In vitro and in vivo protection provided by pinocembrin against lipopolysaccharide-induced inflammatory responses

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A B S T R A C T
Pinocembrin or 5, 7-dihydroxyflavanone is a flavanone, a type of flavonoid. In the present study, we first assessed the anti-inflammatory effects of pinocembrin in RAW macrophage cells; and based on these effects, we investigated the therapeutic effects of pinocembrin in murine model of endotoxin-induced acute lung injury. We found that in vitro pretreatment with pinocembrin remarkably regulated the production of TNF-α, IL-1β, IL-6 and IL-10 via inhibiting the phosphorylation of IκBα, ERK1/2, JNK and p38MAPK. In the mouse model of LPS-induced acute lung injury, pinocembrin (20 or 50 mg/kg, i.p.) attenuated the development of pulmonary edema, histological severities, as well as neutrophil, lymphocyte and macrophage infiltration, which were increased by LPS administration. Additionally, TNF-α, IL-1β and IL-6 concentrations decreased significantly while the concentration of IL-10 was significantly increased after pinocembrin pretreatment. Our results also showed that pinocembrin attenuated LPS-induced lung injury through suppression of IκBα, JNK and p38MAPK activation. These findings suggest that pinocembrin may represent a novel candidate for the modulation of inflammatory responses.

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1. Introduction

Inflammation is a localized protective response elicited by injury or destruction of tissues that serves to destroy, dilute, or sequester both the injurious agent and injured tissue. The acute-phase response is a major pathophysiological phenomenon that accompanies inflammation and is associated with increased activity of proinflammatory cytokines [1]. Macrophages play a critical role, serving as an essential interface between innate and adaptive immunity during inflammatory responses. Following activation, macrophages modulate the expression of accessory molecules such as CD14 and toll-like receptor (TLR) 4 [2,3]. Lipopolysaccharide (LPS), the major ligand for TLR4 [4], has been recognized as a main component in the pulmonary inflammation and sepsis leading to acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) [5,6]. When LPS enters the bloodstream, it elicits inflammatory responses that may lead to shock and ultimately death. These events are mediated by pro-inflammatory cytokines such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6), which are released primarily from monocytes/macrophages and neutrophils [7]. Furthermore, the production of anti-inflammatory cytokine might limit the severity of the inflammatory response without interfering with the beneficial components of host defense and immunity [8].

Common downstream signal transduction pathways that have been shown to mediate inflammatory responses in the lung include nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) [9]. Major signaling pathways regulating cellular growth and response to cytokines and stress occur through the highly conserved mitogen-activated protein kinases family, which consists of p42/44 extracellular signal-regulated kinase, p38 MAPK, and c-Jun N-terminal kinase (JNK) [10]. Nuclear factor-κB is one critical transcription factor required for maximal expression of many cytokines involved in the pathogenesis of acute lung injury. It appears that NF-κB activation is central to the development of pulmonary inflammation and acute lung injury [11]. Although progresses have been made in treating ALI, no suitable therapeutic option exists and treatment is largely supportive [12]. Therefore, the novel effective therapeutic strategy for ALI is required.

Pinocembrin (5, 7-dihydroxyflavanone, C15H12O4, Fig. 1) is a flavanone, a type of flavonoid that has been isolated from the seeds of Alpinia katsumadai Hayata [13]. This plant grows wild mainly in Hainan, Guangxi and Guangdong provinces in Southern China; its seed is a very common traditional Chinese medicine. It is recorded in the Chinese Pharmacopoeia as therapeutic drugs for the treatment of gastric disorders such as epigastric distension, nausea, vomiting and anorexia [14]. In addition, Mee Young Lee et al. have reported that A. katsumadai
Hayata can inhibit the LPS-induced production of interleukin (IL)-6 and tumor necrosis factor (TNF)-α via attenuating the activation of NF-κB signaling pathway in RAW 264.7 cells in vitro [15]. Meanwhile, several pieces of evidence showed that pinocembrin alleviated the ultrastructural changes of cerebral microvessels, astrocyte end-feet and neurons in the GCI/R rats [16], and provided neuroprotection in glutamate-induced cell injury model [17]. Nevertheless, there is no specific study on anti-inflammatory effect of pinocembrin, and the therapeutic potential of the drug for endotoxemia-induced acute lung injury has not been reported. We therefore hypothesized that pinocembrin may have a potential effect against endotoxin-related ALI by suppressing the inflammatory responses. In the present experiment, we first assessed the anti-inflammatory effects of pinocembrin in RAW macrophage cells; and based on these effects, we examined whether pinocembrin could exert protective effects on endotoxin-induced acute lung injury in mice.

2. Materials and methods

2.1. Chemicals and reagents

Pinocembrin (purity > 99.7%) was purchased from Sichuan Research Center of Traditional Chinese Medicine (Chengdu, China); dimethyl sulphoxide (DMSO), LPS (Escherichia coli 055:B5), and 3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin for cell culture were obtained from Invitrogen–Gibco (Grand Island, NY). Mouse TNF-α, IL-6, IL-1β, and IL-10 enzyme-linked immunosorbent assay (ELISA) kits were purchased from BioLegend (CA, USA). The rabbit polyclonal anti-p54 JNK, rabbit polyclonal anti-ERK, rabbit polyclonal anti-p38, mouse monoclonal phospho-specific p46-p54 JNK antibodies, mouse monoclonal anti-phospho-ERK antibodies, mouse monoclonal phospho-specific p38 antibodies and β-actin monoclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit mAb I–κBα and mouse mAb p–I–κBα were purchased from Cell Signaling Technology Inc (Beverly, MA, USA). All other chemicals were of reagent grade.

2.2. In vitro experiments

2.2.1. Cell culture and sample treatment

The RAW 264.7 mouse macrophage cell line was obtained from the China Cell Line Bank (Beijing, China). The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 3 mM glutamine, antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) at 37 °C under a humidified atmosphere of 5% CO2. In all experiments, cells were allowed to acclimate for 24 h before any treatments. Cells were incubated with or without pinocembrin that was always added 1 h prior to LPS treatment.

2.2.2. MTT assay for testing cell viability

Cytotoxicity studies induced by pinocembrin were performed by MTT assay. RAW 264.7 cells were mechanically scraped, plated at a density of 4 × 10^4 cells/ml onto 96-well plates containing 100 μl of DMEM, and incubated in a 37 °C, 5% CO2 incubator overnight. Pinocembrin was dissolved in DMSO, and the DMSO concentrations in all assays did not exceed 0.1%. After overnight incubation, the cells were treated with diverse concentrations of pinocembrin (0–300 μg/ml) in the presence or absence of LPS (1 μg/ml) according to the experimental design. After 18 h, 20 μl of 5 mg/ml MTT was added to each well and the cells were further incubated for an additional 4 h. MTT was removed and cells were lysed with 150 μl/well DMSO. The optical density was measured at 570 nm on a microplate reader (TECAN, Austria).

2.2.3. Measurement of cytokine production

To investigate the effect of pinocembrin on cytokine levels from LPS-treated cells, RAW 264.7 cells (4 × 10^4 cells/ml) seeded into 24-well plates were pretreated with 100, 200, 300 μg/ml of pinocembrin for 1 h prior to 24 h treatment with 1 μg/ml LPS in a 37 °C, 5% CO2 incubator. Cell-free supernatants were collected and stored at −20 °C until assayed for cytokine levels. The concentrations of TNF-α, IL-1β, IL-6 and IL-10 in the supernatants of RAW 264.7 cell culture were determined using an ELISA kit, according to the manufacturer’s instructions (BioLegend, Inc, Camino Santa Fe, Suite E, San Diego, CA, USA).

2.2.4. SDS PAGE and Western blot analysis

RAW 264.7 cells (4 × 10^5 cells/ml) were plated onto 6-well plates and pretreated with 100, 200, 300 μg/ml of pinocembrin for 1 h and then stimulated with 1 μg/ml of LPS for 30 min. The cells were collected by centrifugation and washed twice with ice-cold PBS. The washed cell pellets were resuspended in extraction lysis buffer (50 mM Tris (pH 7.6), 150 mM NaCl, 5 mM EDTA (pH 8.0), 0.6% NP-40, 1 mM Na3VO4, 20 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 mM p-nitrophenyl phosphate and 1:25 Complete Mini Protease Inhibitor cocktail (Boehringer, Mannheim, Germany)). After incubation on ice for 30 min, the lysates were centrifuged (12,000 g at 4 °C) for 5 min to obtain the cytosolic fraction. The protein concentration was determined using the Bradford assay (Bio-Rad, Munich, Germany) according to the manufacturer’s instructions. The samples of cellular protein from treated and untreated cell extracts were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and then electroblotted onto a polyvinylidene difluoride (PVDF) membrane. The immunoblot was incubated overnight with blocking solution (5% (wt/vol) nonfat dry milk, followed by incubation for 4 h with primary antibody in 5% (wt/vol) bovine serum albumin (BSA) dissolved in TWEEN 20/Tris-buffered saline [TTBS, 20 mM Tris–HCl buffer, pH 7.6, containing 137 mM NaCl and 0.05% (vol/vol) TWEEN 20]. Blots were washed with TTBS and incubated with a peroxidase-conjugated secondary anti-mouse antibody. Blots were again washed with TTBS and the bound antibodies were detected using an enhanced chemiluminescence (ECL) Western blotting detection system.

2.3. In vivo experiments

2.3.1. Animal

All studies were performed in accordance with the Health’s Guide for the Care and Use of Laboratory Animals published by US National Institute of Health. Male BALB/c mice, weighing approximately 18 to 20 g, were purchased from the Center of Experimental Animals of Baiqiu Medical College of Jilin University (Jilin, China). The mice were kept in the animal house in a temperature-controlled room with a 12 h light–dark cycle; free access to standard laboratory chow and water was allowed. Laboratory temperature was 24 ± 1 °C and relative humidity was 40–80%. Before experimentation, the mice were left to adapt to the experimental environment for 2 to 3 days.
2.3.2. Experimental design
Mice were randomly divided into four groups. Control mice were given 50 μl PBS intranasally (i.n.) without LPS; in LPS group, 10 μg of LPS was instilled intranasally (i.n.) in 50 μl PBS to induce lung injury. The LPS + PNCB20 and LPS + PNCB50 groups mice were injected with pinocembrin (20 or 50 mg/kg, respectively, dissolved in povidone; i.p.) 1 h prior to LPS administration.

2.3.3. Bronchoalveolar lavage fluid (BALF) collection and cell counting
The lungs were lavaged three times through a tracheal cannula with 0.5 ml of autoclaved PBS, instilled up to a total volume of 1.3 ml. Retrieval volume was maximized by compression of the thorax following the last lavage. The bronchoalveolar lavage fluid was centrifuged (3000 rpm, for 10 min, 4 °C). The cell pellets were resuspended in PBS for total cell counts using a hemacytometer, and cytopsin were prepared for differential cell counts by staining with the Wright-Giemsa staining method.

2.3.4. Cytokine assays in vivo
The tissue sample was centrifuged at 3000 rpm, for 10 min at 4 °C. The cytokine concentrations in the supernatant were determined by enzyme-linked immunosorbent assays (ELISA) using commercial kits specific for mouse according to the manufacturer's instructions.

2.3.5. Lung wet-to-dry weight (W/D) ratio
The mouse lungs were excised, immediately weighed and the dry weight was determined after heating the lungs at 80 °C for 48 h. The W/D ratio was calculated by dividing the wet weight by the dry weight.

2.3.6. Protein analysis
To evaluate vascular permeability in the airways, the BALF was collected and the supernatant was used for protein content determination by bicinchoninic acid (BCA) method. Proteins were expressed in milligram protein per milliliter BALF.

2.3.7. Histopathologic examination of lung
To characterize the histological alterations, the lungs were excised and fixed in 10% buffered formalin. The lungs were dehydrated with graded alcohol, embedded in paraffin, and the sections stained with hematoxylin and eosin were examined by light microscopy. The observations were performed to evaluate alveolar congestion, hemorrhage, infiltration or aggregation of neutrophils in airspace or vessel wall, and the thickness of the alveolar wall/hyaline membrane formation.

2.3.8. Western blot analysis of lung tissue
The lungs were harvested and frozen in liquid nitrogen immediately until homogenization. After centrifugation, protein concentration was assayed using BCA protein assay kit and equal amounts of protein were loaded per well on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel. Subsequently, proteins were transferred onto polyvinylidene difluoride membrane. The membrane was blocked overnight with 5% (w/v) nonfat dried milk to reduce non specific binding, washed in TTBS and then probed with the indicated antibodies including IκB, phosphorylated and non-phosphorylated forms of ERK, JNK, and p38. Membrane was then washed three times for 5 min each with TTBS and a 1:7000 (v/v) dilution of horseradish peroxidase-labeled IgG was added at room temperature for 1 h. Antibody binding was visualized with an enhanced chemiluminescence (ECL) Western blotting detection system.

2.4. Statistical analysis
All values were expressed as mean ± SEM. Differences between mean values of normally distributed data were assessed with one-way ANOVA (Dunnett's t-test) and two-tailed Student's t-test. Statistical significance was accepted at P<0.05 or P<0.01.

3. Results
3.1. Effect of pinocembrin on macrophage viability
To assess the suitable concentration of pinocembrin for the study, RAW 264.7 cells were incubated with pinocembrin at concentrations ranging from 100 to 300 μg/ml and cell viability was measured by MTT test 18 h later. We found that pinocembrin at concentrations from 100 to 300 μg/ml had no cytotoxic effect on RAW 264.7 cells (P<0.05; Fig. 2).

3.2. Effect of pinocembrin on LPS-induced cytokine production in vitro
TNF-α, IL-1β, IL-6 and IL-10 concentrations in the culture supernatants of RAW 264.7 cells were measured by sandwich ELISA (Fig. 3). Treatment of RAW 264.7 cells with LPS alone resulted in significant increases in cytokine production as compared to the control group. However, treatment with pinocembrin inhibited the production of TNF-α and IL-6 at 100, 200 and 300 μg/ml. IL-1β concentration decreased significantly at 200 and 300 μg/ml pinocembrin pretreatment (P<0.01). In contrast, the concentration of IL-10 was significantly increased in groups treated with pinocembrin.

3.3. Effects of pinocembrin on MAPKs and NF-κB signaling pathways in LPS stimulated RAW 264.7 cells
In order to investigate the mechanism by which pinocembrin inhibits LPS-induced production of inflammatory cytokines, we examined the effect of pinocembrin on the LPS-induced phosphorylation of ERK1/2, JNK and p38MAPK in the cytoplasm by Western blotting analysis using three different phospho-specific antibodies (Fig. 4). As shown, the phosphorylation levels of the MAPK increased dramatically after 30 min of stimulation with LPS and pinocembrin markedly inhibited LPS-induced activation of ERK1/2, JNK and p38MAPK in a dose-dependent manner. No changes in the expression of non-phosphorylated MAPKs were observed in cells treated with LPS or LPS and pinocembrin. The major pathway used by a variety of stimuli to activate NF-κB involves the phosphorylation of IκBα and its proteasome-mediated degradation [18]. Thus, we examined the effect of pinocembrin on IκBα phosphorylation and degradation. It was found that pretreatment with pinocembrin prior to LPS induction attenuated IκBα phosphorylation in a dose-dependent manner (Fig. 4).

Fig. 2. The effects of pinocembrin on the viability of RAW 264.7 cells. RAW 264.7 cells were incubated in the presence or absence of pinocembrin (100 to 300 μg/ml) and LPS (1 μg/ml) for 24 h. Cell viability was determined by MTT assay. Data are presented as means ± SEM of three independent experiments.
were increased in the lung tissues in LPS group compared with control group. Our data showed that pretreatment with pinocembrin significantly suppressed LPS-induced activation of JNK, p38/MAPK and NF-κB in a dose-dependent manner. However, there was no significant change in p-ERK1/2 between control and groups treated with pinocembrin.

4. Discussion

To the best of our knowledge, the current study is the first to demonstrate that pinocembrin could 1) attenuate LPS-induced inflammatory responses in RAW 264.7 cells; and 2) significantly protect mice against endotoxemia-induced acute lung injury. Moreover, we found that these protective effects of pinocembrin were due to its influence of varied inflammatory cytokines release, including inflammatory cytokines TNF-α, IL-1β and IL-6 and anti-inflammatory cytokine IL-10.

The study primarily focused on anti-inflammatory effects of pinocembrin on LPS-stimulated RAW 264.7 macrophage cells. Recently, new approaches for the use of Chinese herbal plants to prevent and treat inflammatory responses by inhibiting inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, have become an important area of investigation [19]. Thus, our data revealed that pinocembrin inhibited TNF-α, IL-1β and IL-6 production in a dose-dependent manner in LPS-stimulated RAW 264.7 cells (Fig. 3). It should be noted that this inhibitory effect was not due to cytotoxic activity of pinocembrin, because the cell viability was not affected by pinocembrin treatment alone (Fig. 2). IL-10 cytokine is considered to have an anti-inflammatory role, potentially inhibiting the capacity of monocyte/macrophage cells to secrete inflammatory mediators, including TNF-α, IL-1β, and IL-6 [20]. Thus, in addition to the finding that pinocembrin decreased TNF-α, IL-6 and IL-1β levels, pinocembrin may potentially up-regulate IL-10. The anti-inflammatory actions of IL-10 can interfere with the production of pro-inflammatory cytokines through the suppression of NF-κB activation by preserving the expression of IκB protein. The activation of NF-κB is implicated in MAPK signaling pathway. Inhibition of MAPK family pathway, such as ERK, p38, and JNK, alleviates the production of pro-inflammatory cytokines [21]. The results showed that pinocembrin obviously down-regulated LPS-induced phosphorylation of ERK1/2, JNK

3.4. Effect of pinocembrin on lung inflammatory responses elicited by LPS administration

Six hours after LPS administration, the levels of the cytokines TNF-α, IL-1β and IL-6 in lung homogenate were dramatically increased, while IL-10 was only slightly increased compared with control group (Fig. 5). Pretreatment with pinocembrin (20 or 50 mg/kg) upregulated the IL-10 level and down-regulated TNF-α, IL-1β and IL-6 levels in dose-dependent manner.

To evaluate LPS-induced changes in pulmonary vascular permeability to water, we evaluated the wet weight to dry weight ratio of the lungs. LPS instilled for 6 h caused a significant increase in lung wet/dry weight ratio and total protein concentration compared with control (P<0.01). As shown in Fig. 6, pinocembrin pretreatment significantly reduced the water gain and the total protein concentration during the acute lung injury resulted from LPS challenge in a dose-dependent manner.

Mice exposed to LPS showed an increase in the number of total cells, neutrophils, lymphocytes and macrophages as compared to the control group. Pinocembrin treatment for 1 h prior LPS administration led to a significant lowering of the number of total cells, neutrophils, macrophages and lymphocytes (P<0.01, Fig. 7).

To assess the effects of pinocembrin on ALI, we observed the histological changes after pinocembrin treatment in LPS-exposed mice. After histological examination, the lung tissues from the LPS group were significantly damaged, with interstitial edema, hemorrhage, thickening of the alveolar wall, and infiltration of inflammatory cells into interstitium and alveolar space. However, pinocembrin inhibited these histological changes in a dose response manner (Fig. 8).

3.5. Effects of pinocembrin on MAPKs and NF-κB signaling pathways in the lung tissues of LPS-treated mice

To investigate the anti-inflammatory mechanisms of pinocembrin, we examined the effect of pinocembrin on MAPK and NF-κB activation. As shown in Fig. 9, phosphorylation of ERK, JNK, p38 and IκB were increased in the lung tissues in LPS group compared with control group. Our data showed that pretreatment with pinocembrin significantly suppressed LPS-induced activation of JNK, p38/MAPK and NF-κB in a dose-dependent manner. However, there was no significant change in p-ERK1/2 between control and groups treated with pinocembrin.
and p38 pathways in a dose-dependent manner. In addition, incubation of macrophages with LPS caused marked phosphorylation of cytosolic IkB. However, treatment with pinocembrin was found to significantly inhibit the phosphorylation of IkBα (Fig. 9). This means that pinocembrin might regulate the kinase phosphorylating IkBα. Previous study implies that A. katsumadai Hayata can inhibit the LPS-induced production of interleukin(IL)-6 and tumor necrosis factor (TNF)-α via attenuating the activation of NF-κB signaling pathway in RAW 264.7 cells in vitro. In this study, pinocembrin isolated from this plant was confirmed not only to inhibit NF-κB activation dose-dependently, but also ERK1/2, JNK and P38/MAPK phosphorylation. In addition, TNF-α, IL-1β and IL-6 concentrations decreased significantly while the IL-10 concentration was significantly increased after pinocembrin pretreatment. These results suggest that the effects of pinocembrin on the production of inflammatory cytokines could occur through blocking of the MAPK and NF-κB signaling pathways.

Acute lung injury is characterized by disruption of endothelial and epithelial integrity, lung edema, release of pro-inflammatory mediators and extensive neutrophil infiltration [22]. In our study, pretreatment with pinocembrin markedly reduced the lung inflammatory responses and improved the pulmonary histology. Evidences from several clinical studies indicated that ALI is provoked by a lot of pro-inflammatory cytokines produced by the active neutrophils that accumulated in the lung, such as TNF-α, IL-1β and IL-6, which directly damage pulmonary capillary endothelial cells and induce release of other inflammatory mediators, thereby deteriorating lung injury [23]. It is likely that pro-inflammatory cytokines, notably TNF-α, IL-1β and IL-6, participate in the early development of inflammation; they have been shown to play a crucial role in ALI and ARDS [24]. TNF-α and IL-1β are called primary cytokines due to their role in initiating an acute inflammatory response [25]. It is well known that they up-regulate neutrophil and endothelial adhesive molecular expression and enhance neutrophil–endothelial adherence [26]. Plasma IL-6 has been found to be a significant predictor of morbidity and mortality in patients with ARDS [27]. Although a variety of injurious events and mediators can lead to ALI and ARDS depending on the clinical risk group, cytokine-mediated inflammation is shared in all of these patients [28]. Since some cytokines are potent mediators of potentially damaging tissue responses, several mechanisms exist to ensure that the effects of these cytokines are restricted [29]. In this study, we found that, the levels of the cytokines TNF-α, IL-1β and IL-6 in lung homogenate were dramatically increased
after LPS administration. The pretreatment of pinocembrin (20 or 50 mg/kg) significantly lowered LPS-induced pro-inflammatory cytokines TNF-α, IL-1β and IL-6 production, and it actually was close to the normal level. Anti-inflammatory cytokines like IL-10 are also produced during endotoxemia. They down-regulate production of pro-inflammatory cytokines and provide a key mechanism for limiting the inflammatory response in the lungs [30]. IL-10 concentration treated with pinocembrin significantly increased in a dose-dependent manner compared to the LPS group (P<0.01). According to our results, the inhibition of cytokines and prevention of lung injury might be related to increased BALF levels of IL-10 resulting from pretreatment of mice with pinocembrin 1 h prior to LPS administration. These results showed the same regulatory effects as EtOH [31] and synthetic liver X receptors (LXRs) agonists T0901317 [32] by down-regulating the production of pro-inflammatory mediators TNF-α, IL-1β, IL-6 and simultaneously up-regulating that of anti-inflammatory mediators such as IL-10 in acute lung injury. Since different flavonoids might have different roles in endothelial cells, the structure–activity relationships of flavonoids have become the subject of many investigations [33]. The R form is the stereo structure of pinocembrin in ring A, in which the C2 position

![Image](image1.jpg)

**Fig. 5.** Effect of pinocembrin (PNCB) on the concentrations of inflammatory cytokines in BALF of mice with ALI. Mice were given an intraperitoneal injection of pinocembrin (20 or 30 mg/kg) 1 h prior to an i.n. administration of LPS. BALF was collected at 6 h following LPS challenge to analyze the inflammatory cytokines TNF-α(A), IL-1β(B), IL-6(C) and IL-10(D). The values presented are mean±SEM (n=6). **P<0.01 vs. control group; ***P<0.01 vs. LPS group.

![Image](image2.jpg)

**Fig. 6.** Effects of pinocembrin (PNCB) on the lung W/D ratio and total protein level in the BALF of LPS-induced ALI mice. To induce ALI, LPS was instilled intranasally 1 h after intraperitoneal injection of pinocembrin (20 or 50 mg/kg). The lung W/D ratio (A) and total protein concentration in the BALF (B) were determined at 6 h after LPS given. The values presented are mean±SEM (n=6). **P<0.01 vs. control group; ***P<0.01 vs. LPS group.
Fig. 7. Effects of pinocembrin (PNCB) on LPS-induced inflammatory cell accumulation in BALF. BALF was prepared from mice 6 h after LPS instillation; Total cell counts (A), numbers of neutrophils (B), macrophages (C) and lymphocytes (D) in BALF samples were determined as described previously. The values presented are the mean±SEM (n=4-6 in each group). **P<0.01 vs. control group; *P<0.05, ***P<0.01 vs. LPS group.

Fig. 8. Effect of pinocembrin (PNCB) on LPS-mediated lung histopathologic changes. Mice were given an intraperitoneal injection of pinocembrin (20 or 50 mg/kg) 1 h prior to an i.n. administration of LPS. Lungs (n=3) from each experimental group were processed for histological evaluation at 6 h after LPS challenge. (a) The lung section from the control mouse. (b) The lung section from LPS induced ALI model mouse. (c and d) The lung section from the mice exposed to LPS and then administered with 20 or 50 mg/kg pinocembrin respectively. Representative histological section of the lungs was stained by hematoxylin and eosin (H&E staining, a, b, c, and d magnification 200x; a1, b1, c1, and d1 magnification 400x).
is bound to 5, 7-hydroxyl groups that may be crucial for the anti-inflammatory effect. Taken together, these data imply that pinocembrin may be an important regulator of inflammatory cytokines in septic lung damage. These in vivo results appear to correlate well with in vitro outcomes, which are consistent with previous studies that demonstrate the importance of macrophages as a source of pro-inflammatory cytokines in the lung [34].

Considering the fact that instillation of IL-1β and TNF-α into the lungs leads to neutrophil accumulation, interstitial edema and histological changes consistent with inflammatory lung injury [35], we evaluated the lung W/D ratio, neutrophil, lymphocyte and macrophage accumulation within lung tissue, and we observed the histological changes after pinocembrin treatment in LPS-exposed mice. The histopathologic results showed that there were great improvements in the lung injury of pinocembrin-pretreated groups compared with LPS group. Moreover, we found that pinocembrin pretreatment attenuated the development of pulmonary edema, as well as neutrophil, lymphocyte and macrophage infiltration, which were increased by LPS administration.

To clarify the cellular mechanisms that regulated the cytokine production after endotoxin stimulation, we assessed the effects of pinocembrin on MAPK and NF-κB activation. In endotoxemia, NF-κB released from IκBα translocates into nucleus, where it enhances the transcription of cytokines, such as TNF-α, IL-1β and IL-6. The pivotal role of activation of NF-κB in inflammation during acute lung injury has been well elucidated in previous studies [36,37]. NF-κB is composed of dimers of five different proteins (p50, p52, p65/RelA, RelB, c-Rel). These dimers exist in the cytoplasm in inactive forms bound to the inhibitory protein IκB. A variety of agonists activate IκB kinases. The transmission of LPS signaling through its receptor cluster involving CD14 and TLR4 leads to the activation of the IκB kinase complex (IKK). IKK then phosphorylates the inhibitory IκB protein that normally sequesters NF-κB dimers in the cytoplasm during the inactive state. This phosphorylation targets the IκB proteins for ubiquitination and degradation, leading to the release and subsequent translocation of NF-κB dimers into the nucleus. The binding of NF-κB dimers to the IκB consensus sequences in the promoter facilitates the transcription of specific target genes [39]. Zhou et al. [40] have reported that NF-κB plays an important role in the pathogenesis of lung diseases. In addition, each of the three MAPK pathways, ERK, p38, and JNK, has been demonstrated as participating in LPS-induced ALI [41]. This study demonstrated that MAPK and NF-κB were activated after LPS administration, and pretreatment of pinocembrin prevented the activation of NF-κB through the inhibition of IκBα. The phosphorylation of JNK and p38/MAPK in LPS-induced ALI mouse model was markedly
inhibited by pinocembrin, whereas activation of ERK1/2 was not affected. However, the in vivo effects of pinocembrin on ERK1/2 activation still remained to be elucidated.

In conclusion, we have provided the first evidence that pinocembrin significantly regulated the production of TNF-α, IL-1β, IL-6 and IL-10 through the inhibition of MAPK and NF-κB activation in murine macrophage and endotoxin-induced ALI model. Furthermore, pinocembrin pretreatment attenuated inflammation and reduced lung injury in a murine model of LPS-induced inflammation. These results suggest that this new agent can be a promising therapeutic approach in the treatment of acute lung injury. It might be beneficial in future studies to explore the therapeutic potential of pinocembrin in other inflammatory diseases.

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