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Differential and Reciprocal Regulation between Hypoxia-inducible Factor-α Subunits and Their Prolyl Hydroxylases in Pulmonary Arteries of Rat with Hypoxia-induced Hypertension

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Abstract Hypoxia-inducible factor (HIF)-α subunits (HIF-1α, HIF-2α and HIF-3α), which play a pivotal role during the development of hypoxia-induced pulmonary hypertension (HPH), are regulated through post-translational hydroxylation by their three prolyl hydroxylase domain-containing proteins (PHD1, PHD2 and PHD3). PHDs could also be regulated by HIF. But differential and reciprocal regulation between HIF-α and PHDs during the development of HPH remains unclear. To investigate this problem, a rat HPH model was established. Mean pulmonary arterial pressure increased significantly after 7 d of hypoxia. Pulmonary artery remodeling index and right ventricular hypertrophy became evident after 14 d of hypoxia. HIF-1α and HIF-2α mRNA increased slightly after 7 d of hypoxia, but HIF-3α increased significantly after 3 d of hypoxia. The protein expression levels of all three HIF-α were markedly upregulated after exposure to hypoxia. PHD2 mRNA and protein expression levels were upregulated after 3 d of hypoxia; PHD1 protein declined after 14 d of hypoxia without significant mRNA changes. PHD3 mRNA and protein were markedly upregulated after 3 d of hypoxia, then the mRNA remained at a high level, but the protein declined after 14 d of hypoxia. In hypoxic animals, HIF-1α proteins negatively correlated with PHD2 proteins, whereas HIF-2α and HIF-3α proteins showed negative correlations with PHD3 and PHD1 proteins, respectively. All three HIF-α proteins were positively correlated with PHD2 and PHD3 mRNA. In the present study, HIF-α subunits and PHDs showed differential and reciprocal regulation, and this might play a key pathogenesis role in hypoxia-induced pulmonary hypertension.

Key words hypoxia-inducible factor; prolyl hydroxylase; hypertension; pulmonary; hydroxylation

Hypoxia-inducible factor (HIF) is an αβ-heterodimer consisting of one of three HIF-α subunits (HIF-1α, HIF-2α or HIF-3α), and the aryl hydrocarbon receptor nuclear translocator, also known as HIF1-β. HIF is one of the most important factors in the cellular response to hypoxia, which might regulate the transcription of more than 5% of all human genes (over 70 genes have so far been identified) in pulmonary artery endothelial cells [1]. Heterozygous HIF-1α deficient (Hif-1α$^{+/-}$) mice have impaired pulmonary vascular remodeling after exposure to 10% O_2 for 3 weeks, whereas heterozygous HIF-2α deficient (Hif-2α$^{+/-}$) mice show a complete absence of pulmonary vascular remodeling after exposure to 10% O_2 for 4 weeks [2]. Our previous studies showed that the three HIF-α subunits, with differential dynamic expression [3], play a critical role during the development of rat hypoxia-induced pulmonary hypertension (HPH) together with its target genes such as heme oxygenase-1, vascular endothelial growth factor and inducible nitric oxide synthase [4–6].

HIF-β subunits are constitutive nuclear proteins, whereas HIF-α subunits are inducible by hypoxia in animals and cultured cells. HIF-α subunits are translated constitutively but have a very short half-life under normal oxygen concentration. Regulation of the half-life and activity of

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the HIF-α subunits is dependent on their post-translational hydroxylation by hydroxylases [7]. Prolyl hydroxylation of HIF-α subunits is required for binding of the von Hippel-Lindau tumor suppressor protein, which is the recognition component of an E3 ubiquitin-protein ligase that targets HIF-1α for proteasomal degradation [8]. Three prolyl hydroxylase domain-containing proteins (PHD1, PHD2 and PHD3) that are responsible for this modification have been identified [9]. They are all Fe^{2+}- and 2-oxoglutarate-dependent dioxygenases with an absolute requirement for molecular oxygen, allowing HIF to escape degradation in hypoxia. The enzymatic process splits dioxygen, with one oxygen atom creating the hydroxylated amino acid, and the other oxidizing 2-oxoglutarate to succinate with the release of CO_2 [9]. PHDs are critical oxygen sensors regulating the degradation of oxygen-dependent HIF-α subunits either in normoxia or following exposure to hypoxia, but their roles in HPH have so far not been studied.

To evaluate the differential role of the three PHDs with respect to HIF-α hydroxylation and expression in pulmonary hypertension, we studied the expression patterns of PHDs and HIF-α subunits in pulmonary arteries of rats at different phases of HPH development.

Materials and Methods

Materials

Hypoxia and normoxia rat models were set up as described previously [10]. Briefly, 40 Wistar rats (male, 270±30 g, 9–10 weeks old) from the Animal Experimental Center of Hunan College of Traditional Chinese Medicine (Changsha, China) were randomly divided into five groups, with eight rats in each group. Hypoxic rats were exposed to normobaric hypoxia at 10.0%±0.5% O_2 for 3, 7, 14 and 21 d (8 h per day, intermittently) in a ventilated chamber. The hypoxic condition was established by flushing the chamber intermittently with a gas mixture of room air and nitrogen from a liquid nitrogen reservoir. An HT-6101 oxygen analyzer (Kangda Electrical, Chengdu, China) was used to monitor the chamber environment (the chamber was ventilated by a hole, then a dynamic balance was achieved through the inspiration and expiration of the rats). CO_2 was removed using soda lime. Excess humidity was prevented using anhydrous calcium chloride, and ammonia was kept to a minimum level using boric acid in the chamber. The control rats were kept in a normoxic ventilated chamber (21% O_2) in the same room, and killed after being caged for 10 d because breeding duration has no significant effect on mean pulmonary arterial pressure (*mPAP*), hypoxic pulmonary artery remodeling or right ventricular hypertrophy index (*RVHI*) [4].

mPAP and *RVHI* measurement

mPAP was measured as described previously [10]. After rats were anesthetized with pentobarbital sodium (40 mg/kg intraperitoneally), a specially designed single lumen catheter was inserted into the main pulmonary artery through the right jugular vein. The injecting position was confirmed by the waveform of pressure. Through this catheter, *mPAP* was measured using a Medlab bio-signal operating system (Nanjing MedEase Science and Technology, Nanjing, China). For *RVHI* measurement, each heart was cut open and atria were removed. The right ventricular free wall was dissected, and each chamber weighed. **Equation 1** was used to calculate the *RVHI*, in which *RV* is the weight of the right ventricle, *LV* is the weight of the left ventricle, and *S* is the weight of the septum.

$$RVHI = \frac{RV}{LV + S} \times 100\% \qquad 1$$

Morphometric analysis

Lung slices of 5 μm were embedded with paraffin, stained with hematoxylin-eosin, then by elastic fiber staining, and examined under a light microscope. At least five representative pulmonary arteries (approximately 100 μm in outer diameter) chosen from three different sections of each animal were independently examined. To evaluate hypoxic remodeling by calculating the parameters of pulmonary vascular cross-sections, the ratio of vascular wall area to total vascular area (*WA*) and pulmonary artery media thickness (*PAMT*) were measured. The images of the arteries were captured and analyzed using PIPS-2020 image software (Chongqing Tianhai Company, Chongqing, China).

In situ hybridization of HIF-α subunits and PHD mRNA

In situ hybridization was carried out using an *In situ* hybridization detection kit (Wuhan Boster Biological Technology, Wuhan, China). These oligonucleotide probes were designed by Wuhan Boster Biological Technology according to the sequences of rat HIF-1α, HIF-2α and HIF-3α. The sequences of probes against HIF-1α, HIF-2α and HIF-3α mRNA were the same as those described in Li and Dai [3].

The sequences of probes against PHD1 mRNA were: 5′-GTGTCAGAGTCCTTGGAGCCTAGCCGAGC-CAGGAT-3′; 5′-TGCGGTACTATGGTATCTGCGT-CAAGGACAACTTC-3′; and 5′-TTCAAGTACCCGTGT-CACAGCCAGCTACACCTACC-3′. The sequences of probes against PHD2 mRNA were: 5′-AGCAGATCGGC-GACGAGGTGCGCGCCCTGCACGAC-3′; 5′-ATGAG-CAGCATGGACGACCTGATCCGCCACTGCAG-3′; and 5′-GTTGAACTCAAGCCCAATTCAGTCAGCAAA-GACGT-3′. The sequences of probes against PHD3 mRNA were: 5′-ATCTCCAAAAGGGGCCCTCCGACTTCT-CACTGGGC-3′; 5′-CACGAGGTCGGTTTCTGCTAC-CTGGACAACTTCCT-3′; and 5′-TTCAGGAATCTAAC-TAGAAAAACTGAATCTGCTCT-3′.

Hybridization was carried out on serial lung tissue slices in paraffin fixed by formalin containing 0.1% diethypyrocarbonate according to the manufacturer's instructions. Briefly, slices were digested with pepsin for 15 min at 37 ºC. After 2 h of pre-hybridization, slices underwent hybridization with digoxin-labeled single-stranded oligonucleotide probes for 16 h at 38 ºC. In negative control studies, labeled oligonucleotide probes were substituted by phosphate-buffered saline. After washing off unbound probes, slices were incubated firstly with rabbit antibodies against digoxin, then with biotinylated goat antibodies against rabbit. Slices were then incubated with streptavidin-biotin-peroxidase. Peroxidase activity was visualized by a color reaction using diaminobenzidine (Wuhan Boster Biological Technology) as the substrate. Brown and yellow colors indicated positive results (mainly in cytoplasm). Finally, the sections were counterstained with hematoxylin (resulting in blue nuclei) and mounted. Expression levels of mRNA were quantified by a pathology image analysis system (PIPS-2020; Chongqing Tianhai) using absorbance of positive signal.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis for HIF-α subunits and PHD genes

Total RNA was extracted using Trizol reagent (Sangon, Shanghai, China). Single-stranded cDNA was synthesized according to the manufacturer's instructions with Moloney murine leukemia virus reverse transcriptase (Fermentas, Vilnius, Lithuania) and stored at −70 ºC for further amplifying. Each sample was analyzed in triplicate with specific standards and no-template controls using the GeneAmp 2400 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, USA). Reaction mixtures (25 μl) consisted of 100 ng cDNA, 0.3 μM primers, 10 mM Tris-HCl, 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM dNTPs and 1.25 U *Taq* DNA polymerase (Fermentas). Samples were denatured at 95 ºC for 10 min, and cDNA products were amplified with 30−35 cycles of denaturation at 95 ºC for 30 s, annealing at the optimum temperature for 45 s and extension at 72 ºC for 60 s. PCR products were electrophoretically separated with 1.5% agarose gels. The optical density (*OD*) values of the DNA bands were scanned with a densitometer and quantified with Tanon gel image system version 3.74 (Shanghai Tanon Science and Technology, Shanghai, China). The mRNA expression was evaluated by the ratio of the intensities of the target band to the β-actin band. The primer sequences, optimum annealing temperatures and number of cycles for the amplification, and the predicted length of amplified products, are listed in **Table 1**.

Immunohistochemistry for HIF-α subunits and PHD proteins

A commercial streptavidin-biotin complex kit (Wuhan Boster Biological Technology) was used for immunohistochemistry, which was carried out similar to that described previously [3]. Briefly, serial sections of formalin-fixed paraffin-embedded lung tissues were digested with 3% H_2O_2 for 20 min at room temperature, then preincubated with 10% nonimmunized serum. Sections were incubated with goat anti-HIF-1α, anti-HIF-2α and anti-HIF-3α (Santa Cruz Biotechnology, Santa Cruz, USA) specific polyclonal antibodies (1:200) or rabbit anti-PHD1, anti-PHD2 and anti-PHD3 (Novus Biological, Littleton, USA) specific polyclonal antibodies (1:300) overnight at 4 ºC respectively. Phosphate-buffered saline was used as the negative control in this study. After unbound antibodies were washed off, the sections were incubated with corresponding biotinylated secondary antibodies against rabbit and thereafter incubated with streptavidin peroxidase. Subsequently, peroxidase activity was visualized by a color reaction with diaminobenzidine similar to that of *in situ* hybridization.

Western blot analysis for HIF-α subunits and PHD proteins in lung tissue

Proteins from whole tissue samples were extracted using a modified RIPA homogenization buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% deoxycholic acid, 1 mM EDTA, 1% NP-40, 0.1 μg/ml phenylmethylsulphonyl fluoride and 2 μg/ml leupeptin). Protein concentrations were determined by the Bradford protein assay. Equal amounts of proteins were electrophoresed in 7.5% sodium dodecylsulfate-polyacrylamide gel electrophoresis, and transferred to a Hybond ECL nitrocellulose membrane (Amersham, Piscataway, USA). The membranes were

Table 1 Oligonucleotides, annealing temperatures and number of cycles used for the amplification of each target gene in this study

Target	Oligonucleotide primer (5′→3′)		Product size (bp)	Annealing temperature (°C)	Cycles
HIF-1α	Forward	GCCCCTACTATGTCGCTTTC	433	56	32
	Reverse	GGCCCAAACTAAACTATCTGA			
HIF-2α	Forward	CCCCAGGGGATGCTATTATT	299	56	32
	Reverse	GGCGAAGAGCTTCTCGATTA			
HIF-3α	Forward	AAGTTCACATACTGCGACGAGA	335	55	32
	Reverse	CGAGTATGTTGCTCCGTTTG			
PHD1	Forward	GTGGCGAGGCAAGTTCTAGGC	334	62	30
	Reverse	CCTGCACAGTGGCGGATTAC			
PHD2	Forward	CCATGGTCGCCTGTTACCC	261	57	30
	Reverse	CGTACCTTGTGGCGTATGCAG			
PHD3	Forward	CCTGATGACATTATCGCCTCT	337	55	30
	Reverse	AGCCTAGCGGTATGCGA			
β-actin	Forward	CCTAAGGCCAACCGTGAA	635	55	25
	Reverse	CTAGGAGCCAGGGCAGTAATC			

HIF, hypoxia-inducible factor; PHD, prolyl hydroxylase domain-containing protein.

blocked with 5% (*W*/*V*) instant non-fat milk in Tris-buffered saline , 0.1% Tween-20 for 1 h at room temperature and incubated overnight at 4 °C with specific antibodies of HIF-1α, HIF-1α, HIF-3α, or PHD1, PHD2, PHD3 (Novus Biological), β-actin (Santa Cruz Biotechnology). Immunocomplexes were then labeled with peroxidase-conjugated antimouse or antirabbit immunoglobulin G (Wuhan Boster Biological Technology). An enhanced chemiluminescence detection kit (Santa Cruz Biotechnology) was used for signal detection. Tanon gel image system version 3.74 (Shanghai Tanon Science and Technology) was used for quantification.

Statistical analysis

Data are expressed as mean±standard deviation. One-way ANOVA was used to determine statistically significant difference in more than two groups, and the Newman-Keuls test was used to analyze statistical significance between two groups. $P<0.05$ was considered as a statistically significant difference.

Results

Chronic hypoxia increases *mPAP*

In hypoxic animals, *mPAP* changed as expected: pulmonary hypertension was increased after 7 d of exposure to hypoxia ($P<0.05$), reached its peak after 14 d of hypoxia, then remained unchanged (**Table 2**).

Chronic hypoxia-induced hypoxic pulmonary vascular remodeling and right ventricular hypertrophy

The wall area and medial thickness of pulmonary arterioles were increased after 7 d of hypoxic exposure (**Table 2**). Quantification of these changes, compared with normoxic controls, revealed that *PAMT* increased significantly in hypoxic animals after 7 d of hypoxia and the increase of *WA*% became significant after 14 d of hypoxia. They both increased further with prolonged hypoxia. Right ventricular hypertrophy is a hallmark of pulmonary hypertension resulting from right ventricle pressure overload. After 14 d of hypoxia, *RVHI* was significantly increased compared with the control, and increased further after 21 d of hypoxia. These data indicated that right ventricular hypertrophy developed after 14 d of exposure to hypoxia (**Table 2**).

mRNA and protein levels of three HIF-α subunits in pulmonary arterial walls during normoxia and chronic hypoxia conditions

All three HIF-α mRNA were detected using RT-PCR, but HIF-3α was at a lower level in lungs of control rats (**Fig. 1**). HIF-3α mRNA increased markedly after 3 d of hypoxia, reached its peak at hypoxia day 7, then stabilized. HIF-1α mRNA was slightly upregulated after 7 d of hypoxia. HIF-2α mRNA levels were significantly increased in lungs of hypoxic rats after 7 d and 14 d, but were com-

Table 2 Histological and non-histological parameters for rats exposed to normoxia or hypoxia

Group	*MPAP* (mmHg)	*RVHI* (%)	*WA* (%)	*PAMT* (μm)
Control	16.6±1.6	23.6±2.9	36.3±4.8	8.5±1.3
H3	17.5±1.7	24.4±2.0	38.2±3.2	8.8±1.0
H7	21.7±2.4[ab]	24.6±1.2	43.9±5.3[ab]	10.0±0.7
H14	26.8±3.4[abc]	27.6±1.4[abc]	55.8±5.5[abc]	15.9±1.2[abc]
H21	29.7±3.3[abc]	29.9±1.7[abc]	61.3±7.0[abcd]	20.0±2.4[abcd]
F	43.542	14.714	34.422	102.775
P	<0.01	<0.01	<0.01	<0.01

Data are expressed as mean±standard deviation (n=8). H3, hypoxia for 3 d; H7, hypoxia for 7 d; H14, hypoxia for 14 d; H21, hypoxia for 21 d. [a]P<0.05 versus control group; [b]P<0.05 versus group H3; [c]P<0.05 versus group H7; [d]P<0.05 versus group H14. F, value of one-way ANOVA; *mPAP*, mean pulmonary arterial pressure; *PAMT*, pulmonary artery media thickness; *RVHI*, right ventricular hypertrophy index; *WA*, ratio of vascular wall area to total vascular area.

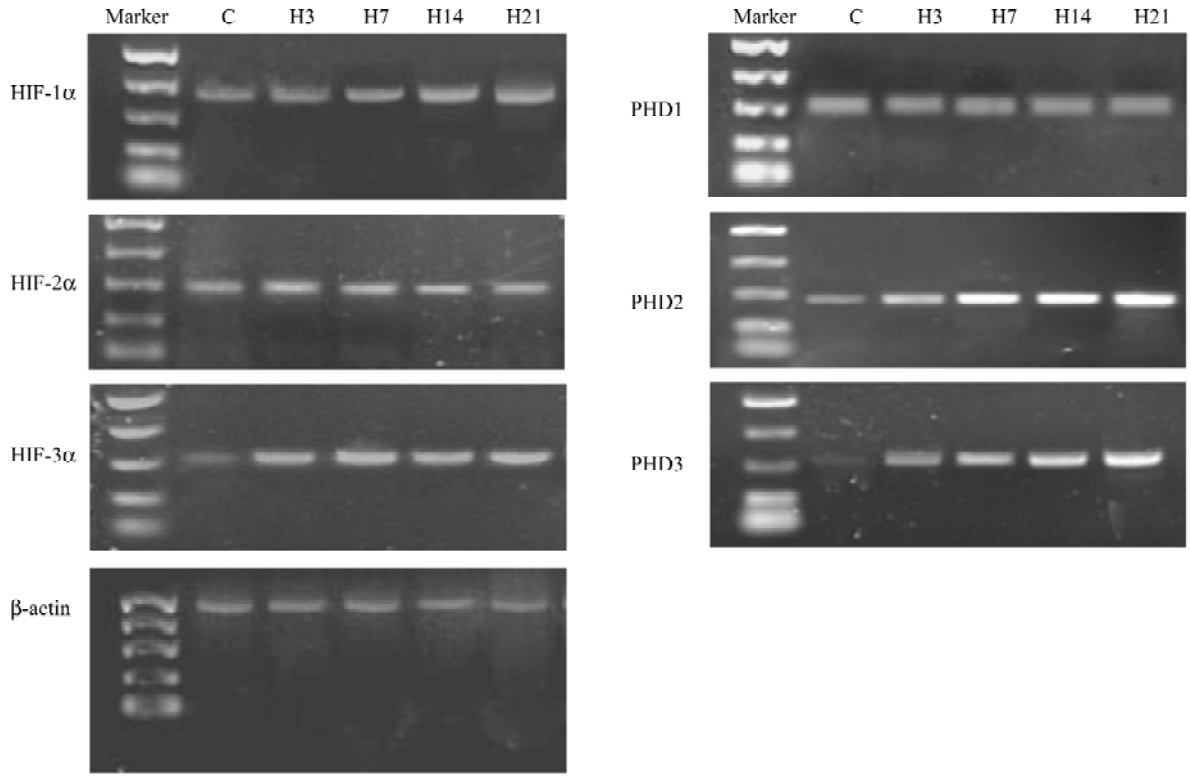

Fig. 1 Reverse transcription-polymerase chain reaction analysis of hypoxia-inducible factor (HIF)-α subunits (HIF-1α, HIF-2α and HIF-3α) and prolyl hydroxylase domain-containing proteins (PHD1–3) mRNA levels in rat lung tissue during normoxia (control) and hypoxia (3, 7, 14 and 21 d)

C, control; H3, hypoxia for 3 d; H7, hypoxia for 7 d; H14, hypoxia for 14 d; H21, hypoxia for 21 d. The amplification of β-actin was used as a control.

parable to that of control rats at hypoxia day 21 (**Fig. 1**). The results of *in situ* hybridization showed the same dynamic expression of HIF-α mRNA in pulmonary arterioles (**Figs. 2**–**4**).

All three HIF-α proteins were hypoxia-inducible in rat lung. In the Western blot results, even in normoxia, the expression of all three HIF-α proteins in the lung tissue could be clearly detected. Expression was enhanced after 3 d of systemic hypoxia and increased further with the prolonged duration of hypoxia, except that HIF-3α proteins declined slightly after 14 d of hypoxia (**Fig. 5**). In normoxic rat lung, all three HIF-α proteins were stained

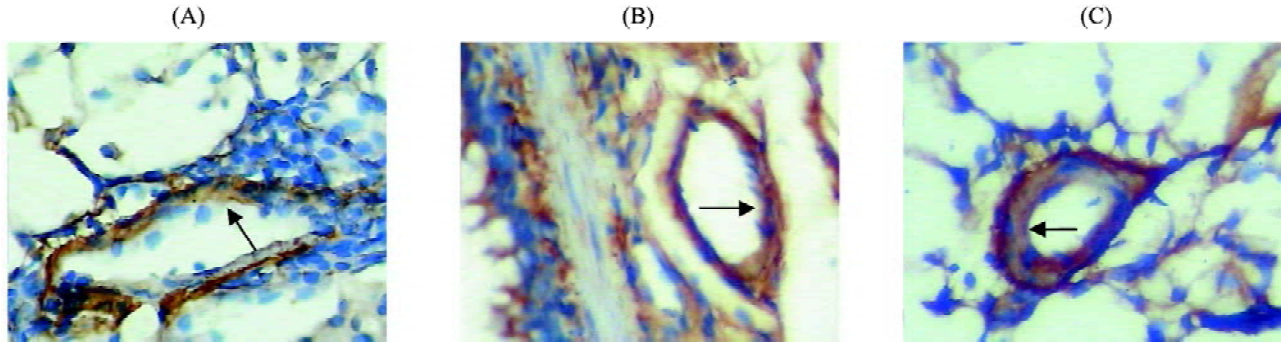

Fig. 2 ***In situ*** **hybridization analysis of hypoxia-inducible factor (HIF)-1α mRNA expression in rat pulmonary arteries after exposure to hypoxia**

HIF-1α mRNA was positively stained in control for 0 d hypoxia (A), increased a little after 7 d of hypoxia (B), then remained unchanged after 21 d of hypoxia (C). Arrow indicates vessel wall. Magnification, 400×.

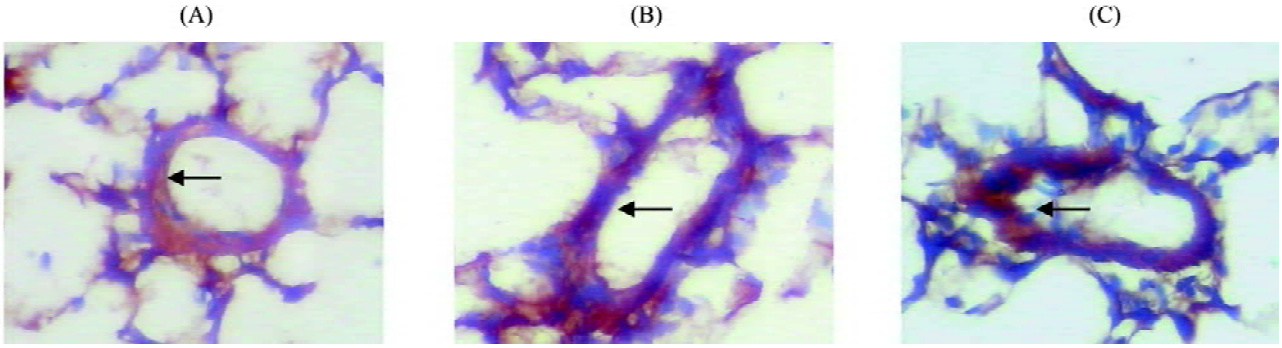

Fig. 3 ***In situ*** **hybridization analysis of hypoxia-inducible factor (HIF)-2α mRNA expression in rat pulmonary arteries after exposure to hypoxia**

HIF-2α mRNA was positively stained in control for 0 d hypoxia (A), increased a little after 7d of hypoxia (B), then remained unchanged after 21 d of hypoxia (C). Arrow indicates vessel wall. Magnification, 400×.

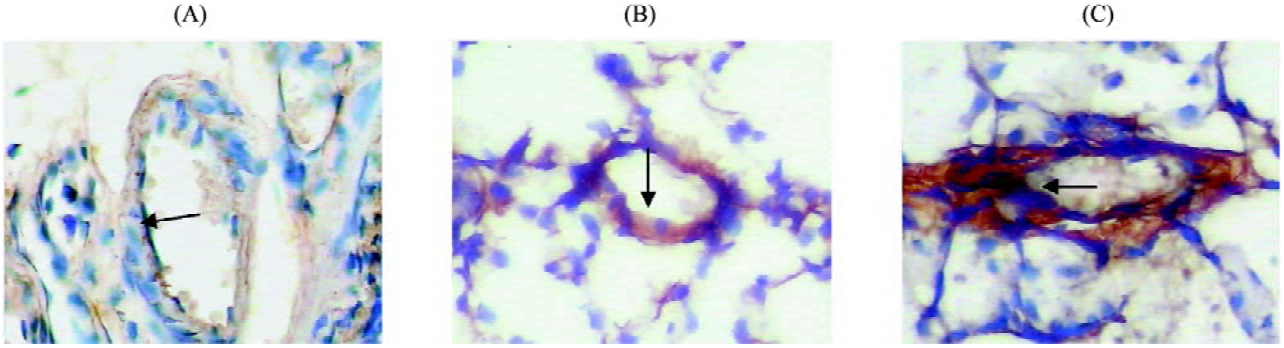

Fig. 4 ***In situ*** **hybridization analysis of hypoxia-inducible factor (HIF)-3α mRNA expression in rat pulmonary arteries after exposure to hypoxia**

HIF-3α mRNA was poorly stained in control for 0 d hypoxia (A), increased after 3 d of hypoxia (B), then increased further after 14 d of hypoxia (C). Arrow indicates vessel wall. Magnification, 400×.

weakly in wall of artery but strongly in the smooth muscle and epithelium of airways. HIF-1α and HIF-2α were positive after 3 d and 7 d of hypoxia. HIF-1α then lessened but HIF-2α increased further till hypoxia day 14, then remained unchanged (**Figs. 6** and **7**). HIF-3α staining became strong after 7 d of hypoxia, and remained stable

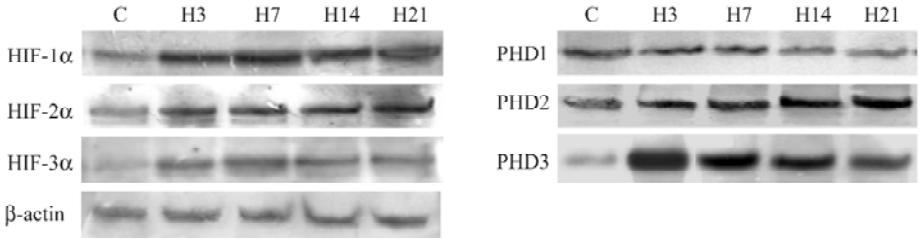

Fig. 5 Western blot analysis of hypoxia-inducible factor (HIF)-α subunits and prolyl hydroxylase domain-containing proteins (PHD1–3) protein levels in rat lung tissue during normoxia (control) and hypoxia (3, 7, 14 and 21 d)

Hypoxia increased the protein levels of all three HIF-α subunits after 3 d of hypoxia. HIF-1α and HIF-2α reached a plateau after 14 d of hypoxia, whereas HIF-3α levels decreased after the same period. PHD1 protein showed a gradual decrease but PHD2 showed a gradual increase after exposure to hypoxia. PHD3 proteins were upregulated after 3 d of hypoxia, then declined after 14 d. C, control; H3, hypoxia for 3 d; H7, hypoxia for 7 d; H14, hypoxia for 14 d; H21, hypoxia for 21 d.

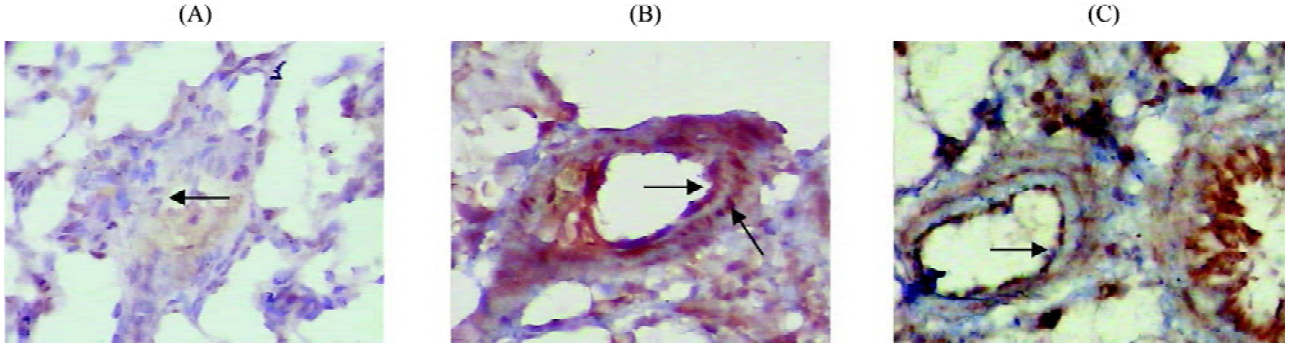

Fig. 6 Immunohistochemistry analysis of hypoxia-inducible factor (HIF)-1α protein expression in rat pulmonary arteries after exposure to hypoxia

HIF-1α protein was negatively stained in control for 0 d hypoxia (A), increased markedly after 3 d of hypoxia (B), then declined slightly after 21 d of hypoxia (C). Arrow indicates vessel wall. Magnification, 400×.

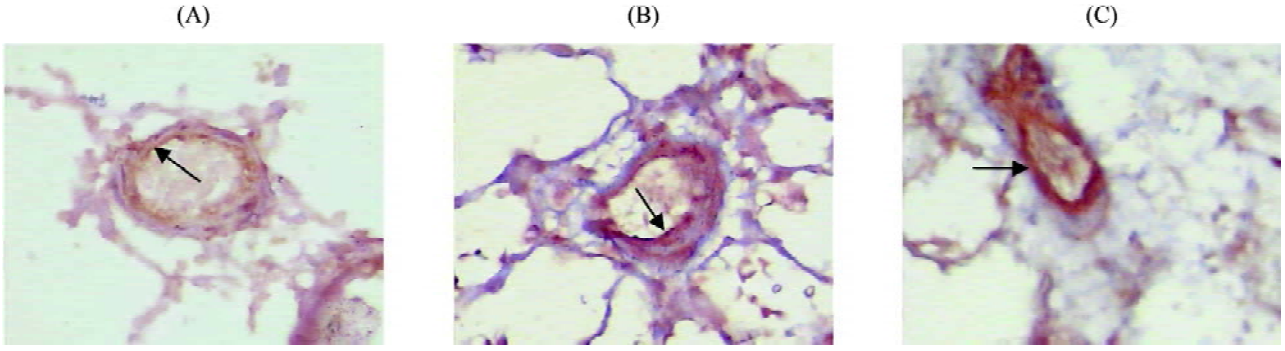

Fig. 7 Immunohistochemistry analysis of hypoxia-inducible factor (HIF)-2α protein expression in rat pulmonary arteries after exposed to hypoxia

HIF-2α protein was poorly stained in control for 0 d hypoxia (A), became positively stained after 3 d of hypoxia (B), then remained at a high level after 21 d of hypoxia (C). Arrow indicates vessel wall. Magnification, 400×.

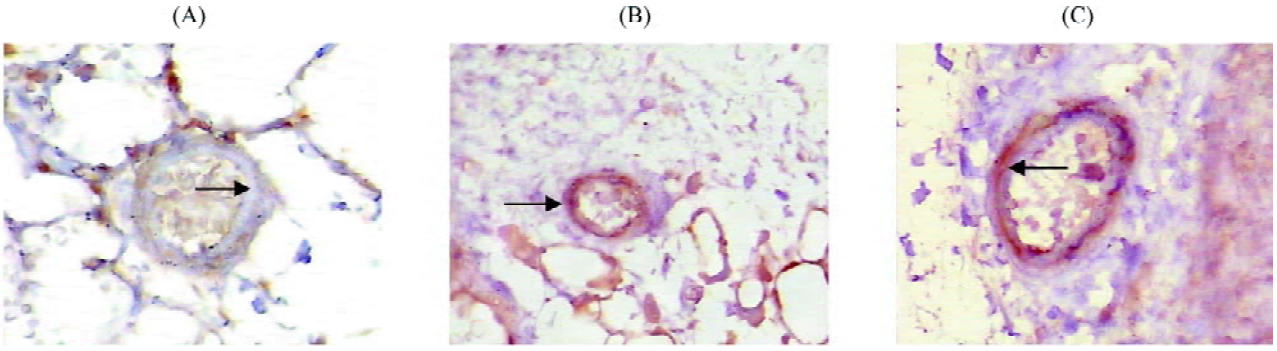

Fig. 8 Immunohistochemistry analysis of hypoxia-inducible factor (HIF)-3α protein expression in rat pulmonary arteries after exposure to hypoxia

HIF-3α protein was negatively stained in control for 0 d hypoxia (A), increased dramatically after 3 d of hypoxia (B). 21 d of hypoxia (C). Arrow indicates vessel wall. Magnification, 400×.

thereafter (**Fig. 8**).

mRNA and protein levels of PHD1, PHD2 and PHD3 in pulmonary arterial walls during normoxia and chronic hypoxia conditions

Although PHD1 mRNA showed little change (P>0.05) according to *in situ* hybridization (**Fig. 9**) and RT-PCR analysis (**Fig. 1**), in hypoxic animals over time, PHD1 protein showed a gradual decrease in hypoxic animals compared with controls. This decrease became significant at hypoxia day 7 (**Fig. 5**). Immunohistochemical analysis revealed that PHD1 was highly expressed in the pulmonary arterial wall of control rats. No significant changes were observed through 3 d and 7 d of hypoxia. However, PHD1-specific staining appeared to be relatively weak in both smooth muscle and endothelium of small pulmonary arteries in rats suffering hypoxic stress for 14 d and 21 d compared with controls (**Fig. 10**).

The result of *in situ* hybridization (**Fig. 11**) and RT-PCR (**Fig. 1**) analysis showed that PHD2 mRNA was clearly expressed in rat lung in normorxia. PHD2 mRNA increased gradually until hypoxia day 14, then remained unchanged. Western blot showed that PHD2 protein expression in rats was increased with prolonged hypoxia. Compared with control values, an increase in PHD2 protein expression was evident at hypoxia day 3 and was significant after hypoxia day 7 (**Fig. 5**). Immunohistochemical analysis revealed that PHD2 was clearly expressed in the pulmonary arterial wall of control rats. Increased staining of PHD2 was found in the smooth muscle layer and endothelium of small pulmonary artery after 7 days of hypoxia **(Fig. 12**).

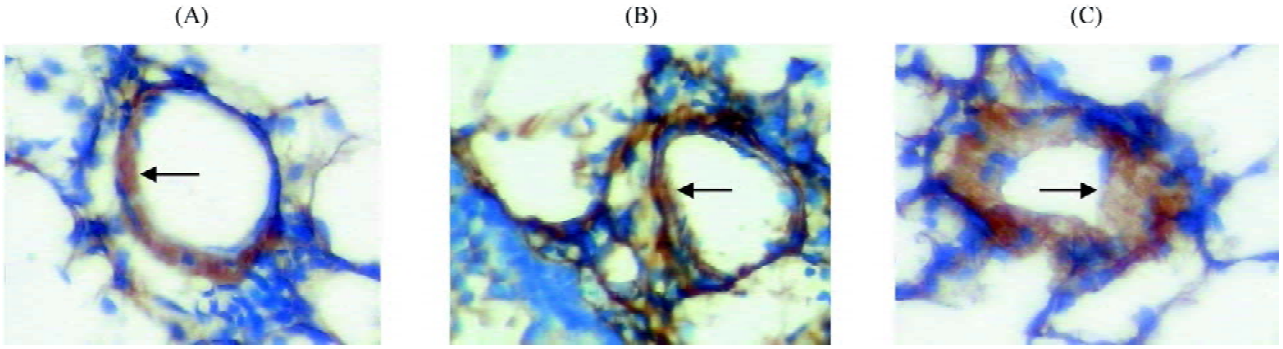

Fig. 9 *In situ* hybridization analysis of hypoxia-inducible factor prolyl hydroxylase domain-containing protein (PHD) 1 mRNA expression in rat pulmonary arteries after exposure to hypoxia

PHD1 mRNA was positively stained in control for 0 d hypoxia (A), but did not show significant changes after exposure to hypoxia (B). 21 d of hypoxia (C). Arrow indicates vessel wall. Magnification, 400×.

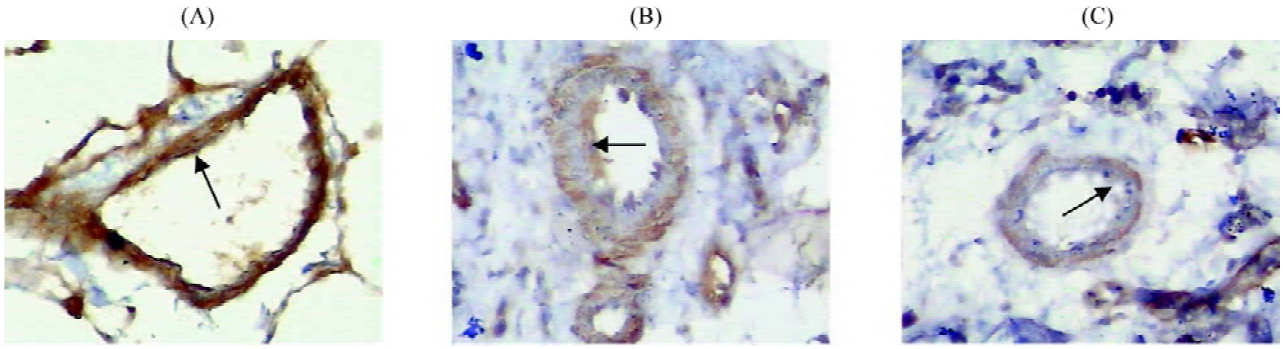

Fig. 10 Immunohistochemistry analysis of hypoxia-inducible factor prolyl hydroxylase domain-containing protein (PHD) 1 protein expression in rat pulmonary arteries after exposure to hypoxia

PHD1 protein was positively stained in control for 0 d hypoxia (A), and became poorly stained after prolonged hypoxia (B). 21 d of hypoxia (C). Arrow indicates vessel wall. Magnification, 400×.

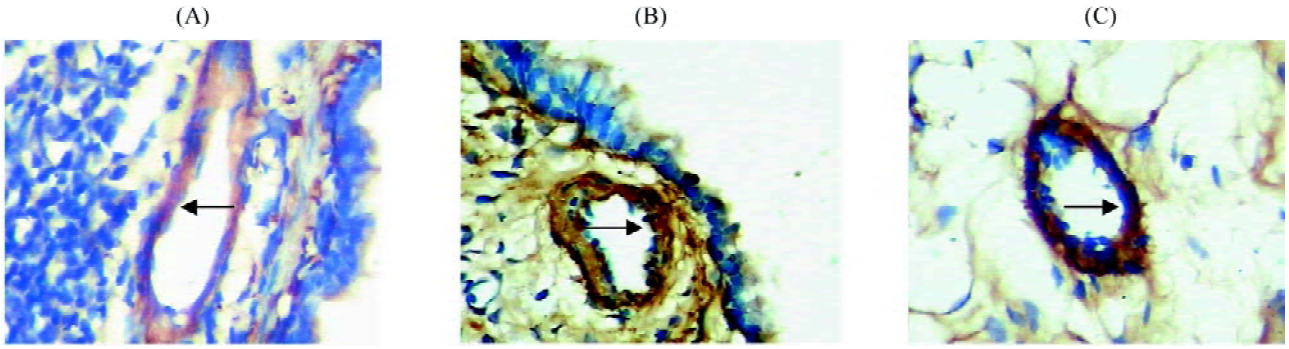

Fig. 11 *In situ* hybridization analysis of hypoxia-inducible factor prolyl hydroxylase domain-containing protein (PHD) 2 mRNA expression in rat pulmonary arteries after exposure to hypoxia

PHD2 mRNA staining was positive in control for 0 d hypoxia (A), and became strongly positive after prolonged hypoxia (B). 21 d of hypoxia (C). Arrow indicates vessel wall. Magnification, 400×.

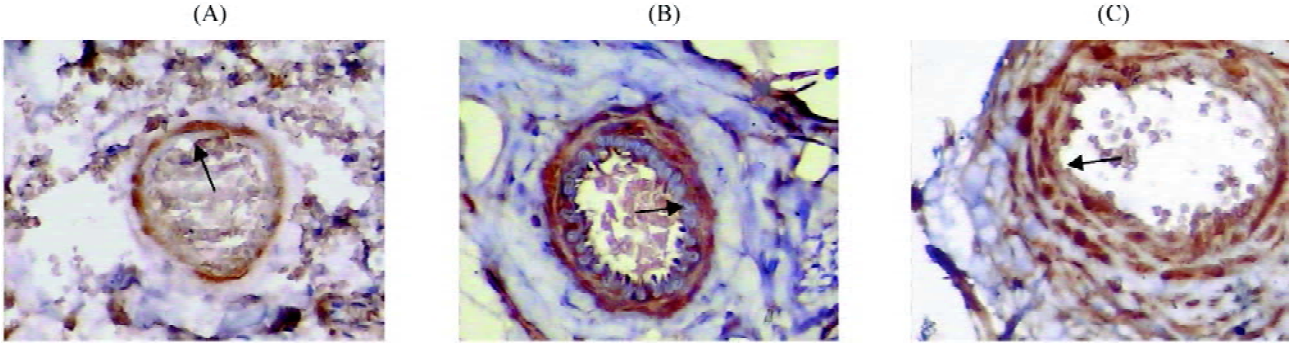

Fig. 12 Immunohistochemistry analysis of hypoxia-inducible factor prolyl hydroxylase domain-containing protein (PHD) 2 protein expression in rat pulmonary arteries after exposure to hypoxia

PHD2 protein staining was positive in control for 0 d hypoxia (A), and become strongly positive after prolonged hypoxia (B). 21 d of hypoxia (C). Arrow indicates vessel wall. Magnification, 400×.

In normorxic rat lung, *in situ* hybridization (**Fig. 13**) and RT-PCR (**Fig. 1**) analysis showed that PHD3 mRNA was expressed at relatively low levels compared with PHD1 and PHD2, but was upregulated more dramatically after exposure to hypoxia. PHD3 protein was also detectable in normoxic rats, and its level was relatively lower than PHD1 or PHD2. PHD3 protein levels were markedly increased in the lung of rats at hypoxia day 3 and day 7 (**Fig. 5**). The levels decreased significantly after 14 d and 21 d of hypoxia, but were still higher than that in the lung of control rats (**Fig. 5**). Immunohistochemical analysis showed that PHD3 was weakly expressed in the lung of control rats. PHD3 staining appeared to be much more intense in both smooth muscle and endothelium of small pulmonary arteries after 3 d and 7 d of hypoxia compared with controls. At hypoxia day 14 and 21, PHD3 level was significantly lower than at hypoxia day 3 and day 7, but significantly higher than in normoxia (**Fig. 14**).

Analysis of linear correlation

Linear correlation analysis showed that there was a significant negative correlation between HIF-1α and PHD2. HIF-2α and HIF-3α showed significant negative correlations with PHD3 and PHD1, respectively, in hypoxic animals. All three HIF-α proteins were positively correlated with PHD2 and PHD3 mRNA, but no correlation was observed between PHD1 mRNA and HIF-α proteins.

Discussion

Chronic hypoxia, due to various obstructive and restric-

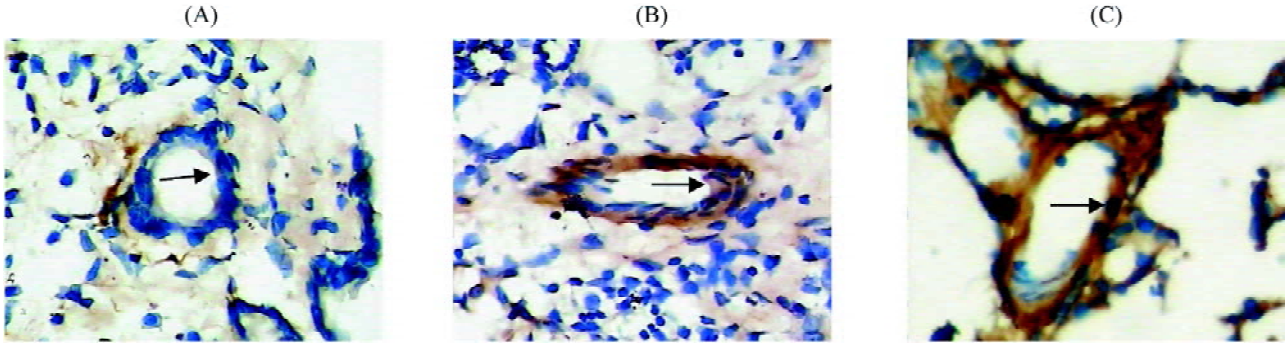

Fig. 13 ***In situ*** **hybridization analysis of hypoxia-inducible factor prolyl hydroxylase domain-containing protein (PHD) 3 mRNA expression in rat pulmonary arteries after exposure to hypoxia**

PHD3 mRNA staining was poorly positive in control for 0 d hypoxia (A), and became strongly positive after prolonged hypoxia (B). 21 d of hypoxia (C). Arrow indicates vessel wall. Magnification,400×.

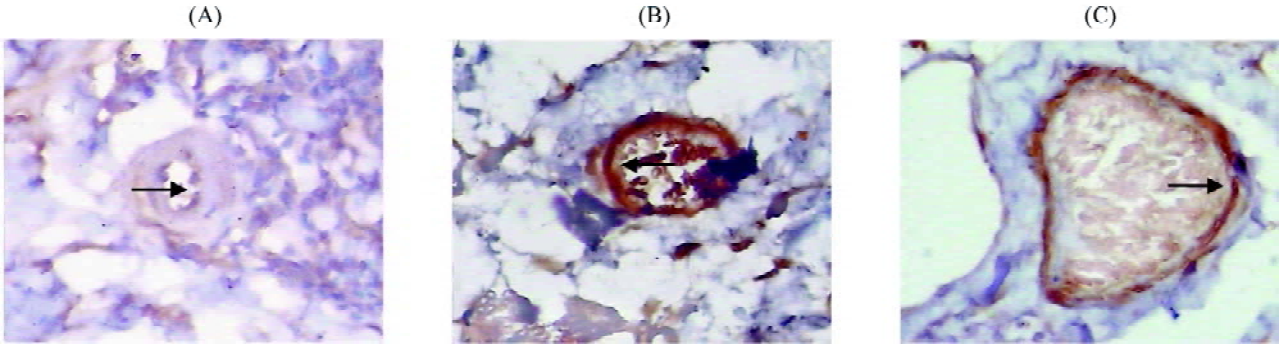

Fig. 14 **Immunohistochemistry analysis of hypoxia-inducible factor prolyl hydroxylase domain-containing protein (PHD) 3 protein expression in rat pulmonary arteries after exposure to hypoxia**

PHD3 protein was poorly stained in control for 0 d hypoxia (A), increased dramatically after 3d and 7d of hypoxia (B), then declined significantly. 21 d of hypoxia (C). Arrow indicates vessel wall. Magnification, 400×.

tive lung diseases, is well known to elicit remodeling of the pulmonary vasculature that is characterized by structural and functional changes in the intima, media and adventitia of the pulmonary artery. These changes cause chronic pulmonary arterial hypertension and subsequent cor pulmonale. HIF plays a pivotal role in vascular remodeling by regulating the expression of its target genes.

Recent studies have defined post-translational modification by PHDs as a key regulatory event that targets HIF-α for proteasomal destruction through the von Hippel-Lindau ubiquitylation complex [7,11]. The present study showed that, when exposed to hypoxia, HIF-α proteins increased dramatically, whereas HIF-α mRNA was upregulated only a little in rat lung. It suggested the post-translational mechanism in rat lung. The K_m values of the three PHDs for O_2 are slightly above atmospheric concentration, so that any decrease in O_2 will result in a reduction in the rate of hydroxylation and an accumulation of HIF protein [12]. This might be why moderate hypoxia (10%) leads to marked accumulation of HIF-α proteins in rat lung. In cultured cells, Appelhoff *et al.* [13] found that HIF-α levels were induced maximally early after hypoxic exposure, then declined significantly due to hypoxia-induced PHD3 and PHD2 levels. The effect was more marked for HIF-2α than for HIF-1α. Interestingly, our data showed, after 14 d of hypoxia, a decline in HIF-1α levels but constant high levels of HIF-2α. The disparity in these results might be attributed to the different expression patterns of PHDs between their study and ours. In our study, PHD1 protein was downregulated after hypoxia exposure and PHD3 protein, in spite of upregulation at earlier stages (hypoxia for 3–7 d), was decreased significantly, compared with hypoxia day 3–7, at later stages (hypoxia for 14 and 7 d).

Although all three PHD proteins contributed to the regulation of HIF-1α and HIF-2α, relative selectivity has also been reported [12–15]. The induction of HIF-2α was affected less than HIF-1α by specific gene silencing of PHD2 using short interfering RNA (siRNA) [13,15]. However, PHD3 suppression alone led to induction of HIF-2α but not HIF-1α [13]. These results indicate that PHD2 has the highest specific activity toward the primary hydroxylation site of HIF-1α, and PHD3 has the highest specificity for hydroxylation of HIF-2α [13,14]. There was also a significant negative correlation between HIF-1α protein and PHD2 protein. In hypoxic rats (control was excluded), HIF-2α and HIF-3α proteins showed significant negative correlations with PHD3 and PHD1 proteins, respectively. These results suggest that the same relative selectivity exists in rat lung after hypoxia exposure. This selectivity contributes, at least in part, to the differential expression patterns of the three HIF-α proteins. It also implies that PHDs retain significant activity in hypoxic rat lungs.

Many studies of cultured cells have shown that the expression of PHD2 and PHD3, not PHD1, is inducible by hypoxia [16–19]. Our data showed that PHD2 mRNA was increased moderately and PHD3 mRNA was upregulated dramatically after exposure to hypoxia. In cell lines with well defined deficiencies in the activation of HIF, no change of PHD3 or PHD2 mRNAs was observed upon exposure to low oxygen tension; and inactivation of pVHL, a protein necessary for oxygen-dependent degradation of HIF-1α, is sufficient to induce PHD3 mRNA in normoxia [17]. Furthermore, Metzen *et al.* [20] have identified the dominant promoter of the *phd2* gene, which contains a functional hypoxia-responsive element and confers hypoxic inducibility. These studies suggested that the hypoxia-induced expression of PHDs is in a HIF-α dependent manner. In the present study, linear correlation analysis showed that HIF-α proteins were positively correlated with PHD2 and PHD3 mRNA, but there was no correlation between PHD1 mRNA and HIF-α protein. This suggested that PHD2 and PHD3 might also be directly upregulated by HIF-α.

Silence of HIF-1α by siRNA results in significant downregulation of hypoxia-induced PHD2 and PHD3 levels [16,19]. Knockdown of HIF-2α by siRNA reduced hypoxic induction of PHD3, but not PHD2 [19]. This indicated relative selectivity for the regulation of PHD isoforms by HIF-α isoforms, but whether this selectivity is applicable in the rat model needs to be investigated further. One published study also showed that PHD1 expression is reduced under hypoxic conditions [21], but the same reduction of PHD1 mRNA could not be observed in the lung of our HPH model. Taken together, in the lung of our HPH model, PHDs and HIF-α would form a feedback loop when exposed to hypoxia: the hypoxia-induced accumulation of HIF-α leads to the increase in the total amount of PHD1 and PHD3 enzymes after a long time. As a result, at least under moderate hypoxia, PHD induction should result in augmented HIF-α degradation and provide a feedback control on HIF signaling.

In the present study, the mRNA expression levels of PHD1 and PHD3 did not parallel their proteins. PHD1 proteins declined gradually without significant changes after the rats were exposed to hypoxia. After 7 d and 14 d of hypoxia, although PHD3 mRNA increased further, PHD3 protein was decreased significantly. This indicated that PHDs might be downregulated by some post-transcriptional mechanism. Nakayama *et al.* recently documented

that Siah1a/2, mammalian homologues of the *Drosophila* seven-in-absentia, targets PHD1 and PHD3 for proteasome-dependent degradation under hypoxic conditions [22]. But further research is needed to clarify whether Siah1a/2 contributes to the regulation of PHDs during the development of HPH.

Considering that HIF-α signaling is fundamental for the homeostasis of pulmonary arteries, we propose that alteration of PHD expression levels and activity, due to decreased availability of oxygen, and subsequent accumulation of HIF-α, is likely to play an important role in the development of the pulmonary vascular remodeling in our model of pulmonary hypertension secondary to increased systemic hypoxic stress. Further understanding of these mechanisms could lead to potential new therapies for the management of hypoxic pulmonary hypertension.

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