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Polyphyllin I alleviates lipopolysaccharide-induced inflammation reduces pyroptosis in BEAS-2B and HPAEC cells by inhibiting NF-κB signaling

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Abstract
Polyphyllin I is an active steroidal saponin isolated from *Paris polyphylla* with anti-cancer and anti-inflammatory properties. The present study investigates the role of polyphyllin I in acute lung injury. Firstly, the human bronchial epithelial cells (BEAS-2B) and human pulmonary artery endothelial cells (HPAEC) were stimulated with increasing concentrations of lipopolysaccharide at 2, 5, and 10 μg/mL. The treatment with lipopolysaccharide reduced the cell viabilities of BEAS-2B and HPAEC, downregulated superoxide dismutase (SOD) and glutathione (GSH), and up-regulated myeloperoxidase (MPO) and malondialdehyde (MDA). Moreover, the levels of TNF-α, IL-1β, and IL-6 were also up-regulated in lipopolysaccharide-treated BEAS-2B/HPAEC cells. Secondly, the lipopolysaccharide-treated cells were then incubated with different concentrations of polyphyllin I. Incubation with polyphyllin I enhanced the cell viabilities of lipopolysaccharide-treated BEAS-2B/HPAEC, up-regulated levels of SOD and GSH, and reduced MPO and MDA. Moreover, polyphyllin I reduced TNF-α, IL-1β, and IL-6 in lipopolysaccharide-treated BEAS-2B/HPAEC cells. Thirdly, the up-regulation of GSDMD-N, pro-caspase-1, and cleaved caspase-1 proteins in lipopolysaccharide-treated BEAS-2B/HPAEC cells were decreased by polyphyllin I. Polyphyllin I increased the protein expression of GSDMD-D in the lipopolysaccharide-treated BEAS-2B/HPAEC cells, and inhibited the translocation of GSDMD from cytoplasm to plasma membrane. Lastly, polyphyllin I reduced the expression of p-p65 in lipopolysaccharide-treated BEAS-2B/HPAEC cells. The over-expression of p65 counteracted with the inhibitory effects of polyphyllin I on oxidative stress and inflammation in lipopolysaccharide-treated BEAS-2B. In conclusion, polyphyllin I repressed the lipopolysaccharide-induced oxidative stress and inflammation in BEAS-2B and HPAEC, and reduced pyroptosis through inhibition of NF-κB signaling.

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Introduction

Acute lung injury and the related complication - acute respiratory distress syndrome - is associated with the development of multiple organ failure, and contributes to the death of patients with sepsis, shock, trauma, and pneumonia.[1] Although multiple approaches, such as immune checkpoint inhibitors,[2,3] have been clinically applied for septic patients, acute lung injury and the related complications still have a high mortality rate. Therefore, innovative strategies are urgently needed for the treatment of this disease.

Distinct risk factors such as virulence pathogens and bacterial infection induce the development of acute lung injury.[4] Acute pulmonary inflammation is the main characteristic of acute lung injury. Neutrophil infiltration and the release of pro-inflammatory cytokines result in lung tissue destruction, leading to deterioration of gas exchange and impairment of alveolar-capillary barrier.[5] Anti-inflammatory strategies have been considered to be beneficial for the clinical therapy of acute lung injury,[6,7] and Lipopolysaccharide - main component of endotoxin - is regarded as the most important pathogen that leads to lung inflammation and sepsis.[8]

Lipopolysaccharide activates a large number of inflammatory cells and induces the release of pro-inflammatory cytokines, thus contributing to acute lung injury through a variety of signaling pathways.[9] Toll-like receptor 4 is the main receptor of lipopolysaccharide, and lipopolysaccharide/toll-like receptor 4 complex triggers the activation of an intracellular signaling pathway, such as NF-κB, to participate in the pathogenesis of acute lung injury.[10] Therefore, the alleviation of lipopolysaccharide-triggered lung inflammation reduces the acute lung injury.[11]

Traditional herbal medicines exert antioxidant and anti-inflammatory properties in diverse metabolic disorders.[12] Moreover, polyphenols also function as plant bioactive compounds to ameliorate metabolic disorders such as non-alcoholic fatty liver disease.[13] Polyphyllin I is a steroid saponin isolated from the rhizome of *Paris polyphylla*, and is widely used as a traditional Chinese medicine to treat fevers and headaches.[14] Polyphyllin I has been reported to show anti-cancer effect in distinct tumors through promoting autophagy, cell apoptosis, and cell cycle, as well as modulation of the inflammatory response.[15] For example, polyphyllin I inhibited tumor development in hepatocellular carcinoma and reduced the expression of phosphorylation of NF-κB and the downstream targets of NF-κB (16). Polyphyllin I reduced the secretion of IL-8 to inhibit keratinocyte cell proliferation and migration,[17] and also provided protection against the left anterior descending coronary artery ligation-induced myocardial cell apoptosis, and inflammation through the inhibition of NF-κB signaling.[18] However, the role of polyphyllin I in acute lung injury-associated inflammation has not been reported.

In this study, the effects of polyphyllin I on inflammation, oxidative stress, and pyroptosis of lipopolysaccharide-treated bronchial epithelial and pulmonary artery endothelial cells were investigated.

Materials and Methods

Cell culture and treatment

BEAS-2B and HPAEC cells were purchased from ATCC (Manassas, VA, USA). The cells were grown in DMEM (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) at a 37°C incubator. To induce the injury, cells were incubated with increasing concentrations of lipopolysaccharide (Sigma-Aldrich) at 2, 5, and 10 μg/mL for 12 hours. To investigate the protective role of polyphyllin I, the cells were treated with 1, 2, 4, 6, 8, or 10 μM polyphyllin I (ChemFaces Biochemical Co., Ltd, Chengdu, China) for 24 hours. The cells were also treated with 5 μg/mL lipopolysaccharide for 12 hours, and then incubated with 0.5, 1, or 2 μM polyphyllin I for another 24 hours.

Cell transfection and viability assays

The BEAS-2B cells were transfected with pcDNA-p65 or the negative control (Invitrogen, Carlsbad, CA, USA) using Lipofectamine 2000 (Invitrogen) for 24 hours, and then incubated with 5 μg/mL lipopolysaccharide and 1 μM polyphyllin I. The BEAS-2B and HPAEC post indicated treatment were incubated with MTT solution (Beyotime, Beijing, China) for 4 hours. Dimethyl sulfoxide was added, and the absorbance at 450 nm was measured by microplate reader (Bio-Rad, Hercules, CA, USA).

ELISA

BEAS-2B and HPAEC cells were lysed in RIPA buffer (Beyotime), and the supernatants were collected and detected by BCA kit (Applygen, Beijing, China) to determine the protein concentration. The levels of SOD, MDA, GSH, and MPO were evaluated using ELISA kits (ExCell Biology, Shanghai, China). The cultured medium of BEAS-2B and HPAEC were also harvested, and the levels of TNF-α, IL-6, and IL-1β were also determined by the ELISA kits (ExCell Biology).

qRT-PCR

BEAS-2B was lysed using TRIzol kit (Invitrogen) to isolate the RNAs. The RNAs were then synthesized into cDNAs using Multiscribe™ Reverse transcription Kit (Applied Biosystems, CA, USA). The mRNA expression of caspase-1 was detected by PreTaq II kit (Takara, Dalian, Liaoning, China) with following primers: caspase-1 (Forward: 5'-GCCTGTTCCTGTGATGTGGAG-3' and Reverse: 5'-TGCCCACAGACATTCATACAGTTTC-3') and GAPDH (Forward: 5'-GGCATGGACTGTGGTCATGAG-3' and Reverse: 5'-TGCACCCCAACTGCTTAGC-3'). The mRNA expression was normalized to GAPDH through $2^{-\Delta\Delta Ct}$ method.

Immunofluorescence

BEAS-2B cells were fixed in 4% paraformaldehyde, and permeabilized with 0.2% Triton X-100. The cells were then

treated with 5% bovine serum albumin, and incubated with rabbit anti-human GSDMD antibody (1:100; Abcam, Cambridge, MA, USA). The cells were then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody, and then stained with diaminobenzidine. The cells were also counterstained with DAPI, and observed under inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Western blot

The proteins isolated from BEAS-2B and HPAEC cells were separated by 10% SDS-PAGE, and transferred onto nitrocellulose membranes. The membranes were blocked in 5% bovine serum albumin, and then probed with specific antibodies: anti-GSDMD and anti-GSDMD-N (1:1500), anti-pro caspase-1 and anti-cleaved caspase-1 (1:2000), anti-β-actin (1:2500), anti-IκBα and anti-p-IκBα (1:3000), anti-p-p65, and anti-p65 (1:3500). The membranes were probed with horseradish peroxidase-conjugated secondary antibody (1:4000), and the immunoreactivities were determined using enhanced chemiluminescence (Sigma-Aldrich), and the protein signals were detected with β-actin as a reference using Image J software. All the antibodies were acquired from Abcam.

Statistical analysis

All the data with at least triple replicates were expressed as mean ± SD, and analyzed by one-way analysis of variance under SPSS software, followed by post hoc analysis. The normality and homogeneity of the data were analyzed by Shapiro-Wilk and Levene test respectively. A p value of < 0.05 was considered as statistically significant.

Results

Polyphyllin I enhanced the cell viability of lipopolysaccharide-treated BEAS-2B/HPAEC.

To investigate the role of polyphyllin I (Figure 1A) in acute lung injury, BEAS-2B and HPAEC cells were first stimulated with lipopolysaccharide. Incubation with lipopolysaccharides reduced the cell viabilities of BEAS-2B and HPAEC in a dosage dependent way (Figure 1B). BEAS-2B and HPAEC

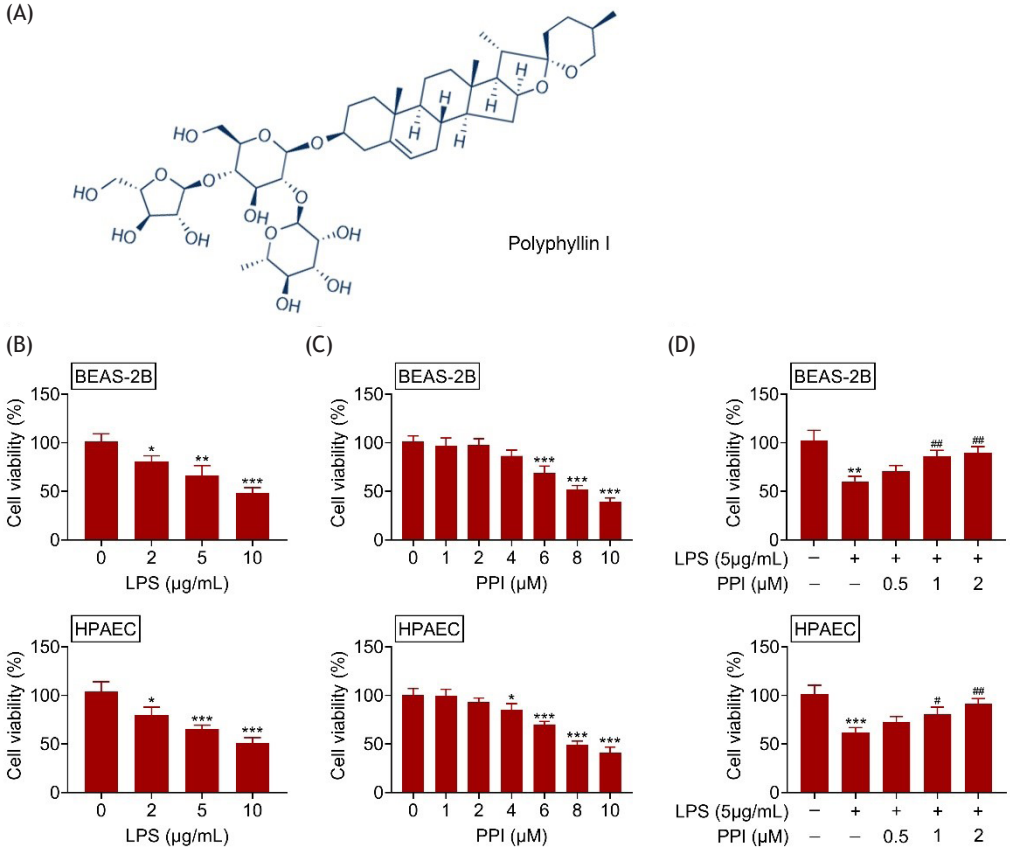

Figure 1 Polyphyllin I enhanced cell viability of lipopolysaccharide-treated BEAS-2B/HPAEC. (A) Chemical structure of polyphyllin I; (B) Incubation with lipopolysaccharide reduced cell viabilities of BEAS-2B and HPAEC in a dosage dependent way; (C) Polyphyllin I below 2 μM did not affect the cell viabilities of BEAS-2B and HPAEC, polyphyllin I more than 4 μM significantly reduced the cell viabilities of BEAS-2B and HPAEC.; (D) Polyphyllin I increased cell viabilities of lipopolysaccharide-treated BEAS-2B/HPAEC. *, **, *** vs. BEAS-2B/HPAEC without lipopolysaccharide and polyphyllin I treatment, $p < 0.05$, $p < 0.01$, $p < 0.001$. #, ## vs. BEAS-2B/HPAEC with lipopolysaccharide treatment, $p < 0.05$, $p < 0.01$.

cells were also treated with polyphyllin I to detect the cytotoxicity. The results showed that polyphyllin I below 2 μM did not affect the cell viabilities of BEAS-2B and HPAEC (Figure 1C). However, polyphyllin I more than 4 μM significantly reduced the cell viabilities of BEAS-2B and HPAEC (Figure 1C). BEAS-2B and HPAEC cells were treated with 5 μg/mL lipopolysaccharide, and then incubated with 0.5, 1, or 2 μM polyphyllin I. Polyphyllin I increased cell viabilities of lipopolysaccharide-treated BEAS-2B/HPAEC (Figure 1D), thus providing protection against lipopolysaccharide-induced cytotoxicity in acute lung injury.

Polyphyllin I reduced the oxidative stress of lipopolysaccharide-treated BEAS-2B/HPAEC cells

The levels of SOD and GSH in BEAS-2B (Figure 2A) and HPAEC (Figure 2B) were down-regulated by lipopolysaccharides. However, MDA and MPO were up-regulated in lipopolysaccharide-treated BEAS-2B/HPAEC cells (Figure 2A and B). Polyphyllin I increased the SOD and GSH levels, and decreased MDA and MPO in lipopolysaccharide-treated BEAS-2B/HPAEC cells (Figure 2A and B), demonstrating anti-oxidant property against acute lung injury.

Polyphyllin I reduced the inflammation of lipopolysaccharide-treated BEAS-2B/HPAEC cells

The levels of TNF-α, IL-1β, and IL-6 in BEAS-2B (Figure 3A) and HPAEC (Figure 3B) were up-regulated by lipopolysaccharides. However, polyphyllin I decreased the TNF-α, IL-1β, and IL-6 in lipopolysaccharide-treated BEAS-2B/HPAEC cells in a dose-dependent manner (Figure 3A and B), demonstrating the anti-inflammatory effects against acute lung injury.

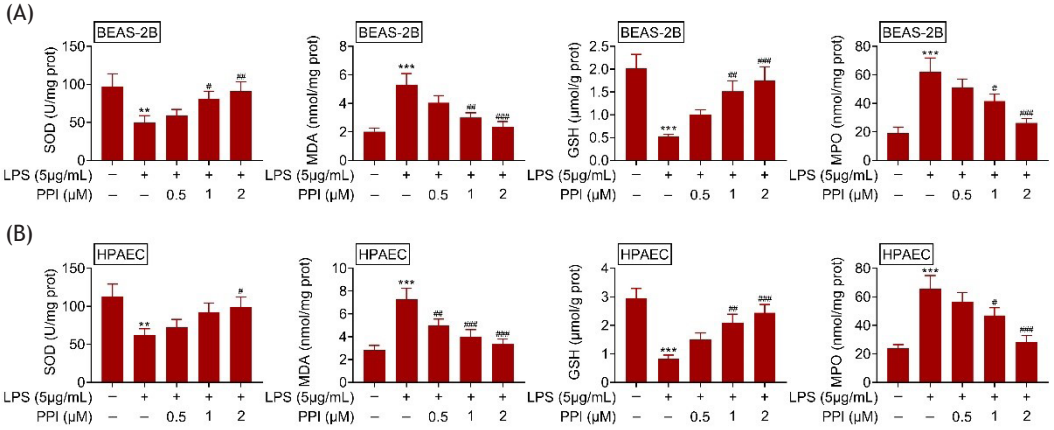

Figure 2 Polyphyllin I reduced oxidative stress of lipopolysaccharide-treated BEAS-2B/HPAEC; (A) Polyphyllin I increased SOD and GSH, while decreased MDA and MPO in lipopolysaccharide-treated BEAS-2B; (B) Polyphyllin I increased SOD and GSH, while decreased MDA and MPO in lipopolysaccharide-treated HPAEC. **, *** vs. BEAS-2B/HPAEC without lipopolysaccharide and polyphyllin I treatment, $p < 0.01$, $p < 0.001$. #, ##, ### vs. BEAS-2B/HPAEC with lipopolysaccharide treatment, $p < 0.05$, $p < 0.01$, $p < 0.001$

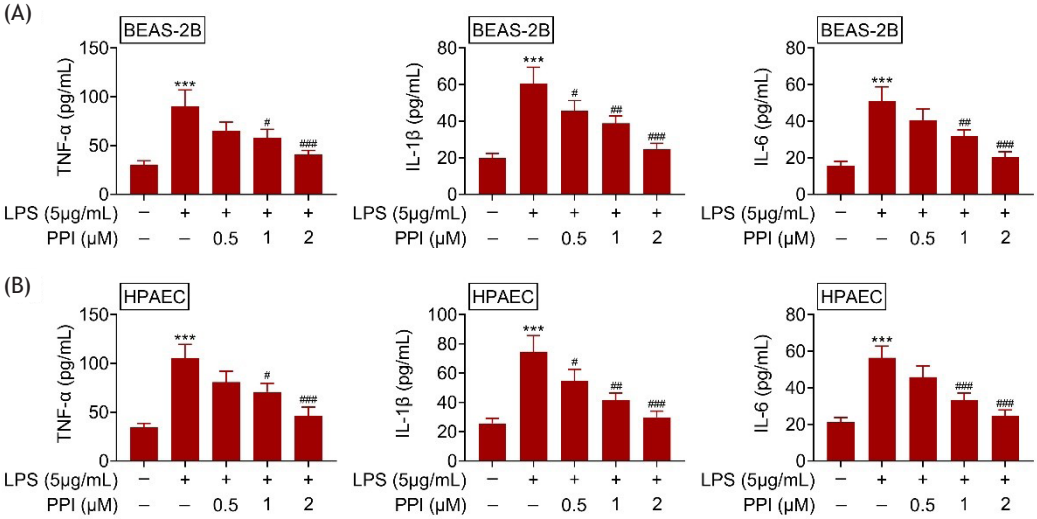

Figure 3 Polyphyllin I reduced the inflammation of lipopolysaccharide-treated BEAS-2B/HPAEC; (A) Polyphyllin I decreased TNF-α, IL-1β, and IL-6 in lipopolysaccharide-treated BEAS-2B; (B) Polyphyllin I decreased TNF-α, IL-1β, and IL-6 in lipopolysaccharide-treated HPAEC. *** vs. BEAS-2B/HPAEC without lipopolysaccharide and polyphyllin I treatment, $p < 0.001$. #, ##, ### vs. BEAS-2B/HPAEC with lipopolysaccharide treatment, $p < 0.05$, $p < 0.01$, $p < 0.001$

Polyphyllin I reduced pyroptosis of lipopolysaccharide-treated BEAS-2B.

Incubation with lipopolysaccharides reduced the protein expression of GSDMD, enhanced GSDMD-N, pro caspase-1, and cleaved caspase-1 in BEAS-2B (Figure 4A). Polyphyllin I increased GSDMD, and decreased GSDMD-N, pro caspase-1 and cleaved caspase-1 in lipopolysaccharide-treated BEAS-2B (Figure 4A). Moreover, polyphyllin I attenuated the lipopolysaccharide-induced increase in caspase-1 mRNA in BEAS-2B (Figure 4B). Lipopolysaccharides also induced the translocation of GSDMD from cytoplasm to plasma membrane (Figure 4C). However, polyphyllin I suppressed the translocation of GSDMD from cytoplasm to plasma membrane (Figure 4C), thus reducing the pyroptosis of lipopolysaccharide-treated BEAS-2B.

Polyphyllin I inhibited NF-κB in lipopolysaccharide-treated BEAS-2B.

Incubation with lipopolysaccharides enhanced protein expression of p-IκBα and p-p65 in BEAS-2B (Figure 5A). However, polyphyllin I reduced the expression of p-IκBα and p-p65 in lipopolysaccharide-treated BEAS-2B (Figure 5A) to inhibit the activation of NF-κB signaling. BEAS-2B was transfected with pcDNA-p65 for the up-regulation of p65 (Figure 5B). The over-expression of p65 attenuated the polyphyllin I-induced decrease in cell viability in lipopolysaccharide-treated BEAS-2B (Figure 5C). Moreover, the up-regulation of SOD and GSH, and down-regulation of MDA and MPO in lipopolysaccharide-treated BEAS-2B were reversed by the over-expression of p65 (Figure 5D). The over-expression of p65 counteracted with the suppressive effects of polyphyllin I on the expression of TNF-α, IL-1β, and IL-6 in BEAS-2B (Figure 5E). These results indicated that Polyphyllin I inhibited the oxidative stress and inflammation of lipopolysaccharide-treated BEAS-2B through inactivation of NF-κB signaling.

Discussion

This study for the first time revealed that polyphyllin I reduced the inflammation and oxidative stress in lipopolysaccharide-treated BEAS-2B/HPAEC cells, exhibiting

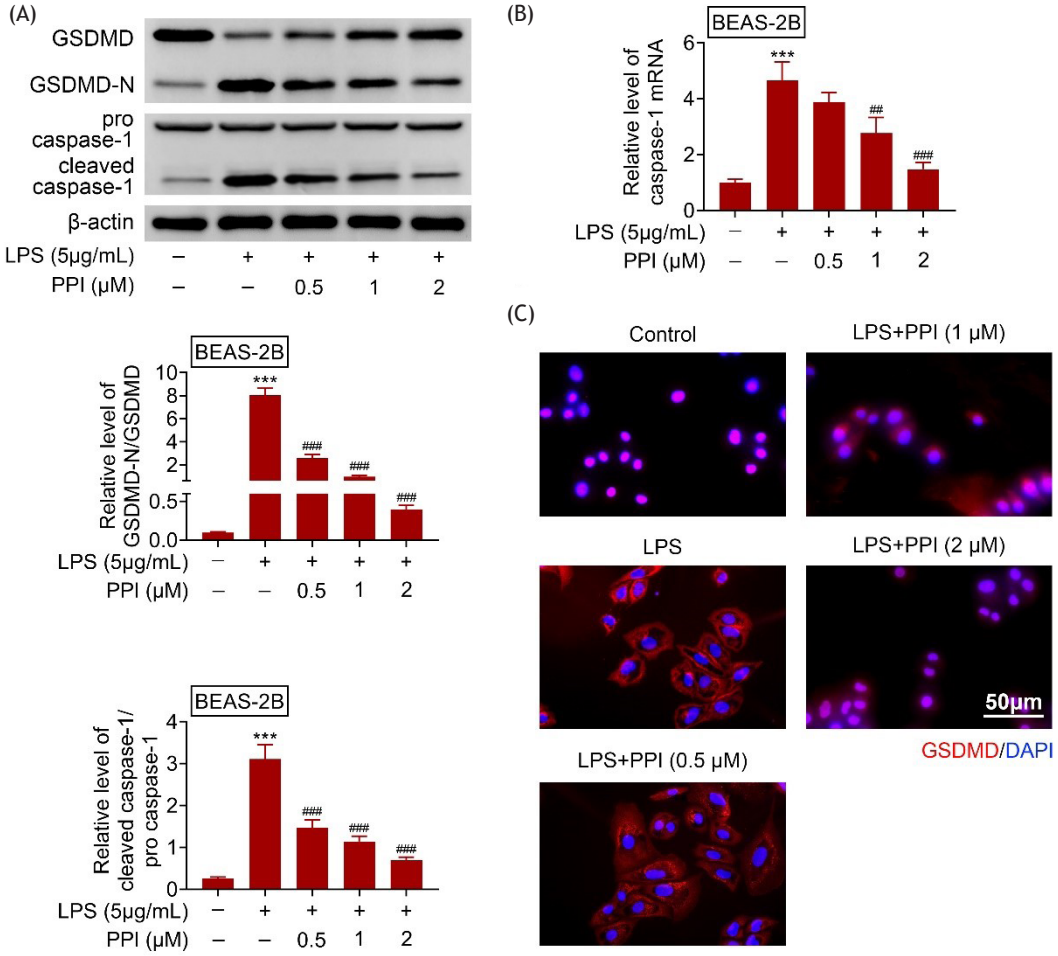

Figure 4 Polyphyllin I reduced pyroptosis of lipopolysaccharide-treated BEAS-2B; (A) Polyphyllin I increased GSDMD, while decreased GSDMD-N, pro caspase-1 and cleaved caspase-1 in lipopolysaccharide-treated BEAS-2B; (B) Polyphyllin I reduced caspase-1 mRNA in lipopolysaccharide-treated BEAS-2B; (C) Polyphyllin I suppressed the translocation of GSDMD from cytoplasm to plasma membrane. *** vs. BEAS-2B without lipopolysaccharide and polyphyllin I treatment, $p < 0.001$. #, ##, ### vs. BEAS-2B with lipopolysaccharide treatment, $p < 0.05$, $p < 0.01$, $p < 0.001$.

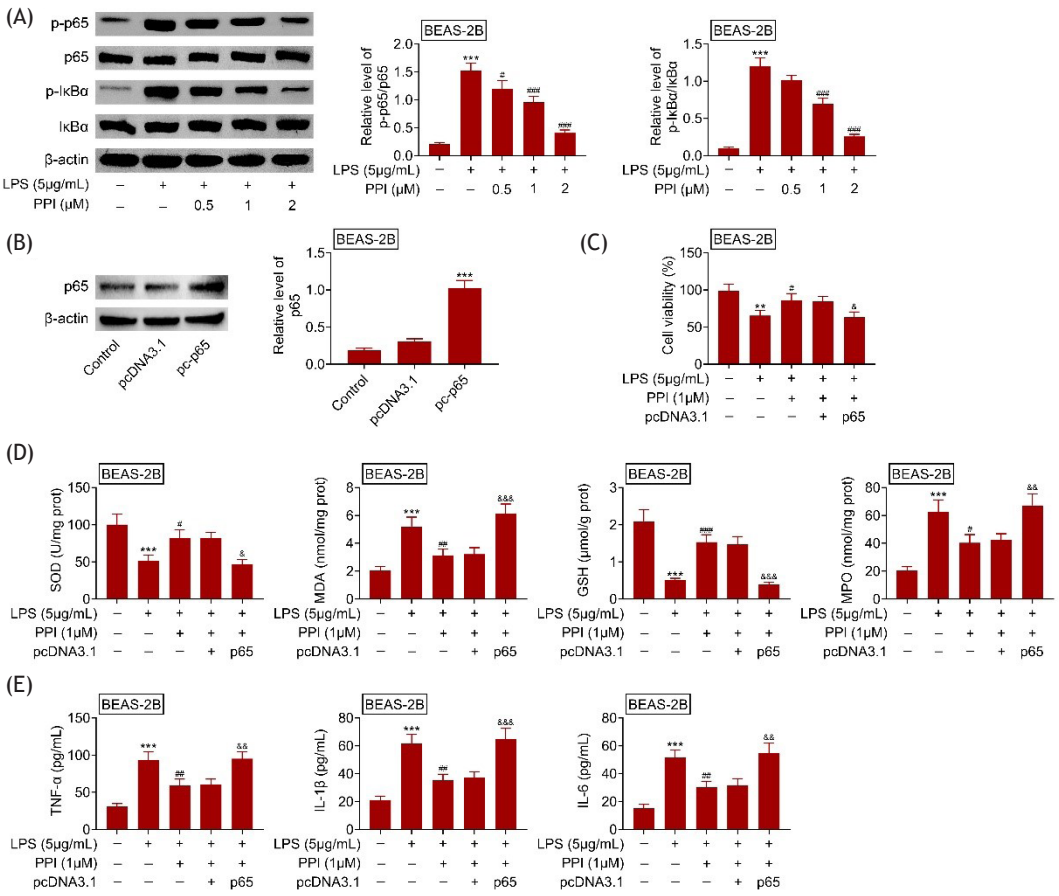

Figure 5 Polyphyllin I inhibited NF-κB in lipopolysaccharide-treated BEAS-2B; (A) Polyphyllin I reduced the expression of p-IκBα and p-p65 in lipopolysaccharide-treated BEAS-2B; (B) BEAS-2B was transfected with pcDNA-p65 for the up-regulation of p65; (C) Over-expression of p65 attenuated polyphyllin I-induced decrease of cell viability in lipopolysaccharide-treated BEAS-2B; (D) Over-expression of p65 attenuated polyphyllin I-induced increase of SOD and GSH, decrease of MDA and MPO in lipopolysaccharide-treated BEAS-2B; (E) Over-expression of p65 attenuated polyphyllin I-induced decrease of TNF-α, IL-1β, and IL-6 in lipopolysaccharide-treated BEAS-2B. **, *** vs. BEAS-2B without lipopolysaccharide and polyphyllin I treatment, $p < 0.01$, $p < 0.001$. #, ## vs. BEAS-2B with lipopolysaccharide treatment, $p < 0.05$, $p < 0.01$. &, &&, &&& vs. BEAS-2B with lipopolysaccharide and polyphyllin I treatment, $p < 0.05$, $p < 0.01$, $p < 0.001$

anti-inflammatory and anti-oxidant effects against acute lung injury.

The lipopolysaccharide-treated bronchial epithelial cell was widely used as a model of acute lung injury.[22] In this study, the lipopolysaccharide was also used to induce injury in BEAS-2B and HPAEC cells through decreasing cell viability and increasing inflammation. Polyphyllin I protected BEAS-2B and HPAEC cells against lipopolysaccharide-induced cytotoxicity by increasing the cell viability. Lipopolysaccharide has been shown to bind with toll-like receptor 4, resulting in the production of inflammatory mediators such as TNF-α, IL-1β, and IL-6, thus contributing to the lung tissue damage during the development of acute lung injury.[23] The suppression of lipopolysaccharide-induced inflammation attenuated the acute lung injury.[23] Polyphyllin I reduced the secretion of IL-1β, TNF-α, IL-6, and iNOS in lipopolysaccharide/IFN-γ activated macrophages.[21] Here, polyphyllin I also decreased the levels of TNF-α, IL-1β, and IL-6 in lipopolysaccharide-treated BEAS-2B/HPAEC cells, indicating the anti-inflammatory property against acute lung injury.

A previous study has shown that lipopolysaccharide stimulates the activation of residential macrophages and recruited neutrophils, promoting the accumulation of reactive oxygen species in lung tissues, and induces extensive tissue damage and destruction, leading to pulmonary fibrosis.[24] Therefore, the oxidative stress has implicated in the pathogenesis of acute lung injury.[25] Polyphyllin I enhanced the levels of antioxidant, SOD, and GSH, and reduced the levels of oxidant, ROS, and MDA, to attenuate myocardial ischemia/reperfusion injury in rats.[18] Here, polyphyllin I increased the SOD and GSH, and decreased MDA and MPO in lipopolysaccharide-treated BEAS-2B/HPAEC cells to suppress the oxidative stress in acute lung injury.

Pyroptosis is induced by inflammatory caspases such as caspase-1, 4, 5, and 11, and it promotes the secretion of inflammatory cytokines and leads to programmed cell death.[26] Pyroptosis and the associated inflammatory caspases have been reported to be involved in the development of acute lung injury.[26] Lipopolysaccharide activates caspase-1, cleaves GSDMD, and induces the translocation of GSDMD from cytoplasm to plasma membrane to promote

the release of cytokines, such as IL-1β and IL-18, thus leading to cell death and pyroptosis of alveolar macrophages and endothelial cells.[26,27] Inhibition of lipopolysaccharide-induced pyroptosis ameliorated the acute lung injury.[28] Our results showed that Polyphyllin I enhanced the level of GSDMD, reduced the levels of caspase-1 and GSDMD-N in lipopolysaccharide-induced BEAS-2B. Moreover, polyphyllin I also suppressed the translocation of GSDMD from cytoplasm to plasma membrane, thus inhibiting pyroptosis to protect against acute lung injury.

NF-κB signaling which is essential for the secretion of pro-inflammatory factors, is activated in lipopolysaccharide-induced acute lung injury.[29] the interference of NF-κB has been used for the treatment of acute lung injury.[30] Polyphyllin I has been shown to reduce the phosphorylation activity of NF-κB.[18-20] The over-expression of p65 eliminated the inhibitory effect of polyphyllin I on collagen-induced arthritis.[21] Here, polyphyllin I reduced the expression of p-IκBα and p-p65 in lipopolysaccharide-treated BEAS-2B. Moreover, the over-expression of p65 attenuated polyphyllin I-induced decrease in cell viability, and increase in oxidative stress and inflammation in lipopolysaccharide-treated BEAS-2B, revealing that polyphyllin I inhibited the activation of NF-κB signaling protection against acute lung injury.

In conclusion, polyphyllin I reduced pyroptosis of lipopolysaccharide-treated BEAS-2B, and exerted anti-oxidant and anti-inflammatory effects against lipopolysaccharide-treated BEAS-2B/HPAEC through the inactivation of NF-κB signaling. Therefore, polyphyllin I might be a promising strategy for the prevention of acute lung injury and the associated complications. However, the effect of polyphyllin I on acute lung injury *in* vivo animal model should be investigated in further research.

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Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

Competing Interests

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

Ethics Approval

Not applicable.

Contribution of Authors

Fangli Mao designed the study and supervised the data collection, Aiping Wu analyzed the data and interpreted the data, Fangli Mao and Aiping Wu prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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