의학박사 학위논문

Alteration of TGF-β-ALK-Smad signaling in hyperoxia-induced bronchopulmonary dysplasia model of newborn rats

신생 쥐 기관지 폐이형성증 모델에서의 **TGF-β-ALK-Smad** 신호전달체계의 변화

2013 년 08 월

서울대학교 대학원

의학과 소아과학 전공

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A thesis of the Degree of Doctor of Philosophy

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August 2013

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by

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A thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Medicine (Major in Pediatrics)

in the Seoul National University, Seoul, Korea

July 2013

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이 논문을 의학박사 학위논문으로 제출함
2013 년 05 월

서울대학교 대학원
의학과 소아과학 전공
김미화

김미화의 의학박사 학위논문을 인준함
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ABSTRACT

Alteration of TGF-β-ALK-Smad signaling in hyperoxia-induced bronchopulmonary dysplasia model of newborn rats

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Background: Bronchopulmonary dysplasia (BPD) is a main chronic lung disease that commonly occurs in preterm infants. BPD is characterized by impaired alveolarization and vascularization of the developing lung. The transforming growth factor-β (TGF-β) signaling pathway is known to play an important role during lung vascular development.

Objective: To investigate whether the regulation of TGF-β-ALK-Smad signaling pathway influences the disruption of pulmonary vascular development in newborn rats using a hyperoxia-induced BPD model.

Methods: Neonatal rats were continuously exposed to 21% or 85% O_2 for 7 days and subsequently kept in normoxic conditions for another 14 days. Lung

tissues were harvested at each time point and were evaluated for the expression of TGF-β1, ALK1, ALK5, phosphorylated Smad1/5, phosphorylated Smad2/3, VEGF and endoglin by both biochemical and immunohistological analyses.

Results: Double-fluorescence immunohistochemical staining indicated that these molecules were mainly expressed in pulmonary endothelial cells. The expression of TGF-β1 and ALK5 mRNA and protein were significantly increased in the D5 hyperoxia group, while the expression of ALK1 mRNA and protein was significantly decreased. The level of phosphorylated Smad1/5 was significantly decreased in the D7 hyperoxia group, whereas the expression of phosphorylated Smad2/3 was increased. In addition, the expression of vascular endothelial growth factor (VEGF) mRNA was increased at D1, with a subsequent decrease in the D7 hyperoxia group. There was no significant difference in endoglin expression over the entire experimental period.

Conclusion: Our results indicate that exposure to hyperoxia altered the balance between the TGF-β-ALK1-Smad1/5 and TGF-β-ALK5-Smad2/3 signaling pathways in pulmonary endothelial cells, which may ultimately lead to the development of BPD.

Keywords: Angiogenesis; endothelial cell; VEGF; endoglin

Student Number: 2011-30825

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LIST OF ABBREVIATION

BPD	bronchopulmonary dysplasia
TGF-β	transforming growth factor-β
TβRⅡ	TGF-β type Ⅱ receptor
TβRⅠ	TGF-β type Ⅰ receptor
ALK1	activin receptor-like kinase-1
ALK5	activin receptor-like kinase- 5
R-Smad	receptor-regulated Smad
Co-Smad	common mediator Smad
VEGF	vascular endothelial growth factor
RT-PCR	reverse-transcription PCR
RDS	respiratory distress syndrome
BMPs	bone morphogenetic proteins
VD_T	tissue volume densities
L_m	mean chord length
S_A	alveolar surface area

INTRODUCTION

In recent years, widespread use of antenatal steroids, different types of mechanical ventilation and early administration of exogenous postnatal surfactant therapy have significantly reduced the mortality of preterm infants. However, despite the advanced perinatal care preterm infants are still at risk for late respiratory morbidity due to development of bronchopulmonary dysplasia (BPD).

The histopathology of ‘old BPD’ was initially characterized by airway epithelial metaplasia, peribronchial fibrosis and airway vascular smooth muscle hypertrophy over 40 years ago [1]. With advanced perinatal therapy, the pathological picture has gradually changed from ‘old BPD’ to ‘new BPD’. ‘New BPD’ is characterized more by impaired alveolarization and abnormal vascular growth and less by parenchymal fibrosis [2]. ‘New BPD’ has been defined as a developmental disorder of the lung and arrested lung vascular development has recently been a focus of research in this context. A recent study has suggested that hyperoxic injury may be associated with the disruption of lung angiogenesis in BPD [3]. Although the regulation of lung angiogenesis is one of the causative factors for BPD pathogenesis, its molecular mechanism remains to be elucidated.

TGF-βs are known to be involved in the regulation of vasculogenesis and

angiogenesis [4]. The TGF-βs family contains three isoforms, known as TGF-β1, TGF-β2 and TGF-β3. TGF-β-mediated signaling is initiated by binding of the TGF-β ligand to its serine/threonine kinase receptors, the type Ⅱ receptor (TβRⅡ), which subsequently complex with the type Ⅰ receptor [5]. Recent studies have shown that TGF-β can bind to two distinct type Ⅰ receptors, ALK1 and ALK5, and transduces signals to endothelial cells [6]. The TGF-β-ALK5 and TGF-β-ALK1 pathways have opposite effects on endothelial cell behavior; TGF-β-ALK5 signaling induces Smad2/3 phosphorylation, which inhibits endothelial cell proliferation and migration, while TGF-β-ALK1 signaling induces Smad1/5 phosphorylation, which promotes both processes [6, 7]. Subsequently, these TGF-β receptor-regulated Smads (R-Smads) form heteromeric complexes with common mediator Smad (Co-Smad) Smad4 and translocate into the nucleus to regulate the expression of target genes [8]. It has also been reported that vascular endothelial growth factor (VEGF) expression increases through enhancement of the TGF-β-ALK1-Smad1/5 signaling pathway [9, 10].

In the present study, we used hyperoxia-exposed newborn rats as a BPD model to evaluate the expression of molecules involved in TGF-β signaling pathway. In addition, we examined the expression of endoglin (CD105), which is a TGF-β accessory receptor also known as the TGF-β type Ⅲ receptor. In particular, we focused on the change in vascularization mediated

by the TGF-β-ALK-Smad signaling pathway during newborn lung development after hyperoxic conditions.

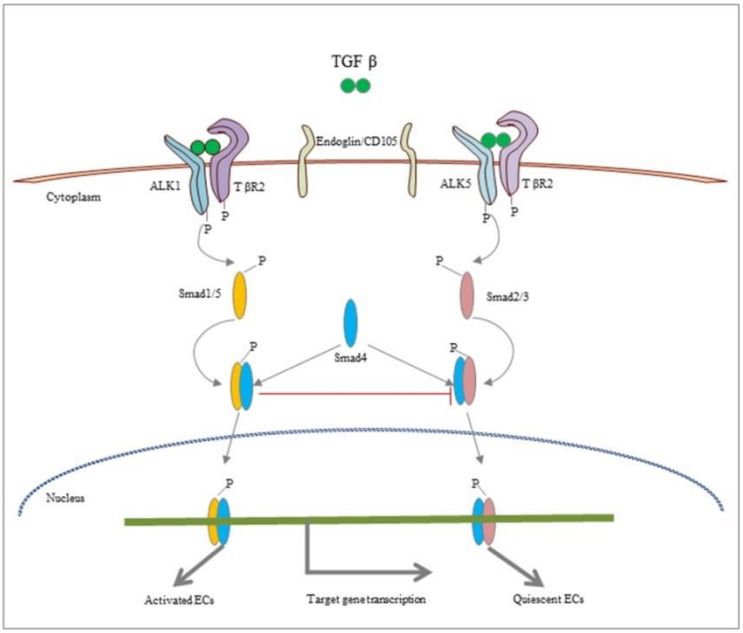

Figure 1 TGF-β transduces signals through the Smad-dependent pathway in endothelial cells [11]. TGF-β signaling is initiated by binding of TGF-β to one of its receptors, the type Ⅱ receptor (TβRⅡ), which subsequently phosphorylates and complexes with the type Ⅰ receptors (ALK1 or ALK5). The TGF-β-ALK5 and TGF-β-ALK1 pathways have opposite effects on endothelial cell behavior; TGF-β-ALK5 signaling induces Smad2/3 phosphorylation, which inhibits endothelial cell proliferation and migration, while TGF-β-ALK1 signaling induces Smad1/5 phosphorylation, which promotes both processes. The activated Smad1/5 and Smad2/3 then form heteromeric complexes with Smad4 and translocate into the nucleus to regulate target gene transcription. Moreover, ALK1-Smad1/5 signaling indirectly inhibits ALK5-Smad2/3 signaling by competing for availability of Smad4.

MATERIALS AND METHODS

Experimental animals

All animal studies were performed at Seoul National University Hospital Biomedical Research Institute. The experimental protocol and procedures were approved by the Animal Care and Use Committee. Four-day-old male Sprague-Dawley rat pups (Orient Bio Inc. Seongnam, Korea) were randomly divided into two groups, control (normoxia) group and hyperoxia group, and used in the experiments. There were fifteen animals per group, and the experiments were repeated three times.

Hyperoxic animal model

Rat pups in the hyperoxia groups were kept with a dam in a cage within a Plexiglas hyperoxic chamber containing 85% oxygen for 7 days. Subsequently, they were kept under normoxic conditions for 14 days. The unexposed control group rats were continuously kept under normoxic conditions for the entire 21 days of the experimental period. Nursing dams were rotated daily between hyperoxia and control cages to prevent oxygen toxicity in the dams. Oxygen concentration, humidity and temperature inside the Plexiglas hyperoxic chamber were continuously monitored with an oxygen sensor (Coy Laboratory Products, Grass Lake, Michigan, USA). Breeding conditions were similar for all animals.

Tissue preparation

Rat pups were respectively sacrificed at D0, D0.5, D1, D3, D5, D7 and D21. The right lungs from each pup were immediately stored at -70℃ for biochemical analysis, and the left lungs were fixed in formalin and stored at 4 ℃ for histological analysis.

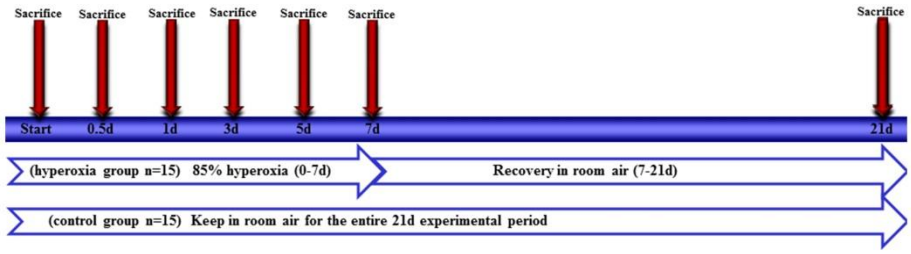

Figure 2 Diagram of experimental protocol. Rat pups in the hyperoxia group were kept in 85% O_2 for 7 days. Subsequently they were kept under normoxic conditions for 14 days. The unexposed control group rats were continuously kept under normoxic conditions for the entire 21 days of the experimental period. Each group consisted of n = 15 animals.

Lung morphometry

Four random, non-overlapping fields per pup were utilized for morphometric examinations. H&E stained sections were photographed using a digital camera system (Leica DFC 280; Leica, Wetzlar, Germany) with a microscope (Olympus BX51; Olympus, Tokyo, Japan) at 100 x magnification. Photographs were analyzed using the morphometric method as described previously [12]. Tissue volume densities (VD_T) were determined using a 10 x 10 grid (grid element side length was approximately 29μm). The mean chord length (L_m) provided an estimate of the distance from one airspace wall to an adjacent airspace wall. Alveolar surface area (S_A) was calculated using S_A=4 x VD_T x lung volume/ Lm. Lung volume was determined by measuring the displacement of water by lungs after fixation.

Real-time RT-PCR

Total RNA was extracted from lung tissue using trizol reagent (Cat No.15596-018; Invitrogen, CA, USA) according to the manufacturer's instructions. The quality and purity of isolated total RNA was determined by spectrophotometry at 260 and 280 nm. Single-stranded cDNA was synthesized from 1 μg of total RNA using a cDNA synthesis kit (Cat No. #170-8891; Bio-Rad, Hemel Hempstead, UK) in a total volume of 20 μl. Real-time PCR reactions with SYBR Green were run on the Chromo4 System (Cat No. CFB-3240; Bio-Rad, USA). After an initial denaturation step (5 min

at 95℃), TGF-β1, ALK5 and endoglin were amplified using 45 cycles of 15 sec at 95℃, 30 sec at 60℃, 30 sec at 72℃. ALK1 was amplified using 45 cycles of 10 sec at 95℃, 10 sec at 60℃, 15 sec at 72℃. VEGF was amplified using 45 cycles of 20 sec at 95℃, 30 sec at 60℃, 30 sec at 72℃. The data were analyzed using Opticon Monitor software (version 3.1, Bio-Rad). The corresponding primer sequences for each gene are shown in Table 1.

Western blot

Lung tissues were homogenized in lysis buffer with a protease inhibitor cocktail (Cat No.11697498001; Roche, IN, USA). After determining the protein concentration by DC protein assays (Bio-Rad, Hercules, CA, USA), total soluble proteins (20 μg) were separated on 4-12% gradient gels (Cat No. NP0335BOX; Invitrogen, CA, USA). The proteins were transferred to a nitrocellulose membrane. Non-specific binding was blocked with 5% Skim Milk (Cat No. 232100; BD, California, USA) at room temperature for 1 h. The protein blots were then probed with specific primary antibodies (1:1000 dilutions) against TGF-β1 (Cat No. NBP1-45891; Novus Biologicals, Littleton, CO, USA), ALK1, ALK5, phospho-Smad1/5 (Cat No. #9516; Cell Signaling, Frankfurt, Germany), phospho-Smad2 (Cat No. #AB384-9; Millipore, MA, USA), phospho-Smad3 (Cat No. A1005; Assay Biotech, CA, USA) and endoglin overnight at 4℃. After incubating with host-specific secondary antibodies (goat anti-rabbit IgG-HRP) for 1 h at room temperature,

the immunoreaction was detected with a chemiluminescent substrate kit (Cat No. #34080; Thermo Scientific, Rockford, IL, USA) and quantitated by densitometric scanning of the X-ray film with SLB MyImager (UVP Inc, Upland, CA, USA). Blots were normalized for protein loading by washing in stripping solution and reprobing with a monoclonal antibody against β-actin (dilution 1:2000; Cat No. #4970, Cell Signaling, Frankfurt, Germany).

Immunohistochemistry (IHC)

ALK1, ALK5 and endoglin were detected by immunohistochemistry after 5 days of 85% oxygen exposure, and PECAM-Ⅰ was detected at D21. Paraffin-embedded sections (4 μm) were deparaffinized with xylene and dehydrated with ethanol. The slides were then exposed to antigen unmasking solution (Cat No. H-3301; Vector Laboratories, CA, USA) in a microwave (98℃) for 15min. After cooling, the slides were placed in a staining dish with 0.3% H_2O_2 to quench for endogenous peroxidase activity for 30 min; then, the slides were washed with PBS three times and subsequently placed in 0.5% triton x 100 (Cat No. T8787; Sigma, Germany) for 15 min. The non-specific binding of antibodies to the lung tissue sections were then blocked by incubating sections with 10% normal goat serum (Cat No. 71-00-27; KPL, Gaithersburg, USA) for 1 h. The sections were incubated overnight at 4℃ with PECAM-Ⅰ(Cat No. MAB1393, dilution 1:200; Millipore, MA, USA), ALK1 (Cat No. BS2555, dilution 1:100; Bioworld Technology, MN, USA),

ALK5 (Cat No. BS3257, dilution 1:100, Bioworld Technology, MN, USA) and endoglin (Cat No. ab107595, dilution 1:100; Abcam, Cambridge, UK) antibodies. The sections were then incubated at room temperature with secondary antibody (Cat No. K5007; Dako Cytomation, Denmark) for 1 h. Binding was detected with 3, 3'-diaminobenzidine tetrachloride (Dako Cytomation, Denmark) for 15 sec. The sections were lightly counterstained with hematoxylin, cleared and mounted with mounting medium. The immunostained sections were examined using a microscope at 200 x magnification, with an attached camera. Five random, non-overlapping fields per section were captured for quantitative imaging (Leica QWin image analysis software).

Normal lung tissue was used for the double fluorescence staining. Goat anti-mouse secondary antibody marked with red fluorochrome Alexa594 (Cat No. A11005, dilution 1:1000; Invitrogen, CA, USA) was used to detect PECAM-Ⅰ. Goat anti-rabbit secondary antibody marked with green fluorochrome Alexa488 (Cat No. A-11008, dilution 1:1000; Invitrogen, CA, USA) was used for the detection of endoglin, ALK1 and ALK5. For nuclear counterstaining, blue-fluorescent DAPI staining was used (Cat No. H-1200; Vector Laboratories, CA, USA). As a negative control, primary antibody was omitted. The sections were captured at 200 x magnification.

Statistical analysis

Statistical analyses were performed using SPSS statistics 18. The data were expressed as the mean ± SEM of three independent experiments. Student's t-test or Mann-Whitney U test was used for comparisons between the hyperoxia and control groups, as appropriate. A p-value of < 0.05 was considered to be statistically significant.

RESULTS

Light microscopy

The unexposed control group showed normal alveolarization, whereas the hyperoxia group displayed impaired alveolarization with fewer, larger and simper alveoli (Figure 3A). The hyperoxia group had a significantly longer L_m (42.08 ± 1.582 vs. $47.24 \pm 1.739\mu m$, $P < 0.05$) and significantly smaller S_A (880.1 ± 34.46 vs. $627.7 \pm 46.74\mu m^2$/lung, $P < 0.001$) compared to the control group (Figure 3B & C). PECAM-Ⅰ, an indicator of angiogenesis, was significantly decreased in the hyperoxia group at D21 ($P < 0.001$) (Figure 4). Quantification of these parameters indicated that the exposure of newborn rats to chronic hyperoxia resulted in impaired alveolarization and vascularization.

Expression of TGF-β1, ALK1, ALK5, VEGF and endoglin mRNA in the hyperoxic groups

The level of TGF-β1 mRNA was significantly increased in the D5 and D7 hyperoxia groups, compared to the expression levels of the control groups ($P < 0.01$ and $P < 0.05$, respectively) (Figure 5A). The level of ALK1 mRNA was significantly decreased in the D5 and D7 hyperoxia groups ($P < 0.001$ and $P < 0.05$, respectively) (Figure 5C), whereas the level of ALK5 was significantly increased ($P < 0.01$ and $P < 0.01$) (Figure 5D), compared to the corresponding control groups. The expression of VEGF mRNA in the D1

hyperoxia group was higher than that of the control. Its expression was markedly reduced in the D7 hyperoxia group relative to the control ($P < 0.05$) (Figure 5E), even though no significant difference was detected between D3 and D5. We observed no significant changes in the expression of endoglin mRNA between the hyperoxia and control groups throughout the entire experimental period (Figure 5B). Collectively, these results indicated that the expression of TGF-β1, ALK1, ALK5, and VEGF were transcriptionally regulated by hyperoxic exposure.

Expression of TGF-β1, ALK1, ALK5 and endoglin protein in the hyperoxic groups

The level of TGF-β1 protein was significantly increased in the D5 hyperoxia group relative to the controls ($P < 0.05$) (Figure 6A). The levels of ALK1 and phospho-Smad1/5 proteins were significantly decreased in the hyperoxia group compared to the control groups at D5 and D7 ($P < 0.05$ and $P< 0.05$, respectively) (Figure 6C & 11A). Meanwhile, the levels of ALK5 and phospho-Smad2/3 were significantly increased in the same hyperoxia group relative to the control groups ($P < 0.05$ and $P < 0.05$, respectively) (Figure 6D & 11B, C), demonstrating that they were oppositely regulated, compared to ALK1 and phosho-Smad1/4, by hyperoxic exposure. There was no statistically significant difference in the level of endoglin protein throughout the entire experimental period (Figure 6B). Taken together, these

results indicate that hyperoxic exposure causes translational regulation of TGF-β1, ALK1, ALK5 and post-translational modification of Smad1, 2, 3 and 5.

Immunohistochemical analysis of endoglin, ALK1 and ALK5 levels in hyperoxic lung tissue

To further investigate the hyperoxia-mediated regulation of endoglin, ALK1 and ALK5 in lung tissue, immunohistochemical analyses were performed. Initially, we examined the tissue distribution of these proteins in normal lungs. As shown in Figure 7, FITC fluorescence staining (green) of endoglin, ALK1 and ALK5 overlapped with positive rhodamine staining (red) for the PECAM-Ⅰ, which is the marker for endothelial cells, indicating that endoglin, ALK1 and ALK5 were mainly expressed in endothelial cells. Next, we examined the changes in their expression during D5 of hyperoxic exposure, as assessed by measuring the density of positive staining. The immunoreactive intensity of ALK1 was significantly decreased in the hyperoxic lung compared with the control group (2.5880 ± 0.2751 vs. 1.7207 ± 0.2293, $P < 0.05$) (Figure 8), while the intensity of ALK5 was significantly increased (1.4686 ± 0.1778 vs. 2.2847 ± 0.2865, $P < 0.05$) (Figure 9). There was no statistically significant difference of endoglin expression between the hyperoxic and control lungs (0.9692 ± 0.1614 vs. 1.3677 ± 0.1291, $P > 0.05$) (Figure 10).

Table1. Primers employed for real-time RT-PCR analysis

Target	Forward primer Reverse primer	References
TGF-β1	5’-TGGCGTTACCTTGGTAACC-3’ 5’-GGTGTTGAGCCCTTTCCAG--3’	[40]
Endoglin	5’-ACCACTTCGGAAAAAGG-3’ 5’-GCTGAAACGTGGGTCG-3’	[41]
ALK1	5’-GTCAAGAAGCCTCCAGCAAC-3’ 5’-CAT CAACTCAGGCTTCGGG-3’	[42]
ALK5	5’-CGTCTGCATTGCACTTATGC-3’ 5’-AGCAGTGGTAAACCTGATCC-3’	[42]
VEGF	5’-AGAAAGCCCTGAAGTGGTG -3’ 5’-ACTCCAGGGCTTCATCATTG-3’	[43]

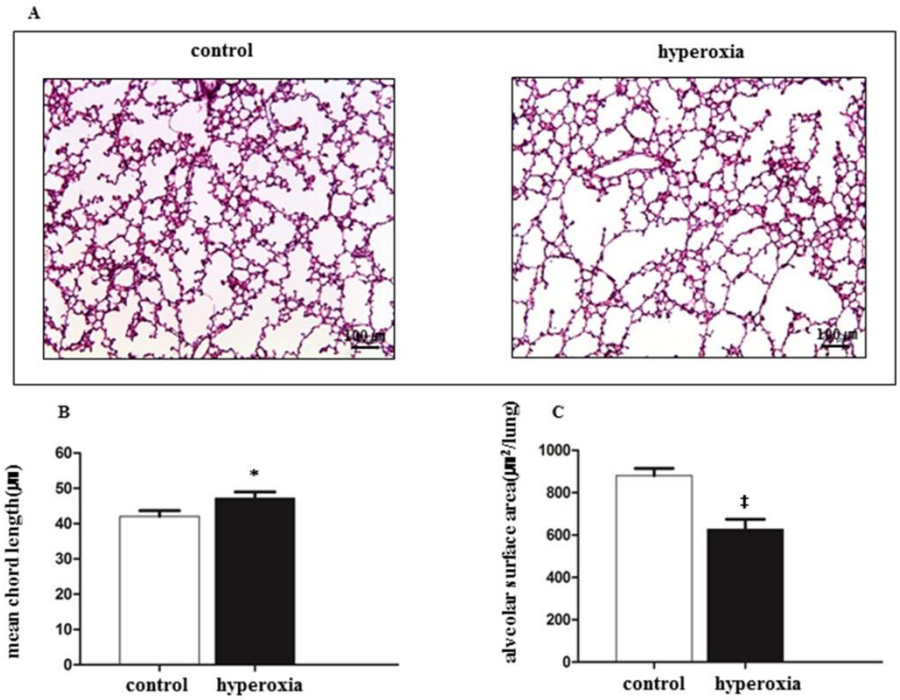

Figure 3 Effect of hyperoxia on the lung alveolarization. The unexposed control group showed normal alveolarization, whereas the hyperoxia group displayed impaired alveolarization with fewer, larger and simper alveoli (**A**). The hyperoxia group had a significantly longer L_m (**B**) (* $p < 0.05$) and significantly smaller S_A compared to the control group (**C**) (‡ $p < 0.001$). The data were expressed as the mean ± SEM and evaluated by Student's t-test. Original magnification: 100 x

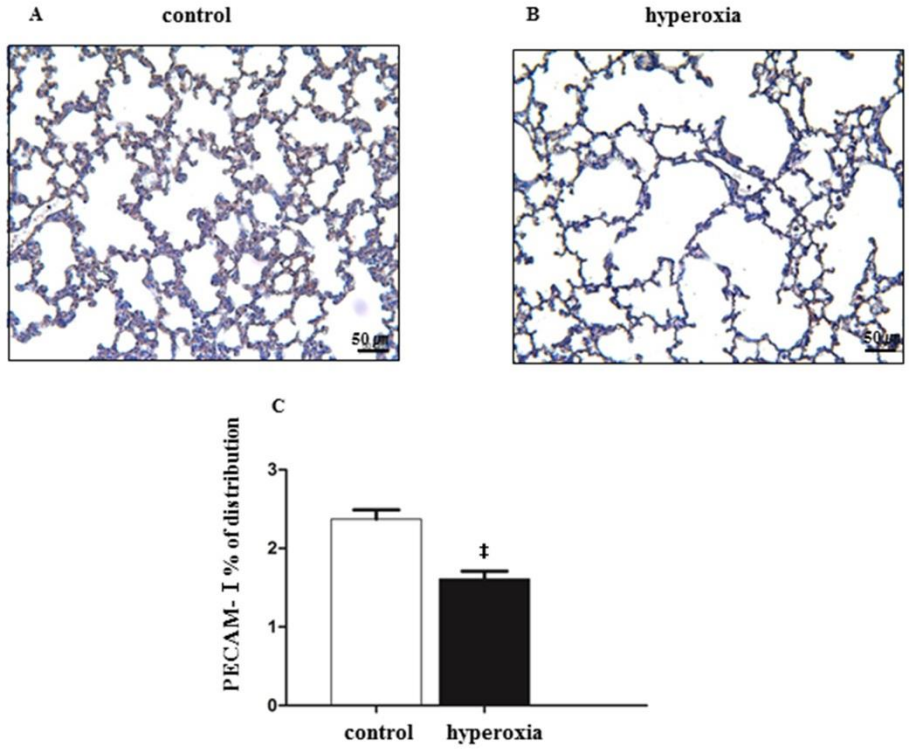

Figure 4 Effect of hyperoxia on the lung angiogenesis. Immunohistochemical staining for PECAM-Ⅰ was significantly decreased in the hyperoxia group (**B**) compared to the control group (**A**) (‡ $p < 0.01$). The data were expressed as the mean ± SEM and evaluated by Student's t-test. Original magnification: 200 x

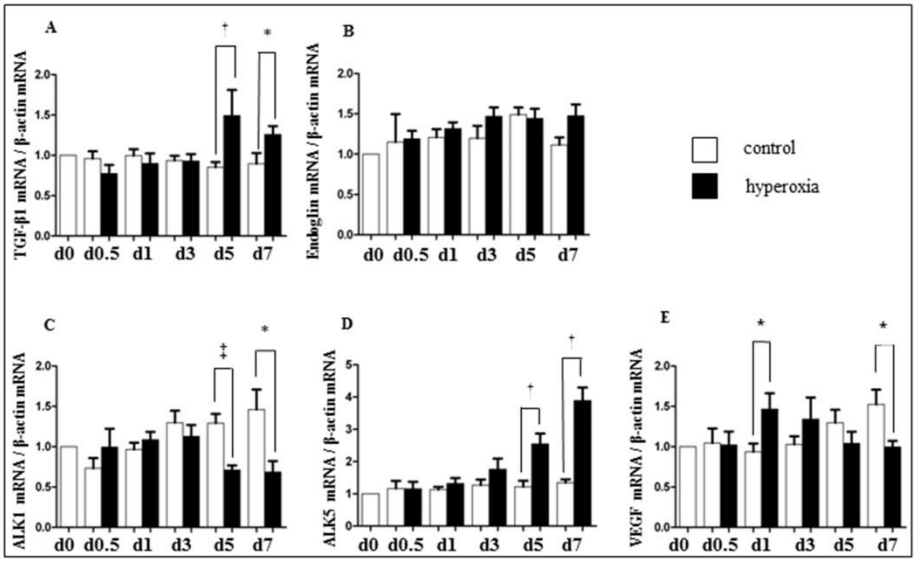

Figure 5 Expression of TGF-β1, ALK1, ALK5, VEGF and endoglin mRNA in the hyperoxic group. Compared with the control group, the mRNA level of TGF-β1 was significantly increased in the D5 and D7 hyperoxia group (**A**) († $p < 0.01$ and * $p < 0.05$, respectively). The mRNA level of ALK1 was significantly decreased in the D5 and D7 hyperoxia group (**C**) (‡ $p < 0.001$ and * $p < 0.05$, respectively), whereas the level of ALK5 was significantly increased compared to the corresponding control groups (**D**) († $p < 0.01$). VEGF mRNA had higher expression at D1 and significantly lower expression at D7 in the hyperoxia group compared to the control group (**E**) (* $p < 0.05$). Endoglin had no statistically significant difference between the control and hyperoxia group throughout the entire experimental period (**B**). The data were expressed as the mean ± SEM (n=15). Groups of data were compared by the corresponding time point by Mann-Whitney U test.

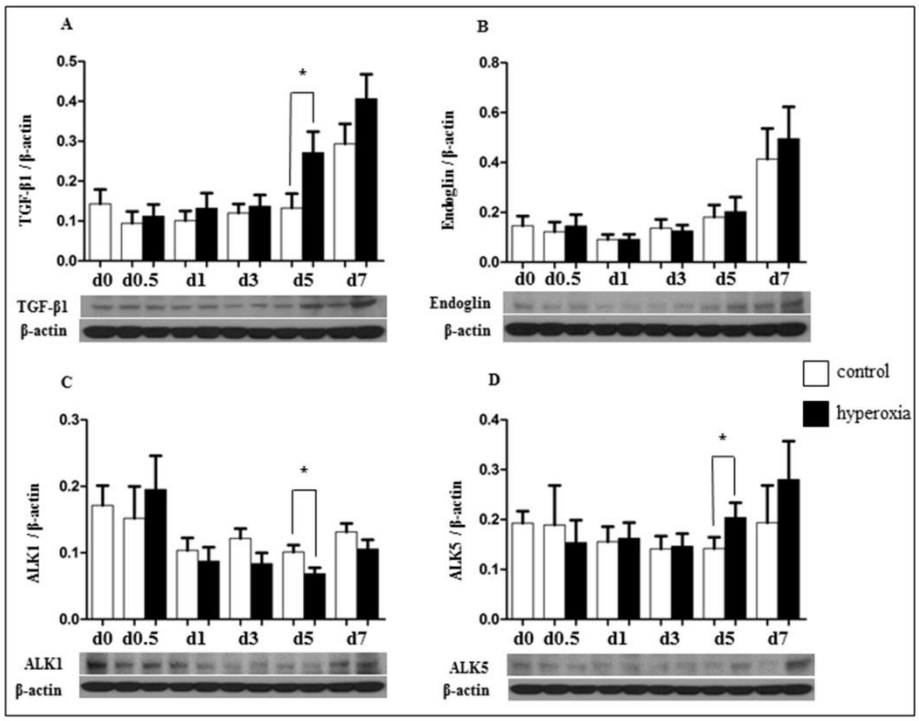

Figure 6 Effects of chronic hyperoxia on TGF-β and TGF-β receptor proteins. The protein level of TGF-β1 was significantly increased in the D5 hyperoxia group relative to the control group (**A**) (* $p < 0.05$). The expression of ALK1 was significantly decreased (**C**) (* $p < 0.05$), whereas the expression of ALK5 was significantly increased in the hyperoxia group compared to the control group at D5 (**D**) (* $p < 0.05$). There was no statistically significant difference in the level of endoglin throughout the entire experimental period (**B**). The data were expressed as the mean ± SEM (n=15). Groups of data were compared by the corresponding time point by Mann-Whitney U test.

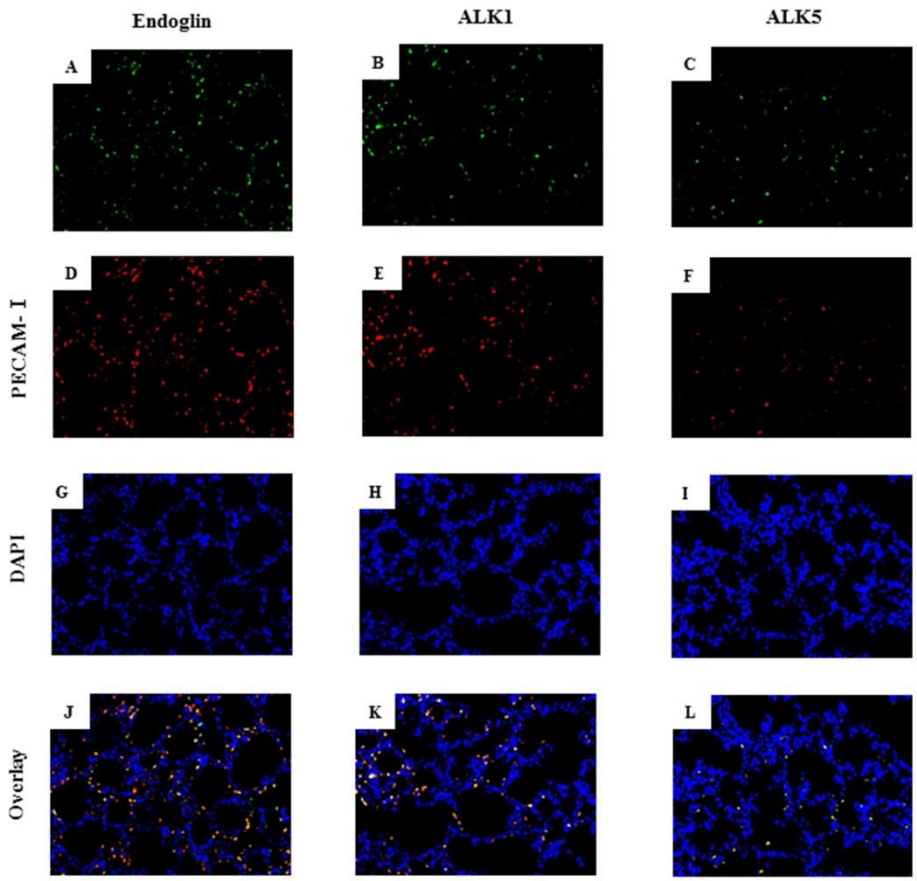

Figure 7 Double-fluorescence immunohistochemical staining. Normal rat lung was double-stained for endoglin, ALK1, ALK5 (A, B, C; green) and PECAM-Ⅰ (D, E, F; red) and then counterstained for DAPI (G, H, I; blue). From a merged image, (J, K, L; orange/yellow), endoglin, ALK1 and ALK5 staining overlapped in the PECAM-Ⅰ-positive endothelial cells, indicating that endoglin, ALK1 and ALK5 were mainly expressed in the endothelium. Original magnification: 200 x.

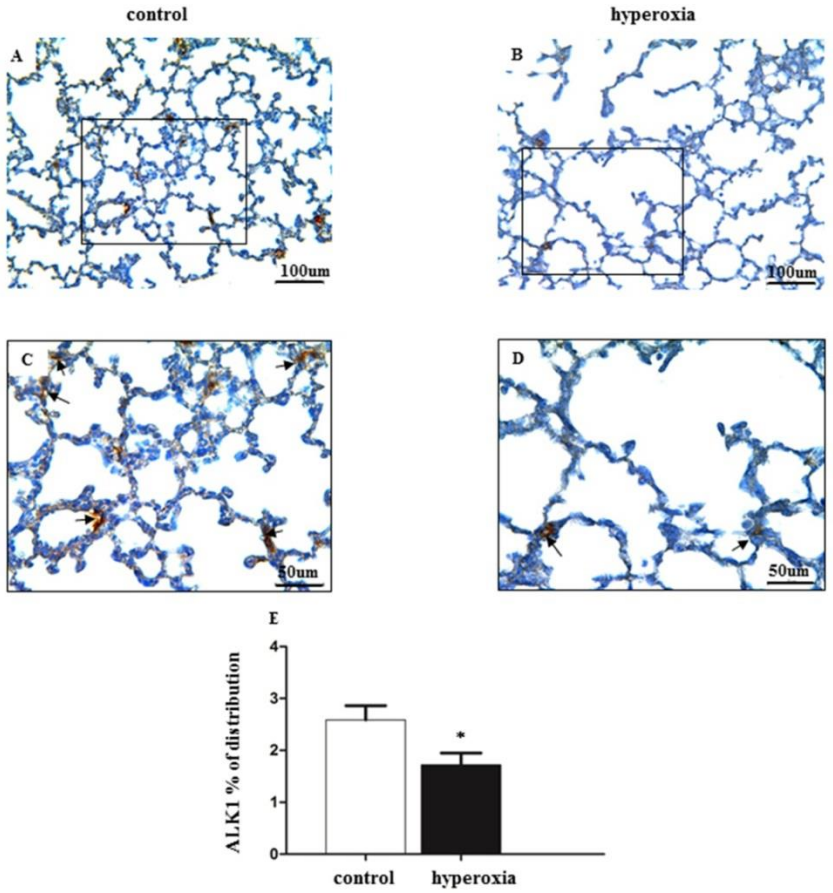

Figure 8 Immunohistochemical analysis of ALK1 in hyperoxic lung tissue. We compared the difference in expression between the two groups by measuring the density of positive staining. There were abundant ALK1 positive-staining cells in the alveolar septum in the control group (**A, C**), while that were significantly decreased in the hyperoxia group (**B, D**). And there was a statistically significant difference between two groups (**E**) (* $P < 0.05$). The data were expressed as the mean ± SEM and evaluated by Student's t-test. Original magnification: (A, B) 200 x; (C, D) 400 x. Arrowheads indicate positive staining.

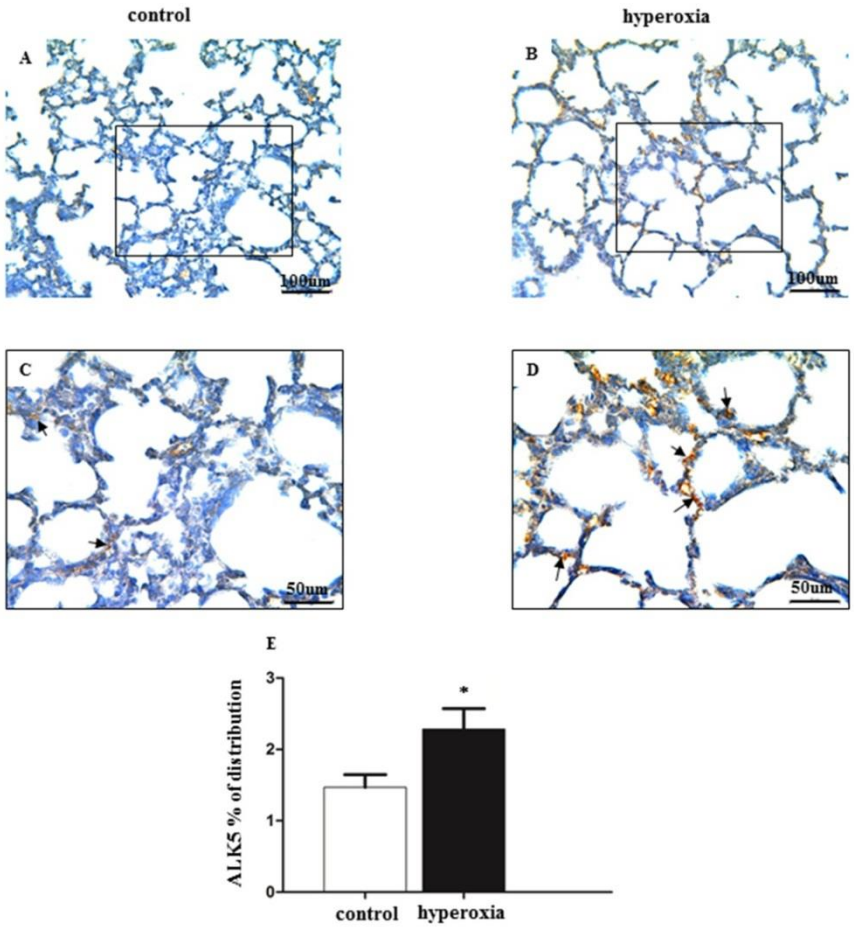

Figure 9 Immunohistochemical analysis of ALK5 in hyperoxic lung tissue. We compared the difference in expression between the two groups by measuring the density of positive staining. ALK5 was increased in the hyperoxia group (**B, D**) compared with the control group (**A, C**) and there was a statistically significant difference between the two groups (**E**) (* $P < 0.05$). The data were expressed as the mean ± SEM and evaluated by Student's t-test. Original magnification: (A, B) 200 x; (C, D) 400 x. Arrowheads indicate positive staining.

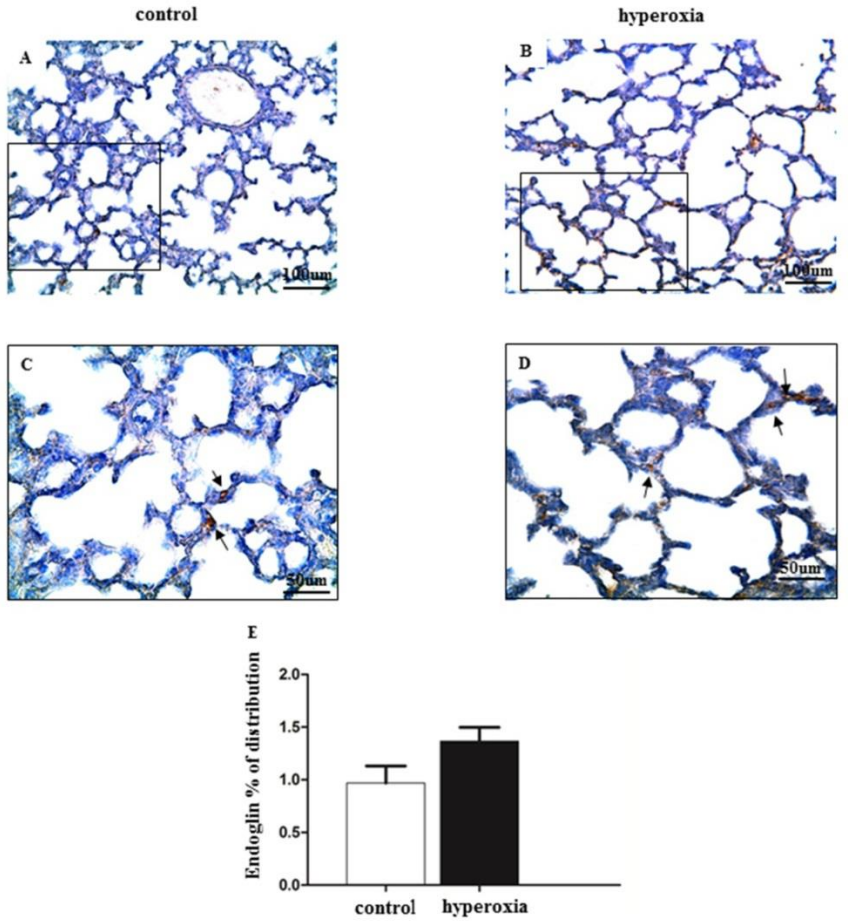

Figure 10 Immunohistochemical analysis of endoglin in hyperoxic lung tissue. We compared the difference in expression between the two groups by measuring the density of positive staining. Endoglin was slightly increased in the hyperoxia group (**B, D**) compared to the control group (**A, C**). However, there was no statistically significant difference (**E**) ($P > 0.05$). The data were expressed as the mean ± SEM and evaluated by Student's t-test. Original magnification: (A, B) 200 x; (C, D) 400 x. Arrowheads indicate positive staining.

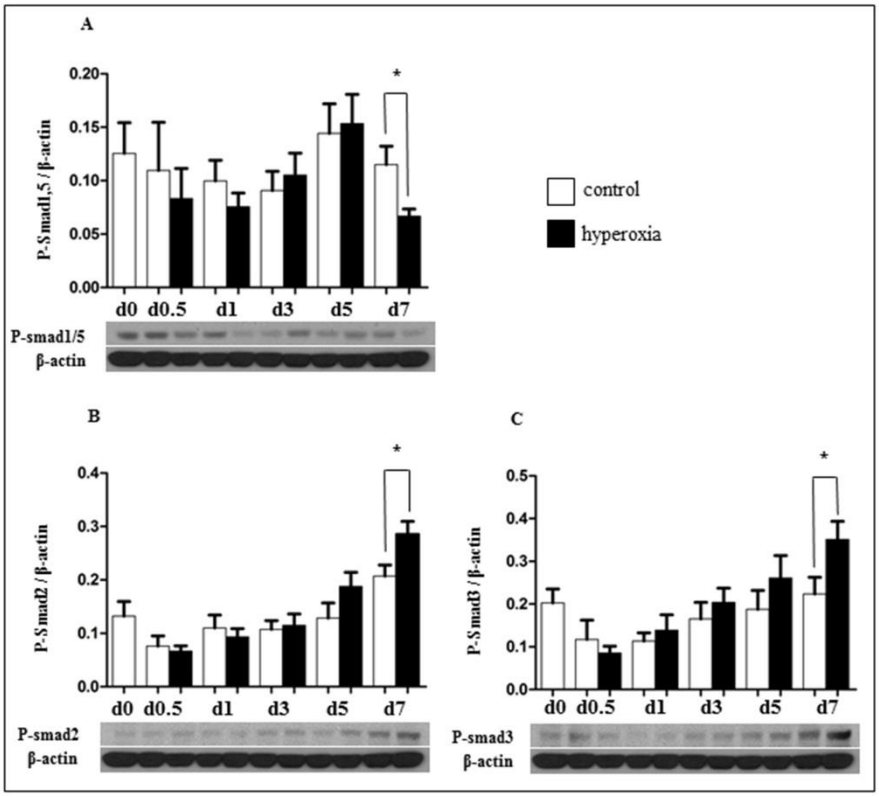

Figure 11 Chronic hyperoxia-induced alteration of Smad proteins. The protein level of phospho-Smad1/5 was significantly decreased (**A**) (* $p < 0.05$), whereas the levels of phospho-Smad2 and phospho-Smad3 were significantly increased in the hyperoxia group compared to the control group at D7 (**B, C**) (* $p < 0.05$). The data were expressed as the mean ± SEM (n=15). Groups of data were compared by the corresponding time point by Mann-Whitney U test.

DISCUSSION

In recent years, BPD is characterized more by simplified alveoli and abnormal vascular growth and less by parenchymal fibrosis [13]. Although the lung is somewhat functional at the time of birth, its structure still in an immature condition [14]. During human lung development, the number of alveoli continues to increase after birth (approximately 9-fold increase) [15]. During the lung development of rodents, septation and angiogenesis occur after term birth, notably between postnatal day 3 and 14 [16, 17]. In the present study, we demonstrated that alveolarization and vascularization were significantly impaired in the hyperoxia-induced BPD model by evaluating the L_m and S_A of lung morphometry, as well as quantitative imaging of the endothelial cell marker PECAM-I.

Hyperoxic lung injury is considered to disrupt critical signaling pathways that regulate lung development, leading to developmental arrest [13]. The TGF-β signaling pathway plays an important role during lung development. Previous studies have revealed the pivotal role of TGF-β signaling pathway on alveolar development, in which the structural changes of the hyperoxia-induced BPD model were accompanied by an increase in the level of TGF-β pathway transducers ALK5 and phospho-Smad3 [13, 18]. Compared to prior studies which mainly focused on alveolization with lung epithelial cells and

fibroblasts [13, 18], our study showed that TGF-β-ALK-Smad signaling molecules were expressed mainly in pulmonary endothelial cells, suggesting their possible role on pulmonary angiogenesis. Because it has been reported that the balance between ALK1-Smad1/5 signaling and ALK5-Smad2/3 signaling plays an important role in the normal angiogenesis process [19], we investigated whether exposure of newborn rats to hyperoxia during the critical period of lung development causes a change in the balance of these two pathways. Consistent with the other studies using human [20] and animal models [21] with BPD, we observed that hyperoxic exposure upregulated the levels of TGF-β1 mRNA and protein at D5. The time-point of hyperoxia-induced changes in TGF-β expression was slightly delayed in our study, most likely due to the different oxygen concentration used for hyperoxia. In another study, rat pups were consecutively exposed to 95% O2 for 7 days, which caused ~40% mortality [22]. The hyperoxic condition in this study was 85% O2 for 7 days, resulting in no mortality (data not shown). Regarding the TGF-β receptors, the expression of ALK1 was downregulated and the expression of ALK5 was upregulated in the D5 hyperoxia group. Additionally, the level of phospho-Smad2/3 was elevated in the D7 hyperoxia group, whereas the level of phospho-Smad1/5 declined. These results indicated that hyperoxia induced the TGF-β-ALK5-Smad2/3 signaling pathway and inhibited TGF-β-ALK1-Smad1/5 signaling, suggesting that the balance between TGF-β-ALK1-Smad1/5 signaling and TGF-β-ALK5-Smad2/3 signaling is disrupted in the

hyperoxia model of BPD.

Vascular endothelial growth factor (VEGF) is probably the most potent and critical regulator of endothelial cells among the factors are associated with angiogenesis [23]. Furthermore, Bhatt et al. showed that the expression of VEGF is decreased in infants with BPD [24]. Of interest, the overexpression of VEGF during normal lung development disrupts the lung architecture [25, 26], exerting the important function of VEGF as a strict regulator for the lung angiogenesis. It is generally accepted that the expression of VEGF appears to be regulated by several factors. For example, TGF-β is involved in the angiogenesis by regulating the activities and the expression of angiogenic factors. For instance, both VEGF and PDGF are transcriptionally regulated by TGF-β–mediated Smad activation [27, 28]. It has been also reported that TGF-β induces endothelial cell proliferation through an ALK1-Smad1/5-VEGF pathway [10]. Other study has shown that overexpression or stimulation of ALK1 results in Smad activation and upregulation of VEGF expression in endothelial cells [29], whereas an ALK5 kinase inhibitor strongly promoted VEGF-induced angiogenesis [30]. In the present study, there was a decrease in VEGF levels, following the initial increase. We speculate that the subsequent decrease in VEGF mRNA, at least in part, may be derived from the hyperoxia-induced disrupted balance between the TGFβ-ALK1-Smad1/5 and the TGFβ-ALK5-Smad2/3 signaling (Figure 5, 6, 11). We found an increased expression of VEGF mRNA with a subsequent

decrease in the D5 and D7 hyperoxic groups. We hypothesized that the initial hyperoxia-induced VEGF increase may potentially contribute to lung injury or pulmonary edema and lead to cellular damage, which in turn results in VEGF reduction; similar changing trend of VEGF expression was found in another study [31]. Conceptually, hyperoxia-induced VEGF increase may have both positive and negative effects on the neonatal lung that is exposed to hyperoxic conditions, i.e., promoting cellular survival and propagating widespread capillary leak into the alveolar space [32].

Endoglin is a homodimeric transmemberane glycoprotein mainly expressed in proliferating vascular endothelial cells and is an auxiliary TGF-β receptor, also as known TGF-β type Ⅲ receptor [33]. Previous studies have shown that endoglin is not directly involved in the TGF-β signaling pathway but modulates the balance between the TGF-β-ALK1 and TGF-β-ALK5 signaling pathways [34]. Previous work has also shown that endoglin-null mice die during utero development (E10-10.5) because of defective angiogenesis [35]. In addition, an increased expression of endoglin results in the stimulation of ALK1-Smad1/5 signaling that has the proangiogenesis effect [36]. Meanwhile, endoglin indirectly attenuates the ALK5-Smad2/3 signaling pathway that has the antiangiogenesis effect [36]. However, we did not find any significant change in the expression of endoglin in the hyperoxic group, which was not consistent with ALK1 regulation as described in previous studies. Recently, the presence of two endoglin isoforms, with a

different binding capacity to TGF-β1, L- and S-type, has been reported in human and mouse tissues [37]. L-endoglin is the predominant form and contains 47 amino acids in the cytoplasmic domain, while S-endoglin contains only 14 amino acids and is significantly expressed in several mouse tissues, such as liver and lung, as well as in endothelial cell lines [37]. S-endoglin expression upregulates the ALK5 signaling pathway, whereas L-endoglin attenuates this pathway [38]. In contrast, L-endoglin, but not S-endoglin, stimulates the ALK1 signaling pathway [38]. Therefore, it is reasonable to predict that the ratio of S-endoglin to L-endoglin in endothelial cells may contribute to balancing the TGF-β signal through ALK5 or ALK1, functioning as an important factor for vascular pathophysiology [39]. Although the presence of S-endoglin in rats is not clearly demonstrated, we could not exclude the possibility of the existence of another endoglin isoform in rat. We cautiously speculate that the role of endoglin in regulating lung angiogenesis is possibly not only by the quantitative change in expression but also by the variation of endoglin forms and their different functions.

We conclude that rat pups exposed to hyperoxia continuously for 7 days, a critical period of postnatal lung development, resulting in alteration of the balance between the TGF-β-ALK1-Smad1/5 and TGF-β-ALK5-Smad2/3 signaling in pulmonary endothelial cells, which may ultimately lead to the development of BPD.

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국문 초록

연구배경: 만성 폐질환으로 알려져 있는 기관지 폐이형성증은 기계 환기나 산소 공급을 필요로 하는 미숙아에서 진행하는 경우가 많다. 기관지 폐이형성증은 폐포와 폐 혈관의 발달 장애를 초래함으로 생기게 되고 TGF-β 신호전달체계는 폐 혈관 발달 과정에서 중요한 역할을 하고 있다고 알려져 있다.

연구방법: 신생 쥐들을 대조군과 연구군으로 나누어 각각 21% 와 85% 농도의 산소에 7 일간 노출 시킨 후 실내에서 14 일간 머물게 한다. 생화학적 분석과 조직학 분석을 통하여 대조군과 연구군 사이에서 TGF-β1, ALK1, ALK5, phospho-Smad1/5, phospho-Smad2/3, VEGF, endoglin 의 차이를 비교하였다.

연구결과: 연구군에서 TGF-β1 과 ALK5 의 mRNA 와 단백질 발현은 5 일째 유의하게 증가하였고 반면 ALK1 의 mRNA 와 단백질 발현은 5 일째 유의하게 감소하였다. 연구군에서 phospho-Smad1/5 의 단백질 발현은 7 일째 유의하게 감소한 반면 phospho-Smad2/3 은 7 일째 유의하게 증가하였다. VEGF 는 연구군에서 1 일째 증가하였고 7 일째 유의하게 감소하였으며 endoglin 의 발현은 두군 사이에서 유의한 차이가 없었다.

결론: 고농도 산소는 폐의 내피 세포에서의 TGF-β-ALK1-Smad1/5와 TGF-β-ALK5-Smad2/3 신호전달체계 사이의 평형을 파괴시킴으로써 궁극적으로 기관지 폐이형성증으로 진행하게 된다.

주요어: 혈관 형성; 내피 세포; 혈관내피세포성장인자; 엔도글린

학 번: 2011-30825