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Original article

Tetrahydroberberrubine attenuates lipopolysaccharide-induced acute lung injury by down-regulating MAPK, AKT, and NF-κB signaling pathways

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ABSTRACT

Acute lung injury (ALI) is a life-threatening syndrome that is characterized by overwhelming lung inflammation and increased microvascular permeability, which causes a high mortality worldwide. Here, we studied the protective effect of tetrahydroberberrubine (THBru), a berberine derivative, on a mouse model of lipopolysaccharide (LPS)-induced acute lung injury that was established in our previous studies. The results showed that a single oral administration of THBru significantly decreased the lung wet to dry weight (W/D) ratio at doses of 2, 10 and 50 mg/kg administered 1 h prior to LPS challenge (30 mg/kg, intravenous injection). Histopathological changes, such as pulmonary edema, infiltration of inflammatory cells and coagulation, were also attenuated by THBru. In addition, THBru markedly decreased the total cell counts, total protein and nitrate/nitrite content in bronchoalveolar lavage fluid (BALF), significantly decreased tumor necrosis factor-α (TNF-α) and nitrate/nitrite content in the plasma, and reduced the myeloperoxidase (MPO) activity in the lung tissues. Additionally, THBru (10 μM) significantly decreased the content of TNF-α and nitric oxide (NO) in LPS-induced THP-1 cells *in vitro*. Moreover, THBru significantly suppressed the activation of the MAPKs JNK and p38, AKT, and the NF-κB subunit p65 in LPS-induced THP-1 cells. These findings confirm that THBru attenuates LPS-induced acute lung injury by inhibiting the release of inflammatory cytokines and suppressing the activation of MAPKs, AKT, and NF-κB signaling pathways, which implicates it as a potential therapeutic agent for ALI or sepsis.

1. Introduction

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), are characterized by disruption of the alveolar-capillary barrier, which results in lung edema, neutrophil accumulation, alveolar fibrin deposition and consequent impairment of arterial oxygenation [1–3]. Although there is an approved therapy with mechanical ventilation to treat ALI/ARDS patients, the mortality has remained high over the last decade [4,5]. Therefore, the development of efficient therapeutic approaches is urgently required. Sepsis is a major cause underlying the development of ALI, wherein gram-negative bacteria are the dominating factor. An injection of lipopolysaccharide (LPS), a major component in gram-negative bacteria, mimics human gram-negative ALI by inducing excessive inflammatory response [6]. It has been widely accepted that the inappropriate activation of inflammatory cells, including polymorphonuclear neutrophils (PMNs), circulating monocytes and tissue resident macrophages, and the increased release of inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and nitric oxide (NO), play a key role in the pathogenesis of sepsis-induced ALI [7–9]. Furthermore, toll like receptor 4 (TLR4), a well-characterized PRR, recognizes the LPS and leads to the activation of the MYD88-mediated nuclear factor-κB (NF-κB), Mitogen-activated protein kinase (MAPK) and AKT/phosphoinositide3-kinase (PI3K) pathways in sepsis-induced ALI [10,11].

Abbreviations: ALI, acute lung injury; ARDS, acute respiratory distress syndrome; BALF, bronchoalveolar lavage fluid; BBR, berberine; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MPO, myeloperoxidase; NF-κB, nuclear factor-κB; NO, nitric oxide; PI3K, phosphoinositide3-kinase; PMNs, polymorphonuclear neutrophils; TGF-β, transforming growth factor beta; THP-1, the human monoblastic leukemia cell line; TLR4, toll like receptor 4; TNF-α, tumor necrosis factor alpha; THBru, tetrahydroberberrubine.

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Due to remarkable synergic therapeutic effects and relatively low toxicity of herbal medicines, considerably more attention has been given to natural anti-oxidant and anti-inflammatory products and their derivatives [12–14]. It has been reported that berberine attenuates bleomycin-induced pulmonary toxicity and fibrosis by suppressing NF-κB-dependent transforming growth factor beta (TGF-β) activation and that it inhibits cigarette smoke- or LPS-induced acute lung inflammation [15–17]. Furthermore, berberine inhibited the LPS-induced over-expression and procoagulant activity of tissue factors by regulating the p38 MAPK and NF-κB/p65 pathways in THP-1 cells [18]. However, accumulating evidence has demonstrated that berberine is characterized by a very low oral bioavailability, which limits the clinical application of this compound [19,20]. Therefore, recent studies have focused on the research and development of berberine derivatives [21–23]. Tetrahydroberberrubine (Fig. 1A), a berberine derivative (Fig. 1B), was synthesized by semi-chemical synthesis, in which berberine chloride undergoes pyrolysis monodemethylation to produce a red compound and then reduction using potassium borohydride [24–26]. Our previous research showed that tetrahydroberberrubine inhibited tissue factor procoagulant activity in LPS-induced THP-1 cells [27] and that its derivative has anxiolytic effects [28]. However, the pharmacological actions of tetrahydroberberrubine on LPS-induced acute lung injury and its possible mechanism have not been clarified. Therefore, our study was designed to assess the protective effects of tetrahydroberberrubine compared with berberine in an LPS-induced acute lung injury mouse model and to elucidate the potential anti-inflammatory mechanism in THP-1 cells *in vitro*.

2. Materials and methods

2.1. Drugs and reagents

Tetrahydroberberrubine sulfate (THBru) was provided by Dr. Haixia Ge, and its purity, as analyzed by high performance liquid chromatography, was 98%. Berberine chloride (BBR) was obtained from Nanjing Qingze Medical Technology Company (Nanjing, China). Dexamethasone (DEX) was obtained from Zhejiang Xianju Pharmaceutical Co. Ltd (Zhejiang, China). LPS (from Escherichia coli O55:B5) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The MPO kit, nitrate/nitrite colorimetric assay kit and TNF-α ELISA kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The BCA assay and BSA were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Lysis buffer and the ECL kit were purchased from Vazyme Biotech Co. Ltd (Nanjing, China). PVDF membrane was acquired from Millipore Company (Shanghai, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies were acquired from Shanghai KangChen Bio-tech Inc. (Shanghai, China). Anti-p65, anti-phospho-NF-κB/p65, anti-JNK, anti-phospho-JNK, anti-p38MAPK, anti-phospho-p38MAPK, anti-AKT, and anti-phospho-AKT antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). Goat anti-mouse IgG antibody and goat anti-rabbit IgG antibody were purchased from Bioworld Technology (St. Louis Park, MN, USA). Alexa Fluor 488-labeled secondary antibody was purchased from Life Technologies (Carlsbad, CA, USA). All other reagents were of analytical grade.

2.2. Cell culture and pharmacological treatments

The human monoblastic leukemia cell line (THP-1) was obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. THP-1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37 °C. THP-1 cells were grown in serum-free medium for 2 h, pretreated with tetrahydroberberrubine (1, 5, 10 μM) for 1 h and stimulated with 500 ng/mL LPS for 5 h or 30 min.

2.3. Animals

Male ICR mice that were 6–8 weeks old were obtained from Shanghai Slac Laboratory Animal Co. Ltd (Shanghai, China). They were kept in plastic cages at 22 ± 2 °C, with free access to pellet food and water on a 12 h light/dark cycle. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and related regulations of China Pharmaceutical University.

2.4. LPS-induced acute lung injury in mice

Mice were randomly divided into seven groups (n = 8 in each group). LPS (30 mg/kg) was administered intravenously to induce lung injury, and sterile saline was used as the control. The solvent (sterile saline with 5% ethanol), THBru (2, 10 and 50 mg/kg), BBR (50 mg/kg) and DEX (3.0 mg/kg) were administered orally 1 h prior to LPS or saline administration. The chosen doses of these drugs were based on our previous studies and preliminary experiments [16,29,30].

2.5. Lung wet to dry weight ratio measurement and histopathological analysis

The left lung was removed and the wet weight was determined at 6 h after LPS injection. Then, the lung tissue was placed in an oven at 60 °C for 24 h to obtain the dry weight. The ratio of the wet lung to the dry lung was calculated to assess tissue edema. Samples of the upper lobe of right lung were harvested at 6 h after LPS injection and fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 24 h at 4 °C. For examination by light microscopy, lung tissues was dehydrated with graded alcohol and then embedded in

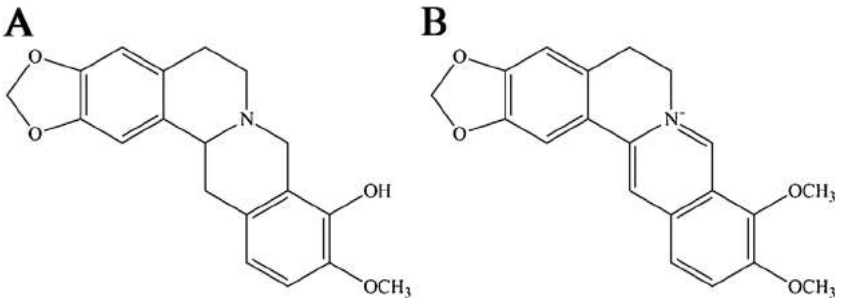

Fig. 1. The chemical structure of tetrahydroberberrubine (A) and berberine (B).

paraffin. Paraffin sections were stained with hematoxylin and eosin. Pathological changes in the lung tissues were observed under a light microscope [31].

2.6. Measurement of total cell counts, total protein and nitrate/nitrite content in bronchoalveolar lavage fluid (BALF)

At 6 h after LPS injection, BALF was collected by cannulating the upper part of the trachea and washing it three times with phosphate buffer solution (pH 7.4) in a total volume of 1.5 mL (0.5 mL × 3). The fluid recovery rate was more than 70%. The lavaged sample from each mouse was kept on ice. BALF was centrifuged at 700*g* for 10 min at 4 °C. The cell-free supernatants were stored at −70 °C for subsequent experiments. The sedimented cells were then resuspended in 1 mL of red blood cell lysis buffer to dismiss red cells. Subsequently, leukocytes were repelleted by centrifugation at 700*g* for 10 min at 4 °C and resuspended in 200 μL PBS. Then, the numbers of total cells were counted using a light microscope [32]. The total protein content in BALF was quantified using the BCA protein assay kit. The nitrate/nitrite content in BALF was measured using a nitrate/nitrite colorimetric assay kit [33].

2.7. Measurement of TNF-α and nitrate/nitrite content in plasma and the MPO content of lung tissue

The remaining lung tissues were removed at 6 h after LPS injection and homogenized in 0.9% saline solution using a homogenizer. The mixtures were centrifuged at 2000*g* for 10 min, and the supernatant was kept at −70 °C before MPO and nitrate/nitrite analysis. MPO activity in lung tissues was determined using an MPO activity kit according to the manufacturer's instructions. Sample protein concentrations were determined by BCA assay, and the results were presented as MPO units per gram of protein. Mice were bled 0.5 mL from the retro-orbital sinus into EP tube at 6 h after LPS injection. The blood were centrifuged at 2000*g* for 15 min, and the plasma was collected. The TNF-α content in plasma was measured by ELISA kits [32]. Nitrate/nitrite in plasma was measured using a nitrate/nitrite colorimetric assay kit.

2.8. Measurement of TNF-α content and NO expression in THP-1 cells

The TNF-α content of the THP-1 cell supernatant was measured using an ELISA kit. NO expression was determined by using a DAF-FM DA fluorescent probe.

2.9. Western blot analysis

Samples of THP-1 cells were homogenized in lysis buffer (containing 20 mM Tris, 150 mM NaCl, 1% Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, Na3VO4, and leupeptin) and lysed for 30 min on ice. The protein concentration was quantified using the BCA protein assay kit. Equal amounts of proteins (40 μg) were separated by 10% SDS-PAGE and transferred to PVDF membranes, which were blocked with 3% bovine serum albumin (BSA) and incubated with the primary antibody. This was followed by incubation with a goat secondary antibody conjugated to horseradish peroxidase for 2 h. Immunoreactive bands were detected by a chemiluminescence system [33].

2.10. Immunofluorescence analysis in THP-1 cells

Treated cells were washed with cold PBS, fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. After blocking, the cells were incubated with the primary antibodies and Alexa Fluor 488-conjugated secondary antibodies. Fluorescence was detected by using a fluorescence microscope (Leica, Germany).

2.11. Statistical analysis

The results were expressed as the mean ± SEM and analyzed using a two-tailed Student's *t*-test (for two groups) or a two-way analysis of variance (ANOVA) followed by Bonferroni's test (for three or more groups). The intensity of fluorescence was measured by Image J. A *P*-value less than 0.05 was considered statistically significant. All results are representative of at least three independent experiments.

3. Results

3.1. Effects of THBru on LPS-induced lung edema and protein content in BALF

LPS challenge produced a significant increase in capillary leakage, as shown by the lung W/D ratio. As illustrated in Fig. 2A, the lung W/D ratio was notably higher after LPS (30 mg/kg) intravenous administration compared with the control mice. Pretreatment with THBru significantly reduced the lung W/D ratio at doses of 10 and 50 mg/kg when given once orally 1 h before LPS injection. The lung W/D ratio was also significantly decreased by the positive control, DEX (3 mg/kg). Additionally, THBru at the same 50 mg/kg dose significantly decreased the lung W/D ratio

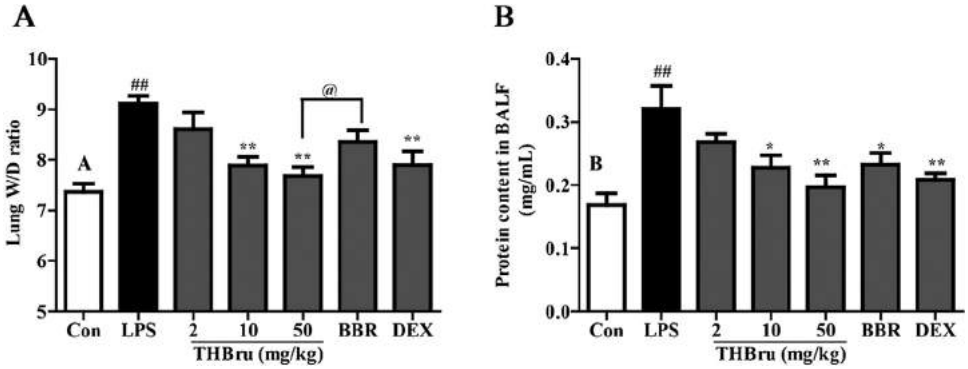

Fig. 2. Effects of THBru on lung edema and protein content in LPS-induced ALI. Mice were orally administered THBru for 1 h prior to LPS injection. Lung wet/dry weight ratio (A) and total protein content in BALF (B) were determined at 6 h after LPS challenge. The results are expressed as the mean ± SEM (n = 8). $^{\#\#}P < 0.01$ vs. Control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. LPS group. $^{@}P < 0.05$ vs. THBru (50 mg/kg) group.

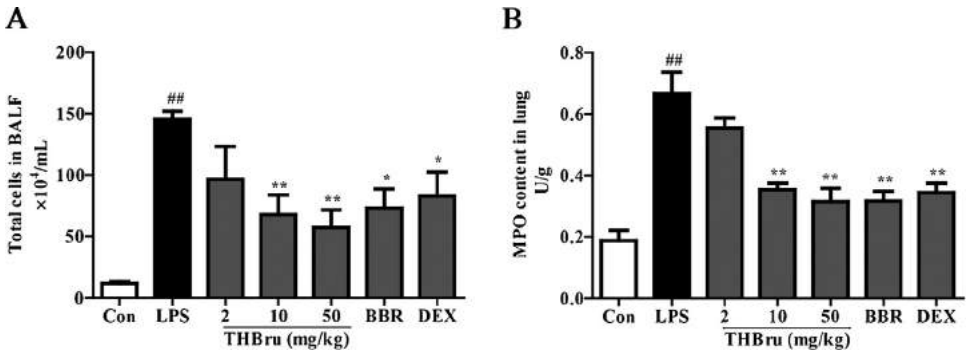

Fig. 3. Effects of THBru on the total cell counts and MPO content in LPS-induced ALI. Mice were orally administered THBru for 1 h prior to LPS injection. Total cell counts in BALF (A) and MPO content of lung tissues (B) were determined at 6 h after LPS challenge. The results are expressed as the mean ± SEM (n = 8). $^{\#\#}P < 0.01$ vs. Control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. LPS group.

compared with BBR. The inhibition rates of THBru and BBR were approximately 82.4% and 43.6%, respectively.

To further identify the protective effect of THBru on the LPS-induced leakage of plasma proteins, the protein content in BALF was measured. The protein content was remarkably increased in BALF after LPS injection. However, THBru treatment dose-dependently reduced the protein content in BALF. The positive controls BBR and DEX also significantly reduced the protein content in BALF (Fig. 2B). Moreover, the effect of THBru (50 mg/kg) on the protein content reduction was superior to that of BBR. The inhibition rates of THBru and BBR were approximately 81.6% and 58.1%, respectively.

3.2. Effects of THBru on LPS-induced total cell counts in BALF and MPO content of lung tissue

To further investigate the anti-inflammatory properties of THBru, total cell counts in BALF and the MPO content of lung tissue were measured. Total cell counts and MPO content were remarkably increased after LPS injection. However, THBru treatments at doses of 10 and 50 mg/kg both significantly reduced the total cell counts in BALF and the MPO content of lung tissue. The positive controls BBR and DEX also significantly reduced total cell counts and MPO content (Fig. 3A and B). Furthermore, THBru was more effective at reducing total numbers of cells in BALF than was BBR at the same dose. The inhibition rates of THBru and BBR were approximately 66.1% and 54.0%, respectively.

3.3. Effects of THBru on LPS-induced TNF-α content in plasma and nitrate/nitrite content in BALF and plasma

To further investigate the effect of THBru on inflammatory cytokine release, the nitrate/nitrite and TNF-α content were determined. Plasma TNF-α content was remarkably increased after exposure to LPS. THBru (50 mg/kg), BBR and DEX treatment significantly decreased the TNF-α content in plasma (Fig. 4A). Furthermore, nitrate/nitrite content was also remarkably increased in BALF and plasma after LPS injection. However, THBru treatments at various doses significantly decreased the nitrate/nitrite content in BALF and plasma. The positive controls BBR and DEX also significantly decreased the nitrate/nitrite content in BALF and plasma, respectively (Fig. 4B and C).

3.4. Effects of THBru on LPS-induced histopathological changes in mice

Histopathological observation revealed normal pulmonary histology in the control group (Fig. 5A). In contrast, lung tissues from the experimental group administered with LPS alone were significantly damaged, with interstitial edema, hyperemia, thickening of the alveolar wall and infiltration of inflammatory cells (Fig. 5B). However, the destruction of lung structure was significantly reduced by pretreatment with THBru (50 mg/kg), BBR (50 mg/kg) and DEX (3 mg/kg), (Fig. 5C–E).

3.5. Effects of THBru on TNF-α content and NO expression in LPS-induced THP-1 cells

TNF-α content and NO expression were remarkably increased after LPS injection. THBru treatments (10 μM) significantly decreased the TNF-α content and NO expression in LPS-induced THP-1 cells (Fig. 6A–C).

3.6. Effects of THBru on the LPS-induced activation of JNK, p38, AKT and p65 in THP-1 cells by western blot

To further determine the mechanism by which THBru inhibits release of inflammatory factors, we investigated the expression and phosphorylation of JNK, p38, AKT and p65 in LPS-induced THP-1 cells. Compared with the control group, the phosphorylations of MAPKs JNK and p38, AKT and the NF-κB p65 subunit were significantly increased after stimulation with LPS. The expression levels of total JNK, p38, AKT and p65 were not altered after stimulation with LPS. Pretreatment with THBru (1, 5, 10 μM) all could significantly suppress LPS-induced phosphorylation of JNK/AKT/p65. Nevertheless, only THBru (10 μM) not THBru (1, 5 μM) could significantly inhibit LPS-induced phosphorylation of p38 (Fig. 7A–D and Supplementary Fig. 1).

3.7. Immunofluorescence analysis of the inhibitory effects of THBru on activation of p38, AKT and p65 in LPS-induced THP-1 cells

Immunofluorescence was performed to further investigate the involvement of MAPK p38, AKT and NF-κB p65 pathways in the effect of THBru on expression of inflammatory factors. As shown in Fig. 7, THBru significantly suppressed the levels of phospho-p38, phospho-AKT and phospho-p65. These results suggest that THBru suppressed the release of inflammatory factors through the above-mentioned pathways (Fig. 7E–G).

4. Discussion

Acute lung injury (ALI) is most often considered to be part of a systemic inflammatory process, particularly systemic sepsis, which is closely related to the excessive release of inflammatory factors, extravasation of protein-rich fluid, and excessive inflammatory cell infiltration [34–36]. Currently, there are no effective clinically available drugs. In the present study, we observed the inhibitory effect of tetrahydroberberrubine, a berberine derivative,

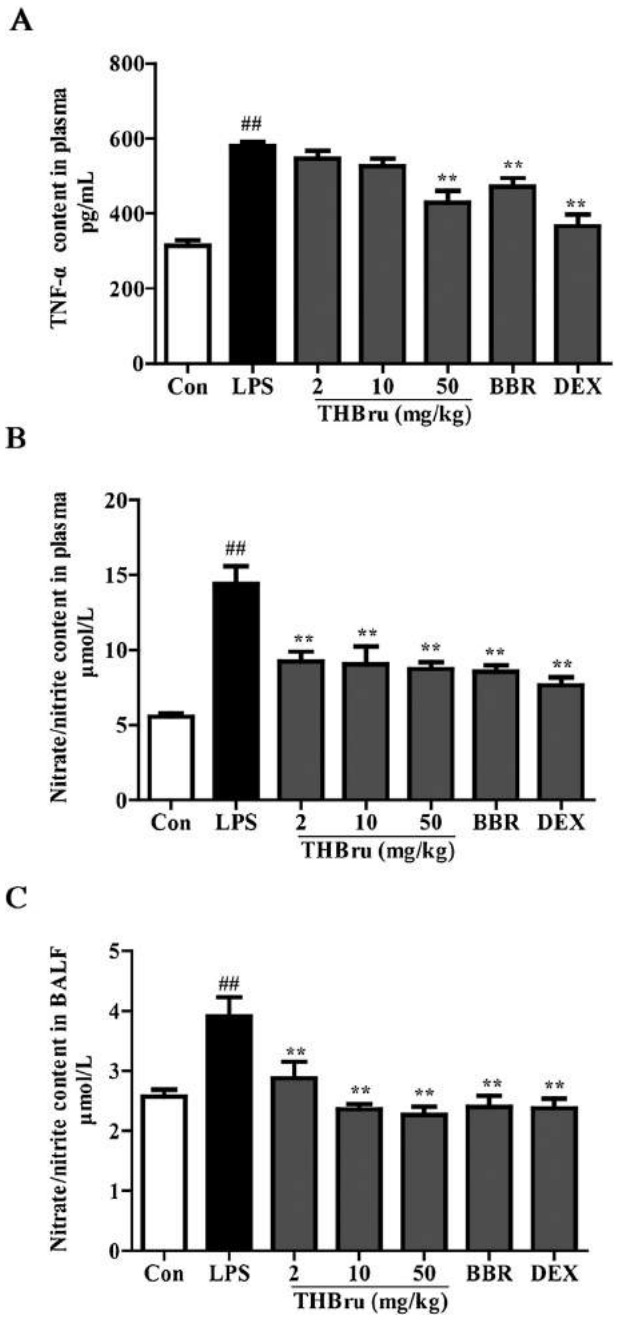

Fig. 4. Effects of THBru on the TNF-α and nitrate/nitrite content in LPS-induced ALI. Mice were orally administered THBru for 1 h prior to LPS exposure. TNF-α content in plasma was determined at 6 h after LPS challenge by ELISA kit (A). Nitrate/nitrite content in plasma (B) and BALF (C) were determined at 6 h after LPS challenge by nitrate/nitrite colorimetric assay kit. The results are expressed as the mean ± SEM (n = 8). $^{\#\#}P < 0.01$ vs. Control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. LPS group.

on a mouse model of LPS-induced acute lung injury that was described in our studies.

One of the initial pathological changes found in ALI is pulmonary edema, which results in reduction of lung compliance and deterioration of pulmonary gas exchange [31]. Our present results showed that THBru dramatically decreased the lung W/D ratio (Fig. 2A), which indicated that THBru could prevent the leakage of liquid into lung tissue. The main contributor to lung edema is considered to be alveolar–capillary barrier destruction [37]. As an index of pulmonary permeability, total protein content was determined in the BALF, and the results indicated that THBru significantly decreased the lung permeability induced by LPS injection(Fig. 2B). These results suggested that THBru might alleviate the development of pulmonary edema.

Leukocyte infiltration is the hallmark of pulmonary inflammation associated with ALI. In particular, neutrophils are considered a primary cellular effector of lung damage [35,38]. However, MPO, an enzyme that is located in the primary granules of neutrophils, reflects the adhesion and migration of neutrophils in the lung. An increase in MPO activity reflects neutrophil accumulation in the lung tissues [39]. In the present study, we found that the LPS-induced elevation of total cell counts in the BALF of mice was reduced by pretreatment with THBru (Fig. 3A). Accordingly, our present data showed that the well-established increase in MPO activity in the lung tissue induced by LPS [40,41] was notably decreased by THBru (Fig. 3B). These results indicated that THBru at doses of 10 and 50 mg/kg protect against LPS-induced ALI by dramatically reducing the migration of leukocytes, particularly neutrophils, into lung tissue.

Excessive release of pro-inflammatory cytokines plays a critical role in ALI. TNF-α, a multifunctional cytokine that is mainly produced by LPS-stimulated monocytes, activates the initial inflammatory cascade. A consistently elevated level of TNF-α is associated with poor outcome in patients with ALI [40,42]. In our study, LPS induced a remarkable increase in the TNF-α levels in plasma compared with control group. THBru downregulated the expression of TNF-α compared with the LPS group (Fig. 4A). This result indicates that the protective effects of THBru on ALI may be due to its ability to inhibit the release of TNF-α.

The over-production of NO by iNOS may lead to cytotoxic effects on endothelial cells via the formation of peroxynitrite, which in turn lead to increased pulmonary microvascular permeability. Accordingly, inhibiting NO production could ameliorate endotoxin-induced acute lung injury [43,44]. Our finding demonstrated that pretreatments with THBru at three different doses could all significantly suppress the increase in NO production in BALF and plasma induced by LPS (Fig. 4B and C). This result suggests that THBru ameliorates LPS-induced acute lung injury in part by protecting the lung from NO damage.

The above results were also supported by histological analysis that LPS-induced increases in pulmonary edema, neutrophil infiltration and coagulation were reduced by THBru (Fig. 5A–E). These findings confirm that THBru attenuates LPS-induced ALI by decreasing protein-rich interstitial liquid, leukocyte infiltration into lung tissue and inflammatory factor release.

Of note, on the one hand, our research results indicate that the effect of THBru on the release of anti-inflammatory factors is equivalent to that of BBR. Moreover, the protective effect of THBru against LPS-induced alveolar–capillary hyper-permeability is improved over BBR at the same dose. On the other hand, it has been reported that the absolute oral bioavailability of berberine was poor (0.36%) [19]. However, its derivative named berberrubine has a higher bioavailability of 31.6% [45]. THBru and berberrubine both have the free hydroxyl group on C-9. Therefore, the oral bioavailability of THBru is improved over that of BBR due to the substitution of the free hydroxyl group on C-9, which provides an active site for further esterification or glycosylation [19]. Simultaneously, it has been reported that the oral bioavailability of THBru significantly improved by esterification of the 9-hydroxy group [46]. These features indicate that the drug candidate THBru has some prospects for treatment of LPS-induced lung injury.

Monocytes are central players in the pathophysiology of ALI. An early elevation of mononuclear phagocyte counts has been observed in the BALF of ALI patients, which was primarily attributable to a rapid influx of monocytes from the vascular compartment into pulmonary alveoli [9,47,48]. It has also been reported that monocytes exhibit a substantial ability to generate

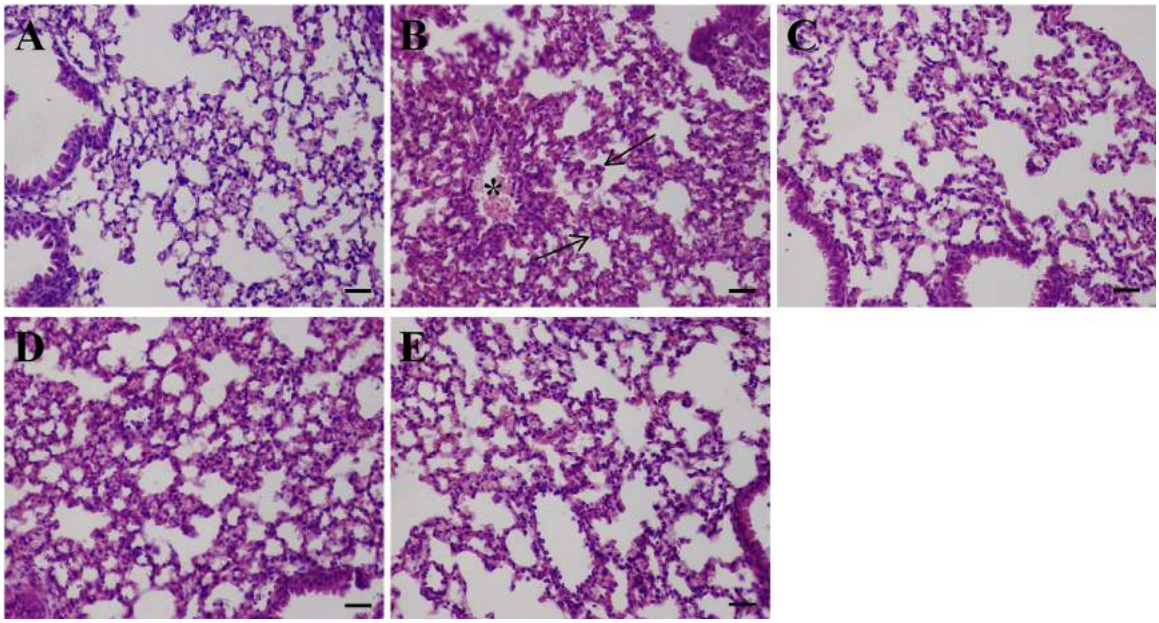

Fig. 5. Effects of THBru on lung histopathological changes in LPS-induced ALI. Mice were orally administered THBru (50 mg/kg), BBR (50 mg/kg) or DEX (3 mg/kg) for 1 h prior to LPS injection (30 mg/kg). The histological changes were determined at 6 h after LPS challenge. Lung tissue sections were stained with hematoxylin and eosin (Original magnification × 200, Scale bar = 50 μm). (A) Control group; normal histopathology was observed, (B) LPS group; The arrow indicated prominent inflammatory cells infiltration and the asterisk indicated alveolar hemorrhage, (C) THBru + LPS group, (D) BBR+ LPS group, (E) DEX+ LPS group; The lung injury was greatly attenuated by THBru, BBR and DEX.

inflammatory factors in response to LPS [49]. Therefore, to explore the possible mechanisms of the protective action of THBru against LPS-induced ALI, we established a model of LPS-stimulated inflammation using THP-1 cells *in vitro*. Our results showed that the enhancement of the TNF-α levels and NO expression in LPS-induced THP-1 cells was reduced by THBru at the concentration of 10 μM (Fig. 6A–C). The results demonstrate that THBru partially alleviates lung injury by suppressing the release of inflammatory

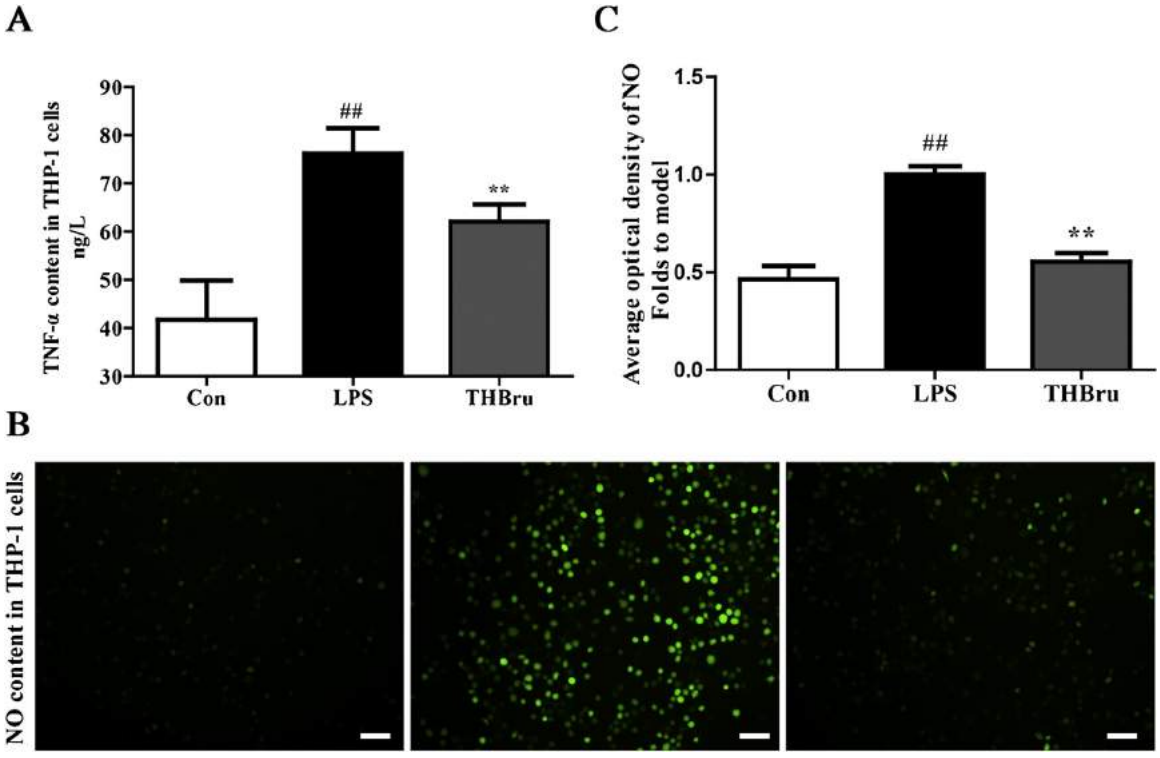

Fig. 6. Effects of THBru on TNF-α content and NO expression in LPS-stimulated THP-1 cells. THP-1 cells were pretreated with THBru (10 μM) for 1 h prior to LPS exposure. TNF-α content (A) in the THP-1 cell supernatant was determined at 5 h after LPS challenge by ELISA kit. NO expression (B) in THP-1 cells was determined at 5 h after LPS challenge using a DAF-FM DA fluorescent probe (Original magnification × 100, Scale bar = 100 μm). Quantitative analysis of NO fluorescence intensity (C). All data presented are the mean ± SEM (n = 3), $^{\#}P < 0.05$ vs. Control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. LPS group.

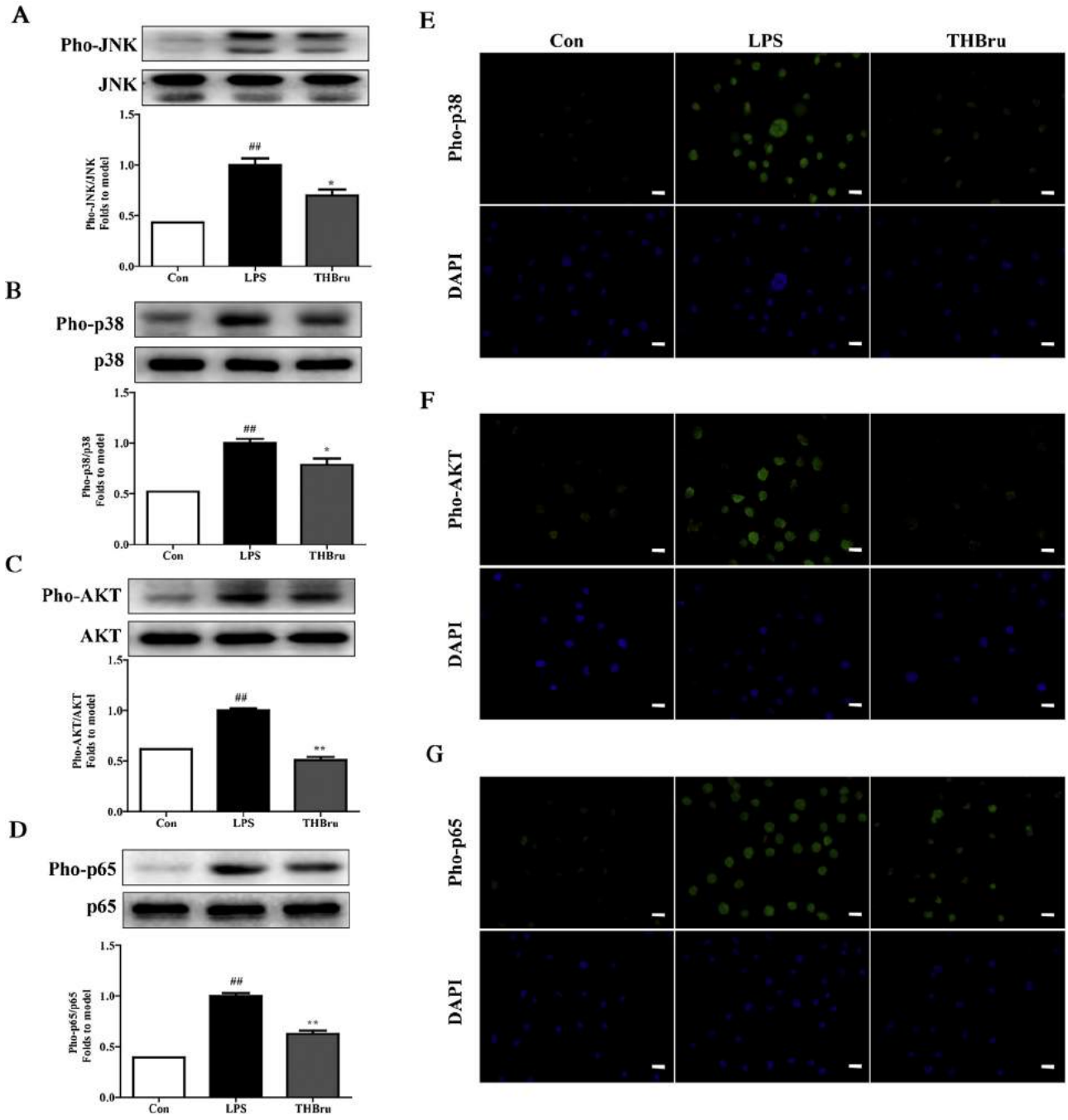

Fig. 7. Effects of THBru on activation of JNK, p38, p65 and AKT in LPS-stimulated THP-1 cells. THP-1 cells were pretreated with THBru (10 μM) for 1 h prior to LPS exposure. The activation of JNK and phospho-JNK (A), p38 and phospho-p38 (B), AKT and phospho-AKT (C) and p65 and phospho-p65 (D) was detected at 30 min after LPS challenge by western blotting. Additionally, phospho-p38 (E), and phospho-AKT (F) and phospho-p65 (G) were detected at 30 min after LPS challenge by immunofluorescence (Original magnification × 400, Scale bar = 20 μm). All data presented are the mean ± SEM (n = 3), $^{\#}P < 0.05$ vs. Control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. LPS group.

factors derived from LPS-induced monocytes. However, protective mechanism of THBru against LPS-induced ALI is still unclear.

It has been demonstrated that NF-κB, MAPK and AKT signaling pathways is closely relationship with sepsis-induced ALI [50–54]. Meanwhile, it has been reported that BBR attenuates cigarette smoke-induced acute lung inflammation and LPS-induced endometritis by inhibiting activation of NF-κB *in vivo* [16,55]. Another, parts of literatures indicated that BBR has a beneficial effect against diabetic neuropathy or arthritis via the MAPK signaling pathway *in vivo* [56,57]. And it has been shown that BBR attenuates myocardial ischemia reperfusion injury by suppressing the activation of PI3 K/AKT signaling *in vivo* [58]. Therefore, according to above literatures we suggest that THBru has protective effect on LPS-induced ALI via down-regulating MAPK, AKT, and NF-κB signaling pathways. Moreover, other researcher results have been shown that BBR inhibited LPS-induced expression of proinflammatory genes via suppressing the phosphorylation of p38/JNK/ERK in peritoneal macrophages and RAW 264.7 cells [59,60]. Simultaneously, our previous results indicated that berberine inhibited activation of MAPK, AKT, and NF-κB signaling pathways in LPS-induced THP-1 cell [18]. Combination literatures with our previous results, we further focus on whether THBru alleviate LPS-induced ALI via suppressing activation of MAPK, AKT, and NF-κB pathways to reduce release of inflammatory cytokines in THP-1 cell.

Toll-like receptor 4, which plays an important role in LPS recognition, triggers the activation of an intracellular signaling pathway that involves the NF-κB p65 subunit, which is a key transcription factor that is involved in regulating the expression of proinflammatory mediators [61]. Once exposed to LPS, the degradation and phosphorylation of IκBα will increase, which results in the increase of p65 phosphorylation and translocation from the cytoplasm to the nucleus and leads to the transcription of target genes, such as TNF-α [62]. Our results showed that different concentrations of THBru all significantly suppressed the LPS-induced activation of the NF-κB p65 subunit (Fig. 7D and Supplementary Fig. 1). And the results were further confirmed by fluorescence data (Fig. 7G).

MAPKs also play an important role in inducing cytokine production. The LPS stimulation of monocytes has been known to induce the phosphorylation and activation of ERK1/2, JNK, and p38 MAPKs [61,63]. To further elucidate the molecular mechanisms of THBru on LPS-induced ALI, we investigated whether the anti-inflammatory activity of THBru was exerted through MAPK signaling pathways. Western blot and immunofluorescence analysis demonstrated that THBru (10 μM) dramatically reduced the increase in JNK and p38 MAPK phosphorylation that was stimulated by LPS (Fig. 7A, B and E). And THBru (1, 5 μM) also could inhibit LPS-induced activation of JNK in THP-1 cell (Supplementary Fig. 1).

Moreover, AKT is the effector of the PI3 K family of enzymes, which participate in various cellular functions. It has been reported that the PI3 K/AKT pathway could be activated in response to LPS [64]. However, the function of PI3 K/AKT signaling in the regulation of inflammatory responses remains under debate. For example, some studies have suggested that the PI3 K/AKT pathway negatively regulates the release of inflammatory factors induced by LPS [65,66], whereas other studies have shown that LPS leads to inflammation in monocytes by activating the PI3 K and AKT signals [67,68]. Our western blot data indicate that THBru (1, 5, 10 μM) all significantly attenuated the increase in AKT phosphorylation stimulated by LPS, which is in agreement with other studies (Fig. 7C and Supplementary Fig. 1) [62,69]. The above results were further confirmed by fluorescence data (Fig. 7F). The above results suggest that the THBru-mediated transcriptional regulation of TNF-α and NO may be due in part to the anti-inflammatory effects associated with inhibiting the activation of JNK/p38 MAPKs and AKT and NF-κB p65 signaling pathway *in vitro*.

In conclusion, we have demonstrated that pretreatment with tetrahydroberberrubine significantly attenuated LPS-induced acute lung injury in mice primarily by suppressing the release of inflammatory factors, infiltration of leukocytes, and presence of protein-rich liquid in pulmonary alveoli. The possible protective mechanism of tetrahydroberberrubine against acute lung injury induced by LPS primarily involved the inactivation of MAPK, AKT and NF-κB signaling pathways. These findings support its potential application for ALI or sepsis.

Conflict of interest

The authors declare that there are no conflicts of interest.

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