Laboratory Investigation (2019) 99:1770–1783
https://doi.org/10.1038/s41374-019-0289-7

ARTICLE

Ang-(1-7) treatment attenuates lipopolysaccharide-induced early pulmonary fibrosis

Yongmei Cao[1] · Yujing Liu[1] · Jiawei Shang[1] · Ziming Yuan[1] · Feng Ping[1] · Sijia Yao[1] · Yong Guo[1] · Yingchuan Li[1]

Received: 20 April 2019 / Revised: 13 May 2019 / Accepted: 24 May 2019 / Published online: 5 July 2019
© The Author(s), under exclusive licence to United States and Canadian Academy of Pathology 2019

Abstract

Early pulmonary fibrosis is the leading cause of poor prognosis in patients with acute respiratory distress syndrome (ARDS). However, whether the renin-angiotensin system (RAS) can serve as a therapeutic target is unknown. In this study, an animal model of early pulmonary fibrosis was established via the LPS three-hit regimen. Afterwards, the animals were treated with intraperitoneal injections of Ang-(1-7), AVE0991, or A779 once per day for 20 days. The plasma and BALF AngII levels of the animals were increased, while there were no significant changes in Ang-(1-7) levels in lung tissue after LPS treatment. Furthermore, the AT1R protein levels were significantly increased and the Mas levels were significantly decreased on days 14 and 21. Administration of Ang-(1-7) downregulated LPS-induced AT1R mRNA expression, which was upregulated by A779. The expression of Mas mRNA responded in the opposite direction relative to AT1R. Moreover, LPS caused decreased levels of Mas and E-cadherin and increased AT1R, Vimentin, and Src phosphorylation levels. Ang-(1-7) or AVE0991 blocked these effects but was counteracted by A779 treatment. Our findings suggested that AngII and AT1R levels exhibit opposite dynamic trends during LPS-induced early pulmonary fibrosis, as do Ang-(1-7) and Mas. Ang-(1-7) exerts protective effects against early pulmonary fibrosis, mainly by regulating the balance between AngII and AT1R and between Ang-(1-7) and Mas and by inhibiting Src kinase activation.

Introduction

Acute respiratory distress syndrome (ARDS) is a kind of acute respiratory failure that involves diffuse interstitial and alveolar edema, exudation of inflammatory factors and refractory hypoxemia, which are caused by various factors [1, 2]. Recent studies have shown that early pulmonary fibrosis is the leading cause of poor prognoses in patients with ARDS [3, 4]. Therefore, investigating the mechanism of pulmonary fibrosis and exploring interventions for the early stage of this disease may improve its prognosis and reduce mortality.

In recent years, clinical and animal experiments have shown that the renin-angiotensin system (RAS) plays an important role in the development of ARDS [5, 6]. The administration of LPS induced an increase in AngII level and a decrease in Ang-(1-7) level in animal experiments [7]. AT1R and Mas mRNA levels were significantly elevated in a bleomycin-mediated pulmonary fibrosis animal model [8, 9]. Importantly, AngII can promote inflammation, fibrosis, and apoptosis by binding to its specific receptor (angiotensin II type 1 receptor, AT1R) [10–12], while Ang-(1-7) antagonizes the effects of AngII by binding to its receptor Mas [13–15]. LPS administration has also been shown to induce increased levels of AngII and cause severe damage to endothelial cells, both of which were reversed in the lung tissue by the use of AT1R blockers [16]. Ang-(1-7) can reduce ARDS that induced by mechanical ventilation or acid inhalation and can attenuate bleomycin-induced pulmonary inflammation and extracellular matrix accumulation, which are blocked by the use of the Mas receptor antagonist A779 [13, 17].

The epithelial-mesenchymal transition (EMT) is considered to be the basis of pulmonary fibrosis [18, 19]. At present, the EMT of alveolar epithelial type II cells is the

These authors contributed equally: Yongmei Cao, Yujing Liu

✉ Yingchuan Li
yingchuan.li@sjtu.edu.cn

[1] Department of Critical Care Medicine, Shanghai Jiaotong University Affiliated to The Sixth People's Hospital, Shanghai, China

key pathophysiological mechanism for the development of pulmonary fibrosis. Studies have shown that RAS plays an important regulatory role in EMT [20]. AngII can induce EMT in human renal tubular epithelial cells [21] and reduce myocardial hypertrophy and fibrosis by promoting EMT [22]. Ang-(1-7) can inhibit AngII-mediated EMT in hepatocytes [23] and high-glucose-induced EMT in renal proximal tubular epithelial cells [24]. In addition, Ang-(1-7) can suppress the metastasis of non-small-cell lung cancer by inhibiting EMT [25].

Src kinase, a non-receptor tyrosine protein kinase, is located in the cytoplasm and is involved in EMT and fibrosis [26]. Src is activated in the EMT of lung cancer and renal cancer cells [27, 28]. Src kinase inhibitors prevent the activation of renal fibroblasts and attenuate renal fibrosis [29]. In addition, Src kinase is involved in the anti-inflammatory effect of Ang-(1-7) in macrophages [30]. Therefore, we speculate that Src kinase may be involved in the anti-fibrotic effect of Ang-(1-7).

Ang-(1-7)/Mas antagonizes the effect of AngII/AT1R and may be an effective measure for the treatment of pulmonary fibrosis. However, the mechanism of Ang-(1-7)/Mas against pulmonary fibrosis has not yet been elucidated. In this study, we investigated the effects and mechanisms of Ang-(1-7) in the process of LPS-induced early pulmonary fibrosis.

Materials and methods

Animals and reagents

Sprague Dawley rats weighing ~200–250 g were obtained from the Department of Laboratory Animal Science of Shanghai Jiaotong University and all experiments were performed in accordance with the protocol approved by the Ethics Committee of Animal Research at the College of Medicine, Shanghai Jiaotong University (Shanghai, China). Shanghai, China. LPS (*Escherichia coli* 055:B5), Ang-(1-7) and a Mas receptor agonist (AVE0991) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and a Mas receptor blocker (A779) was purchased from AbBiotech (San Diego, CA, USA). Mouse anti-AT1R, rabbit anti-Vimentin, and mouse anti-E-cadherin primary antibodies were purchased from Abcam (Cambridge, UK). Rabbit anti-Mas primary antibody was procured from Alomone Labs (Jerusalem, Israel). Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were both purchased from Cell Signaling Technology (Danvers, MA, USA). TGF-β, AngII, and Ang-(1-7) ELISA Kits were procured from the Kamiya Biomedical Company (Seattle, WA, USA).

Animal model

The animal model for early pulmonary fibrosis that is caused by LPS-induced ARDS was established through a modified version of previously published methods [31]. In brief, 36 rats were randomly divided into 6 groups and each group of 6 rats were anesthetized via an intraperitoneal injection of pentobarbital (0.8%) at a dosage of 200 μL/10 g body weight and were subjected to an oral endotracheal intubation using an arterial puncture needle (18 G). LPS (1.5 mg/kg) was injected into the lung via an endotracheal tube; afterwards, the rat was rotated vertically in order to distribute the liquid drug within the lung evenly. The endotracheal tube was then removed. LPS (3 mg/kg) was administered via an intraperitoneal injection after 24 h and via an endotracheal tube after 48 h. An equivalent amount of normal saline was used for a solvent comparison, and the control group received nothing. The rats were killed on the 3rd, 7th, 14th, and 21st days following the LPS treatment. Alveolar lavage was performed before the lung tissues were harvested.

Experimental protocols

The LPS-induced ARDS animal model was generated with several modifications. Ang-(1-7) (600 μg/kg/d), the Mas receptor agonist AVE0991 (600 μg/kg/d) or the Mas receptor inhibitor A779 (600 μg/kg/d) was intraperitoneally administered once per day for 20 days continuously. The intervention started the next day following the LPS three-hit treatment. Rats were killed, and the lung tissues were harvested on the 21st day after LPS stimulation. In brief, the animals were randomly divided into the following groups ($n = 6$ for each group): (1) the control group; (2) the LPS group; (3) the LPS + Ang-(1-7) group; (4) the LPS + Ang-(1-7) + A779 group; (5) the LPS + AVE0991 group; and (6) the LPS + AVE0991 + A779 group.

Pathological staining

The left lobes of lung were immersed in 4% paraformaldehyde for 24 h, dehydrated with ethanol, embedded in paraffin, and sliced at a thickness of 3 μm. Before immunostaining, the sections were dewaxed in xylene, rehydrated, and washed in PBS. The sections were then stained with either hematoxylin and eosin (HE) or Masson's trichrome and analyzed by light microscopy. Semi-quantitative scoring of lung injuries for edema, inflammation and hemorrhage was performed according to previous methods [32], and pulmonary fibrosis was assayed via the standard Aschoff scoring method [33]. Degrees of lung injury and pulmonary fibrosis were graded on a scale of 0–4: 0, absent, and lung appears to be normal (−); 1, light injury (+); 2, moderate injury ++); 3, strong injury

(+++); and 4, intense injury (++++). Three sections collected from each lung were analyzed, and analyze was conducted by three independent observers.

ELISA

The serum and supernatants of the bronchoalveolar lavage fluid (BALF) were collected. TGF-β, AngII, and Ang-(1-7) levels were detected using ELISA kits according to the manufacturer's protocol. Absorbance was determined at 450 nm with a microplate reader (Bio-Rad Laboratory), and concentrations were calculated from a linear standard curve. ELISA measurements were performed in triplicate.

Western blotting

Total protein was extracted with a PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL, USA) in accordance with the manufacturer's instructions. Equivalent amounts of the protein samples (100 μg) were separated via gel electrophoresis on a 10% SDS-PAGE gel and transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The blots were blocked at room temperature for 2 h with 5% bovine serum albumin (BSA). The membranes were subsequently incubated with specific primary antibodies (1:500 dilution for mouse anti-AT1R; 1:500 dilution for rabbit anti-Mas; 1:1000 dilution for rabbit anti-Vimentin; 1:1000 dilution for mouse anti-E-cadherin; 1:2000 dilution for rabbit anti-Scr; and 1:1000 dilution for rabbit anti-p-Scr) at 4 °C overnight, followed by an appropriate HRP-conjugated secondary antibody (1:2000 dilution) at room temperature for 2 h. Afterwards, ECL Plus detection reagents (Pierce, Rockford, IL, USA) were used to develop the signal. The total protein and cytoplasm levels were normalized to β-actin and the membrane protein levels were normalized to Na-K ATPase.

Immunohistochemical analysis

The lung tissue sections were pretreated with heat-induced antigen retrieval for optimal immunostaining and probed with a mouse anti-AT1R antibody (1:200 dilution) and a rabbit anti-Mas antibody (1:100 dilution). Finally, the sections were incubated with a peroxidase-conjugated goat-anti-rabbit IgG and visualized via the streptavidin-biotin-peroxidase complex system using an SABC kit (Boster, Wuhan, China). As a negative control, the primary antibodies were replaced with irrelevant immunoglobulin.

Real-time PCR

Total RNA was extracted from the lung tissue using TRIzol Reagent (15596-018, Invitrogen, OR, USA). RNA was reverse transcribed via the PrimeScript™ RT Master Mix kit (RR036A, Takara, Japan) for AT1R and Mas mRNAs, per the manufacturer's instructions. SYBR Green quantitative RT-PCR was performed using the Applied Biosystems 7500 RT-PCR system. For each sample, the amplification reactions were performed in triplicate. The primers were purchased from the Bio-TNT Company (Shanghai, China). The AT1R and Mas primers used in this study were as follows: AT1R forward, 5′-TAACAACTGCCTGAACCCTC-3′; AT1R reverse, 5′-GCGTGCTCATTTTCGTAGAC-3′; Mas forward, 5′-GTGTATTGACAGCGGAGAAG-3′; Mas reverse, 5′-CGGAGTGAAGACCAAGAAG-3′; β-actin forward, 5′-CCTCTATGCCAACACAGT-3′; β-actin reverse, 5′-AGCCACCAATCCACACAG-3′. Relative RNA quantification was performed via the comparative $2^{-\Delta\Delta Ct}$ methods. AT1R and Mas mRNA levels were normalized to β-actin mRNA levels.

Statistical analysis

All of the data are presented as the mean ± standard deviation (SD). Comparisons between the treatment groups and the control group were performed via a one-way analysis of variance followed by Dunnett's test. Comparisons among multiple groups were performed via a one-way analysis of variance followed by *t*-tests with the Bonferroni correction. Statistical analyses were performed with GraphPad Prism version 5.0 (San Diego, CA, USA). A value of $p < 0.05$ was considered statistically significant.

Results

LPS induces increased pulmonary inflammation and fibrosis in rat lung tissue

The HE staining results showed that the morphological structure of the lung tissue was normal in the control group and that there was little structural damage in the saline group. On the third day after the LPS three-hit regimen, however, the alveolar walls were slightly widened, with severe hyperemia and edema, and inflammatory cells had leaked into the alveolar cavities in the rat lung tissue. Thickened alveolar septa, severe hemorrhage, and exudation occurred in the alveolar cavities and interstitial space, and a large number of inflammatory cells had infiltrated on the 7th day. As of the 14th and 21st days, the inflammatory response had decreased, and the alveolar structure had been destroyed (Fig. 1a). Compared to the control group, the LPS-treated rats had significantly increased lung injury scores on the 3rd and 7th days ($p < 0.05$). The lung injury scores were gradually reduced on the 14th and 21st days in the LPS-treated rats, but they

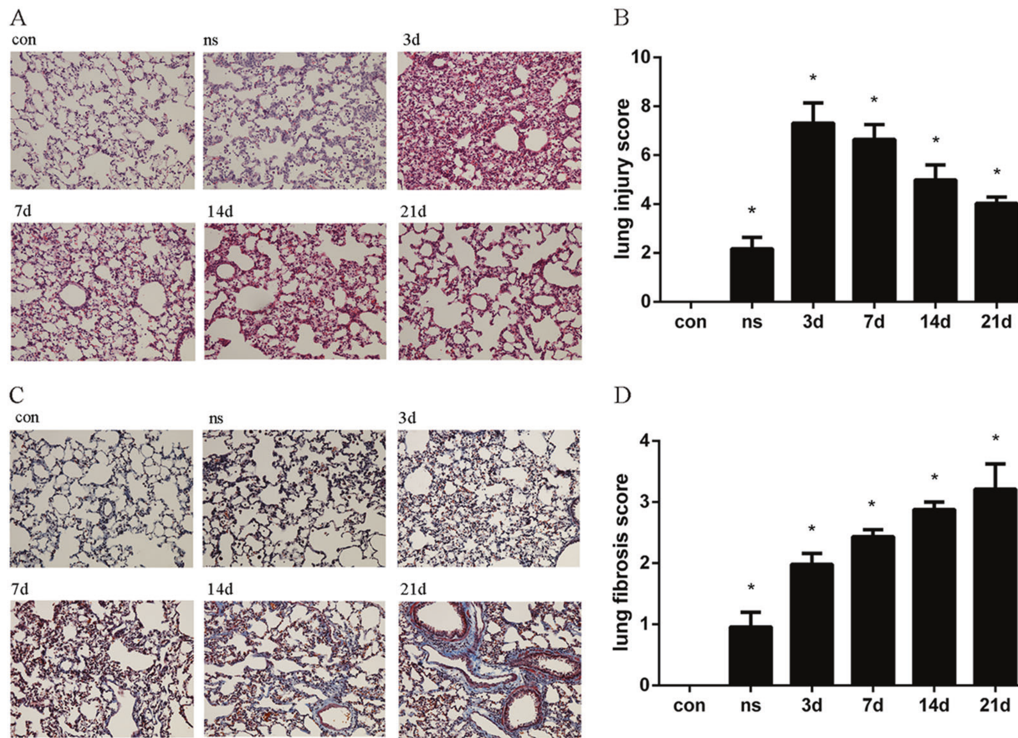

Fig. 1 Pathological changes in rat lung tissue in the development of early pulmonary fibrosis. Representative photographs of the lung tissues with HE staining (**a**) and Masson staining (**c**) in an animal model of early pulmonary fibrosis (original magnification: × 20). Morphological changes in the lung section were examined semi-quantitatively using the lung injury score (**b**) and the Ashcroft score (**d**). The data are expressed as the mean ± SD from at least three replicate experiments. $^{*}P < 0.05$ versus the control group

were still statistically higher than those in the control group ($p < 0.05$). (Fig. 1b).

The Masson staining results indicated that a small quantity of blue-stained collagen fibers, the main components of the extracellular matrix, was observed in the control group. There was no significant change in the lung tissue of the saline group. A few collagen fibers appeared in the lung tissue on the third day after the LPS treatment. The quantity of collagen fibers significantly increased and was concentrated around the vessels and bronchus on the 7th day. The quantity of blue-stained collagen fibers increased, and fibrosis was gradually aggravated over time. A large quantity of collagen fibers was deposited, and typical pulmonary fibrosis formed on the 21st day (Fig. 1c). Similarly, the fibrosis scores showed a progressive increase in the development of LPS-induced early pulmonary fibrosis (Fig. 1d).

The BALF and plasma levels of TGF-β, AngII, and Ang-(1-7) in the development of early pulmonary fibrosis

The ELISA results showed that LPS induced a significant gradual increase in plasma TGF-β levels. The BALF TGF-β levels increased on the 3rd and 7th days and then decreased on the 14th and 21st days, but they were still significantly higher than those of the control group ($p < 0.05$; Fig. 2a, b). The plasma levels of AngII slightly increased on the third day, while the BALF levels of AngII significantly increased after the LPS treatment ($p < 0.05$; Fig. 2c, d). There was no significant difference in the plasma levels of Ang-(1-7) between the LPS group and the control group, and the BALF Ang-(1-7) levels increased slightly on the 21st day group alone ($p < 0.05$; Fig. 2e, f). These results indicated that the balance between AngII and Ang-(1-7) in the plasma and the local lung tissue is destroyed during the development of early pulmonary fibrosis.

The expression of AT1R and Mas protein in the lung tissue during the development of early pulmonary fibrosis

The western blot results showed that the expression of AT1R protein was increased, while the expression of Mas protein was significantly decreased, in LPS-treated rat lung tissue on the 14th and 21st days ($p < 0.05$ vs control; Fig. 3a, b). Immunohistochemical staining exhibited the same results (Fig. 3c, d). These results indicated that AT1R and Mas protein expression levels followed opposing trend

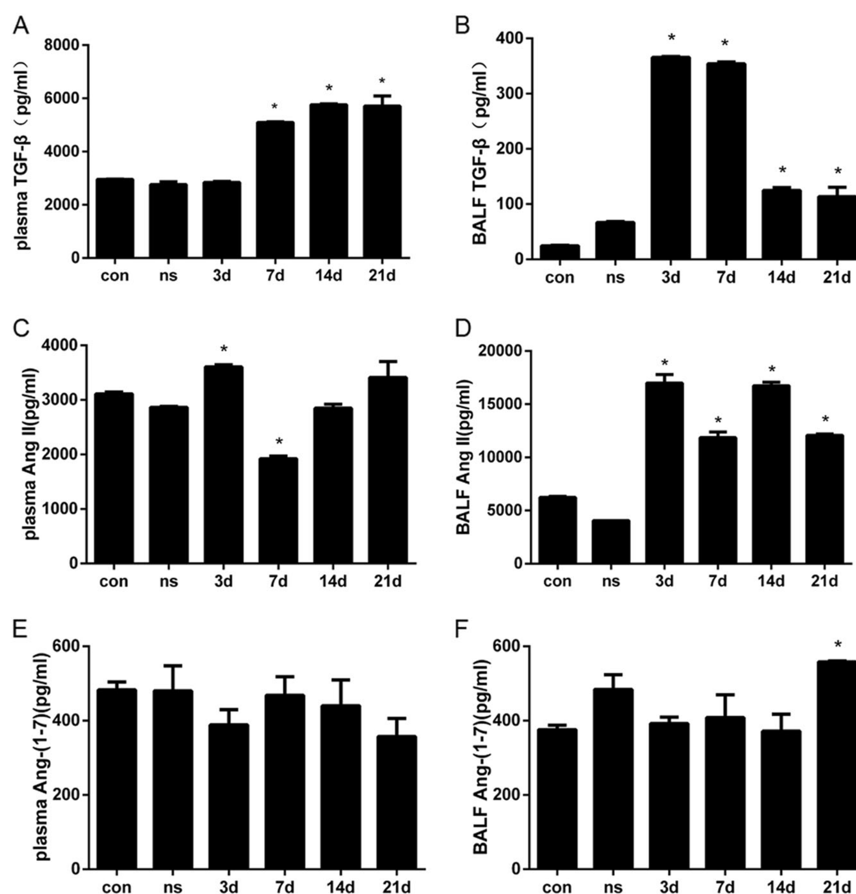

Fig. 2 The BALF and plasma levels of TGF-β, AngII and Ang-(1-7) in the development of early pulmonary fibrosis. The TGF-β levels in plasma (**a**) and BALF (**b**) were significantly increased after LPS treatment. LPS treatment caused a significant increase in AngII levels in BALF (**c**), while the AngII levels in plasma (**d**) were increased only on day 3. The Ang-(1-7) levels in BALF (**e**) were increased on day 21 after LPS treatment, while the Ang-(1-7) levels in plasma (**f**) had no obvious changes. The data are represented as the mean ± SD from at least three replicate experiments. *$P < 0.05$ versus the control group

during the development of LPS-induced early pulmonary fibrosis.

The effects of Ang-(1-7) and Mas receptor agonists and inhibitors on lung injury and early pulmonary fibrosis

HE staining of the lung tissue showed that the LPS treatment resulted in the infiltration of inflammatory cells, thickening and rupture of the alveolar wall and damage of the alveolar structure, and the lung injury score of the LPS group was significantly higher than that of the control group ($p < 0.05$). The LPS-induced lung injury score was significantly improved by Ang-(1-7) or AVE0991 treatment, and the lung injury score was also lower than that of the LPS group ($p < 0.05$). However, the Mas receptor antagonist A779 blocked the protective effects of Ang-(1-7) and AVE0991; the lung injury was significantly aggravated, and the lung injury score was also significantly higher compared with the LPS + Ang-(1-7) or LPS + AVE0991 groups ($p < 0.05$; Fig. 4a, b).

Masson staining showed that LPS increased the deposition of collagen fibers in the lung tissue and caused pulmonary fibrosis, resulting in the fibrosis score of the LPS group to be much higher than that of the control group ($p < 0.05$). Ang-(1-7) or AVE0991 treatment significantly reduced the deposition of collagen fibers, and the fibrosis score was significantly lower than that of the LPS group ($p < 0.05$). A779 treatment blocked the protective effects of Ang-(1-7) and AVE0991, and the deposition of collagen fibers in the lung tissue, along with the fibrosis score, was significantly increased ($p < 0.05$; Fig. 4c, d).

These results indicated that Ang-(1-7) or AVE0991 can reverse LPS-induced pulmonary inflammation and fibrosis. A779 can antagonize the effects of Ang-(1-7) and AVE0991, thus resulting in an increased degree of inflammation and fibrosis in the local lung tissue.

Effects of Ang-(1-7) and Mas receptor agonists and inhibitors on the plasma and BALF levels of TGF-β, AngII, and Ang-(1-7) in LPS-induced early pulmonary fibrosis rats

LPS induced an increase in the plasma and BALF TGF-β levels, and the plasma TGF-β levels were significantly

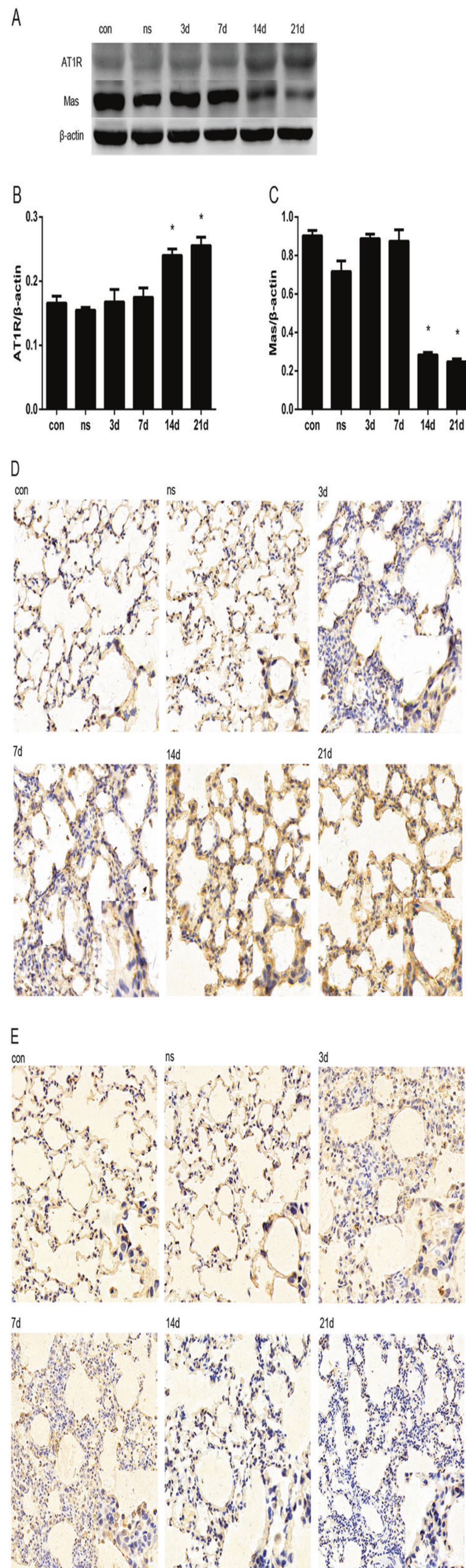

Fig. 3 AT1R and Mas protein levels in the lung tissue in the development of early pulmonary fibrosis. LPS induced a significant increase in AT1R protein levels and a significant decrease in Mas protein levels. The expression levels of AT1R and Mas protein in the lung tissue in an animal model of early pulmonary fibrosis were detected by western blotting (**a**–**c**). Furthermore, the expression levels of AT1R (**d**) and Mas (**e**) proteins in the lung tissue in an animal model of early pulmonary fibrosis were detected via immunohistochemical analysis. AT1R or Mas protein was dyed brown, while blue represented nucleus. All of the data are expressed as the mean ± SD from at least three replicate experiments. *$P < 0.05$ versus the control group

higher than those of the control group ($p < 0.05$). Ang-(1-7) or AVE0991 treatment reduced the BALF TGF-β levels slightly ($p > 0.05$), while the plasma TGF-β levels were decreased significantly more ($p < 0.05$). A779 treatment blocked the effects of Ang-(1-7) and AVE0991, and increased the BALF levels of TGF-β significantly ($p < 0.05$; Fig. 5a, b). LPS also upregulated the AngII levels, while Ang-(1-7) treatment blocked the increase of AngII level in BALF, and AVE0991 reversed the increase of AngII level in plasma and BALF. Furthermore, A779 treatment reversed the effect of LPS + Ang-(1-7) in BALF significantly ($p < 0.05$; Fig. 5c, d). Exogenous Ang-(1-7) treatment contributed to the increases in plasma Ang-(1-7) levels significantly ($p < 0.05$) and increases BALF Ang-(1-7) levels slightly ($p > 0.05$). However, A779 treatment decreased the plasma and BALF Ang-(1-7) levels and as compared with LPS + Ang-(1-7) group, the difference was not statistically significant ($p >$ 0.05; Fig. 5e, f).

These results indicated that LPS led to an imbalance of systemic and local AngII and Ang-(1-7) levels. Either Ang-(1-7) or AVE0991 treatment was able to restore this balance, and these effects were again blocked by the Mas inhibitor A779.

The effects of Ang-(1-7) and Mas receptor agonists and inhibitors on the expression of E-cadherin, Vimentin, AT1R and Mas in the lung tissue of LPS-induced early pulmonary fibrosis rats

The western blot data showed that the LPS treatment downregulated the expression of E-cadherin levels and upregulated the levels of Vimentin protein in the lung tissue ($p < 0.05$, vs control). Ang-(1-7) or AVE0991 treatment significantly reversed the decrease in E-cadherin levels and the increase in Vimentin levels ($p < 0.05$). However, A779 treatment blocked the effects of Ang-(1-7) and AVE0991, and the expression of E-cadherin protein was decreased, while the Vimentin protein levels were increased ($p < 0.05$; Fig. 6a–c). These results indicated that LPS induced epithelial-mesenchymal transition in the lung tissue, and Ang-(1-7) or AVE0991 treatment inhibited the process, while A779 treatment blocked the effects of Ang-(1-7) and AVE0991.

There was no significant difference in AT1R mRNA expression levels between the LPS and control groups.

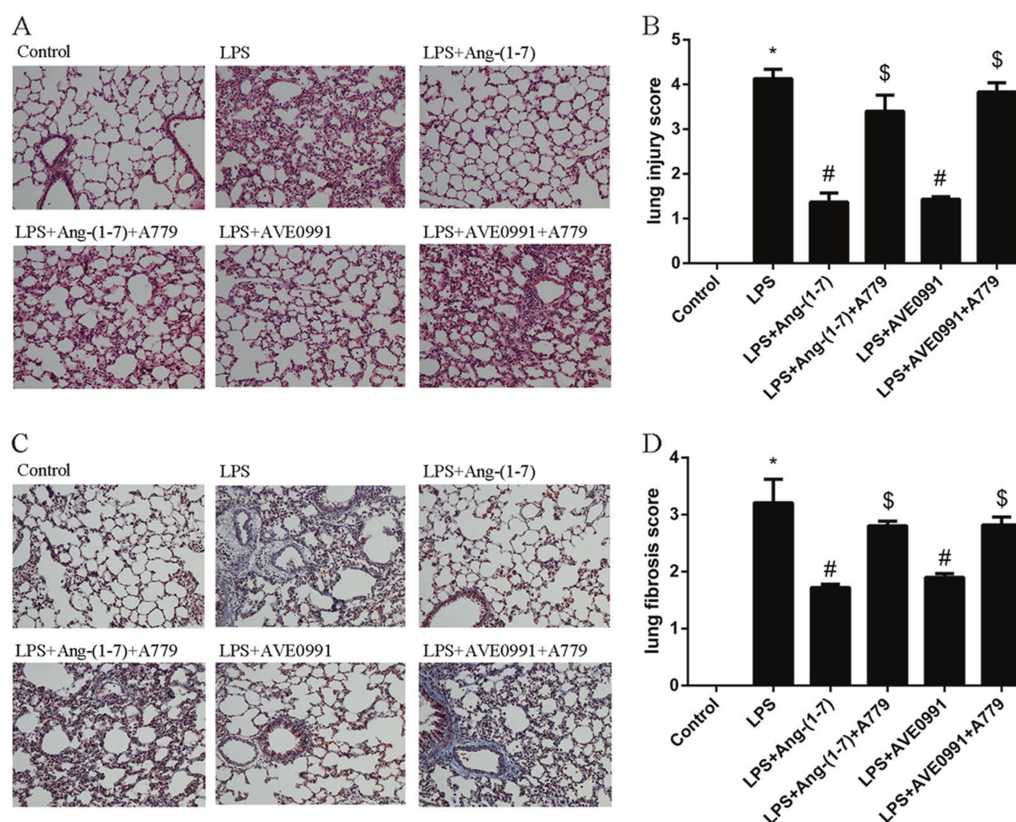

Fig. 4 The effects of Ang-(1-7) and Mas receptor agonists and inhibitors on lung injury and early pulmonary fibrosis. Representative photomicrographs of the lung tissues with HE staining (**a**) and Masson staining (**c**) in Ang-(1-7), A779, or AVE0991 intervention groups. Morphological changes in the lung section were examined semiquantitatively using the lung injury score (**b**) and the Ashcroft score (**d**). All of the data are expressed as the mean ± SD from three replicate experiments. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the LPS group; $^{\$}P < 0.05$ versus the LPS + Ang-(1-7) or LPS + AVE0991 groups

Ang-(1-7) or AVE0991 treatment decreased AT1R mRNA expression levels ($p < 0.05$), while A779 treatment blocked the effects of Ang-(1-7), thus resulting in increased AT1R mRNA levels ($p < 0.05$). LPS induced a decrease in Mas mRNA levels, but there was no significant difference between the LPS and control groups. Ang-(1-7) treatment significantly increased Mas mRNA expression levels ($p < 0.05$), while A779 treatment reversed this increase ($p < 0.05$). AVE0991 treatment did not affect Mas expression levels (Fig. 6d, e). These results indicated that Ang-(1-7) decreased AT1R mRNA expression levels and increased Mas mRNA expression levels, while A779 blocked the effects of Ang-(1-7).

The total AT1R protein levels were increased in the LPS group compared with the control group. Ang-(1-7) or AVE0991 treatment decreased total AT1R protein levels, and A779 treatment antagonized the effect of Ang-(1-7; $p < 0.05$). The total Mas protein levels also significantly increased in the LPS-treated lung tissue, which was decreased by Ang-(1-7) treatment and increased by A779 treatment ($p < 0.05$; Fig. 6e–g).

The effects of Ang-(1-7) and Mas receptor agonists and inhibitors on the levels of AT1R and Mas in the cell membranes and cytoplasm of the lung tissue in the LPS-induced early pulmonary fibrosis rat models

For further investigation, we measured the expression levels of AT1R and Mas protein in the membrane and cytoplasm. LPS slightly increased the expression levels of AT1R protein in the membrane ($p > 0.05$). However, the LPS-induced AT1R protein levels of the membrane were downregulated by Ang-(1-7) treatment, which were then increased by A779 treatment. Interestingly, there was no significant difference in the AT1R expression levels in the cytoplasm between the LPS and control groups, and Ang-(1-7) treatment did not affect the AT1R expression levels in the cytoplasm (Fig. 7a, c, e). In addition, compared with the control group, there was no significant difference in the Mas expression levels of the membranes in the LPS group, while Ang-(1-7) treatment induced a significantly increased expression of membrane Mas protein levels ($p < 0.05$). LPS upregulated the expression levels of Mas protein in the cytoplasm, which was

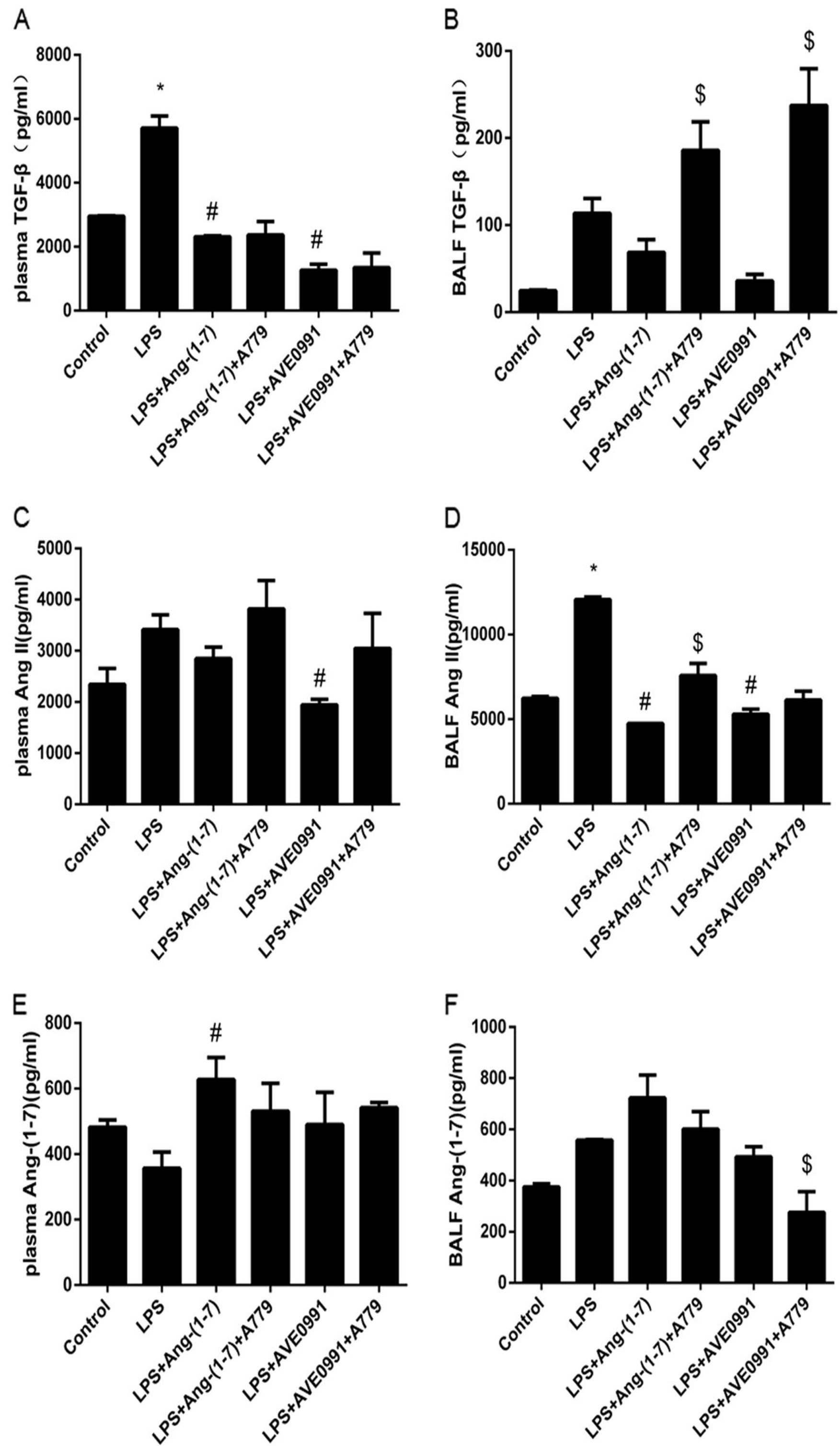

Fig. 5 The effects of Ang-(1-7) and Mas receptor agonists and inhibitors on the plasma and BALF levels of TGF-β, AngII, and Ang-(1-7) in rats with LPS-induced early pulmonary fibrosis in rats. The TGF-β levels in plasma (**a**) and BALF (**b**) were significantly decreased after Ang-(1-7) or AVE0991 treatment and restored by A779 treatment. Ang-(1-7) or AVE0991 treatment caused a significant decrease in plasma and BALF AngII levels, whereas these effects were abolished by A779 administration (**c**, **d**). The Ang-(1-7) levels in plasma (**e**) and BALF (**f**) were significantly increased after Ang-(1-7) treatment, which were restored by A779 treatment. The data are represented as the mean ± SD from at least three replicate experiments. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the LPS group; $^{\$}P < 0.05$ versus the LPS + Ang-(1-7) or LPS + AVE0991 groups

blocked by Ang-(1-7) treatment. A779 treatment restored the Mas protein levels of the cytoplasm ($p < 0.05$; Fig. 7b, d, f). There was no significant difference in AT1R and Mas protein expression levels between the LPS + AVE0991 and LPS groups.

These results indicated that LPS induced increased AT1R and decreased the Mas protein ratio of the membrane and cytoplasm, which were reversed by Ang-(1-7). A779 antagonized the effect of Ang-(1-7). AVE0991 did not affect LPS-induced AT1R and Mas protein expression,

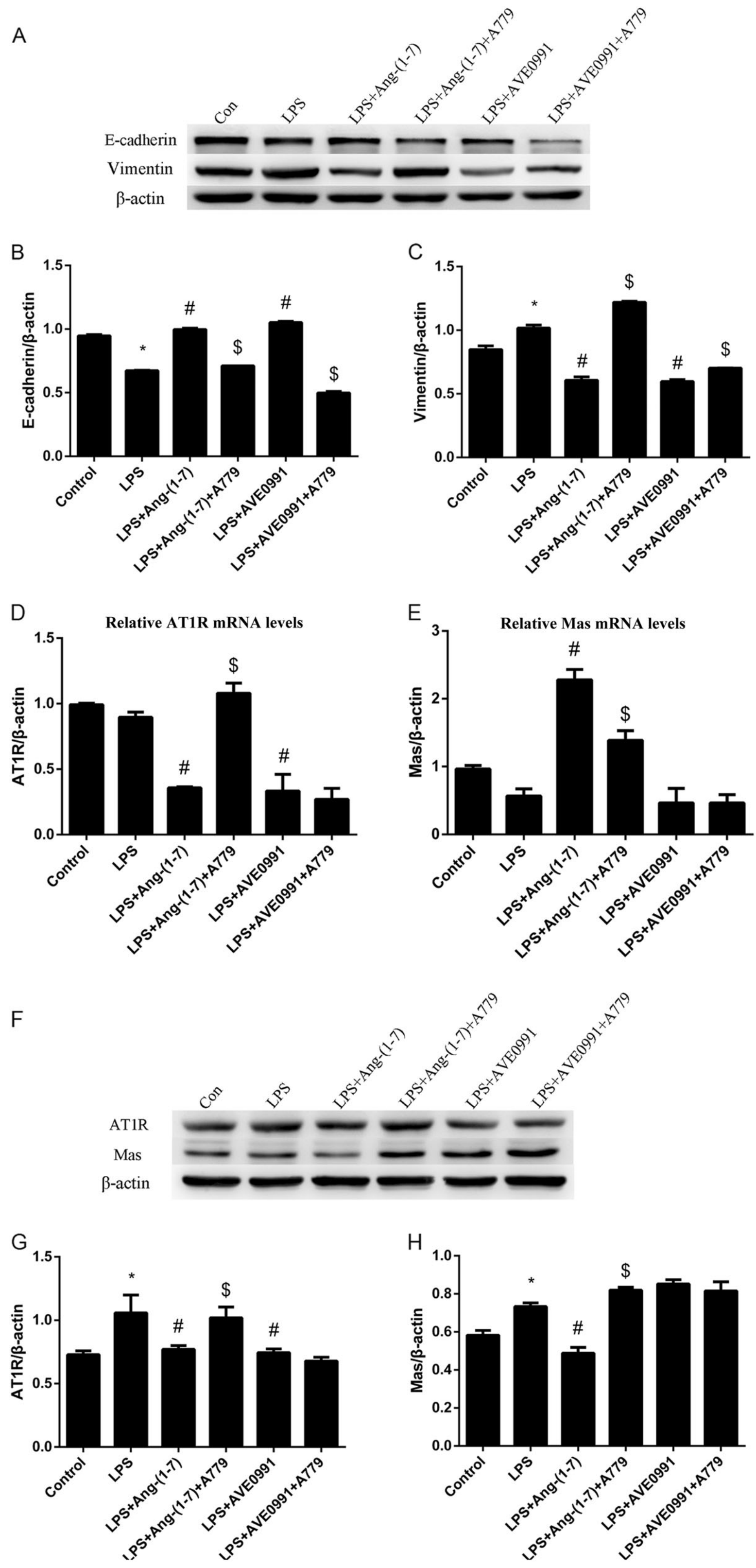

Fig. 6 The effects of Ang-(1-7) and Mas receptor agonists and inhibitors on the expression levels of E-cadherin, Vimentin, AT1R and Mas in the lung tissue of rats with LPS-induced early pulmonary fibrosis. The expression levels of E-cadherin and Vimentin proteins in the lung tissue in the Ang-(1-7), A779, or AVE0991 intervention groups in an animal model of early pulmonary fibrosis were detected via western blotting (Fig. 6a–c). The expression levels of AT1R mRNA and protein, as well as Mas mRNA and protein, in the lung tissue in Ang-(1-7), A779, or AVE0991 intervention groups in an animal model of early pulmonary fibrosis were quantitatively analyzed using real-time PCR (Fig. 6d, e) and western blotting (Fig. 6f, g). The data are represented as the mean ± SD from six replicate experiments. *$P < 0.05$ versus the control group; #$P < 0.05$ versus the LPS group; \$$P < 0.05$ versus the LPS + Ang-(1-7) or LPS + AVE0991 group

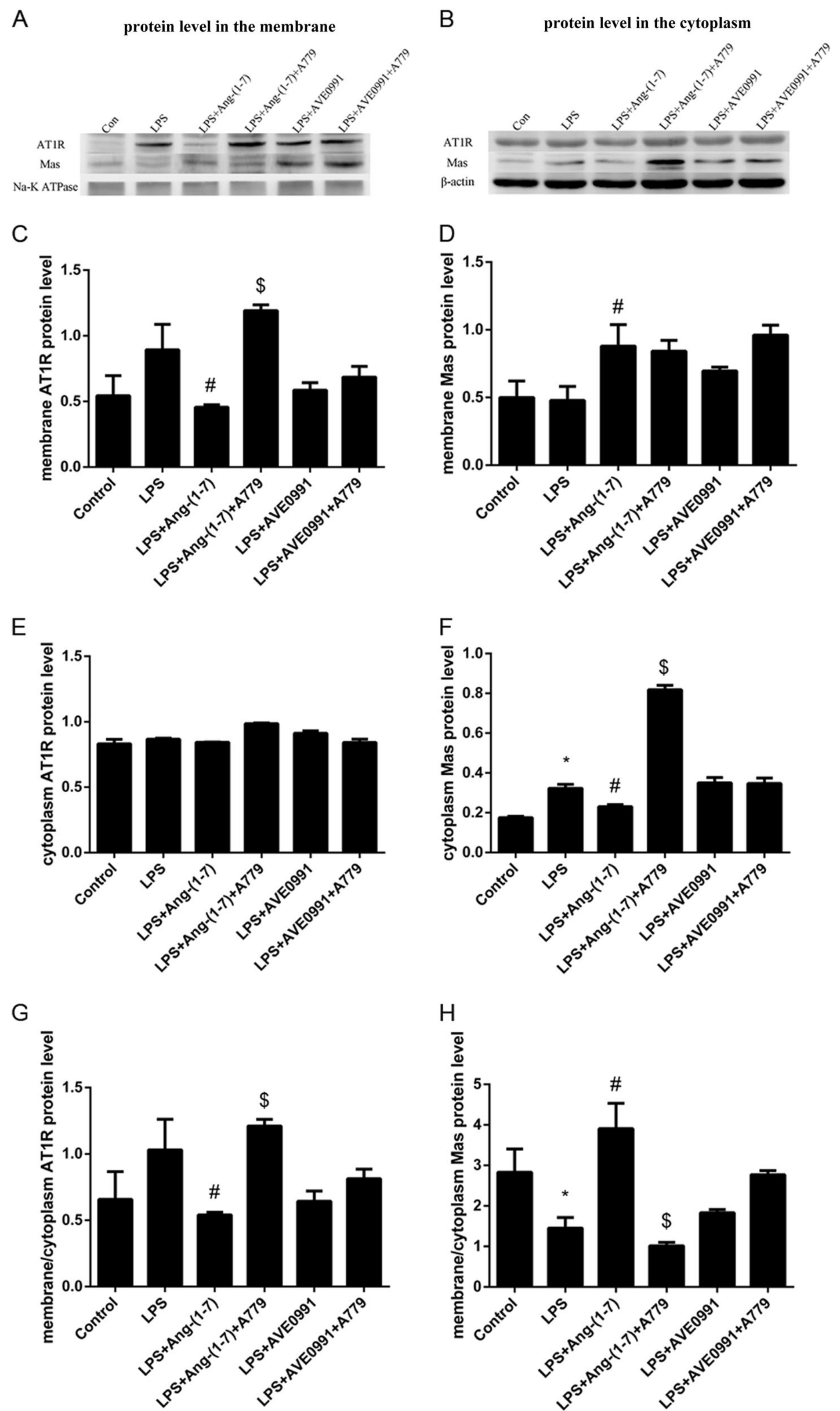

Fig. 7 The effects of Ang-(1-7) and Mas receptor agonists and inhibitors on the AT1R and Mas proteins in the membranes and cytoplasm of the lung tissue in rats with LPS-induced early pulmonary fibrosis. The expression levels of AT1R and Mas proteins of the membranes and cytoplasm in the lung tissue in Ang-(1-7), A779, or AVE0991 intervention groups in an animal model of early pulmonary fibrosis were detected via western blotting (Fig. 7a–h). The expression levels of AT1R and Mas proteins in the membrane (**a**) and cytoplasm (**b**) respectively, and the membrane protein levels were normalized to Na-K ATPase and cytoplasm protein were normalized to β-actin. All of the data are expressed as the mean ± SD from at least three replicate experiments. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the LPS group; $^{\$}P < 0.05$ versus the LPS + Ang-(1-7) or LPS + AVE0991 groups

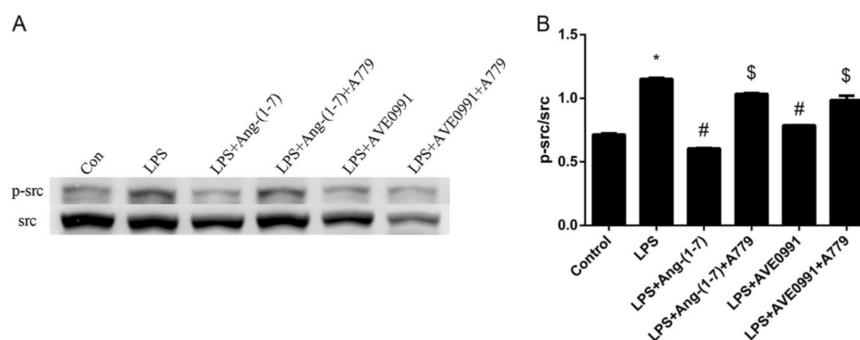

Fig. 8 The effects of Ang-(1-7) and Mas receptor agonists and inhibitors on the phosphorylation of Src kinase in the lung tissue of rats with LPS-induced early pulmonary fibrosis. The expression levels of Src kinase phosphorylation in the lung tissue in the Ang-(1-7), A779, or AVE0991 intervention groups in an animal model of early pulmonary fibrosis were detected via western blotting. All of the data are expressed as the mean ± SD from six replicate experiments. $^{*}P < 0.05$ versus the control group; $^{\#}P < 0.05$ versus the LPS group; $^{\$}P < 0.05$ versus the LPS + Ang-(1-7) or LPS + AVE0991 groups. Ang-(1-7) inhibits LPS-induced early pulmonary fibrosis by binding to the Mas receptor, and these effects of Ang-(1-7) are related to RAS activity in local lung tissues. The mechanism may primarily act to restore the balance of local AngII/AT1R and Ang-(1-7)/Mas expression and to block the activation of the Src signaling pathway

regardless of whether this expression was in the membrane or cytoplasm.

The effects of Ang-(1-7) and Mas receptor agonists and inhibitors on the phosphorylation of Src kinase in the lung tissue of LPS-induced early pulmonary fibrosis in rats

The western blot results showed that LPS induced an increased phosphorylation of Src kinase. Ang-(1-7) or AVE0991 treatment significantly inhibited the LPS-induced phosphorylation of Src kinase ($p < 0.05$); however, A779 treatment blocked the effects of Ang-(1-7) and AVE0991, and the phosphorylation of Src kinase was significantly increased ($p < 0.05$; Fig. 8). These results indicated that Src kinase is activated in LPS-induced ARDS and that Src kinase may be involved in the role of Ang-(1-7).

Discussion

Sepsis is an important factor in ARDS, which is one of the most common diseases in the intensive care unit (ICU) [34]. In previous animal studies, bleomycin or LPS alone was used to establish a chronic pulmonary fibrosis model [35, 36]. However, the LPS three-hit method was used to induce early pulmonary fibrosis following ARDS [31]. In the present study, the HE and Masson staining results showed that the inflammation response and pulmonary fibrosis significantly increased in an LPS-induced early pulmonary fibrosis animal model and that the lung injury and fibrosis scores in the LPS group were significantly higher than those of the control group. Therefore, our results demonstrated that LPS causes significant lung injury and early pulmonary fibrosis.

TGF-β can promote the pulmonary epithelial to mesenchymal transition and plays an important role in the process of pulmonary fibrosis [37–39]. In this study, LPS induced increased plasma TGF-β levels in the development of early pulmonary fibrosis, while BALF TGF-β levels increased on days 3 and 7, after which these levels declined on days 14 and 21. Interestingly, plasma TGF-β levels were significantly higher than those of BALF. It may be that plasma TGF-β, but not local TGF-β, plays an important role in the development of early pulmonary fibrosis.

EMT plays an important role in pulmonary fibrosis, which is further aggravated through the increased collagen fibers that are secreted by myofibroblasts [40]. Yttria has been shown to promote EMT, thus leading to pulmonary fibrosis in a model of rat pulmonary fibrosis induced by cerium oxide [41]. miR-221 has been shown to inhibit bleomycin-induced pulmonary fibrosis by regulating TGF-β1/Smad3-mediated EMT [42]. In this study, LPS decreased the expression of the epithelial marker E-cadherin and increased the expression of the interstitial marker Vimentin, thus indicating that LPS induced EMT in lung tissue.

The RAS, an important part of the neuroendocrine system, not only regulates blood pressure, water, and electrolyte balance but also plays an extremely important role in the regulation of inflammation and fibrosis [43]. Two major axis systems (ACE/AngII/AT1R and ACE2/Ang-(1-7)/Mas) compose the RAS and play opposing biological roles [44]. The former system promotes inflammation, fibrosis and vasoconstriction, while the latter system inhibits inflammation and fibrosis [45]. The balance of these two axes maintains the steady state of the body, and an imbalance leads to the development of disease [46]. Our results showed that LPS significantly increased AngII levels and slightly increased Ang-(1-7) levels in BALF, thus indicating that the balance of AngII and Ang-(1-7) was disrupted in

the process of LPS-induced pulmonary fibrosis. In addition, we found the increase of AngII and Ang-(1-7) levels in BALF are more significant than that of plasma, which may be due to that the plasma AngII level reflects the state of whole body while the BALF AngII level indicates a state of the local lung tissue. The western blot and immunohistochemistry results showed that AT1R protein expression gradually increased, while Mas protein expression gradually decreased, in the lung tissue in the development of early pulmonary fibrosis. Importantly, we also found that LPS increased AT1R and Mas protein expression, but had no effect on its mRNA levels, which may be ascribe to the post-transcriptional mechanism of AT1R and Mas expression. Therefore, we conclude that LPS activates AngII/AT1R and inhibits Ang-(1-7)/Mas in local lung tissues, which may be closely related to the occurrence and progression of early pulmonary fibrosis.

AngII/AT1R and Ang-(1-7)/Mas are reciprocal, antagonistic axes of the RAS, and the imbalance of these axes has an important impact on the occurrence and progression of early pulmonary fibrosis [9]. Previous studies have found that Ang-(1-7) can reduce AT1R mRNA and protein expression levels and increase Mas mRNA expression levels in a bleomycin-induced animal model of pulmonary fibrosis [8, 9, 17]. In addition, A779 has been shown to antagonize the effects of Ang-(1-7) in acid aspiration-induced lung injury [13], and AVE0991 has been shown to exert a similar protective effect as Ang-(1-7) and elevate the levels of AT1R and Mas proteins in an ovalbumin-induced asthma animal model [47]. Our study showed that Ang-(1-7) increased the expression levels of E-cadherin, reduced the expression levels of Vimentin and TGF-β and mitigated lung injury and pulmonary fibrosis. Ang-(1-7) also decreased AngII levels and increased Ang-(1-7) levels in the plasma and BALF, decreased AT1R mRNA expression levels and increased Mas mRNA levels in the lung tissue in LPS-induced rat model. AVE0991, a non-peptide analog of Ang-(1-7), plays a similar role in the process of early pulmonary fibrosis. However, the Mas receptor antagonist A779 can block the effects of Ang-(1-7) and AVE0991 and decrease the expression of E-cadherin, as well as increase the levels of Vimentin and TGF-β, thus aggravating inflammation and fibrosis. These results indicate that Ang-(1-7) exerts a pulmonary protective effect by binding to the Mas receptor.

Interestingly, our experimental results showed that Ang-(1-7) reduced AT1R mRNA levels and elevated Mas mRNA levels, which were blocked by A779 and then reversed by AVE0991. However, AT1R and Mas protein expression levels were more complicated. LPS induced an increased expression of AT1R and Mas protein levels, which were significantly decreased by Ang-(1-7) and significantly increased by A779 co-administration. Afterwards, we investigated the expression levels of AT1R and Mas proteins in the membrane and cytoplasm, respectively. LPS increased the levels of membrane AT1R protein, which were decreased by Ang-(1-7) and increased again by A779 treatment. AT1R protein expression levels in the cytoplasm did not significantly change. Mas protein expression levels in the membrane were not significantly changed after LPS treatment but were increased by Ang-(1-7). The Mas protein levels in the cytoplasm were increased by LPS, decreased by Ang-(1- 7) treatment and increased again by A779. The ratio of Mas protein in the membrane and cytoplasm was decreased by LPS, increased by Ang-(1-7), and decreased again by A779. These results indicated that Ang-(1-7) reduced AT1R levels in the cell membrane, while Mas levels were increased, AT1R levels in the cytoplasm remained unchanged and Mas levels were decreased, thus indicating that Ang-(1-7) may act through agonistic effects on Mas. It reduces the transport of AT1R to the cell membrane and promotes the transport of Mas to the cell membrane, while AVE0991 may directly act as an agonist.

LPS increased AT1R and Mas protein levels, and the biological effects of AT1R protein in the membrane increased and the Mas protein levels decreased. Ang-(1-7) induced a decrease in the total levels of AT1R and Mas proteins but reduced the levels of AT1R and Mas proteins in the membrane, which were eliminated by A779. In addition, A779 promoted the synthesis of Mas protein, which may be due to the combination of A779 and Mas receptors affecting Mas expression by some related signal pathways.

In addition, we found that the phosphorylation of Src kinase was induced by LPS, decreased by Ang-(1-7) and increased by A779. This suggests that Ang-(1-7) may inhibit the phosphorylation of Src kinase by binding to the Mas receptor, thereby inhibiting the occurrence and progression of early pulmonary fibrosis.

Src kinase is involved in signal transduction and is activated by extracellular factors, such as TGF-β1, AngII, epidermal growth factor (EGF), and the extracellular matrix (ECM) [26]. Activated Src kinase can induce the phosphorylation of signal transducers and activators of transcription 3 (STAT3), protein kinase B (PKB/Akt), and epidermal growth factor receptor (EGFR) signaling proteins, and can also promote the TGF-β1-mediated activation of Smad3, thereby promoting EMT and the metastasis of tumor cells and tissue fibrosis [29]. We found that LPS increased the phosphorylation levels of Src kinase and that the phosphorylation levels were decreased by Ang-(1-7) and increased by A779. This suggests that Ang-(1-7) may inhibit the phosphorylation of Src kinase by binding to the Mas receptor, thereby inhibiting the occurrence and progression of early pulmonary fibrosis after ARDS.

In summary, Ang-(1-7) inhibits LPS-induced early pulmonary fibrosis by binding to the Mas receptor, and these effects of Ang-(1-7) are related to RAS activity in local lung tissues. The mechanism may primarily act to restore the balance of local AngII/AT1R and Ang-(1-7)/Mas expression and to block the activation of the Src signaling pathway.

Acknowledgements This work was supported by the Natural Science Foundation of Shanghai (No.18ZR1428800).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

1. Marshall RP, Webb S, Bellingan GJ, Montgomery HE, Chaudhari B, McAnulty RJ, et al. Angiotensin converting enzyme insertion/deletion polymorphism is associated with susceptibility and outcome in acute respiratory distress syndrome. Am J Respir Crit Care Med. 2002;166:646–50.
2. Jerng JS, Yu CJ, Wang HC, Chen KY, Cheng SL, PC. Y. Polymorphism of the angiotensin-converting enzyme gene affects the outcome of acute respiratory distress syndrome. Crit Care Med. 2006;34:1001–6.
3. Marshall R, Bellingan G, Webb S, Puddicombe A, Goldsack N, McAnulty R, et al. Fibroproliferation occurs early in the acute respiratory distress syndrome and impacts on outcome. Am J Respir Crit Care Med. 2000;162:1783–8.
4. Burnham EL, Janssen WJ, Riches DW, Moss M, Downey GP. The fibroproliferative response in acute respiratory distress syndrome: mechanisms and clinical significance. Eur Respir J. 2014;43:276–85.
5. Satar M, Taşkın E, Özlü F, Tuli A, Özcan K, Yıldızdaş HY. Polymorphism of the angiotensin-converting enzyme gene and angiotensin-converting enzyme activity in transient tachypnea of neonate and respiratory distress syndrome. J Matern Fetal Neonatal Med. 2012;25:1712–5.
6. Marshall RP, McAnulty RJ, Laurent GJ. Angiotensin II is mitogenic for human lung fibroblasts via activation of the type 1 receptor. Am J Respir Crit Care Med. 2000;161:1999–2004.
7. Wösten-van Asperen RM, Lutter R, Specht PA, Moll GN, van Woensel JB, van der Loos CM, et al. Acute respiratory distress syndrome leads to reduced ratio of ACE/ACE2 activities and is prevented by angiotensin-(1-7) or an angiotensin II receptor antagonist. J Pathol. 2011;225:618–27.
8. Meng Y, Li T, Zhou GS, Chen Y, Yu CH, Pang MX, et al. The angiotensin-converting enzyme 2/angiotensin (1-7)/Mas axis protects against lung fibroblast migration and lung fibrosis by inhibiting the NOX4-derived ROS-mediated RhoA/Rho kinase pathway. Antioxid Redox Signal. 2015;22:241–58.
9. Meng Y, Yu CH, Li W, Li T, Luo W, Huang S, et al. Angiotensin-converting enzyme 2/angiotensin-(1-7)/Mas axis protects against lung fibrosis by inhibiting the MAPK/NF-kappaB pathway. Am J Respir Cell Mol Biol. 2014;50:723–36.
10. Marshall R, Gohlke P, Chambers R, Howell D, Bottoms S, Unger T, et al. Angiotensin II and the fibroproliferative response to acute lung injury. Am J Physiol Lung Cell Mol Physiol. 2004;286:L156–164.
11. Okada M, Suzuki K, Matsumoto M, Takada K, Nakanishi T, Horikoshi H, et al. Effects of angiotensin on the expression of fibrosis-associated cytokines, growth factors, and matrix proteins in human lung fibroblasts. J Clin Pharm Ther. 2009;34:288–99.
12. Santos-Silva MA, Pires KM, Trajano ET, Martins V, Nesi RT, Benjamin CF, et al. Redox imbalance and pulmonary function in bleomycin-induced fibrosis in C57BL/6, DBA/2, and BALB/c mice. Toxicol Pathol. 2012;40:731–41.
13. Klein N, Gembardt F, Supé S, Kaestle SM, Nickles H, Erfinanda L, et al. Angiotensin-(1-7) protects from experimental acute lung injury. Crit Care Med. 2013;41:e334–43.
14. Simoes e Silva AC, Silveira KD, Ferreira AJ, Teixeira MM. ACE2, angiotensin-(1-7) and Mas receptor axis in inflammation and fibrosis. Br J Pharmacol. 2013;169:477–92.
15. Uhal BD, Li X, Xue A, Gao X, Abdul-Hafez A. Regulation of alveolar epithelial cell survival by the ACE-2/angiotensin 1-7/Mas axis. Am J Physiol Lung Cell Mol Physiol. 2011;301:L269–74.
16. Zhang H, Sun GY. LPS induces permeability injury in lung microvascular endothelium via AT(1) receptor. Arch Biochem Biophys. 2005;441:75–83.
17. Shenoy V, Ferreira AJ, Qi Y, Fraga-Silva RA, Diez-Freire C, Dooies A, et al. The angiotensin-converting enzyme 2/angiogenesis-(1-7)/Mas axis confers cardiopulmonary protection against lung fibrosis and pulmonary hypertension. Am J Respir Crit Care Med. 2010;182:1065–72.
18. Tellez CS, Juri DE, Do K, Bernauer AM, Thomas CL, Damiani LA, et al. EMT and stem cell-like properties associated with miR-205 and miR-200 epigenetic silencing are early manifestations during carcinogen-induced transformation of human lung epithelial cells. Cancer Res. 2011;71:3087–97.
19. Kim YS, Yi BR, Kim NH, Choi KC. Role of the epithelial-mesenchymal transition and its effects on embryonic stem cells. Exp Mol Med. 2014;46:e108.
20. Chapman HA. Epithelial-mesenchymal interactions in pulmonary fibrosis. Annu Rev Physiol. 2011;73:413–35.
21. Rodrigues-Díez R, Carvajal-González G, Sánchez-López E, Rodríguez-Vita J, Rodrigues Díez R, Selgas R, et al. Pharmacological modulation of epithelial mesenchymal transition caused by angiotensin II. Role of ROCK and MAPK pathways. Pharm Res. 2008;25:2447–61.
22. Murdoch CE, Chaubey S, Zeng L, Yu B, Ivetic A, Walker SJ, et al. Endothelial NADPH oxidase-2 promotes interstitial cardiac fibrosis and diastolic dysfunction through proinflammatory effects and endothelial-mesenchymal transition. J Am Coll Cardiol. 2014;63:2734–41.
23. Zhang LL, Huang S, Ma XX, Zhang WY, Wang D, Jin SY, et al. Angiotensin-(1–7) attenuated angiotensin II-induced hepatocyte EMT by inhibiting NOX-derived H2O2-activated NLRP3 inflammasome IL-1β Smad circuit. Free Radic Biol Med. 2016;9:531–43.
24. Zhou L, Xue H, Wang Z, Ni J, Yao T, Huang Y. et al. Angiotensin-(1-7) attenuates high glucose-induced proximal tubular epithelial-to-mesenchymal transition via inhibiting ERK1/2 and p38 phosphorylation. Life Sci. 2012;90:454–62.
25. Qian YR, Guo Y, Wan HY, Fan L, Feng Y, Ni L, et al. Angiotensin-converting enzyme 2 attenuates the metastasis of non-small cell lung cancer through inhibition of epithelial-mesenchymal transition. Oncol Rep. 2013;29:2408–14.
26. Roskoski R Jr. Src protein-tyrosine kinase structure and regulation. Biochem Biophys Res Commun. 2004;324:1155–64.
27. Cunningham AJ. Acute respiratory distress syndrome-two decades later. Yale J Biol Med. 1991;64:387–402.

28. Shao K, Chen ZY, Gautam S, Deng NH, Zhou Y, Wu XZ. Post-translational modification of E-cadherin by core fucosylation regulates Src activation and induces epithelial-mesenchymal transition-like process in lung cancer cells. Glycobiology. 2016;26:142–54.
29. Yan Y, Ma L, Zhou X, Ponnusamy M, Tang J, Zhuang MA, et al. Src inhibition blocks renal interstitial fibroblast activation and ameliorates renal fibrosis. Kidney Int. 2016;89:68–81.
30. Souza LL, Costa-Neto CM. Angiotensin-(1-7) decreases LPS-induced inflammatory response in macrophages. J Cell Physiol. 2012;227:2117–22.
31. Li H, Du S, Yang L, Chen Y, Huang W, Zhang R, et al. Rapid pulmonary fibrosis induced by acute lung injury via a lipopolysaccharide three-hit regimen. Innate Immun. 2009;15:143–54.
32. Li Y, Zeng Z, Li Y, Huang W, Zhou M, Zhang X, et al. Angiotensin-converting enzyme inhibition attenuates lipopolysaccharide-induced lung injury by regulating the balance between angiotensin-converting enzyme and angiotensin-converting enzyme 2 and inhibiting mitogen-activated protein kinase activation. Shock. 2015;43:395–404.
33. Chen Q, Yang Y, Huang Y, Pan C, Liu L, Qiu H. Angiotensin-(1-7) attenuates lung fibrosis by way of Mas receptor in acute lung injury. J Surg Res. 2013;185:740–7.
34. Ning F, Wang X, Shang L, Wang T, Lv C, Qi Z, et al. Low molecular weight heparin may prevent acute lung injury induced by sepsis in rats. Gene. 2015;557:88–91.
35. Ma B, Zhou PY, Ni W, Wei W, Ben D, Lu W, et al. Inhibition of activin receptor-like kinase 5 induces matrix metallopeptidase 9 expression and aggravates lipopolysaccharide-induced pulmonary injury in mice. Eur Rev Med Pharmacol Sci. 2013;17:1051–9.
36. Lieber GB, Fernandez X, Mingo GG, Jia Y, Caniga M, Gil MA, et al. Mineralocorticoid receptor antagonists attenuate pulmonary inflammation and bleomycin-evoked fibrosis in rodent models. Eur J Pharmacol. 2013;718:290–8.
37. Goodwin A, Jenkins G. Role of integrin-mediated TGFbeta activation in the pathogenesis of pulmonary fibrosis. Biochem Soc Trans. 2009;37:849–54.
38. Breen E, Shull S, Burne S, Absher M, Kelley J, Phan S, et al. Bleomycin regulation of transforming growth factor-beta mRNA in rat lung fibroblasts. Am J Respir Cell Mol Biol. 1992;6:146–52.
39. Azuma A, Li YJ, Abe S, Usuki J, Matsuda K, Henmi S, et al. Interferon-{beta} inhibits bleomycin-induced lung fibrosis by decreasing transforming growth factor-{beta} and thrombospondin. Am J Respir Cell Mol Biol. 2005;32:93–8.
40. Kalluri R, Neilson EG. Epithelial-mesenchymal transition and its implications for fibrosis. J Clin Invest. 2003;112:1776–84.
41. Ma J, Bishoff B, Mercer RR, Barger M, Schwegler-Berry D, Castranova V. Role of epithelial-mesenchymal transition (EMT) and fibroblast function in cerium oxide nanoparticles-induced lung fibrosis. Toxicol Appl Pharmacol. 2017;323:16–25.
42. Wang YC, Liu JS, Tang HK, Nie J, Zhu JX, Wen LL, et al. miR221 targets HMGA2 to inhibit bleomycininduced pulmonary fibrosis by regulating TGFbeta1/Smad3-induced EMT. Int J Mol Med. 2016;38:1208–16.
43. Weir MR, Dzau VJ. The renin-angiotensin-aldosterone system: a specific target for hypertension management. Am J Hypertens. 1999;12:205S–13S.
44. de Man FS, Tu L, Handoko ML, Rain S, Ruiter G, Francois C, et al. Dysregulated renin-angiotensin-aldosterone system contributes to pulmonary arterial hypertension. Am J Respir Crit Care Med. 2012;186:780–9.
45. Santos RA, Ferreira AJ, Verano-Braga T, Bader M. Angiotensin-converting enzyme 2, angiotensin-(1-7) and Mas: new players of the renin-angiotensin system. J Endocrinol. 2013;216:R1–17.
46. Sarzani R, Salvi F, Dessì-Fulgheri P, Rappelli A. Renin-angiotensin system, natriuretic peptides, obesity, metabolic syndrome, and hypertension: an integrated view in humans. J Hypertens. 2008;26:831–43.
47. Rodrigues-Machado MG, Magalhaes GS, Cardoso JA, Kangussu LM, Murari A, Caliari MV, et al. AVE 0991, a non-peptide mimic of angiotensin-(1-7) effects, attenuates pulmonary remodelling in a model of chronic asthma. Br J Pharmacol. 2013;170:835–46.