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SB-431542, a specific inhibitor of the TGF-β type I receptor inhibits hypoxia-induced proliferation of pulmonary artery adventitial fibroblasts

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The vascular remodeling process plays an important role in the pathology of hypoxia-induced pulmonary hypertension, and it includes cell proliferation, cell motility, cell synthesis and collagen coagulation. Due to their proliferation and synthesis ability, the adventitial fibroblasts are thought to be critical in the vascular remodeling process initiated in response to hypoxia. However, the factors driving hypoxia-induced fibroblast proliferation and synthesis have yet to be elucidated, and the treatment regimens to treat hypoxia remain ineffective. As for this study, its purpose was to examine the effects exerted by SB-431542, a small-molecule antagonist of transforming growth factor-β-receptor, on the proliferation, synthesis and collagen coagulation in cultured adventitial fibroblasts. Another aim of this study was to assess the inhibitory ability of SB-431542 on pulmonary vascular remodeling in chronic hypoxia *in vivo*.The cell morphology and proliferation of cultured adventitial fibroblasts was assessed by laser confocal microscopy and the MTT assay, respectively. Additionally, collagen synthesis was determined by hydroxyproline chromatography, while the expression of cytokines in adventitial fibroblasts and lung tissues was evaluated by immunohistochemical and reverse transcription PCR analyses. The results indicated that the exposure of cultured fibroblasts to 1% oxygen led to the up regulation of cell proliferation, cell synthesis. In addition, increased expression of cytokines and collagen was detected *in vivo* in the pulmonary artery adventitia of rats exposed to chronic hypoxia. Conversely, SB-431542 inhibited fibroblast proliferation and synthesis in the process of hypoxia-induced pulmonary hypertension ($P < 0.01$). Thus, the results suggested that by reducing cell proliferation, cell synthesis of vascular adventitia, small molecule inhibitors of the TGF-β1 receptors may offer a novel therapy for pulmonary hypertension.

1. Introduction

In most chronic obstructive pulmonary diseases (COPDs), chronic exposure to hypoxia leads to pulmonary hypertension, and vascular remodeling plays an important role in the pathology. Additionally, extensive fibro-proliferative changes have been noted within the adventitia of pulmonary arteries after chronic exposure to hypoxia (Stenmark et al. 1997). Adventitial fibroblasts are able to rapidly respond to low oxygen tension and modulate its own function, so as to adapt to local vascular needs (Stenmark et al. 2000). Moreover, early and sustained proliferation of adventitial fibroblasts in response to hypoxic conditions has been demonstrated in previous studies (Belknap et al. 1997; Das et al. 2002). Such response of fibroblasts has been found to exceed that observed in smooth muscle or the endothelium under identical partial pressure of oxygen (Das et al. 1995). Despite this finding, the factors driving the hypoxia-induced proliferation remain to be fully characterized, and the treatment regimens available to treat this disease are still ineffective. In addition, it has been found that adventitial fibroblasts are activated during the early stages of vascular injury or stress (Xu et al. 2007a, b), and secrete a largenumber of cytokines, enzymes and collagen (Yuan et al. 2014; Laping et al. 2002; Hu and Xu (2011)). Furthermore, cell proliferation, migration and differentiation were revealed to be influenced by a variety of factors, including TGF-β1 and MMP-1 (Ait-Oufella et al. 2011; Guo et al. 2009). Additional factors associated with the progression of the pathology include the expression SM α-actin (α-SMA) in fibroblasts and their differentiation into myofibroblasts. The migration, synthesis and differentiation of fibroblasts may be mediated through their activated NF-κB pathway. Accordingly, by determining the TGF-β1, MMP-I, α-SMA, NF-κB and collagen levels in adventitial fibroblasts and lung tissues, this study aims to evaluate the anti-proliferation and anti-fibrotic effects of SB431542 on hypoxia-induced pulmonary vascular remodeling.

SB-431542, a small molecule inhibitor of the type I TGF-β receptor, blocks intracellular mediators of TGF-β1 signaling, which leads to decreased TGF-β1–mediated proliferation, cytokines and collagen expression (Laping et al. 2002; Matsuyama et al. 2003). In clinical settings, SB-431542 is widely used to treat respiratory asthma, thus the finding in our study that SB-431542 inhibits proliferation and synthesis of adventitial fibro in the process of pulmonary vascular remod-

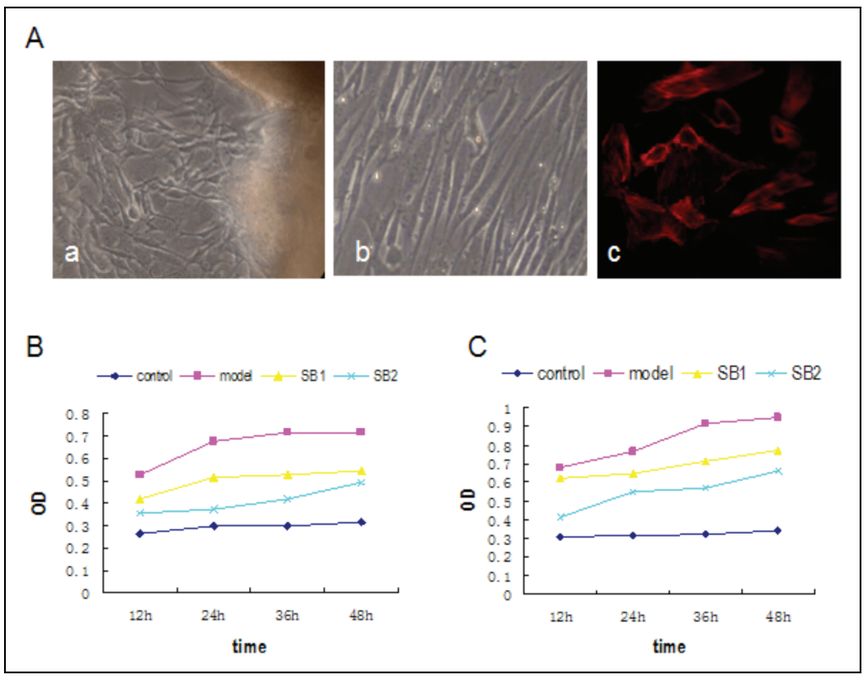

Fig. 1: Characteristics of cultured vascular adventitial fibroblasts. A: Morphological observation and representative photographs of fibroblasts. (a)Fibroblasts grown form tissue pieces of vascular adventitia (×200). (b) The 2-3 generations of fibroblasts showed a robust growth (×200). (c) Fibroblasts stained with Cy3 antibody (red) indicating intracellular vimentin (×400). B: Growth curve of fibroblasts in various groups. Maximal growth inhibition rates of the SB2 group (45.7% of those model groups) were observed following 48 h ($P<0.01$). C: Collagen synthesis curve of fibroblasts in various groups. Collagen expression decreased as a function of increasing concentrations of SB-431542, with maximal effects being obtained in the SB2 group ($P<0.01$).

eling can be readily translated into a novel therapeutic approach for hypoxia-induced pulmonary hypertension.

2. Investigations and results

2.1. Morphology and immunofluorescence of fibroblasts

After about 2 to 3 d of culture, fibroblasts, observed under inverted microscopy, transformed from the edges of adventitial tissue pieces exhibiting adherent growth (Fig. 1A, a). Additionally, primary cultured adventitial fibroblasts showed an irregular polygonal shape, while a robust growth of fibroblasts, which proliferated rapidly with a multilayer overlapping crest shape (Fig. 1A, b). Immunohistochemical analysis with anti vimentin and anti desmin revealed a rate of 95% fibroblasts staining (Fig. 1A, c). However, staining of vascular adventitial fibroblasts with anti -SM α-actin was negative.

2.2. Effect of SB-431542 on adventitial fibroblasts proliferation and collagen synthesis

SB-431542 inhibited the proliferative activity as a function of exposure time and concentration, as shown in Fig. 1B. Specifically, compared with the respective model groups, the growth inhibition rates of fibroblasts treated with SB-431542 for 48 h at concentrations of 50 and 100 μg/ml groups were 36.5 and 45.7% respectively. Regarding collagen, as determined by hydroxyproline content, compared with the controls, the group subjected to hypoxia shows a greater amount of hydroxyproline content, as illustrated in Fig. 1C. However, after SB-431542 administration, hydroxyproline content was significantly reduced ($P<0.01$). Meanwhile, cell proliferation and collagen synthesis with statistically significant differences were observed among each of the four groups of 12, 24, 36 and 48 h as functions of culture duration, and decreased gradually as a function of increasing concentrations of SB-431542.

2.3. SB-431542 down regulates TGF-β1, MMP-1, α-SMA and NF-κB expressions in adventitial fibroblasts

The immunocytochemical analysis revealed that the expression of TGF-β1, MMP-1, α-SMA and NF-κB in fibroblasts was up regulated by hypoxia. In contrast, treatment of fibroblasts with 50 or 100 μg/ml of SB-431542 for 48 h significantly reduced the expression levels of TGF-β1, MMP-1. In addition, α-SMA, a representative specific marker of myofibroblasts differentiation, was also down-regulated by SB-431542 (Fig. 2A). Furthermore, little or very weak intracellular staining of NF-κB was detected by the immunofluorescence assays following the treatment with SB-431542. Thus, the comparison of the expression of TGF-β1, MMP-1, α-SMA and NF-κB in the four different treatment groups ostensibly shows that SB-431542 was effective in inhibiting the increased expression of cell synthesis proteins which would normally be indicated by the expression of these cytokines.

2.4. SB-431542 down regulates MMP-1, TGF-β1, α-SMA and NF-κB mRNA expression in adventitial fibroblast

The PCR fragment analysis was performed manually by agarose gel electrophoresis and each band of the agarose gel was evaluated using a gel imaging system. Additionally, to ensure a reliable comparison the grey values were calculated relative to ß-actin. The fibroblasts in the model groups cultured under hypoxia exhibited up regulated expression of the TGF-β1, MMP-1, α-SMA and NF-κB mRNAs compared with the

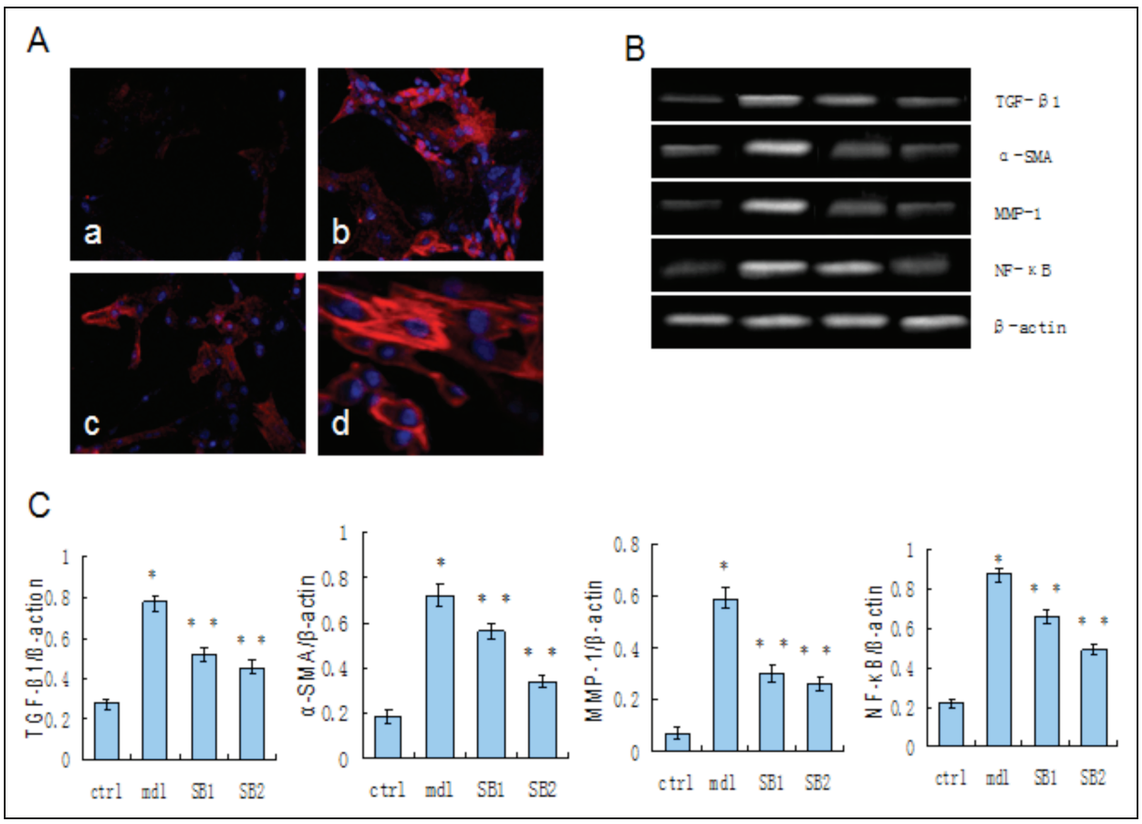

Fig. 2: Effects of SB-431542 on expressions of TGF-β1, TNF-α, α-SMA, and NF-κB of adventitial fibroblasts. A: Expressions of cell synthesis in adventitial fibroblasts by immunocytochemistry staining. (a) fibroblasts stained with TGF-β1 in control group. (b) fibroblasts stained with TGF-β1 in model group. (c) fibroblasts stained with TGF-β1 in SB2 group.(d) fibroblasts stained with α-SMA. B: Expressions of α-SMA, TGF-β1, MMP-1 and NF-κB mRNA in adventitial fibroblasts in the model groups or the control group with or without SB-431542. C: Significant differences among groups were indicated by RT-PCR. Averages of at least three independent experiments (n = 3). Bars indicated the mean ± SD. Statistical analysis was performed using one-way ANOVA followed by multiple comparison test ($^{*}P < 0.01$). $^{*}P < 0.01$ compared to the control (model vs. control, $^{*}P < 0.01$; SB1 group or SB2 group vs model, $^{**}P < 0.01$).

control groups, whereas the SB-431542-treated groups showed significantly down regulated mRNA expression of these genes compared with the model groups. Besides, there were significant differences between the SB1 and SB2 groups ($P < 0.01$) (Fig. 2B, 2C). Together these expression data demonstrated that the SB-431542 was able to inhibit the expressions of MMP-1, TGF-β1, α-SMA and NF-κB at the transcriptional and translational levels.

2.5. Morphological analysis and histochemical staining of vascular adventitia

In order to evaluate the changes in the vascular adventitia morphology, samples from serial sections of the pulmonary tissue were stained with hematoxylin and eosin. In addition, some sections were stained with light green, by Masson staining, so as to identify collagen fibers. Admittedly, collagen fibers were mainly found in the vascular adventitia. A comparative analysis of the pulmonary arteries, pulmonary arteriole and lung tissue from the groups is presented in Fig. 3. The histological analysis revealed that, compared with the control groups and the SB1 or SB 2 groups, the hypoxia-induced model groups displayed more pathological changes in vascular adventitia, and the severity of the changes varied from slight to moderate. In addition, the pulmonary arteries in the hypoxia-induced model groups had greater amounts of collagen fibers than that of the control groups. In comparison, collagen fibers were significantly reduced after treatment with SB-431542 ($P < 0.01$).

2.6. Effect of SB431542 on the expression of TGF-β1, MMP-1, α-SMA and NF-κB mRNA in vascular adventitia tissue

The gene expression of TGF-β1, MMP-1, α-SMA and NF-κB were evaluated in vascular adventitia tissue of pulmonary arteries by quantitative RT-PCR analysis (Fig. 4A, 4B). The vascular adventitia of hypoxia-induced rats in the model groups showed significantly increased ($P < 0.01$) levels of TGF-β1, MMP-1, α-SMA and NF-κB mRNA expression compared with those of rats in the control groups. On the other hand, treatment of rats in the SB groups with SB-431542 significantly decreased the mRNA levels compared with the model groups ($P < 0.01$). This effect appeared to be dose-dependent, since the higher dose of SB-431542 was associated with a more substantial reduction in the mRNA levels. It is believed that the expression of these genes in the vascular adventitia could be affected by the SB-431542 at the transcriptional and translational levels.

3. Discussion

The vascular remodeling process in response to injury or stress is a common complication of many cardiovascular diseases including pulmonary hypertension, systemic hypertension, atherosclerosis and restenosis following interventional surgery (Schulze-Bauer et al. 2002; Kantachuvesiri et al. 2001). As the main pathogenesis of COPDs, the hypoxia-induced vascular remodeling is involved in pulmonary hypertension through multiple mechanisms. Indeed, vascular remodeling can repre-

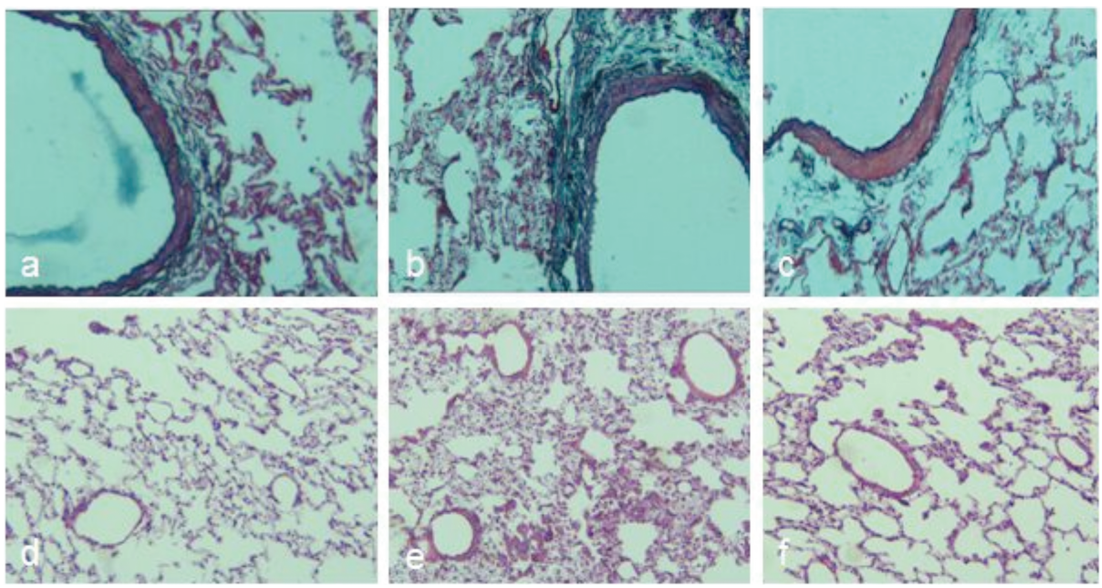

Fig. 3: Expression of collagen in pulmonary artery vascular adventitia and pathologic fibrosis of pulmonary arteriole and lung tissue. Representative examples of Masson's staining(a∼c, × 200) and Hematoxylin-Eosin(d∼f, × 100).(a) Collagen fibers were found mainly to exist in the vascular adventitia. Less Collagen fibers (light green) in pulmonary artery adventitia was evaluated by Masson's staining of air condition rats in control groups with normal saline treatment. (b) Amount of collagen fibers showed in hypoxia-induced model groups with normal saline treatment. (c)Less collagen fibers showed in SB-431542 treatment SB2 groups. Photographs showed the distribution of collagen in the three groups described above. Rats in the hypoxia-induced model groups showed greater amounts of collagen content in the vascular adventitia than that of control rats. However, collagen content was significantly reduced after SB-431542 treatment of SB2 groups ($P < 0.01$). (d) Pulmonary arteriole and lung tissue in control groups with normal photographs. (e) Pulmonary arteriole and lung tissue in hypoxia-induced model groups with obvious fibrosis pathological morphology. (f) Pulmonary arteriole and lung tissue in SB2 groups after treatment with high dose SB-431542 with decreasing pathologic fibrosis (× 100). Photographs showed the significantly distribution of pathologic fibrosis in the three groups described above.

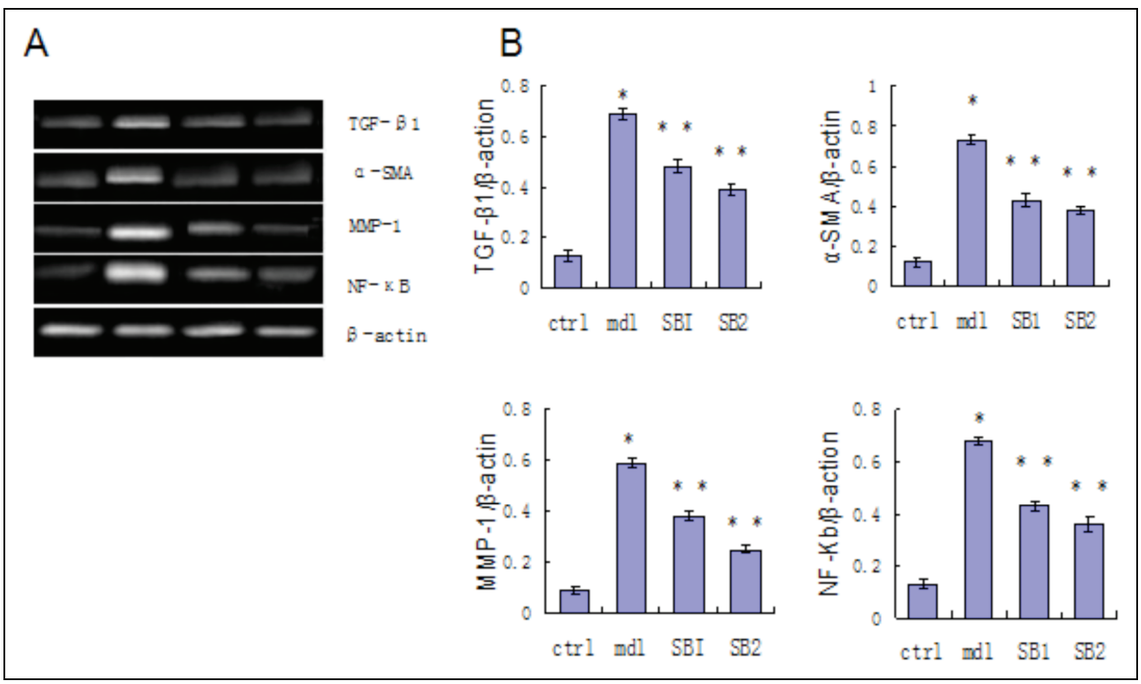

Fig. 4: Expressions of α-SMA, TGF-β1, MMP-1 and NF-κB mRNA of pulmonary vascular adventitia. A: Representative images of significant differences among groups are indicated by RT-PCR. B. Quantitative analysis of TGF-β1, TNF-α, α-SMA, and NF-κB. Averages of at least three independent experiments (n = 3). Bars indicate the mean ± SD. β-actin was used as internal control.

sent a major component of cardiovascular diseases due to its association with aorta and pulmonary hypertension. Each cell type in the vascular wall plays a specific role in response to injury. Several earlier studies have shown that adventitial fibroblasts may serve as a critical cell in the vascular response to injury due to pulmonary hypertension, especially under hypoxic conditions (Das et al. 2000, 2001; Stenmark et al. 1987, 2002). Our previous studies indicated that during the early stages of vascular remodeling, the vascular adventitial fibroblasts were activated (Xu et al. 2007a, b). Importantly though, it has been

demonstrated that adventitial fibroblasts have the capacity to perform multiple functions, such as rapid proliferation, migration, and synthesis of cytokines in response to an activation stimulus. Besides, activated adventitial fibroblast show a number of specific histological, biochemical, and functional characteristics associated with the vascular proliferation responses, including the expressions of TGF-β, MMPs, α-SMA and NF-κB, all of which could contribute to regulate vascular remodeling. Furthermore, as one of the most important cytokines during remodeling (Ryan et al. 2003; Siow et al. 2007), TGF-β1 seems to promote vascular proliferation or migration while at the same time increasing the production of collagen and elastin in the adventitia. According to the study of Liu et al. (2008), adventitial fibroblasts proliferation and synthesis are associated with the Smad signaling pathway. Thus this pathway is useful to inhibit cell proliferation by blocking the TGF-β1 receptor signaling pathway at various levels. The MMP-1 protein participates in the regulation of cell migration by serving as an extracellular matrix protein hydrolysis enzyme. Results from some studies have shown that vascular remodeling is dependent on the role of the MMPs (Amalinei et al. 2007; Raffetto et al. 2008). Additionally, Zhang et al. (2009) reported that TGF-β, *via* the activation of the NF-κB pathway, can induce the expression of MMP-9, which promotes in myofibroblast migration through the degradation of extracellular matrix. In fact, inhibition of the activity of MMPs can significantly inhibit vascular remodeling. Activation of the nuclear transcription factor NF-κB can lead to increased expression of a number cytokines (Hogan et al. 2013). The promoter regions of many genes which encode cytokines contain binding sites for various transcription factors of the NF-κB family. Thus, NF-κB activation can affect a variety of gene expressions and pathways regulations. For example, the study from Brand et al. (1996) demonstrated that vascular lesions accompanying atherosclerosis are associated with the presence of the activated form of NF-κB.

Pulmonary hypertension encompasses important pathological responses, including vascular remodeling and lung tissue fibrosis, and adventitial fibroblasts are activated by altering the expressions of fibrotic cytokines in the injury process. Long et al. (2009) reported that and TGF-β1signaling in models of pulmonary hypertension had the potential for being active in receptor-like kinase-5 inhibition. Besides, the release of adventitial fibroblasts has been shown to induce vascular proliferation at local tissue sites in response to hypoxia-induced pulmonary vascular remodeling (Stenmark et al. 2000). Our studies demonstrated that intact TGF-β1 signaling was required for the expression of NF-κB-dependent genes in vascular fibroblasts. Furthermore, our results suggested that a convergence of these pathways in the nucleus rather than the cytoplasm may be critical for the regulation of vascular remodeling pathways by TGF-β1.

Regarding the development of therapeutic strategies for human diseases, small molecule inhibitors of signaling pathways have been proven to be extremely useful. Blocking the fibrosis-promoting effects of TGF-β1 represents a potentially interesting drug target for therapeutic intervention. As a novel small molecule inhibitor, SB-431542 has recently been used in the treatment of respiratory asthma. Inhibitors of the TGF-β1 receptors are recognized as important in blocking TGF-β1/Smads signal pathways in vascular remodeling. SB-431542 was shown to decrease TGF-β1-mediated transcription by inhibiting the phosphorylation and nuclear translocation of the SMADs and blocking intracellular mediators of TGF-β signaling (Hasegawa et al. 2005; Tanaka et al. 2010). Furthermore, SB-431542 was found to exert no effect on components of the ERK, JNK, or p38 MAP kinase pathways (Inman et al. 2002), or on components of the signaling pathways activated in response to serum.

Our results revealed that hypoxia-induced vascular remodeling can significantly increase the amount of cytokines and collagen in vascular adventitia. In addition, attenuation of the fibrosis-promoting effects of TGF-β1, including TGF-β-induced cell proliferation, cell motility, cell migration and cell synthesis, was observed by treatment with SB-431542. Furthermore, the study demonstrated that SB-431542 has the capacity to inhibit TGF-β-induced gene expression. Therefore, a foundation is provided by this study not only for future work on the mechanism of pulmonary hypertension but also for the identification of the potential of SB-431542 for the treatment of hypoxia-induced pulmonary hypertension.

4. Experimental

4.1. Animals and reagents

Male Sprague-Dawley rats aged 5 weeks and weighing 200-220 g were provided by the Experimental Animal Center of Binzhou Medical University, Shandong, China. All rats were treated according to guidelines approved by the Institutional Animal Care and Use Committee in Binzhou Medical University. SB-431542 was purchased from the HanX Biological Technology Co (Shanghai, China). The hydroxyproline assay kit was purchased from the Jiancheng Co (Nanjing, China). Trizol and the two-step PCR detection kits were purchased from the TaKaRa Co (Dalian, China). Anti-α-SMA antibody, anti-TGF-β1antibody, anti-MMP-1antibody, and anti-NF-κBp65 antibody for immunohistochemical were bought from Beyotime, Shang-hai, China. All other analytical grade reagents for histology and biochemical assays were bought from Sigma Chemical Co (Nanjing, China). The light microscope and camera were purchased from the Nikon Corporation (Japan). All other chemicals and reagents were of analytical grade.

4.2. Adventitial fibroblasts cultivation and experimental groups

The SD rats were euthanized with an overdose of 3% pentobarbital anesthesia and then sterilized by immersion within 75% alcohol. The adventitia of the pulmonary arteries was stripped and isolated from the media and intima, and then was cut into small tissue blocks and incubated in DMEM culture medium (containing 15% fetal bovine serum) in a humidified atmosphere of 5% CO_2 in air at 37 °C. The medium was changed every two days until the cells surrounded tissue blocks by adherent the bottom of the bottle. The cells were harvested and subcultured, and the 2-3 generations of cells were cultured on 25-mm cover slips and used in the experiments. Vascular adventitial fibroblasts were identified by immunocytochemistry staining using the primary antibodies vimentin, desmin and SM-a-actin (1:100). The 2-3 generations of cells seeded at a density of 3×10^4 cells/well in 6-well plates (containing 10% fetal bovine serum). When cells approached 80% confluence, an model of hypoxia-induced vascular injury *in vitro* was simulated in this study as exposure to 1% oxygen to cell medium to active adventitial fibroblasts, then the fibroblasts were aliquoted into the following groups: control groups (cells cultured in 5% CO_2 in air at 37 °C), model groups (cells cultured in 5% CO_2 in 1% O_2 and N_2 balance at 37 °C), SB1 groups (cells cultured in 1% O_2 with culture medium containing 50 ug/ml SB-431542), SB2 groups (cells cultured in 1% O_2 with culture medium containing 100 ug/ml SB-431542). All cells were cultured for 48 h.

4.3. MTT assay for cell proliferation

The fibroblasts were harvested during the logarithmic growth phase and seeded in 96-well plates at a density of 1 × 104 cells/ml, and then were divided into the four treatment groups described above and all cells were cultured for 12 h, 24 h, 36 h, 48 h, respectively. At each of the time points, 20 μl of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, 5 mg/ml) was added to each well and the cells were then incubated for additional 4 h. The culture supernatant was removed and 150 μl of dimethyl sulfoxide was added to each well to fully dissolve the MTT-formazan crystals. Cell growth inhibition was determined and evaluated by measuring

4.4. Hydroxyproline assay for cell collagen synthesis

The fibroblasts were evaluated for their *in vitro* extraction of hydroxyproline from collagen and oxidation to pyrrole by chloramine-T with use of the hydroxyproline assay (Fu et al. 2014), for production of color with para-dimethyl benzaldehyde. The adventitial fibroblast were harvested and seeded in 24-well plates at a density of 1×10^4 cells/mL, and were cultured at 37 °C in a humidified incubator for 24 h (5% CO_2). The cells were then divided into the four treatment groups, as described above. A sample from

Table 1: PCR primers used in this study

Gene Name	Sequence
TGF-β1	forward: 5′-GTCATAGATTGCATTGTTGC-3′ reverse: 5′-AAGGAGACGGAATACAGGG-3′
MMP-1	forward: 5′-GATGGATCCCAAGCCATATATGGACGTTCC-3′ reverse: 5′-TTGGAATTCCGGACTTCATCTCTGTCGG-3′
α-SMA,	forward: 5′-CGAGAAGCTGCTCCAGCTATGTG-3′ reverse: 5′-CTCTCTTGCTCTGCGCTTCGT-3′
NF-κB	forward: 5′-GAAGAAGCGAGACCTGGAG-3′ reverse: 5′-TCCGGAACACAATGGCCAC-3′
ß-actin	forward: 5′-GGAGATTACTGCCCTGGCTCCTA-3′ reverse: 5′-GACTCATCGTACTCCTGCTTGCTG-3′

the supernatant was removed for the hydroxyproline assay. The color signals were measured in a spectrophotometer at 550 nm, and compared to a standard curve.

4.5. Adventitial fibroblast morphological analysis and immunocytochemistry staining

The cells were fixed in 4% paraformaldehyde, and incubated with primary antibodies overnight at 4 °C, and the cell were washed 3X in PBS, and were incubated with the secondary antibody in a 37 °C incubator for 30 min. The primary antibodies TGF-β1 (1:500), α-SMA (1:200), MMP-1 (1:100) and NF-κB (1:100) were used to identify the cells with IgGCy3 (1:200) as the fluorescence labeling goat as the second antibody and labeling with the nuclear dye DAPI (0.02 g/L fluorescent dye 4, 6 - acetyl 2-2 - phenyl indole, Santa Cruz Biotech). Laser confocal microscopy was used for fluorescent analysis. Ten different horizons of cells were randomly selected, and the rates of positive cell conversions were calculated.

4.6. Adventitial fibroblast mRNA extraction and reverse transcription PCR

Reverse transcription PCR (RT-PCR) was used to determine mRNA expression of TGF-β1, MMP-1, α-SMA and NF-κB in the adventitial fibroblasts of the four groups as described above. Total RNA extraction was performed according to the instructions of Trizol reagent kit and subsequently according to the manual of two-step PCR detection kit of the TaKaRa Company. PCR primers were synthesized by the TaKaRa Company (Table 1). A RT-PCR amplification system in a total of 12.5 μl were applied to the following PCR program: 5 min at 94 °C (pre denaturation), 30 s at 94 °C (initial denaturation), 30 s at 60 °C, 50 s at 70 °C, repeated 30 times, and 5 min at 70 °C (amplification). The PCR products were assessed on 1.5% agarose gels. Results of the electrophoresis for quantitative analysis were scanned by a Gel imaging system

4.7. Animal model and morphological analysis and histochemical staining

A total of 24 males, 5-week-old SD rats (200-230 g) were randomly divided into the following groups. Rats lived in air served as control groups, and rats lived in an air condition incubator containing 10% O_2 to simulate chronic hypoxia animal model, and served as model groups. In experiments using chemical inhibitors, SB431542 was given intraperitoneally to chronic hypoxic rats. Rats in SB1 groups treated daily with a single dose of 10 mg/kg, and rats in SB2 treated daily with a single dose of 20 mg/kg. Rats in control groups and model groups treated daily with normal saline. After 28 days treatment with daily intraperitoneal injections of the drugs as described above, rats were sacrificed. Lung tissues sections were fixed with 10% paraformaldehyde solution and embedded in paraffin wax. Sections were cut at 5 μm thickness and were subjected to staining for histological examination. Lung tissues were stained with both hematoxylin-eosin (HE) staining to observe morphology changes, and light green by Masson staining to identify collagen fibers, and the slides were examined by light microscopy and photographed.

4.8. Vascular adventitia mRNA extraction and RT-PCR

Samples from the pulmonary arteries were isolated, and the adventitia was stripped. Total RNA was extracted from adventitial tissue using Trizol reagent according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA using reverse transcriptase. PCR was then performed in a final volume of 25 μl using a PCR reagent kit (Takara Co). The ß-actin gene was used as an internal control. The cycling program involved preliminary denaturation at 95 °C for 10 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final elongation step at 72 °C for 5 min

4.9. Statistic methods

All experiments were conducted 3-6 times and significant differences were calculated by t-test or one-way ANOVA as $*p < 0.01$ compared to control using SPSS software.

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