Overexpression of Krüppel-Like Factor 4 (KLF4) Inhibits the Proliferation of Pulmonary Arterial Smooth Muscle Cells Through ERK1/2 Signaling Under Hypoxia Environment

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Abstract

**Background** This study is designed to examine the role of Krüppel-like factor 4 in mediating the proliferation of pulmonary artery smooth muscle cells (PASMCs) in hypoxic pulmonary hypertension and the underlying mechanisms.

**Methods** Experiments were conducted using PASMCs isolated from male Sprague-Dawley rats. The cells were divided into 24 h group, 48 h group and 72 h group according to different hypoxia treatment time. For specific inhibition of the ERK1/2 and p38 signaling pathways, PASMCs were treated with the ERK1/2-specific inhibitor. KLF4 was cloned from plasmid into the eukaryotic expression vector and transfected into PASMCs for KLF4 overexpression and was transfected into PASMCs for KLF4 knockdown.

**Results** PASMCs dedifferentiation and proliferation phenotypes under hypoxia conditioned culture were not synchronous. Overexpression of KLF4 attenuated extracellular signal-regulated kinase (ERK)1/2-mediated proliferative signals in PASMCs. Knockdown of KLF4 expression resulted in increased ERK1/2 phosphorylation and significantly increased hypoxia-induced PASMCs proliferation, while p38 did not change and showed no influence on proliferation during the above process.

**Conclusions** Our results indicate for the first time that KLF4 plays inhibitor role through activating ERK1/2 in PASMCs proliferation under hypoxia condition, expanding existing reports on the participation of KLF4 in the development of HPH. Our findings may provide pharmacological targets for therapy of HPH.

Introduction

Pulmonary hypertension (PH) is a devastating disease with an estimated global prevalence of 1%[1]. It is characterized by sustained increase of pulmonary arterial pressure and eventually leading to right heart failure[2]. Although current treatments have alleviated some symptoms and prolonged survival, PH remains incurable and fatal. The development of PH involves complex and heterogeneous cells of multiple genetic, molecular, and humoral abnormalities. The included interaction behaves as a complicated manner, presenting a manifestation of vascular remodeling in which major between fibroblasts, smooth muscle and endothelial cells[3].

Hypoxic pulmonary hypertension (HPH) is one of the common clinical types of PH [4]. Hypoxic ultimately leads to the occurrence of HPH, a series of pathophysiological have pulmonary artery vasoconstriction, pulmonary artery smooth muscle cells (PASMCs) proliferation and migration, vascular matrix reconstruction and changes[5]. Numerous studies have indicated that proliferation of PASMCs play an important role in the pathological development of HPH, eventually lead to vascular remodeling[6]. Other studies have shown that after a hypoxic injury, endothelial cells become dysfunctional and begin to secrete extracellular matrix as well as growth factors, which may stimulate PASMCs proliferation. In addition, it has also been reported that direct stimulation by hypoxia results in PASMCs proliferation through an autocrine pathway[7]. The idea that hypoxia alone can cause PH and significant structural
remodeling of pulmonary arteries (PAs) in human has long been supported[8], however, the specific mechanisms remain unclear.

Recent evidence suggests that KLF4 is closely associated with the proliferation of PASMCs. KLF4 is an extensively expressed zinc finger transcription factor, participates in cell differentiation, cell proliferation, and apoptosis by regulating the expression of downstream target genes[9]. KLF4 expression cannot be detected in mature PASMCs in vivo. However, its expression level sharply increases in the early stage of in vivo vascular injury[10]. Additionally, KLF4 is expressed at a low level in PASMCs in vitro, but stimulation with all-trans retinoic acid increases its expression, suggesting that KLF4 plays an important role in PASMCs proliferation[11, 12]. Therefore, the specific effect of KLF4 on PASMCs proliferation and whether KLF4 could participate in the development HPH deserves to be further elucidated.

In this study, we aimed to establish an external model of HPH and to clarify the mechanism that KLF4 participates in the process of the hypoxia-induced proliferation of cultured PASMCs. Our results demonstrated that KLF4 plays inhibitor role through activating ERK1/2 in PASMCs proliferation under hypoxia condition, while p38 MAPK signaling does not directly involve in PASMCs proliferation. These findings may further uncover the molecular mechanism of pulmonary vascular remodeling, providing a theoretical basis for its prevention and treatment.

**Materials And Methods**

**Isolation and culture of rat PASMCs**

Twenty male Sprague-Dawley rats (age, 50 days; weight, 80-100 g) purchased from the Experimental Animal Center of Shanxi Medical University (Taiyuan, China) were maintained in a temperature (25±2˚C) and humidity (60 - 65%) controlled room on a 12 h light/dark cycle with free access to food and water for 1 week prior to use. The current study was reviewed and approved by the Animal Management Guidelines of the Ministry of Health of the People's Republic of China, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rat PASMCs were isolated and cultured as previously reported[13]. Rats were anaesthetized by intraperitoneal injection of pentobarbital sodium (Sinopharm Chemical Reagent Co., Ltd., Beijing, China; 50 mg/kg body weight), then the main trunk of pulmonary arteries and the right and left branches were isolated under a dissecting light microscope (Olympus Corporation, Tokyo, Japan). After connective tissues of arteries were cleaned and vessels cut open longitudinally, luminal endothelia were removed by gentle scraping with cotton swabs. The isolated pulmonary arteries were dissected into small pieces of 1x1 mm, maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F12 media (Thermo Scientific Hyclone, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) and 1% SM cell growth supplement (SMCGS) (Science Research Laboratories, Carlsbad, CA, USA) and incubated in a humidified atmosphere with 5% CO2 at 37˚C. Culture medium was changed twice per week and cells were harvested with trypsin (0.25%; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing EDTA. Morphological assay and
immunocytochemistry were used to detect SM-actin expression to identify PASMCs. Cells passage between three and six times were used in the experiments.

**Treatment of rat PASMCs**

All experiments were carried out as follows. The cells were seeded at a density of $5 \times 10^4$ cells/well in 6-well plates or at $1.5 \times 10^3$ cells/well in 96-well plates and cultured in DMEM/F12 media supplemented with 10% FBS and 1% SMCGS, in 5% CO$_2$ at 37°C for 24 h. The cells were then starved in serum-free media for 24 h, so that they subsided into the resting stage. Thereafter, the media were changed to DMEM/F12 supplemented with 2% FBS and 1% SMCGS, and the cells were cultured at 37°C under hypoxic conditions (5% O$_2$, 5% CO$_2$, and 90% N$_2$) for 24 h, 48 h or 72 h. Cells in the control group were cultured under normal conditions (21% O$_2$, 5% CO$_2$, and 74% N$_2$).

For specific inhibition of the ERK1/2 and p38 signaling pathways, cells in the resting stage were treated with the ERK1/2-specific inhibitor U0126 (5 mM; Cell Signaling Technology, Boston, MA, USA) or the p38-specific inhibitor SB203580 (20 mM; Sigma-Aldrich, Louis, MO, USA) in DMEM/F12 supplemented with 5% FBS and 1% SMCGS and the cells were cultured at 37°C under hypoxic conditions.

**KLF4 overexpression and knockdown**

Cells were seeded in 6-well plates. The plasmid pCMV.SPORT6 were used, which contains KLF4 coding sequences and was obtained from the Hematology Department of the Second Affiliated Hospital, Shanxi Medical University. For KLF4 overexpression, KLF4 was cloned from this plasmid into the eukaryotic expression vector pIRES2-ZsGreen1 and transfected into resting-stage PASMCs with the Attractene Transfection Reagent (Qiagen, Dusseldorf, Germany) according to the manufacturer’s instructions. The pIRES2-ZsGreen1 plasmid was used as a negative control. After culture under hypoxic conditions for 72 h, cells were collected for further study.

For KLF4 knockdown, FlexiTube GeneSolution GS9314 for KLF4 (Qiagen, Dusseldord, Germany) or non-specific siRNA as a negative control was transfected into resting-stage PASMCs with the HiPerFect Transfection Reagent (Qiagen, Dusseldord, Germany) according to the manufacturer’s instructions. After culture under hypoxic conditions for 72 h, cells were collected for further study.

**Western blot analysis**

Cells were collected and subjected to lysis with lysis buffer (Beyotime, Shanghai, China) supplemented with 1 mM phenylmethylsulfonyl fluoride. Lysis buffer (50 μl) was used for the cells in each well of a 6-well plate. Bicinchoninic acid assay (Beyotime, Shanghai, China) was used for protein quantification. The proteins (50 μg) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto nitrocellulose (NC) membranes (GE Healthcare, Buckinghamshire, UK). The NC membranes were blocked in phosphate-buffered saline with Tween 20 (PBST) containing 5% nonfat milk powder at room temperature for 1 h and incubated overnight with primary antibody at 4°C. Primary
antibodies were diluted with PBST as follows: 1:200 mouse anti-human proliferating cell nuclear antigen (PCNA) antibody (Boster, Wuhan, China); 1:400 rabbit anti-human smooth muscle (SM) α-actin antibody (Abcam, Cambridge, MA, USA); 1:200 rabbit anti-human KLF4 antibody (Abcam, Cambridge, UK); 1:200 rabbit anti-human ERK1/2 antibody (Cell Signaling Technology, Boston, MA, USA); and 1:1000 rabbit anti-human β-actin antibody (Boster, Wuhan, China). Next, the NC membrane was incubated with horseradish peroxidase–labeled secondary antibody (Boster, Wuhan, China) diluted with PBST at a ratio of 1:10,000 at room temperature for 1 h, and subjected to development with electrochemiluminescence reagent (Thermo Fisher Scientific, Waltham, MA, USA). Signal intensity was quantified and analyzed with GeneSnap (Syngene, Cambridge, UK).

**Statistical methods**

SPSS 21.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. Data are presented in the form of Mean ± SD. Test of normality and homogeneity of variance was carried out. We used the t-test and one-way analysis of variance for comparisons between two groups and among three of more groups, respectively. The Student-Newman-Keuls method was used when variance was homogeneous, while Dunnetts’s T3 method was used when variance was not homogeneous. Differences were considered significant at \( P<0.05 \).

**Results**

**Hypoxia induced proliferation of rat PASMCs**

Immunofluorescence staining determined that PASMCs stain positively for smooth muscle markers (Fig. 1). To address the effects of hypoxia on rat PASMCs proliferation, the cells were divided into 24 h group, 48 h group and 72 h group. According to western blotting results, SM α-actin protein expression levels in hypoxic group for 24 h, 48 h and 72 h were 3.621 ± 0.270, 4.162 ± 0.540 and 9.405 ± 2.216, respectively. On the other hand, SM α-actin protein level in cells exposed to normoxia for 72 h was 6.540 ± 0.162. Compared with the normoxia for 72 h group, SM α-actin protein expression levels in PASMCs exposed to hypoxia for 24 h were significantly decreased and thereafter increased in PASMCs exposed to hypoxia for 48 h and 72 h \((P<0.05; \text{Fig. 2A-B})\). PCNA protein expression levels in hypoxic group for 24 h, 48 h and 72 h were 0.159 ± 0.056, 0.339 ± 0.105 and 0.646 ± 0.286, respectively. On the other hand, PCNA protein levels in cells exposed to normoxia for 72 h was 0.251 ± 0.070. Compared with the normoxia for 72 h group, PCNA protein expression levels in PASMCs exposed to hypoxia for 72 h were remarkably increased \((P<0.05; \text{Fig. 2C-D})\). Compared with cells exposed to hypoxia for 24 h, SM α-actin and PCNA protein expression levels of PASMCs in hypoxic exposure for 72 h were outstandingly increased \((P<0.05; \text{Fig. 2})\). Taken together, these results indicated that hypoxia promoted the proliferation of PASMCs, and this effect was positively correlated to the duration of culture under hypoxic conditions.

**Overexpress Klf4 In Pasmcs Suppresses Proliferation**
To explore the role of KLF4 in mediating hypoxia-induced proliferation of PASMCs, the cells were transfected with plasmid pCMV.SPORT6 which contains KLF4 coding sequences. Western blotting results indicated that PCNA protein expression levels in normoxia 72 h, hypoxia 72 h, hypoxic KLF4 untransfected and hypoxic KLF4 transfected groups were 0.356 ± 0.061, 0.812 ± 0.037, 0.782 ± 0.019 and 0.152 ± 0.033, respectively. Compared with KLF4 untransfected cells, PASMCs transfected with the KLF4-expressing plasmid showed significantly decreased PCNA expression, which was even lower than the level in cells cultured under normal conditions (P < 0.05; Fig. 3A-B). These data suggested that KLF4 inhibited hypoxia-induced PASMCs proliferation.

**Overexpression Or Knock-down Of Klf4 Effects Erk1/2 Activity**

To elucidate whether the ERK1/2 and p38 signaling pathways involves in the hypoxia-induced biological function in PASMCs, the changes in ERK1/2 and p38 activity under hypoxic conditions were detected. According to the western blotting results, pERK1/2 expression levels in cells exposed to hypoxia for 24 h, 48 h and 72 h were 0.226 ± 0.033, 1.505 ± 0.098 and 1.689 ± 0.085, respectively; compared with cells exposed to normoxia for 72 h, which was 0.821 ± 0.045. Compared with the 72 h normoxia group, pERK1/2 expression in PASMCs after hypoxia exposure for 48 h and 72 h was markedly upregulated, interestingly, it was significantly decreased after exposure to hypoxia for 24 h (P < 0.05, Fig. 4A-B). While the expression of p-ERK1/2 changed, the expression of p-p38 showed no significant changes (Fig. 4A-B). These data demonstrated that changes in ERK1/2 activity might mediate hypoxia-induced PASMCs proliferation. ERK1/2 showed low activity in the early stage of hypoxic injury, while in the late stage of the cellular proliferation process, the activity of ERK1/2 increased.

When PASMCs exposed to hypoxia for 72 h were pretreated with the ERK1/2-specific inhibitor U0126 and p38-specific inhibitor SB203580, PCNA protein expression levels of PASMCs in the normoxia 72 h, hypoxia 72 h, hypoxic U0126 control and hypoxic SB203580 control groups were 0.333 ± 0.032, 0.597 ± 0.019, 0.370 ± 0.027 and 0.519 ± 0.054, respectively. Compared with the hypoxia for 72 h group, the PCNA protein expression in the hypoxic U0126 control group was notably attenuated (P < 0.05; Fig. 5A-B). However, treatment with the p38-specific inhibitor SB203580 had no significant influence on PASMCs proliferation (Fig. 5A-B). Thus, the suppression of the ERK1/2 signaling pathway by ERK1/2 inhibitor significantly inhibited hypoxia-induced PASMCs proliferation, and this is similar to the effect of KLF4 overexpression. To further clarify whether the suppression of hypoxia-induced PASMCs proliferation by KLF4 is related to ERK1/2 activity, the influence of KLF4 expression levels on ERK1/2 expression levels and activity under hypoxic conditions were investigated by using KLF4 overexpression or knockdown by RNA interference.

Western blotting results indicated that pERK1/2 protein expression levels in the hypoxic KLF4 untransfected, hypoxic KLF4 transfected, hypoxic NS-siRNA, and hypoxic KLF4-siRNA groups were demonstrated to be 1.926 ± 0.059, 1.551 ± 0.110, 1.951 ± 0.127 and 2.352 ± 0.170, respectively. pERK1/2
protein expression levels in the presence of the overexpression of KLF4 was greatly reduced compared with the KLF4 untransfected group ($P < 0.05$; Fig. 6A-B). While compared with the hypoxic NS-siRNA group, ERK1/2 phosphorylation in the presence of knockdown of endogenous KLF4 was significantly increased ($P < 0.05$; Fig. 6A-B). Furthermore, the changes in KLF4 expression level showed no influence on the expression of total ERK1/2 (Fig. 6A-B).

In summary, these results indicated that KLF4 overexpression downregulated the activity of ERK1/2, while knockdown of KLF4 by RNA interference upregulated the activity of ERK1/2.

**Discussion**

Our study demonstrated that KLF4 plays inhibitor role through activating ERK1/2 in PASMCs proliferation under hypoxia condition, while p38 MAPK signaling does not directly involve in PASMCs proliferation.

Numerous researches have indicated that hypoxia is the most common injury in a variety of vascular pathogenetic mechanisms and might eventually lead to vascular remodeling[14]. However, the direct impact of hypoxia has not been clearly investigated[15]. In this study, we unveil that hypoxia directly induce the PASMCs proliferation under hypoxia condition *in vitro*, which is inhibited by KLF4. Unlike terminally differentiated skeletal muscle cells and cardiac muscle cells, PASMCs in mature animals remain plastic, and retain the capability to undergo phenotypic transformation in response to a multitude of local environment cues, which play important roles in pulmonary vascular remodeling[16]. In normal mature vessels, PASMCs are in a low proliferative state, express PASMCs differentiation markers, and exhibit a contractile phenotype[17]. However, in the case of vascular injury or under pathological conditions, stimulation by a variety of pathological factors causes PASMCs to exhibit the synthetic phenotype, with remarkably decreased PASMCs differentiation markers expression and enhanced cell proliferation, cell migration, and extracellular matrix protein synthesis[18]. PASMCs differentiation markers include SM22α, SM α-actin, and SM-myosin heavy chain. Here we found that after 24 h of hypoxic culture, the expression of SM α-actin in PASMCs significantly decreased, and this might have led to the transformation of PASMCs from the differentiated phenotype to the undifferentiated phenotype. Subsequently, the expression of SM α-actin increased, and the PASMCs differentiated once again, exhibiting increased proliferative activity. These results differ from the generally accepted theory that in PASMCs, cell proliferation is inversely related to cell differentiation. Studies have demonstrated that dedifferentiation and proliferation are not synchronized in some situations: in the late embryonic development and postnatal stages, PASMCs exhibit high proliferative activity, with peak expression of PASMCs differentiation markers. Furthermore, platelet-derived growth factor-BB (PDGF-BB) can inhibit both the mitosis of PASMCs and the expression of SMCs differentiation markers[19]. Taken together, these reports demonstrate that the relation between proliferation and differentiation of PASMCs is not simply mutually exclusive but might be related to different environmental stimuli as well as different signaling pathways and regulatory factors.
To further clarify the effects of KLF4 on the cell proliferation and differentiation process, we used KLF4 overexpression or knock-down approach to investigate the biological functions we mentioned above. KLF4 was originally found to be highly expressed in epithelial cells and to regulate the differentiation and proliferation of epithelial cells[20]. KLF4 was also found to play mediator role during proinflammatory process in different cell types, such as in endothelial cells, growth of cancer cells and stem cells[21]. Increased KLF4 expression can inhibit cell growth, block the cell cycle, and affect DNA synthesis[22]. It has been reported that conditional deletion of KLF4 downregulates of smooth muscle cell differentiation markers but accelerates neointimal formation following vascular injury through the induction of p53 and p21WAF1/Cip1 expression. In accordance with these reports, we found that in PASMCs cultured in vitro, overexpression of KLF4 inhibited hypoxia-induced cell proliferation and led to G1-S block, indicating that KLF4 is an inhibitor of hypoxia-induced PASMCs proliferation in vitro.

The blockage of cell growth by KLF4 is related to a series of complicated signal transduction pathways, with KLF4 playing multiple roles in this process[23]. Studies have demonstrated that KLF4 might be both an upstream factor and a downstream factor of the MAPK pathway, depending on the type of cell and environmental stimulus[24]. Among members of the MAPK family, p38 and ERK1/2 have been found to be related to SMCs phenotype transformation and proliferation, and stimulation with a variety of substances can affect SMCs proliferation, via the regulation of the p38 and ERK1/2 signaling pathways[25–27]. For instance, TGF-β1 can induce the phosphorylation of KLF4 through the activation of the p38 signaling pathway to inhibit the growth and proliferation of PASMCs, at the same time, KLF4 can regulate the activity of p38 via a positive feedback cycle, through the activation of TGF type I receptors[28]. It has also been reported that hypoxia can increase phosphorylated ERK1/2 levels and the activity of p38 kinase in fibroblasts and SMCs[29]. Further studies of the downstream signaling pathways have found that in response to stimulation with hypoxia, the ERK1/2 signaling pathway participates in the regulation of mitosis and cell proliferation in fibroblasts and SMCs, while p38 is related to the proliferation of SMCs only[30]. In line with previous studies, our research indicated that ERK1/2 participated in hypoxia-induced PASMCs proliferation. After 24 h of hypoxic culture, the activity of ERK1/2 decreased, however, this activity then increased gradually as the duration of hypoxic culture increased. Furthermore, the proliferative activity of PASMCs was significantly reduced after 72 h of hypoxic culture using ERK1/2 pathway inhibitor. Under hypoxic conditions, inhibition of the ERK1/2 pathway resulted in a similar effect to that caused by KLF4 overexpression. Our experiment also indicated that the activity of p38 did not change during the above process, and inhibition of p38 showed no significant influence on hypoxia-induced PASMCs proliferation. Therefore, we concluded that p38 MAPK signaling does not directly involve in PASMCs proliferation. Finally, we extensively studied the relationship between KLF4 and ERK1/2 and found that KLF4 overexpression downregulated the activity of ERK1/2, while knockdown of KLF4 by RNA interference upregulated the activity of ERK1/2. These results indicate that ERK1/2 might participate in the process of hypoxia-induced PASMCs proliferation as a signal transduction molecule downstream of KLF4.
Owing to its function in cell proliferation and differentiation, KLF4 has been widely studied. However, few reports have focused on the role of KLF4 in the abnormal, hypoxia-induced proliferation of the cells that compose the vessel walls, especially PASMCs. In this study, we found that through the downregulation of ERK1/2 activity, KLF4 overexpression can inhibit hypoxia-induced proliferation of PASMCs cultured *in vitro*.

**Conclusions**

Taken together, our research demonstrates that growth and differentiation are not mutually exclusive in hypoxia-induced rat PASMCs, and KLF4 can inhibits PASMCs proliferation by activating ERK1/2. Although our understanding of the HPH disease process has advanced over the past few decades, therapeutic targets and evidence-based interventions remain limited, and novel approaches to modify the underlying disease process continue to be pursued. Since KLF4 played a regulatory role in hypoxia-induced PASMCs proliferation, it may therefore represent a potential target for HPH treatment, which will warrant further study.

**Abbreviations**

KLF4
Krüppel-like factor; PASMCs: Pulmonary artery smooth muscle cells; HPH, Hypoxic pulmonary hypertension; PH, Pulmonary hypertension; PDGF-BB: Platelet-derived growth factor-BB; ERK: Extracellular signal-regulated kinase.

**Declarations**

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**Authors’ contributions**

Yiwei Shi, Xiaojiang Qin were involved in conception and design of the study. Xiaomin Hou, Yuxuan Hao, 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. Chao Zeng, Xiaojiang Qin and Xiaomin Hou critically reviewed and revised the manuscript. All authors reviewed and approved the final version of the manuscript.

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Ethics approval and consent to participate

All experiments in this study were approved by the Animal Care and Use Committee of Shanxi Medical University and performed in accordance with the Guide for the Care and Use of Laboratory Animals (SXMU-0062). Because no patient participated in this study, we did not require permission to access and use the individual data/video files according to Ethical Guidelines for Medical and Health Research Involving Human Subjects of the Ministry of Health and Welfare in China.

A statement for ARRIVE Guidelines

This research does not involve human materials or human data. Our research strictly complies with the requirements of the Ethics Committee. In the research process, we often review the ethical approval. Moreover, we are also very concerned about whether our basic research will be clinically applied in the future. It is important that the clinical research we do must also be conducted in accordance with the ARRIVE guidelines.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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**Figures**

Figure 4

The changes in ERK1/2 and p38 activity under hypoxic conditions. Rat PASMCs were serum-starved and then cultured in DMEM/F12 (2% FBS and 1% SMCGS) under hypoxic conditions for the indicated times. Total protein lysates were collected and analyzed by Western blot for p-ERK1/2, ERK1/2, p-p38, p38. (A) Blots from a representative experiment and (B) Band intensities of phosphorylated molecules. Data are presented as the Mean ± SD deviation of three independent experiments. *P<0.05 vs. the normoxia group.