Amelioration of Bleomycin-induced Pulmonary Fibrosis of Rats by an Aldose Reductase Inhibitor, Epalrestat

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INTRODUCTION

Pulmonary fibrosis is an interstitial lung disease induced by various etiological factors. It has been demonstrated that several factors contribute to the pathogenesis of pulmonary fibrosis including inflammation, epithelial mesenchymal transition, oxidative stress and immune dysfunction, which result in alveolar epithelial cell injury and fibroblast proliferation that consequently leads to abnormal deposition of the extracellular matrix (EMC) and tissue remodelling [1]. However, the mechanism of pulmonary fibrosis is not completely understood, and the effects of drugs on fibrosis are not satisfactory [2]. Therefore, it is crucial to find new therapeutic strategies for pulmonary fibrosis.

Aldose reductase (AR) is known to play a crucial role in the mediation of diabetic and cardiovascular complications. Recently, several studies have demonstrated that allergen-induced airway remodeling and ovalbumin-induced asthma is mediated by AR. Epalrestat is an aldose reductase inhibitor that is currently available for the treatment of diabetic neuropathy. Whether AR is involved in pathogenesis of pulmonary fibrosis and whether epalrestat attenuates pulmonary fibrosis remains unknown. Pulmonary fibrosis was induced by intratracheal instillation of bleomycin (5 mg/kg) in rats. Primary pulmonary fibroblasts were cultured to investigate the proliferation by BrdU incorporation method and flow cytometry. The expression of AR, TGF-β1, α-SMA and collagen I was analyzed by immunohistochemistry, real-time PCR or western blot. In vivo, epalrestat treatment significantly ameliorated the bleomycin-mediated histological fibrosis alterations and blocked collagen deposition concomitantly with reversing bleomycin-induced expression up-regulation of TGF-β1, AR, α-SMA and collagen I (both mRNA and protein). In vitro, epalrestat remarkably attenuated proliferation of pulmonary fibroblasts and expression of α-SMA and collagen I induced by TGF-β1, and this inhibitory effect of epalrestat was accompanied by inhibiting AR expression. Knockdown of AR gene expression reversed TGF-β1-induced proliferation of fibroblasts, up-regulation of α-SMA and collagen I expression. These findings suggest that AR plays an important role in bleomycin-induced pulmonary fibrosis, and epalrestat inhibited the progression of bleomycin-induced pulmonary fibrosis is mediated via inhibiting of AR expression.

Key Words: Aldose reductase, Epalrestat, Pulmonary fibroblasts, Pulmonary fibrosis

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ABBREVIATIONS: EPS, Epalrestat; PF, Pulmonary fibrosis; ECM, extracellular matrix; α-SMA, α-smooth muscle actin; COPD, chronic obstructive pulmonary diseases; SMC, smooth muscle cells; TGF-β1, transforming growth factor-β1; DME, Dulbecco’s modified Eagle’s medium; BLM, bleomycin.
acute lung and kidney injury, tumorigenesis and meta-
stasis, and renal and ovarian abnormalities [9-12]. An ear-
lier study indicated that AR plays an important role in 
TGF-β1-induced proliferation of rat mesangial cells (MCs) 
and deposition of ECM [13-16]. It has also been shown that 
AR mediates early airway inflammatory response in rag-
weed pollen extract and ovalbumin-induced asthma and 
IL-13-induced mucous cell metaplasia [17-19]. Other stud-
ies have demonstrated that allergen-induced airway re-
modeling is mediated by AR and its inhibition blocks the 
progression of remodeling via inhibiting TGF-β1-induced 
Smad-independent pathway [20]. Further, increased ex-
pression of AR was observed in the lungs of chronic ob-
structive pulmonary diseases (COPD) patients [21]. These 
studies indicate that AR may be an important role in respi-
ratory disease. Whether AR is involved in the pathogenesis 
of pulmonary fibrosis and pulmonary fibroblast proliferation 
and differentiation remains unknown.

If AR is involved in pathogenesis of pulmonary fibrosis, 
AR inhibitor may be a promising avenue for the therapeutic 
intervention of pulmonary fibrosis. Nowadays, clinical tri-
als with AR inhibitor such as, Tolrestat, Ponalrestat, Zopol-
restat, Zenarestat, Fidarestat and Ranirestat have yielded 
mixed results, showing either an apparent lack of efficacy 
or adverse effects [22]. Only AR inhibitor, epalrestat, is in 
market in Japan to treat patients with diabetic neuropathy 
[23]. Epalrestat [(5-([1Z, 2E]-2-methyl-3-phenyl propenyl-
diene)-4-oxo-2-thioxo-3-thiazolidine acetic acid; EPS), a 
carboxylic acid derivative, is an inhibitor of aldose reductase, 
a rate-limiting enzyme in the polyol pathway [24]. Under 
hyperglycemic conditions, EPS reduces intracellular sorbi-
tol accumulation, which is implicated in the pathogenesis 
of diabetic complications [25]. Except diabetic neuropathy, 
other study has shown that epalrestat prevents human cor-
onary artery smooth muscle cells migration potentiated by 
high glucose treatment and Ang II-stimulated ECM biosyn-
thesis in MCs [25,26]. It has also demonstrated that long-term 
oral administration of epalrestat could reverse vascular re-
modeling of spontaneously hypertensive rats from the in-
hibition of aldose reductase expression [27,28]. However, 
whether epalrestat attenuates pulmonary fibrosis is still 
unclear.

By using models of bleomycin-induced pulmonary fibrosis 
of rats and proliferation of primary rat pulmonary fibro-
blasts, we performed the present study with two related 
hypotheses. First, we designed to investigate the role of AR 
in bleomycin-induced pulmonary fibrosis in vivo and in vitro. 
Second, we tried to explore protective effects of epal-
restat on pulmonary fibrosis induced by bleomycin, and 
whether these protective effects are achieved via inhibiting 
of AR expression.

**METHODS**

**Chemicals and reagents**

Epalrestat (EPS) was purchased from Sigma Chemical Co. (SML0527, St. Louis, MO, USA). Transforming growth 
factor-β1 (TGF-β1) was purchased from PeproTech (New 
Jersey, USA). Masson’s trichrome stain kit was purchased from the Nanjing KeyGEN Biotech (Nanjing, China). The 
BrdU cell proliferation assay kit was provided by Roche 
(Mannheim, Germany). Dulbecco’s modified Eagle’s me-
dium (DMEM) was provided by Gibco (New York, N.Y., 
USA). The primers were purchased from Shanghai Sangon 
Biological Engineering Co. Ltd. (Shanghai, China). The 
PrimeScript reverse transcription reagent Kit and SYBR® 
Premix Ex Taq™ were obtained from TakaraBiotechnology 
Co., Ltd. (Dalian, China). Primary antibodies against α-SMA, 
collagen I and collagen III were purchased from Abcam 
(Hong Kong, China) and against AR and GAPDH were ob-
tained from Santa Cruz (CA, USA).

**Animals**

Male Sprague-Dawley (SD) rats (aged 6-8 weeks, weighing 
180-220 g) were obtained from the Nanjing Qinglongshan 
Experimental Animal Company (certificate No: SCXK (jun) 
2007-012; Nanjing, China). All experiments were conducted in 
accordance with the US National Institutes of Health 
Guide for the Care and Use of Laboratory Animals, and 
the experimental protocol was approved by the Medicine 
Animal Welfare Committee of Wannan Medical College, 
China.

**Animal experiments**

Forty eight rats were randomly divided into four groups, 
with twelve rats per group, as follows: 1) the control group, 
SD rats were anesthetized intraperitoneally with sodium 
pentobarbital (P3761, 30 mg/kg; Sigma) followed by intra-
tracheal instillation of 0.9% saline; 2) the bleomycin (BLM) 
group, rats were anesthetized intraperitoneally with so-
dium pentobarbital followed by intratracheal instillation of 
5 mg/kg bleomycin (Nippon Kayaku, Tokyo, Japan) in 1 mL 
of saline; and 3) the BLM treated with EPS (50,100 mg/kg) 
group. Bleomycin was chosen as 5 mg/kg according to the 
previous study [29] and epalrestat was chosen as 500 and 
100 mg/kg based on our pilot study. Epalrestat was dis-
solved in double-distilled water and administered via oral 
gavage daily from day 1 to day 28 after BLM or saline treat-
ment (day 0) and all rats were sacrificed with exsanguination 
on day 29. Pulmonary fibrosis was assessed by lung histol-
ogy as described in the following section [30].

**Lung tissue histology, Masson’s trichrome staining and Immunohistochemistry**

For light microscopic investigation, right lungs were 
fixed by inflation with freshly prepared 4% paraformal-
dehyde in PBS (pH 7.4) for 24 h and embedded in 
paraffin. Tissue sections (5 μm) from the apex to bottom 
longitudinal of the right lung were stained with hematox-
ylin and eosin (H&E) and Masson’s trichrome stain to en-
able histological evaluation of lung fibrosis. Masson’s tri-
chrome stain was used to demonstration collagen deposi-
tion, and collagen fiber is stained blue, nuclei are stained 
dark red/purple, and cytoplasm is stained red/pink. The 
procedure is according to the manufacturer’s instructions 
(KeyGEN Biotech, Nanjing, China). For Lung AR immuno-
histochemistry staining, serial sections of formalin-fixed 
paraffin-embedded lung tissues were digested with 3% 
H2O2 for 20 min at room temperature, and then preincubated 
with 10% non-immunized serum. Sections were incubated with mouse anti-AR antibody (1 : 100) overnight at 4°C. 
After unbound antibodies were washed off, the sections 
were incubated with biotinylated goat anti-rabbit secon-
dary antibody (1 : 500, Santa Cruz, California, USA), and 
thereafter incubated with streptavidin-HRP. Subsequently,
sections were visualized by a color reaction with diaminobenzidine as the substrate. Brown and yellow colors indicated positive results (mainly cytoplasm). All the histological assays were performed blind to the interventions.

**Cell experiments**

Primary rat pulmonary fibroblasts were prepared from the lung tissue of male 10-week-old healthy SD rats using the trypsin digestion method as described previously [31]. The cells were cultured at 37°C under 5% CO2 in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum, 100 U/mL penicillin and 100 g/mL streptomycin. Fibroblasts were identified by immunofluorescence staining with the antibody of Vimentin (ab8978, 1 : 50; Abcam, Hong Kong, China). The cells between passages 3 and 6 were used for the experiments. Two series of experiments were designed. The first series of experiments were to explore the effect of EPS on proliferation of fibroblasts via inhibiting of AR expression. The cells were divided into 6 groups as follows: i) control, cells were incubated with double distilled water (TGF-β1 solvent) for 24 h; ii) TGF-β1, cells were incubated with TGF-β1 (5 ng/mL) for 24 h; iii-iv) + EPS (1,10,100 μM): cells were pre-treated with EPS (1,10,100 μM) for 1 h, and then subjected to TGF-β1 (5 ng/mL) for 24 h; and v) EPS (100 μM) alone for 1 h, and then incubated with double distilled water for 24 h. The second series of experiments were to evaluate the role of AR in TGF-β1-induced proliferation of rat fibroblasts. The cells were divided into 4 groups as follows: i) control, cells were incubated with double distilled water (TGF-β1 solvent) for 24 h; ii) TGF-β1, cells were incubated with TGF-β1 (5 ng/mL) for 24 h; iii) + scrambled: cells were pre-treated with AR siRNA negative control for 24 h before treated with TGF-β1 (5 ng/mL) for 24 h; iv) AR siRNA: cells were pre-treated with AR siRNA for 24 h before treated with TGF-β1 (5 ng/mL) for 24 h. Cell proliferation assays were performed. The expressions of AR, α-SMA and collagen I were analyzed. The 24 h duration of TGF-β1 was based on our pilot study.

**Small interfering RNA Transfection**

Small interfering RNA (siRNA) against AR gene (Gene-Pharma CO, Shanghai, China) was generated against the following rat AR sequences: sense, 5'-GUGCCCAAACACA-AGGAAUATT-3'; and antisense, 5'-UAUACCUGUGUGU-UGGACATT-3'. Fibroblasts grown to 70% to 80% confluence were transfected with Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). The transfection efficiency was evaluated by AR mRNA and protein expression using real-time PCR and Western blot analysis, respectively. Twenty-four hours after transfection, the cells were used for the experiments as mentioned above.

**Cell Proliferation Assays**

Cell proliferation was measured by 2 methods (the DNA synthesis and cell cycle) were analysed by BrdU marking and flow cytometry, respectively) as we described previously [29].

**Real-time PCR analysis**

Total RNA was extracted by using TRIzol reagent (Invi-}

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thickening of the interalveolar septa and dense interstitial infiltration by inflammatory cells and fibroblasts (Fig. 1A). Myofibroblasts are generally considered to be key effector cell in the development of pulmonary fibrosis, the hallmark of which is the expression of \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA). In line with previous studies [5], bleomycin dramatically increased the expression of \(\alpha\)-SMA (both mRNA and protein) in lung tissues of rats (Fig. 1B and C). All these effects of bleomycin were significantly alleviated by treatment of rats with epalrestat (\(p<0.05\)).

**Effect of epalrestat on lung collagen accumulation in bleomycin-induced pulmonary fibrosis rats**

Masson’s trichrome staining of lung specimens demonstrated that bleomycin instillation induced severe dis-

![Fig. 1. Lung histology and \(\alpha\)-SMA expression in bleomycin-induced pulmonary fibrosis of rats. (A) Hematoxylin-eosin staining of lung tissue. (B and C) The expression of \(\alpha\)-SMA mRNA and protein were determined by real-time PCR and Western blot. Data are means±S.E.M. n=8. **\(p<0.01\) vs. Control; #\(p<0.05\), ##\(p<0.01\) vs. Bleomycin. EPS, Epalrestat; \(\alpha\)-SMA, \(\alpha\)-smooth muscle actin.](image1)

![Fig. 2. Collagen expression of lung tissue in bleomycin-induced pulmonary fibrosis rats. (A) Masson’s trichrome staining of lung tissue. (B) The expression of collagen I mRNA and protein were determined by real-time PCR and Western blot. (C) The expression of collagen I protein was determined by Western blot. Data are means±S.E.M. n=8. **\(p<0.01\) vs. Control; #\(p<0.05\), ##\(p<0.01\) vs. Bleomycin. EPS, Epalrestat; \(\alpha\)-SMA, \(\alpha\)-smooth muscle actin.](image2)
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Fig. 3. Effect of Epalrestat on the expression of TGF-$\beta_1$ and AR in bleomycin-induced pulmonary fibrosis rats. (A and B) The expression of TGF-$\beta_1$ mRNA and protein in lung tissue were determined by real-time PCR and Western blot. (C and D) The expression of AR mRNA and protein in lung tissue were determined by real-time PCR and Western blot. (E) The expression of AR in lung tissue was determined with immunohistochemistry staining (arrows indicate AR positive staining). Data are means±S.E.M. n=8. **p<0.01 vs. Control; p<0.05, **p<0.01 vs. Bleomycin. EPS, Epalrestat; AR, aldose reductase.

Effect of epalrestat on the expression of AR in lungs from bleomycin-induced pulmonary fibrosis rats

TGF-$\beta_1$ seems to play a major profibrotic role, inducing fibroblast into myofibroblast differentiation and increasing collagen expression. In line with previous studies [32], bleomycin dramatically increased the expression of TGF-$\beta_1$ (both mRNA and protein) in lungs of rats (Fig. 3A and B). On this basis, we further found that the expression of AR was obviously increased in lungs from bleomycin-induced pulmonary fibrosis rats (Fig. 3C~E). And importantly epalrestat obviously inhibited bleomycin-induced upregulation of TGF-$\beta_1$ and AR expression (both mRNA and protein) (p<0.05) (Fig. 3).

Effect of epalrestat on TGF-$\beta_1$-induced expression of AR and proliferation of pulmonary fibroblasts

TGF-$\beta_1$ can induce the excessive proliferation and accumulation of pulmonary fibroblasts and promote the synthesis and deposition of collagen, which plays a crucial role in fibrotic diseases [33]. To investigate whether epalrestat inhibits AR expression directly, pulmonary fibroblasts were stimulated with TGF-$\beta_1$ (5 ng/ml) in the presence or absence of epalrestat (1, 10, 100 $\mu$M) for the indicated time. As shown in Fig. 4A and B, exposure of fibroblasts to TGF-$\beta_1$ for 24 h significantly increased mRNA and protein levels of AR, and epalrestat significantly inhibited the TGF-$\beta_1$-induced upregulation of AR expression (both mRNA and protein) (p<0.05). However, epalrestat (100 $\mu$M) alone had no effect on AR expression in cultured pulmonary fibroblasts. We also found that exposure of pulmonary fibroblasts to TGF-$\beta_1$ (5 ng/ml) for 24 h significantly increased the percentage of cells in the S+G2 phase and BrdU incorporation,

tortion of lung structure and accumulation of collagen fiber (blue) in rat lungs, whereas a well-alveolized normal histology was seen in rats treated with saline (Fig. 2A). And bleomycin also markedly up-regulated the expression of collagen I mRNA and protein in lung tissue of rats (Fig. 2B and C). All these effects of bleomycin were significantly alleviated by treatment of rats with epalrestat (p<0.05).
and epalrestat (1, 10, 100 μM) significantly inhibited the TGF-β₁-induced proliferation of pulmonary fibroblasts (p<0.05). Epalrestat (100 μM) alone had no effect on proliferation of pulmonary fibroblasts (Fig. 4C~E).

**Effect of epalrestat on expression of α-SMA and collagen I expression induced by TGF-β₁ in cultured pulmonary fibroblasts**

As shown in Fig. 5, exposure of TGF-β₁ (5 ng/ml) for 24 h significantly increased α-SMA and collagen I expression in cultured pulmonary fibroblasts, whereas epalrestat (1, 10, 100 μM) obviously inhibited the expression of α-SMA and collagen I (both mRNA and protein) (p<0.05). Epalrestat (100 μM) alone had no effect on α-SMA and collagen I expression.

**Effect of AR knockdown on TGF-β₁-induced cell proliferation and expression of α-SMA and collagen I in cultured pulmonary fibroblasts**

To confirm the role of AR in mediating TGF-β₁-induced the expression of AR in pulmonary fibroblasts, we developed AR specific siRNA. In our pilot study, we used three siRNA TargetSeq against AR to establish the AR siRNA pulmonary fibroblasts, and compared the effects of the three siRNA TargetSeq. The result showed that transfection with the first sequence for 24 h had the best efficiency to inhibit AR expression (data not shown). We therefore used the first siRNA TargetSeq for the sequent experiments. As shown in Fig. 6A and B, AR siRNA inhibited TGF-β₁-induced up-regulation of AR expression. Importantly, we found that AR siRNA reversed the effect of TGF-β₁-induced proliferation of pulmonary fibroblasts as shown by an increase in BrdU incorporation and the percentage of cells in S+G2 phase (Fig. 6C~E). Accordingly, AR siRNA also reversed the effect of TGF-β₁-induced up-regulation

**Fig. 4. Effect of epalrestat on TGF-β₁-induced expression of AR and proliferation of pulmonary fibroblasts.** (A) The expression of AR mRNA was determined by real-time PCR. (B) The expression of AR protein was determined by Western blot. (C) Cell proliferation was measured by BrdU incorporation assay. (D) The percentage of cells in S+G2 phase. (E) Cell cycle distribution was monitored by flow cytometry using a propidium iodide staining assay. The values are means±S.E.M. from three independent experiments in vitro. *p<0.05 vs. Control; #p<0.05, ##p<0.01 vs. TGF-β₁. EPS, Epalrestat; AR, aldose reductase.
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Fig. 5. Effect of Epalrestat on TGF-β₁-induced expression of α-SMA and collagen I in cultured pulmonary fibroblasts. (A and B) The expression of α-SMA mRNA and protein were determined by real-time PCR and Western blot. The values are means±S.E.M. from three independent experiments in vitro. **p < 0.01 vs. Control; #p < 0.05, ##p < 0.01 vs. TGF-β₁. EPS, Epalrestat; α-SMA, α-smooth muscle actin.

DISCUSSION

Pulmonary fibrosis, the final step of a group of lung disorders known as interstitial lung diseases, has been described to follow three stages: injury, inflammation and tissue repair [3]. Injury and inflammation cause the destruction of the alveolar epithelium, which activates lung fibroblasts, leading to their proliferation and differentiation into myofibroblasts [5]. The fibroproliferative process is precisely regulated by several cytokines and growth factors, such as TGF-β₁ and tumor necrosis factor-α [34,35]. Failure to regulate this process results in excessive extracellular matrix production and accumulation in lung connective tissue.

TGF-β₁ is one of the most important profibrotic cytokines and is produced by various types of cells, including macrophages, epithelial cells and fibroblasts [36]. Clinical researches reveal that TGF-β₁ is upregulated in lungs and plasma of patients with pulmonary fibrosis [37]. Furthermore, overexpression of active TGF-β₁ results in pulmonary fibrosis characterized by extensive deposition of extracellular matrix proteins collagen, fibronectin and elastin, and the emergence of cells with a myofibroblast phenotype [38]. AR is an enzyme responsible for conversion of glucose to sorbitol in the polyol pathway of glucose metabolism [39]. Recent study has shown that allergen-induced airway remodeling is mediated by AR and its inhibition blocks the progression of remodeling via inhibiting TGF-β₁-induced Smad-independent pathway [20]. Further, increased expression of AR was observed in the lungs of COPD patients [21]. These studies indicate that AR may be an important role in respiratory disease. In the present study, we have found that bleomycin dramatically increased the expression of AR in lungs of rats and exogenous TGF-β₁ markedly up-regulated expression of AR in cultured pulmonary fibroblast concomitantly with proliferation of cell for the first time. Of note, knockdown of AR gene expression reversed TGF-β₁-induced up-regulation of AR expression. In addition, we also found that AR siRNA reversed the effect of TGF-β₁-induced proliferation of fibroblasts and up-regulation of α-SMA and collagen I, and epalrestat (100 μM) had no effect on α-SMA and collagen I expression in TGF-β₁+AR siRNA cells. These findings suggest that AR is related to the development of pulmonary fibrosis induced by bleomycin and is involved in the regulation of fibroblasts proliferation.

Epalrestat (EPS) approved in Japan in 1992, is the only aldose reductase inhibitor currently available for the treatment of diabetic neuropathy. EPS is easily absorbed and inhibits aldose reductase with minimum adverse effects [40]. A recent study showed that treatment with EPS at an early stage delayed the progression of diabetic neuropathy and prevented the onset/progression of retinopathy and nephropathy [41]. Except diabetic neuropathy, other study has shown that EPS might be a new strategy useful for improving oxidative stress-related diverse diseases including neurodegenerative diseases and atherosclerosis as well as diabetes [42]. It has also demonstrated that long-term oral administration of epalrestat could reverse vascular remodeling of spontaneously hypertensive rats from the inhibition of aldose reductase expression [27,28]. Further, epalrestat could also prevent human coronary artery SMC migration potentiated by high glucose treatment and Ang
Fig. 6. Effect of AR knockdown on TGF-β1-induced cell proliferation in cultured pulmonary fibroblasts. (A and B) The expression of AR mRNA and protein were determined by real-time PCR and Western blot. (C) Cell proliferation was measured by BrdU incorporation assay. (D) The percentage of cells in S+G2 phase. (E) Cell cycle distribution was monitored by flow cytometry using a propidium iodide staining assay. The values are means±S.E.M. from three independent experiments in vitro. **p<0.01 vs. Control; #p<0.05, ##p<0.01 vs. TGF-β1. AR, aldose reductase; siRNA, small interfering RNA.

II-stimulated ECM biosynthesis in mesangial cells [25,26]. In this study, histological examination showed that epalrestat alleviated the interalveolar septa and dense interstitial infiltration by inflammatory cells and fibroblasts, and Masson’s trichrome staining showed that epalrestat treatment reduced collagen accumulation in lung of rats with bleomycin-induced pulmonary fibrosis. Further, epalrestat treatment also decreased bleomycin-induced AR, α-SMA and collagen I expression. We also found that epalrestat remarkably attenuated cultured pulmonary fibroblast proliferation and α-SMA and collagen I expression induced by TGF-β1, and this inhibitory effect of epalrestat was accompanied by inhibiting of AR expressions. These results suggest that epalrestat inhibited the progression of bleomycin-induced pulmonary fibrosis in rats is mediated via inhibiting of AR expression.

In summary, according to our studies the antifibrotic effect of epalrestat in bleomycin-induced pulmonary fibrosis rats may be related to its antiproliferative activity, which is mediated by inhibiting of AR expression. These findings indicate that epalrestat may be a potential candidate compound to current therapies for pulmonary fibrosis. Of course, the roles of the AR in the development of pulmonary fibrosis in vivo require further rigorous investigation. Similarly to
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Fig. 7. Effect of AR knockdown on TGF-β1-induced expression of α-SMA and collagen I in cultured pulmonary fibroblasts. (A, B and E, F) The expression of α-SMA mRNA and protein were determined by real-time PCR and Western blot. (C, D and G, H) The expression of collagen I mRNA and protein were determined by real-time PCR and Western blot. The values are means±S.E.M. from three independent experiments in vitro. (A, B and C, D) **p < 0.01 vs. Control; p < 0.05, ***p < 0.01 vs. TGF-β1; (E, F and G, H) **p < 0.01 vs. Control; ***p < 0.01 vs. EPS. EPS, Epalrestat; α-SMA, α-smooth muscle actin; siRNA, small interfering RNA.
most drugs, epalrestat has an antifibrotic effect that is diffi-
cult to explain by a simple mechanism of action.

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