Osthole Alleviates Bleomycin-Induced Pulmonary Fibrosis via Modulating Angiotensin-Converting Enzyme 2/Angiotensin-(1–7) Axis and Decreasing Inflammation Responses in Rats

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Regular Article

Pulmonary fibrosis (PF) is a fatal interstitial lung disease significantly correlating with a dismal outcome, which exact etiological mechanisms are unknown. PF is characterized by progressive scarring of lung tissue, exaggerating of fibroblast proliferation, excessively deposition of extracellular matrix proteins, exacerbating of lung inflammation, and diffused proliferation, excessively deposition of extracellular matrix progressive scarring of lung tissue, exaggerating of fibroblast etiological mechanisms are unknown. PF is characterized by significantly correlating with a dismal outcome, which exact could protect against bleomycin (BLM)-induced fibrosis. On the other hand, studies showed that the traditional Chinese herbal extract, osthole, had beneficial effects on lipopolysaccharide (LPS) induced acute lung injury (ALI) via an ACE2 pathway. Here we further investigated the protective effects of osthole on bleomycin induced pulmonary fibrosis and attempted to determine the underlying mechanism. PF mode rats were induced by bleomycin (BLM) and then subsequently administered osthole. Histopathological analyses were employed to identify PF changes. The results showed that BLM resulted in severe PF and diffuse lung inflammation, together with significant elevation of inflammatory factors and a marked increase in expression of angiotensin II (ANG II) and transforming growth factor-beta 1 (TGF-β1). ACE2 and angiotensin-(1–7) [ANG-(1–7)] were both greatly reduced after BLM administration. Meanwhile, osthole treatment attenuated BLM induced PF and inflammation, decreased the expression of these inflammatory mediators, ANG II, and TGF-β1, and reversed ACE2 and ANG-(1–7) production in rat lungs. We conclude that osthole may exert beneficial effects on BLM induced PF in rats, perhaps via modulating the ACE2/ANG-(1–7) axis and inhibiting lung inflammation pathways.

Key words pulmonary fibrosis; transforming growth factor beta; osthole; angiotensin-converting enzyme 2; angiotensin II (ANG II); ANG-(1–7)

MATERIALS AND METHODS

Chemicals and Instruments ANG-II Mouse Monoclonal Antibody (Abcam, Cambridge, U.K.) - Osthole was obtained from YiLe Bio-Tech laboratory (Xi’an, Shanxi, China). BLM and Sirius red F3B were bought from Sigma-Aldrich (St. Louis, MO, U.S.A.). Light microscope was purchased from Olympus (BX50, Tokyo, Japan). ACE2, tumor growth factor-beta 1 (TGF-β1), and ANG-(1–7) mouse monoclonal antibodies were from Abcam (Cambridge, U.K.). Enzyme-linked immunosorbent assay (ELISA) kits for ANG-(1–7), ANG II, tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) were purchased from R&D Corporation (R&D Systems Inc., MN, U.S.A.). Radio immunoprecipitation assay (RIPA) lysis buffer was bought from Beyotime Inc. (Jiangsu, China). Enhanced chemiluminescence (ECL) kit was purchased from BestBio Inc. (Shanghai, China). Primers of quantitative polymerase chain reaction (qPCR) were designed and purchased from Qiagen Company (Germany). Image analysis system was from Media Cybernetics (Image Pro Plus version 6.0; Bethesda, MD, U.S.A.).

Experimental Grouping All the animals’ experiments were verified and approved by the Animal Care and Use Committee of the Fourth Military Medical University and complied with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1996). Thirty male Sprague-Dawley (SD) rats (body weight 176±14 g) were purchased from the ani-
mal center of the Fourth Military Medical University (Xi’an, China), and the rats were randomly grouped into 3 groups, 1) Control group, 2) BLM group, and 3) BLM+osthale (OSTH) group, 10 rats for each group. Optimal dose of osthale was chosen by our preliminary experiments (details are provided in the supplemental data). After anesthetization, the BLM group rats were weighted and a dose of 5 mg/kg (50 μL) BLM was instilled intratracheally. In the BLM+OSTH group, osthale (40 mg/kg) was administered (the first dose was after instilling BLM for 1 h) by gavage daily until four weeks. In the control group, animals were injected intratracheally with 50 μL of sterile saline of 0.9%. The dose of BLM and saline was administered just once.

**Lung Coefficient Investigation** Lung edema was analyzed by lung coefficient [weight of wet lung (mg)/body weight]. Briefly, at three end time points (1 week, 2 weeks, and 4 weeks, separately), sternotomy surgery was done after the rats being anesthetized, and lungs were then carefully harvested. After the lungs being washed with cold phosphate buffered saline (PBS), the lungs were absorbed by filter paper and the lung coefficient was then obtained. At the same part of the lower lobe of right lungs were cut and immersed in 10% formalin solution (pH=7.4) for next histological assays. The remaining lungs were maintained in a −80°C refrigerator for subsequent experiments.

**Morphological Investigation** After soaked in 10% formalin for 3 d, the slices were embedded in paraffin and sectioned into 4-μm-thick sections and hematoxylin and eosin (HE) staining and Trichrome Masson’s staining were performed. A modified picrosirius procedure was performed to investigate the collagen and fibrosis changes during PF. In a word, after being regularly deparaffinized and rehydrated, the lung sections were dipped in 1% phosphomolybdic acid for 5 min. Then a 0.1% (w/v) Sirius red F3B in saturated picric acid solution was added in for 60 min at room temperature (r.t.). After being dehydrated and hylainized in graded xylene, the sections were mounted with neutral resin. A light microscope was employed to analyze the collagen and the fibrosis changes in the sections under normal and polarized light. The inflammatory and PF changes in lung tissue were evaluated by the HE staining and the Trichrome Masson’s staining according to previous report. The inflammatory cells, fibroblasts and collagen were scored as follows: ‘none,’ score 0; ‘few,’ score 0.5; ‘moderate,’ score 1; ‘many,’ score 2; and ‘considerable,’ score 3. Newly formed capillaries were counted by randomly selecting 10 fields distributed over at least three separate sections. The mean number of newly formed capillaries was calculated.

**Immunohistochemical Staining** Lung sections were deparaflinized, rehydrated in graded alcohols, and incubating in 0.3% H2O2 at 37°C for 30min. Microwave antigen retrieval method was performed, and the slides were incubated for 1 h with normal goat serum. The slides were then incubated with ACE2 mouse monoclonal antibody, TGF-β1 mouse monoclonal antibody, ANG-(1–7) mouse monoclonal antibody, and ANG-II mouse monoclonal antibody at 4°C overnight. After being completely washed in PBS, the tissues were incubated in corresponding antibody for 30 min at 37°C. The signal was detected using diaminobenzidine (DAB). Positive staining was showed in a brown color. All sections were evaluated using an Olympus optical microscope equipped with an image analysis program (Image Pro Plus version 6.0; Media Cybernetics) and analyzed quantitatively. The integrated optical density (IOD) was calculated by measuring continuous 10 fields for each sample using a 40× objective. Mean values were then obtained by gathering all groups of data. A statistical analysis was processed to compare different experimental groups.

**Western-Blot (WB) Analyses** Total protein of rat lungs was got after the rat lungs tissues being homogenated and lysed by a RIPA lysis buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done to separate those protein samples by adding equivalent amounts of protein. The samples were then transferred onto 0.22 μm nitrocellulose filter membranes. After being blocked in 5% skimmed milk, the bands were then incubated with the relevant primary antibodies (ACE2, TGF-β). The signals were detected by an ECL kit.

**Real-Time PCR Investigation** Real-time PCR was performed to assess mRNA expression of the following genes. The qPCR primers were ACE2 (Cat. No. PPR55748G), TGF-β (Cat. No. PPR06430B), IL-6 (Cat. No. PPR06483B), TNF-α (Cat. No. PPR06411F), and IL-1β (Cat. No. PPR06480B).

**ELISA Assays** Commercial ELISA kits were used to detect the concentrations of ANG-(1–7), ANG II, IL-6, TNF-α and IL-1β in rat lung tissues according to the manufacturer’s instructions.

**Statistical Analyses** All values are expressed as the mean±standard deviation (S.D.). Statistical analyses were processed by ANOVA following by a post hoc of Bonferroni’s multiple comparisons test. Differences were considered significant when p〈0.05.

### RESULTS

**Effect of Osthale on BLM-Induced Lung Injury and Pulmonary Fibrosis** As the data showed, BLM exposure resulted in great loss of rat weight as time went by, and significant increase of lung coefficient compared to those of control group (*p〈0.05, ***p〈0.001, Table 1). However, osthale treatment notably halted the weight loss and lung coefficient increase in rats (†p〈0.05, ‡‡‡p〈0.001, vs. BLM group, Table 1).

| Table 1. Changes of Weight and Lung Coefficient between Each Group of Rat |
|----------------------------------------|----------------------------------------|
|                                       | Weight (g)                              | Lung coefficient (mg/g)  |
|                                       | 1 week       | 2 weeks      | 4 weeks      | 1 week       | 2 weeks      | 4 weeks      |
| Control                                | 182±7.8      | 218±4.9      | 247±6.9      | 3.74±0.16    | 3.3±0.21     | 4.28±0.31    |
| BLM                                    | 171±4.5*     | 181±6.5***   | 194±5.8***   | 4.66±0.26*** | 7.54±0.30*** | 8.92±0.45*** |
| BLM+OSTH                               | 179±5.6*     | 206±5.7***   | 218±4.2***   | 3.51±0.17*** | 5.14±0.26*** | 6.2±0.59***  |

* p〈0.05, ***p〈0.001, vs. Control group; †p〈0.05, ‡‡‡p〈0.001, vs. BLM group.
In the control group lungs, the HE staining exhibited uninjured and clear alveoli, intact interstitium, and very few inflammatory cells at each time point (Fig. 1). On the other hand, BLM exposure resulted in progressive lung injury, showing as gradual damages of lung alveoli, exacerbating infiltration of inflammatory cells, and incrassation of lung interstitium with prolongation of time (***p<0.001 vs. Control group, Fig. 1, Table 2). In the meanwhile, osthole treatment significantly prevented those changes in rat lungs after BLM exposure (**p<0.01 vs. BLM group, Fig. 1, Table 2).

Table 2. Alveolar Inflammation and Pulmonary Fibrosis Changes Analyses

<table>
<thead>
<tr>
<th></th>
<th>1 week</th>
<th>2 weeks</th>
<th>4 weeks</th>
<th>1 week</th>
<th>2 weeks</th>
<th>4 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00±0.00</td>
<td>0.06±0.009</td>
<td>0.04±0.006</td>
<td>0.03±0.007</td>
<td>0.06±0.003</td>
<td>0.03±0.001</td>
</tr>
<tr>
<td>BLM</td>
<td>2.58±0.19***</td>
<td>3.88±0.34***</td>
<td>6.58±0.29***</td>
<td>1.12±0.1***</td>
<td>3.87±0.17***</td>
<td>5.36±0.22***</td>
</tr>
<tr>
<td>BLM+OSTH</td>
<td>1.67±0.09***</td>
<td>2.55±0.23***</td>
<td>3.05±0.14***</td>
<td>0.89±0.09***</td>
<td>2.49±0.15***</td>
<td>3.57±0.24***</td>
</tr>
</tbody>
</table>

**p<0.01, vs. Control group; ***p<0.001, vs. BLM group.
Sirius red staining, ×200, Type I collagen fibers are stained in orange to red color, and type III collagen fibers are appeared yellow to green color. BLM=bleomycin; OSTH=osthole.

Table 3. Collagen Contents Analyses

<table>
<thead>
<tr>
<th></th>
<th>Collagen I</th>
<th>Collagen III</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1 week</td>
<td>2 weeks</td>
</tr>
<tr>
<td>Control</td>
<td>0.09±0.005</td>
<td>0.10±0.01</td>
</tr>
<tr>
<td>BLM</td>
<td>0.24±0.02***</td>
<td>0.52±0.05***</td>
</tr>
<tr>
<td>BLM+ OSTH</td>
<td>0.18±0.02###</td>
<td>0.33±0.03###</td>
</tr>
</tbody>
</table>

***p<0.001, vs. Control group; ###p<0.001, vs. BLM group.
Normal lung structures were also showed by the Trichrome Masson’s staining in the lungs of control group at the three time points (Fig. 2). However, BLM exposure caused progressive lung alveoli destruction and inflammation, interstitium thickening, and fibroblasts extensive infiltration in rat lungs (***p<0.001 vs. Control group, Table 2, Fig. 2). But treatment with osthole significantly prevented those changes in rat lungs at each time point (**p<0.01 vs. BLM group, Table 2, Fig. 2).

**Effect of Osthole on BLM-Induced Collagen Fibers Production**  Abundant type I collagen fibers (stained in orange to red color) and very little type III collagen fibers (stained in yellow to green color) were found in the Control group demonstrated by the Sirius red staining (Fig. 3). No significant changes were found in the Control group between each time point. Nevertheless, BLM resulted in proliferated accumula-

**Fig. 5. Effect of Osthole on ANG II Expression in Rat Lungs**
(A) Expression of ANG II determined by immunohistochemical staining; (a) IOD analyses of ANG II. (b) ELISA assays of ANG II protein level. ***p<0.001 vs. Control group, ^^^p<0.001 vs. BLM group. (A’) Control group; (B’) BLM group; (C’) BLM+OSTH group.

**Fig. 6. Effect of Osthole on ACE2 Expression in Rat Lungs**
(A) Expression of ACE2 determined by immunohistochemical staining; (a) Integral optical density (IOD) analyses of ACE2. (B) Representative Western blotting analysis of ACE2 protein level; (b) ACE2 expression analyses. (c) Real-time PCR analysis of ACE2 mRNA level. ***p<0.001 vs. Control group, ^^^p<0.001 vs. BLM group. (A’) Control group; (B’) BLM group; (C’) BLM+OSTH group.
tion of type III collagen fibers, which gradually congested the rat lungs as time elapsing (**p<0.001 vs. Control group, Fig. 3, Table 3). Osthole treatment significantly alleviated all those changes (##p<0.001 vs. BLM group, Fig. 3, Table 3).

**Effect of Osthole on BLM-Induced Inflammatory Factors Expression** IL-6, TNF-α, and IL-1β, mRNA and protein levels in the lung tissues were analyzed by real-time PCR and ELISA, respectively (Fig. 4). The three different cytokines were all exponentially increased in rats after BLM exposure for 4 weeks (**p<0.001 vs. Control group). Osthole administration significantly decreased those inflammation-related genes expression (###p<0.001 vs. BLM group). Similarly, the ELISA data showed that all three cytokines were significantly elevated after BLM exposure for 4 weeks (**p<0.001 vs. Control group), and treatment with osthole notably inhibited those factors expression (###p<0.001 vs. BLM group).

**Effect of Osthole on ANG II Expression** The immunohistochemical staining results showed that a great increase

![Image](image1.png)

**Fig. 7. Effect of Osthole on ANG-(1–7) Expression in Rat Lungs**

Expression of ANG-(1–7) detected by immunohistochemical staining; (a) IOD analyses of ANG-(1–7), (b) ELISA assays of ACE2 protein level. **p<0.001 vs. Control group, ###p<0.001 vs. BLM group. (A') Control group; (B') BLM group; (C') BLM+OSTH group.

![Image](image2.png)

**Fig. 8. Effect of Osthole on TGF-β Expression in Rat Lungs**

(A) Expression of TGF-β analyzed by immunohistochemical staining; (a) IOD analyses of TGF-β, (B) Representative Western blotting analysis of TGF-β protein level, (b) TGF-β expression analyses, (c) Real-time PCR analysis of TGF-β mRNA level. **p<0.001 vs. Control group, ###p<0.001 vs. BLM group. (A') Control group; (B') BLM group; (C') BLM+OSTH group.
of ANG II in the BLM group compared with that of Control group after BLM exposure for 4 weeks (**p<0.001, Fig. 5Aa). However, treatment with osthole apparently reduced the elevated ANG II (***p<0.001 vs. BLM group, Fig. 5Aa). ELISA data further demonstrated that 4 weeks of BLM exposure notably increased the protein level of ANG II in rat lungs (**p<0.001 vs. Control group, Fig. 5Bb), and osthole administration significantly decreased the expression of ANG II (**p<0.001 vs. BLM group, Fig. 5Bb).

Effect of Osthole on ACE2 Expression Immunohistochemical staining showed that BLM exposure significant decreased ACE2 expression in rat lungs versus that of Control group at the endpoint of 4 weeks (**p<0.001, Fig. 6Aa). Osthole administration notably increased the expression of ACE2 in rat lungs compared with that of BLM group (**p<0.001, Fig. 6Aa). WB assays showed that the protein levels of ACE2 were evidently reduced after BLM exposure (Fig. 6Bb). However, osthole treatment apparently reversed the lessened ACE2 expression (Fig. 6Bb).

Similarly, the mRNA levels of ACE2 were also notably decreased after BLM administration versus those of Control group (**p<0.001, Fig. 6c), and treatment with osthole significantly increased ACE2 mRNA expression in rat lungs (**p<0.001, Fig. 6c).

Effect of Osthole on ANG-(1–7) Expression ANG-(1–7) expression in rat lungs was greatly reduced after BLM administration compared to the Control group at the endpoint of 4 weeks (**p<0.001, Fig. 7Aa). But osthole treatment significantly reversed the expression of ANG-(1–7) (###p<0.001 vs. BLM group, Fig. 7Aa).

ANG-(1–7) protein level was further tested by ELISA assays. BLM administration also resulted in great decrease of ANG-(1–7) in rat lungs (**p<0.001 vs. Control group, Fig. 7b). Osthole treatment greatly reversed the reduced ANG-(1–7) expression (**p<0.001 vs. BLM group, Fig. 7b).

Effect of Osthole on TGF-β1 Expression Significant increase of TGF-β1 expression was observed in BLM group versus the Control group at the endpoint of 4 weeks (**p<0.001, Fig. 8Aa), yet treating of osthole evidently reduced TGF-β1 expression (**p<0.001 vs. BLM group, Fig. 8Aa). WB assays also showed that the protein levels of TGF-β1 were greatly elevated after 4 weeks BLM exposure (Fig. 8Bb), and treatment with osthole dramatically inhibited its expression (Fig. 8Bb).

Similar to the protein levels, BLM exposure also resulted in significant increase of TGF-β1 mRNA level in rat lungs (**p<0.001 vs. Control group, Fig. 8c). But osthole treatment notably reduced TGF-β1 mRNA expression (**p<0.001 vs. BLM group, Fig. 8c).

DISCUSSION

It is well known that PF can be resulted from various lung insults or injuries, and the etiologies including idiopathic pulmonary fibrosis, sarcoidosis, irradiation-induced pneumonitis, acute respiratory distress syndrome (ARDS), and drug-related lung disorders (Maharaj et al.15) and Kuba et al.16). BLM induced PF animal model was duplicated early in 1970,15) and now is a commonly accepted and classic model for PF researching.

In the present study, we successfully established PF rat models induced by BLM, which caused proliferation and progressive accumulation of connective tissue replacing normal parenchyma, and significant alveolar inflammation, exacerbated fibroblast proliferation, and diffused destruction of alveolar in rat lungs. Moreover, BLM exposure also resulted in dramatic elevation of ANG II and various inflammatory factors, augmented expression of TGF-β, and significant decrease of ACE2 and ANG-(1–7) in rat lungs. However, it is interesting for us to find that treatment with osthole significantly prevented BLM induced PF and alveolar inflammation, decreased ANG II and those inflammatory factors, inhibited augmentation of TGF-β1, and reversed expression loss of ACE2 and ANG-(1–7) in rat lungs.

A recent study showing that capillary blood vessels in the lung are a central site of high-level expression of ACE, including a homologue of ACE, ACE2.16) ACE removes dipeptides from ANG I to produce ANG II, while ACE2 cleaves a single residue from ANG II to generate ANG-(1–9), and a single residue from ANG II to generate ANG-(1–7).17) Thus, ACE2 counterbalances the functions of ACE and negatively regulating the production of ANG II. Studies demonstrated that ACE2 and ANG-(1–7) may exert protective effects in kinds of lung diseases,5,13,14) which effects during the progression of PF were being intensively investigated.18,19) Rey-Parra et al. showed in their study that treatment with intraperitoneal recombinant ACE2 improved survival, exercise capacity, lung function, and decreased lung inflammation and fibrosis in mice.5) Indeed, BLM exposure resulted in significant decrease expression of ACE2 and ANG-(1–7), together with great increase of ANG II and TGF-β1 in rat lungs in the present study. However, treatment with osthole prevented the expression reduction of both ACE2 and ANG-(1–7) in rat lungs, together with morphological improvement. Herein, we demonstrated the protective effects of osthole on BLM induced PF. The present study is partly in accordance with those researches mentioned above.

BLM-induced lung injury in rodents demonstrated as an acute phase showing as inflammatory lung injury, and a chronic phase showing as progressive PF.20) Inflammatory cytokines play important roles in recruiting cells to the site of lung injury, promoting extracellular matrix accumulation, and even participating in epithelial to mesenchymal transition.13,21,22) On the other hand, ANG II plays as a powerful inducer of DNA synthesis and fibroblast proliferation.23) Moreover, ANG II promotes extracellular matrix (ECM) synthesis by activating fibroblasts to produce excessive type I collagen.23) Additionally, ANG II upregulates TGF-β expression and then promotes the conversion of fibroblasts to myofibroblasts and the accumulation of collagen.14,24) Besides, there is crosstalk between ANG II and TGF-β1, which synergistically boost ECM production, fibrocytes to myofibroblast transition, and lung scarring development.25) Furthermore, ANG II is also demonstrated to be associated with inflammatory responses and cell apoptosis during kinds of lung diseases.26) Thus, those molecules mentioned above affect each other and synergistically promote the development of PF.

Being an active constituent in C. monnieri, osthole possesses various biological effects, such as anti-cancer,27) anti-hepatitis,28) anti-oxidative stress,29) and anti-osteoporosis.30) Studies also showed that osthole attenuated intestinal, myocardial, and spinal cord ischemia–reperfusion injury in animal models.31,32) Besides, osthole could effectively inhibit TGF-β1 expression...
in fibroblasts and protect against cardiac and hepatic fibrosis. Furthermore, there are studies revealed that osthole also exerts anti-inflammatory effects and attenuated lung injuries in kinds of animal models. The present study showed that osthole indeed possesses anti-inflammatory effects, which is in concert with those studies aforementioned.

CONCLUSION

Taken together, the present experimental results suggested protective effects of osthole on BLM induced PF in rats. These protective effects may be via modulating the ACE2/ANG-(1–7) axis and antagonizing the ANG II and TGF-β, and decreasing inflammation responses. This study furthered our knowledge about the lung protective effects and anti-PF mechanisms of osthole, and may offer new targets for treating PF.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


