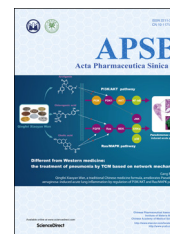Chinese Pharmaceutical Association
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ORIGINAL ARTICLE

Andrographolide sulfonate ameliorates lipopolysaccharide-induced acute lung injury in mice by down-regulating MAPK and NF-κB pathways

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Shuang Peng[a], Nan Hang[a], Wen Liu[a], Wenjie Guo[a], Chunhong Jiang[b,c], Xiaoling Yang[b,c], Qiang Xu[a,*], Yang Sun[a,*]

[a]State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing 210093, China
[b]Jiangxi Qingfeng Pharmaceutical Co., Ltd., Ganzhou 341000, China
[c]State Key Laboratory of Innovative Nature Medicine and TCM Injections, Jiangxi Qingfeng Pharmaceutical Co., Ltd., Ganzhou, 341000, China

Abstract Acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) is a severe, life-threatening medical condition characterized by widespread inflammation in the lungs, and is a significant source of morbidity and mortality in the patient population. New therapies for the treatment of ALI are desperately needed. In the present study, we examined the effect of andrographolide sulfonate, a water-soluble form of andrographolide (trade name: Xi-Yan-Ping Injection), on lipopolysaccharide (LPS)-induced ALI and inflammation. Andrographolide sulfonate was administered by intraperitoneal injection to mice with LPS-induced ALI. LPS-induced airway inflammatory cell recruitment and lung histological alterations were significantly ameliorated by andrographolide sulfonate. Protein levels of pro-inflammatory cytokines in bronchoalveolar lavage fluid (BALF) and serum were reduced by andrographolide sulfonate administration. mRNA levels of pro-inflammatory cytokines in lung tissue were also suppressed. Moreover, andrographolide sulfonate markedly suppressed the activation of mitogen-activated protein kinase (MAPK) as well as p65

subunit of nuclear factor-κB (NF-κB). In summary, these results suggest that andrographolide sulfonate ameliorated LPS-induced ALI in mice by inhibiting NF-κB and MAPK-mediated inflammatory responses. Our study shows that water-soluble andrographolide sulfonate may represent a new therapeutic approach for treating inflammatory lung disorders.

1. Introduction

Inflammatory lung diseases, including acute lung injury (ALI), acute respiratory distress syndrome, and pneumonia, are life-threatening diseases which cause high morbidity and mortality world-wide[1,2]. Many factors such as sepsis, shock, trauma and burns can lead to ALI. It is well established that lipopolysaccharide (LPS)-induced inflammation in the lung is mediated by several pro-inflammatory mediators, and is one of the main reasons for ALI[3-5]. After stimulation by LPS, TLR4 recruits TIRAP, MyD88 and IRAK kinases, forming a multi-molecular complex which triggers a signaling cascade leading to activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) and controls the production of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6)[6,7]. Despite the elucidation of the pathogenic factors of ALI through extensive investigations, current treatments fail to reduce lung injury and mortality[8,9]. Therefore, new and effective strategies are still required for ALI patients.

Andrographolide sulfonate, a water-soluble form of andrographolide made through a sulfonating reaction, is an andrographolide sulfate mixture and has been made into an injection dosage form for intramuscular injection and intravenous drip. It has been used in China for many years, mainly for therapy of bronchitis, paristhmitis and bacillary dysentery. The effect of andrographolide sulfonate on LPS-induced sepsis and trinitrobenzenesulfonic acid (TNBS)- or dextran sulfate sodium (DSS)-induced colitis has been demonstrated[10-12]. In the present study, we examined the anti-ALI effect of andrographolide sulfonate and showed that andrographolide sulfonate significantly inhibited MAPK and NF-κB pathways and reduced the susceptibility to LPS-induced ALI in mice.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice, aged 6–8 weeks, were purchased from Model Animal Research Center of Nanjing University (Nanjing, China). Mice were maintained in an animal facility under standard laboratory conditions for 1 week prior to experiments, and provided water and standard chow. Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals. All efforts were made to reduce the number of animals used and to minimize animals' suffering.

2.2. Agents

Andrographolide sulfonate (trade name: Xi-Yan-Ping Injection) was provided by Jiangxi Qingfeng Pharmaceutical Co., Ltd.

(Ganzhou, China). LPS (from *Escherichia coli* (0111:B4)) was purchased from Sigma-Aldrich (St. Louis, MO, USA). ELISA kits for TNF-α, IL-6 and IL-1β were purchased from Dakewei (Beijing, China). Anti-phosphorylation of c-Jun N-terminal kinase (JNK) (Thr183/Tyr185), anti-phosphorylation of ERK1/2 (Thr202/Tyr204), anti-phosphorylation of p38 (Thr180/Tyr182), anti-phosphorylation of p65 were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-mouse CD3-FITC, CD11b-PE and CD11c-APC antibodies were bought from eBioscience. (San Diego, CA, USA) GTVisin™ anti-mouse/anti-rabbit immunohistochemical analysis KIT was purchased from Gene Company (Shanghai, China). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Induction of acute lung injury by LPS inhalation

ALI in mice were induced by inhaling atomized LPS (500 μg/mL) for 30 min at 2 h intervals over a 4 h period. Mice were randomly divided into 5 groups ($n=8$ per group): control group inhaled atomized PBS, model group inhaled atomized LPS. Andrographolide sulfonate (2.5, 5 and 10 mg/kg) dissolved in saline was given to mice once per day (i.p.) for 3 days before inhalation of LPS. Mice were killed 1 h after administration of LPS. Bronchoalveolar fluid (BALF), blood plasma and tissue samples were collected. For BALF collection, mice were perfused with ice cold PBS. The lungs were lavaged for three times with 1 mL of saline, and the resultant BALF was centrifuged to separate the cellular components from the supernatants. Total BALF cell number was determined and the BALF cells were stained with anti-mouse CD3-FITC, CD11b-PE and CD11c-APC antibodies and the composition was evaluated by fluorescence-activated cell sorting (FASC) analysis.

2.4. Histological analysis

To examine histological changes, lungs from animals in each group were taken and fixed in 10% formalin, embedded in paraffin, and then sectioned to reveal the maximum longitudinal view of the main intrapulmonary bronchus of the left lung lobe. A histopathologic study was conducted using hematoxylin & eosin (H&E)-stained lung sections. Alveolar congestion, hemorrhage, infiltration or aggregation of inflammatory cells in airspaces or vessel walls, and the thickness of the alveolar walls were assessed.

2.5. Cytokine analysis by ELISA

Serum was collected from blood of mice by centrifugation at $3500 \times g$ for 15 min. Serum cytokine levels were measured by specific ELISA kits (Dakewe, Beijing, China) as instructed.

2.6. Real-time PCR

Real-time PCR was performed as follows: RNA samples from lung tissues of mice in each group were extracted and reversed to cDNA and subjected to quantitative PCR, which was performed with the BioRad CFX96 Touch™ Real-Time PCR Detection System (BioRad , USA) using iQ™ SYBR® Green Supermix, and threshold cycle numbers were obtained using BioRad CFX Manager software. The program for amplification was 1 cycle of 95 °C for 2 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 95 °C for 10 s. The primer sequences used in this study were as follows: Tnf-α forward 5′-CGAGTGACAAGCCTG-TAGCCC-3′; Tnf-α reverse 5′-GTCTTTGAGATCCATGCCGT TG-3′; Il-1β forward 5′-CTTCAGGCAGGCAGTATCACTC-3′; Il-1β reverse 5′-TGCAGTTGTCTAATGGGAACGT-3′; Il-4 forward 5′-GTCTGCTGTGGCATATTCTG-3′; Il-4 reverse 5′-GGC ATTTCTCATTCAGATTC-3′; Il-5 forward 5′-GGCTACACA-GAGAAACCCTGT-3′; Il-5 reverse 5′-CATGCATACACAGG TAGTTCA-3′; Il-6 forward 5′-ACAACCACGGCCTTCCCTAC-3′; Il-6 reverse 5′-TCTCATTTCCACGATTTCCCAG-3′; Il-$17a$ forward 5′-TCGAGAAGATGCTGGTGGGT-3′; Il-$17a$ reverse 5′-CTCTGTTTAGGCTGCCTGGC-3′; β-actin forward 5′-TGCTG TCCCTGTATGCCTCT-3′; β-actin reverse 5′-TTTGATGTCAC GCACGATTT-3′.

2.7. Western blot

Protein from lung tissue was extracted in lysis buffer (30 mmol/L Tris, pH 7.5, 150 mmol/L sodium chloride, 1 mmol/L phenyl-methylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 1% Nonidet P-40, 10% glycerol, and phosphatase and protease inhibitors). The protein content of the supernatant was determined by BCA protein assay Kit (Pierce, Rochford, IL). The proteins were then separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membrane was blocked with 5% skimmed milk for 1 h at room temperature. The blocked membrane was incubated with the indicated primary antibodies overnight at 4 °C, and then incubated with horseradish peroxidase (HRP)-coupled secondary antibody. Detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).

2.8. Immunohistochemical analysis

Immunohistochemical analysis was performed on paraffin-embedded lung tissue sections (5 μm) as described previously[10].

2.9. Statistical analysis

Data are expressed as mean ± SEM. ANOVA with *post hoc* comparisons was used to evaluate the differences between experimental and control groups. *P* values less than 0.05 were considered significant.

3. Results

3.1. Andrographolide sulfonate inhibited infiltration of inflammatory cells and pathological changes of LPS-induced lung injury in mice

In order to verify whether andrographolide sulfonate can improve lung inflammation, we used a mouse model of LPS-induced acute lung injury to evaluate the therapeutic effect of andrographolide sulfonate. Mice were challenged with LPS and lung tissue samples were collected. The sections stained with H&E was shown in Fig. 1. Lung tissues from the LPS group demonstrated significantly pathological alterations, including notable inflammatory cells infiltration, interstitial and intra-alveolar edema and patchy
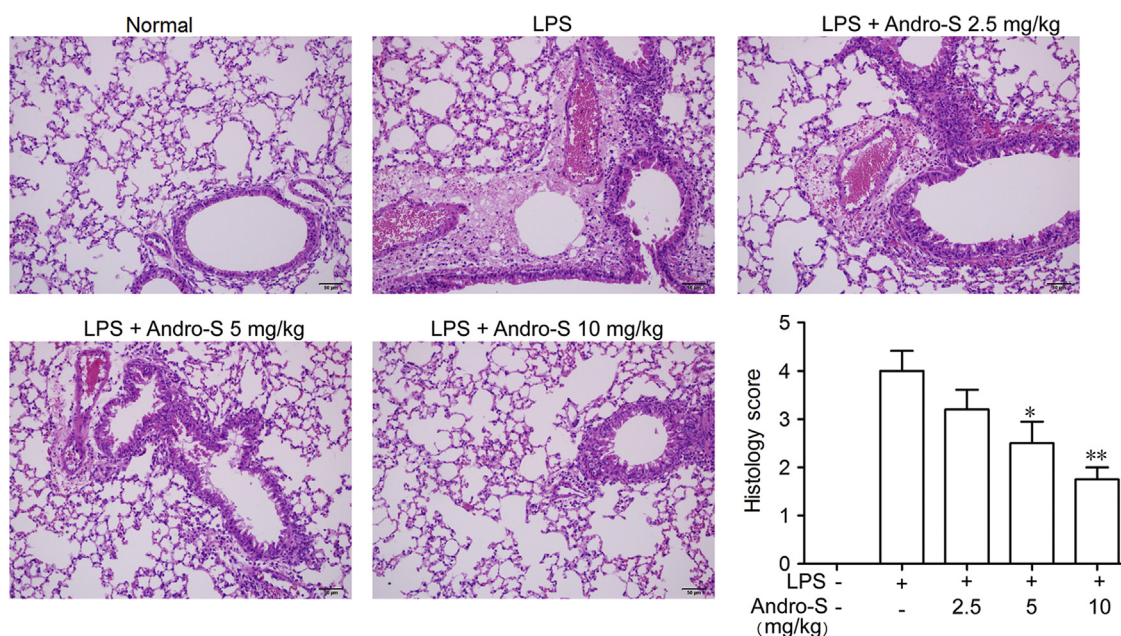

Figure 1 Andrographolide sulfonate treatment ameliorated LPS-induced acute lung injury in mice. Mice were given by andrographolide sulfonate by intraperitoneal injection (2.5, 5 and 10 mg/kg) for days 0–2. After LPS inhalation on day 2, lung tissue was fixed in 4% formalin and subjected to H&E staining. Values are shown as mean ± SEM of 8 mice. *P < 0.05, **P < 0.01 *vs.* mice treated with LPS alone. Andro-S: andrographolide sulfonate.

hemorrhage, hyaline membrane formation and some collapsed alveoli. Against these changes, treatment with andrographolide sulfonate markedly reduced the extent of tissue damage. The histology score also showed that andrographolide sulfonate dose-dependently alleviated lung tissue damage. Next, the infiltration of inflammatory cells in BALF was quantified. The number of total infiltrated cells, T cells ($CD3^+$), macrophages ($CD11b^+$) and neutrophils ($Gr1^+$), in BALF were examined by FACS. As shown in Fig. 2, LPS stimulation-induced infiltration of inflammatory cell was dose-dependently reduced with andrographolide sulfonate treatment.

3.2. Andrographolide sulfonate inhibited levels of cytokines in BALF and serum

After LPS inhaled-administration, the infiltration of inflammatory cells significantly increased and levels of inflammatory cytokines in BALF and serum were all elevated; however, these effects were attenuated by andrographolide sulfonate treatment. As shown in Fig. 3, andrographolide sulfonate significantly reduced the concentrations of TNF-α, IL-6 and IL-1β both in BALF and serum.

3.3. Andrographolide sulfonate inhibited mRNA expression of inflammatory cytokines induced by LPS in the injured lung

Since andrographolide sulfonate significantly inhibited the BALF and serum levels of TNF-α and IL-1β, the mRNA levels of various proinflammatory cytokines in the injured lung was measured. As shown in Fig. 4, the mRNA levels of Il-4, Il-5, Il-1β, Tnf-α and Il-6 were remarkably increased after LPS challenge while there was no change of Il-17a level. Andrographolide sulfonate

significantly inhibited the elevated expression of cytokines in a dose-dependent manner.

3.4. Andrographolide sulfonate reduced LPS-induced activation of MAPK and NF-κB signaling

After administration of LPS, MAPK and NF-κB were activated leading to the transcription of various genes involved in inflammation as shown in Fig. 4. LPS administration caused phosphorylation of p38, ERK, JNK, as well as p65 in the injured lung from mice with ALI (Fig. 5B and C). Andrographolide sulfonate treatment markedly reduced the phosphorylation of p38, JNK and NF-κB as well as ERK to some extent. Immunohistochemical staining revealed an increased level of p-p65 after LPS stimulation, and andrographolide sulfonate administration markedly inhibited the phosphorylation of p65 (Fig. 5A).

4. Discussion

Clinical treatment of acute lung injury is based on both ventilatory and nonventilatory strategies. Lung-protective strategies that include mechanical ventilation therapies have an established survival advantage[13]. Although numerous pharmacologic therapies have been provided, all have failed to demonstrate benefit in multicenter clinical trials[14,15].

Acute lung injury is a serious illness characterized by acute inflammation and disruption of the lung endothelial and epithelial barriers. Excess production of inflammatory cytokines and increased permeability of the alveolar-capillary barrier occurred following infection or trauma. When mice inhaled LPS, they exhibited these
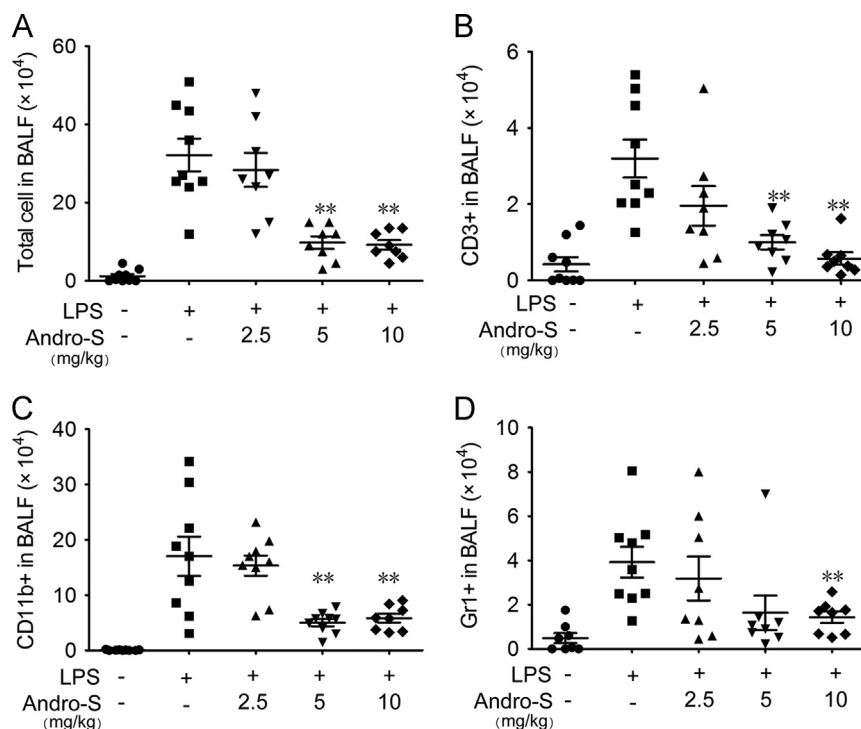

Figure 2 Andrographolide sulfonate treatment prevented LPS-induced recruitment of inflammatory cells in lung tissue. BALF from each group was collected and the cells present in BALF were counted (A). Cells were stained with antibodies to CD3-FITC (B), CD11b-PE (C) and CD11c-APC (D) and analyzed by FACS. Values are shown as the mean $\pm$ SD of 8 mice. $^*P < 0.05$, $^{**}P < 0.01$ vs. mice treated with LPS group. Andro-S: andrographolide sulfonate.
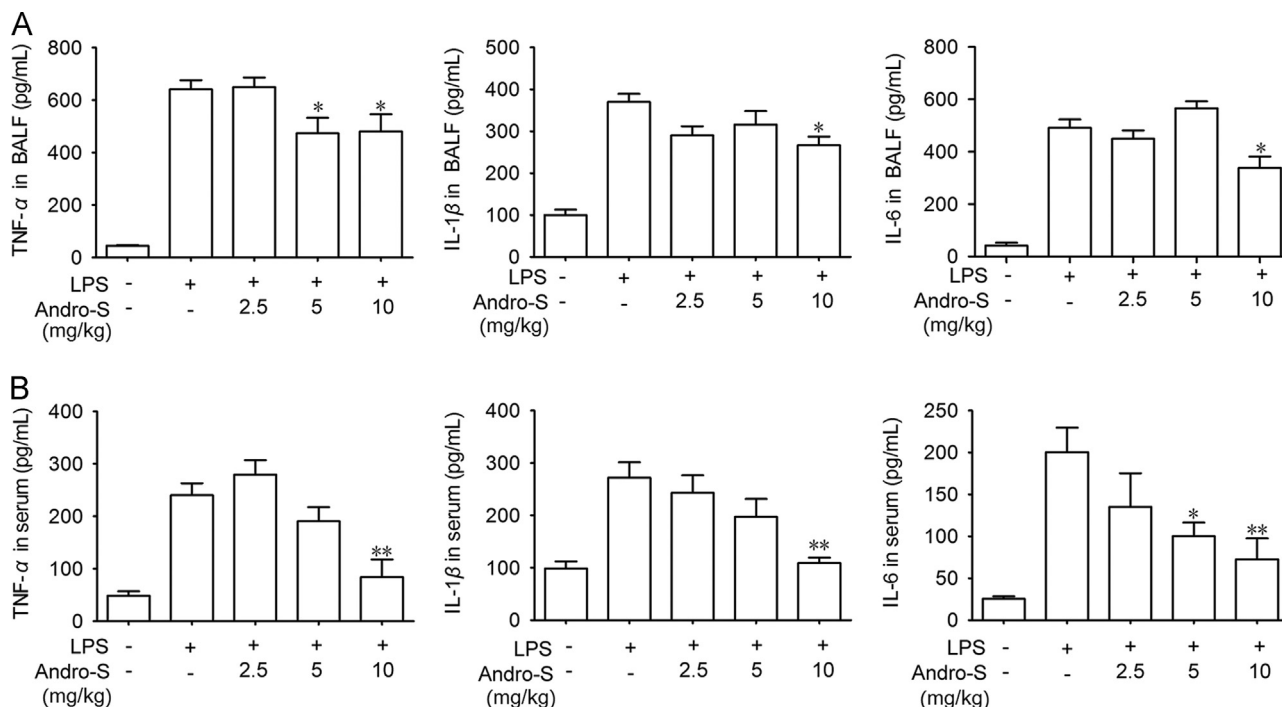
Figure 3 Andrographolide sulfonate treatment suppressed LPS-induced cytokine secretion. Cytokine levels in BALF supernatant (A) and serum (B) from each group were determined by ELISA. Values are shown as the mean $\pm$ SD of 8 mice. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. mice treated with LPS group. Andro-S: andrographolide sulfonate.
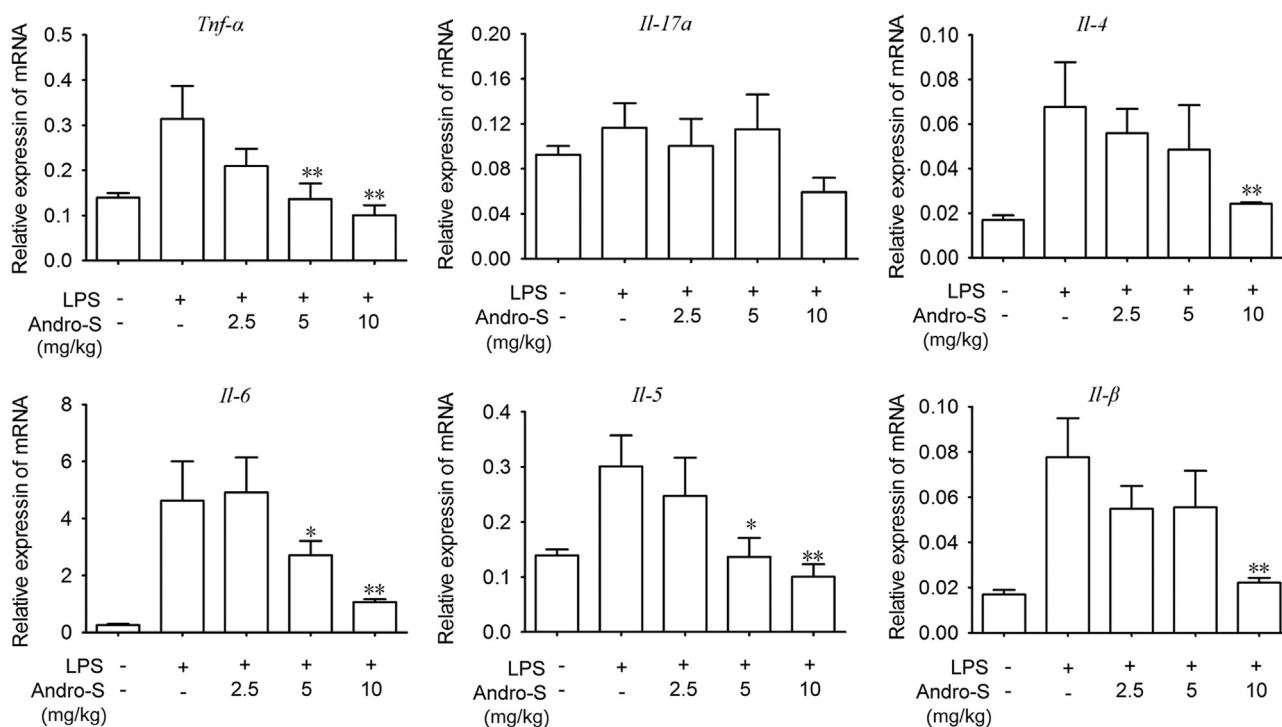

Figure 4 Andrographolide sulfonate suppressed mRNA levels of inflammatory cytokines in lung of mice with LPS-induced ALI. RNA of lung tissue was extracted. The mRNA expression of Il-6, Tnf-α, Il-1β, Il-4, Il-5, Il-17a was examined by real-time PCR. Values were shown as the mean $\pm$ SEM of 6 mice. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. mice treated with LPS alone. Andro-S: andrographolide sulfonate.

symptoms[16,17]. In the current study, our data revealed that andrographolide sulfonate attenuated the LPS-induced lung histopathologic changes, lung edema and inflammatory cytokine production and activation of NF-κB and thus protected mice against LPS-induced induced acute lung injury. These results indicated that andrographolide sulfonate exerted potential protective effects against LPS-induced ALI.
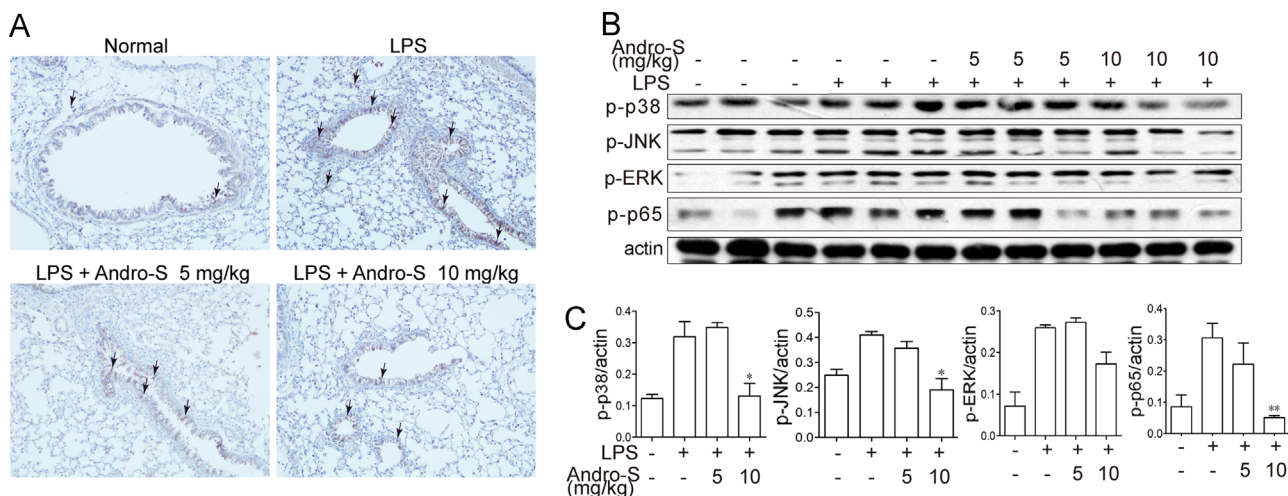
Figure 5 Andrographolide sulfonate decreased activation of MAPK and NF-κB in the lungs of mice with LPS-induced ALI. (A) Paraffin-embedded lung tissue sections from vehicle- and andrographolide sulfonate-treated groups were stained for p-p65. Original magnification, 100×; (B) lung tissue protein was extracted from the treated mice and protein levels of p-p38, p-ERK, p-JNK and p-p65 were examined by western blot. Andro-S: andrographolide sulfonate.

Andrographolide, a natural diterpenoid, is the major constituent of *Andrographis paniculata*, which has been used in China for many years. It has been reported that andrographolide possesses antibacterial, anti-inflammatory and antiviral activities[18,19]. Andrographolide sulfonate is a water-soluble mixture made from andrographolide through a sulfonating reaction. The main constituents are 17-hydro-9-dehydroandrographolide-3,19-disodium sulfonate, 17-hydro-9-dehydroandrographolide-3-sodium sulfonate, 17-hydro-9-dehydroandrographolide-19-sodium sulfonate, and 17-hydro-9-dehydroandrographolide[10]. Although we have not yet purified any of these components, intraperitoneally injection of total andrographolide sulfonate at the dose of 2.5, 5 and 10 mg/kg significantly reduced the infiltration of prominent inflammatory cells (including alveolar macrophages, neutrophils and T cells) (Figs. 1 and 2), inhibited the release of cytokines (such as TNF-α, IL-1β and IL-6) in BALF and serum (Fig. 3) thus alleviating the lung damage.

It is known that inflammatory stimuli such as LPS led to the activation of MAPK and the transcription factor NF-κB, which mediates the expression of several pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, playing an important role in many inflammatory disease processes[20–22]. The possibility that andrographolide inhibits NF-κB activity has been investigated *in vitro* in that it can bind to p50[23]. Our study showed that andrographolide sulfonate treatment can inhibit phosphorylation of both MAPK (p38, JNK and ERK) and NF-κB, suggesting that andrographolide sulfonate may work on the upstream portion of LPS-TLR4-induced signaling.

Above all, our data here showed that andrographolide sulfonate can inhibit LPS-induced cytokine production with a possible mechanism for negative regulation NF-κB and MAPK activation, and thus attenuate acute lung injury in mice. The detailed mechanism needs further exploration. These results provide evidence for the possible clinical application of andrographolide sulfonate for ALI.

Acknowledgment

References

1. Ochiai R. Mechanical ventilation of acute respiratory distress syndrome. *J Intensive Care* 2015;**3**:25.
2. Nair GB, Niederman MS. Year in review 2013: critical care-respiratory infections. *Crit Care* 2014;**18**:572.
3. Chen H, Bai CX, Wang XD. The value of the lipopolysaccharide-induced acute lung injury model in respiratory medicine. *Expert Rev Respir Med* 2010;**4**:773–83.
4. Han S, Mallampalli RK. The acute respiratory distress syndrome: from mechanism to translation. *J Immunol* 2015;**194**:855–60.
5. Dreyfuss D, Ricard JD. Acute lung injury and bacterial infection. *Clin Chest Med* 2005;**26**:105–12.
6. Sender V, Stamme C. Lung cell-specific modulation of LPS-induced TLR4 receptor and adaptor localization. *Commun Integr Biol* 2014;**7**: e29053.
7. Płóciennikowska A, Hromada-Judycka A, Borzecka K, Kwiatkowska K. Co-operation of TLR4 and raft proteins in LPS-induced pro-inflammatory signaling. *Cell Mol Life Sci* 2015;**72**:557–81.
8. Johnson ER, Matthay MA. Acute lung injury: epidemiology, pathogenesis, and treatment. *J Aerosol Med Pulm Drug Deliv* 2010;**23**: 243–252.
9. Silversides JA, Ferguson ND. Clinical review: acute respiratory distress syndrome-clinical ventilator management and adjunct therapy. *Crit Care* 2013;**17**:225.
10. Guo WJ, Liu W, Chen G, Hong SC, Qian C, Xie N, et al. Water-soluble andrographolide sulfonate exerts anti-sepsis action in mice through down-regulating p38 MAPK, STAT3 and NF-κB pathways. *Int Immunopharmacol* 2012;**14**:613–9.
11. Guo WJ, Sun Y, Liu W, Wu XX, Guo LL, Cai PF, et al. Small molecule-driven mitophagy-mediated NLRP3 inflammasome inhibition is responsible for the prevention of colitis-associated cancer. *Autophagy* 2014;**10**:972–85.
12. Liu W, Guo WJ, Guo LL, Gu YH, Cai PF, Xie N, et al. Andrographolide sulfonate ameliorates experimental colitis in mice by inhibiting Th1/Th17 response. *Int Immunopharmacol* 2014;**20**:337–45.
13. The Acute Respiratory Distress Syndrome Network. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. *N Engl J Med* 2000;**342**:1301–8.
14. Calfee CS, Matthay MA. Nonventilatory treatments for acute lung injury and ARDS. *Chest* 2007;**131**:913–20.

15. Levitt JE, Matthay MA. Treatment of acute lung injury: historical perspective and potential future therapies. *Semin Respir Crit Care Med* 2006;**27**:426–37.

16. Håkansson HF, Smailagic A, Brunmark C, Miller-Larsson A, Lal H. Altered lung function relates to inflammation in an acute LPS mouse model. *Pulm Pharmacol Ther* 2012;**25**:399–406.

17. Mouratis MA, Magkrioti C, Oikonomou N, Katsifa A, Prestwich GD, Kaffe E, et al. Autotaxin and endotoxin-induced acute lung injury. *PLoS One* 2015;**10**:e0133619.

18. Zhou B, Zhang DY, Wu XM. Biological activities and corresponding SARs of andrographolide and its derivatives. *Mini Rev Med Chem* 2013;**13**:298–309.

19. Lim JCW, Chan TK, Ng DSW, Sagineedu SR, Stanslas J, Wong WSF. Andrographolide and its analogues: versatile bioactive molecules for combating inflammation and cancer. *Clin Exp Pharmacol Physiol* 2012;**39**:300–10.

20. Rahman A, Fazal F. Blocking NF-κB: an inflammatory issue. *Proc Am Thorac Soc* 2011;**8**:497–503.

21. McKenna S, Wright CJ. Inhibiting IκBβ–NFκB signaling attenuates the expression of select pro-inflammatory genes. *J Cell Sci* 2015;**128**:2143–55.

22. Lee IT, Yang CM. Inflammatory signalings involved in airway and pulmonary diseases. *Mediators Inflamm* 2013;**2013**:791231.

23. Xia YF, Ye BQ, Li YD, Wang JG, He XJ, Lin XF, et al. Andrographolide attenuates inflammation by inhibition of NF-κB activation through covalent modification of reduced cysteine 62 of p50. *J Immunol* 2004;**173**:4207–17.