Pachymic acid inhibits inflammation and cell apoptosis in lipopolysaccharide (LPS)-induced rat model with pneumonia by regulating NF-κB and MAPK pathways

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Abstract
Pneumonia is a common infectious disease with high morbidity and mortality. It is caused by a variety of pathogenic microorganisms that infect the lung parenchyma. Anti-infective drugs are one of the preferred choices for the treatment of pneumonia. Pachymic acid (PA) is a lanolin triterpene compound from Poria cocos, which has antiemetic, anti-inflammatory, and anticancer properties. Although PA inhibits inflammatory response in a variety of diseases, its role in pneumonia is not clear. In this study, we established that PA improved histopathological changes in the lungs of rats with pneumonia. PA inhibited the expression of inflammatory cytokines in the serum of rats having pneumonia. In addition, PA inhibited the apoptosis of cells from rat lung tissues. Mechanically, PA inhibited inflammation and cell apoptosis via NF-κB and MAPK pathways. Therefore, PA could serve as a promising drug for treating pneumonia.

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KEYWORDS
apoptosis; inflammation; MAPK; NF-κB; pachymic acid (PA); pneumonia

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Introduction

Pneumonia is a common infectious disease, characterized by the lower respiratory tract infection by pathogens such as viruses, bacteria, and fungi. The morbidity and mortality rates because of pneumonia are increasing worldwide. Typical symptoms of pneumonia are chills, fever, pleurisy, cough, and purulent sputum. Pneumonia has become a leading cause of death in children aged less than 5 years, with about 1.3 million child deaths worldwide every year. A better understanding of the underlying mechanism of childhood pneumonia could contribute to the development of an effective and novel treatment to overcome this burden. Fighting infection is the primary need toward treating pneumonia. Since pneumonia is a disease caused by a variety of pathogenic microorganisms infecting the lung parenchyma, anti-infective drugs are the first choice for its treatment.

Pachymic acid (PA) is a lanolin triterpene compound from Poria cocos. Studies have demonstrated that PA has antiiptemic, anti-inflammatory, and anticancer properties. Previous investigations have shown that Poria could reduce inflammatory response to septic kidney and lung injuries. In particular, PA has an anti-inflammatory effect in the pulpitis model. Besides, PA protects cardiomyocytes from LPS-induced inflammation and apoptosis by regulating the extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38 MAPK) pathways. PA also competitively inhibits the biological activity of phospholipase A2 (PLA2) and reduces PLA2-mediated cell damage. In addition, PA inhibits the invasion of breast cancer by regulating nuclear factor kappa B (NF-kB) pathway.

NF-kB and mitogen-activated protein kinase (MAPK) signaling are important pathways of inflammation. Signaling from pathogens or host cells, such as pathogen-associated molecular patterns (PAMP), is recognized by various pattern recognition receptors and eventually triggers the activation of MAPK (p38, ERK, and c-JUN N-terminal kinase [JNK]) and NF-kB signaling pathways. These pathways activate gene transcription and promote the expressions of various cytokines and amplify the inflammatory response through positive feedback cascades. Several studies have provided evidence showing that NF-kB could serve as a target for treatment of pneumonia.

Although previous studies have shown that PA has anti-inflammatory effects and could inhibit the inflammatory response in a variety of diseases, its role in pneumonia is not clear. The purpose of this study was to elucidate the potential effects of PA on pneumonia and explore relevant regulatory mechanisms.

Materials and methods

Animals and treatment

The animal study was performed according to the National Institutes of Health Laboratory Animal Care and Use Guidelines. The study was approved by the Institutional Animal Care and Use Committee of the Dingxi People’s Hospital. A total of 20 male rats weighing approximately 180-220 g were brought from Shanghai Slack Laboratory Animal Co. Ltd. The rats were randomly divided into the following four groups (n = 5 in each group): control group, Lipopolysaccharide (LPS) group (2 mg/kg LPS), LPS+PA group (10 mg/kg), and LPS+PA group (20 mg/kg). PA was purchased from BioCrick Biotech (Chengdu, Sichuan Province, China) and administered to rats intraperitoneally. To induce acute lung injury, rats were injected light anesthesia with 0.5% pentobarbital sodium intraperitoneally and intranasal administration of 2 mg/kg of LPS in sterile saline. After 3 days, the blood samples were collected and rats were sacrificed. Lungs were subjected to bronchoalveolar lavage fluid (BALF) collection.

Hematoxylin and Eosin (HE) staining

Freshly excised lung tissues were fixed with 10% paraformaldehyde and embedded with paraffin and cut into 4-μm slices. Then these sections were deparaffinized and subjected to HE (Sigma-Aldrich, Kenilworth, USA) staining. The total lung injury score was calculated as the sum of the scores of each variable (edema, neutrophilic infiltration, interstitial inflammation, and congestion). The scores ranged between 0 (normal) and 4 (severe), with the mean value recorded as a semi-quantitative histological indicator of lung injury.

Lung wet-to-dry weight ratio measurement

The lung samples were isolated after sacrifice of rats and weighed immediately. The samples were dried until the weight was stable. The wet-to-dry weight (W/D) ratio was obtained.

Enzyme linked immunosorbent assay (ELISA)

The lung tissues were lysed with phosphate buffer solution (PBS) and minced with magnetic beads. Then lysates were collected and centrifuged. Concentrations of TNF-α, Interleukin (IL)-1β, IL-6, and monocyte chemotactic protein (MCP) in lung tissues were measured by using the ELISA kit (R&D Systems, Minneapolis, USA).

Cell number in BALF

The BALF samples were centrifuged at 1700 g for 10 min at 4°C and cell pellets were dissolved in saline. Cell numbers in the suspension were measured with an automatic blood cell counter (Sysmex E-25000; Toua-iyoudenshi Co. Ltd, Japan).

TUNEL assay

The slices were deparaffinized in xylene for 5 min, hydrated, and rinsed in distilled water. Then the sections were incubated with 3% H2O2 to block endogenous peroxidase activity and terminal deoxyribonucleotidyl transferase (TdT) reaction mixture for 1-2 h in a humidified chamber. Then reaction was stopped by rinsing sections
PA inhibits pneumonia progression

In order to explore the therapeutic effects of PA in LPS-induced lung injury in rats, four groups of rats were pre-treated with or without PA. After induction of LPS in rats, histological changes in each group were analyzed through HE staining. LPS treatment induced lung injury, such as alveolar exudate, edema, and inflammatory cell infiltration, in rats compared with the control group (Figure 1A). However, PA treatment significantly alleviated these phenomena in a dose-dependent manner (Figure 1A). Consistently, PA treatment reversed the elevated W/D ratio of lung tissue induced by LPS (Figure 1B). Taken together, these results indicated that PA could attenuate lung injury induced by LPS.

**Immunoblot analysis**

Protein samples from tissues were separated through electrophoresis on sodium dodecylsulfate-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. After blocking with 5% milk in TBST (20-mM tris-buffered saline, 500-mM NaCl, pH 7.5, and 0.1% Triton X-100) for 1 h at room temperature, the membranes were incubated with anti-Bax antibody (1:1000 dilution, ab32503, Abcam plc, Cambridge, UK), anti-GAPDH (1:5000 dilution, ab8245, Abcam plc), anti-B-cell lymphoma protein 2 (Bcl-2) (1:1000 dilution, ab32124, Abcam plc), anti-p65 (1:2000 dilution, ab32536, Abcam plc), anti-p-p65 (1:1000 dilution, ab76302, Abcam plc), anti-p38 (1:1000 dilution, ab170099, Abcam plc), anti-p-p38 (1:1000 dilution, ab178867, Abcam plc), anti-ERK (1:1000 dilution, ab184699, Abcam plc), and anti-p-ERK (1:1000 dilution, ab201015, Abcam plc) at 4°C overnight. Then membranes were washed for three times in TBST and conjugated with secondary antibody with horseradish peroxidase (Sigma, Kenilworth, USA) at room temperature for 1 h. The membranes were analyzed with enhanced chemiluminescence (ECL) reagent (Sigma, Kenilworth, USA).

**Statistics**

GraphPad 5.0 software was used for statistical analysis. Data were represented as mean ± SD. Student’s t-test was used for comparisons, and P < 0.05 was considered as significant.

**Results**

**PA alleviates Lipopolysaccharide (LPS)-induced lung injury in Rats**

In order to explore the therapeutic effects of PA in LPS-induced lung injury in rats, four groups of rats were treated with different doses of PA. After induction of LPS, histological changes in each group were analyzed through HE staining. LPS treatment induced lung injury, such as alveolar exudate, edema, and inflammatory cell infiltration, in rats compared with the control group (Figure 1A). However, PA treatment significantly alleviated these phenomena in a dose-dependent manner (Figure 1A). Consistently, PA treatment reversed the elevated W/D ratio of lung tissue induced by LPS (Figure 1B). Taken together, these results indicated that PA could attenuate lung injury induced by LPS.

**PA relieves inflammation induced by LPS in rats**

Induction of LPS was accompanied with elevated level of TNF-α, IL-6, IL-1β, and MCP-1. To delineate the role of PA in LPS-induced inflammatory cytokine production, the respective concentrations of TNF-α, IL-6, MCP-1, and IL-1β were detected in the lung tissues of rats. As shown in Figure 2, LPS stimulation significantly elevated the levels of TNF-α, IL-6, MCP-1, and IL-1β in lung tissues. However, administration of PA lowered TNF-α level to half of that in the LPS group, and the levels of IL-6, MCP-1, and IL-1β in the PA group were decreased to less than 50% that in the LPS group. These results suggested that PA treatment could relieve LPS-induced inflammation in mice.

**PA relieves cell numbers in BALF of rats induced by LPS**

In order to analyze cell numbers in BALF, the total cell number, neutrophil number, and white cell number were measured. Clear elevation in the total cell number, neutrophil number, and white cell number in the BALF of LPS-induced rats were observed. After PA treatment, the total cell number, neutrophil number, and white cell number were decreased in a dose-dependent manner (Figures 3A–3C). Taken together, the PA treatment effectively reduced the total cell number, neutrophil number, and white cell number in the BALF of LPS-induced rats.

![Figure 1](https://example.com/fig1.png)

**Figure 1** PA alleviates LPS-induced lung injury in Rats. (A) The histological changes of lung tissue in control, LPS, LPS+10 mg/kg PA, and LPS+20 g/kg PA groups. (B) The wet/dry weight (W/D) ratio of the lung tissues in control, LPS, LPS+10 mg/kg PA, and LPS+20 g/kg PA groups. **P < 0.01 versus the control group; *P < 0.05, #P < 0.01 versus the LPS group.
**PA inhibits cell apoptosis in rats induced by LPS.**

The cell apoptosis in lung tissues was detected by TdT-mediated dUTP Nick-End Labeling (TUNEL) assay. As expected, LPS induction in rats led to increased number of apoptotic cells in rats, which was evidenced by increased number of TUNEL positive cells (Figure 4A). PA treatment significantly reduced the number of TUNEL positive cells (Figure 4A). Also, the expression levels of Bax and Bcl-2 were detected in lung tissues. LPS treatment enhanced the Bax level and reduced Bcl-2 level. However, alterations in the levels of Bax and Bcl-2 were reversed by PA treatment (Figure 4B). Thus these results suggested that PA could inhibit cell apoptosis in rats induced by LPS.

**PA represses NF-κB and MAPK signaling pathways**

NK-kB and MAPK signaling pathways were involved in LPS-induced inflammation model. The possible association between PA and these signaling pathways was analyzed. LPS treatment elevated the levels of p-p65, p-p38, and p-ERK1/2 in lung tissues of rats as compared to that from the control group. However, PA challenge significantly reduced the expression levels of p-p65, p-p38, and p-ERK1/2 in the lung tissues of LPS-induced lung injury model (Figure 5). Thus, these findings suggested that PA could suppress NF-κB and MAPK signaling pathways.

**Discussion**

Pneumonia is a major disease threatening human life. Inflammatory response and apoptosis of lung epithelial cells have important effects on the development and prognosis of pneumonia. Overexpression of cytokines is also an important cause of pneumonia; hence, regulation of body’s inflammatory response is an effective means to treat pneumonia. Based on the importance of apoptosis and inflammatory response in the progression of pneumonia, many studies have focused on investigating targets related to apoptosis and inflammatory response so as to...
Figure 4  PA inhibits cell apoptosis in rats induced by LPS. (A) TUNEL staining of lung tissues in control, LPS, LPS+10 mg/kg PA, and LPS+20 g/kg PA groups. (B) Immunoblot assay detected the protein levels of Bax and Bcl-2 in control, LPS, LPS+10 mg/kg PA, and LPS+20 g/kg PA groups. The relative expression related to the GAPDH was used as 1 for comparison. **P < 0.01 versus the control group; ##P < 0.01 versus the LPS group.

Figure 5  PA represses NF-κB and MAPK signaling pathways. Immunoblot assay detected the protein levels of p-p65, p65, p-p38, p38, p-ERK1/2, and ERK1/2 in control, LPS, LPS+10 mg/kg PA, and LPS+20 g/kg PA groups. The relative expression related to the GAPDH was used as 1 for comparison. **P < 0.01 versus the control group; ##P < 0.01 versus the LPS group.
improve the prognosis of patients and improve their quality of life.18 Importantly, PA, a lanolin triterpene compound, was identified as a potential factor for the treatment of pneumonia. However, the precise mechanism needs further investigation.

The effect of PA on the progression of pneumonia in vitro was determined by HE, ELISA, TUNEL, and Immunoblot assays. The multiple biological activities of PA have been widely revealed in a variety of studies.7,11 PA suppressed TGF-β1-induced renal fibrosis and proliferation via MAPK pathway.19 Similarly, the effect of PA on this pathway in lung tissue cells was also observed. Also, PA could activate AMP-activated protein kinase (AMPK) pathway to attenuate fibroblast activation and defects of extracellular matrix remodeling in renal fibrosis.11 It could also protect against cerebral ischemia injury by targeting PI3K/Akt pathway.19 Also, PA enhanced melatonin by inhibition of AKI-to-CKD transition through mediating growth arrest-specific 6 and nuclear factor-erythroid-2-related factor 2 (Gas6/Nrf2) axis.10 Another study showed that PA impaired glucose metabolism and stimulated mitochondrial apoptosis.4 In the present study, the alterations of Bax and Bcl-2 were reversed by PA treatment, suggesting that PA suppressed cell apoptosis in rats with lung injury. On the contrary, PA plays its roles in cancer treatment due to because of its pro-apoptotic activity. This different result could be due to different diseases. Besides, PA challenge significantly reduced the expression levels of p-p65, p-p38, and p-ERK1/2 in the lung tissues of LPS-induced lung injury model. These studies provided the evidence that PA affected multiple cellular processes via multiple mechanisms.

The effect of NF-κB/MAPK pathway on the progression of pneumonia have been clearly revealed.20–22 NF-κB pathway could serve as a potential target for the treatment of pneumonia. Several drugs have been used for treating pneumonia through targeting NF-κB pathway.21 For example, knockdown of circ-UQCCRC2 ameliorated LPS-induced injury in lung MRC-5 cells by NF-κB pathway.24 Another study has indicated that mir-143-3p affected pulmonary inflammatory factors and cell apoptosis in mice with mycoplasmal pneumonia through NF-κB pathway.13 Notably, MAPK pathway also had critical effects on progression of pneumonia.21 A study has indicated that cardiolipin-mediated PPARγ S112 phosphorylation impaired IL-10 production and inflammation resolution via MAPK pathway during bacterial pneumonia. The nucleotide-binding oligomerization domain (NOD)-like receptors could mediate inflammatory lung injury during plateau hypoxia exposure via MAPK pathway, suggesting the effects on pneumonia.23,25 Although several studies have confirmed the effects of PA on the phenotypes of inflammation and apoptosis via NF-κB and MAPK pathways, this is the first evidence showing that PA affected and interfered with lung injury through this signaling pathway. To some extent, this study explained the effect of PA on the apoptosis and inflammation in lung injury in model rats. Our study innovatively found that PA could be used as a potential therapeutic agent for lung injury, and its precise mechanism of action is still worthy of further study. These studies, together with our findings, confirmed that both NF-κB and MAPK pathways could serve as promising therapeutic targets for the treatment of pneumonia.

In summary, the present study demonstrated that PA improved the histopathological alterations in lung tissues in model rats with pneumonia. PA inhibited the expression levels of inflammatory cytokines in rat serum and suppressed cell apoptosis from rat lung tissue. Mechanically, PA inhibited inflammation and cell apoptosis via NF-κB and MAPK pathways. Therefore, PA could serve as a promising drug for the treatment of pneumonia.

Competing interests
The authors state that there are no conflicts of interest to disclose.

Ethics approval
Ethical approval was obtained from the Ethics Committee of Dingxi People’s Hospital.

Contribution of authors
Yanjun Gui and Lijuan Sun designed the study and supervised data collection. Rui Liu analyzed and interpreted the data. Jinzhu Luo reviewed the draft of manuscript and prepared it for publication. All authors have read and approved the final manuscript.

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