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Differential Expression of Three Hypoxia-inducible Factor-α Subunits in Pulmonary Arteries of Rat with Hypoxia-induced Hypertension

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Abstract Hypoxia inducible transcription factor (HIF)-1α plays an important role in the development of hypoxic pulmonary hypertension, but little is known about HIF-2α and HIF-3α with respect to transcriptional regulation by hypoxia. To examine the expression patterns of all HIF-α subunits (HIF-1α, HIF-2α and HIF-3α) in pulmonary arteries of rats undergoing systemic hypoxia, five groups of healthy male Wistar rats were exposed to normoxia (N) and hypoxia for 3 (H_3), 7 (H_7), 14 (H_{14}) and 21 (H_{21}) d respectively. Mean pulmonary arterial pressure (*mPAP*), vessel morphometry and right ventricular hypertrophy index were measured. Lungs were inflation fixed for immunohistochemistry and *in situ* hybridization, and homogenized for Western blot. *mPAP* increased significantly after 7 d of hypoxia [(18.4±0.4) vs. (14.4±0.4) mmHg, H_7 vs. N], reached its peak after 14 d of hypoxia, then remained stable. Pulmonary artery remodeling and right ventricular hypertrophy developed significantly after 14 d of hypoxia. During normoxia, HIF-1α and HIF-3α staining were slightly positive regarding mRNA levels. A substantial alteration of HIF-1α and HIF-3α staining occurred in pulmonary arteries after 14 d and 7 d of hypoxia, respectively, but HIF-2α staining showed an inversed trend after 14 d of hypoxia. Protein levels of all HIF-α subunits except HIF-3α showed a marked increase corresponding to the duration of hypoxia, which was obtained by Western blot. Our study found that HIF-1α, HIF-2α and HIF-3α may not only confer different target genes, but also play key pathogenetic roles in hypoxic-induced pulmonary hypertension.

Key words hypoxia inducible factor-α; hypoxia; hypertension; lung

Acute hypoxia constricts reversibly the pulmonary arteries and dilates systemic arteries, a process known as hypoxia pulmonary vasoconstriction. Chronic hypoxia leads to structural remodeling of the vessels, comprising increased thickness of the adventitial and medial layers, and perhaps more importantly, the muscularization of precapillary vessels that either have poor muscularization or are devoid of muscle under normal conditions. The combination of vasoconstriction and vascular remodeling, as well as an increase in hematocrit, result in pulmonary hypertension and, subsequently, right ventricular hypertrophy [1,2]. Hypoxia inducible transcription factor-1 (HIF-1) gene and its dependent target genes, with the hypoxia-responsive element as the regulatory component, are strongly activated in chronic hypoxia [3]. HIF-1 is a heterodimeric bHLH transcription factor that consists of an oxygen-regulated functional α-subunit, and a constitutively expressed β-subunit also known as aryl hydrocarbon receptor nuclear translocator (ARNT). HIF-1 mediates complex cellular and systemic adaptive responses to a reduced oxygen supply, stimulating transcriptions of a large array of glycolytic and vasoactive genes, iron metabolism genes, growth factor genes, erythropoietin genes and many angiogenic factor genes [4,5]. Besides the well-established HIF-1α, two other members of the

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bHLH-PAS superfamily have also been described: HIF-2α [6] and HIF-3α [7], which bear similar functions to HIF-1α regarding hypoxic stabilization and binding to ARNT. The role of HIF-1α in cellular response to hypoxia is well established, whereas little is known about HIF-2α and HIF-3α with respect to dynamic expression in pulmonary arteries during chronic hypoxia.

To unravel the roles of HIF-2α and HIF-3α, and the susceptibility of HIF-α subunits to chronic hypoxic conditions, we studied the expression patterns of HIF-1α, HIF-2α and HIF-3α in pulmonary arteries of rats at different phases of hypoxia-induced pulmonary hypertension development.

Materials and Methods

Materials

Hypoxia and normoxia rat models were set up as described previously [8]. 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 Company Limited, 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 with soda lime, excess humidity was prevented by anhydrous calcium chloride, and ammonia was kept to a minimum level by boric acid in the chamber. For hypoxia models, 40 Wistar rats (male, 220±10 g, 6–8 weeks old) from the Animal Experimental Center of Center South University (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 (H_3), 7 (H_7), 14 (H_{14}) and 21 (H_{21}) d (8 h per day, intermittently), respectively, in a ventilated chamber. The control rats were kept in a normoxic ventilated chamber (21% O_2) in the same chamber, and were 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*) [8].

mPAP and *RVHI* measurements

mPAP was measured as described previously [9]. Rats were intraperitoneally anesthetized with 40 mg pentobarbital sodium per 1 kg body weight. A specially designed single-lumen catheter was then inserted into the main pulmonary artery through the right jugular vein; the injecting position was decided through the waveform of pressure. Through this catheter, *mPAP* was measured using PowerLab monitoring equipment (AD Instruments Pty Limited, Milford, USA). 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 and eosin, followed by elastic fiber staining, then examined with light microscopy. At least five representative pulmonary arteries (100–150 μm in outer diameter) chosen from three different sections from 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-1α, HIF-2α and HIF-3α

In situ hybridization was performed using an *In situ* hybridization detection kit (Wuhan Boster Biological Technology Company, Wuhan, China). The oligonucleotide probes were designed by Boster according to the sequences of rat *HIF-1α*, *HIF-2α* and *HIF-3α*.

The sequences of probes against *HIF-1α* mRNA were: (1) 5′-TTATGAGCTTGCTCATCAGTTGCCACTTCC-3′; (2) 5′-CTCAGTTTGAACTAACTGGACACAGTGTGT-3′; (3) 5′-GGCCGCTCAATTTATGAATATTATCATGCT-3′.

The sequences of probes against *HIF-2α* mRNA were: (1) 5′-CGAACACATAAACTCCTGTCTTCAGTGTGC-3′; (2) 5′-ATCCGAGAGAACCTGACACTCAAAACTGGC-3′; (3) 5′-GGGCAAGTGAGAGTCTACAACAACTGCCCC-3′.

The sequences of probes against *HIF-3α* mRNA were: (1) 5′-CGCATGCACCGCCTCTGCGCTGCAGGGGAG-3′; (2) 5′-ACATGGCTTACCTGTCGGAAAATGTCAGCA-3′; (3) 5′-ATATGAGGGCCTACAAGCCCCCTGCACAGA-3′.

Hybridization was performed 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 20 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 (PBS). After washing off unbound probes, slices were incubated first with rabbit antibodies against digoxin, then with biotinylated goat antibodies against rabbit. Slices were then incubated with SABC-peroxidase. Peroxidase activity was visualized by a color reaction using diaminobenzidine (Boster) 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.

Immunohistochemistry for HIF-1α, HIF-2α and HIF-3α

A commercial SABC kit (Boster) was used for immunohistochemistry, which was performed similar to that described previously with minor modification [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% non-immunized serum. Sections were incubated with rabbit anti-HIF-1α (Boster), anti-HIF-2α and anti-HIF-3α (Santa Cruz Biotechnology, Heidelberg, Germany) specific polyclonal antibodies (1:150) overnight at 4 °C. In negative control studies, the antibodies were substituted by PBS. After unbound antibodies were washed off, the sections were incubated with corresponding biotinylated second 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-1α, HIF-2α and HIF-3α in lung tissue

Proteins from whole tissue samples were extracted as described previously [10] using a modified homogenization buffer containing 10 mM Tris (pH 8.0), 1 mM EDTA, 400 mM NaCl, 0.1% igepal CA-630, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin (Sigma, Deutschland, Germany). Protein concentrations were determined by the Lowry method (Merck, Darmstadt, Germany). Equal amount of proteins were separated in 7.5% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a polyvinylidene difluoride membrane (Millipore, Eschborn, Germany) and stained by Coomassie blue. Detection of the target proteins was accomplished using specific antibodies of HIF-2α, HIF-3α, β-actin (Santa Cruz), or HIF-1α. Immunocomplexes were then labeled with peroxidase-conjugated anti-mouse or anti-rabbit IgG (Boster). An enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK) was used for signal detection. Fotolook and NIH image software programs were used for quantification.

Statistical analysis

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

Results

mPAP increase induced by chronic hypoxia

mPAP was measured as an indicator of pulmonary artery pressure in conscious rats. In normoxic rats, *mPAP* was 14.4±0.4 mmHg. In hypoxic animals, *mPAP* changed as expected: the pulmonary hypertension was increased after 7 d exposed to hypoxia ($P<0.05$), reached its peak after 14 d of hypoxia, then remained stable as the hypoxia condition was prolonged (**Table 1**).

Table 1 Histological and nonhistological parameters for rats exposed to normoxia or hypoxia

Group	*mPAP* (mmHg)	*RVHI* (%)	*WA* (%)	*PAMT* (μm)
Control	14.4±0.4	23.6±0.5	35.5±1.3	11.9±0.6
H_3	14.6±0.3	23.3±1.7	37.0±0.8	12.0±0.5
H_7	18.4±0.4 [ab]	24.0±1.0	47.8±0.8 [ab]	12.3±0.5
H_{14}	21.2±0.2 [abc]	25.0±1.8 [ab]	60.3±0.4 [abc]	15.0±0.3 [abc]
H_{21}	22.2±0.2 [abc]	27.7±1.0 [abc]	65.0±0.7 [abcd]	23.0±0.8 [abcd]

Data are represented as mean±SD (n=8). H_3, hypoxia for 3 d; H_7, hypoxia for 7 d; H_{14}, hypoxia for 14 d; H_{21}, hypoxia for 21 d. [a] $P<0.05$ versus control group; [b] $P<0.05$ versus group H_3; [c] $P<0.05$ versus group H_7; [d] $P<0.05$ versus group H_{14}. *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.

Chronic hypoxia induced hypoxic pulmonary vascular remodeling and right ventricular hypertrophy

Increased thickness of pulmonary arteries (*PAMT* and *WA*) due to smooth muscle cell hypertrophy and hyperplasia is the structural hallmark of pulmonary hypertension. As shown in **Table 1**, pulmonary arteries 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 slices from all three hypoxia groups (H_7, H_{14} and H_{21}) revealed significantly increased medial thickness of pulmonary arteries in hypoxic animals compared with normoxic controls. 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 value, and increased further after 21 d of hypoxia. These data indicated that right ventricular hypertrophy was developed after 14 d exposed to hypoxia (**Table 1**).

mRNA level of HIF-1α, HIF-2α and HIF-3α in pulmonary arterial wall during normoxia and chronic hypoxia conditions

To determine dynamic changes at the transcriptional level, the mRNAs of HIF-1α, HIF-2α and HIF-3α were monitored during normoxia and after different hypoxic periods. The changes of the HIF α-subunits were quite different to each other in the investigated arteries (**Figs. 1–3**). In all arteries, distinct mRNA staining of HIF-1α (**Fig. 1**) and HIF-3α (**Fig. 3**) were detected after 14 d and 7 d of hypoxia respectively; they were faintly traceable during normoxia, even after 3 d of hypoxia. In contrast to HIF-1α and HIF-3α, HIF-2α presented a completely different expression pattern. mRNA staining of HIF-2α lessened drastically after 14 d of hypoxia, whereas strong *HIF-2α* mRNA staining was detected during normoxia and after 3 d and 7 d of hypoxia (**Fig. 2**). Notably, with regard to time periods, HIF-2α mRNA staining lessened while *HIF-3α* mRNA was strongly stained. In summary, moderate O_2 concentration induced significant changes in the mRNAs of HIF-1α, HIF-2α and HIF-3α.

Protein level of HIF-1α, HIF-2α and HIF-3α in pulmonary arterial wall and lung tissue during normoxia and chronic hypoxia conditions

In immunohistochemical analysis, antibodies against HIF-1α, HIF-2α and HIF-3α produced different signals respectively (**Figs. 4–6**). All HIF-α protein levels enhanced gradually during hypoxia, but the expression pattern differs to each other, implying the specific stabilization of HIF-α protein by hypoxia. All HIF-α stainings were slightly positive in control rats. But staining of HIF-1α was positive after 3 d and 7 d of hypoxia, then lessened (**Fig. 4**). HIF-2α was stained strongly after 14 d of hypoxia, then remained unchanged (**Fig. 5**). HIF-3α staining became strong after 7 d of hypoxia, and remained stable thereafter (**Fig. 6**).

In Western blot analysis, interestingly, even in normoxia, all HIF-α proteins except HIF-3α in the lung tissue could be clearly detected, but HIF-1α and HIF-2α proteins enhanced gradually after 3 d of systemic hypoxia and increased further as the duration of hypoxia prolonged (**Fig. 7**), indicating the stabilization of the two proteins by hypoxia.

Discussion

The main finding of this study is that the expression patterns of HIF-1α, HIF-2α and HIF-3α are quite different during hypoxic pulmonary hypertension development. Chronic hypoxia induced *mPAP* increases pulmonary vascular muscularization and right ventricular hypertrophy. Importantly, we are able to document the dynamic expression of HIF-2α and HIF-3α during prolonged hypoxia. These data show that HIF-1α, HIF-2α and HIF-3α, which may confer different target genes, also play key pathogenetic roles in the mechanisms of hypoxic pulmonary hypertension.

Previously we have shown that HIF-1α 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 [8,9]. In this study, the rise in pulmonary artery pressure was most pronounced during the first week of hypoxia and reached a plateau after 14 d, with only a small further increase during prolonged hypoxia until 21 d. The sustained elevation in pulmonary artery pressure in chronic hypoxia results, for the most part, from architectural changes in the pulmonary vascular bed as reflected by the significant increase in *WA* and *PAMT*. Pulmonary vascular remodeling in chronic hypoxia has many features of injury repair. Accumulation of vascular smooth muscle has long been recognized as a typical feature of hypoxic pulmonary vascular remodeling [11]. As homologs to HIF-1α, there are very limited data about the expression of the HIF-2α and HIF-3α subunits

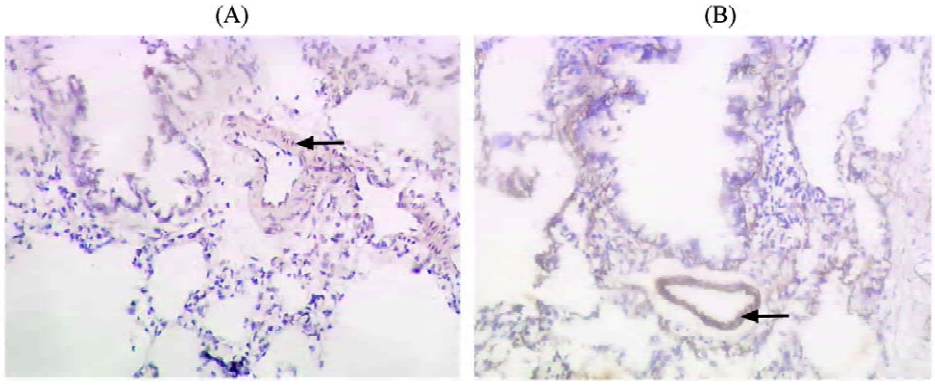

Fig. 1 Representative hypoxia inducible factor (HIF)-1α mRNA staining in pulmonary artery wall after normoxia (A) and 14 d of hypoxia (B) obtained by *in situ* hybridization in lung sections from rats undergoing systemic hypoxia over 3 weeks

Prolonged hypoxia (H_{14}) resulted in strong staining of *HIF-1α* mRNA. Magnification, 200×.

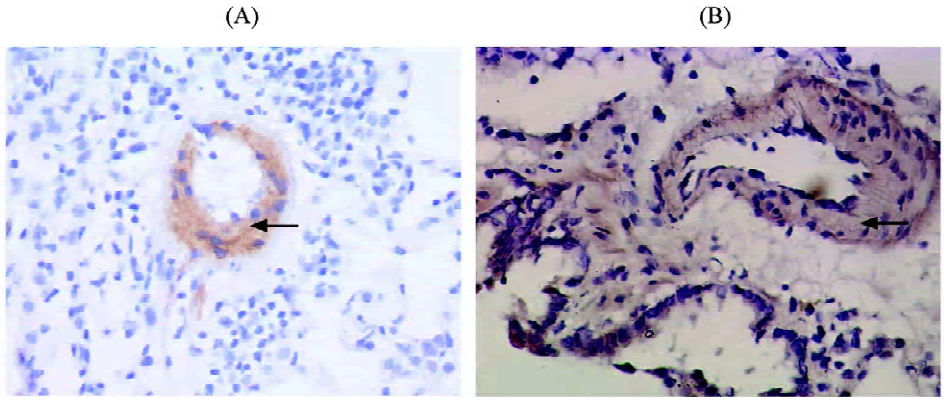

Fig. 2 Representative hypoxia inducible factor (HIF)-2α mRNA staining in pulmonary artery wall after 7 d (A) and 14 d of hypoxia (B) obtained by *in situ* hybridization in lung sections from rats undergoing systemic hypoxia over 3 weeks

HIF-2α mRNA was strongly stained during normoxia and acute hypoxia (H_7), but prolonged hypoxia (H_{14}) led to faint staining of HIF-2α mRNA. Magnification, 400×.

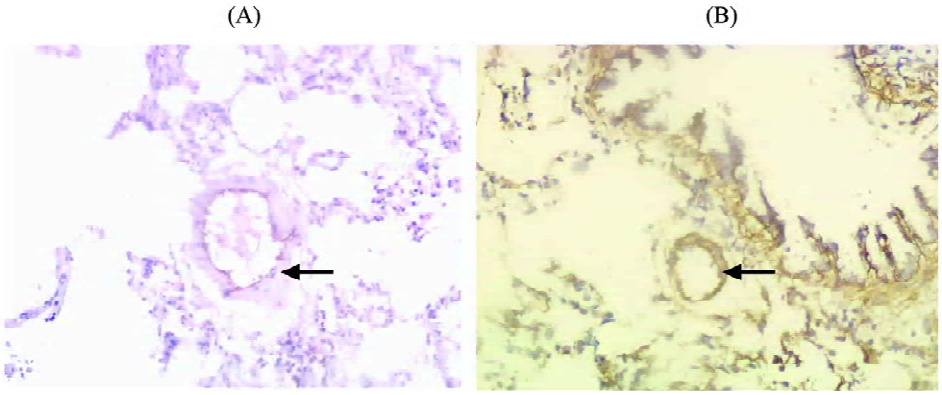

Fig. 3 Representative hypoxia inducible factor (HIF)-3α mRNA staining in pulmonary artery wall after 3 d (A) and 7 d of hypoxia (B) obtained by *in situ* hybridization in lung sections from rats undergoing systemic hypoxia over 3 weeks

HIF-3α mRNA was strongly stained after 7 d of hypoxia. Magnification, 200×.

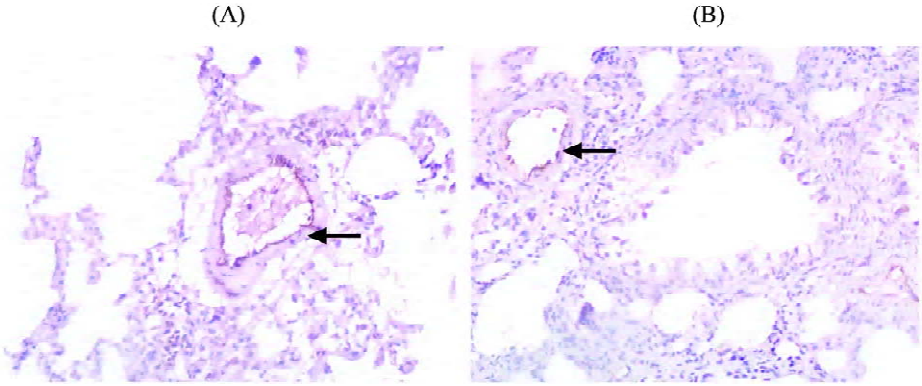

Fig. 4 Representative hypoxia inducible factor (HIF)-1α immunoreactivity in pulmonary artery wall after 3 d (A) and 21 d of hypoxia (B) obtained by immunohistochemistry in lung sections from rats undergoing systemic hypoxia over 3 weeks

The pulmonary artery wall showed strong HIF-1α immunoreactivity after acute hypoxia (H_3), but showed faint staining after prolonged hypoxia (H_{21}). Magnification, 200×.

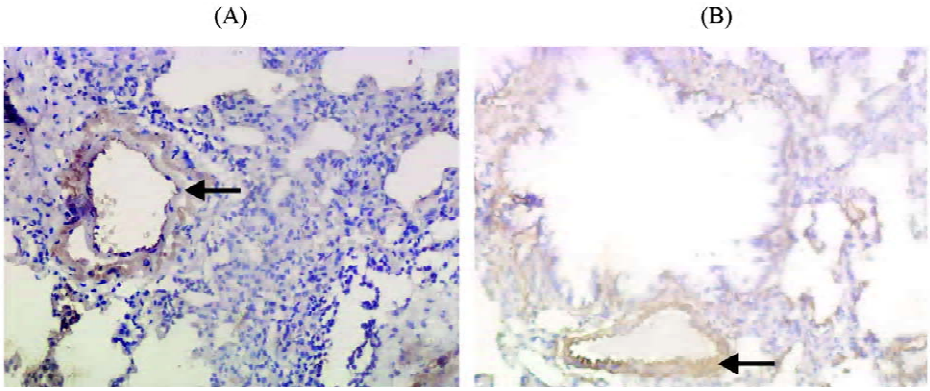

Fig. 5 Representative hypoxia inducible factor (HIF)-2α immunoreactivity in pulmonary artery wall after 7 d (A) and 14 d of hypoxia (B) obtained by immunohistochemistry in lung sections from rats undergoing systemic hypoxia over 3 weeks

The pulmonary artery wall showed strong HIF-2α immunoreactivity after prolonged hypoxia (H_{14}). Magnification, 200×.

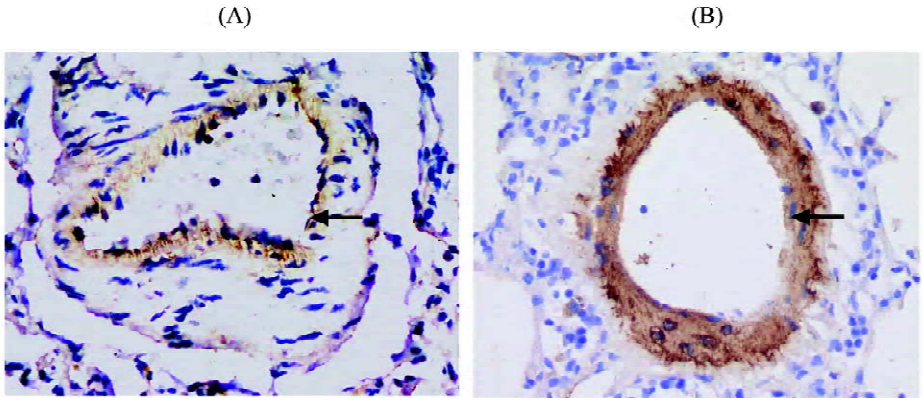

Fig. 6 Representative hypoxia inducible factor (HIF)-3α immunoreactivity in pulmonary artery wall after 3 d (A) and 21 d of hypoxia (B) obtained by immunohistochemistry in lung sections from rats undergoing systemic hypoxia over 3 weeks

The pulmonary artery wall showed strong HIF-3α immunoreactivity after prolonged hypoxia (H_{21}). Magnification, 400×.

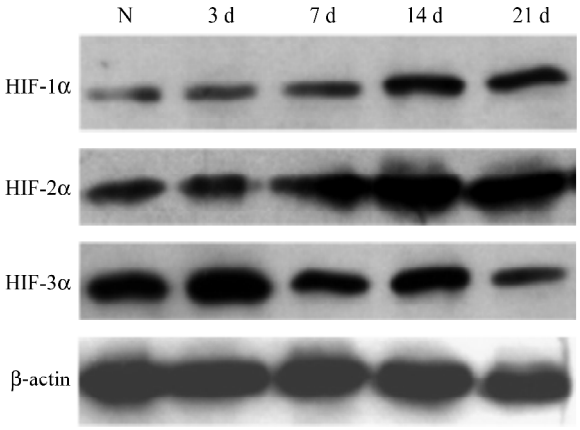

Fig. 7 Analysis of hypoxia inducible factor (HIF)-1α, HIF-2α and HIF-3α protein levels in lung tissue during normoxia (N) and hypoxia (3, 7, 14 and 21 d) by Western blot

Hypoxia increased the protein levels of HIF-1α and HIF-2α, reaching a plateau after 14 d of hypoxia, whereas HIF-3α level decreased after 3 d of hypoxia.

at the levels of mRNA and the protein in pulmonary arteries during normoxic and hypoxic conditions. In one earlier study, specific mRNAs of HIF-1α, HIF-2α and HIF-3α were clearly detectable by real-time reverse transcription (RT)-PCR in rat lung under normoxic and acute hypoxic conditions [10]. In this study, the staining of *HIF-1α* and *HIF-2α* mRNA were not affected by acute hypoxia (7 d of hypoxia) in pulmonary arteries. Regarding HIF-1α, this is in line with earlier suggestions that other factors except hypoxia may increase the expression of *HIF-1α* mRNA. In that study, at no time point did an increase in HIF-1α appear, even after 12 h of severe hypoxia [12]. Another study on rat brain revealed by Northern blot analysis that an increase in HIF-1α could be achieved only after permanent occlusion of the middle cerebral artery up to 20 h [13]. It may be concluded that: (1) the hypoxic protocol we used was not sufficiently severe; and/or (2) other or additional stimuli are required to induce an increase in mRNA. The HIF-α subunits are structurally similar in their DNA binding and dimerization domains but different in their transactivation domains, implying that they may have unique target genes. From studies of two cell lines (HEK293 and 768-O), Hu *et al.* demonstrated that HIF-2α regulated a variety of broadly expressed hypoxia-inducible genes and only HIF-1α (not HIF-2α) regulated the expression of the glycolytic gene during hypoxia [14]. By RNA interference, Warnecke *et al.* demonstrated that erythropoietin is a HIF-2α target gene in Hep3B and Kelly cells [15]. High steady-state staining of *HIF-2α* mRNA was detected in the pulmonary artery. Therefore we suggest that HIF-2α plays an important role in homeostasis in adult rat lung artery. The importance of HIF-2α versus HIF-1α in hypoxic adaptation needs to be further investigated by specifically inactivating HIF-1α and HIF-2α.

Surprisingly, in contrast to other α-class subunits, a significant increase in *HIF-3α* mRNA occurred after 7 d of hypoxia. Unlike HIF-1α and HIF-2α, the moderate decrease in O_2 concentration used in our model was therefore sufficient to induce *HIF-3α* mRNA synthesis. It was demonstrated in COS-1 cells that HIF-3α also interacts with HIF-1β, although at that time, the role of HIF-3α in the cellular response to hypoxia had not been established [16]. There is little evidence that HIF-3α is linked to conditions with low O_2 concentration. By transfecting rat hepatocytes with *HIF-3α*, mRNA analysis could be shown that *HIF-3α* mRNA is expressed predominantly in previous areas of the liver, a fact that was attributed to the formation of an O_2 gradient with lower oxygen concentration in perivenousareas of the liver caused by the unidirectional flow of blood from portal to central veins in this organ [17]. Despite the structural similarities of HIF-3α with other α-class subunits, HIF-3α seems to constitute a more sensitive and rapidly reacting response component to systemic hypoxia compared with HIF-1α and HIF-2α, as reported previously [10]. But the exact functional properties of HIF-3α are still unknown. It can not be excluded that HIF-3α itself may also be involved in the pathogenesis of hypoxic pulmonary hypertension, by inducing target genes of *HIF-1α*, such as *HO-1* and *VEGF* in pulmonary artery [12,13], especially during early phases of systemic hypoxia. On the other hand, it may be speculated that HIF-3α acts as a counterpart to other HIF-α subunits. This is supported by transfection experiments that HIF-3α of human suppresses HIF-α mediated target gene expression [18]. Therefore, it appears more likely that HIF-3α represents a subtle and sensitive mechanism that modulates the HIF response even in short and/or moderate hypoxia. In addition to protein stability, the transactivation activity of HIF-3α was different from those of HIF-1α and HIF-2α. The transfection of the HIF-3α protein suppressed HRE-driven gene expression when the expression of ARNT was limited [7]. The importance of HIF-3α during hypoxia is further supported by the very recent finding that the *HIF-3α* gene can be spliced alternatively in mice [19]. The resulting *HIF-3α* variant could be detected only when mice were exposed to severe hypoxia (6% O_2), where it was expressed predominantly in myocardial tissues and lungs. Our results obtained by Western blot analysis indicate that HIF-2α also undergoes

hypoxic stabilization, which therefore supports a role for it in hypoxia. Regarding HIF-3α, inconsistent results arose between immunohistochemistry and Western blot. These controversial results may have risen because of the use of different sources (pulmonary artery vs. lung homogenate) or because HIF-3α protein underwent cell-specific stabilization.

In conclusion, this study has, for the first time, directly compared the dynamic expression of HIF-α subunits in pulmonary arteries of rat with hypoxia-induced hypertension. The findings indicate that additional regulatory steps appear to operate to determine which alternative subunit is induced. Base on the differential expression patterns of HIF-1α, HIF-2α and HIF-3α during prolonged hypoxia, it is tempting to speculate that target gene specificity plays an important role in hypoxia-induced pulmonary hypertension development.

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