Korean J Physiol Pharmacol
Vol 19: 401−411, September, 2015
http://dx.doi.org/10.4196/kjpp.2015.19.5.401

pISSN 1226-4512
eISSN 2093-3827

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

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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-β_1, α-SMA and collagen I was analyzed by immunohistochemisty, 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-β_1, 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-β_1, and this inhibitory effect of epalrestat was accompanied by inhibiting AR expression. Knockdown of AR gene expression reversed TGF-β_1-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

INTRODUCTION

Pulmonary fibrosis is an interstitial lung disease induced by various etiological factors. It has been demonstrated that several factors contributes 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 remodeling [1]. However, the mechanism of pulmonary fibrosis is not completely understood, and the effects of drugs on pulmonary fibrosis, are not satisfactory [2]. Therefore, it is crucial to find new therapeutic strategies for pulmonary fibrosis.

Fibroblasts have been implicated as a major participant in pulmonary fibrosis and are currently being studied as new therapeutical targets [3]. Histologic sections of diseased lung from patients with PF show clusters of proliferating fibroblasts termed "fibroblastic foci" [4]. These clusters of fibroblasts are composed primarily of myofibroblasts, contractile cells that express both fibroblast and smooth muscle cell markers such as α-smooth muscle actin (α-SMA) [5]. Myofibroblasts are largely responsible for the excess production of extracellular matrix components, such as collagen and fibronectin [6]. Unfortunately, the underlying molecular mechanisms responsible for fibroblast proliferation and excessive deposition of collagen in fibrotic lesions are not fully understood.

Aldose reductase (AR), a member of the aldo-keto reductase super family, catalyzes the conversion of glucose to sorbitol dependent on NADPH in the first step of the polyol pathway [7]. AR is known to play a crucial role in the mediation of diabetic and cardiovascular complications [8]. A growing of evidence shows that AR plays a crucial role in

Received February 4, 2015, Revised May 11, 2015,
Accepted June 29, 2015

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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-β_1, transforming growth factor-β_1; DMEM, Dulbecco's modified Eagle's medium; BLM, bleomycin.

acute lung and kidney injury, tumorigenesis and metastasis, and renal and ovarian abnormalities [9-12]. An earlier study indicated that AR plays an important role in TGF-β_1-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 ragweed pollen extract and ovalbumin-induced asthma and IL-13-induced mucous cell metaplasia [17-19]. Other studies have demonstrated that allergen-induced airway remodeling is mediated by AR and its inhibition blocks the progression of remodeling via inhibiting TGF-β_1-induced Smad-independent pathway [20]. Further, increased expression of AR was observed in the lungs of chronic obstructive pulmonary diseases (COPD) patients [21]. These studies indicate that AR may be an important role in respiratory 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 trials with AR inhibitor such as, Tolrestat, Ponalrestat, Zopolrestat, 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 propenylidene]-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 sorbitol accumulation, which is implicated in the pathogenesis of diabetic complications [23]. Except diabetic neuropathy, other study has shown that epalrestat prevents human coronary artery smooth muscle cells migration potentiated by high glucose treatment and Ang II-stimulated ECM biosynthesis in MCs [25,26]. 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]. 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 fibroblasts, 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 epalrestat 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-β_1 (TGF-β_1) 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 medium (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 TaKaRa Biotechnology 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 obtained 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 intratracheal instillation of 0.9% saline;2) the bleomycin (BLM) group, rats were anesthetized intraperitoneally with sodium 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 dissolved in double-distilled water and administered via oral gavage daily from day 1 to day 28 after BLM or saline treatment (day 0) and all rats were sacrificed with exsanguination on day 29. Pulmonary fibrosis was assessed by lung histology as described in the following section [30].

Lung tissue histology, Masson's trichrome staining and Immunohistochemisty

For light microscopic investigation, right lung tissues were fixed by inflation with freshly prepared 4% paraformaldehyde 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 hematoxylin and eosin (H&E) and Masson's trichrome stain to enable histological evaluation of lung fibrosis. Masson's trichrome stain was used to demonstration collagen deposition, 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 immunohistochemisty staining, serial sections of formalin-fixed paraffin-embedded lung tissues were digested with 3% H_2O_2 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 secondary 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% CO_2 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-β_1 solvent) for 24 h; ii) TGF-β_1, cells were incubated with TGF-β_1 (5 ng/mL) for 24 h; iii-v) + EPS (1,10,100 μM): cells were pre-treated with EPS (1,10,100 μM) for 1 h, and then subjected to TGF-β_1 (5 ng/mL) for 24 h; and vi) EPS (100 μM): cells were pre-treated with 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-β_1-induced proliferation of rat fibroblasts. The cells were divided into 4 groups as follows: i) control, cells were incubated with double distilled water (TGF-β_1 solvent) for 24 h; ii) TGF-β_1, cells were incubated with TGF-β_1 (5 ng/mL) for 24 h; iii) + Scrambled: cells were pre-treated with AR siRNA negative control for 24 h before treated with TGF-β_1 (5 ng/mL) for 24 h; iv)+ AR siRNA: cells were pre-treated with AR siRNA for 24 h before treated with TGF-β_1 (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-β_1 was based on our pilot study.

Small interfering RNA Transfection

Small interfering RNA (siRNA) against AR gene (GenePharma CO, Shanghai, China) was generated against the following rat AR sequences: sense, 5'-GUGCCAAACACAAGGAUUATT-3'; and antisense, 5'-UAAUCCUUGUGUUUGGCACTT-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 (Invitrogen). RNA (0.2～0.5 μg) was subjected to reverse transcription reaction using the PrimeScript reverse transcription reagent Kit (DRR037S; TaKaRa, Dalian, China). Quantitative analysis of the change in expression levels was performed using SYBR® Premix Ex Taq™ (DRR42OA; TaKaRa) by the ABI 7300 system. PCR cycling conditions were an initial incubation at 95°C for 15 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing at 60°C for 31 s. Primers for TGF-β_1 were: forward 5'-TGGCGTTACCTTGGTAACC-3', reverse 5'-GGTGTTGAGCCCTTTCCAG-3'. Primers for eIF3a were: forward 5'-TCAAGTCGCCGGGACGATA-3', reverse 5'-CCTGTCATCAGCACGTCTCCA-3'. Primers for AR were: forward 5'-TCCCAGGATCAAGGAAATTG-3', reverse 5'-ACAACAG GAACTGGAGGGTG-3'. Primers for α-SMA were: forward 5'-CTATTCCTTCGTGACTACT-3', reverse 5'-ATGCTGTTATAGGTGGTT-3'. Primers for collagen I were: forward 5'-CCAACTGAACGTGACCAAAAACCA-3', reverse 5'-GAAGGTGCTGGGTAGGGAAGTAGGC-3'. Primers for collagen III were: forward 5'-ATTCTGCCACCCTGAACTCAAGAGC-3', reverse 5'-TCCATGTAGGCAATGCTGTTTTTGC-3'. Primers for GAPDH were: forward 5'-TGGCCTCCAAGGAGTAAGAAAC-3', reverse 5'-GGCCTCTCTCTTGCTCTCAGTATC-3'. Data analysis was performed by comparative Ct method using the ABI software. GAPDH was used to normalize the expression of mRNA.

Western blot analysis

Protein was extracted from left lung tissues and primary cultured lung fibroblast with RIPA buffer (containing 0.1% PMSF), and equal amounts of protein from each sample (50 μg) were separated by 10% SDS/PAGE and transferred to polyvinylidene fluoride membranes. The membranes were then incubated with primary antibodies overnight at 4°C, and horseradish peroxidase (HRP)-coupled goat anti-mouse or goat anti-rabbit secondary antibody (sc-2005, 1 : 2,000, sc-2030, 1 : 5,000; Santa Cruz, CA, USA). The chemiluminescence signals were detected with the EasySee Western Blot Kit (Beijing TransGen Biotech, Beijing, China). The densitometric analysis was conducted with Image J 1.43 (National Institutes of Health). Primary antibodies against α-SMA (ab5694, 1 : 2000) and collagen I (ab34710, 1 : 1000) were purchased from Abcam (Hong Kong, China), and primary antibodies against AR (sc-33219, 1 : 500) and GAPDH (sc-137179, 1 : 2000) were obtained from Santa Cruz (CA, USA).

Statistical analysis

The results were presented as means±S.E.M. (standard errors). Statistical analysis was performed by ANOVA followed by the Newman-Student-Keuls test for multiple comparisons. Results were considered statistically significant when $p < 0.05$.

RESULTS

Effect of epalrestat on lung histological and α-SMA expression in bleomycin-induced pulmonary fibrosis rats

In keeping with previous study [31], after 4 weeks of exposure to bleomycin induced rat pulmonary fibrosis, as shown by a significant disturbed alveolar structure, marked

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 α-smooth muscle actin (α-SMA). In line with previous studies [5], bleomycin dramatically increased the expression of α-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-

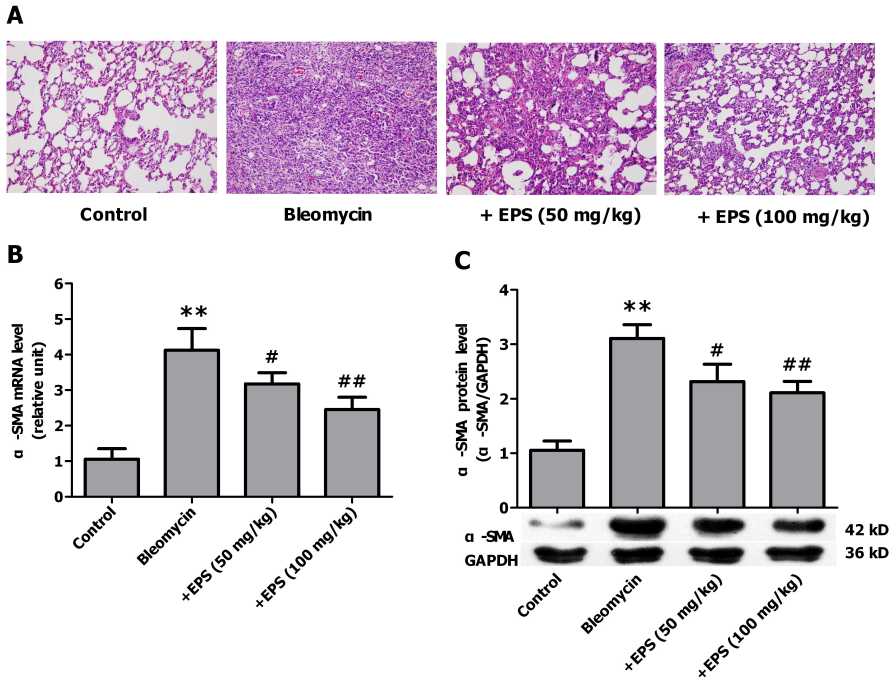

Fig. 1. Lung histology and α-SMA expression in bleomycin-induced pulmonary fibrosis of rats. (A) Hematoxylin-eosin staining of lung tissue. (B and C) The expression of α-SMA mRNA and protein were determined by real-time PCR and Western blot. Data are means±S.E.M. n=8. **$p<0.01$ vs. Control; $^{\#}p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

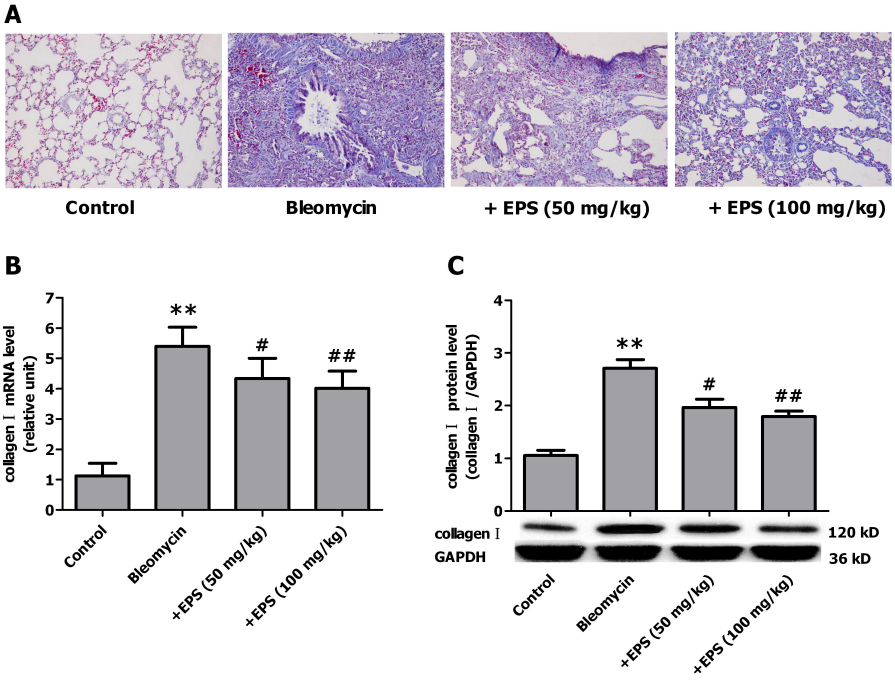

Fig. 2. Collagen expression of lung tissue in bleomycin-induced pulmonary fibrosis rats. (A) Masson's trichrome staining of lung tissue. (B) The expression of collagen I mRNA was determined by real-time PCR. (C) The expression of collagen I protein was determined by Western blot. Data are means±S.E.M. n=8. **$p<0.01$ vs. Control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. Bleomycin. EPS, Epalrestat; α-SMA, α-smooth muscle actin.

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 ($p < 0.05$).

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

TGF-β_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-β_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-β_1 and AR expression (both mRNA and protein) ($p < 0.05$) (Fig. 3).

Effect of epalrestat on TGF-β_1-induced expression of AR and proliferation of pulmonary fibroblasts

TGF-β_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-β_1 (5 ng/ml) in the presence or absence of epalrestat (1, 10, 100 μM) for the indicated time. As shown in Fig. 4A and B, exposure of fibroblasts to TGF-β_1 for 24 h significantly increased mRNA and protein levels of AR, and epalrestat significantly inhibited the TGF-β_1-induced upregulation of AR expression (both mRNA and protein) ($p < 0.05$). However, epalrestat (100 μM) alone had no effect on AR expression in cultured pulmonary fibroblasts. We also found that exposure of pulmonary fibroblasts to TGF-β_1 (5 ng/ml) for 24 h significantly increased the percentage of cells in the S+G2 phase and BrdU incorporation,

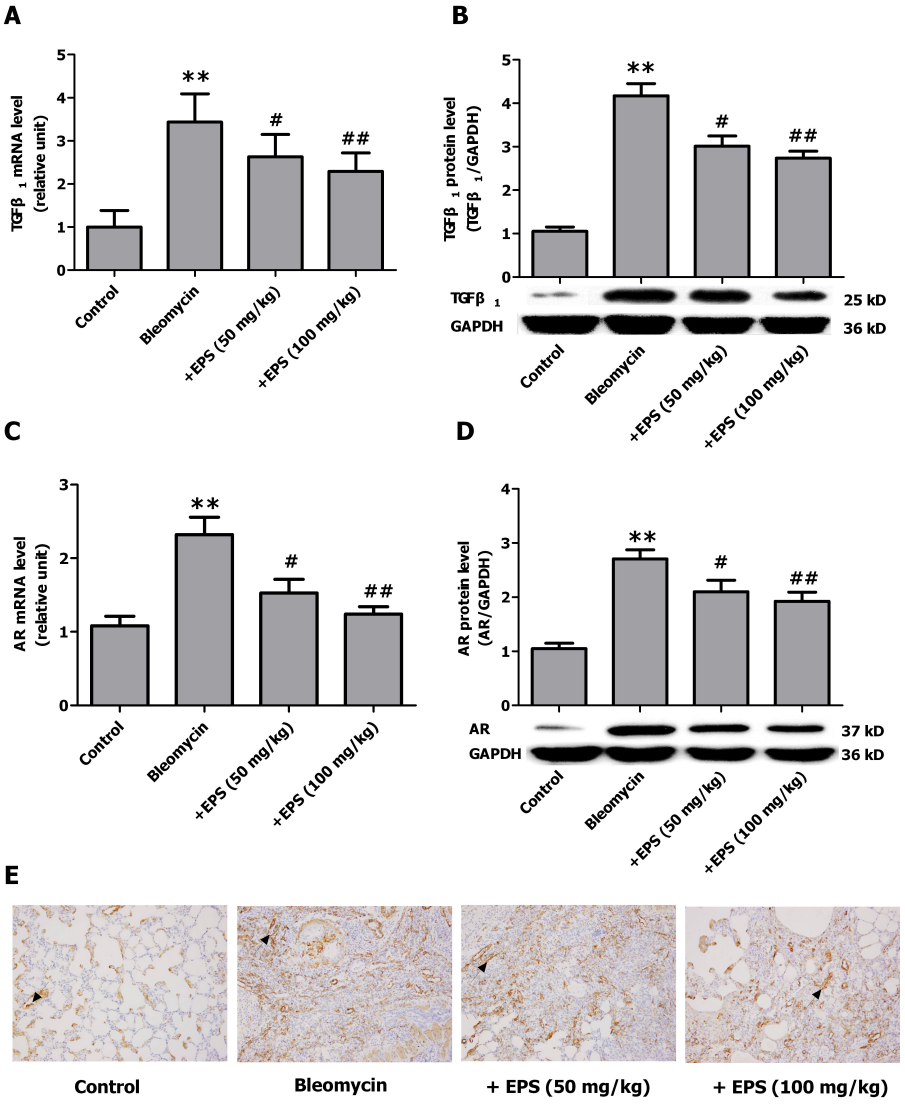

Fig. 3. Effect of Epalrestat on the expression of TGF-β_1 and AR in bleomycin-induced pulmonary fibrosis rats. (A and B) The expression of TGF-β_1 mRNA and protein in lung tissue were determined by real-time PCR and Western blot. (C and D) The expression of AR mRNA and protein in lung tissue were determined by real-time PCR and Western blot. (E) The expression of AR in lung tissue was determined with immunohistochemisty 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.

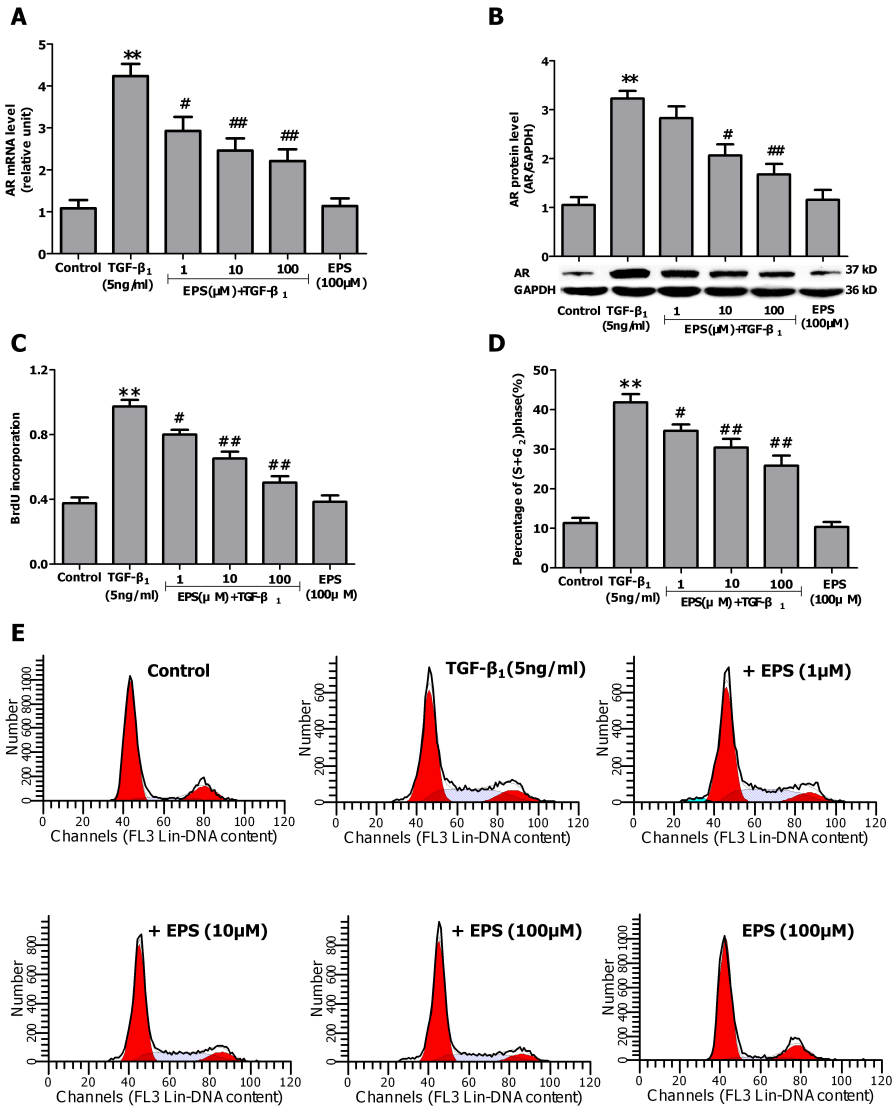

Fig. 4. Effect of epalrestat on TGF-β_1-induced expression of AR and proliferation of pulmonary fibroblasts. (A) The expression of AR mRNA was determined by real-time PCR. (B) The expression of AR protein was determined by Western blot. (C) Cell proliferation was measured by BrdU incorporation assay. (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. $^{**}p<0.01$ vs. Control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. TGF-β_1. EPS, Epalrestat; AR, aldose reductase.

and epalrestat (1, 10, 100 μM) significantly inhibited the TGF-β_1-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-β_1 (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-β_1-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-β_1-induced up-regulation of AR expression. Importantly, we found that AR siRNA reversed the effect of TGF-β_1-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-β_1-induced up-regulation

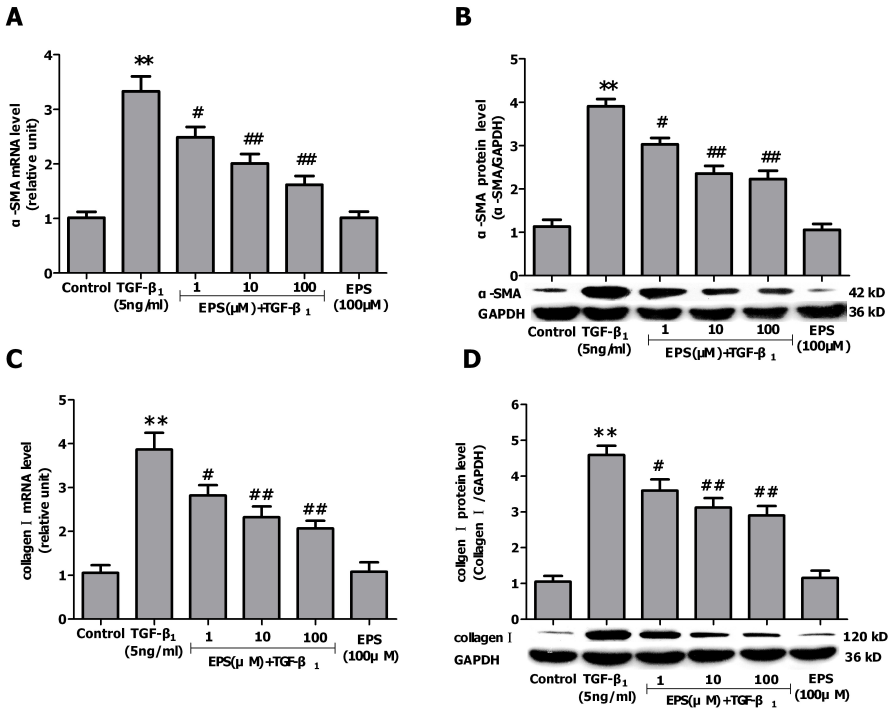

Fig. 5. Effect of Epalrestat on TGF-β_1-induced expression of α-SMA and collagen I in cultured pulmonary fibroblasts. (A and B) The expression of α-SMA mRNA and protein were determined by real-time PCR and Western blot. (C and D) The expression of collagen I mRNA and protein were determined by real-time PCR and Western blot. The values are means±S.E.M. from three independent experiments in vitro. **$p < 0.01$ vs. Control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. TGF-β_1. EPS, Epalrestat; α-SMA, α-smooth muscle actin.

of α-SMA and collagen I (both mRNA and protein) ($p < 0.05$) (Fig. 7A~D). But compared with TGF-β_1+AR siRNA, epalrestat (100 μM) had no effect on α-SMA and collagen I expression in TGF-β_1+AR siRNA cells (Fig. 7E~H). These results strongly suggested that epalrestat reduced pulmonary fibrosis via inhibition of AR.

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-β_1 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-β_1 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-β_1 is upregulated in lungs and plasma of patients with pulmonary fibrosis [37]. Furthermore, overexpression of active TGF-β_1 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-β_1-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-β_1 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-β_1-induced up-regulation of AR expression. In addition, we also found that AR siRNA reversed the effect of TGF-β_1-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-β_1+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

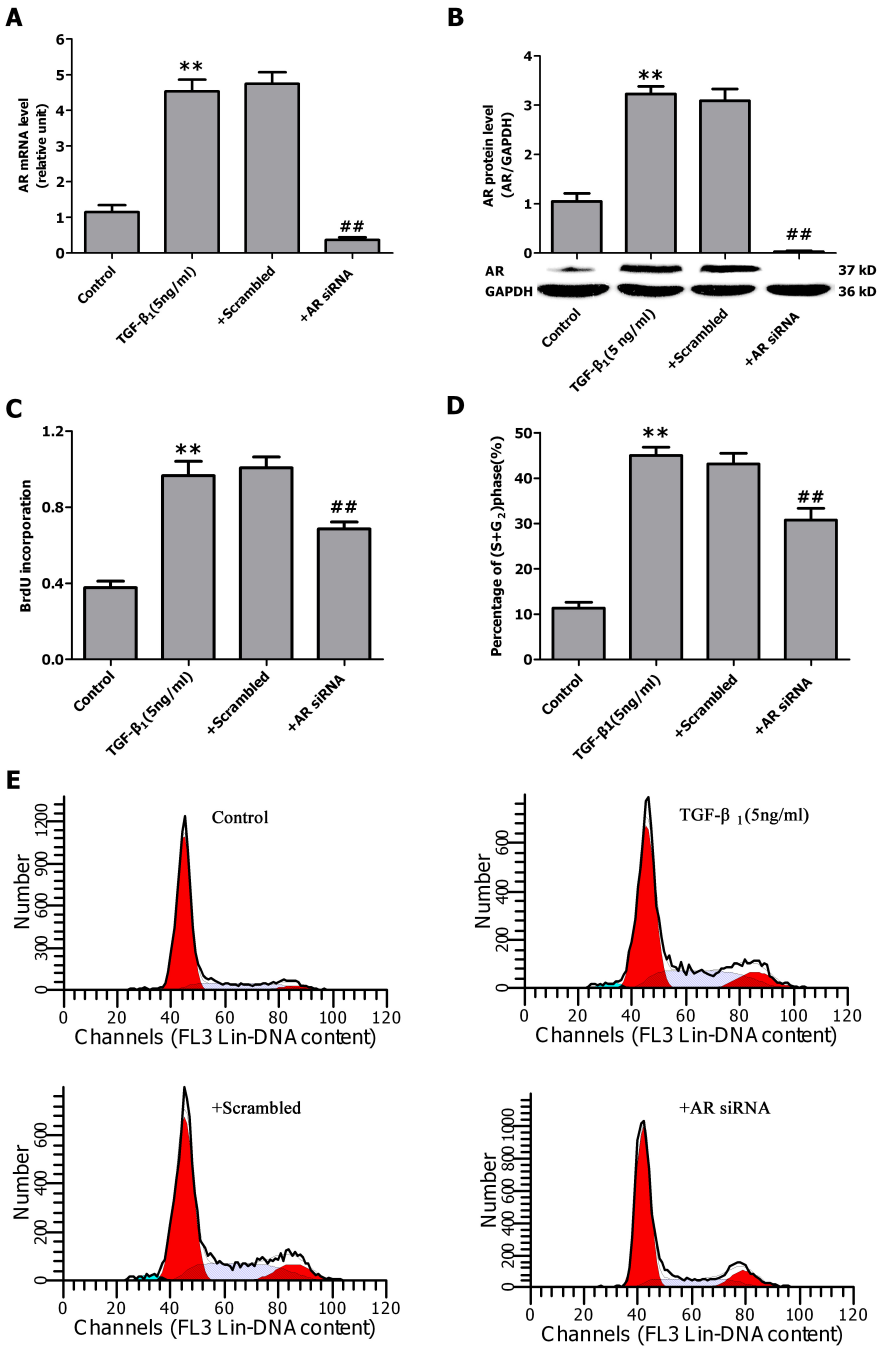

Fig. 6. Effect of AR knockdown on TGF-β_1-induced cell proliferation in cultured pulmonary fibroblasts. (A and B) The expression of AR mRNA and protein were determined by real-time PCR and Western blot. (C) Cell proliferation was measured by BrdU incorporation assay. (D) The percentage of cells in S+G2 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-β_1. 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-β_1, 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

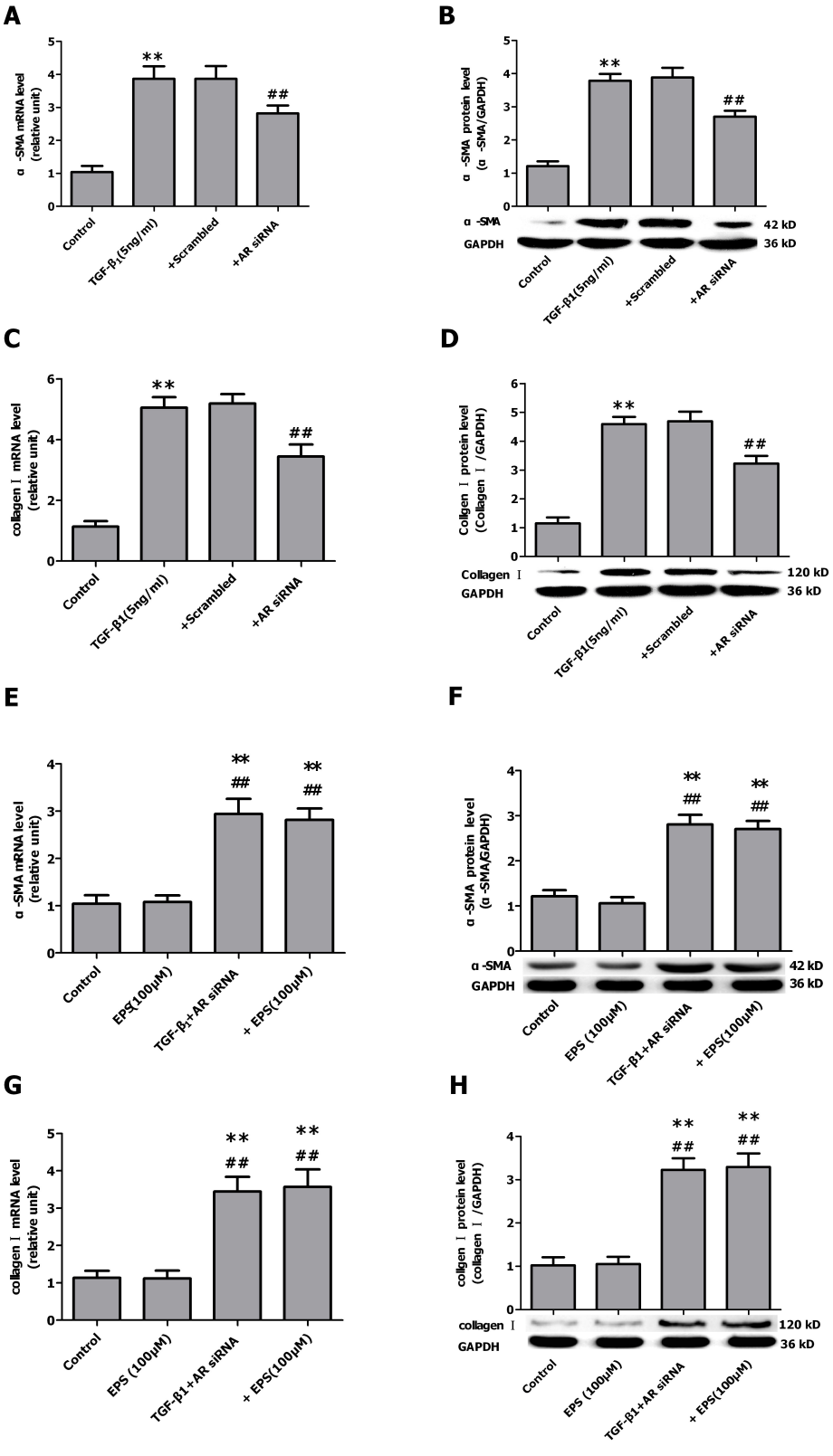

Fig. 7. Effect of AR knockdown on TGF-β_1-induced expression of α-SMA and collagen I in cultured pulmonary fibroblasts. (A, B and E, F) The expression of α-SMA mRNA and protein were determined by real-time PCR and Western blot. (C, D and G, H) The expression of collagen I mRNA and protein were determined 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-β_1; (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 difficult to explain by a simple mechanism of action.

ACKNOWLEDGMENTS

This work was supported by the grant from the Natural Science Foundation of Anhui Province (No. 1408085QH168).

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