Increased *SUMO-1* expression in response to hypoxia: Interaction with HIF-1α in hypoxic pulmonary hypertension

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Abstract. Pulmonary hypertension (PH) develops in 30-70% of chronic obstructive pulmonary disease patients and increases morbidity and mortality. The present study aimed to investigate the regulation of small ubiquitin-related modifier-1 (*SUMO-1*) expression in response to hypoxia. The experiments were carried out *in vitro* in rat pulmonary arterial smooth muscle cells (PASMCs) and *in vivo* using a rat hypoxic PH (HPH) model. A significant increase in *SUMO-1* mRNA and protein levels was observed following hypoxic stimulation *in vivo* and *in vitro*. SUMO-1 is known to interact with various transcription factors, including hypoxia-inducible factor-1α (HIF-1α) *in vitro*. Notably, the expression of HIF-1α and its target gene, vascular endothelial growth factor, was increased by hypoxia in HPH. In addition, the present data suggest that *SUMO-1* regulated HIF-1α in response to hypoxia (gene silencing and overexpression). Finally, the co-immunoprecipitation assays suggest a direct and specific interaction between SUMO-1 and HIF-1α. In conclusion, *SUMO-1* may participate in the modulation of HIF-1α through sumoylation in HPH. However, further studies are required to confirm this.

Introduction

Pulmonary hypertension (PH) is a serious complication of chronic obstructive pulmonary disease (COPD) and develops in 30-70% of COPD patients, increasing morbidity and mortality (1,2). The pathophysiology underlying PH development in COPD is poorly understood and is possibly multifactorial. A consistent finding in COPD patients is the close association between the severity of hypoxemia and pulmonary artery pressure (PAP), supporting a major role for alveolar hypoxia (1-3). Alveolar hypoxia causes constriction of pulmonary arteries, and sustained alveolar hypoxia induces pulmonary vascular remodeling. Pathological studies of lung specimens from COPD patients have shown extensive pulmonary vascular remodeling with prominent intimal thickening, medial hypertrophy and muscularization of small arterioles (4-6).

Hypoxia-inducible factor (HIF)-1 is a heterodimeric transcription factor composed of α and β subunits, and regulates the expression of hundreds of genes in response to hypoxia, including numerous genes associated with hypoxic PH (HPH) (7). HIF-1β is ubiquitously expressed, whereas HIF-1α expression is O_2-regulated (8,9). Under normoxic conditions, HIF-1α is hydroxylated on two proline residues by prolyl hydroxylase domain proteins, which use O_2 as a substrate, marking the protein for ubiquitination and proteasomal degradation (10,11). Under hypoxic conditions, HIF-1α accumulates and dimerizes with HIF-1β, allowing for the transcription of target genes. The heterodimeric complex regulates the expression of genes involved in the pathology of numerous diseases, including HPH. Our previous study (12) indicated that HIF-1α upregulates the expression of its downstream target genes, including vascular endothelial growth factor (*VEGF*), by transcriptional activation during hypoxia. Its involvement in the remodeling of rats with HPH suggests that HIF-1α acts as a 'molecular switch' in hypoxic pulmonary vascular remodeling (12-14).

The ubiquitin system is a key intracellular signaling pathway for post-translational modification of proteins (15). Small ubiquitin-related modifier-1 (SUMO-1) is an 11.5-kDa ubiquitin-related protein with 18% similarity with ubiquitin (16-18). SUMO-1 expression has been described in the nuclear pore complex and within nuclei in a wide range of tissues and cell types (16,19,20). Previous studies have demonstrated that the *SUMO-1* gene is highly conserved between humans and mice (20,21), with mRNA and protein expressed in the brain and heart (22-24). SUMO-1 has been suggested to primarily prevent proteasome-mediated degradation of proteins, in contrast to ubiquitin (15,25). Previous data have shown that SUMO-1 stabilizes nuclear proteins by a process known as sumoylation (26). In addition, several studies have demonstrated that SUMO-1 interacts with various transcription factors and further modulates their activities (16,17,20,25). For instance, SUMO-1 modifies HIF-1α and further regulates its transcription activity *in vitro* (27). Of note, it was recently shown that increased levels

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of SUMO-1 participate in the modulation of HIF-1α function through sumoylation in the adult mouse brain and heart (28). However, the interaction between SUMO-1 and HIF-1α has not been studied in the context of HPH, either *in vivo* or *in vitro*. We hypothesized that another post-translational modification other than ubiquitination may serve as a regulatory factor to protect HIF-1α from degradation.

In the present study, *SUMO-1* expression was assessed under hypoxic conditions and the interaction between *SUMO-1* and HIF-1α was investigated. SUMO-1 expression was markedly upregulated at gene and protein levels under hypoxia in a rat HPH model and *in vitro* (in pulmonary artery smooth muscle cells, PASMCs). In addition, results suggested that SUMO-1 interacted with HIF-1α, which resulted in modulation of HIF-1α function by the sumoylation pathway.

Materials and methods

Animals. Forty adult male Wistar rats (150-250 g, 8 weeks old) were randomly divided into 5 groups (n=8 in each group). The animals were kept in a specific pathogen-free class experimental animal room, at 15-28˚C and 45-55% relative humidity. The animals were used for experiments after 7 days of adaptive feeding.

All the animals, procedures and experiments were approved by the Animal Care and Use Committee of the Zhongnan University (Changsha, China).

Rat model of chronic hypoxia-induced PH. The protocol for the exposure of the animals to hypoxia and normoxia was identical to that reported previously by our laboratory (13). Wistar rats were placed in custom-made plexiglas chambers continuously supplied with a mixture of air and N_2 (10% O_2) for 3, 7, 14 and 21 days (H3, H7, H14 and H21, respectively). Normoxic animals were kept under normal room air conditions. Oxygen levels were measured with a gas analyzer (MB80 oxygen transmitter; Zhuhai S.E.Z. Hangto Science & Tech. Co., Ltd., Zhuhai, China), and the animals were allowed free access to food and water. Rats were removed from chambers for <5 min twice weekly to replenish food and water, and to clean cages. At the end of the exposure period, animals were anesthetized with sodium pentobarbital (50 mg/kg) administered intraperitoneally. PH was evaluated in rats by measuring right ventricular (RV) systolic pressure (RVSP) and RV hypertrophy. RV pressure was measured using a SPR-671 Mikro-Tip pressure catheter (Model SPR-671, size 1.4F; Millar Instruments, Inc., Houston, TX, USA) via the cannulation of the right jugular vein. Heart and lungs were removed following exsanguination. RV was separated from the left ventricle and septum (LV + S). The mass ratio of RV/(LV + S) was determined as the right ventricular hypertrophy index (RVHI), as previously described (14).

Isolation of intralobar pulmonary arteries. Intralobar pulmonary arteries were isolated from normoxic and hypoxic male Wistar rats (29). Following exsanguination, the lungs were removed and transferred into petri dishes filled with HEPES-buffered salt solution (HBSS) containing 130 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES and 10 mM glucose (pH 7.4). Third- and fourth-generation intrapulmonary arteries (200-800 μm) were isolated.

PASMC isolation and culture. PASMCs were enzymatically isolated and transiently cultured (30). Briefly, lungs were extracted from untreated animals following sacrifice, and intrapulmonary arteries were dissected in HBSS containing 130 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES and 10 mM glucose (pH 7.2). Pulmonary arteries were carefully cleaned from the connective tissue, and the endothelial layer was removed by gently rubbing the luminal surface with a cotton swab. Subsequently, the clean arteries were sequentially incubated in ice-cold HBSS (30 min) and reduced Ca^{2+} (20 μM) HBSS (20 min, room temperature), following which they were digested in reduced Ca^{2+} HBSS containing collagenase (Type I, 1,750 U/ml), papain (6.4 U/ml), bovine serum albumin (2 mg/ml) and dithiothreitol (1 mM) at 37˚C for 20 min. Following washing with Ca^{2+}-free HBSS, single smooth muscle cells (identified as expressing α-actin at ≥95%) were gently dispersed from tissues by trituration in Ca^{2+}-free HBSS, and cultured (16-24 h, 37˚C, 5% CO_2) on 25-mm glass coverslips in Ham's F-12 medium supplemented with 0.5% fetal calf serum, 100 U/ml streptomycin and 0.1 mg/ml penicillin in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA, USA) under 1% O_2-5% CO_2 for 2, 6, 12 and 24 h for hypoxia, and 21% O_2-5% CO_2 for 2, 6, 12 and 24 h for normoxic analysis, respectively, prior to reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis.

Semi-quantitative RT-PCR. Pulmonary arteries and PASMCs of normoxic and hypoxic conditions were mechanically homogenized. Subsequently, total RNA was extracted using an RNeasy Fibrous Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Genomic DNA contamination was removed with TURBO DNA-free DNase (Ambion, Austin, TX, USA). Total RNA concentrations were measured by spectrophotometry (DU-70; Beckman Coulter Inc., Brea, CA, USA) at 260 nm, and 1 μg of RNA was used for reverse transcription with random hexamer primers and SuperScript III RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The resulting first-strand cDNA samples were used as templates for PCR amplification. Sense and antisense primers specific for *SUMO-1*, *HIF-1α*, *VEGF* and β-actin (Table I) were used in PCR reactions containing PCR SuperMix (Gibco, Invitrogen). PCR conditions were: Denaturation at 94˚C for 30 sec, annealing at 60˚C for 45 sec and extension at 72˚C for 90 sec for a total of 35 cycles; and final extension at 72˚C for 10 min. PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized by staining with ethidium bromide. Parallel reactions were run for each RNA sample in the absence of SuperScript II to access the degree of genomic DNA contamination.

Western blot analysis and co-immunoprecipitation. Pulmonary arteries from rats or PASMC primary cultures were washed in phosphate-buffered saline (PBS). Pulmonary arteries were snap-frozen in liquid nitrogen, crushed, homogenized using a mortar and pestle and resuspended in ice-cold cell lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% deoxycholic acid, 0.1% SDS, 0.5% Nonidet P-40 (NP-40) and protease inhibitor cocktail. The

Table I. Primer sequences for RT-PCR.

Gene	Accession number	Source	Primer pair sequence, sense/antisense	Product size, bp	Location in sequence
HIF-1α	NM024359.1	Rat	5'-GCCCCTACTATGTCGCTTTC-3' 5'-GGCCCAAACTAAACTATCTGA-3'	433	2247-2679
SUMO-1	NM001009672.1	Rat	5'-ATTGCCCTTCTTCCTTTA-3' 5'-TTCCACAGTTCGGTTCTC-3'	218	560-777
VEGF	NM053653.1	Rat	5'-CATCCACCATGCACTTGCTGT-3' 5'-GGCTGCTCCAAACTCCTTCCA-3'	490	80-569
β-actin	NM031144	Rat	5'-CCTAAGGCCAACCGTGAA-3' 5'-CTAGGAGCCAGGGCAGTAATC-3'	635	223-205

RT-PCR, reverse transcription-polymerase chain reaction; bp, base pairs.

homogenates were centrifuged at 1,000 x g for 5 min (4˚C) and the supernatants were collected. Protein concentration was estimated in supernatants by the bicinchoninic acid assay and 10 μg of protein was resolved on 8% SDS-PAGE gel and electrotransferred onto nitrocellulose membranes (Shanghai Jining Co., Ltd., Shanghai, China). Subsequent to blocking in 5% (w/v) skimmed dry milk in PBS containing 0.05% Tween-20 (PBST) for 1 h at room temperature, membranes were incubated at 4˚C overnight with specific primary antibodies raised in rabbits against SUMO-1 (Cat. no. SC-9060), HIF-1α (Cat. no. sc-10790), VEGF (Cat. no. sc-507) and β-actin (Cat. no. sc-130619) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Subsequently, membranes were washed with PBST and incubated with peroxidase-conjugated goat-anti-rabbit secondary antibodies (Cat. no. BA1003; Wuhan Boster Bio., Co., Ltd., Wuhan, China) at room temperature for 1 h. Following washing, signals were detected with enhanced chemiluminescence, and membranes were imaged on a Gel Logic 200 image system (Tanon Gis-2010; Tanon Science and Technology Co., Ltd., Shanghai, China).

Co-immunoprecipitation experiments were performed using a commercial kit (Wuhan Boster Bio., Co., Ltd.). Total proteins were extracted with ice-cold lysis buffer containing 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP-40, 1% SDS, 200 μM sodium deoxycholate, 1 mM dithiothreitol, 5 mM EDTA, 0.5 mM phenylmethanesulfonylfluoride, 10 mM N-ethylmaleimide (NEM), 10 mM iodoacetamide and a cocktail of protease inhibitors. Anti-SUMO-1 antibodies (Santa Cruz Biotechnology, Inc.) were added to 500 μg of protein extracts and incubated for 4 h at room temperature. Immune complexes were obtained by addition of 50 μl of pansorbin cells. The resulting immobilized immune complexes were washed in radioimmunoprecipitation assay buffer, supplemented with 10 mM NEM and 10 mM iodoacetamide. The bound proteins were eluted by boiling in 30 μl of SDS sample-reducing loading buffer for 5 min. Immunoprecipitation complexes were analyzed by western blot analysis using the anti-HIF-1α antibody, as described above.

Immunohistochemistry. Immunohistochemical analysis (Wuhan Boster Bio., Co., Ltd.) was performed as previously described (14). In brief, paraffin-embedded lung sections were mounted on poly-L-lysine slides. Slides (6 μm) were dewaxed and sections rehydrated by immersion in ethanol gradient and distilled water. After antigen retrieval, endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 30 min. Sections were pre-incubated in PBS supplemented with 1% bovine serum albumin and 10% normal horse serum for 1 h. Endogenous biotin was blocked using an avidin/biotin-blocking kit and slides were incubated overnight with primary antibodies raised in rabbits against SUMO-1 or HIF-1α. Negative controls were incubated with PBS only. Subsequent to washing, sections were sequentially incubated with biotinylated goat-anti-rabbit secondary antibodies and streptavidin-horseradish peroxidase (HRP). Subsequently, sections were visualized by a color reaction with diaminobenzidine as the substrate. Brown and yellow cells indicated positive staining. Finally, the sections were counterstained with hematoxylin and mounted, and protein expression levels were quantified by a pathology image analysis system (PIPS-2020; Chongqing Thme Co, Ltd., Chongqing, China). Eight rats per group were studied.

In situ hybridization. In situ hybridization was performed using a commercial kit (Wuhan Boster Bio., Co., Ltd.). Sections (4 μm) were mounted on charged slides, dewaxed, dehydrated and washed. Slides were placed in Declere (Wuhan Boster Bio., Co., Ltd.) for 15 min, washed and treated with proteinase K (25 μg/ml) for 1 min. Subsequently, endogenous peroxidase was blocked using 3% H_2O_2. To block biotin, sections were incubated with avidin for 15 min, and avidin was blocked by incubation with biotin for 15 min. Sections were covered with 10 μl of the cagA probe (4 μg/μl). For DNA denaturation, slides were incubated at 95˚C for 5 min, 4˚C for 10 min, and hybridized overnight at 37˚C in a humid chamber. Subsequent to washing with HWb (Research Genetics Inc., Huntsville, AL, USA) at 60˚C for 10 min, slides were sequentially incubated in protein-blocking buffer for 30 min and streptavidin-HRP for 30 min. Following the reaction with diaminobenzidine, positive staining appeared brown and yellow. Finally, the sections were counterstained with hematoxylin and mounted. Expression levels of mRNA were quantified by a pathology image analysis system (JD801; Beijing Time Technologies Co., Ltd., Beijing, China).

Table II. Histological and non-histological parameters of rats exposed to normoxia or hypoxia.

Group	mPAP, mmHg	RVHI, %	WA, %	WT, %	LA, %
Control	15.9±1.3	23.2±2.1	35.0±2.2	15.7±1.6	65.0±2.2
H3	17.1±1.4	23.6±2.1	36.6±2.0	16.5±1.7	63.4±2.0
H7	21.3±1.6[a,b]	24.7±1.7	41.4±2.8[a,b]	19.0±1.8[a,b]	58.6±2.8[a,b]
H14	26.5±1.7[a-c]	27.0±1.8[a,c]	52.0±4.0[a-c]	23.1±1.9[a-c]	48.1±4.0[a-c]
H21	28.0±2.0[a-c]	29.0±1.5[a-d]	58.5±4.7[a-d]	26.1±1.6[a-d]	41.5±4.7[a-d]
F	91.188	13.866	76.366	52.715	76.351
P-value	<0.01	<0.01	<0.01	<0.01	<0.01

Data are expressed as mean ± standard deviation (SD) (n=8). P<0.05 vs. [a]control group; [b]group H3; [c]group H7; [d]group H14. mPAP, mean pulmonary arterial pressure; RVHI, right ventricular hypertrophy index; WA, ratio of vascular wall area to total vascular area; WT, ratio of vascular wall thickness to external diameter; LA, ratio of lumen area to total vascular area; H3, hypoxia for 3 days; H7, hypoxia for 7 days; H14, hypoxia for 14 days; H21, hypoxia for 21 days; F, F-value derived from one-way analysis of variance.

Lentiviral vector construction and transfection. According to the nucleotide sequence of the rat *SUMO-1* gene (NM024359.1, NM001009672.1 and NM053653.1), 4 small interfering RNAs (*SUMO-1*-RNAi-LV2 at $5x10^8$ TU/ml, *SUMO-1*-RNAi-LV3 at $5x10^8$ TU/ml, *SUMO-1*-RNAi-LV4 at $2x10^8$ TU/ml and pGC FU-RNAi-NC-LV at $1x10^9$ TU/ml as the negative control) and 2 *SUMO-1* overexpression fragments (*SUMO*-1-LV at $2x10^8$ TU/ml and pGC FU-GFP-LV at $2x10^9$ TU/ml as the negative control) were constructed and synthesized by Shanghai GeneChem Co. (Shanghai, China). The sequences were transfected into PASMCs in 6-well cell culture plates, including blank control, no-load lentivirus and small interference/overexpression groups, and cultured for 4-6 h in a modular incubator chamber. Lentiviral transfection rates were observed by fluorescence microscopy (Olympus, Tokyo, Japan). Cells were grown to 90% confluency and the media replaced for synchronization, followed by further culture for 12 h under normoxia (21% O_2-5% CO_2) and hypoxia (1% O_2-5% CO_2), respectively. Finally, mRNA and protein levels of SUMO-1, HIF-1α, VEGF were tested by RT-PCR and western blot analysis.

Statistical analysis. The statistical package SPSS 19.0 (SPSS Inc., Chicago, IL, USA) was used for all the analyses. Data are expressed as mean ± standard deviation. The group t-test was used to compare data between two groups. Analysis of variance was used to determine statistically significant differences among multiple groups, with Newman-Keuls test comparing differences between two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Verification of the hypoxia-induced PHP model. Rats exhibited profound pulmonary arterial hypertension and right ventricular hypertrophy when examined 21 days after hypoxia induction. Mean PAP (mPAP) was measured as an indicator of PAP in conscious rats (mPAP in normoxic animals was 15.9±1.3 mmHg). As expected, hypoxic animals developed PH after 7 days of hypoxia (P<0.05). PH peaked at 14 days, and remained at high levels (Table II). In addition, pulmonary arterioles in normoxic animals were thin, whereas after 7 days of hypoxia, they developed increased medial thickness characteristic of PH (Table II). Quantification of the structural changes in several lung sections from animals exposed to different hypoxia time periods (3, 7, 14 or 21 days) revealed significantly increased medial thickness of pulmonary arterioles in comparison with normoxic controls. Right ventricular hypertrophy resulting from right ventricle pressure overload is a hallmark of PH. After 14 days of hypoxia, the RVHI was significantly increased in comparison with controls (P<0.05), and further increased at 21 days of hypoxia. These results indicate that right ventricular hypertrophy had developed after 14 days of hypoxia.

SUMO-1, HIF-1α and VEGF levels in rat pulmonary arteries following exposure to hypoxia. The mRNA levels of *SUMO-1*, *HIF-1α* and *VEGF* in pulmonary arteries from control and hypoxia-treated rats were assessed using *in situ* hybridization and RT-PCR (Fig. 1). *SUMO-1* mRNA levels were extremely low in the control group (Fig. 1A), increased by day 3 of hypoxia (Fig. 1B) and peaked at 14 days (Fig. 1D). Although *SUMO-1* mRNA levels were reduced in the H21 group, they were higher compared with the values obtained from the control group (Fig. 1E). The same trend was obtained for pulmonary artery *SUMO-1* mRNA (Fig. 1L and M) as assessed by RT-PCR and *in situ* hybridization (Fig. 1K). The SUMO-1 protein was hardly detected in the pulmonary arteries from control animals (Fig. 1F). However, the H3 group (Fig. 1G) showed overtly increased protein levels, which peaked in the H14 group (Fig. 1I) and remained high at 21 days (H21 group; Fig. 1J). SUMO-1 expression in lung arteries assessed by immunoblotting (Fig. 1O and P) was consistent with immunohistochemistry data (Fig. 1N).

Subsequently, *in situ* hybridization and immunohistochemistry were used to evaluate the expression of HIF-1α mRNA and protein, respectively (Fig. 2). Detectable amounts of *HIF-1α* mRNA were found in the pulmonary arteries from the controls (Fig. 2A), but no clear change was evident at days 3 and 7 of hypoxia (P>0.05). However, the H14 and H21 groups showed higher mRNA levels compared with the control, H3 and H7 groups. The trend for *HIF-1α* gene expression in lung arteries (Fig. 2L and M) observed by RT-PCR was consistent with *in situ* hybridization data (Fig. 2K). Immunohistochemistry

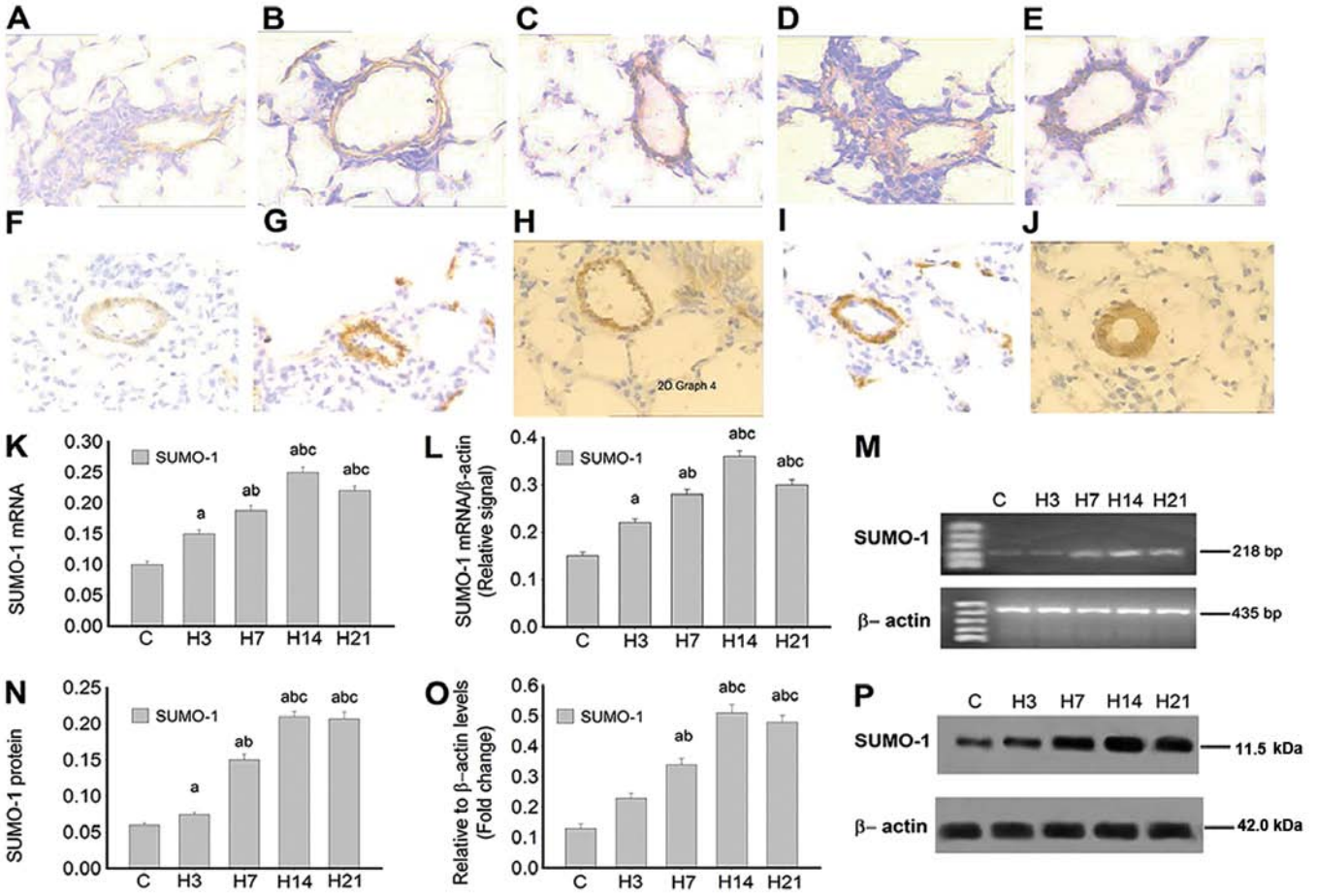

Figure 1. Hypoxia induces ubiquitin-related modifier-1 (*SUMO-1*) expression in rat lung arteries. Time course of *SUMO-1* mRNA expression by *in situ* hybridization in rat lung arteries exposed to (A) normoxia or hypoxia for (B) 3, (C) 7, (D) 14 and (E) 21 days (magnification, x400). Immunohistochemistry staining of SUMO-1 protein in rat lung arteries exposed to (F) normoxia or hypoxia for (G) 3, (H) 7, (I) 14 and (J) 21 days (magnification, x400). Detection of *SUMO-1* mRNA and protein by (M) RT-PCR and (P) western blot analysis; β-actin was used as reference. Densitometric analyses: (K) (N), (L) and (O) represent (A-E), (F-J), (M) and (P), respectively. Data are expressed as mean ± standard deviation (n=5). P<0.05 vs. [a]control group; [b]group H3; [c]group H7. RT-PCR, reverse transcription-polymerase chain reaction.

and immunoblotting analyses revealed no clear HIF-1α protein signal in the controls, while a statistical significance was observed at day 3 of hypoxia in the intima and media of pulmonary arterioles, compared with the controls (P<0.05). A peak was obtained by day 7 (H7 group) and by day 14, protein levels began to drop, a trend that continued to day 21 (Fig. 2N and O).

Fig. 3A-E and F-J present the time course analysis of *VEGF* mRNA and protein expression, respectively, in rat lung arteries. *VEGF* mRNA was hardly detected in the control and H3 groups. However, samples collected after 7 days of hypoxia showed that *VEGF* mRNA levels were markedly higher compared with that of the controls, with a statistical significance at P<0.05. Notably, mRNA expression peaked at day 14 and remained high until day 21 (Fig. 3K). *VEGF* mRNA was also mainly expressed at the intima and media. Accordingly, the levels of VEGF protein were extremely low in the control and H3 groups, significantly raised in the H7 group, peaked at day 14 and remained high at day 21 of hypoxia (Fig. 3L).

SUMO-1, HIF-1α and VEGF expression levels in rat PASMCs following hypoxia. The results presented suggest that hypoxia induces *SUMO-1*, *HIF-1α* and *VEGF* gene expression in animals. However, it is not clear whether this is true at the cell level, *in vitro*. Therefore, RT-PCR and western blot analysis were used to assess the expression levels of SUMO-1, HIF-1α and VEGF in PAMSCs, at mRNA and protein levels (Fig. 4). *SUMO-1* mRNA levels began to increase in PAMSCs after hypoxia for 2 h (H2h), compared with normoxia (control group), peaked after 12 h (H12h), and remained higher than the control values over time, until 24 h (H24h) (Fig. 4C). *HIF-1α* mRNA levels immediately increased in PAMSCs after 2-h hypoxia compared with the normoxia group; however, no significant difference was found between all the hypoxia groups (H2h, H6h, H12h and H24h) and controls (P>0.05) as shown in Fig. 4D. As for *VEGF*, the mRNA expression levels gradually increased with hypoxia time, reaching a peak after 12 h, and still exhibiting high levels at 24 h (Fig. 4E).

Western blot data showed that SUMO-1 protein levels began to increase in PAMSCs after 6 h of hypoxia, compared with the normoxia group, peaked after 12 h but tended to decrease at 24 h (Fig. 4B and F). However, HIF-1α protein levels steadily increased and maintained higher levels from 6 to 24-h hypoxia, peaking after 12 h (Fig. 4G), consistent with VEGF protein levels (Fig. 4H). Therefore, we may conclude that hypoxia overtly enhances mRNA and protein

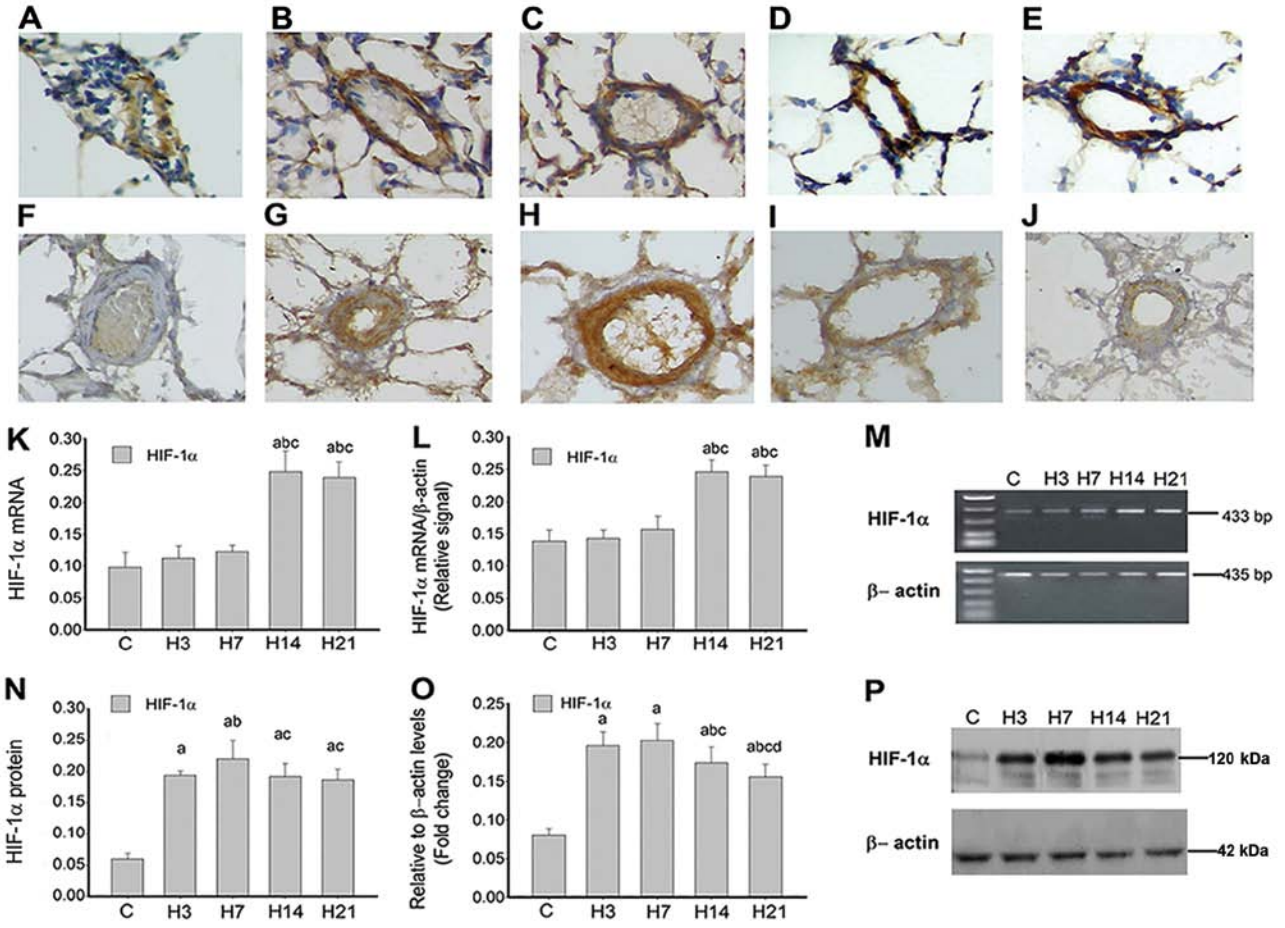

Figure 2. Expression of HIF-1α in rat lung arteries following exposure to normoxia and hypoxia. Time course analysis of *HIF-1α* (A to E) mRNA and (F-J) protein expression in rat lung arteries. N, H3, H7, H14 and H21 represent rat lung exposure to normal room air (normoxia) as control or 10% O_2 (hypoxia) for 3, 7, 14 and 21 days, respectively (magnification, x400). Analysis of HIF-1α mRNA and protein by (M) RT-PCR and (P) western blot analysis; β-actin was used as a reference. Densitometric analyses in (K), (N), (L), and (O) represent (A-E), (F-J), (M) and (P), respectively. Data are expressed as mean ± standard deviation (n=4). P<0.05 vs. [a]control group; [b]group H3; [c]group H7; [d]group H14. Hypoxia-inducible factor-1α, HIF-1α; RT-PCR, reverse transcription-polymerase chain reaction.

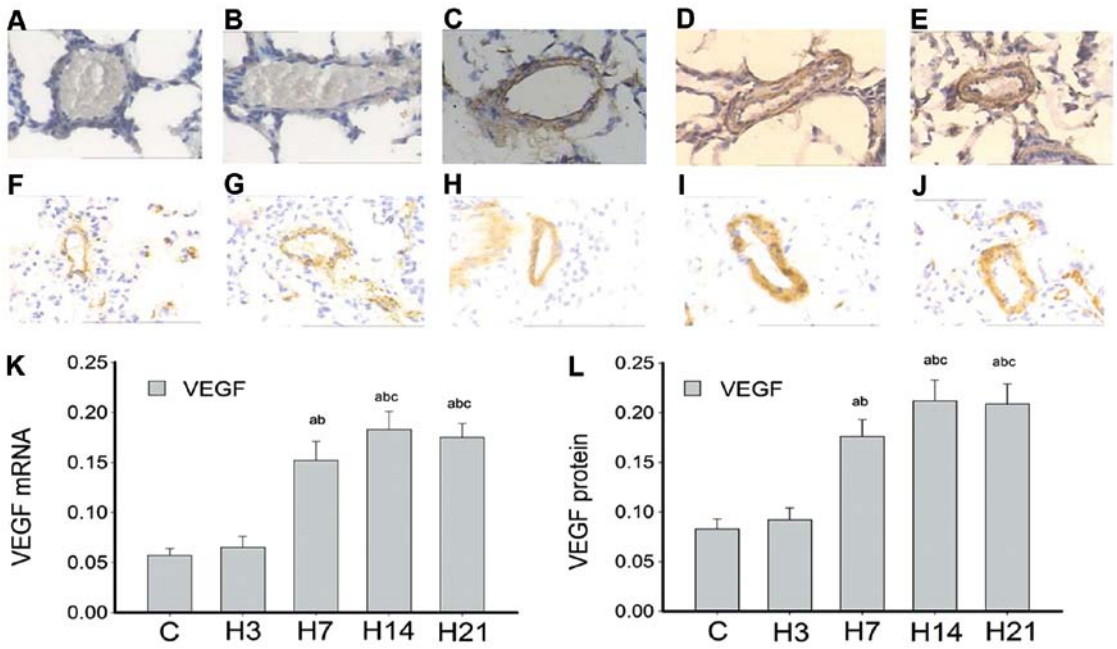

Figure 3. Vascular endothelial growth factor (VEGF) expression in rat lung arteries during normoxia and hypoxia. Time course analysis of VEGF (A-E) mRNA and (F-J) protein expression in rat lung arteries. N, H3, H7, H14 and H21 represent rat lung exposure to normal room air (normoxia) as control or 10% O_2 (hypoxia) for 3, 7, 14 and 21 days, respectively (magnification, x400). Column graphs (K) and (L) represent (A-E) and (F-J) quantification. Data are expressed as mean ± standard deviation (n=5). P<0.05 vs. [a]control group; [b]group H3; [c]group H7.

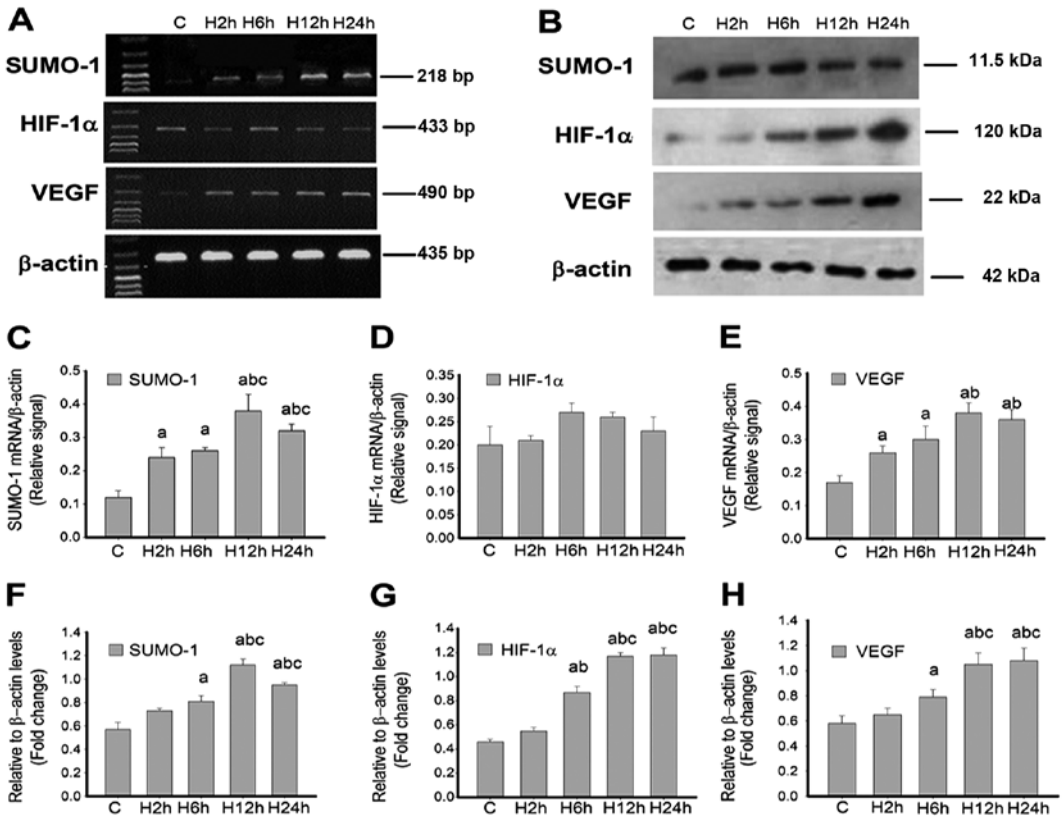

Figure 4. Ubiquitin-related modifier-1 (SUMO-1), HIF-1α and vascular endothelial growth factor (VEGF) are increased by hypoxia in pulmonary arterial smooth muscle cells (PASMCs) isolated from rat lungs. Time-course analysis of SUMO-1, HIF-1α and VEGF expression by (A) RT-PCR and (B) western blot analysis in rat PASMCs cultured in complete medium equilibrated with either 21% O_2 (normoxia) or 1% O_2 (hypoxia) for 2, 6, 12 and 24 h (H2h, H6h, H12h and H24h, respectively). β-actin was used as a reference. Densitometric analyses in (C and F), (D and G) and (E and H) represent the levels of SUMO-1, HIF-1α and VEGF mRNA and protein, respectively. Data are expressed as mean ± standard deviation from four independent experiments. P<0.05 vs. [a]control group; [b]group H2h; [c]group H6h. RT-PCR, reverse transcription-polymerase chain reaction.

expression of SUMO-1, HIF-1α and VEGF in rat PASMCs in a time-dependent manner.

Effect of rat SUMO-1 gene regulation in PASMCs. As shown previously, hypoxia promotes the expression of *SUMO-1*, *HIF-1* and *VEGF* in PASMCs. To confirm the role of SUMO-1 in sumoylational modification of HIF-1α, gene silencing (*SUMO-1*-RNAi-LV) and overexpression (*SUMO-1*-LV) were carried out in rat PASMCs.

Following *SUMO-1* gene silencing, RT-PCR and immunoblotting revealed a more pronounced decrease in the expression of HIF-1α and VEGF, compared with that of the control groups and cells transfected with pGC FU-RNAi-NC-LV (Fig. 5A and B). Compared with the corresponding normoxia groups (control, pGC FU-RNAi-NC-LV and *SUMO-1*-RNAi-NC-LV groups), *SUMO-1* and *VEGF* mRNA expression were increased in all hypoxia groups (Fig. 5C and E). Whether under normoxia or hypoxia, SUMO-1, HIF-1α and VEGF in the *SUMO-1*-RNAi-NC-LV group were clearly reduced, with a statistically significant difference (P<0.05) (Fig. 5C-H).

Subsequent to *SUMO-1* gene overexpression, RT-PCR and immunoblotting data demonstrated that the expression of HIF-1α and VEGF (mRNA and protein) was increased compared with that of the control and pGC FU-RNAi-NC-LV groups (Fig. 6A and B). Compared with the corresponding normoxia groups (control, pGC FU-RNAi-NC-LV and *SUMO-1*-LV groups), *SUMO-1α* and *VEGF* expression was increased in all the hypoxia groups (Fig. 6C and E). In addition, whether under normoxia or hypoxia, SUMO-1, HIF-1α and VEGF levels in the *SUMO-1*-LV group were all clearly enhanced, with a statistically significant difference (P<0.05), as shown in Fig. 6C-H. Therefore, *SUMO-1* gene silencing and overexpression assays confirmed that SUMO-1 may participate in the modulation of HIF-1α through sumoylation and upregulate *HIF-1α* expression, as well as *HIF-1α* downstream target genes, including *VEGF*, in rat PASMCs.

There was no difference between pGC FU-RNAi-NC-LV or pGC FU-GFP-LV and controls under normoxia and hypoxia (all P>0.05) (Figs. 5D, E, G and H, and 6D, E, G and H), suggesting that the negative controls of *SUMO-1* knockdown and overexpression did not affect hypoxia-induced HIF signaling.

HIF-1α sumoylational modification in rat lung arteries and PASMCs. To further confirm the findings, HIF-1α sumoylational modification of rat lung arteries was compared after exposure to hypoxia for 0, 3, 7, 14 and 21 days, and PASMCs after 2, 6, 12 and 24 h of exposure to hypoxia (Fig. 7). The data clearly demonstrated that HIF-1α in the control group did not bear any sumoylational modification. However, hypoxia-treated cells showed significant sumoylational modification from day 3, which peaked by 14 days and remained high until 21 days (Fig. 7A). Similarly, HIF-1α in the control cells

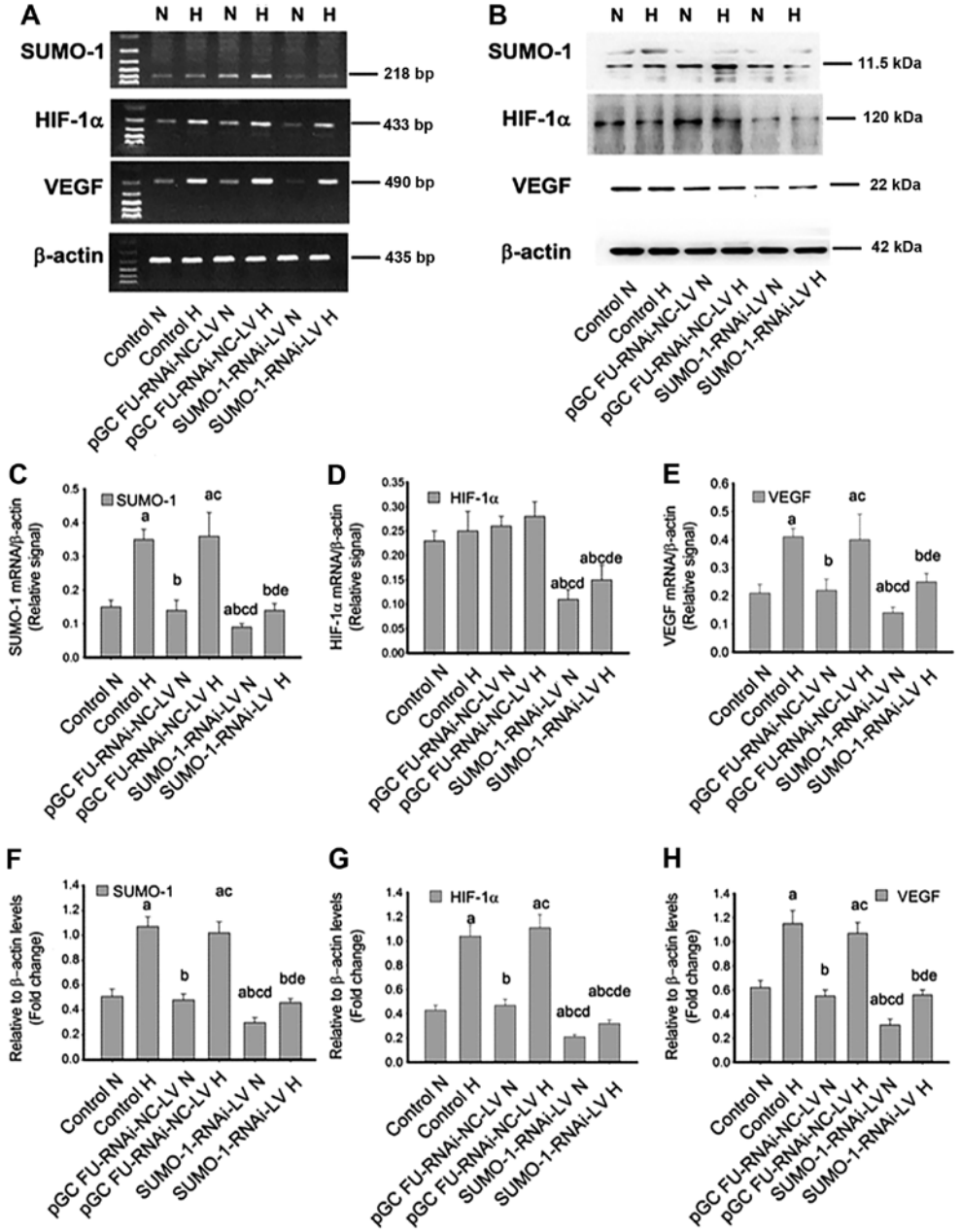

Figure 5. Ubiquitin-related modifier-1 (*SUMO-1*) silencing reduced SUMO-1, HIF-1α and vascular endothelial growth factor (VEGF) levels in rat pulmonary arterial smooth muscle cells (PASMCs). Analysis by (A) RT-PCR and (B) western blot analysis of SUMO-1, HIF-1α and VEGF mRNA and protein in rat PASMCs after transfection with *SUMO-1*-RNAi-NC-LV in normoxia or hypoxia at 1% O_2 for 12 h. Non-transfected cells and cells transfected with pGC FU-RNAi-NC-LV were used as controls. β-actin served as mRNA and protein loading control. Column graphs (C and F), (D and G) and (E and H) represent the levels of SUMO-1, HIF-1α and VEGF mRNA and protein, respectively. Data are expressed as mean ± standard deviation from five independent experiments. $P<0.05$ vs. [a]control N group; [b]control H group; [c]pGC FU-RNAi-NC-LV N group; [d]pGC FU-RNAi-NC-LV H group; [e]*SUMO-1*-RNAi-LV N group. N, normoxia; H, hypoxia; RT-PCR, reverse transcription-polymerase chain reaction.

exhibited no sumoylational modification, and hypoxia groups showed overt sumoylational modifications, more evident at 12 and 24 h (Fig. 7B).

Discussion

In the present study, the results suggest that *SUMO-1* gene expression is enhanced by hypoxic stimulation in rat PASMCs and the rat HPH model. Furthermore, there may be an association between SUMO-1 and HIF-1α in response to hypoxic stimulation *in vivo* and *in vitro*, suggesting that sumoylation of HIF-1α may be important in the stabilization and action of the HIF-1α protein.

HIF-1 is a heterodimer of two basic helix-loop-helix/PAS proteins, HIF-1α and HIF-1β (8), and its activation plays an important role in tissue preservation as a response to regional hypoxia (31). HIF-1α is rapidly degraded under normoxic conditions by the ubiquitin-proteasome system; HIF-1β is constitutively expressed in the nucleus and its level is not significantly affected by oxygen levels (32,33). It is known that hypoxia stabilizes HIF-1α, and nucleus-bound translocation of the stabilized HIF-1α allows for the formation of the HIF-1αβ

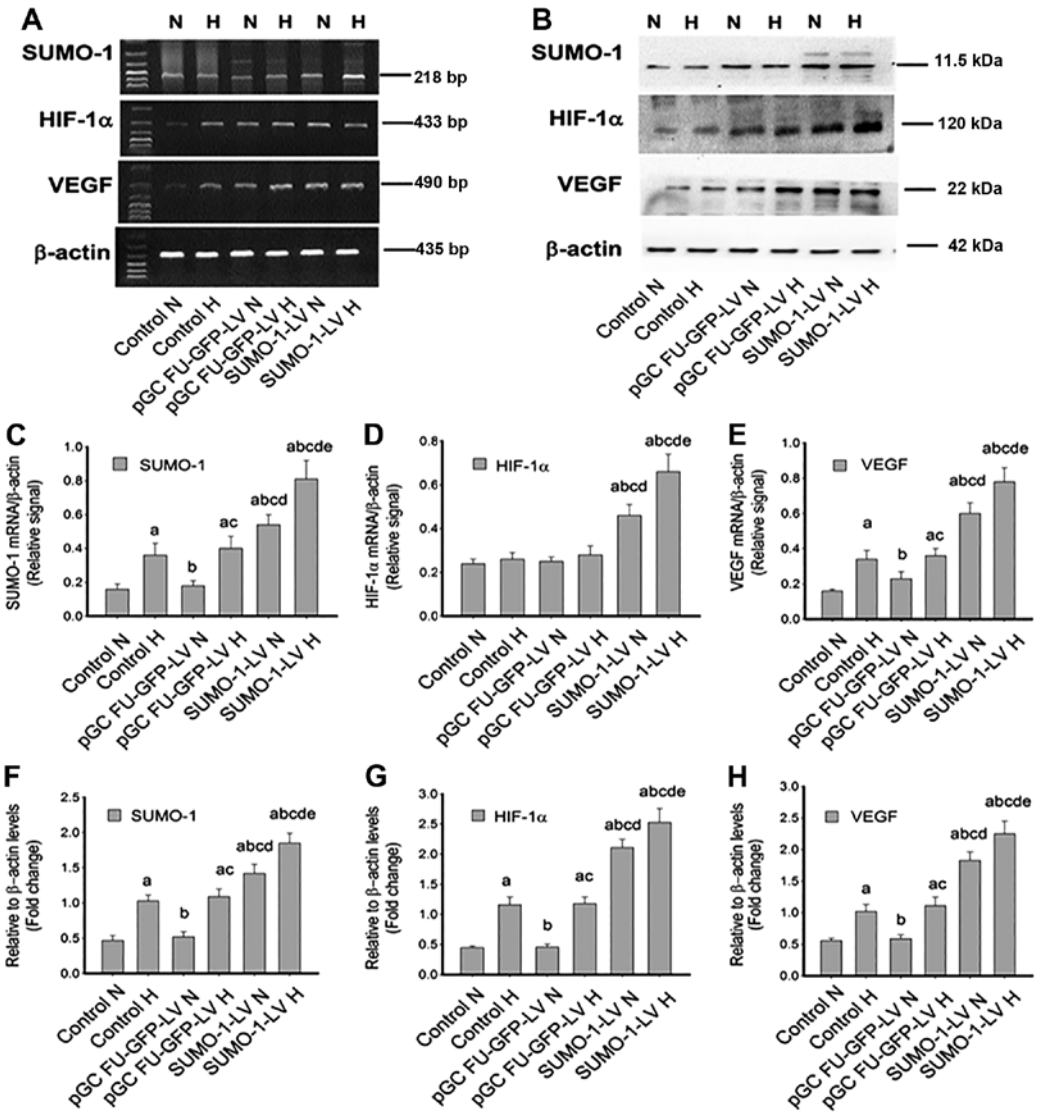

Figure 6. Ubiquitin-related modifier-1 (*SUMO-1*) overexpression increased SUMO-1, HIF-1α and vascular endothelial growth factor (VEGF) levels in rat pulmonary arterial smooth muscle cells (PASMCs). (A) RT-PCR and (B) western blot analysis of SUMO-1, HIF-1α and VEGF mRNA and protein in rat PASMCs with *SUMO-1*-LV transfection in normoxia or hypoxia at 1% O_2 for 12 h. Non-transfected cells and cells transfected with pGC FU-GFP-NC-LV were used as controls. β-actin served as mRNA and protein loading control. Densitometric analysis of (C and F), (D and G) and (E and H) represent the levels of SUMO-1, HIF-1α and VEGF mRNA and protein, respectively. Data are expressed as mean ± standard deviation from five independent experiments. P<0.05 vs. [a]control N group; [b]control H group; [c]pGC FU-GFP-LV N group; [d]pGC FU-GFP-LV H group; [e]*SUMO-1*-LV N group. N, normoxia; H, hypoxia; RT-PCR, reverse transcription-polymerase chain reaction.

heterodimer that becomes transcriptionally active (33). HIF-1α, as a transcription factor induced by hypoxia, regulates the expression of >100 genes involved in cellular adaptation and survival (34,35). It has been shown that HIF-1α can be regulated by various post-translational modifications, including hydroxylation, acetylation and phosphorylation. Therefore, HIF-1α plays a central role in the cellular response to hypoxia, and its expression levels are tightly controlled through synthesis and degradation (34,35).

The present study indicates an important role for sumoylation of HIF-1α in hypoxic PH. The covalent conjugation of SUMOs to proteins has received increasing attention since its discovery (36,37), due to the involvement of target proteins in gene expression, chromatin structure, signal transduction or maintenance of the genome (38). SUMO modification has emerged as an important regulatory mechanism for protein function and localization. Sumoylation is a dynamic process, catalyzed by SUMO-specific E1, E2 and E3s (39). In mammals, SUMO is first activated by an E1 heterodimer comprised of SAE1 and SAE2, transferred to an E2-conjugating Ubc9 enzyme and conjugated by an E3 ligase to target proteins. Three mammalian E3 enzymes have been described: Ran-binding protein2, protein inhibitor of activated STAT and polycomb protein 2 (40). For instance, transient global cerebral ischemia induces significant increases in the sumoylation of proteins, including transcription factors (41). In particular, hypoxia increases SUMO-1 mRNA and protein levels in the brain, and the induced SUMO-1 is co-expressed with HIF-1α in neurons (39).

However, controversy exists regarding the cellular mechanism of HIF-1α modulation through sumoylation. It is generally believed that sumoylation of transcription factors blocks their activation (26,42). Thus, conjugation of SUMO to HIF-1α

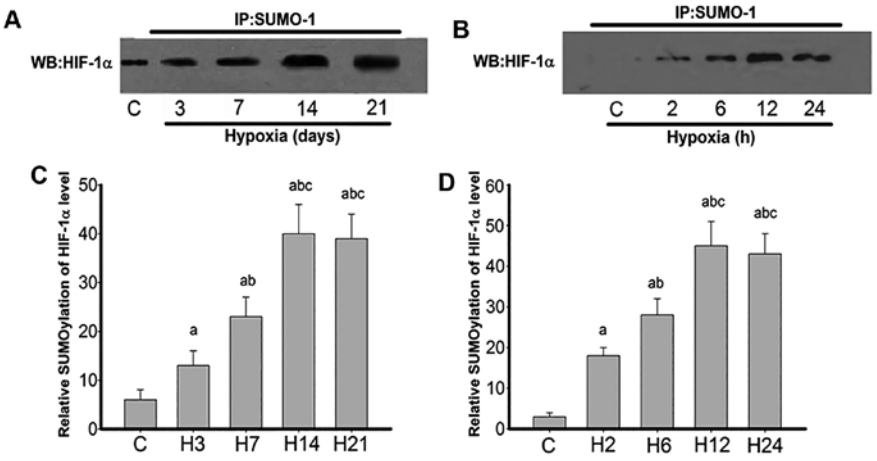

Figure 7. Co-immunoprecipitation of ubiquitin-related modifier-1 (SUMO-1) with HIF-1α in rat lung arteries and pulmonary arterial smooth muscle cells (PASMCs) exposed to hypoxia. Immunoprecipitation (IP) was performed with the anti-SUMO-1 antibody. For western blot analysis (WB), the primary antibody against HIF-1α was used. (A) SUMO-1 and HIF-1α co-immunoprecipitation at lung arteries exposed to hypoxia 3, 7, 14 and 21 days (H3, H7, H14 and H21, respectively). (C) Quantification of (A). Data are expressed as mean ± standard deviation (SD) (n=4). P<0.05 vs. [a]control group; [b]group H3; [c]group H7. (B) SUMO-1 and HIF-1α co-immunoprecipitation in PASMCs exposed to hypoxia for 0, 2, 6, 12 and 24 h. (D) Quantification of (B). Data are expressed as mean ± SD from four independent experiments. P<0.05 vs. [a]control group; [b]group H2; [c]group H6. Hypoxia-inducible factor-1α, HIF-1α.

decreases its activity (43), and hypoxia-induced sumoylation of HIF-1α leads to its ubiquitination and degradation (44). By contrast, Bae *et al* (45) reported that HIF-1α upregulation through SUMO-1 modification at Lys(391)/Lys(477) residues increases its stability and enhances transcriptional activity, and sumoylation of HIF-1α blocks its degradation (41). However, sumoylation of HIF-1α in the pathogenesis of hypoxia-induced PH remains poorly understood.

The present results suggest that hypoxia induces *SUMO-1* expression and promotes HIF-1α and VEGF mRNA and protein expression in PASMCs. Increased SUMO-1 mRNA and protein levels following hypoxia stimulation and involvement in HIF-1α post-translational modifications is a well-described process (39). Therefore, an increase in SUMO-1 may contribute to HIF-1α stabilization under hypoxic conditions, which is important for HIF-1α-dependent gene activation (such as activation of *VEGF*, erythropoietin, glucose transporters and endothelial cell proliferation) involved in vascular biology, cellular metabolism and tissue tolerance to hypoxia (46). VEGF has been shown *in vivo* and *in vitro* to be the principal mediator of hypoxia-induced angiogenesis (46-49). In the present study, sumoylation does not appear to be the only mechanism responsible for increased HIF-1α expression. In addition to increased protein levels due to a decreased degradation (8,9), *HIF-1α* mRNA expression is also increased. Previous studies suggest that hypoxia *per se* increases *HIF-1α* mRNA expression through a number of mechanisms, including the mTOR pathway (50), changes in redox status within the cells (51) and lipopolysaccharide exposure (52). Further studies are required to examine the exact mechanisms involved in *HIF-1α* mRNA expression, particularly in HPH.

In order to further verify the association between sumoylation modification and HIF-1α, gene silencing and overexpression were used to modulate SUMO-1 in rat PASMCs. The present data suggested that HIF-1α and VEGF expression, at mRNA and protein levels, was decreased following *SUMO-1* gene knockdown, and increased upon overexpression. These findings also suggested that *SUMO-1* plays a critical role in HIF-1α sumoylational modification. In addition, the co-immunoprecipitation experiment suggests an association between SUMO-1 and HIF-1α, but did not allow the assessment of the exact nature of this association. Further studies are required regarding this point.

Notably, Shao *et al* (39) reported that RWD-containing sumoylation enhancer (RSUME) can be induced by hypoxia, enhancing HIF-1α sumoylation and promoting its stabilization and transcriptional activity during hypoxia. Disruption of the RWD domain of RSUME demonstrated that RWD is critical for RSUME action. Therefore, we can speculate that RSUME plays a central role in the regulation of sumoylation, having indirectly demonstrated that sumoylation of HIF-1α stabilizes HIF-1α and enhances its transcriptional activity.

In conclusion, to the best of our knowledge, this is the first study investigating the effect of hypoxia on *SUMO-1* gene expression in PASMCs and rat HPH models. There was an association between the hypoxia-induced increase in *SUMO-1* expression and HIF-1α induction in response to hypoxia, but our results did not allow us to determine the extent and the exact nature of this association. Further studies are required to explore this association.

Acknowledgements

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