Deoxyelephantopin alleviates lipopolysaccharide-induced septic lung injury through inhibiting NF-κB/STAT3 axis

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Abstract
Sepsis induces multiple organ dysfunction syndromes, such as acute kidney, liver, or lung injury. Septic lung injury is associated with excessive apoptosis and inflammatory responses in hepatocytes. Deoxyelephantopin is a sesquiterpene lactone found in \textit{Elephantopus scaber} L, and has immunomodulatory, antibacterial, anti-inflammatory, and antifungal properties. The role of deoxyelephantopin in sepsis-associated lung injury was investigated. First, human bronchial epithelial cells (BEAS-2B) and human pulmonary artery endothelial cells (HPAEC) were treated with lipopolysaccharide to induce cytotoxicity. Treatment with lipopolysaccharide reduced cell viability of BEAS-2B and HPAEC, and promoted cell apoptosis through down-regulation of poly (ADP-ribose) polymerase (PARP) and B-cell lymphoma 2 (Bcl-2), and up-regulation of cleaved PARP and B-cell lymphoma-associated X protein (Bax). Second, lipopolysaccharide-treated BEAS-2B and HPAEC were incubated with increasing concentrations of deoxyelephantopin, that is, 1, 5, or 10 μM. Deoxyelephantopin enhanced cell viability and reduced cell apoptosis of lipopolysaccharide-treated BEAS-2B and HPAEC. Third, deoxyelephantopin attenuated lipopolysaccharide-induced decrease of superoxide dismutase and glutathione, and increase of malondialdehyde and myeloperoxidase in BEAS-2B and HPAEC. Moreover, deoxyelephantopin also weakened lipopolysaccharide-induced increase of tumor necrosis factor-α, interleukin (IL)-1β, and IL-6. Finally, deoxyelephantopin decreased protein expression of p-p65 and p-signal transducer and activator of transcription 3 (STAT3) in lipopolysaccharide-treated BEAS-2B and HPAEC. In conclusion, deoxyelephantopin exhibited anti-oxidative and anti-inflammatory effects against lipopolysaccharide-treated BEAS-2B and HPAEC through inactivation of nuclear factor kappa B/STAT3 signaling.

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Introduction

Sepsis is generally induced by pathogen infections, and causes multiple organ dysfunction, such as damage to the liver, lung, brain, or kidney. The lung is a vulnerable target organ of sepsis, and approximately 50% of the patients with sepsis develop into acute lung injury and severe acute respiratory distress syndrome. Considering that acute lung injury contributes to the death of patients with sepsis, shock, trauma, and pneumonia, strategies to ameliorate sepsis-associated acute lung injury are urgently required.

Acute lung injury is initiated by disruption in alveolar-capillary membrane barrier, which induces the infiltration of inflammatory cells and neutrophil accumulation to promote the secretion of pro-inflammatory cytokines, thus contributing to the lung tissue damage. Strategies to counterbalance the secretion of pro-inflammatory cytokines have demonstrated benefits in septic children. Lipopolysaccharide, an endotoxin that stimulates the activation of innate immunity, is implicated in the pathogenesis of inflammatory diseases. Lipopolysaccharide induces damage to alveolar capillary barrier and secretion of pro-inflammatory cytokines, which is regarded as a potential model of acute lung injury. Therefore, inhibition of lipopolysaccharide-induced inflammatory responses is a promising strategy for treating septic lung injury.

Traditional herbs, such as Urtica dioica (stinging nettle), has exerted beneficial anti-inflammatory effects in diabetic rats. Deoxyelephantopin is a sesquiterpene lactone from Elephantopus scaber L. and has pharmacological properties, including antitumor, hepatoprotective, antiprototozoal, and anti-inflammatory effects. Deoxyelephantopin reduced aerobic glycolysis and inhibited the secretion of inflammatory cytokines in macrophages, thus providing a novel mechanism for anti-inflammatory therapy. Moreover, deoxyelephantopin exerted neuroprotective effect in memory impairment by suppressing lipopolysaccharide-induced secretion of hippocampal chemokines. However, the role of deoxyelephantopin in lipopolysaccharide-induced septic lung injury has not been reported yet.

In this study, the effects of deoxyelephantopin on lipopolysaccharide-induced oxidative stress and inflammation were investigated in human bronchial epithelial cells (BEAS-2B) and human pulmonary artery endothelial cells (HPAEC). The result might provide a potential antiseptic agent for the treatment of septic lung injury.

Materials and methods

Cell culture and treatment

BEAS-2B cells were obtained from the cell bank of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA). HPAEC cells were obtained from Cell Applications (San Diego, CA, USA), and cultured in endothelial growth medium (Thermo Fisher Scientific). BEAS-2B and HPAEC were treated with 0.2-, 1-, 2.5-, 5-, 10-, and 20-μM deoxyelephantopin (Chengdu Ruifensi Co. Ltd, Chengdu, China) for 24 h. BEAS-2B and HPAEC were also treated with 5-μg/mL lipopolysaccharide (Sigma-Aldrich, St. Louis, MO, USA) and 1-, 5-, and 10-μM deoxyelephantopin for 24 h.

Assays for detection of cell viability and apoptosis

BEAS-2B and HPAEC were seeded into 96-well plates, and incubated with different concentrations of deoxyelephantopin with or without lipopolysaccharide for 24 h. Cells were treated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (Beyotime, Beijing, China) for another 4 h. Dimethyl sulfoxide (DMSO) was added, and absorbance at 450 nm was measured by microplate reader (Thermo Fisher Scientific). In order to detect cell apoptosis, BEAS-2B and HPAEC were harvested through trypsin digestion, and resuspended in binding buffer of BD Cytofix™ plus DNA reagent kit (BD Biosciences, San Jose, CA, USA). Cells were then stained with fluorescein isothiocyanate-conjugated annexin V and propidium oxide. Apoptotic cells were analyzed under fluorescence activated cell sorting (FACS) flow cytometry (Life Technologies, Darmstadt, Germany).

Enzyme-linked-immunosorbent serologic assay (ELISA)

BEAS-2B and HPAEC were lysed in radioimmunoprecipitation assay (RIPA) buffer (Beyotime), and the levels of malondialdehyde (MDA), myeloperoxidase (MPO), superoxide dismutase (SOD), and glutathione (GSH) were determined by ELISA kits (Thermo Fisher Scientific). The levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 in supernatants of cultured medium were also detected by ELISA kits (Thermo Fisher Scientific).

Western blot

Proteins isolated from BEAS-2B and HPAEC were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The membranes were blocked in 5% bovine serum albumin (BSA) and probed with specific antibodies, including anti-β-actin, anti-B-cell lymphoma-associated X protein (Bax), and anti-poly(ADP-ribose) polymerase (PARP) (1:2000), anti-cleaved PARP and anti-B-cell lymphoma 2 (Bcl-2; 1:2500), anti-inducible nitric oxide synthase (iNOS) and anti-cyclooxygenase-2 (COX-2) (1:3000), anti-p-κBα and anti-IκBα (1:3500), anti-p-p65 and anti-p65 (1:4000), and anti-p-signal transducer and activator of transcription 3 (STAT3) and anti-STAT3 (1:4500). Then the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:5000). Immunoreactivities were visualized using enhanced chemiluminescence (Sigma-Aldrich), quantified by using...
Deoxyelephantopin in sepsis

ImageQuant LAS500, and normalized to β-actin. All the antibodies were acquired from Abcam, Waltham, MA, USA.

Statistical analysis

All data with at least triple replicates were expressed as mean ± standard error of mean (SEM). The normality and homogeneity of data were detected by Shapiro-Wilk and Levene tests. The data were analyzed by Student’s t-test or one-way analysis of variance with Tukey’s post hoc test using the SPSS software. P < 0.05 was considered as statistically significant.

Results

Deoxyelephantopin enhanced cell viability of lipopolysaccharide-treated BEAS-2B/HPAEC

In order to investigate the cytotoxicity of deoxyelephantopin in pulmonary cells, BEAS-2B and HPAEC were treated with different concentrations of deoxyelephantopin (Figure 1A). Deoxyelephantopin at a concentration lower than 20 μM did not affect the cell viability of BEAS-2B and HPAEC (Figure 1B). The cell viability of BEAS-2B and HPAEC was reduced by 20-μM deoxyelephantopin (Figure 1B). Lipopolysaccharide-treated BEAS-2B and HPAEC were incubated with 1-, 5-, and 10-μM deoxyelephantopin to investigate the role of deoxyelephantopin in septic lung injury. Treatment with deoxyelephantopin increased the cell viability of lipopolysaccharide-treated BEAS-2B and HPAEC in a dose-dependent manner (Figure 1C), suggesting the proliferative effect of deoxyelephantopin on lipopolysaccharide-treated BEAS-2B and HPAEC.

Deoxyelephantopin reduced lipopolysaccharide-induced cell apoptosis of BEAS-2B/HPAEC

Lipopolysaccharide induced down-regulation of Bcl-2 and PARP, up-regulation of p-PARP and Bax in BEAS-2B and HPAEC (Figure 2A) to promote cell apoptosis (Figures 2B and C). However, deoxyelephantopin increased the protein expression of Bcl-2 and PARP, and decreased p-PARP and Bax in lipopolysaccharide-treated BEAS-2B and HPAEC (Figure 2A) to inhibit cell apoptosis (Figures 2B and C), indicating the antiapoptotic effect of deoxyelephantopin in septic lung injury.

Deoxyelephantopin reduced lipopolysaccharide-induced oxidative stress in BEAS-2B/HPAEC

Levels of SOD and GSH in BEAS-2B and HPAEC were down-regulated, while that of MDA and MPO were up-regulated by lipopolysaccharide (Figure 3). However, deoxyelephantopin increased SOD and GSH, and decreased MDA and MPO in lipopolysaccharide-treated BEAS-2B and HPAEC (Figure 3), revealing the antiapoptotic effect of deoxyelephantopin in septic lung injury.

Deoxyelephantopin reduced lipopolysaccharide-induced inflammation in BEAS-2B/HPAEC

Lipopolysaccharide stimulated secretion of TNF-α, IL-1β, and IL-6 in BEAS-2B and HPAEC (Figure 4). However, deoxyelephantopin attenuated lipopolysaccharide-induced increase of TNF-α, IL-1β, and IL-6 in BEAS-2B and HPAEC (Figure 4), demonstrating the anti-inflammatory effect of deoxyelephantopin in septic lung injury.

Figure 1 Deoxyelephantopin enhanced cell viability of lipopolysaccharide-treated BEAS-2B and HPAEC. (A) Chemical structure of deoxyelephantopin. (B) Deoxyelephantopin at a concentration lower than 20 μM did not affect the cell viability of BEAS-2B and HPAEC, but 20-μM deoxyelephantopin reduced 20% of cell viability of BEAS-2B and HPAEC. (C) Treatment of deoxyelephantopin increased cell viability of lipopolysaccharide-treated BEAS-2B and HPAEC in a dosage-dependent manner. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control. #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. LPS. DEX: deoxyelephantopin; LPS: lipopolysaccharide.
Figure 2 Deoxyelephantopin reduced lipopolysaccharide-induced cell apoptosis of BEAS-2B and HPAEC. (A) Treatment with deoxyelephantopin increased protein expressions of Bcl-2 and PARP, decreased p-PARP and Bax of lipopolysaccharide-treated BEAS-2B and HPAEC in a dosage-dependent manner. (B) Treatment with deoxyelephantopin decreased lipopolysaccharide-induced cell apoptosis of BEAS-2B and HPAEC in a dosage-dependent manner. (C) Relative cell apoptosis of lipopolysaccharide-treated BEAS-2B and HPAEC with deoxyelephantopin incubation. ***P < 0.001 vs. control. #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. LPS.

**Deoxyelephantopin suppressed lipopolysaccharide-induced activation of iNOS/COX-2 signaling in BEAS-2B/HPAEC**

Lipopolysaccharide stimulated up-regulation of iNOS and COX-2 proteins in BEAS-2B and HPAEC (Figure 5), while the up-regulation of iNOS and COX-2 proteins in BEAS-2B and HPAEC driven by lipopolysaccharide was reduced by deoxyelephantopin (Figure 5), indicating that deoxyelephantopin suppressed lipopolysaccharide-induced inflammation in BEAS-2B and HPAEC through inactivation of iNOS/COX-2 signaling.
Deoxyelephantopin in sepsis

Deoxyelephantopin reduced lipopolysaccharide-induced oxidative stress in BEAS-2B and HPAEC. Treatment with deoxyelephantopin increased levels of SOD and GSH, and decreased MDA and MPO of lipopolysaccharide-treated BEAS-2B and HPAEC in a dosage-dependent manner. ***P < 0.001 vs. control. #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. LPS. DEX: deoxyelephantopin; LPS: lipopolysaccharide.

Deoxyelephantopin reduced lipopolysaccharide-induced inflammation in BEAS-2B/HPAEC. Treatment with deoxyelephantopin decreased levels of TNF-α, IL-1β, and IL-6 in lipopolysaccharide-treated BEAS-2B and HPAEC in a dosage-dependent manner. ***P < 0.001 vs. control. #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. LPS. DEX: deoxyelephantopin; LPS: lipopolysaccharide.

Deoxyelephantopin reduced lipopolysaccharide-induced activation of NF-κB/STAT3 in BEAS-2B/HPAEC

Lipopolysaccharide induced up-regulation of p-IκBα, p-p65, and p-STAT3 in BEAS-2B and HPAEC, which was reversed by deoxyelephantopin (Figure 6).

Discussion

Elephantopus scaber L. has been extensively screened for wound-healing ability, and antiplatelet, analgesic, antioxidant, hepatoprotective, antimicrobial, antidiabetic, anti-asthmatic, anticancer, and anti-inflammatory activities in a variety of diseases, including eczema, leukemia, diabetes, diarrhea, hepatitis, and arthritis. The protective effect of sesquiterpene lactone in septic shock has been widely reported. This study found that deoxyelephantopin, sesquiterpene lactone from Elephantopus scaber L., attenuated lipopolysaccharide-induced apoptosis, oxidative stress, and inflammation in BEAS-2B and HPAEC through inactivation of NF-κB/STAT3 pathway.

Lipopolysaccharide induces cytotoxicity on pulmonary cells through excessive release of inflammatory cytokines and accumulation of reactive oxygen species, which has
Deoxyelephantopin suppressed lipopolysaccharide-induced activation of INOS/COX-2 signaling in BEAS-2B and HPAEC. Treatment with deoxyelephantopin decreased INOS and COX-2 proteins of lipopolysaccharide-induced BEAS-2B/HPAEC in a dosage-dependent manner. ***P < 0.001 vs. control. #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. LPS. DEX: deoxyelephantopin; LPS: lipopolysaccharide.

Deoxyelephantopin inhibited lipopolysaccharide-induced activation of NF-κB/STAT3 signaling in BEAS-2B/HPAEC. Treatment with deoxyelephantopin decreased expression of p-IκBα, p-p65, and p-STAT3 proteins of lipopolysaccharide-treated BEAS-2B/HPAEC in a dosage-dependent manner. ***P < 0.001 vs. control. #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. LPS. DEX: deoxyelephantopin; LPS: lipopolysaccharide.

been widely used to treat BEAS-2B and HPAEC for establishing cell model of septic lung injury.16,17 In this study, lipopolysaccharide also decreased cell viability and promoted the cell apoptosis of BEAS-2B and HPAEC. Moreover, levels of SOD and GSH in BEAS-2B and HPAEC were reduced, while that of MDA, MPO, TNF-α, IL-1β, and IL-6 were enhanced by lipopolysaccharide, suggesting that lipopolysaccharide induced oxidative stress and inflammatory injury in BEAS-2B and HPAEC.

Endotoxin induces apoptosis and destruction of alveolar epithelial cells through up-regulation of Bax and down-regulation of Bcl-2 to activate apoptotic pathways in alveolar epithelial cells, thus leading to increased epithelial permeability and edema fluid inflow into alveolar cavity during the development of septic lung injury.18 In this study, deoxyelephantopin increased expressions of Bcl-2 and PARP but decreased expressions of cleaved PARP and Bax to suppress lipopolysaccharide-induced apoptosis of BEAS-2B and HPAEC, and exerted antiapoptotic effect against septic lung injury.

The lung serves as an innate immune organ. The infections of allergens, toxicants, or pathogens in the lung
can induce inflammatory responses in innate lymphoid cells, alveolar macrophages, and pulmonary epithelial cells, thus leading to the excessive secretion of pro-inflammatory cytokines to promote pulmonary injury in sepsis. Moreover, the infiltrated neutrophils and activated lung macrophages can secret pro-inflammatory cytokines to initiate oxidative stress in lung epithelial and endothelial cells, thus resulting in tissue damage and organ dysfunction. Therefore, oxidative stress and inflammation are implicated in the pathogenesis of septic lung injury. Suppression of oxidative stress and inflammation contributed to amelioration of lipopolysaccharide-induced lung injury. Previous study has demonstrated shown that deoxyelephantopin reduced lipopolysaccharide-induced release of IL-1β in activated macrophages and protected mice from lipopolysaccharide-induced septic shock through attenuation of aerobic glycolysis. Results in this study also established that deoxyelephantopin attenuated lipopolysaccharide-induced increase of MDA, MPO, TNF-α, IL-1β, IL-6, iNOS, and COX-2, and decrease of SOD and GSH in BEAS-2B and HPAEC, suggesting the anti-oxidant and anti-inflammatory effects against in septic lung injury.

NF-κB signaling is essential for the secretion of pro-inflammatory cytokines, and STAT3 is also associated with lung innate immunity through regulation of neutrophil production and recruitment. Inhibition of NF-κB/STAT3 signaling demonstrated protective effect in lipopolysaccharide-induced lung injury. Deoxyelephantopin reduced lipopolysaccharide-induced neuroinflammatory response through inactivation of NF-κB signaling. Deoxyelephantopin also reduced expression of pSTAT3-Tyr705 to inhibit cell growth of cervical carcinoma. Here, deoxyelephantopin reduced the expression of p-p65 and p-STAT3 in lipopolysaccharide-treated BEAS-2B and HPAEC, suggesting that inhibition of NF-κB/STAT3 signaling was involved in the protective effect of deoxyelephantopin in septic lung injury.

In summary, deoxyelephantopin exerted antiapoptotic, anti-oxidant, and anti-inflammatory effects on lipopolysaccharide-treated BEAS-2B and HPAEC through inactivation of NF-κB/STAT3 signaling. However, the role of deoxyelephantopin in animal model of septic lung injury must be investigated in the future research. Moreover, preclinical and clinical trials must also be investigated to determine the entire spectrum of antisepsis activity of deoxyelephantopin to endorse the further utility in treating septic lung injury.

Competing interests
The authors stated that there were no conflicts of interest to disclose.

Ethics approval
This article does not contain any research conducted by any author with human participants or animals.

Author Contributions
Shu Wang and Yuefeng Chen designed and carried the study. Both authors supervised data collection, and analyzed and interpreted the data. Both prepared and reviewed the draft of manuscript for publication. Both authors had read and approved the final manuscript.

References


