ACE2 inhibits lipopolysaccharide-caused lung fibrosis via downregulating the TGF-β1/Smad2/Smad3 pathway

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Running title: ACE2 inhibits LPS-caused lung fibrosis

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The number of text pages: 23;
The number of tables: 0;
The number of figures: 7;
The number of references: 49;
The number of words in the Abstract: 254;
The number of words in Introduction: 751
The number of words in Discussion: 1360

Non-standard abbreviation list

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>ACE2</td>
<td>angiotensin-converting enzyme 2</td>
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<td>ACE</td>
<td>angiotensin-converting enzyme</td>
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<tr>
<td>ALI</td>
<td>acute lung injury</td>
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<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
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<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
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<td>DIZE</td>
<td>diminazene aceturate</td>
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<td>EMT</td>
<td>epithelial–mesenchymal transition</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>RAS</td>
<td>renin-angiotensin system</td>
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<td>α-SMA</td>
<td>alpha-smooth muscle actin</td>
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Recommended section assignment: Cellular and Molecular.
Abstract

Background: In our previous studies, angiotensin-converting enzyme 2 (ACE2) was shown to alleviate the severity of acute lung injury (ALI), but its effects on the development of lung injury-caused lung fibrosis have not been studied. Methods: In the present study, the effects of ACE2 on lipopolysaccharide (LPS)-induced fibrosis in lung were studied. The role of epithelial–mesenchymal transition (EMT) and that of the transforming growth factor-beta1 (TGF-β1)/Smad2/Smad3 pathway in LPS-induced fibrosis in lung were investigated. Results: ACE2 expression in the mouse model of LPS-induced lung fibrosis was significantly increased. ACE2 activator diminazene aceturate (DIZE) significantly reduced pulmonary fibrosis, decreased α-SMA expression, collagen I, hydroxyproline, and TGF-β1 in the lung. DIZE significantly decreased TGF-β1 expression and the activation of Smad2 and Smad3. ACE2 overexpression inhibited the LPS-induced EMT in MLE-12 cells (lung epithelial cells) and siRNA treatment of ACE2 stimulated EMT. ACE2 overexpression also inhibited TGF-β1 expression and activation of Smad2 and Smad3 in MLE-12 cells. Finally, after MLE-12 cells were treated with both ACE2 and TGF-β1 plasmid, TGF-β1 plasmid significantly abolished the effect of ACE2 plasmid on the EMT in MLE-12 cells. Conclusion: Combined with the in vivo study, it was revealed that ACE2 can suppress the TGF-β1/Smad2/Smad3 pathway in lung type II epithelial cells, thus reversing their EMT and lung fibrosis. The present study provides basic research data for the application of ACE2 in lung injury-caused lung fibrosis treatment and clarifies the intervention mechanism of ACE2 in pulmonary fibrosis, which has potential value for clinical application.

Keywords: ACE2; pulmonary fibrosis; TGF-β1; Smad2; Smad3; epithelial–mesenchymal transition

Significance Statement

ACE2 can inhibit the EMT in lung type II epithelial cells and lung fibrosis. ACE2 can regulate the TGF-β1/Smad2/Smad3 pathway in lung type II epithelial cells, which may be the underlying mechanism of ACE2’s effect on EMT and lung fibrosis.

1. Introduction
Acute lung injury (ALI) is secondary diffuse lung parenchyma of the inflammation cascade involving a variety of inflammatory media and effector cells, which share the same pathophysiological changes (Wang et al., 2014). They are common clinical respiratory diseases with complex etiology and pathogenesis and high mortality rates (Butt et al., 2016). Acute respiratory distress syndrome (ARDS) in the later stage can cause interstitial fibrosis and bronchofibrosis and can even form honeycomb lung due to the progress of pulmonary interstitial fibrosis and extensive destruction of lung parenchyma, which leads to serious and irreversible damage to lung function (Antunes et al., 2014). In addition, after the novel severe acute respiratory syndrome (SARS) coronavirus 2 (SARS-CoV-2) broke out around the world, there have been reports regarding the post-COVID-19 pneumonia pulmonary fibrosis (Tale et al., 2020; Shi et al., 2020). Even though there are still some debate on whether survivors of COVID-19 will fully recover or have progressive fibrosis after the COVID-19 infection (McDonald, 2021), previous data from previous coronavirus infections such as SARS outbreak in 2003 and Middle East respiratory syndrome and the subsequent ALI/ARDS indicated there could be substantial fibrotic consequences following SARS-CoV-2 infection (George et al., 2020). Therefore, exploring the mechanism of lung fibrosis would potentially provide new molecule targets for the treatment of lung fibrosis occurred in the COVID-19 pandemic.

Transforming growth factor beta1 (TGF-β1) is an extracellular matrix (ECM) deposition promoter, which can increase synthesis of the ECM and reduce degradation by upregulating the transcription and translation of matrix component genes, thus causing fibrosis (Liu, 2006). TGF-β1 expression in normal lung tissue is very low but can be stimulated by endotoxins and participates in increasing the permeability of pulmonary capillaries and the transition from lung injury to early pulmonary fibrosis (Mackinnon et al., 2012). Smad proteins are the main signal transduction molecules of TGF-β1 (Attisano and Wrana, 2002). TGF-β1 was confirmed to increase the permeability of endothelial cells by phosphorylation of Smad2, and Smad2 siRNA reversed the effects of TGF-β1 on endothelial cell permeability, indicating Smad2 plays a leading role in how TGF-β1 affects
endothelial cell permeability (Lu et al., 2006). TGF-β1 can mediate the proliferation of pulmonary fibroblasts and production of the ECM, which contributes to pulmonary fibrosis in the later stage (Lu et al., 2006). Smads are a family of cytoplasmic signal transduction proteins regulated by TGF-β1 and are divided into receptor-regulated Smads (such as Smad2/3), universal Smads and inhibitory Smads (Wu et al., 2012).

The classic renin-angiotensin system (RAS) members include renin, angiotensinogen, and angiotensin converting enzyme (ACE) (White et al., 2015). Improper activation of the RAS produces excessive Ang II and causes cell injury (Kobori et al., 2007). In the in vitro studies, Ang II could significantly aggravate the inflammatory damage of LPS on pulmonary microvascular permeability in rats by mediating Ang II 1 receptor (AT1R) (Zhang and Sun, 2005). ACE2 is an ACE homologous compound, which hydrolyzes Ang II to angiotensin 1-7 (Ang1-7) (Li et al., 2012). Ang1-7 plays a protective role in the body through its main receptor, Mas receptor (MasR) (Santos et al., 2003). It can antagonize most effects of Ang II, such as inducing vasodilation, increasing organ perfusion, and improving myocardial remodeling as well as preventing renal fibrosis and lung tumor proliferation (Simko et al., 2021). In our previous studies, ACE2 exhibits ability to alleviate ALI symptoms (Fang et al., 2019; Huang et al., 2020). Diminazene aceturate (DIZE) is a widely used drug against Trypanosoma and was recently shown to be an ACE2 agonist (Tao et al., 2016). Recently, in animal models of pulmonary hypertension and myocardial infarction, DIZE was shown to activate ACE2, promote Ang II degradation to Ang 1-7, and play a protective role (Qaradakhi et al., 2020). In our previous experiments, DIZE treatment was confirmed to increase ACE2 protein expression in the lung and effectively reduce lung injury in animals (Fang et al., 2019). Hence, whether activation of ACE2 can inhibit lung injury-related fibrosis and the mechanism requires further study.

As the effects and mechanism of the ACE2/Ang1-7/Mas axis in the development of lipopolysaccharide (LPS)-caused lung fibrosis have not yet been investigated, the effects of ACE2 on LPS-caused lung fibrosis were evaluated in the study. The epithelial–mesenchymal transition (EMT) is associated with embryonic
development, inflammation, wound healing, tumor progression, and organ fibrosis (Puisieux et al., 2014). Whether the EMT is involved in LPS-caused lung fibrosis is unknown; thus, the contribution of the EMT to LPS-caused lung fibrosis and the mechanism of ACE2 on lung fibrosis were investigated.

**Materials and Methods**

**Animals and groups**

C57BL/6 mice (male, 6–8 weeks, 20–25 g) were purchased from the Animal Center of Fujian Medical University and housed with freely accessed water/food and 12-h light/12-h dark cycles. In Part I, mice were randomly grouped into two groups, control and model (N = 12). After mice were injected with ketamine (100 mg/kg, i.p.) and acepromazine (5.0 mg/kg, i.p.), mice in the control group received a single intratracheal injection of phosphate-buffered saline (PBS; 30 μL). Mice in the model group were treated with intratracheal administration of LPS in PBS (5.0 mg/kg, Sigma-Aldrich) diffused in 30 μL of PBS. In Part II, mice were randomly grouped into the following groups: control, model+vehicle, model+DIZE-10 mg/kg, model+DIZE-20 mg/kg, and model+DIZE-40 mg/kg (N = 12). After mice were injected with ketamine/acepromazine, mice in the model+vehicle group received intratracheal administration of LPS and were continuously infused with saline. Mice in the model+DIZE-10 mg/kg, model+DIZE-20 mg/kg, and model+DIZE-40 mg/kg groups received intratracheal administration of LPS and were continuously infused with DIZE (10–40 mg/kg/d). The whole study was approved by Fujian Medical University.

**LPS-caused lung fibrosis and DIZE treatment**

Similar to Li et al (2018), male C57BL/6 mice received a single intratracheal administration of LPS (5.0 mg/kg, Sigma-Aldrich) diffused in 30 μL of PBS. After mice were injected with ketamine/acepromazine, they received orotracheal intubation with a 20-G intravenous cannula. The same volume of PBS was injected as control. Mice were treated with the ACE2 activator DIZE as previously described (Fang et al., 2019). Briefly, mice received daily i.p. injection of DIZE (10, 20, or 40 mg/kg, Santa Cruz) for 7 days after LPS administration.
Specimen collection

At day 28 after LPS injection, mice were injected with ketamine/acepromazine and sacrificed by quick dislocation of cervical vertebra. The bronchoalveolar lavage fluid (BALF) was collected. After the trachea was exposed, it was cannulated with 20-G cannulas and lavaged with PBS (containing 2 mM ethylene diamine tetraacetic acid) three times to collect BALF. Next, the left lower lobe of lung was collected and kept in 10% formaldehyde for the following experiments; the right lung tissue (150 mg) was kept at −80°C for further examination.

Pathological examination of lung tissue

The left lower lobe of lung was cut into sections and stained with H&E method. The following pathological changes were recorded under a light microscope: alveolar edema, inflammatory cell infiltration, alveolar hemorrhage and microthrombosis, fibroblast infiltration, and type II alveolar epithelial cell proliferation.

Masson staining was performed in paraffin sections and then washed with water. The mixture of ferro hematoxylin A and B was added to the tissue at a ratio of 1:1 for 3 min, treated with 1% hydrochloric acid for 5 s, and washed with water. Next, the slices were placed in Ponceau dye for 7 min and cleaned with water. They were treated with molybdophosphoric acid for 3 min and stained with aniline blue for 5 min. Finally, slices were treated with glacial acetic acid for 1 min followed by dehydration (95% alcohol for 3 min, 95% alcohol again for 3 min, anhydrous ethanol for 5 min, anhydrous ethanol again for 5 min, xylene for 5 min, and xylene again for 5 min) and sealed with neutral gum.

The level of alpha-smooth muscle actin (α-SMA) was detected using immunohistochemistry based on the method of Wang et al (2019). The lung tissue was deparaffinized, incubated with 1% albumin solution, blocked with goat serum, then incubated with rabbit anti-α-SMA primary antibody (1:2000, Sigma) and goat anti-rabbit IgG secondary antibody (1:1000, Sigma).

Measurement of collagen I in lung tissue

After the lung tissues were weighed, the prepared lysis solution (mass: volume ratio 1:50) was added in proportion. After the tissues were homogenized using an
ultrasonic processor and centrifuged for 10 min (12,000 ×g, 4°C). The supernatant was then gathered and added to 96-well plates. After diluting the standard solution, 100 μL was added to the 96-well plates, which were transferred to 37°C for 60 min. 100 μL working liquid of test solution was added. The microplates were tightly sealed with film and kept at 37°C (60 min). The microplates were rinsed with prepared washing solution, and 100 μL of the working liquid test solution B was added into each well. The microplates were then kept at 37°C (30 min). The liquid was discarded again, the microplates washed with washing solution, and 90 μL of 3,3′,5,5′-Tetramethylbenzidine solution was added. The microplates were placed at 37°C in the dark for 10 min. Next, 50 μL of stop reaction solution was mixed. The preheated enzyme standard instrument was used for reading at 450 nm. The light density of each well was detected and the values saved for analysis.

**Determination of hydroxyproline in lung tissue**

A lung tissue sample (50 mg) was put in a clean microfuge tube. Next, 1 mL of hydrolysate was added to each tube. The tubes were covered, tightly sealed, and placed in boiling water for 20 min. Then, 30 mg of activated carbon was added to 3 mL of diluted hydrolysate and mixed well. The tubes were centrifuged 5 min at 3500 ×g and 1 mL of supernatant removed for analysis. Next, 0.5 mL reagent was mixed in each sample tube and placed for 10 min at 25°C. Then, 20.5 mL of reagent was added and kept for 5 min, then kept with 30.5 mL of reagent addition for 5 min. The microfuge tube was sealed with film, incubated in a 60°C water bath for 15 min, cooled naturally, centrifuged for 5 min at 3500 ×g, and 100 μL supernatant was carefully removed. 100 μL sample from each group was added to 96-well plates and 550 nm wave length was used for analysis. The optical density value of each well was measured and the data recorded and saved for analysis.

**Determination of TGF-β1 in BALF using enzyme-linked immuno sorbent assay (ELISA)**

The standard was diluted, the sample activation reagent prepared, and 100 μL of mice alveolar lavage solution was taken from each group. Next, 20 μL of activation reagent A was added. After 10 min later, 20 μL of activating reagent B solution was
added and placed at 37°C for 90 min. Then, the liquid was discarded and 100 μL of prepared biotin-labeled anti-mouse TGF-β1 antibody was added. The plates were incubated at 37°C for 60 min. The liquid was discarded; the plates were washed five times with washing liquid. The working solution of avidin peroxidase complex was prepared in advance and placed at 37°C for 30 min. The liquid was discarded; the plates were washed five times with washing solution, and 90 μL of TMB substrate was added. The plates were sealed, placed in at 37°C in the dark for 15 min, and 50 μL stop reaction solution was added. The enzyme standard was immediately used at 450 nm for the detection of light density in each well. The values for each group were recorded and the concentration of TGF-β1 was calculated.

Quantitative polymerase chain reaction (PCR)

The procedure was previously described by Jun et al (2015). Briefly, firstly, the RNA was separated from the lung tissue with a RNA extraction kit (Promega). The primers used were the following: ACE2, forward: 5′-CACCATGTCAAGCTCTTCC-3′, reverse: 5′-AAAGGAGGTCTGAACTCATCAG-3′; TGF-β1, forward: 5′-GACTCTCCACCTGCAAGACC-3′, reverse: 5′-GGGACTGGAGCCTGATTTG-3′; Smad2, forward: AAGCCATCACCAGCTCAGTTG, reverse: CACTGATCTACCGTATTTGCTG; Smad3, forward: 5′-GAGTAGAGACGCAGTTTGGC-3′, reverse: 5′-GGCTTCTGGGACAGCTGCGA-3′; and GAPDH, forward: AGGGAGTAAGAAACCCTGGAC, reverse: CTGGGATGGAATTGTGAG. The PCR conditions were set as 95°C for 10 min, 95°C for 15 s, 40 cycles, 60°C for 60 s. The 2^{-ΔΔCt} method was adopted to measure the mRNA content.

Transfections and siRNA interference

Cells were transfected with plasmid DNA carrying the transcriptional target sequence of ACE2 promoter according to Zhang et al. (2018). The cells were also transfected with empty plasmid to establish a plasmid control. For ACE2 siRNA, cells were treated with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) following the instruction. The following primers were used for ACE2 siRNA: forward,
5'-GAGGAGACUAUGAAGUAAATT-3' and reverse, 5'-UUUACUUCAUAGUCUCCUCTT-3'.

**Western blot**

As previously described (Zhang et al., 2019), the expression of ACE2, TGF-β1, phosphorylated Smad2 (p-Smad2), Smad2, p-Smad3, Smad3, E-cadherin, α-SMA, vimentin, or GAPDH (Sigma-Aldrich) were examined by Western blot.

**Statistical analyses**

All the data are shown as Mean± standard deviation (SD). The statistical analyses were performed with one-way analysis of variance (ANOVA) followed by Newman–Keuls test. P value<0.05 was considered statistically significant.

**Results**

**ACE2 activator DIZE attenuates LPS-caused lung fibrosis**

After mice were treated with LPS (5 mg/kg) and different dosages of ACE2 activator DIZE (10-40 mg/kg/day), the degree of pulmonary fibrosis was measured using H&E staining, Masson staining, and Ashcroft scores. As shown in Figure 1A, in the model+vehicle and model+DIZE-10 mg/kg groups, the alveoli structure was destroyed, the alveoli cavity was reduced, and exfoliated alveolar epithelium and protein exudates were present in the cavity. Areas with obvious collagen deposition and fibrosis were observed. Collagen fibers proliferated in strips, forming diffuse pulmonary fibrosis. In the model+DIZE-20 mg/kg and model+DIZE-40 mg/kg groups, fibroblasts exhibit slight proliferation, collagen fibers were deposited in the pleura and alveolar septum, and changes of mild and moderate pulmonary fibrosis occurred; the scope of the lesions was limited. As shown in Figure 1B, in the model+vehicle and model+DIZE-10 mg/kg groups, the alveoli structure was destroyed and fascicular or large collagenous fibers were deposited in the lung interstitium (blue), forming typical fibrosis changes. In the model+DIZE-20 mg/kg and model+DIZE-40 mg/kg groups, the alveolar space was widened and blue collagen fibers were found in the alveolar septum and lung interstitial space; however, the color was lighter than in the model+vehicle group and the collagen fibers were significantly reduced. Figure 1C shows the Ashcroft scores. The Ashcroft scores in the model+vehicle and
model+DIZE-10 mg/kg groups were increased compared to control (P < 0.05, N = 12). The Ashcroft scores in the model+DIZE-20 mg/kg and model+DIZE-40 mg/kg groups were significantly decreased in the model+vehicle group (P < 0.05, N = 12).

Effects of DIZE on α-SMA expression and collagen I, hydroxyproline, and TGF-β1 in the lung

Figure 2A shows the α-SMA expression measured with immunohistochemistry method. Figure 2B shows the α-SMA expression scores. The level of α-SMA was significantly raised in the model+vehicle and model+DIZE-10 mg/kg groups compared to control (P < 0.05, N = 12) but was decreased by 20 mg/kg and 40 mg/kg DIZE treatments (P < 0.05 compared to control, N = 12). Figure 2C and 2D show the collagen I and hydroxyproline concentrations in lung tissue, which were significantly raised in the model+vehicle and model+DIZE-10 mg/kg groups compared to control (P < 0.05, N = 12). In the model+DIZE-20 mg/kg and model+DIZE-40 mg/kg groups, collagen I and hydroxyproline concentrations in lung tissue were significantly decreased (P < 0.05 compared to control, N = 12). Figure 2E shows TGF-β1 in BALF was significantly increased in the model+vehicle and model+DIZE-10 mg/kg groups (P < 0.05 compared to control, N = 12) but decreased in the model+DIZE-20 mg/kg and model+DIZE-40 mg/kg groups (P < 0.05 compared to model+vehicle group, N = 12).

The expression of ACE2, TGF-β1 and Smad2/3 in the mouse model of LPS-induced lung fibrosis

Firstly, the mRNA levels of ACE2, TGF-β1 and Smad2/3 in the lung tissue were measured. The results are shown in Figure 3A–D. Compared to control, the ACE2 mRNA level was significantly decreased in the model group (P < 0.05, N = 12). By contrast, the TGF-β1 mRNA level was significantly increased in the model group (P < 0.05, N = 12). However, the Smad2 and Smad3 mRNA levels were not dramatically changed in the model group (P > 0.05, N = 12). Secondly, protein levels of ACE2, TGF-β1, p-Smad2, p-Smad3, Smad2, and Smad3 were measured in the lung tissue of mice (Fig.3E–G). ACE2 protein expression was dramatically decreased in the model group and TGF-β1 protein expression was dramatically increased (P < 0.05,
The p-Smad2/Smad2 and p-Smad3/Smad3 ratios were adequately increased in the model group (P < 0.05, N = 12), indicating activation of the TGF-β1/Smad2/Smad3 pathway following LPS stress in vivo.

**Effects of DIZE on the level of TGF-β1 and Smad2/3 in mice**

To measure the effects of ACE2 on the TGF-β1/Smad2/3 pathway, mice were treated with DIZE and the mRNA and protein expression of TGF-β1 and Smad2/3 in lung was measured. The TGF-β1 mRNA levels were adequately decreased by 20 mg/kg and 40 mg/kg DIZE treatments (Fig. 4A), while Smad2 and Smad3 mRNA levels were not significantly changed between groups (Fig. 4B, C). Figure 4D shows the western blot results of ACE2, TGF-β1, p-Smad2/3, and Smad2/3 expression in the lung tissue; 20 mg/kg and 40 mg/kg DIZE treatments significantly decreased the expression of TGF-β1 and p-Smad2/3 but did not affect Smad2 and Smad3 expression, indicating DIZE inhibited the activation of TGF-β1/Smad2/Smad3 pathway in vivo.

**Effects of ACE2 overexpression or siRNA on the EMT in MLE-12 cells**

To examine the anti-fibrosis effects of ACE2 on lung, the type II epithelial cell line MLE-12 cells were treated with LPS and ACE2 plasmid or ACE2 siRNA. Then, the expression of E-cadherin, α-SMA and vimentin was determined. Figure 5A shows that the expression of ACE2 was significantly increased in the ACE2 plasmid group, but significantly decreased in the ACE2 siRNA group, indicating that the ACE2 plasmid or ACE2 siRNA was successful. Figure 5B–D show the protein expression of E-cadherin, α-SMA, and vimentin. The E-cadherin protein expression level was dramatically reduced in the model group, increased by ACE2 plasmid, and reduced by ACE2 siRNA. By contrast, the expression of α-SMA and vimentin were significantly escalated in the model group, decreased by ACE2 plasmid, and increased by ACE2 siRNA. These results suggested that ACE2 inhibited the EMT in MLE-12 cells.

**Effects of ACE2 overexpression or siRNA on the levels of TGF-β1 and Smad2/3 in MLE-12 cells**

To examine the effects of ACE2 on the TGF-β1/Smad2/Smad3 pathway, MLE-12 cells were treated with ACE2 plasmid or ACE2 siRNA and the mRNA and protein expression of TGF-β1 and Smad2/3 in lung were measured. As shown in
Figure 6A, the TGF-β1 mRNA level was decreased by ACE2 plasmid but increased by ACE2 siRNA. As shown in Figure 6B and 6C, the mRNA levels of Smad2 and Smad3 were not significantly changed between groups. Figure 6D shows TGF-β1, p-Smad2, p-Smad3, Smad2, and Smad3 expression in lung tissue. ACE2 plasmid significantly decreased the expression of TGF-β1, p-Smad2/3 but did not affect the expression of Smad2/Smad3 expression, indicating that ACE2 inhibited the TGF-β1/Smad2/Smad3 pathway in cells.

**TGF-β1 overexpression abolished the effects of ACE2 plasmid on the EMT**

To explore the involvement of TGF-β1 in the anti-EMT effects of ACE2, MLE-12 cells were treated with LPS and ACE2+TGF-β1 plasmid. Figure 7A shows that the expression of TGF-β1 was significantly increased in the TGF-β1 plasmid group, indicating that the expression of TGF-β1 using the plasmid vector was successful. Figure 7B–D shows the expression of E-cadherin, α-SMA, and vimentin. E-cadherin expression was dramatically decreased in the model+ACE2+TGF-β1-plasmid group compared to model+ACE2-plasmid group. By contrast, α-SMA and vimentin expression was dramatically increased with the ACE2+TGF-β1-plasmid treatment. Taken together, these results indicated ACE2 inhibited the EMT in MLE-12 cells by downregulating TGF-β1.

**Discussion**

Although our understanding of ARDS has increased in recent years, the mortality rate of ARDS remains 35–46%. Currently, the high mortality rate of patients with ARDS is thought to be due to pulmonary fibrosis, which is also a key factor affecting the prognosis of ARDS (Zhang et al., 2015). In the acute inflammatory phase of ARDS, the cytotoxic mediators (including reactive oxygen species and nitrogen substances) released by infiltrating leukocytes and proteolytic enzymes lead to the injury (Zemans et al., 2009). Persistent injury and untimely injury repair are the main factors leading to a pathological fibroproliferative reaction (Burnham et al., 2014). In some patients, macrophages, fibroblasts, and myofibroblasts persistently accumulate, resulting in excessive deposition of components of the ECM (Fahy et al., 2003), which is accompanied by the imbalance between pro-fibrosis mediators and...
anti-fibrosis mediators and leads to fibro proliferative response (Fahy et al., 2003; White et al., 2008). In recent studies, epithelial cells were shown to be involved in the formation of renal fibrosis and liver fibrosis (Zhao et al., 2019; Zou et al., 2019). The damage and repair of lung epithelial cells also play a key role in pulmonary fibrosis (Zhang et al., 2015). If lung epithelial cells are continuously damaged and excessively repaired, pulmonary fibrosis may occur (Quesnel et al., 2012). The present study results showed that LPS treatment could cause pulmonary fibrosis. H&E and Masson staining of lung tissue showed the integrity of lung structure was significantly damaged after LPS treatment (Figure 1). The alveolar septum was thickened and a large amount of blue-stained collagen was observed in the thickened area, indicating significant collagen deposition. The amount of collagen I and hydroxyproline in lung tissue as well as TGF-β1 levels in BALF were also significantly increased by LPS treatment (Figure 2). These results suggested that LPS treatment successfully caused lung fibrosis.

Discovery of the ACE2 gene in recent years was a major breakthrough in the prevention and treatment of new targets of hypertension, diabetes, and other diseases (Liu et al., 2011). ACE2 can degrade Ang II to Ang (1-7) and Ang I to Ang (1-9), which can be further degraded to Ang (1-7). Ang (1-7) and its main receptor (MasR) constitute the ACE2/Ang (1-7)/MasR axis. Firstly, the results of the present study showed that ACE2 mRNA and protein expression in the mouse model of LPS-induced lung fibrosis were significantly decreased (Figure 3), although the level of significance is not very high. The relative low level of significance may indicate that the ACE2 expression was regulated by multiple factors, which prevent the dramatic decrease of ACE2 in the LPS-induced lung fibrosis model. Secondly, the ACE2 activator DIZE attenuated LPS-caused lung fibrosis based on H&E and Masson staining and Ashcroft scores (Figure 1). DIZE also significantly decreased the expression of α-SMA, collagen I, hydroxyproline and TGF-β1 in lung (Figure 2), indicating that ACE2 can inhibit formation of collagen and activation of the TGF-β1 pathway, which is consistent with previous studies. Reportedly, ACE2 can slow down the pathological progression of organ fibrosis by regulating the balance of angiotensin...
peptide and the production of inflammatory mediators and ECM, and by inhibiting the process of oxidative stress. Consequently, ACE2 has become a novel target for intervention and prevention of organ fibrosis (Liu et al., 2011), which is also supported by our study.

TGF-β is a member of a group of cytokines with complex biological functions (Chin et al., 2004). TGF-β1 is the main factor that promotes fibrosis in vivo as well as the proliferation and aggregation of fibroblasts; it also stimulates the growth of immature fibroblasts (Xu et al., 2003). TGF-β1-monoclonal antibody can partially inhibit the proliferation of fibroblasts and synthesis of collagen (Alsafadi et al., 2017). TGF-β1 can also promote the transformation of fibroblasts to myofibroblasts, an essential cell in the pathogenesis of pulmonary fibrosis (Giménez et al., 2017; Salgado et al., 2017). When pulmonary fibrosis occurs, TGF-β1 is distributed in macrophages, eosinophils, alveolar type II epithelial cells, small bronchioles, bronchial epithelial cells, fibroblasts, and myofibroblasts in the lung (Sadar et al., 2016; Bamberg et al., 2018). Reportedly, TGF-β, Smad, MAPK, PI3K, and JNK signaling pathways all participate in the occurrence of pulmonary fibrosis (Miyazawa et al., 2002; Tojo et al., 2005), in which the Smad signaling pathway is the classical pathway that mediates the signal transduction of TGF-β. The present study shows that the level of TGF-β1 in mice was increased (Figure 3). The levels of Smad2 and Smad3 were unchanged, but the p-Smad2/Smad2 and p-Smad3/Smad3 ratios were significantly increased in the LPS group (Figure 3). Thus, in the LPS-induced lung fibrosis mouse model, the TGF-β1/Smad2/Smad3 pathway was activated. Smad2 and Smad3 are TGF-β1 receptor substrates. In previous studies, upon bleomycin treatment, Smad3 in the cytoplasm continuously decreased and its level in the nucleus transiently increased. Smad2/Smad3 phosphorylation and nuclear aggregation continuously increased and Smad4 in the cytoplasm and nucleus transiently increased (Tatler et al., 2016). Smad3 deficiency can inhibit TGF-β1 overexpression and the progress of lung fibrosis. The regulation of Smad2/Smad3 protein expression was suggested to significantly participate in the advancement of pulmonary fibrosis. The mRNA and protein levels of TGF-β1 were both significantly inhibited by ACE2 inhibitor DIZE (Figure 4).
Although DIZE did not affect Smad2 and Smad3 mRNA and protein expression, it significantly inhibited the expression of TGF-β1 and the phosphorylation of Smad2/3 (Figure 4), indicating that ACE2 suppressed the TGF-β1/Smad2/Smad3 pathway in vivo.

The EMT is closely associated with organ fibrosis (Puisieux et al., 2014). Characteristics of the EMT mainly include the disappearance of tight junctions between cells, loss of normal polarity, deformed and remolded cytoskeleton, and increased α-SMA expression (Nieto et al., 2016; Dos Santos, 2008). Therefore, to examine the possible role of ACE2 on LPS-induced lung fibrosis, MLE-12 cells were treated with LPS and ACE2 overexpression or siRNA before the EMT changes were measured. LPS significantly reduced E-cadherin level and increased α-SMA and vimentin expression in MLE-12 cells (Figure 5), indicating LPS treatment could induce EMT in MLE-12 cells. ACE2 overexpression increased E-cadherin expression but decreased α-SMA and vimentin expression (Figure 5); siRNA treatment of ACE2 resulted in opposite changes (Figure 5), indicating ACE2 inhibited the EMT. These results indicated the EMT of lung type II epithelial cells may contribute to LPS-induced lung fibrosis, which can be reversed by ACE2.

To further confirm the role of the TGF-β1/Smad2/Smad3 pathway, cells were treated with ACE2 plasmid or ACE2 siRNA before the TGF-β1, Smad2, and Smad3 mRNA and protein expression measured. Similar to the in vivo study, the TGF-β1 levels were both significantly reduced by ACE2 plasmid but increased by ACE2 siRNA (Figure 6). ACE2 plasmid significantly decreased the p-Smad2/3 levels, indicating that ACE2 suppressed the TGF-β1/Smad2/Smad3 pathway (Figure 6). We have conducted a series of experiments to explore the effect of ACE2 on the expression of TGF-β1. The result of Figure 2E, Figure 4A and D, Figure 6A and D indicated that ACE2 downregulate TGF-β1 in the lung tissue of mice, but didn’t indicate the anti-EMT effect of ACE2 is dependent on TGF-β1. Therefore, we conducted the experiments of Figure 7 to see whether ACE2 inhibited EMT via TGF-β1. We used the TGF-β1 overexpressing plasmid vector to restore the expression of TGF-β1, then observed the expression of EMT-related proteins (E-cadherin,
α-SMA, and vimentin). As the results showed that overexpression of TGF-β1 abolished the effect of ACE2 overexpressing plasmid vector on the expression of these proteins, indicating that the anti-EMT effect of ACE2 is dependent on TGF-β1. Combined the previous experiments (Figure 2E, Figure 4A and D, Figure 6A and D), we can conclude that ACE2 inhibited EMT by downregulating TGF-β1. Combined with the in vivo study, it was indicated that ACE2 can inhibit the TGF-β1/Smad2/Smad3 pathway in lung type II epithelial cells, thus reversing their EMT and lung fibrosis.

In conclusion, the present study provides basic research data for the application of ACE2 in the lung fibrosis and clarifies the intervention mechanism of ACE2 in pulmonary fibrosis, which may be clinically relevant. The results also improve our understanding of TGF-β1/Smad2/Smad3 signaling in lung fibrosis. The regulatory mechanism is of theoretical significance for determining the molecular mechanism of pulmonary fibrosis induced by ALI.

Authors’ contributions
Participated in research design: X, Lin and Gao.
Conducted experiments: W Lin.
Contributed new reagents or analytic tools: Zhuang.
Performed data analysis: Zhuang.
Wrote or contributed to the writing of the manuscript: XS, Lin and Gao.

Ethics approval
Approval for all experimental protocols was granted by the Animal Care Committee of Fujian Medical University.

Consent to participate (include appropriate statements)
Not applicable.

Consent for publication (include appropriate statements)
Not applicable.
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George PM, Wells AU, Jenkins RG (2020) Pulmonary fibrosis and COVID-19: the


Mas. Proc Natl Acad Sci U S A 100:8258-8263.


Footnotes

a) Source of financial support: This work was supported by Fujian Provincial Natural Science Foundation of China [Grant 2018J01261]; Scientific Research Projects of Shanghai Municipal Health and Family Planning Commission [Grant 201840263].

b) Unnumbered footnote providing thesis information, citation of meeting abstracts where the work was previously presented: None.

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d) Numbered footnotes, using superscript numbers, beginning with those (if any) to authors’ names and listed in order of appearance.

e) Conflicts of interest/Competing interests: The authors declare no competing interests.
Legends for Figures

Figure 1. Effects of DIZE on LPS-induced histological and fibrosis changes. Fig. 1A shows the lung histological changes demonstrated by H&E staining. The dosage of LPS was 5.0 mg/kg. Fig. 1B shows the lung fibrosis demonstrated by Masson staining. Figure 1C shows the results of Ashcroft scores. LPS, lipopolysaccharide; DIZE, Diminazene aceturate. #, p<0.05 compared to control; *, p<0.05 compared to the LPS+vehicle group. N=12.

Figure 2. Effects of DIZE on LPS-induced changes on fibrosis related biomarkers. Fig.2A shows the expression of α-SMA in lung measured by immunohistochemistry. Fig.2B shows the results of α-SMA scores calculated from immunohistochemistry. Fig.2C and D show the levels of collagen I and hydroxyproline in lung tissue. Fig.2E shows the levels of TGF-β1 in BALF. LPS, lipopolysaccharide; DIZE, Diminazene aceturate. #, p<0.05 compared to control; *, p<0.05 compared to the LPS+vehicle group. N=12.

Figure 3. The mRNA and protein expression of ACE2, TGF-β1, Smad2, and Smad3 in the mouse model of LPS-induced lung fibrosis. Fig.3A-D show the mRNA levels of ACE2, TGF-β1, Smad2, and Smad3 in control mice and model mice. Fig.3E shows the representative images of Western blot; Fig.3F shows the relative changes of ACE2 and TGF-β1; Fig.3G shows the relative ratios of p-Smad2/Smad2 andp-Smad3/Smad3. #, p<0.05 compared to control. N=12.

Figure 4. Effects of DIZE on mRNA and protein expression of TGF-β1, Smad2 and Smad3 in mice lung. After mice were treated with LPS and DIZE, the mRNA and protein expression of TGF-β1, Smad2 and Smad3 in mice lungs were measured. Fig.4A-C show the mRNA levels of TGF-β1, Smad2, and Smad3 in control mice and model mice. Fig.4D shows the representative images of Western blot. #, p<0.05 compared to control; *, p<0.05 compared to the LPS+vehicle group. N=12.

Figure 5. Effects of ACE2 overexpression or siRNA on the EMT in MLE-12 cells. The mouse lung type II epithelial cell line MLE-12 was treated with LPS and ACE2 plasmid or ACE2 siRNA. Then, expression of E-cadherin, α-SMA, and vimentin was measured. Figure 5A shows the representative images of Western blot of ACE2 after
cells were treated with ACE2 plasmid or ACE2 siRNA. Figure 5B-D show the representative images of Western blot of E-cadherin, α-SMA, and vimentin and their relative changes. #, p<0.05 compared to control; *, p<0.05 compared to vehicle. N=12.

**Figure 6. Effects of ACE2 overexpression or siRNA on mRNA and protein levels of TGF-β1, Smad2, and Smad3 in MLE-12 cells.** The MLE-12 cells were treated with ACE2 plasmid or ACE2 siRNA and the mRNA and protein expression of TGF-β1, Smad2, and Smad3 in lung were measured. Fig.6A-C show the mRNA levels of TGF-β1, Smad2, and Smad3 in cells. Fig.6D shows the representative images of Western blot. #, p<0.05 compared to control; *, p<0.05 compared to vehicle. N=12.

**Figure 7. Effects of TGF-β1 overexpression on the EMT in MLE-12 cells.** MLE-12 cells were treated with LPS and ACE2+TGF-β1 plasmid and expression of E-cadherin, α-SMA, and vimentin was measured. Figure 7A shows the expression of TGF-β1 after cells were treated with TGF-β1 plasmid. Figure 7B-D show the representative images of Western blot of E-cadherin, α-SMA, and vimentin and their relative changes. #, p<0.05 compared to control; *, p<0.05 compared to vehicle; @, p<0.05 compared to ACE2-plasmid; &, p<0.05 compared to control-plasmid. N=12.
Figure 2

A  
<table>
<thead>
<tr>
<th>Control</th>
<th>Vehicle</th>
<th>DIZE-10</th>
<th>DIZE-20</th>
<th>DIZE-40 (mg/kg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-SMA</td>
<td></td>
<td></td>
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</tbody>
</table>

B  
- Relative α-SMA scores
- Control, Vehicle, DIZE-10, DIZE-20, DIZE-40 (mg/kg/d)

C  
- Collagen-I in lung tissue (mg/g)
- Control, Vehicle, DIZE-10, DIZE-20, DIZE-40 (mg/kg/d)

D  
- Hydroxyproline in lung tissue (mg/g)
- Control, Vehicle, DIZE-10, DIZE-20, DIZE-40 (mg/kg/d)

E  
- TGF-β1 in BALF (pg/ml)
- Control, Vehicle, DIZE-10, DIZE-20, DIZE-40 (mg/kg/d)
Figure 3

A) Expression of ACE2 (% of Control) in Control vs Model.
B) Expression of TGF-β1 (% of Control) in Control vs Model.
C) Expression of Smad2 (% of Control) in Control vs Model.
D) Expression of Smad3 (% of Control) in Control vs Model.

E) Western Blot images showing ACE2, TGF-β1, Smad2, Smad3, and GAPDH proteins.

F) Relative expression of ACE2 and TGF-β1 proteins in Control vs Model.
G) Relative ratio of p-Smad2/Smad2 and p-Smad3/Smad3 in Control vs Model.
Figure 7

A) TGF-β1

B) E-Cadherin

C) α-SMA

D) Vimentin

Relative TGF-β1 expression

Relative expression of E-Cadherin

Relative expression of α-SMA

Relative expression of Vimentin

Control, Vehicle, ACE2-plasmid, Control-plasmid, TGF-β1-plasmid vs. Control-plasmid

* p < 0.05, @ p < 0.01, # p < 0.001, & p < 0.0001