up experimental conditions.

Growing evidence of increased cPKC expression in hypoxia-induced PASMCs has been reported. It has been confirmed that PKCα can be upregulated by hypoxia in bovine PASMCs[19]. Equally, hypoxia induces upregulation of PKCα in rat PASMCs, but the cell proliferation is inhibited when iptakalim, a novel ATP-sensitive potassium channel opener that inhibits PKCα expression is used. In line with previous studies, we have also detected the upregulation of PKCα in isolated rat PASMCs induced by hypoxia[18]. Moreover, we further investigated the expression levels of four types of nPKC under hypoxic conditions. From the results of flow cytometry, the expression levels of PKCδ and PKCε increased significantly with the extending of hypoxic treatment time, while the expressions of PKCη and PKCθ remained unchanged. In parallel with flow cytometry, we then gained the same conclusion from Western blot. It has also been reported that hypoxia in rat PASMCs promotes the change of PKCβ content, from water-soluble dissociation state in cytoplasm to lipid soluble activation state in membrane structure[20]. In addition, hypoxia induces the activation of PKCα and PKCγ in the cytoplasm of carotid body chemoreceptor cells, while PKCβ is not activated[21]. Given the above investigations, we identify that different subtypes of PKC family may have different activation modes. To illustrate, not all subtypes of PKC can be activated by hypoxia, either because some cells do not contain all PKC subtypes, or because different subtypes of PKC play different roles in the same cell and are therefore activated in different ways, which needs to be explored.

To further investigate the relationship between up-regulation of nPKC and cell proliferation, we used agonists and inhibitors of PKCδ and PKCε. According to the results of Brdu, hypoxia promoted PASMCs proliferation, but this effect disappeared when inhibitors of PKCδ and PKCε were used. While normoxia did not promote cell proliferation, agonizing PKCδ and PKCε under normoxia substantially increased cell proliferation and was at the same level as that induced by hypoxia. These results indicated that hypoxia induced upregulation of PKCδ and PKC expression to promote the proliferation of PASMCs. Several studies have also documented that hypoxia accelerates PASMCs proliferation via up-regulating PKC. For example, hypoxia promotes potassium channel activation by upregulation of PKC resulting in the proliferation of smooth muscle cells[22]. In bovine PASMCs, agonizing PKC with PMA induces cell proliferation, which is inhibited by using PKC inhibitors[23]. Taken together with the available studies and our results, it is almost certain that hypoxia-induced upregulation of PKC promotes PASMCs proliferation, but it is unclear whether each subtype is involved due to the large number of PKC types, which needs to be further studied.

After clarifying that hypoxia promotes PASMCs proliferation through activation of PKCδ and PKCε, we went on to further investigate whether there are some signaling pathways associated with cell proliferation. According to research, among numerous cellular pathways, AKT and ERK pathways play important roles in cell proliferation[24]. AKT is a kind of Ser/Thr protein kinase at the center of many signaling pathways[25]. A number of researchers have demonstrated the close relationship between AKT and cell proliferation. To illustrate, in tumor cells AKT can promote cell proliferation by inhibiting Caspase-9 through activating downstream signaling pathways to promote the degradation p53 protein[26]. In myocytes, activation of AKT inhibits muscle creatine kinase to promotes myocyte proliferation[27]. ERK also belong to Ser/Thr protein kinase family, which are signal transduction proteins

that transmit mitogen signals[28]. Its non-activated state is located in the cytoplasm, and when activated, it translocates to nucleus, regulates transcription factor activity to produce cellular effects[29, 30]. Several studies have shown the close link between ERK activation and cell proliferation. For instance, in vascular smooth muscle cells, angiotensin II promotes cell proliferation by upregulating ERK expression, conversely, angiotensin (□-□) has an inhibitory effect on cell proliferation, probably by inhibiting ERK expression[31, 32]. It can be concluded that AKT and ERK play crucial roles in the proliferation of both tumor cells and normal cells. In our study, we confirmed that hypoxia could induce the activation of AKT and ERK, whereas this induction disappeared after the inhibition of PKCδ and PKCε. Then we further demonstrated that with the knockdown of PKCδ and PKCε, the hypoxia-induced phosphorylation of AKT and ERK again was inhibited. As shown by Brdu results, the decreased expression of PKCδ and PKCε led to the inhibition of cell proliferation, which was promoted in the normal hypoxia-induced group. To summarize, hypoxia induced upregulation of PKCδ and PKCε expression resulting to the proliferation of PASMCs via up-regulating the phosphorylation of AKT and ERK.

Conclusions

With this study we showed that increased nPKC expression contributes to PASMCs proliferation and uncovers the correlation between AKT and ERK pathways and nPKC-mediated proliferation of PASMCs. Moreover, PKCδ and PKCε contribute to the development of PH. nPKC, as PKC family plays a critical role in PH, it may therefore represent a potential pharmacological target. Further study on the relationship between PKC family and PH is the basis for drug design and therapy, which may provide novel targets for molecular therapy of pulmonary hypertension.

Abbreviations

PKC: Protein Kinase C; PH: Pulmonary Hypertension; PAH: Pulmonary arterial hypertension; cPKC: Conventional PKC; nPKC: Novel PKC; PASMCs: Pulmonary artery smooth muscle cells.

Declarations

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Authors’ contributions

Yiwei Shi, Rui Jiang, and Xiaojiang Qin were involved in conception and design of the study. Xiaomin Hou, Liangjing Chen, Xinrong Xu, and Lina Chai performed the analyses. Yunting Guo, Liangyuan Zhao and Xuefeng Du interpreted the results. Anqi Gao and Xiaojiang Qin prepared figures and drafted the

manuscript. Rui Jiang, Xiaojiang Qin and Xiaomin Hou critically reviewed and revised the manuscript. All authors read and approved the final version of the manuscript.

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Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

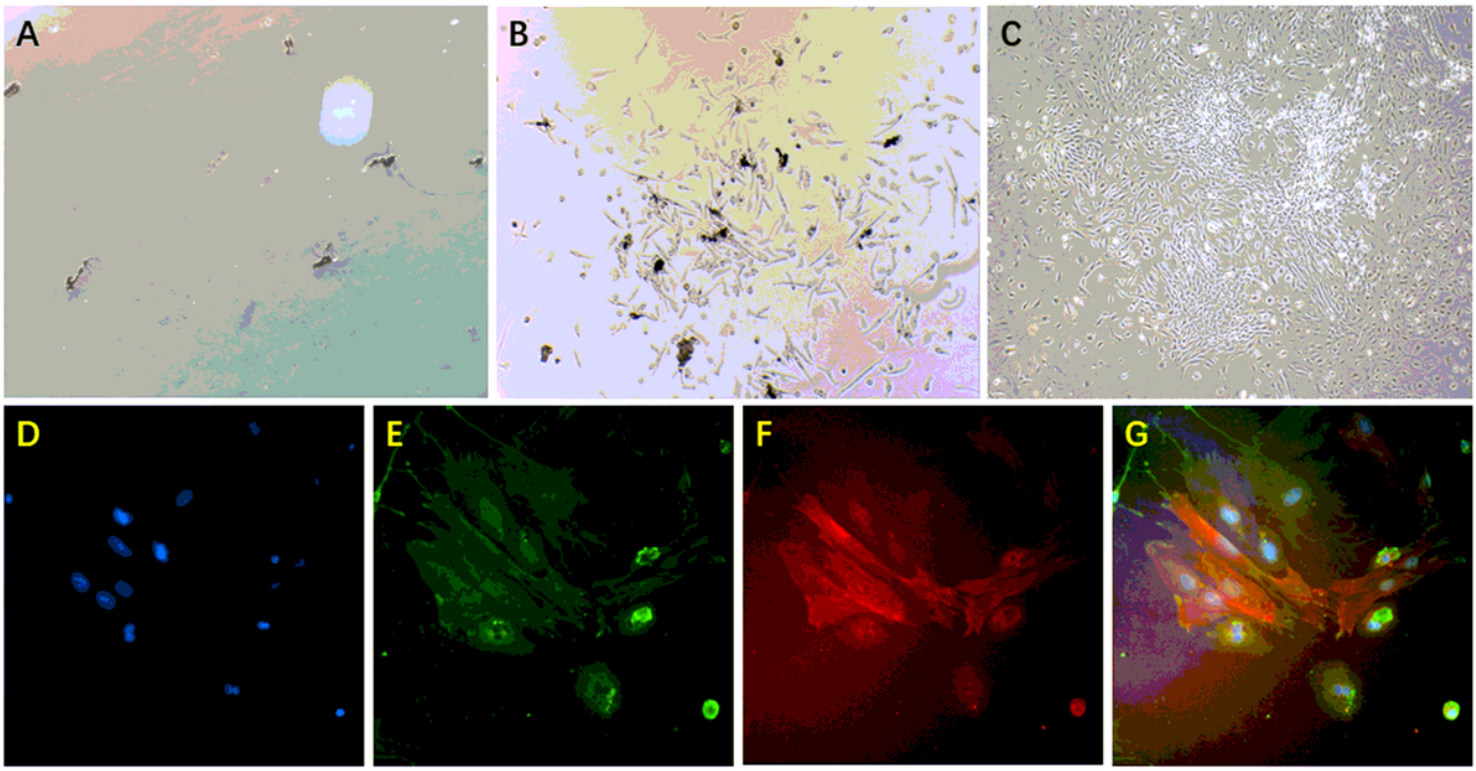

Figure 1

PASMCs isolated by magnetic separation were in good condition. (A) Iron-containing vascular fragments (×100). (B) Cells crawled out from iron-containing tissues and adhered to the medium for division and proliferation (×100). (C) After passages and magnet purifications, the cells were virtually free of iron particles and were in growth condition (×100). (D-G) Immunofluorescence staining of pulmonary arterial smooth muscle cells (×600). D. Blue is the nucleus, E. Green fluorescence is positive for smoothlin expression, F. red fluorescence is positive for smooth muscle myosin heavy chain expression, G. a superimposition of D, E, and F graphs.

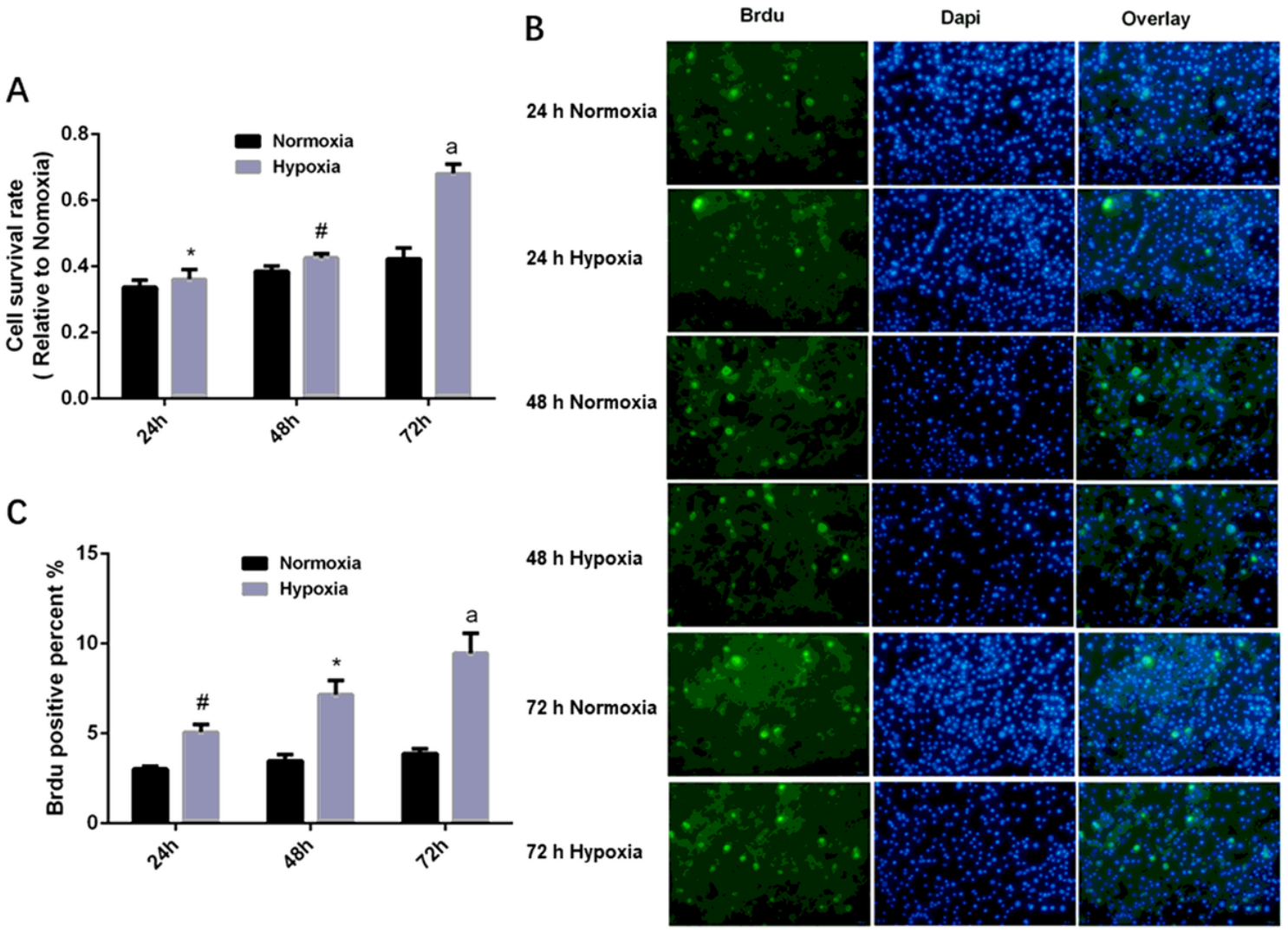

Figure 2

Hypoxia induced proliferation of PASMCs in mice. (A) CCK8 assays to detect the proliferation of PASMCs under different hypoxic treatment times. Mean±SD, n=6, *P>0.05 vs Normoxia 24 h, #P>0.05 vs Normoxia 48 h, aP<0.05 vs Normoxia 72 h. (B) Brdu assays to detect the proliferation of PASMCs under different hypoxic treatment times. Scale bar=100 μm. (C) Brdu assays to detect the proliferation of PASMCs under different hypoxic treatment times. Mean±SD, n=6, #P>0.05 vs Normoxia 24 h, *P<0.05 vs Normoxia 48 h, aP<0.05 vs Normoxia 72 h.

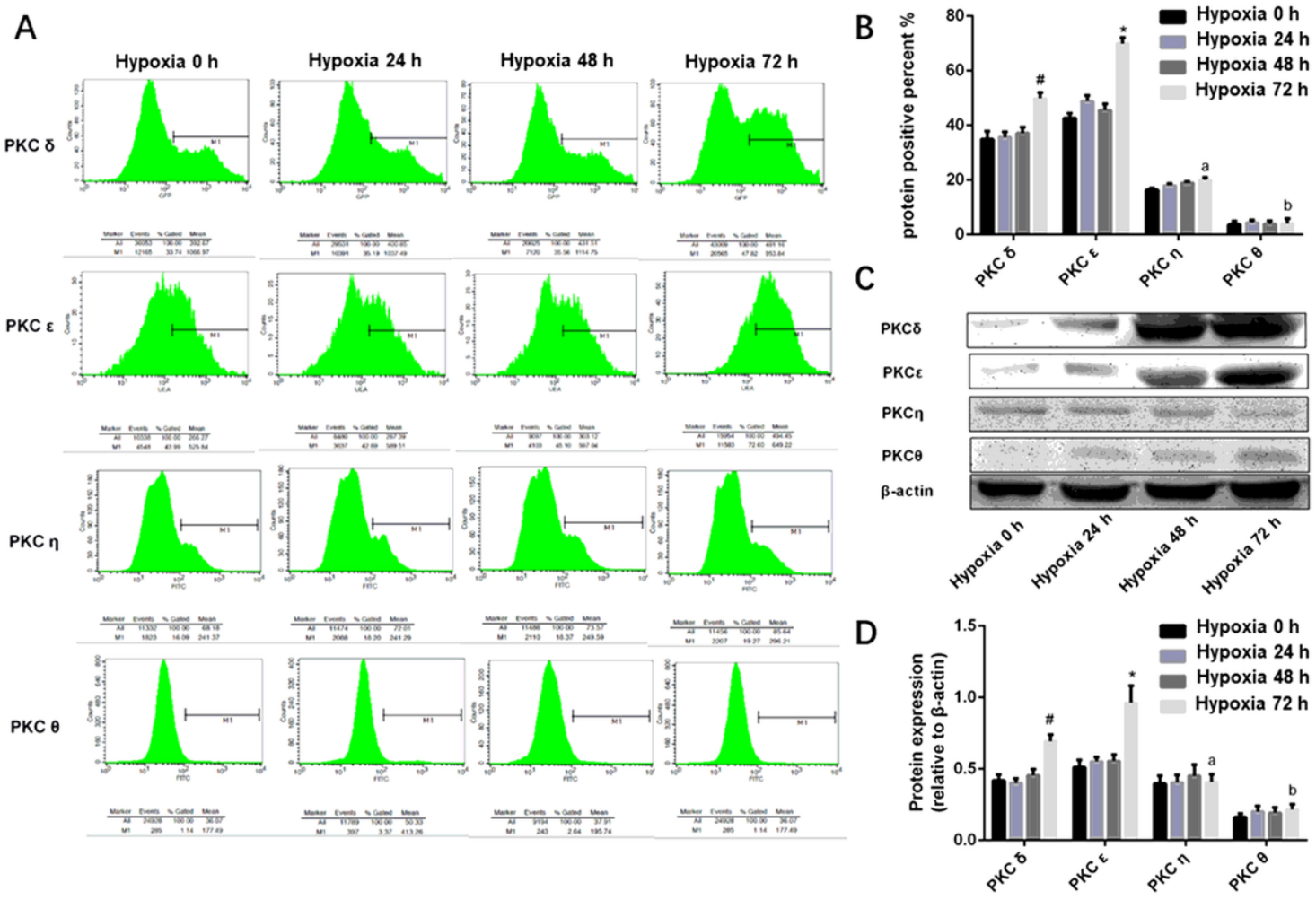

Figure 3

PKCδ and PKC□ were up-regulated in hypoxia-exposed PASMCs. (A-B) Flow cytometry detection of PKCδ, PKCε, PKCη and PKCθ expression in PASMCs under different hypoxic treatment times. Mean±SD, n=6, #P<0.05 vs PKCδ Hypoxia 0 h, *P<0.05 vs PKCε Hypoxia 0 h, aP>0.05 vs PKCη Hypoxia 0 h, bP>0.05 vs PKCθ Hypoxia 0 h. (C-D) Western blot detection of PKCδ, PKCε, PKCη and PKCθ expression in PASMCs under different hypoxic treatment times. Mean±SD, n=6, #P<0.05 vs PKCδ Hypoxia 0 h, *P<0.05 vs PKCε Hypoxia 0 h, aP>0.05 vs PKCη Hypoxia 0 h, bP>0.05 vs PKCθ Hypoxia 0 h.

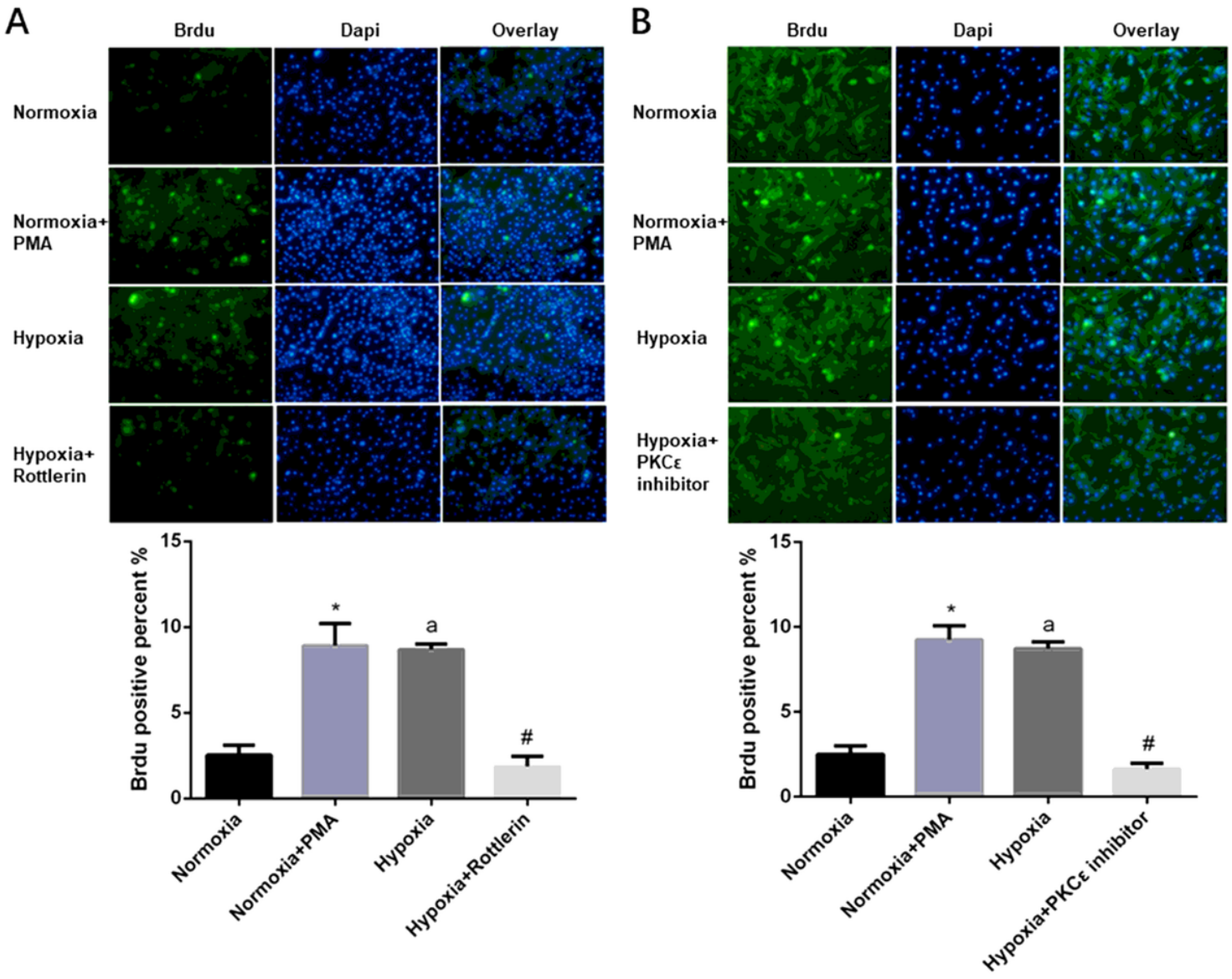

Figure 4

Hypoxia induced up-regulation of PKCδ and PKC resulting to PASMCs proliferation. (A) Brdu assays to detect the proliferation of PASMCs under different interventions of PKCδ. Mean±SD, n=6, Scale bar=100 μm, *P<0.05 vs Normoxia, #P<0.05 vs Hypoxia, aP<0.05 vs Normoxia. (B) Brdu assays to detect the proliferation of PASMCs under different interventions of PKC. Mean±SD, n=6, Scale bar=100 μm, *P<0.05 vs Normoxia, #P<0.05 vs Hypoxia, aP<0.05 vs Normoxia.

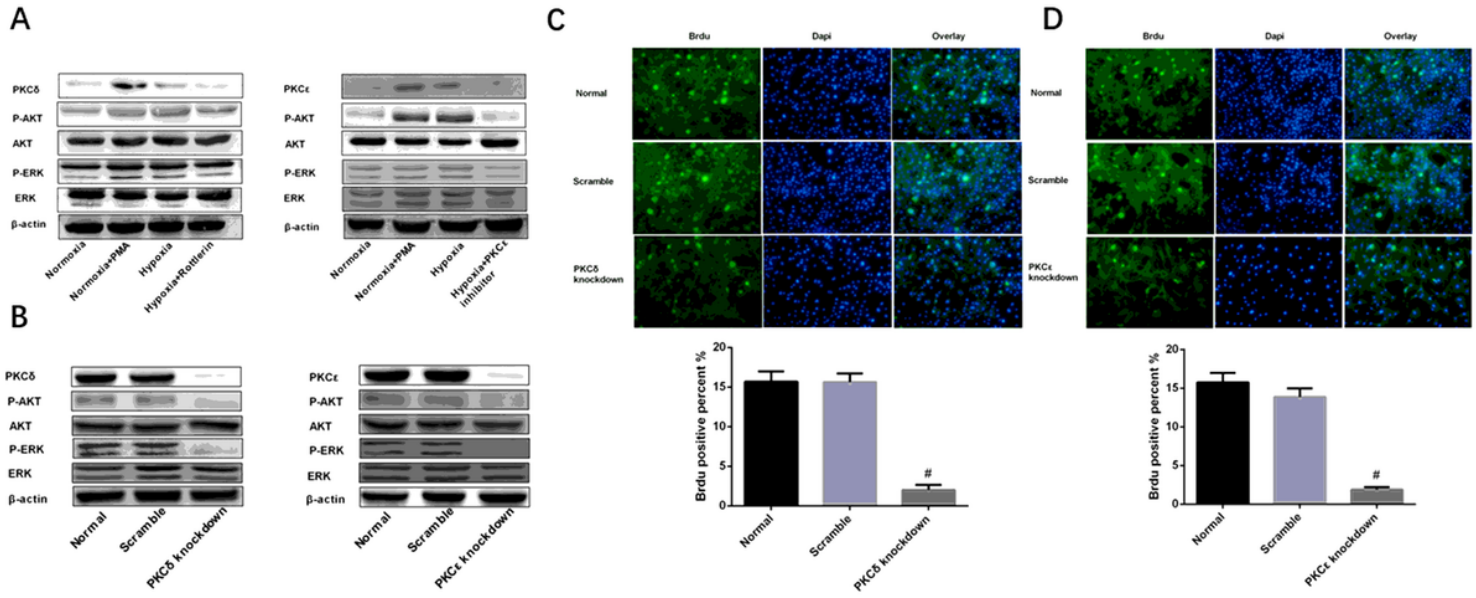

Figure 5

Hypoxia induced AKT and ERK phosphorylation by upregulating PKCδ and PKCε. (A) Western blot detection of PKCδ, PKCε, AKT, ERK, P-AKT and P-ERK expression in each group. (B) Western blot detection of PKCδ and PKCε expression in each group. Mean±SD, n=6, *P<0.05 vs Normoxia, #P<0.05 vs Normoxia, aP<0.05 vs Hypoxia. (C) Western blot detection of AKT phosphorylation in each group. Mean±SD, n=6, *P<0.05 vs Normoxia, #P<0.05 vs Normoxia, aP<0.05 vs Hypoxia. (D) Western blot detection of ERK phosphorylation in each group. Mean±SD, n=6, *P<0.05 vs Normoxia, #P<0.05 vs Normoxia, aP<0.05 vs Hypoxia. (E) Western blot detection of PKCδ, PKCε, AKT, ERK, P-AKT and P-ERK expression in each group. (F) Western blot detection of PKCδ and PKCε expression in each group. Mean±SD, n=6, *P<0.05 vs Normal. (G) Western blot detection of AKT phosphorylation in each group. Mean±SD, n=6, *P<0.05 vs Normal. (H) Western blot detection of ERK phosphorylation in each group. Mean±SD, n=6, *P<0.05 vs Normal. (I) Brdu assays to detect the proliferation of PASMCs in each group. Mean±SD, n=6, #P<0.05 vs Normal. (J) Brdu assays to detect the proliferation of PASMCs in each group. Mean±SD, n=6, #P<0.05 vs Normal.