Original Article

Sivelestat protects against acute lung injury by up-regulating angiotensin-converting enzyme 2/angiotensin-(1–7)/Mas receptors

Changqing He, Ruoxin Li, Jia Zhang, Wenshu Chai

Department of Respiratory Medicine, First Affiliated Hospital of Jinzhou Medical University, Jinzhou, China

Contributions: (I) Conception and design: W Chai, C He; (II) Administrative support: W Chai; (III) Provision of study materials or patients: W Chai, C He, R Li; (IV) Collection and assembly of data: C He, R Li, J Zhang; (V) Data analysis and interpretation: C He, J Zhang; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Wenshu Chai, MD. Department of Respiratory Medicine, First Affiliated Hospital of Jinzhou Medical University, No. 2, Section 5, Renmin Street, Guta District, Jinzhou 121000, China. Email: cws1964@163.com.

Background: Acute lung injury (ALI) and its most severe manifestation of acute respiratory distress syndrome (ARDS) is a disease with a clinical mortality rate of up to 40% and is one of the most dangerous and common complications of severe coronavirus disease 2019 (COVID-19). Sivelestat (SIV) is the only licensed therapeutic medicine in the world for ALI/ARDS treatment. The angiotensin-converting enzyme 2 (ACE2)/angiotensin (Ang)-(1–7)/Mas receptor axis is critical in the prevention of ALI/ARDS. This study aims to investigate whether SIV alleviates lipopolysaccharides (LPS)-induced ALI by inhibiting the down-regulation of ACE2/Ang-(1–7)/Mas receptor axis expression.

Methods: *In vivo*, 90 male Sprague-Dawley rats were randomized into 5 groups. Then, we pretreated different groups of rats with dexamethasone (DEX) or SIV. Rats were sacrificed at three different time points (3, 6, and 12 hours) following LPS instillation. In vitro, RAW264.7 cells were divided into 11 groups. Different groups of cells were pretreated with DEX or SIV. And then added with LPS for 3, 6, and 12 hours. Next, we introduced A779, a potent Ang-(1–7) receptor antagonist, and DX600 as the ACE2 antagonist in different groups. Then the protein and messenger RNA (mRNA) expression levels of ACE2 in rat lung tissue and the expression levels of tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and Ang-(1–7) in the rat serum and the cell culture supernatant were measured. And the data were statistically analyzed.

Results: *In vivo*, the rats pretreated with SIV or DEX had significantly lower lung wet/dry (W/D) ratios and lung pathological alterations than those exposed to LPS only. Both *in vivo* and *in vitro*, we observed that SIV or DEX significantly attenuated the LPS-induced up-regulation of IL-6 and TNF-α levels, and the down-regulation of ACE2 and Ang-(1–7) levels. *In vitro*, the pretreatment of the RAW264.7 cells with DX600 and A779 significantly reduced and even abolished the protective effects of SIV.

Conclusions: Therefore, it was concluded that SIV protected against LPS-induced ALI and decreased inflammatory cytokine release by up-regulating the ACE2/Ang-(1–7)/Mas receptor axis. Our results enrich the theoretical foundation for the clinical application of SIV and provide fresh ideas for the treatment of ALI/ARDS.

Keywords: Sivelestat (SIV); angiotensin-converting enzyme 2 (ACE2); angiotensin-(1–7) [Ang-(1–7)]; acute lung injury (ALI); lipopolysaccharide (LPS)

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Introduction

Acute lung injury (ALI) is the pulmonary manifestation of multiple organ dysfunction syndromes caused by systemic inflammatory response syndrome, and acute respiratory distress syndrome (ARDS) is one of the most severe forms of this largely pathogenic process (1). Sepsis, aspiration, smoking, pathogenic infections, such as severe acute respiratory syndrome (SARS) and severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2), and other factors contribute to ALI/ARDS (2,3). Currently, the prevention and treatment of ALI/ARDS remain a complex clinical challenge, and no specific therapeutic drugs are available. The mortality rate of ALI/ARDS is up to 40% (4). It is also one of the most serious and common complications of severe coronavirus disease 2019 (COVID-19) (5).

The main component of endotoxin [lipopolysaccharide (LPS)] can cause a systemic acute inflammatory response and has been widely used to establish animal models of ALI (6,7). LPS stimulation has been shown to increase the secretion of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and other pro-inflammatory factors both *in vitro* and *in vivo* (8), and to down-regulate the expression of angiotensin-converting enzyme 2 (ACE2) and angiotensin (Ang)-(1–7) (9). LPS infusion leads to lung inflammation and sepsis, which can subsequently develop into ARDS.

Dexamethasone (DEX), a synthetic corticosteroid, can decrease the production of inflammatory factors and lung edema, while also protecting alveolar epithelial and endothelial cells (10,11). It dramatically up-regulates ACE2 expression both *in vivo* and *in vitro*. DEX has also been applied to treat ALI/ARDS, SARS, COVID-19, and their accompanying hyperinflammatory or cytokine storms (12-14). A study has shown that ACE2 is a negative regulator of the renin-angiotensin system (RAS), which transforms Ang I and Ang II into Ang-(1–9) and Ang-(1–7), respectively (15). Ang-(1–7) binds to the Mas receptor to establish the ACE2/Ang-(1–7)/Mas receptor axis, the beneficial RAS axis, which exerts anti-inflammatory and vasodilatory effects (16). ACE2 is not only the major cell entrance receptor of SARS-CoV-2, it is also the key enzyme that regulates the body's RAS (17,18). This dual identity indicates that ACE2 plays a central role in ALI/ARDS. Many studies have shown that the up-regulation of the ACE2/Ang-(1–7)/Mas receptor axis *in vitro* and *in vivo* can delay or even prevent the development of ALI/ARDS, pulmonary fibrosis, pulmonary hypertension, and other lung disorders (19,20).

Sivelestat (SIV) is a selective neutrophil elastase inhibitor, which can significantly improve the indicators of lung injury by intravenous administration, including reducing inflammatory cell infiltration, serum protein concentration, and pulmonary vascular permeability (21,22). SIV can be used to lower the number of animals dying from respiratory distress (23). Additionally, it is the only medicine authorized worldwide for the treatment of ALI/ARDS (24,25). In recent years, a study has shown that SIV alleviates inflammation and treats ALI/ARDS via a variety of mechanisms (26). Nonetheless, to date, no studies have investigated the relationship between SIV and the ACE2/Ang-(1–7)/Mas receptor pathway.

Given the central position of ACE2 in the prevention and treatment of ALI/ARDS, as well as the therapeutic potential of SIV, we hypothesized that the protective effect of SIV was owing to the up-regulation of the ACE2/Ang-(1–7)/Mas receptor pathway. Thus, we established a rat ALI model via the intratracheal instillation of LPS

Highlight box

Key findings

- This study is the first to unveil the impact of sivelestat (SIV) on the angiotensin-converting enzyme 2 (ACE2)/angiotensin (Ang)-(1–7)//Mas receptor targeting axis, and it pioneers in investigating the therapeutic efficacy of SIV on acute lung injury (ALI) in rats in a dose-dependent manner. SIV inhibits the increased secretion of tumor necrosis factor alpha and interleukin-6 in cells induced by lipopolysaccharide (LPS). SIV can reduce the inflammatory response by upregulating the expression levels of ACE2 and Ang-(1–7), thus exerting a pulmonary protective effect.

What is known and what is new?

- SIV is currently the only drug approved for the treatment of ALI/acute respiratory distress syndrome (ARDS) in the world and one of the first urgently approved clinical trial drugs for the treatment of coronavirus disease 2019 in China.
- The results of this study revealed for the first time that the treatment of SIV on LPS-induced ALI is associated with the up-regulation of ACE2 and Ang-(1–7) expression. The ACE2/Ang-(1–7)/Mas receptor axis provides an underlying explanation for the therapeutic effects of SIV *in vitro* and *in vivo*.

What is the implication, and what should change now?

- This study provides a new theoretical basis for the clinical treatment of ALI/ARDS.
- Further experiments strictly verified whether the up-regulation of ACE2 expression by SIV was tissue-specific, and whether SIV could play a lung protective role in the up-regulation of Ang-(1–7) expression at messenger RNA level. It is helpful for the clinical application of SIV in the treatment of ALI/ARDS.

and an inflammatory model of ALI in RAW264.7 cells, were obtained from Wuhan Pricella Biotechnology Co., Ltd. (Wuhan, China), to test our hypothesis. We present this article in accordance with the ARRIVE reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-24-1281/rc).

Methods

Animals and reagents

Male Sprague-Dawley rats, weighing 220±10 g, were obtained from the Department of Laboratory Animals of Jinzhou Medical University (Jinzhou, China). Animal experiments were performed under a project license (No. 241092) approved by ethics board of Jinzhou Medical University, in compliance with Care and Use of Laboratory Animals of Jinzhou Medical University guidelines for the care and use of animals. A protocol was prepared before the study without registration. SIV was purchased from Huilun Biotechnology Co., Ltd. (Shanghai, China). The DEX used for the injection was purchased from China National Pharmaceutical Group Corporation (Beijing, China). LPS was acquired from Sigma Chemical Company (St. Louis, MO, USA). The polymerase chain reaction (PCR) assay kit was purchased from Novoprotein Technology Co., Ltd. (Shanghai, China). TNF-α and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were obtained from Camilo Biological Engineering Co., Ltd. (Nanjing, China). A779 [an Ang-(1–7) receptor potent antagonist] was purchased from Top Science Co., Ltd. (Shanghai, China). The anti-ACE2 polyclonal antibody was purchased from ABclonal Technology Co., Ltd. (Wuhan, China). The anti-β-actin monoclonal antibody was purchased from Bioworld Technology, Inc. (Nanjing, China). Hyclone (Logan, UT, USA) provided the fetal bovine serum (FBS) and Dulbecco's modified Eagle medium (DMEM).

Animal grouping and experimental protocol

The rats were housed in a room with controlled temperature and 12/12 light cycles and had access to a standard rodent diet and water ad libitum. After three days of acclimatization, all the Sprague-Dawley rats were randomized into the following five groups (n=18): control (CON) group; LPS model group; LPS + 5 mg/kg DEX group; LPS + 10 mg/kg SIV group; and LPS + 30 mg/kg SIV group. The rats in the corresponding groups were pretreated with SIV (10 or 30 mg/kg) or DEX (5 mg/kg) intraperitoneally for 30 minutes before being anesthetized with urethane (3). Subsequently, an ALI model was established by the intratracheal instillation of LPS: the rats were immobilized in a supine position, and their windpipes were then exposed after a neck operation. Except for those in the CON group, the rats received and intratracheal instillation of LPS (5 mg/kg) via a 1-mL syringe toward the lung. Next, the rats were gently shaken horizontally in an upright position so that the medication could be distributed evenly throughout the lungs. Meanwhile, the rats in the CON group were intratracheally dripped with identical volumes of normal saline. The general condition of the rats was observed. The rats were sacrificed at three different time points (3, 6, and 12 hours) following the LPS or saline injection (11). Each group comprised six rats at each time point.

Cell culture and grouping

The RAW264.7 macrophage cells were maintained in high-glucose DMEM supplemented with 10% FBS in a humidified incubator with 5% carbon dioxide at 37 ℃. The logarithmic growth cells were inoculated into six-well plates and cultured in a serum-free medium at 80–90% fusion. The cells were divided into the following groups: (I) CON group; (II) LPS model group; (III) LPS + 0.51 mM DEX group; (IV) LPS + 10^{-8} M SIV group; (V) LPS + 10^{-6} M SIV group; (VI) LPS + 0.51 mM DEX + A779 group; (VII) LPS + 10^{-8}M SIV + A779 group; (VIII) LPS + 10^{-6} M SIV + A779 group; (IX) LPS + 0.51 mM DEX + DX600 group; (X) LPS + 10^{-8} M SIV + DX600 group; and (XI) LPS + 10^{-6} M SIV + DX600 group (27). Groups (IV), (VII), and (X) were administered with 10^{-8} M of SIV, groups (V), (VIII), and (XI) with 10^{-6} M of SIV, and groups (III), (VI) and (IX) with 0.51 mM of DEX for 1 hour at the same time. Subsequently, groups (VI), (VII), and (VIII) were co-incubated with A779 [an antagonist of Ang-(1–7)] with a final concentration of 10^{-5} M for 1 hour. Simultaneously, groups (IX), (X), and (XI) were co-incubated with DX600 (a specific inhibitor of ACE2) with a final concentration of 100 nM. Next, the LPS-induced ALI model was established *in vitro*; all the groups except the CON group were treated with 1 μg/mL of LPS for 3, 6, and 12 hours. Cell culture supernatants and cells were extracted respectively at all three time points. The experiment was repeated three times.

Preparation of serum and supernatants of cultured cells

At the corresponding time points, blood was collected by

cardiac puncture under urethane anesthesia. After being kept for 30 minutes at room temperature, the blood samples were centrifuged at 3,000 rpm for 10 minutes at 4 ℃ to separate the serum. Similarly, the cell culture medium was collected at three time points and centrifuged for 20 minutes at 3,000 rpm. Next, the serum samples and supernatants were extracted and stored at –80 ℃ for the subsequent assays.

Lung wet/dry (W/D) ratio

The middle lobes of the right lungs were separated at the end of the experiment at each time point, and the residual exudate and blood were absorbed with filter paper. The wet weight was then recorded. The lung tissues were processed in a dryer at 80 ℃ for 72 hours to achieve a constant weight that was taken as the dry weight, and the W/D weight ratio was calculated to quantify the magnitude of lung edema.

Lung historical and immunohistochemistry (IHC) examination

Half of the left lung tissues of the rats in each group were fixed in 4% paraformaldehyde for 48 hours, embedded in paraffin, and then sliced into sections. Next, hematoxylin and eosin staining was performed. The pathological alterations in the lung tissues were observed under a light microscope. For IHC, the sections were dewaxed, exposed to 3% hydrogen peroxide for 10 minutes at room temperature to inhibit endogenous peroxidase activity, and then blocked with phosphate buffer saline containing 10% normal goat serum for 10 minutes. Subsequently, the sections were incubated with anti-ACE2 (1:100 dilution) at 4 ℃ overnight and then treated with biotinylated secondary antibody for 30 minutes at 37 ℃, followed by color development with 3,3N-Diaminobenzidine Tertrahydrochloride (DAB) horseradish peroxidase chromogenic kits. Next, the sections were redyed, dehydrated, and mounted. The brown-yellow granules were detected as a positive expression under light microscopy.

Quantitative real-time PCR

Total RNA was isolated from the lung tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions, and quantitative real-time PCR was used to detect ACE2 expression products. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control. Specific primers for ACE2 and GAPDH were synthesized through the National Center for Biotechnology Information online primer design tool. The primer sequences were as follows: ACE2 (forward: GTTGGAACGCTGCCATTTAC and reverse: GCTTCATCTCCCACCACTTT); GAPDH (forward: ACTCCCATTCTTCCACCTTTG and reverse: CCCTGTTGCTGTAGCCATATT). The relevant expression level of ACE2 messenger RNA (mRNA) was standardized to the GAPDH gene using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

All the homogenized lung tissues and cells were lysed in Radio Immunoprecipitation Assay lysis buffer on ice. The samples were then centrifuged at 4 ℃ for 20 minutes at 12,000 rpm. Next, the clear supernatants were collected as protein extracts, and total protein concentrations were measured using bicinchoninic acid assay protein quantitation kits as directed. The proteins were separated by 8–10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were blocked with Tris buffered Saline Tween containing 5% skim milk for 2 hours at room temperature, and then incubated overnight at 4 ℃ with the first antibodies, anti-ACE2 (1:1,000 dilution) and anti-β-actin (1:10,000 dilution). Subsequently, the membranes were incubated with the goat anti-rabbit Immunoglobulin G-Horseradish Peroxidase (Wanleibio, Shenyang, China) second antibody for 1.5 hours at room temperature and detected using hypersensitive enhanced chemiluminescence reagent (Biosharp, Labgic Technology Co., Ltd., Bengbu, China). ImageJ software was used to measure the grayscale alues, and each experiment was performed at least three times.

ELISA

The Ang-(1–7), TNF-α, and IL-6 levels in the serum, lung tissues, and cell culture supernatant samples were detected in strict accordance with the ELISA kits' (Camilo, Nanjing, China) operation instructions. Next, the optical density values were determined at 450 nm using a microplate reader (Biotek Corporation, Beijing, China). The concentrations were then calculated using the plotted standard curves.

Statistical analysis

The statistical analyses were performed using the GraphPad

Prism software, version 9.0 (GraphPad Software, San Diego, California, USA). The results are expressed as the mean ± standard deviation. A one-way analysis of variance was used to analyze differences among groups, and a P value <0.05 was considered statistically significant.

Results

Effect of SIV on LPS-mediated lung histopathologic changes and lung edema

Under the light microscope, the normal lung tissue revealed no obvious organizational structure changes, or inflammation. Conversely, after LPS infusion, the lung tissues exhibited significant diffuse congestion, edema, massive exudation of inflammatory cells, and considerable thickening of the alveolar septa. However, the administration of SIV or DEX to the rats improved the pathological changes in the lung tissues (*Figure 1A*). The W/D ratio was used to assess lung edema and was markedly higher in the LPS group than the CON group (P<0.05). This increase was largely prevented by the SIV or DEX treatment (P<0.05). Additionally, the reduction was more prominent in the LPS + 30 mg/kg SIV group than the LPS + 10 mg/kg SIV group (*Figure 1B*).

Effect of SIV on the concentrations of TNF-α and IL-6 in serum

The TNF-α and IL-6 levels in the serum of the rats exposed to LPS for 3, 6, and 12 hours were notably higher than those in the CON group. SIV or DEX considerably decreased the release of TNF-α and IL-6 induced by LPS. Further, the inhibiting effect was stronger in the LPS + 30 mg/kg SIV group than in the LPS + 10 mg/kg SIV group (*Figure 2*).

Effect of SIV on the expression of ACE2 and Ang-(1–7) protein in lung tissues

The protein expression of ACE2 in the lung tissues was detected by western blotting and IHC. The Ang-(1–7) content was evaluated by ELISA as a parameter for the protein expression of Ang-(1–7). The IHC analysis of ACE2 was carried out after LPS infusion in the lung for 3, 6, and 12 hours. The brown-yellow granules, which indicated a positive expression of ACE2, were detected in the alveolar epithelial cells of rats in the CON group. Conversely, the positive cells were barely detectable in the LPS group. However, they were markedly increased in the SIV or DEX pretreatment groups to almost the same level as that in the CON group (*Figure 3A*). Moreover, the western blotting and ELISA results revealed that the expression of ACE2 (*Figure 3B*) and Ang-(1–7) (*Figure 3C*) was considerably decreased in the LPS group compared to the CON group (P<0.05). The pretreatment of rats with SIV or DEX significantly prevented this decrease (P<0.05).

Effect of SIV on the expression of ACE2 mRNA expression in lung tissues by real-time PCR

We used real-time PCR to analyze ACE2 expression in rat lung tissues at the mRNA level to investigate the underlying mechanism of SIV's protective effect. This result, which was almost identical to that of the IHC analysis, was confirmed by a quantitative assessment of the ACE2 mRNA. The expression of ACE2 mRNA was significantly decreased in the LPS-treated tissues compared to those in the CON group. However, this decrease as dramatically improved by pretreatment with SIV (P<0.01) or DEX (P<0.05) in rats (*Figure 3D*).

Effect of SIV on the concentrations of TNF-α and IL-6 in RAW264.7 cells

The TNF-α and IL-6 levels in the supernatants of the RAW264.7 cells exposed to LPS for 3, 6, and 12 hours were substantially higher than those in the CON group (P<0.05). The LPS-induced TNF-α and IL-6 secretions were significantly inhibited by SIV or DEX. However, the administration of A779, the Ang-(1–7) receptor antagonist, to the RAW264.7 cells in the antagonist stimulation groups significantly increased inflammatory cytokine levels, which almost reached the same level as that of the LPS group (*Figure 4*, P<0.05). The results indicated that the anti-inflammatory effect of SIV was associated with Ang-(1–7).

Effect of SIV on the expression of ACE2 in RAW264.7 cells

The Western blotting examination indicated that the expression of ACE2 protein in the RAW264.7 cells after exposure to LPS for 3, 6, and 12 hours was considerably lower than that in the CON group (P<0.05). Conversely, SIV or DEX significantly ameliorated the decrease of ACE2 protein expression caused by LPS stimulation in the RAW264.7 cells, which was prevented by pretreatment with

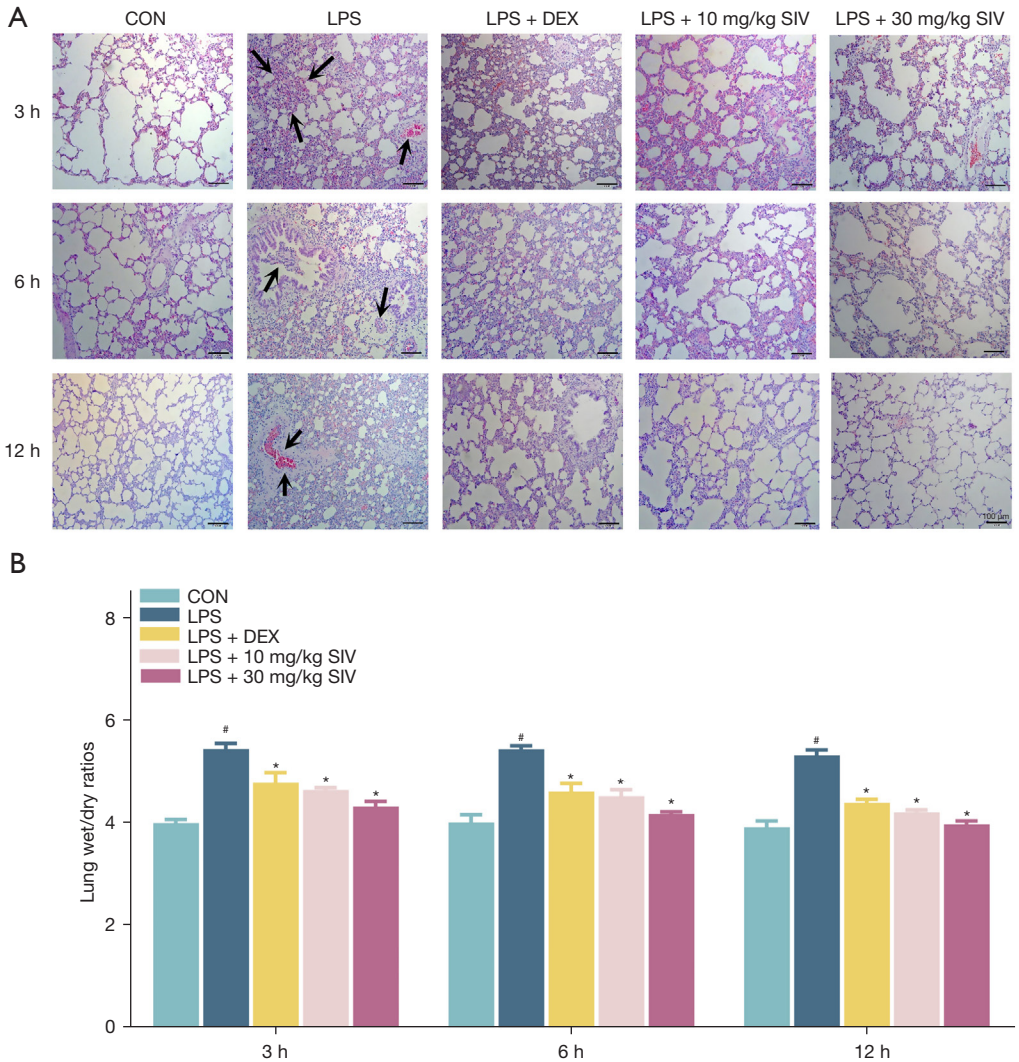

Figure 1 Effect of SIV on the LPS-induced lung histopathologic changes and lung edema of rats. (A) In the CON groups, the normal lung tissues had a clear organizational structure, and no obvious edema or inflammation. In the LPS groups, the lung tissues exhibited substantial diffuse congestion and edema, the massive exudation of inflammatory cells, and considerable thickening of the alveolar septa (as indicated by the black arrows in the pictures) compared with the CON groups. The administration of SIV or DEX administration significantly improved the above pathological changes (representative image of HE staining, scale bar =100 μm). (B) Pretreatment with SIV or DEX noticeably decreased the lung wet/dry ratios induced by LPS. The results are presented as the mean ± standard deviation. #, P<0.05 compared with CON group; *, P<0.05 compared with LPS group. CON, control group; LPS, lipopolysaccharide; DEX, dexamethasone; SIV, sivelestat; HE, hematoxylin and eosin.

A779 (*Figure 5A*, P<0.05). These findings showed that the beneficial effect of SIV was linked to Ang-(1–7).

Effect of SIV on the expression of Ang-(1–7) in RAW264.7 cells

The expression of Ang-(1–7) in the RAW264.7 cell supernatants after 3, 6, and 12 hours of LPS exposure was substantially lower than that in the CON group (P<0.05). SIV or DEX pretreatment of the RAW264.7 cells significantly increased the Ang-(1–7) expression (P<0.01). Nevertheless, DX600, an ACE2 antagonist, noticeably prevented this increase (P<0.05). The expression of Ang-(1–7) in the antagonist stimulation groups almost reached

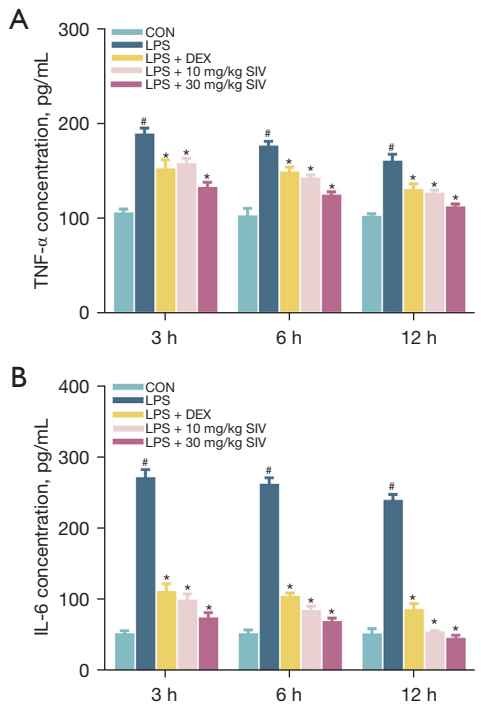

Figure 2 Effect of SIV on the concentrations of TNF-α and IL-6 in serum. (A) The levels of TNF-α in serum were examined 3, 6, and 12 hours after LPS administration. (B) The levels of IL-6 in serum were examined 3, 6, and 12 hours after LPS administration. The results are presented as the mean ± standard deviation. #, P<0.05 compared with CON group; *, P<0.05 compared with LPS group. CON, control group; LPS, lipopolysaccharide; DEX, dexamethasone; SIV, sivelestat; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6.

the same level as that of the LPS group (*Figure 5B*). These results revealed that SIV improved the decrease of Ang-(1–7) expression caused by the LPS stimulation in the RAW264.7 cells, and the ACE2 antagonist significantly attenuated this protective effect. The positive effect of SIV was associated with ACE2.

Discussion

In this study, we discovered that SIV increased the expression of the ACE2/Ang-(1–7)/Mas receptor axis in rat lung tissues, reduced LPS-induced lung histopathological injury, alveolar vascular permeability, and serum TNF-α and IL-6 production. These findings may be related to the anti-inflammatory characteristics of SIV, including the up-regulation of ACE2 and Ang-(1–7) expression to inhibit the production of inflammatory cytokines TNF-α and IL-6 in LPS-stimulated RAW264.7 cells *in vitro*.

SIV is a selective neutrophil elastase inhibitor that can inhibit the degradation of the extracellular matrix protein (the main component of the air-blood barrier), thereby reducing alveolar edema, pulmonary endothelial and epithelial permeability, and neutrophil infiltration (28,29).

SIV is the only medicine approved for the treatment of ALI/ARDS in the world (24,25) and was one of China's first emergency-approved clinical trial medications for COVID-19 therapy (30). In recent years, studies have confirmed that SIV effectively treats ALI/ARDS induced by ischemia-reperfusion injury (31), oleic acid inhalation (32), gastric aspiration, (33) and severe burns (34). Further, the therapeutic potential of SIV has also been investigated in other acute and chronic diseases in many organs and tissues, such as acute pancreatitis (35), extracorporeal shock wave lithotripsy-related kidney damage (36), diabetes mellitus (37), and the prevention of atherosclerosis (38). As a medication undergoing clinical trials, SIV has great research potential and shows promising clinical applications in the treatment of ALI caused by various factors.

LPS, the main component of endotoxin, can elicit a systemic acute inflammatory response and has frequently been used to establish animal models of ALI (39). The pathophysiology of ALI is extremely complicated and is not yet fully understood (40). Studies have shown that LPS-induced ALI is associated with increased inflammatory cell infiltration and pro-inflammatory cytokine production, with the main characteristics of the changes in histopathological characteristics and other indicators in ALI. In pathological sections, it was observed that the structure of most alveolar endothelial and epithelial cells was destroyed, and a large number of neutrophils and other inflammatory cells exudated and aggregated, accompanied by obvious bleeding and congestion. Moreover, the ratio of lung wet weight to lung dry weight increased significantly in the ALI model group, indicating severe pulmonary edema (41). Based on previous research, we established the ALI model in the rats using the intratracheal instillation of LPS, which has been shown to better replicate the usual features of lung injuries in humans (38). LPS stimulation enhances the production of TNF-α, IL-6, and other pro-inflammatory factors while diminishing the expression of ACE2 and Ang-(1–7) (42). LPS infusion causes lung inflammation and apoptosis and then progresses to ARDS (43).

DEX, a synthetic corticosteroid, has been widely used to treat ALI/ARDS and does so by lowering the

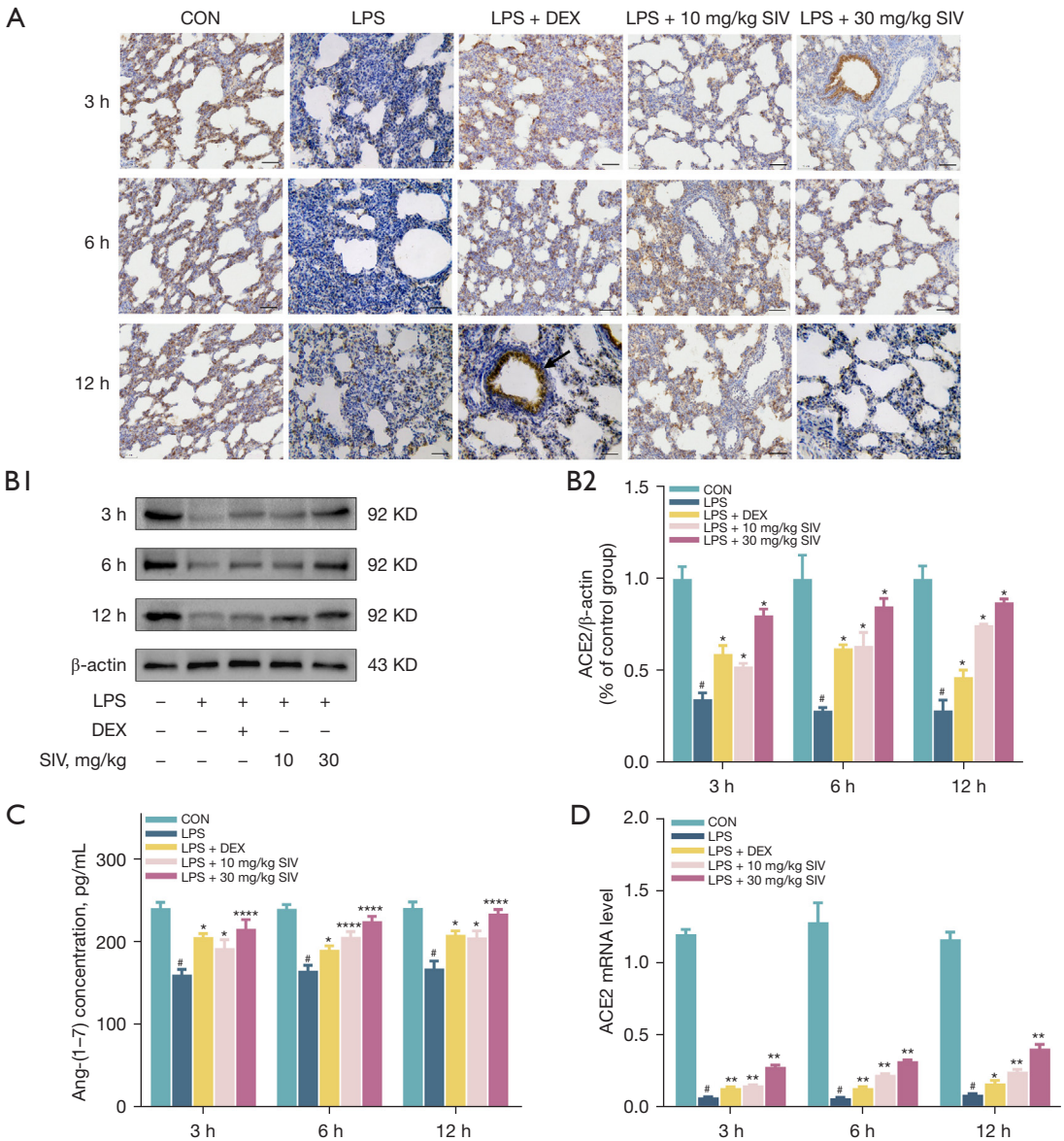

Figure 3 Effect of SIV on the expression of ACE2 and Ang-(1–7) in lung tissues. (A) Representative images of positive expression of ACE2 in lung tissues by immunohistochemical analysis (the black arrows in the pictures indicate positive expression, scale bar =50 μm). (B1) Representative western blotting bands of ACE2 expression in lung tissues. (B2) The quantitative analysis showed the ACE2 protein levels. ACE2 expression was considerably decreased in the LPS groups compared to the CON groups. Pretreatment with SIV or DEX significantly prevented this decrease, and the LPS + SIV groups performed much better than the LPS + DEX groups. (C) Ang-(1–7) expression was significantly decreased in the LPS groups compared with the CON groups. Pretreatment with SIV or DEX markedly prevented this decrease. Further, the LPS + SIV groups performed much better than the LPS + DEX groups. (D) ACE2 mRNA levels in lung tissues were examined 3, 6, and 12 hours after LPS administration. ACE2 mRNA expression was detectable in the CON groups but was significantly reduced in the LPS groups. SIV or DEX administration markedly boosted the levels. The results are presented as the mean ± standard deviation. #, P<0.05 compared with CON group; *, P<0.05 compared with LPS group; **, P<0.01 compared with LPS group; ****, P<0.0001 compared with LPS group. CON, control group; LPS, lipopolysaccharide; DEX, dexamethasone; SIV, sivelestat; ACE2, angiotensin-converting enzyme 2; Ang, angiotensin; mRNA, messenger RNA.

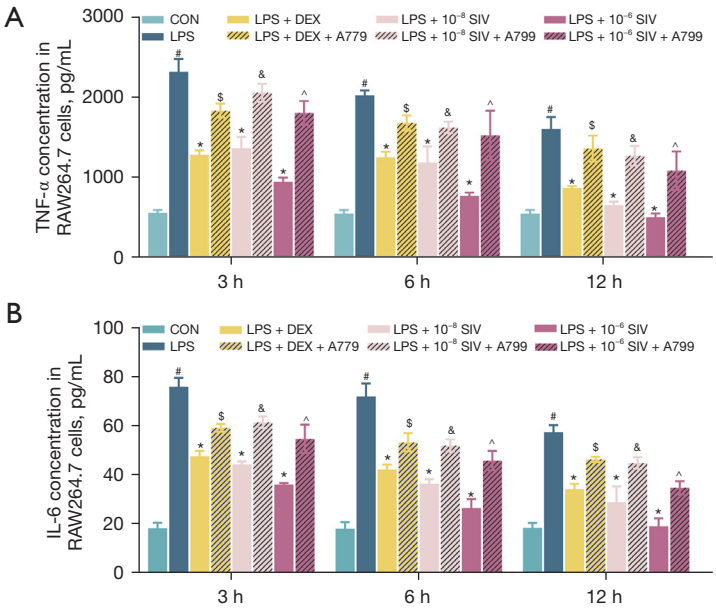

Figure 4 Effect of SIV on the concentrations of TNF-α and IL-6 in the RAW264.7 cells. (A) The level of TNF-α in the supernatants of the RAW264.7 cells exposed to LPS for 3, 6, and 12 hours; (B) The level of IL-6 in the supernatants of the RAW264.7 cells exposed to LPS for 3, 6, and 12 hours. The results are presented as the mean ± standard deviation. #, P<0.05 compared with CON group; *, P<0.05 compared with LPS group; $, P<0.05 compared with LPS + DEX group; &, P<0.05 compared with LPS + 10^{-8} SIV group; ^, P<0.05 compared with LPS + 10^{-6} SIV group. CON, control group; LPS, lipopolysaccharide; DEX, dexamethasone; SIV, sivelestat; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6.

production of pro-inflammatory factors and pulmonary edema while also attenuating alveolar epithelial and endothelial cell damage (44). DEX has also been applied to treat SARS and COVID-19, as well as their accompanying hyperinflammatory or cytokine storms (13). Further, both *in vitro* and *in vivo*, DEX has been shown to significantly increase ACE2 and Ang-(1–7) levels (45). The aforementioned conclusion serves as a positive control drug for DEX and offers robust evidence to support our findings. In the current study, we found that SIV or DEX pretreatment in rats attenuated LPS-induced alveolar hemorrhage, alveolar septum widening, inflammatory cell exudation, and lung edema (*Figure 1*). These findings showed that SIV can be used to alleviate lung pathological injury. Conversely, SIV substantially inhibited LPS-induced inflammatory cytokine release in a dose-dependent manner (*Figure 2*). The aforementioned experimental results are consistent with those of the previous study (46). As is well known, renin cleaves to Ang to produce Ang I, which can be decomposed by ACE to generate Ang II. ACE2, the homolog of ACE, is a RAS counter-regulator (47). Ang-(1–7) then binds to the Mas receptor to form the ACE2/Ang-(1–7)/Mas receptor axis, the beneficial axis of RAS that counteracts the harmful effect of the ACE/Ang II/AT1R axis by increasing vascular permeability and promoting ALI (48).

Many studies have confirmed that activating the ACE2/Ang-(1–7)/Mas axis *in vitro* and *in vivo* can delay or even prevent the development of ALI/ARDS, pulmonary fibrosis, pulmonary hypertension, and other lung diseases (19,49,50). Knocking out the ACE2 gene has been shown to exacerbate ALI in animal models (51). Further, the ACE2/Ang-(1–7)/Mas receptor axis has been shown to play a vital role in protecting pulmonary microvascular endothelial cells against LPS-induced apoptosis and inflammation by inhibiting the c-Jun N-terminal kinase/nuclear factor kappa-B pathways, while the Ang-(1–7) receptor inhibitor (A779) has been shown to reverse the protective effect of ACE2 (52). Similarly, research has shown that fraxinol attenuates LPS-induced ALI in RAW264.7 cells by decreasing inflammatory cytokine generation, which is connected to the regulation of the ACE2/Ang-(1–7)/Mas receptor axis (19). Our results also showed that the absence of LPS-induced ACE2 raised lung vascular permeability, led to pulmonary edema, and increased lung pathological alterations in rats. Similarly, the

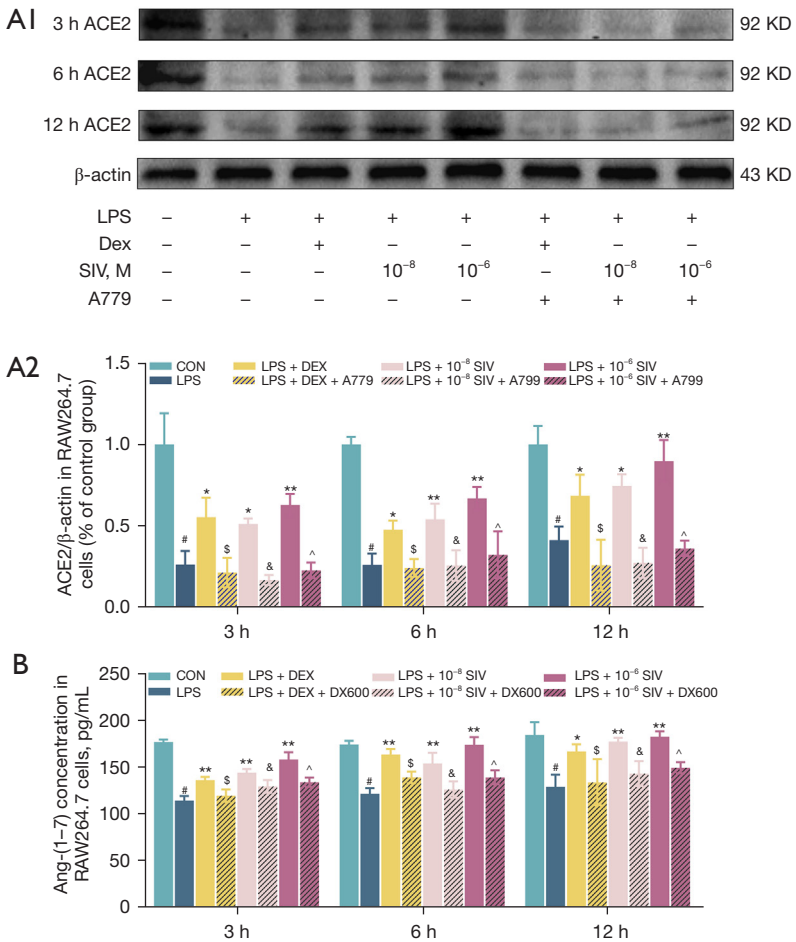

Figure 5 Effect of SIV on the expression of ACE2 and Ang-(1–7) in the RAW264.7 cells. (A1) Representative western blotting bands of ACE2 expression in the RAW264.7 cells. (A2) The quantitative analysis showed the ACE2 protein levels. The expression of ACE2 was considerably decreased in the LPS groups compared to the CON groups. Pretreatment of the RAW264.7 cells with SIV or DEX significantly prevented this decrease, while the administration of Ang-(1–7) receptor antagonist had the reverse effect. (B) SIV or DEX ameliorated the decrease of Ang-(1–7) expression caused by the LPS stimulation in the RAW264.7 cells, and DX600 significantly attenuated this protective effect. The results are presented as the mean ± standard deviation. #, P<0.05 compared with CON group; *, P<0.05 compared with LPS group; **, P<0.01 compared with LPS group; $, P<0.05 compared with LPS + DEX group; &, P<0.05 compared with LPS + 10^{-8} SIV group; ^, P<0.05 compared with LPS + 10^{-6} SIV group. CON, control group; LPS, lipopolysaccharide; DEX, dexamethasone; SIV, sivelestat; ACE2, angiotensin-converting enzyme 2; Ang, angiotensin.

decreased expression of ACE2 and Ang-(1–7) in the ALI inflammation model of RAW264.7 cells also resulted in a considerable increase of inflammatory cytokine expression in the cell culture supernatant.

The cytokine storm, including TNF-α, IL-6, interleukin-8 (IL-8), and interferon gamma, has been confirmed to be involved in the occurrence and progression of ALI (53). TNF-α is the primary promoter of cytokine storm, and it can produce IL-6 and IL-8, and increase pulmonary vascular permeability (54). IL-6, as a core pro-inflammatory cytokine, promotes inflammatory cell movement into and out of the lungs while also enhancing neutrophil recruitment and activation (55). It simultaneously stimulates the release of vascular endothelial growth factor, which increases the pulmonary vascular permeability of inflammatory cells, resulting in pulmonary edema and systemic inflammatory response (56). IL-6 is an essential laboratory indicator of the inflammatory response to LPS-induced ALI because it

represents the degree of inflammatory damage (57). It has also been linked to pulmonary infiltration areas in severe COVID-19 patients (58). TNF-α promotes the activation and release of these pro-inflammatory cytokines, resulting in an uncontrolled inflammatory response and a cascade of an amplified "waterfall effect", which destroys the normal lung structure and aggravates lung injury severity (59).

In recent years, several studies have demonstrated that SIV alleviates inflammation and treats ALI/ARDS via a variety of mechanisms, including by activating Tyrosine-protein kinase Mer (60), and inhibiting the NF-κB pathway (46). Similar to a previous study, our findings indicated that SIV prevented LPS-induced inflammation both inside and outside the lungs by lowering inflammatory cytokine levels (46). Our data also illustrated that SIV decreased lung histopathological changes while also dramatically increasing ACE2 and Ang-(1–7) expression levels. Moreover, A779 and DX600, the antagonists of the ACE2/Ang-(1–7)/MAS receptor axis, reversed all the benefits of SIV, including the anti-inflammation effect in ALI. This suggests that SIV protected against ALI by activating the ACE2/Ang-(1–7)/Mas receptor axis and exerting a substantial anti-inflammatory effect. Based on these findings, SIV-induced ACE2 up-regulation was dose- and time-dependent, but tissue specificity remains unknown. This study only confirmed the protective effect of SIV by up-regulating Ang-(1–7) at the protein level. As is well known, changes in protein level are not necessarily consistent with changes in mRNA. Therefore, it is still unclear whether SIV regulates Ang-(1–7) expression at the mRNA level. We intend to explore these questions further in our future research.

Conclusions

In conclusion, no previous study had investigated the relationship between the pharmacological effects of SIV and the ACE2/Ang-(1–7)/Mas receptor axis. For the first time, our results revealed that the treatment of SIV on LPS-induced ALI is associated with the up-regulation of ACE2 and Ang-(1–7) expression. We also confirmed that the ACE2/Ang-(1–7)/Mas receptor axis provides an underlying explanation for the therapeutic effects of SIV *in vitro* and *in vivo*.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-24-1281/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were performed under a project license (No. 241092) approved by ethics board of Jinzhou Medical University, in compliance with Care and Use of Laboratory Animals of Jinzhou Medical University guidelines for the care and use of animals.

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