

## Hypoxia Induces Transforming Growth Factor- $\beta$ 1 Gene Expression in the Pulmonary Artery of Rats via Hypoxia-inducible Factor-1 $\alpha$

Yongliang JIANG, Aiguo DAI\*, Qifang LI, and Ruicheng HU

*Department of Respiratory Medicine, Hunan Institute of Gerontology, Hunan Province Geriatric Hospital, Changsha 410001, China*

**Abstract** The present study was undertaken to investigate the dynamic expression of hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) in hypoxia-induced pulmonary hypertension of rats. It was found that mean pulmonary arterial pressure (mPAP) increased significantly after 7 d of hypoxia. Pulmonary artery remodeling index and right ventricular hypertrophy became evident after 14 d of hypoxia. HIF-1 $\alpha$  mRNA staining was less positive in the control, hypoxia for 3 d and hypoxia for 7 d, but began to enhance significantly after 14 d of hypoxia, then remained stable. Expression of HIF-1 $\alpha$  protein in the control was less positive, but was up-regulated in pulmonary arterial tunica intima of all hypoxic rats. TGF- $\beta$ 1 mRNA expression in pulmonary arterial walls was increased significantly after 14 d of hypoxia, but showed no obvious changes after 3 or 7 d of hypoxia. In pulmonary tunica adventitia and tunica media, TGF- $\beta$ 1 protein staining was less positive in control rats, but was markedly enhanced after 3 d of hypoxia, reaching its peak after 7 d of hypoxia, and then weakening after 14 and 21 d of hypoxia. Western blotting showed that HIF-1 $\alpha$  protein levels increased significantly after 7 d of hypoxia and then remained at a high level. TGF- $\beta$ 1 protein level was markedly enhanced after 3 d of hypoxia, reaching its peak after 7 d of hypoxia, and then decreasing after 14 and 21 d of hypoxia. Linear correlation analysis showed that HIF-1 $\alpha$  mRNA, TGF- $\beta$ 1 mRNA, TGF- $\beta$ 1 protein were positively correlated with mPAP, vessel morphometry and right ventricular hypertrophy index. TGF- $\beta$ 1 protein (tunica adventitia) was negatively correlated with HIF-1 $\alpha$  mRNA. Taken together, our results suggest that changes in HIF-1 $\alpha$  and TGF- $\beta$ 1 expression after hypoxia play an important role in hypoxia-induced pulmonary hypertension of rats.

**Key words** hypoxia inducible factor-1 $\alpha$ ; transforming growth factor- $\beta$ 1; hypertension; lung; hypoxia

Hypertension occurs as a result of narrowing of the lumen of the pulmonary arteries due to vasoconstriction or hyperplasia of pulmonary vascular smooth muscle cells. Primary pulmonary hypertension (PPH) is a condition of unknown etiology, whereas secondary pulmonary hypertension can accompany a number of chronic hypoxic lung disorders [1]. Vascular smooth muscle cells and endothelial cells play important roles in the development of pulmonary hypertension [2]. Increased plasma levels of several

factors that are responsible for the regulation of pulmonary vascular tone and smooth muscle cell proliferation have been associated with pulmonary hypertension, including interleukin (IL)-1, IL-6, endothelin-1, and prostanoids [3, 4]. The production of prostanoids can act as a negative-feedback mechanism, in the same way that prostaglandin (PG) E<sub>2</sub> and PGI<sub>2</sub> are potent vasodilators and inhibitors of vascular remodeling [5,6].

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a polypeptide cytokine that exists in three isoforms: TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3. TGF- $\beta$  isoforms, particularly TGF- $\beta$ 1, can regulate smooth muscle cell proliferation and vascular remodeling [7]. In experimental settings systemic administration of TGF- $\beta$ 1 resulted in rich in extracellular matrix proteins [8]. Conversely, anti-TGF- $\beta$ 1 neutralizing anti-

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\*Corresponding author: Tel, 86-731-4762793; Fax, 86-731-4735215; E-mail, daiaiguo2003@163.com

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bodies reduced extracellular matrix proteins, which is further evidence of the important role of TGF- $\beta$ 1 in vascular repair [9]. Hypoxia inducible factor 1 (HIF-1) is a potential mediator of pulmonary responses to hypoxia [10]. HIF-1 is a heterodimeric transcription factor composed of a hypoxia inducible factor 1 alpha (HIF-1 $\alpha$ ) functional subunit and hypoxia-inducible factor 1 beta (HIF-1 $\beta$ ) constitutonal subunit. Hypoxia induces the expression of HIF-1 $\alpha$ , which then activates the transcriptions of some hypoxia-responsive genes. However, the role of HIF-1 $\alpha$  and TGF- $\beta$ 1 in the development of hypoxia-induced pulmonary hypertension and the accompanying vascular remodelling is not completely understood. In this study, we investigated the expression of the HIF-1 $\alpha$  and TGF- $\beta$ 1 genes, as well as their relationship to each other, in pulmonary arterial walls of rats at different phases of hypoxia-induced pulmonary hypertension development.

## Materials and Methods

### Animals and hypoxia model

The protocol for exposure of rats to hypoxia and normoxia was identical to that reported previously by our laboratory [11]. In the present study, we used 40 male Wistar rats purchased from the Animal Experimental Centre of Central South University (Changsha, China). The animals weighed 220 $\pm$ 10 g and the average age was 6–8 weeks. They were randomly divided into five groups (eight rats in each group). Each group of hypoxic rats was exposed for a specified time period (3, 7, 14, or 21 d) with 8 h per day intermittently to normobaric hypoxia (10.0% $\pm$ 0.5% oxygen) in a ventilated chamber. Age- and weight-matched control rats were maintained in normobaric 21% oxygen (fresh air). To establish the hypoxic conditions the chamber was flushed with a mixture of room air and nitrogen from a liquid nitrogen reservoir. An oxygen analyzer (HT-6101; Kanda Electrical, Chengdu, China) was used to monitor the chamber environment. Carbon dioxide was removed with soda lime, excess humidity removed by anhydrous calcium chloride, and boric acid was used to keep ammonia levels within the chamber to a minimum. The normoxic control rats were not kept in the chamber but they were housed in the same room and treated in the same way as the hypoxia rats.

### Mean pulmonary arterial pressure measurement

Mean pulmonary arterial pressure (mPAP) was measured as described previously [12]. 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, at which point the position of the catheter was judged by the waveform of the pressure signal. The mPAP was measured with PowerLab monitoring equipment (AD Instruments, Milford, USA).

### Right ventricular hypertrophy index

After the measurement of mPAP, the rats were killed and their lungs were collected for morphometry analysis, *in situ* hybridization and immunohistochemical examination; their hearts were collected for measurement of right ventricular hypertrophy index (RVHI). For right ventricular hypertrophy measurement, hearts were excised and atria were removed. The right ventricular free wall was dissected, and each chamber weighed. The ratio of right ventricular (RV) weight to the weight of left ventricle (LV) plus septum (S) ( $W_{RV}/W_{LV+S}$ ) was used as an index of right ventricular hypertrophy.

### Vessel morphometric analysis

Lung sections (4- $\mu$ m thick) were embedded in paraffin, stained with hematoxylin-eosin, then examined using light microscopy. At least five representative pulmonary arterioles (outer diameter approximately 100–150  $\mu$ m), chosen from three different sections from each animal, were independently examined. The images of the arterioles were captured and analyzed with PIPS-2020 Image software (Tianhai Co., Chongqing, China). To evaluate hypoxic pulmonary vascular remodeling, the ratio of vascular wall area to external diameter, the ratio of vascular lumen area to total area, the number of smooth muscle cell nuclei in pulmonary arteriole tunica media (SMC, per 1000  $\mu$ m<sup>2</sup>) and pulmonary artery media thickness were obtained.

### *In situ* hybridization of HIF-1 $\alpha$ and TGF- $\beta$ 1

*In situ* hybridization was carried out using a detection kit (Boster Biological Technology Co., Wuhan, China). The oligonucleotide probes (Boster Biological Technology Co.) were designed according to the HIF-1 $\alpha$  and TGF- $\beta$ 1 sequences of rat. The sequences of probes against HIF-1 $\alpha$  mRNA were: 5'-TTATGAGCTTGCTCATC-AGTTGCCACTTCC-3'; 5'-CTCAGTTTGAACACTG-GACACAGTGTGT-3'; 5'-GGCCGCTCAATTTATGAAT-ATTATCATGCT-3'. The sequences of probes against TGF- $\beta$ 1 mRNA were: 5'-ACCTGCAAGACCATCGACA-TGGAGCTGGTG-3'; 5'-TGTACAACAGCACCCGCGAC-CGGGTGGCAG-3'; 5'-CTACCAGAAATATAGCAACAA-TTCCTGGCG-3'.

Hybridization was carried out on serial sections of formalin-fixed (containing 0.1% diethylpyrocarbonate) paraffin-embedded lung tissues according to the manufacturer's instructions. Briefly, sections were digested with pepsin for 20 min at 37 °C. After 2 h of prehybridization, sections were incubated with digoxin-labelled single-stranded oligonucleotide probes for 16 h at 38 °C (the negative control was incubated with blank probes solution). After unbound probes were washed off, sections were incubated with rabbit antibodies against digoxin and with biotinylated goat anti-rabbit secondary antibodies. Afterwards, sections were incubated with streptavidin-horseradish peroxidase (HRP) and visualized by a color reaction with diaminobenzidine (Boster Biological Technology Co.). Brown and yellow colors indicated positive results. Finally, the sections were counterstained with hematoxylin and mounted. Expression levels of mRNA were quantified by the pathology image analysis system (PIPS-2020).

#### Immunohistochemistry analysis of HIF-1 $\alpha$ and TGF- $\beta$ 1

A streptavidin-biotin complex kit (Boster Biological Technology Co.) was used for immunohistochemistry, which was carried out similar to that described previously with minor modifications. Briefly, serial sections of formalin-fixed paraffin-embedded lung tissues were digested with 3% H<sub>2</sub>O<sub>2</sub> for 20 min at room temperature, then preincubated with 10% non-immunized serum. Sections were incubated with rabbit anti-HIF-1 $\alpha$  or anti-TGF- $\beta$ 1 antibody (at a working dilution of 1:100) overnight at 4 °C (the negative control was incubated with PBS only). After unbound antibodies were washed off, the sections were incubated with biotinylated goat anti-rabbit secondary antibodies and thereafter incubated with streptavidin-HRP. Subsequently, sections were visualized by a color reaction with diaminobenzidine as the substrate. Brown and yellow colors indicated positive results (mainly cytoplasm). Finally, the sections were counterstained with hematoxylin (resulting in blue nuclei) and mounted. Expression levels of protein were quantified by a pathology image analysis system (PIPS-2020).

#### Western blot analysis of HIF-1 $\alpha$ and TGF- $\beta$ 1

Rat lung tissues were homogenized (50 g/L) in lysis buffer (10 mM Tris, 50 mM NaCl, 0.03  $\mu$ M sodium pyrophosphate, 50 mM sodium fluoride, and 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride, 20 mg/L aprotinin, and 1 mM sodium vanadate [13]. After homogenization, samples were centrifuged at 15,000 g for 15 min. The supernatants were taken for further analysis.

Protein concentration was determined by the Bradford method [14]. Approximately 10  $\mu$ g of the total protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were probed with polyclonal anti-HIF-1 $\alpha$  or anti-TGF- $\beta$ 1 rabbit antibody (Cell Signaling, Beverly, USA). Immunoreactive bands were visualized by the addition of horseradish peroxidase-conjugated antibodies against rabbit Fab and chemiluminescent substrates (Pierce, Rockford, USA), according to the manufacturer's instructions.

#### Statistical analysis

Data were expressed as mean $\pm$ SD. The group *t*-test was used to compare data between two groups. ANOVA was used to determine statistically significant differences among multiple groups, with Newman-Keuls test comparing the statistical significance between the two groups. *P*<0.05 was considered as statistically significant.

## Results

### Chronic hypoxia increased mPAP

Mean pulmonary arterial pressure was measured as an indicator of pulmonary artery pressure in conscious rats. mPAP in normoxic rats was 14.02 $\pm$ 0.41 mmHg. As expected, the hypoxic animals developed pulmonary hypertension after 7 d of exposure to hypoxia (*P*<0.05), reaching its peak level after 14 d of hypoxia, and thereafter remaining at a high level (Table 1).

### Chronic hypoxia led to hypoxic pulmonary vascular remodeling and right ventricle hypertrophy

As shown in Table 1, pulmonary arterioles in normoxic animals were thin, whereas after 7 d of hypoxic exposure, they developed increased medial thickness characteristic of pulmonary hypertension. Quantification of these structural changes in several lung sections of all of the animals exposed to each of the different hypoxia time periods (3, 7, 14 or 21 d) revealed significantly increased medial thickness of pulmonary arterioles in hypoxic animals in comparison with normoxic controls. Right ventricular hypertrophy resulting from right ventricle pressure overload is a hallmark of pulmonary hypertension. After 14 d of hypoxia, RVHI was significantly increased in comparison with the control (*P*<0.05). RVHI had increased further after 21 d of hypoxia. This result indicated right ventricular hypertrophy had developed after 14 d of

**Table 1** Effects of different time periods of hypoxia on mean pulmonary arterial pressure (mPAP), right ventricle (RV) hypertrophy, and pulmonary arteries remodeling in rats

Group	mPAP (mmHg)	LA (%)	WA (%)	SMC	PAMT ( $\mu\text{m}$ )	$W_{\text{RV}}/W_{\text{LV+S}}$ (%)
Control	14.02 $\pm$ 0.41	64.5 $\pm$ 1.3	35.5 $\pm$ 1.3	5.5 $\pm$ 1.0	11.9 $\pm$ 0.6	23.6 $\pm$ 0.5
3 d	14.58 $\pm$ 0.28	63.0 $\pm$ 0.9	37.0 $\pm$ 0.8	5.8 $\pm$ 0.8	12.0 $\pm$ 0.5	23.3 $\pm$ 1.7
7 d	18.41 $\pm$ 0.37 <sup>ac</sup>	52.2 $\pm$ 0.8 <sup>ac</sup>	47.8 $\pm$ 0.8 <sup>ac</sup>	6.1 $\pm$ 0.8	12.3 $\pm$ 0.5	24.0 $\pm$ 0.9
14 d	21.17 $\pm$ 0.23 <sup>acd</sup>	39.7 $\pm$ 0.4 <sup>acd</sup>	60.3 $\pm$ 0.4 <sup>acd</sup>	7.1 $\pm$ 0.8 <sup>acd</sup>	15.0 $\pm$ 0.3 <sup>acd</sup>	25.0 $\pm$ 1.8 <sup>ac</sup>
21 d	22.24 $\pm$ 0.21 <sup>bcd</sup>	35.0 $\pm$ 0.7 <sup>bcd</sup>	65.0 $\pm$ 0.7 <sup>acde</sup>	9.1 $\pm$ 0.8 <sup>acde</sup>	23.0 $\pm$ 0.8 <sup>acde</sup>	27.7 $\pm$ 1.0 <sup>bcd</sup>

<sup>a</sup>  $P < 0.05$  compare with the control group, <sup>b</sup>  $P < 0.01$  compare with the control group, <sup>c</sup>  $P < 0.05$  compared with hypoxia for 3 d, <sup>d</sup>  $P < 0.05$  compared with hypoxia for 7 d, <sup>e</sup>  $P < 0.05$  compared with hypoxia for 14 d. LA, the ratio of vascular lumen area to total area; PAMT, pulmonary artery media thickness; SMC, the number of smooth muscle cell nuclei in pulmonary arteriole media per 1000  $\mu\text{m}^2$ ; WA, the ratio of vascular wall area to external diameter. Data are represented as mean $\pm$ SD ( $n=8$ ).

exposure to hypoxia.

### Hypoxia induces HIF-1 $\alpha$ and TGF- $\beta$ 1 mRNA expression in pulmonary arterial walls

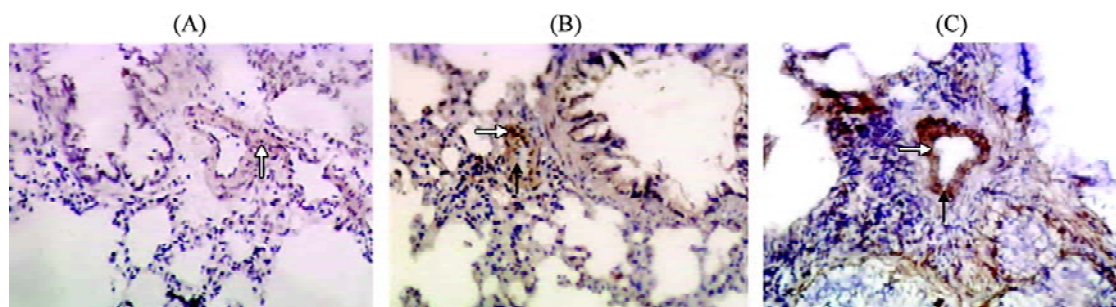
**Table 2** shows that HIF-1 $\alpha$  mRNA levels in pulmonary arterial walls had increased significantly by 14 d of hypoxia and remained stable, but had shown no obvious changes by 7 d of hypoxia or normoxia (**Fig. 1**). HIF-1 $\alpha$  mRNA

was located mainly in the tunica intima and tunica media. Control pulmonary arterioles displayed low-level expression of TGF- $\beta$ 1 transcripts in medial SMCs. Adventitial fibroblasts also showed a paucity of TGF- $\beta$ 1 transcripts, TGF- $\beta$ 1 mRNA expression in pulmonary arterial walls was increased significantly after 14 and 21 d of hypoxia, but showed no obvious changes after 3 or 7 d of hypoxia (**Fig. 2**).

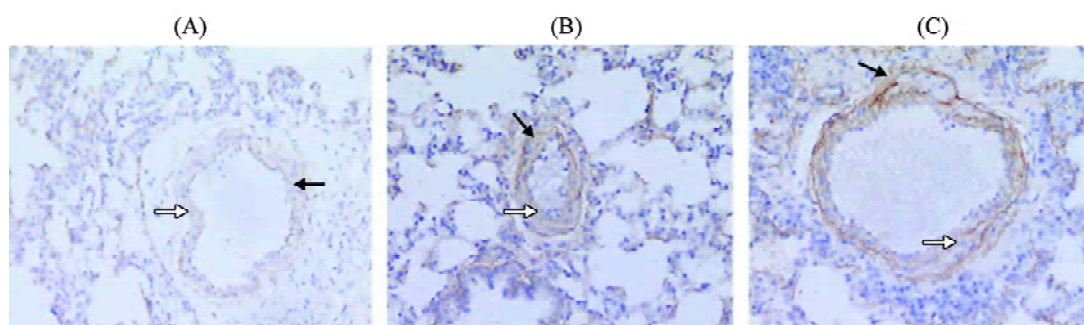
**Table 2** Effects of different hypoxia time periods of hypoxia on expression of hypoxia inducible factor (HIF)-1 $\alpha$  and transforming growth factor (TGF)- $\beta$ 1 gene in the pulmonary arteries of rats

Group	Absorbance of different sample			
	HIF-1 $\alpha$ protein	HIF-1 $\alpha$ mRNA	TGF- $\beta$ 1 protein	TGF- $\beta$ 1 mRNA
Control	0.05 $\pm$ 0.01	0.05 $\pm$ 0.01	0.042 $\pm$ 0.012	0.145 $\pm$ 0.018
3 d	0.20 $\pm$ 0.02 <sup>a</sup>	0.06 $\pm$ 0.02	0.198 $\pm$ 0.031 <sup>a</sup>	0.163 $\pm$ 0.021
7 d	0.22 $\pm$ 0.02 <sup>ab</sup>	0.05 $\pm$ 0.02	0.267 $\pm$ 0.035 <sup>ab</sup>	0.176 $\pm$ 0.026
14 d	0.16 $\pm$ 0.01 <sup>abc</sup>	0.20 $\pm$ 0.02 <sup>abc</sup>	0.143 $\pm$ 0.026 <sup>abd</sup>	0.385 $\pm$ 0.028 <sup>abd</sup>
21 d	0.10 $\pm$ 0.01 <sup>abc</sup>	0.18 $\pm$ 0.01 <sup>abc</sup>	0.125 $\pm$ 0.015 <sup>abd</sup>	0.413 $\pm$ 0.025 <sup>abd</sup>

<sup>a</sup>  $P < 0.01$  compared with control group, <sup>b</sup>  $P < 0.01$  compared with 3 d of hypoxia, <sup>c</sup>  $P < 0.05$  compared with 7 d of hypoxia, <sup>d</sup>  $P < 0.01$  compared with 7 d of hypoxia. Data are represented as mean $\pm$ SD ( $n=8$ ).



**Fig. 1** *In situ* hybridization of hypoxia inducible factor (HIF)-1 $\alpha$  mRNA expression in the pulmonary arteries of rats (A) Control. (B) Hypoxia for 7 d. (C) Hypoxia for 14 d. Blank arrow, arterial intima; black arrow, arterial media. Magnification, 200 $\times$ .

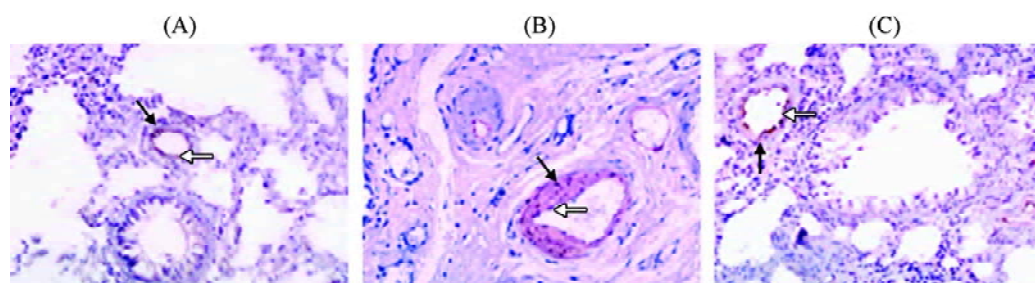


**Fig. 2** *In situ* hybridization of transforming growth factor (TGF)- $\beta$ 1 mRNA expression in the pulmonary arteries of rats (A) Control. (B) Hypoxia for 7 d. (C) Hypoxia for 14 d. Blank arrow, arterial intima; black arrow, arterial media. Magnification, 200 $\times$ .

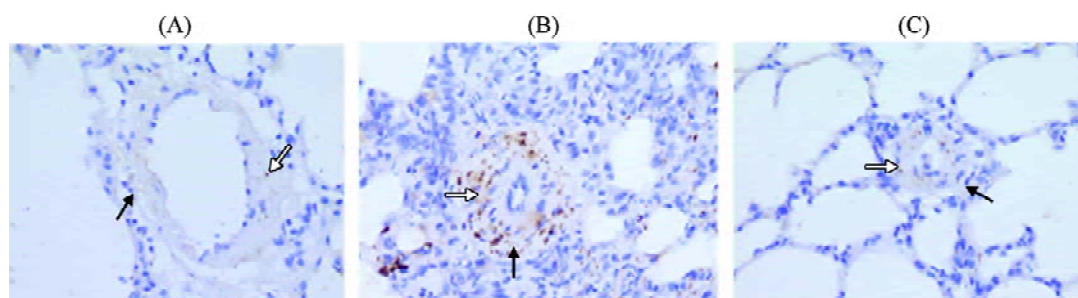
### Hypoxia induces HIF-1 $\alpha$ and TGF- $\beta$ 1 protein expression in pulmonary arterial walls

The HIF-1 $\alpha$  protein level varied between the pulmonary arterial tunica media and tunica intima. In pulmonary arterial tunica media, HIF-1 $\alpha$  protein levels were poorly positive in control rats, but had increased markedly by 3 d and further by 7 d of hypoxia, then lessened by 14 d and even further by 21 d of hypoxia. In pulmonary arterial tunica intima, however, HIF-1 $\alpha$  staining was poorly

positive in control rats, but strongly positive in all hypoxic rats (Table 2 and Fig. 3). In pulmonary arterioles tunica adventitia and tunica media, TGF- $\beta$ 1 protein staining was poorly positive in control rats, but was markedly enhanced after 3 and 7 d of hypoxia, then weakened after 14 and 21 d of hypoxia (Fig. 4). In Western blot analysis, HIF-1 $\alpha$  protein levels increased significantly after 7 d of hypoxia then remained at a high level. TGF- $\beta$ 1 protein levels were markedly enhanced after 3 d of hypoxia, reached a peak after 7 d of hypoxia, and then decreased after 14 and 21 d



**Fig. 3** Immunohistochemistry of hypoxia inducible factor (HIF)-1 $\alpha$  protein expression in the pulmonary arteries of rats (A) Control. (B) Hypoxia for 14 d. (C) Hypoxia for 21 d. Blank arrow, arterial intima; black arrow, arterial media. Magnification, 200 $\times$ .

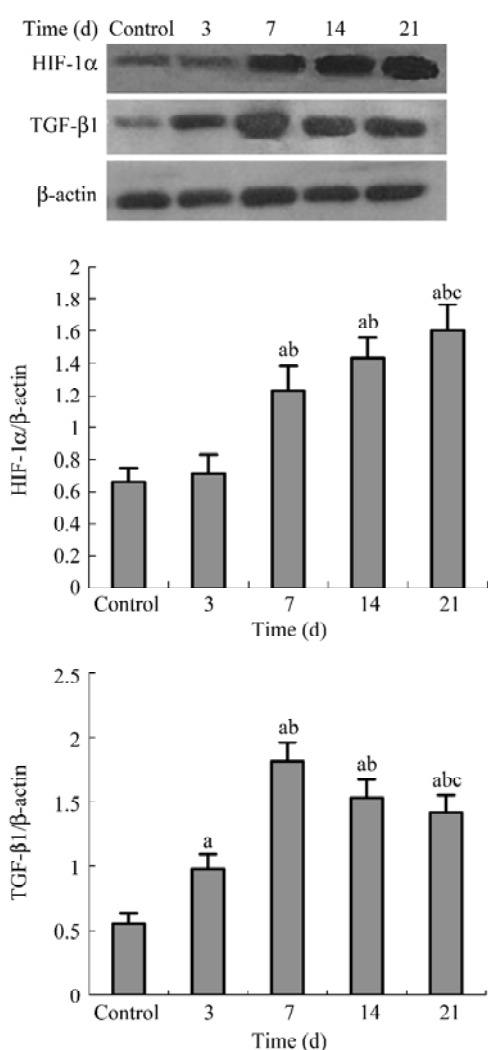


**Fig. 4** Immunohistochemistry of transforming growth factor (TGF)- $\beta$ 1 protein expression in the pulmonary arteries of rats (A) Control. (B) Hypoxia for 7 d. (C) Hypoxia for 14 d. Blank arrow, arterial intima; black arrow, arterial media. Magnification, 200 $\times$ .

of hypoxia (Fig. 5).

### Analysis of linear correlation

The linear correlation analysis was carried out between different parameters for hypoxia rats. Linear correlation analysis showed that HIF-1 $\alpha$  mRNA, TGF- $\beta$ 1 mRNA and TGF- $\beta$ 1 protein were positively correlated with mPAP, vessel morphometry and RVHI ( $r=0.811-0.931$ ,  $P<0.05$ ). TGF- $\beta$ 1 protein (tunica adventitia) was negatively correlated with HIF-1 $\alpha$  mRNA ( $r=-0.836$ ,  $P<0.05$ ).



**Fig. 5** Western blot detection of hypoxia inducible factor (HIF)-1 $\alpha$  and transforming growth factor (TGF)- $\beta$ 1 levels respectively ( $n=3$ )

The histogram showed the comparisons of optical density odds among different groups. 1, control group; 2, hypoxia for 3 d group; 3, hypoxia for 7 d group; 4, hypoxia for 14 d group; 5, hypoxia for 21 d group. <sup>a</sup> $P<0.01$  compared with the control group; <sup>b</sup> $P<0.01$  compared with hypoxia for 3 d; <sup>c</sup> $P<0.05$  compared with hypoxia for 7 d.

### Discussion

Chronic hypoxia in the pulmonary vasculature is known to result in vascular remodeling characterized by proliferation and migration of smooth muscle cells, as well as by an increased accumulation of extracellular matrix. The present results demonstrate that the hypoxic animals developed pulmonary hypertension after 7 days of exposure to hypoxia and hypoxia groups (hypoxia for 14 and 21 d) revealed significantly increased medial thickness of pulmonary arterioles and the muscularization of non-muscular pulmonary arterioles in hypoxic animals compared with normoxic controls.

Several factors induced by hypoxia have been implicated as modulators or mediators in the vascular remodeling of hypoxia-induced pulmonary hypertension. These include endothelin-1 [15], vascular endothelial growth factor [16], angiotensin II [17], and nitric oxide (NO) [11], HIF-1 $\alpha$  [11,12,16] and TGF- $\beta$ 1 [18]. Of these, TGF- $\beta$ 1 is a member of the TGF- $\beta$  cytokine superfamily that coordinates differentiation of mesenchymal stem cells during such distinct processes as organogenesis, bone and neuronal tissue formation, and myofibroblast activation [19]. Furthermore, TGF- $\beta$ 1 gene polymorphisms are associated with chronic obstructive pulmonary disease in the Chinese population [20]. Recently we have shown that TGF- $\beta$ 1 can induce transdifferentiation of fibroblasts into myofibroblasts, which is an important cause in hypoxic pulmonary vascular remodeling [21]. In this study, hypoxia induced dynamic changes in TGF- $\beta$ 1 expression, with the initial changes involving the adventitia and media, as reflected by *in situ* hybridization and immunohistochemistry findings. The increase in TGF- $\beta$ 1 mRNA in adventitial and medial cells was apparent as early as 3 d after hypoxia. The question can be raised as to the mechanism(s) of TGF- $\beta$ 1 induction after vascular hypoxia, in that normal adventitial fibroblasts are devoid of this cytokine. The ability of TGF- $\beta$ 1 to induce its own expression suggests that its release from degranulated platelets and activated macrophages might initiate TGF- $\beta$ 1 upregulation in adventitial fibroblasts [22]. Furthermore, platelet-derived growth factor released from platelets early after vascular insult could contribute to the induction of TGF- $\beta$ 1 [23]. Interestingly, the TGF- $\beta$ 1 mRNA was not significantly increased until 14 d after hypoxia, yet TGF- $\beta$ 1 protein was markedly increased after only 3 d of hypoxia. Moreover, 14 and 21 d of hypoxia, when the TGF- $\beta$ 1 mRNA levels are at their highest, protein levels, in fact, start to drop. This suggests a major alteration in



post-transcriptional regulation.

Previously we have shown that HIF-1 $\alpha$  is one of the pivotal mediators in the pathogenesis of hypoxia-induced pulmonary hypertension development in rat, and most presumably through target genes such as the inducible nitric oxide synthase gene, vascular endothelial growth factor gene and heme oxygenase-1 gene [11,12,16]. Recently we have shown that HIF-1 $\alpha$ , HIF-2 $\alpha$  and HIF-3 $\alpha$  may not only confer different target genes, but also play key pathogenetic roles in hypoxic-induced pulmonary hypertension [24]. In this study we detected steady-state levels of HIF-1 $\alpha$  mRNA up to hypoxia for 7 d, but the levels increased dramatically after 14 and 21 d of hypoxia. This accumulation of mRNA was limited to the tunica intima and media, whereas HIF-1 $\alpha$  protein levels in pulmonary artery tunica media increased significantly by 3 d of hypoxia, reaching a peak around 7 d of hypoxia and then declining to a lower level as the hypoxia continued. HIF-1 $\alpha$  protein in tunica intima was strongly positive in all hypoxic groups. Low O<sub>2</sub> tension is known to regulate the expression of a number of genes, such as growth factors and cytokines [25]. In addition, the responses of a particular gene to low O<sub>2</sub> tension have also been shown to be dependent on the cell type [26]. The mechanisms by which low O<sub>2</sub> levels regulate gene expression have recently been investigated. Cis-acting sequences responsible for the induction of gene transcription by hypoxia for the erythropoietin gene have been identified. The transacting factor HIF-1 binds to an enhancer located in the 38-flanking region of the erythropoietin gene and is required for induction by hypoxia [27, 28]. This DNA binding protein is a heterodimer composed of HIF-1 $\alpha$  and HIF-1 $\beta$  subunits [26]. Both subunits are induced by hypoxia and rapidly decay on return to normoxia [29]. HIF-1 DNA binding activity has been shown to be phosphorylation and redox dependent [30]. Functionally important binding sites for HIF-1 (consensus, 58-RCGTG-38) have been found in a number of genes known to be regulated by hypoxia, including those encoding vascular endothelial growth factor [16]; the glycolytic enzymes aldolase A, enolase-1, lactate dehydrogenase A, and phosphoglycerate kinase-1 [31]; and heme oxygenase-1 [12].

The regulation of TGF- $\beta$ 1 gene expression in response to low O<sub>2</sub> tension may be important in several physiological and pathological conditions in which O<sub>2</sub> availability is compromised. In the present study, TGF- $\beta$ 1 expression has been shown to correlate with the development of the remodeling process. The mechanism by which TGF- $\beta$ 1 is increased in chronic hypoxia-induced models of pulmonary hypertension is unknown. Our studies indicate

that hypoxia induces HIF-1 $\alpha$  in this lung model and that the increased expression of HIF-1 $\alpha$  may result in the transcriptional activation of TGF- $\beta$ 1 gene expression. TGF- $\beta$ 1 may also upregulate HIF-1 $\alpha$  gene expression via trans-activation. It has been shown that TGF- $\beta$ 1 upregulated HIF-1 $\alpha$  protein in cultured and native vascular smooth muscle cells [32].

Although elucidation of the pathophysiologic importance of HIF-1 $\alpha$  in these conditions awaits the availability of specific HIF-1 $\alpha$  antagonists, the present study provides new information about variations in local synthesis and distribution of HIF-1 $\alpha$  and TGF- $\beta$ 1 in pulmonary arteries. Further elucidation of the effects of these proteins might reveal clues for approaches to curing hypoxia-induced pulmonary hypertension.

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Edited by  
**Minghua XU**