ORIGINAL ARTICLE

Pulmonary AngII promotes LPS-induced lung inflammation by regulating microRNA-143

Shenglan Wang · Yan Tan · Tingting Yang · Chen Liu · Rufang Li

Abstract Angiotensin converting enzyme 2 (ACE2) is a terminal carboxypeptidase, which cleaves single terminal residues from several bioactive peptides such as Angiotensin II (AngII). Many investigations indicated that ACE2 functions in angiotensin system and plays a crucial role in inflammatory lung diseases. However, the mechanism behind the involvement of ACE2 in inflammatory lung disease has not been fully elucidated. In this study, BEAS-2B cells were treated with gradient concentration of AngII and lipopolysaccharide (LPS) to induce inflammatory condition. Quantitative RT-PCR was performed to detect the level of ACE2 and miR-143-3p. Western blotting and immunofluorescence assays were performed to measure the expression of related proteins. The levels of inflammatory cytokines and cell viability were respectively measured by ELISA and CCK-8 kits. And ACE2 activity was detected by corresponding commercial kits. Bioinformatics methods were employed to predict the potential microRNA targeting ACE2, which was then confirmed by dual luciferase reporter assay. The results showed that ACE2 expression and activity were time-dependently decreased in LPS group for the first 12 h, after which this tendency was reversed. AngII addition enhanced these effects, compared with LPS group. Additionally, the lowest ACE2 activity level was found in both LPS and AngII + LPS groups at 6 h. The number of nuclei and the ACE2 expression were decreased in LPS groups at 6 h and further reduced by addition of AngII, detected by immunofluorescence. Moreover, ACE2 was validated to be a direct target of miR-143-3p. Pretreatment of AngII and LPS regulated the activity of ACE2, increased the expression of proinflammatory cytokines and cell apoptosis and regulated the expression of Bax, Bcl-2 and cleaved caspase-3 in BEAS-2B cells, which could be reversed by transfecting miR-143-3p inhibitor. The results collectively suggest that AngII promotes LPS-induced inflammation by regulating miR-143-3p in BEAS-2B cells. Therefore, miR-143-3p is considered a potential molecular target for the treatment of lung inflammation.

Keywords ACE2 · AngII · MicroRNA-143 · Inflammation · Bronchial epithelial cell

Shenglan Wang and Yan Tan make equal contributions for this work.

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Introduction

Lung inflammation is a nonnegligible pulmonary response to many kinds of external stimulation (Khair et al. 1996). It, as is known, can be caused by pneumonias, sepsis, immune deficiency or trauma. However, the common instigating events remain unknown (Li et al. 2011). Lung inflammation usually occurs with pathogen exposure, which is often associated with molecular patterns such as lipopolysaccharide, LPS (Boskabadi et al. 2019). Acute LPS exposure is a trigger of respiratory symptoms or inflammation, which can be observed in vitro as increased levels of pro-inflammatory cytokines and neutrophil infiltration (Arora et al. 2011; Hu et al. 2019). The investigation of lung inflammation mechanism should be proceeded and deepened, which is a must for discovering unique and effective therapeutic targets for lung inflammation.

The role of Angiotensin II (AngII) has been reported in the development of atherosclerosis and in the arterial response of injury and restenosis. And the mechanisms above all include extracellular matrix production, cytokine induction and vascular hypertrophy (Silva et al. 2020). The angiotensin-converting enzyme (ACE) is a metallo monocarboxypeptidase, the only one of several enzymes that can produce AII and is generally considered as a component of the renin angiotensin system (Zhang et al. 2015). The participation of ACE2 in lung function has attracted research attention in recent years because of the findings supporting that this ectoenzyme is also the receptor of the SARS and NL63 human coronaviruses, CoVs (Li et al. 2003). Moreover, the discord of pulmonary ACE2 function plays an important role in respiratory diseases including severe infections such as SARS-CoV, sepsis and acid aspiration (Sodhi 2018). Recently, some studies found that miRNAs were closely associated with the pathogenesis of coronavirus disease (Matarese et al. 2020; Tang et al. 2020; Gallicano et al. 2020).

However, the role and the potential mechanism of pulmonary ACE2 in inflammation are still unknown and in need of exploration. The recognition of differently expressed miRNAs through bioinformatics database and transcriptome data could help us to understand and investigate pathological mechanisms in sepsis and associated lung injury (Ahmad et al. 2019, 2020). A study report that miRNA-34a

inhibition was able to improve lung injury and reduce the levels of inflammatory markers in LPS-induced lung injury (Khan et al. 2020). MicroRNAs (miRs) are approximately 22 nucleotides RNA molecules which regulate gene expression at the posttranscriptional level by escalating mRNA degradation or translational repression (Kim et al. 2009). miRs participate in most basic biological processes, whose abnormal expression has been found in many human diseases (Bartel and Bartel 2009).

miR-143/145 was previously reported to be expressed in cardiomyocytes and smooth muscle cells, SMCs (Wang et al. 2014). A study demonstrated that inhibiting ACE and/or angiotensin type 1 (AT1) receptor in mice with loss of miR-143/145 rescued the vascular dysfunction and the aberrant gene expression (Boettger et al. 2009). Further, it is known that miR-143 has close contact with human immune response and may become in future a therapeutic target for immunization in view of its adjustment for the anti-inflammatory effect of mesenchymal stem cells (Ponsuksili et al. 2017). According to the latest study, miR-143 is intimately related to lung sepsis in mouse, and human leukocytes after inducement of LPS (Han et al. 2016). However, little is known about the relationship between ACE2 and miR-143 in the lung immune response. The purpose of the present study was to investigate the exact role of AngII in lung inflammation, and further elucidate the underlying mechanism.

Materials and methods

Cell culture and reagent

The human bronchial epithelial cells BEAS-2B were acquired from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F12 (DMEM/F12, Gibco, Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Life Technologies) and 1% penicillin–streptomycin–glutamine (Gibco, Life Technologies). Flask was placed at 37 °C in a humidified incubator (Thermo Fisher Scientific, USA) with 5% CO_2.

LPS and AngII were purchased from Sigma-Aldrich (St Louis, MO, USA). The serum-free DMEM/F12 was used to dilute LPS to 1 mg/L, and

AngII solution to 1, 5, 10 or 50 µmol/L before experiments. BEAS-2B cells were treated with 1 mg/L LPS for 24 h, and/or 0, 1, 5, 10 or 50 µmol/L AngII every 24 h for 96 h in this research.

Cell transfection

One day prior to transfection, BEAS-2B cells (5×10^4 cells/well) were seeded into 6-well plates and cultured at 37 °C in an atmosphere containing 5% CO_2. Subsequently, the cells were transfected with small interference RNA targeting miR-143-3p mimic, miR-143-3p inhibitor or negative control (NC) using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Cells of the blank control group (control) did not receive any treatment. Following incubation for 48 h, these cells were collected for the following experiments. Transfection efficiency was detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

Real-time quantitative PCR

qRT-PCR was performed to detect the expression level of miR-143-3p and ACE2 in BEAS-2B cells after corresponding treatment or transfection. Briefly, total RNAs in BEAS-2B cells were extracted using TRIzol Plus RNA Purification kit (Invitrogen). cDNA was reversely transcribed using SuperScript Reverse Transcription Reagents (Invitrogen). The expression level of miR-143-3p and ACE2 were then measured at 0, 3, 6, 12 and 24 h after AngII and/or LPS treatment using TaqMan MicroRNA Assay (Applied Biosystems), with U6 as the endogenous control. The primer sequences were as follows: miR-143-3p forward: 5′-CTGGCGTTGAGATGAAGCAC-3′, and reverse: 5′-CAGAGCAGGGTCCGAGGTA-3′; ACE2 forward: 5′-TCTGCCACCCCACAGCTT-3′, and reverse: 5′-GGCTGTCAAGAAGTTGTCCATTG-3′; U6, forward: 5′-GCGAGCACAGAATTAATACGAC-3′, and reverse: 5′-AACGCTTCACGAATTTGCGT-3′. Data were quantified using the classic $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Cell viability assay

Viability of BEAS-2B cells was evaluated using cell counting kit-8 (CCK-8) assay (Beyotime Biotechnology, Shanghai, China) in this research. Briefly, BEAS-2B cells were seeded into 96-well plates (2×10^3 cell/well) respectively after AngII (1, 5, 10 or 50 µmol/L) and/or LPS (1 mg/L) treatment. And 10 µL CCK-8 solution was added into each well followed by incubation at 37 °C. Subsequently, the absorbance of each well was recorded at 450 nm after 24 h, 48 and 96 h of incubation using Micro-plate Reader (Bio-Tek Instruments, Winooski, VT, USA). Cell viability (%) was calculated as the ratio of average absorbance of treatment group/average absorbance of control group × 100%.

Measurement of ACE2 activity

Measurement of ACE2 activity (SensoLyte 390 ACE2 activity assay kit; AnaSpec, USA) was conducted in accordance with the supplied manual. Briefly, cells were incubated for 15 min at 4 °C and then centrifugated at $20,000 \times g$ for 10 min. The supernatants were collected and stored at − 80 °C for ACE2 activity measurement. Total protein concentration was quantified using the Bradford protein assay.

Immunofluorescence

BEAS-2B cells were incubated with polyclonal antisera against ACE-1 (catalog ab39172; Abcam). Secondary antibodies used were appropriate fluorescent labeled secondary antibodies (1:1000, Life Technologies, Inc.) as well as the nuclear marker DAPI (Biolegend). The slides were then mounted using Gelvatol (Sigma-Aldrich) solution prior to imaging using a Zeiss LSM 710 confocal microscope (Carl Zeiss, Jena, Germany) under appropriate filter sets.

Enzyme-linked immunosorbent assay (ELISA)

In supernatant samples, IL-1β, IL-6 and tumor necrosis factor α (TNF-α) were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer 's instructions.

Western blot analysis

The AngII and/or LPS-induced cells were collected and lysed at 24 h after treatment using RIPA buffer. Identical amounts of proteins were resolved by 10–15% SDS-PAGE and then transferred to a PVDF

membrane (Millipore Corporation, Billerica, MA, USA). The membrane was incubated with specific antibodies (Bcl-2, Bax, cleaved caspase3, ACE2 and β-actin; Cell Signaling Technology, Inc.), and peroxidase-conjugated secondary antibodies (Gaithersburg, MD, USA) at 4 °C overnight. Chemiluminescence (Millipore Corporation) and densitometry analysis (ImageJ software) were applied to the protein expression detection.

Dual luciferase reporter assay

It was predicted by bioinformatics software (http://www.targetscan.org) that ACE2 can be a potential target of miR-143-3p. To confirm this prediction, the 3′-UTR of ACE2 containing the miR-143-3p binding site was cloned into pmirGLO dual luciferase miRNA reporter vectors (Promega, Madison, WI, USA). A mutated 3′-UTR of ACE2 was introduced into the potential miR-143-3p binding site. The reporter vectors containing the wild type or mutant of ACE2 3′-UTR and miR-143-3p mimic or miR-NC were co-transfected into BEAS-2B cells using Lipofectamine 3000 (Invitrogen). The relative luciferase activity was detected right after 48 h of transfection with a dual-luciferase reporter assay system (Promega Corporation, Madison, WI, USA).

Statistical analysis

Data are expressed as the mean ± standard deviation. SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. Comparisons between groups were analyzed by Student's t-test or one-way analysis of variance followed by Tukey's test. $P < 0.05$ is considered to indicate a statistically significant difference.

Results

ACE2 is up-regulated in BEAS-2B cells treated with AngII in low concentrations

To investigate the role of ACE2 and AngII in lung inflammation, we measured ACE2 expression in the human bronchial epithelial cells. CCK-8 assay showed that AngII (0, 1, 5, 10 or 50 μmol/L) significantly reduced cell growth and viability of BEAS-2B in a dose-dependent manner after 24 h (Fig. 1A). As shown by RT-qPCR analysis (Fig. 1B), the expression level of ACE2 was increased most significantly by 5 μmol/L AngII compared with the control group without AngII treatment and other groups treated with different concentrations of AngII. Therefore, we chose 5 μmol/L AngII for our further studies.

AngII promotes LPS-induced inflammation in BEAS-2B cells

Next, we used LPS to further investigate AngII biology and its possible role in mediating inflammation. Compared with the control group without LPS and AngII, 1 mg/L LPS significantly reduced cell viability of BEAS-2B, which could be further reduced by adding AngII (Fig. 2A). Moreover, we examined whether the ACE2 activity was altered by LPS and AngII in BEAS-2B cells using the SensoLyte 390 ACE2 activity assay kit. As shown in Fig. 2B, ACE2 activity was time-dependently decreased in LPS group within 12 h compared with control group, and this tendency could be reversed after 12 h. By adding AngII, the effects mentioned above could be enhanced compared with LPS group. As shown by RT-qPCR analysis (Fig. 2C), after interfering with LPS, the expression level of ACE2 mRNA was significantly increased in the first 3 h, then decreased from 3 to 12 h, and noticeably increased again after 12 h. After pretreatment with AngII, the increasing ACE2 was significantly receded in 3 h compared with LPS group. And from 3 to 12 h, the weakening trend of ACE2 in AngII + LPS group was also inferior to LPS group. These results showed that the change of ACE2 mRNA expression fell behind with the change of activity. ACE2 showed the minimum activity in both LPS and AngII + LPS groups at 6 h. In addition, the results of immunofluorescence showed that the number of nuclei and the ACE2 expression were decreased in LPS groups at 6 h, which could be further reduced by adding AngII (Fig. 2D).

The consumption of ACE2 in LPS-induced BEAS-2B cells indicated that AngII may function as an enhancer of lung inflammation. To test this hypothesis, we investigated whether inhibition of AngII affects inflammation and cell apoptosis in bronchial epithelial BEAS-2B cells treated with LPS. After
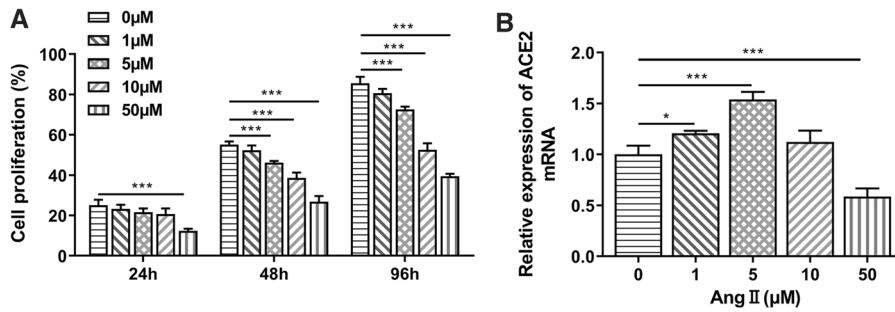
Fig. 1 After human bronchial epithelial cells underwent the treatment of different concentration of AngII (0, 1, 5, 10, 50 μmol/L), the detection of cell proliferation through CCK-8 assay (**A**) and the detection of ACE2 mRNA through RT-qPCR (**B**) were performed, respectively. *p < 0.05 and ***p < 0.001
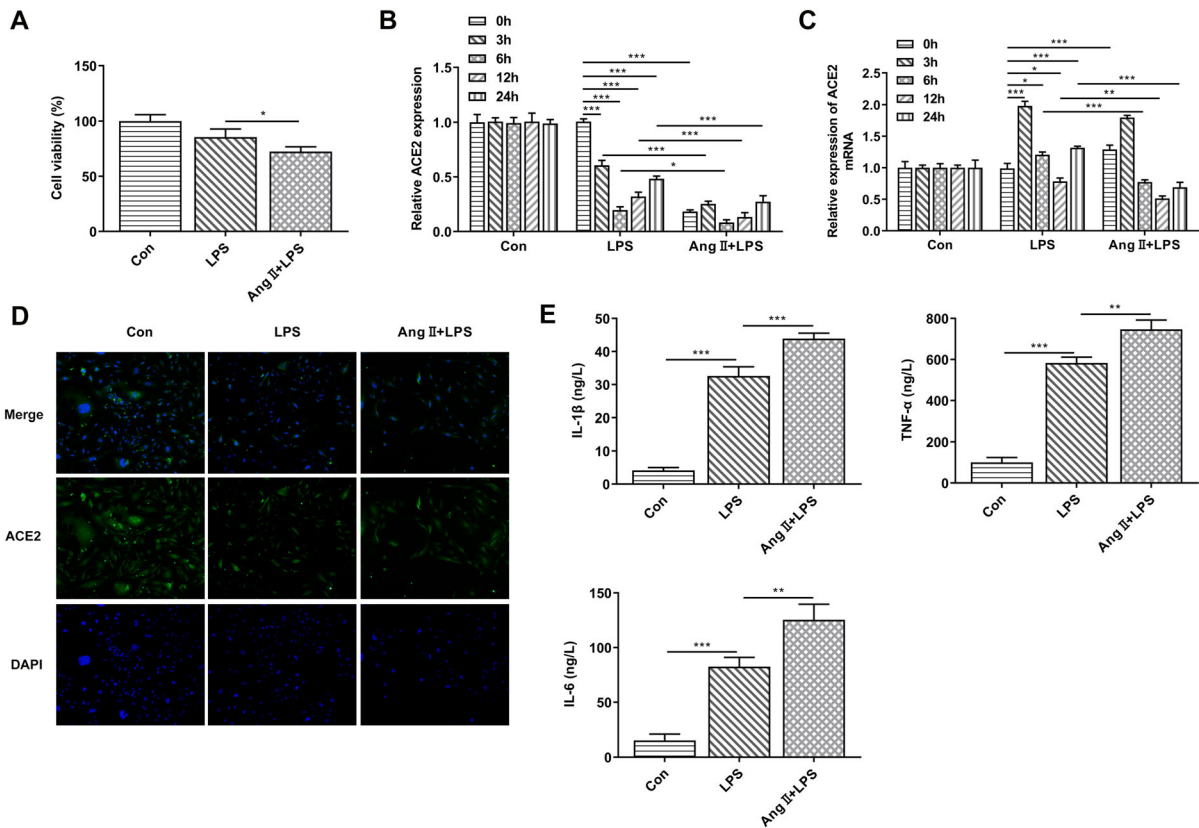

Fig. 2 After LPS or AngII treatment, human bronchial epithelial cells were used to perform the detection of cell viability through CCK-8 assay (**A**), the analysis of ACE2 activities (**B**) and the measurement of ACE2 mRNA through qPCR (**C**), immunofluorescence assay for the analysis of ACE2 expression (**D**), and qPCR assay for the analysis of expression of IL-1β, IL-6 and TNF-α (**E**). *p < 0.05, **p < 0.01 and ***p < 0.001

24 h, in Fig. 2E, the proinflammatory cytokines, including IL-1β, IL-6 and TNF-α, induced by LPS were all significantly enhanced by AngII. Further, in western blot assay (Fig. 3A, B), compared with control group, the expression of Bax, cleaved-caspase-3 and ACE2 were upregulated, and the expression of Bcl-2 was downregulated, and these tendencies could be further strengthened by adding AngII. These results indicate that AngII enhances proinflammatory cytokine production and cell apoptosis induced by LPS.
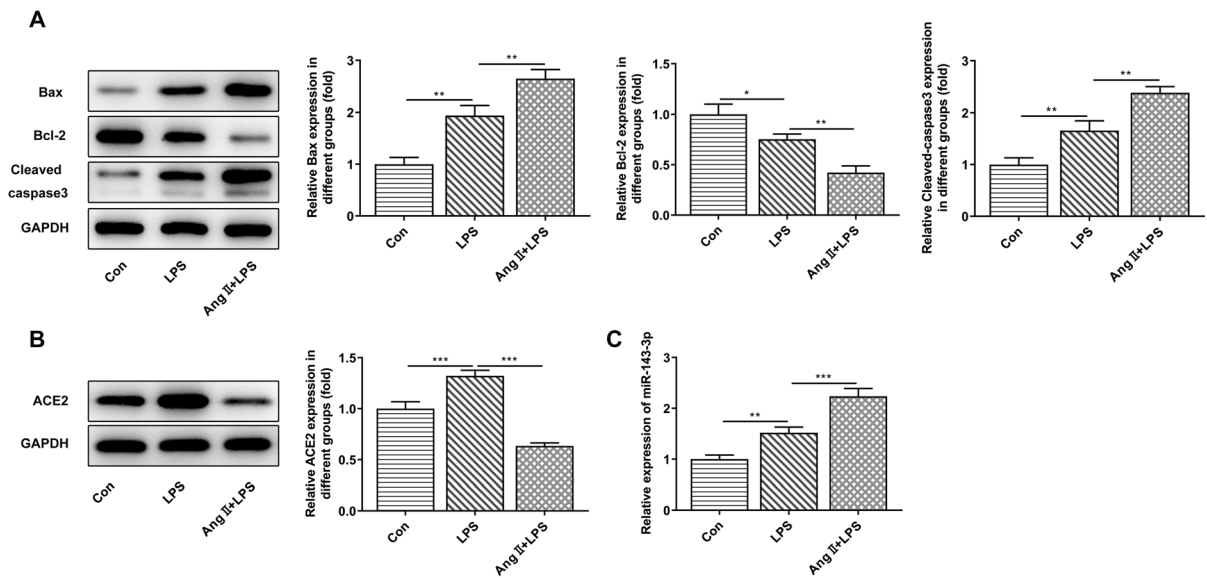
Fig. 3 After LPS or AngII stimulation, human bronchial epithelial cells were used to perform western blot assay for the expression analysis of Bax, cleaved-caspase-3 and Bcl-2 (**A**) and ACE (**B**), and qPCR for the analysis of miR-143-3p levels (**C**). *p < 0.05, **p < 0.01 and ***p < 0.001

AngII promotes LPS-induced inflammation by regulating miR-143 in BEAS-2B cells

miRNAs are important regulators in lung inflammation. To further verify the underlying mechanism of the effect of AngII in LPS-induced BEAS-2B cells, we utilized multiple target prediction programs to identify the potential miRNAs that ACE2 targets. miR-143-3p was highlighted for further investigation.

RT-PCR was performed to investigate the expression of miR-143-3p in BEAS-2B cells, shown in Fig. 3C. We observed an increase in miR-143-3p expression in LPS-induced BEAS-2B cells compared with control group, which could be enhanced by AngII. In addition, a transfection plasmid, miR-143-3p inhibitor or negative control RNAs (miR-NC) was transfected to BEAS-2B cells. Compared with the control and the miR-NC groups, the expression of miR-143-3p was significantly decreased in miR-143-3p inhibitor group (Fig. 4A).

Next, we investigated whether miR-143-3p is critical for the function of AngII in BEAS-2B cells. CCK-8 assay showed that AngII pretreatment significantly inhibited the viability of LPS-induced BEAS-2B cells, and that this inhibitory effect was reversed by miR-143-3p inhibitor transfection (Fig. 4B). ACE2

activity was significantly decreased in AngII + LPS and AngII + LPS + NC groups compared with control group in 6 h, and this tendency could be reversed by transfecting miR-143-3p inhibitor (Fig. 4C). The expressions of proinflammatory cytokines, including IL-1β, IL-6 and TNF-α, induced in AngII + LPS and AngII + LPS + NC groups were reversed by miR-143-3p inhibitor. Western blot assay (Fig. 4D) showed that compared with the control group, the expressions of Bax, cleaved-caspase-3 and ACE2 were upregulated while that of Bcl-2 was downregulated in AngII + LPS and AngII + LPS + NC groups, and that these tendencies could be reversed by adding miR-143-3p inhibitor (Fig. 5A, B).

The dual-luciferase reporter assay confirmed the relationship between ACE2 and miR-143-3p predicted by bioinformatics methods. Firstly, RT-PCR showed increased expression of miR-143-3p in AngII and LPS-induced BEAS-2B cells compared with control group, which could be reversed by miR-143-3p inhibitor (Fig. 5C). Next, dual-luciferase reporter results showed that cells co-transfected with pDNA-ACE2-wild type and the miR-143-3p mimic (the wt-ACE2 + miR-143-3p mimic group) had significantly lower luciferase activity than cells co-transfected with pDNA-ACE2-wild type and the miR-NC (the wt-
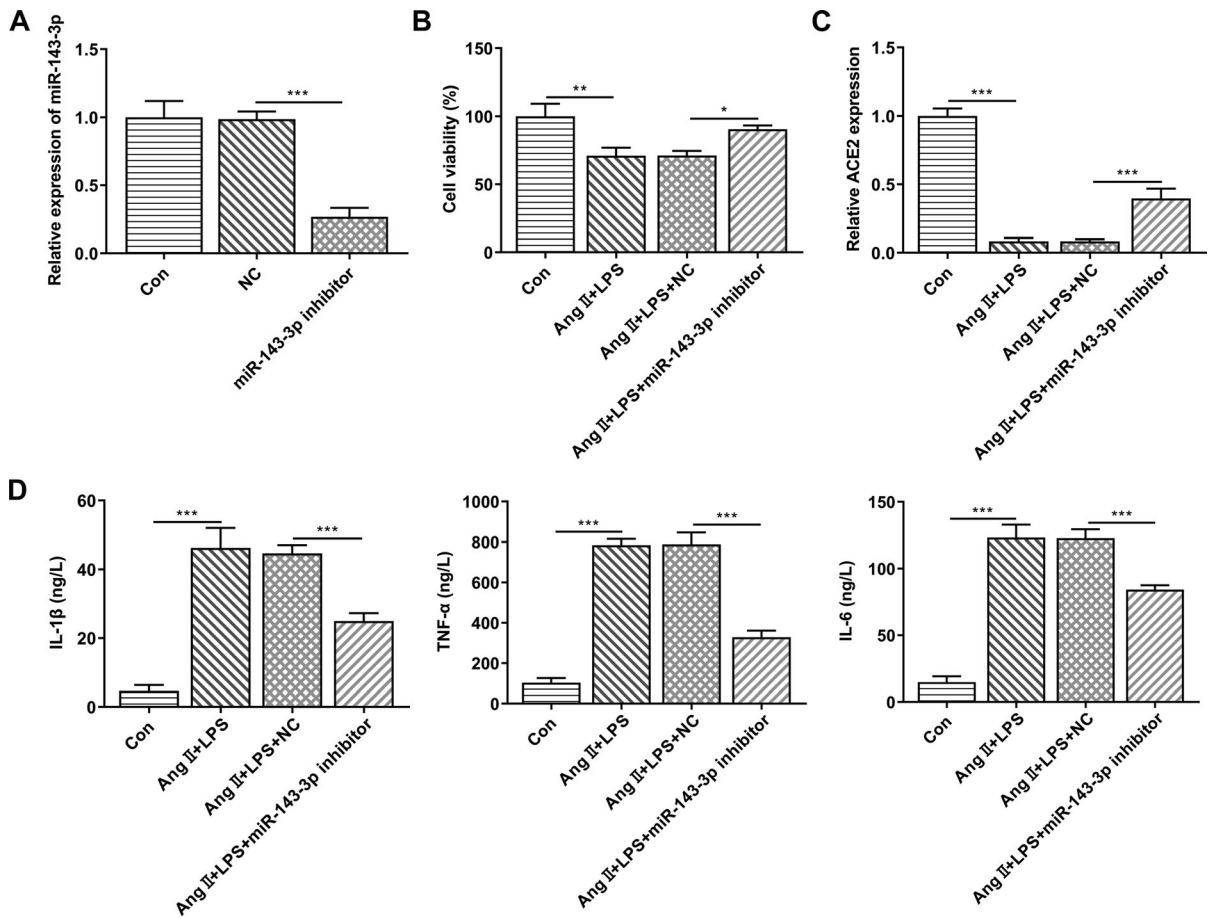
Fig. 4 miR-143-3p inhibitor interfered the effects of AngII on inflammatory markers in BEAS-2B cells under LPS stimulation. The analysis of miR-143-3p expression through qPCR (**A**), cell viability through CCK-8 assay (**B**), ACE2 activities (**C**), the detection of IL-1β, IL-6 and TNF-α levels through ELISA (**D**). *p < 0.05, **p < 0.01 and ***p < 0.001

ACE2 + NC mimic group). In contrast, we found no significant difference in the relative luciferase activity between the mut-ACE2 + miR-143-3p and the mut-ACE2 + NC mimic groups (Fig. 5E). These data strongly suggest that ACE2 targets and has a negative correlation with miR-143-3p expression in lung inflammation.

Discussion

Pneumonia has been regarded as a leading cause of infectious disease-related morbidity and mortality worldwide (Ravi Kumar et al. 2018). In our study, we used LPS and AngII to establish the bronchial cell inflammatory injury model. We found that 1 mg/L LPS and 5 μmol/L AngII treatment successfully

induced BEAS-2B cell apoptosis, inhibited cell viability, and enhanced the expression of proinflammatory cytokines. In this study, the important role of miR-143-3p in lung inflammation is identified. miR-143-3p expression upregulated in AngII and LPS-induced injury induced cell apoptosis and inflammation and decreased the expression and the activity of ACE2 by targeting it in AngII and LPS-induced BEAS-2B cells. Therefore, miR-143-3p may be a promising therapeutic target for pneumonia treatment.

ACE2, whose function can be generally explained by its name, converts angiotensin and thereby regulates the renin–angiotensin system, RAS (Mowry et al. 2019). RAS regulates blood pressure, fluid dynamics, and electrolyte balance. Additionally, it plays a role in modulating the innate immune system and is a potent regulator of inflammation (Sodhi et al. 2019). In our
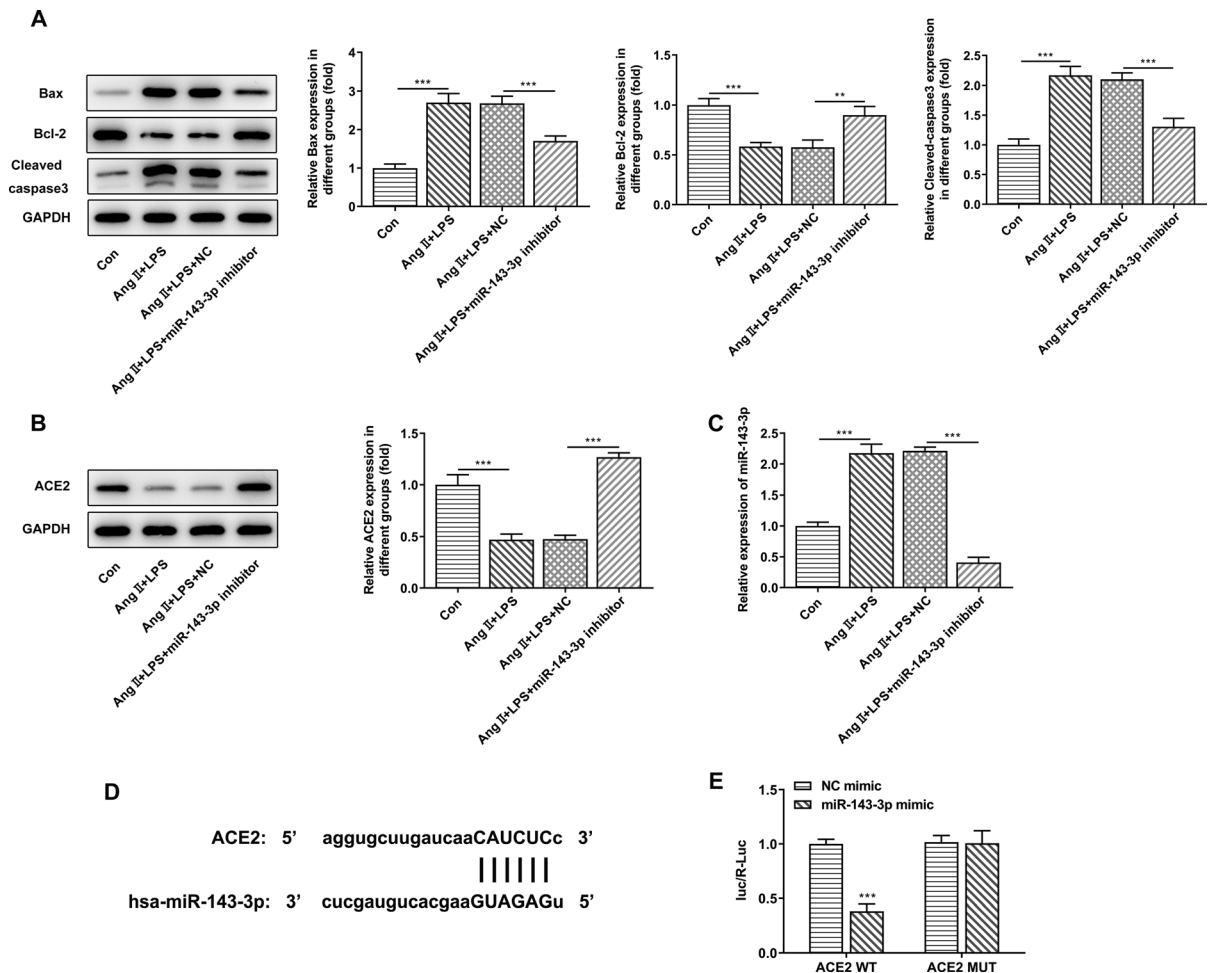
Fig. 5 miR-143-3p inhibitor counteracted the effects of AngII apoptosis-related proteins. The detection of Bax, cleaved-caspase-3, Bcl-2 and ACE2 proteins through western blot (**A**, **B**), the analysis of miR-143-3p mRNA through qPCR (**C**), the prediction of binding sites between ACE2 and miR-143-3p (**D**) and dual-luciferase reporter assay (**E**). **p < 0.01 and ***p < 0.001

study, ACE2 expression and activity were time-dependently decreased in LPS-induced group compared with the control group within the first 12 h, which started to increase after 12 h. Pretreatment of LPS significantly increased the expression of proinflammatory cytokines, enhanced cell apoptosis and regulated the expression of Bax, Bcl-2 and cleaved caspase-3 in BEAS-2B cells. Again, in view of ACE2's ability to convert AngII to Ang 1-7, blocking AngII/AGT1R and activating Ang 1-7/Mas1, it thereby alleviates the inflammatory response induced by RAS. (Shi et al. 2013). Moreover, Ang 1-7 can act against AngII and dampen its effects (Liu et al. 2016). In our study, ACE2 expression was significantly

increased after pretreatment of AngII in 3 h compared with LPS group, which might owe to the positive feedback of AngII. In fact, in other diseases such as sepsis and acid aspiration-induced lung injury, ACE2 has been shown to be beneficial for modulating the inflammatory response. And studies have shown that pulmonary ACE2 is impaired in several lung diseases, including viral and aspiration pneumonia, pulmonary hypertension, and LPS-induced lung injury (Imai et al. 2005; Shao et al. 2019). Additionally, ACE2, which was abundantly expressed in the lung cells including vascular endothelium and respiratory epithelium, could mediate the access of SARS-CoV-2 to host cells (Amraei et al. 2020; Sardu et al. 2020; Pearce

et al. 2020). Systemic inflammatory response caused by SARS-CoV-2 is the most important pathological basis in novel coronavirus pneumonia patients, especially in critical patients who died due to complicated sepsis. Recently, a study proposed that the decreased ACE2 receptor expression was engaged in keeping off SARS-CoV-2 invasion of pulmonary epithelial cell (Hasanagic et al. 2020). Our work suggested that ACE was reduced by AngII via miR-143-3p, which provides a new sight for into pathogenesis of COVID-19 induced lung inflammation. Latest studies revealed that pneumonia and lung cancer are significant hazard factors connected to the mortality of patients of certain age with hypertension (Shen et al. 2016). Hypertension is commonly seen in non-cardiovascular diseases, including severe Coronavirus Disease 2019 (COVID-19) pneumonia and acute respiratory distress syndrome, ARDS (Pranata et al. 2020). Interestingly, in our study, the ACE2 expression was largely consumed in AngII + LPS group between 3 and 12 h, which was consistent with prior studies and might explain the relationship between hypertension and lung inflammation.

miRs are small non-coding RNAs, which are approximately 19 to 25 nucleotides long and act as posttranscriptional regulators of gene expression (Lagos-Quintana and Lagos-Quintana 2001). A large number of studies have proven that miRNAs can regulate various cellular processes such as differentiation, proliferation, and cell–cell interaction (Chen and Meister 2005). Likewise, some miRNAs are dysregulated in lung inflammation and functionally implicated in the pathogenesis of lung inflammation (Xue et al. 2018; Yun et al. 2020). In previous study, serum miR-143 was found to be increased in patients suffering from sepsis and thought to be a potentially valuable biomarker for distinguishing sepsis and systemic inflammatory response syndrome, SIRS (Han et al. 2016). Decreased expression of miR-143-3p has been observed in fibromyalgia, further suggesting its association with inflammatory pain (Wang et al. 2020). Our results showed that pretreatment of AngII and LPS regulated the activity of ACE2, increased the expression of proinflammatory cytokines, and promoted cell apoptosis in BEAS-2B cells, which could be reversed by transfection of miR-143-3p inhibitor. Above all, the present study revealed that AngII promotes LPS-induced inflammation by regulating miR-143-3p in BEAS-2B cells.

In conclusion, our results proved that miR-143-3p inhibits ACE2 by targeting the 3′-UTR of ACE2. Functional experiments in our study showed that AngII promotes apoptosis and inflammation by regulating miR-143-3p in BEAS-2B cells. These results suggest that the regulation of ACE2 by miR-143-3p may become a novel strategy for the treatment of lung inflammation.

Declarations

Conflict of interest The authors declare no conflict of interest.

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