

Leonurine: A compound with the potential to prevent acute lung injury

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Abstract. Sepsis is an intense immune response to infection that contributes to the pathophysiological process of acute lung injury (ALI). Inflammation and oxidative stress serve an important role in the development of ALI. Leonurine (LEO) is a natural phenolic alkaloid extracted from *Leonurus cardiaca*, which possesses anti-inflammatory and antioxidative properties. Therefore, the aim of the present study was to explore the effect of LEO on sepsis-induced ALI and to investigate its underlying mechanism. MTT and Cell Counting Kit-8 assays were performed to measure cell viability. The levels of reactive oxygen species, lactate dehydrogenase and malondialdehyde, as well as the activity of superoxidase dismutase, were quantified using commercial assay kits. The expression levels of specific inflammatory cytokines were measured by using ELISA. In addition, western blotting was employed to assess the expression levels of cytokines, including TNF- α , IL-6, nuclear factor erythroid 2-related factor 2 (Nrf2) and heme oxygenase-1. The findings demonstrated that LEO increased the viability of lipopolysaccharide (LPS)-stimulated BEAS-2B human lung epithelial cells in a dose-dependent manner. Additionally, LEO suppressed LPS-induced oxidative stress and inflammatory cytokine release in BEAS-2B cells. Treatment with Nrf2 inhibitor reversed the effects of LEO treatment on LPS-induced oxidative stress and inflammatory response in BEAS-2B cells. Taken together, the data of the present study indicated that LEO attenuated LPS-induced ALI through the inhibition of oxidative stress and inflammation regulated by the Nrf2 signaling pathway. Therefore, LEO may be a novel and effective agent for the prevention of sepsis-induced ALI.

Introduction

Sepsis is an intense immune response to infection that contributes to 19.7% of all global deaths according to an analysis published in 2020 (1). Currently, sepsis remains a leading cause of mortality in intensive care units, as it may lead to multiple organ system dysfunction and even multiorgan failure (2-4). It was previously reported that the lung may be one of the organs in the human body most vulnerable to sepsis, and sepsis contributes to ~40% of the cases of acute lung injury (ALI) (2). Despite significant advances in the overall treatment strategies, no specific treatment has yet been developed for sepsis-induced ALI (5). Therefore, the development of novel effective agents is crucial for optimizing the management, prevention and treatment of patients with sepsis-mediated ALI.

Sepsis-induced lung damage promotes the hypersecretion of inflammatory cytokines, leading to pathological damage of the alveolar epithelium and vascular endothelial cells, and to the development of ALI (6). In addition, oxidative stress also serves a key role in the development of ALI (7,8). Therefore, inhibition of inflammation and oxidative stress may be a promising preventive and/or therapeutic strategy for sepsis-induced ALI. Leonurine (LEO) is a natural phenolic alkaloid extracted from *Leonurus cardiaca* that has been shown to possess anti-inflammatory and antioxidant properties in numerous studies (9-12). It has been reported that LEO alleviates lipopolysaccharide (LPS)-induced myocarditis through anti-inflammatory and antioxidant mechanisms via the inactivation of the NF- κ B signaling pathway (13). In addition, LEO reduced acute kidney injury and protected renal function against LPS-induced inflammation (14). However, the effects of LEO on ALI have not yet been determined, to the best of our knowledge. Xu *et al* (15) reported that LEO exerted no cytotoxic effects on human lung epithelial cells. Moreover, LEO was reported to attenuate the aging process in mice through activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway (16). A recent study demonstrated that the activation of the Nrf2 signaling pathway suppressed inflammation and oxidative stress in ALI (17). Therefore, it was hypothesized that LEO may have a role in sepsis-induced ALI.

The aim of the present study was to explore the effects of LEO on oxidative stress and on the inflammatory response in LPS-induced BEAS-2B human lung epithelial cells. Notably,

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LEO led to the activation of the Nrf2 signaling pathway. Therefore, LEO may be an effective agent for the prevention of ALI, and the findings of the present study may provide a theoretical basis for the application of LEO in the prevention of ALI.

Materials and methods

Cell culture and treatment. Human lung epithelial cells (BEAS-2B; American Type Culture Collection) were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO₂ at 37°C. BEAS-2B cells were pretreated with various doses of LEO (0-120 µg/ml) for 6 h and subsequently stimulated with LPS (1 µg/ml) for 48 h. To block the Nrf2 signaling pathway, ML385 (Abmole Bioscience, Inc.), an Nrf2 inhibitor, was used to treat the cells before the LEO treatment.

MTT assay. The viability of treated BEAS-2B cells was measured using an MTT assay, as described previously (18). Briefly, treated cells (3×10³ cells/well) were cultured in a 96-well plate for 48 h and incubated for 4 h at 37°C with 20 µl MTT solution (Beijing Solarbio Science & Technology Co., Ltd.). Subsequently, the formazan particles were dissolved in 200 µl DMSO and the absorbance was measured at 580 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Cell Counting Kit-8 (CCK-8) assay. BEAS-2B cells were transferred into 96-well plates at a density of 3×10³ cells/well. Following 24 h of incubation at 37°C, 10 µl CCK-8 reagent (Beyotime Institute of Biotechnology) was added to each well and cultured at 37°C for 1 h. The absorbance of each sample was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Detection of reactive oxygen species (ROS), lactate dehydrogenase (LDH), malondialdehyde (MDA) and superoxide dismutase (SOD) levels. The levels of ROS (cat. no. CS-E64644; Shanghai C-reagent Biotechnology Co., Ltd.), LDH (cat. no. A020-2-2; Nanjing Jiancheng Bioengineering Institute), MDA (cat. no. A003-2-2; Nanjing Jiancheng Bioengineering Institute) and the activity of SOD (cat. no. A001-1-2; Nanjing Jiancheng Bioengineering Institute) in cell culture medium were quantified using commercial assay kits, following the manufacturers' instructions. The absorbance of each sample was measured using a microplate reader (Bio-Rad Laboratories, Inc.).

Western blot analysis. BEAS-2B cells were lysed using Lysis Buffer (Promega Corporation) to extract the total protein. The concentration of the protein samples were quantified using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). The cell lysates (20 µg/lane) were separated by 10% SDS-PAGE and subsequently transferred to PVDF membranes. The membranes were blocked with 5% non-fat dried milk diluted in PBS at room temperature for 1 h and further incubated with primary antibodies overnight at 4°C. The primary antibodies used were as follows: Anti-TNF-α (cat. no. ab183218; 1:1,000), anti-IL-6 (cat. no. ab233706; 1:1,000),

anti-Nrf2 (cat. no. ab62352; 1:1,000), anti-heme oxygenase (HO)-1 (cat. no. ab52947; 1:2,000) and anti-β-actin (cat. no. ab8227; 1:3,000) (all from Abcam). The HRP-conjugated secondary antibody used was a goat anti-rabbit IgG (cat. no. ab6721; 1:10,000, Abcam). Finally, the protein bands were visualized using ECL Western Blotting Substrate (cat. no. PE0010; Beijing Solarbio Science & Technology Co., Ltd.) and quantified using ImageJ software, version 1.x (National Institutes of Health).

ELISA. The expression levels of the inflammatory cytokines, including TNF-α (cat. no. 589201-480; AmyJet Scientific, Inc.) and IL-6 (cat. no. ab178013; Abcam) were detected in the culture supernatant of BEAS-2B cells using the corresponding ELISA kits, according to the manufacturer's instructions.

Statistical analysis. The data are presented as the mean ± standard deviation of three independent experiments and were analyzed using GraphPad Prism version 5.01 (GraphPad Software, Inc.). Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

LEO increases the viability of LPS-stimulated BEAS-2B cells. The chemical structure of LEO is presented in Fig. 1A. To investigate the potential cytotoxic effects of LEO, cell viability was measured using an MTT assay. As shown in Fig. 1B, no significant changes were noted in the viability of BEAS-2B cells treated with various doses of LEO (0, 1, 10, 20, 40, 80 and 120 µg/ml), suggesting that this compound was not cytotoxic to BEAS-2B cells. In addition, the results of the CCK-8 assay indicated that cell viability was reduced following LPS stimulation, whereas it was increased by LEO treatment in a dose-dependent manner in LPS-treated cells (Fig. 1C). Owing to the higher viability, 40 µg/ml LEO was selected for subsequent experiments. These results suggested that LEO exerted potential therapeutic effects on epithelial cells with LPS-induced injury.

LEO suppresses LPS-induced oxidative stress and inflammation. To investigate the mechanism underlying the function of LEO, the induction of oxidative stress and inflammation were analyzed in LPS-stimulated BEAS-2B cells. LPS stimulation increased ROS, LDH and MDA levels, and decreased SOD activity in BEAS-2B cells (Fig. 2); subsequent LEO treatment abolished these effects. In addition, the results of the western blot analysis and ELISA demonstrated that LPS induced the upregulation of TNF-α and IL-6 in BEAS-2B cells, whereas LEO treatment suppressed the expression levels of TNF-α and IL-6 (Fig. 3). These results indicated that LEO treatment suppressed the LPS-induced oxidative stress and inflammatory response in BEAS-2B cells.

Effect of LEO on the Nrf2 signaling pathway. To investigate the mechanisms underlying the effects of LEO, proteins involved in the Nrf2 signaling pathway were examined by western blot analysis. LPS stimulation led to a reduction in

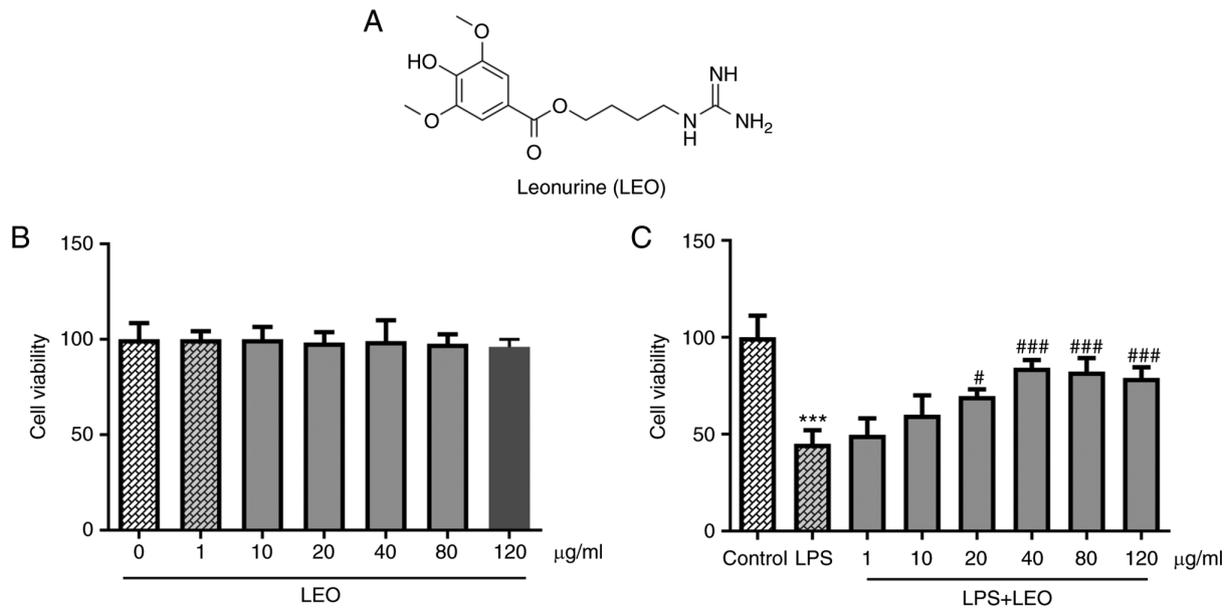


Figure 1. LEO increases the viability of LPS-stimulated BEAS-2B cells. (A) Chemical structure of LEO. BEAS-2B cell viability was quantified using (B) MTT and (C) CCK-8 assays following pretreatment with various concentrations of LEO and subsequent LPS stimulation. Data are presented as the mean \pm standard deviation of three independent experiments. *** P <0.001 vs. control; # P <0.05, *** P <0.001 vs. LPS. CCK-8, Cell Counting Kit-8; LEO, leonurine; LPS, lipopolysaccharide.

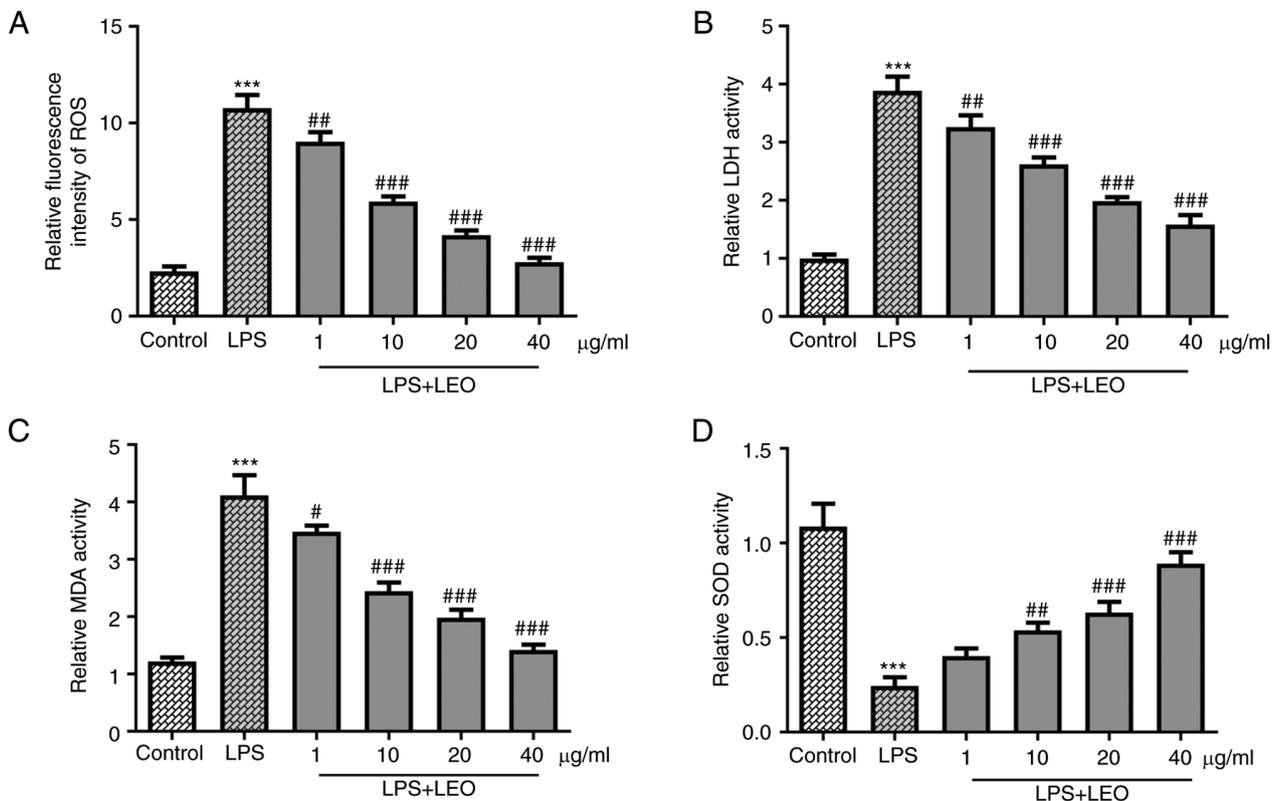


Figure 2. LEO suppresses LPS-induced oxidative stress in BEAS-2B cells. (A) ROS, (B) LDH and (C) MDA levels, as well as (D) SOD activity in BEAS-2B cells were measured using specific assay kits. Data are presented as the mean \pm standard deviation of three independent experiments. *** P <0.001 vs. control; # P <0.05, ** P <0.01, *** P <0.001 vs. LPS. LDH, lactate dehydrogenase; LEO, leonurine; LPS, lipopolysaccharide; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase.

the expression levels of Nrf2 and HO-1 in BEAS-2B cells, whereas LEO treatment exerted the opposite effects on the expression levels of these markers (Fig. 4). These results

suggested that LEO treatment partially reversed the suppressive effects of LPS stimulation on the activation of the Nrf2 signaling pathway.

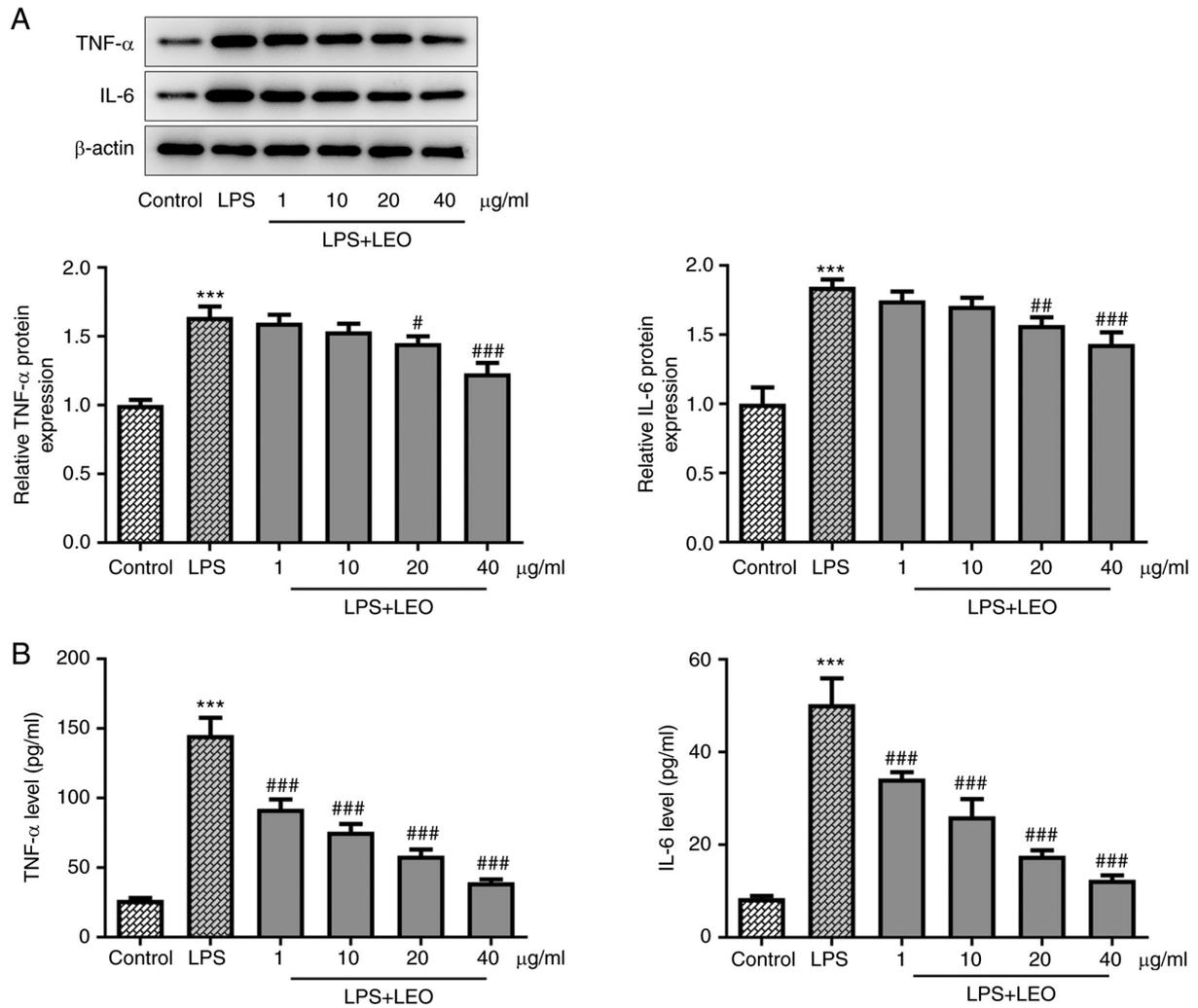


Figure 3. LEO attenuates the LPS-induced inflammatory response in BEAS-2B cells. (A) Western blot analysis was used to determine the protein expression levels of TNF- α and IL-6. (B) ELISA was used to quantify the concentrations of TNF- α and IL-6. Data are presented as the mean \pm standard deviation of three independent experiments. ***P<0.001 vs. control; *P<0.05, **P<0.01, ***P<0.001 vs. LPS. LEO, leonurine; LPS, lipopolysaccharide.

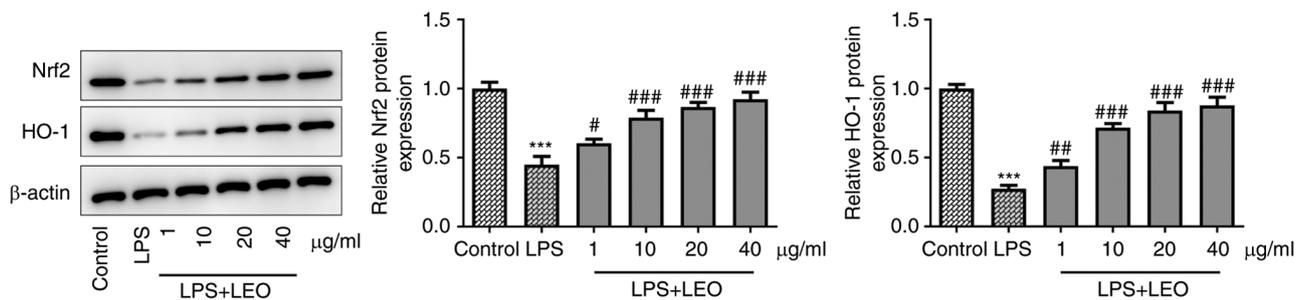


Figure 4. Effect of LEO on the Nrf2 signaling pathway. The protein expression levels of Nrf2 and HO-1 were determined by western blot analysis. Data are presented as the mean \pm standard deviation of three independent experiments. ***P<0.001 vs. control; *P<0.05, **P<0.01, ***P<0.001 vs. LPS. HO-1, heme oxygenase-1; LEO, leonurine; LPS, lipopolysaccharide; Nrf2, nuclear factor erythroid 2-related factor 2.

LEO enhances viability and inhibits LPS-induced oxidative stress and inflammatory response in BEAS-2B cells via activation of the Nrf2 signaling pathway. To confirm whether the Nrf2 signaling pathway mediates the effects of LEO on LPS-induced epithelial cell injury, ML385 (an Nrf2 inhibitor) was used. BEAS-2B cells were pretreated with 10 μ M ML385 to induce downregulation of Nrf2 expression. The viability

of BEAS-2B cells in the LPS + LEO + ML385 group was lower compared with that observed in the LPS + LEO group (Fig. 5A). In addition, the inhibitory effects of LEO treatment on ROS, LDH and MDA levels were reversed by ML385 treatment (Fig. 5B-D, respectively). Furthermore, treatment with ML385 significantly reduced SOD activity in BEAS-2B cells co-treated with LPS and LEO (Fig. 5E). Furthermore,

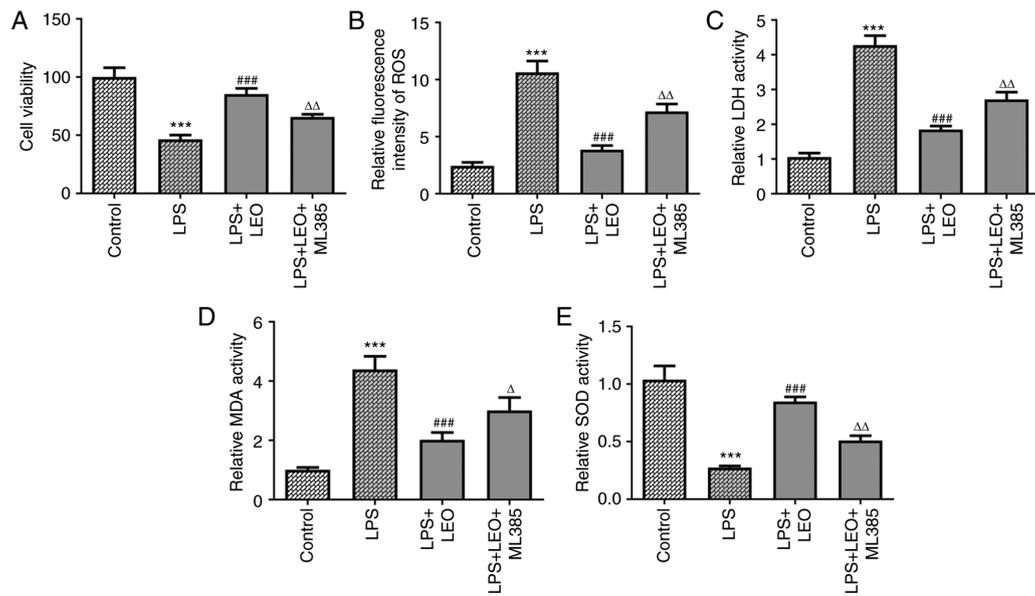


Figure 5. ML385 Nrf2 inhibitor treatment ameliorates the effects of LEO on LPS-induced oxidative stress in BEAS-2B cells. (A) BEAS-2B cell viability was quantified using a Cell Counting Kit-8 assay. (B) ROS, (C) LDH and (D) MDA levels, as well as (E) SOD activity in BEAS-2B cells were measured using specific assay kits. Data are presented as the mean \pm standard deviation of three independent experiments. *** P <0.001 vs. control; ### P <0.001 vs. LPS; Δ P <0.05, $\Delta\Delta$ P <0.01 vs. LPS + LEO. LDH, lactate dehydrogenase; LEO, leonurine; LPS, lipopolysaccharide; MDA, malondialdehyde; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; SOD, superoxide dismutase.

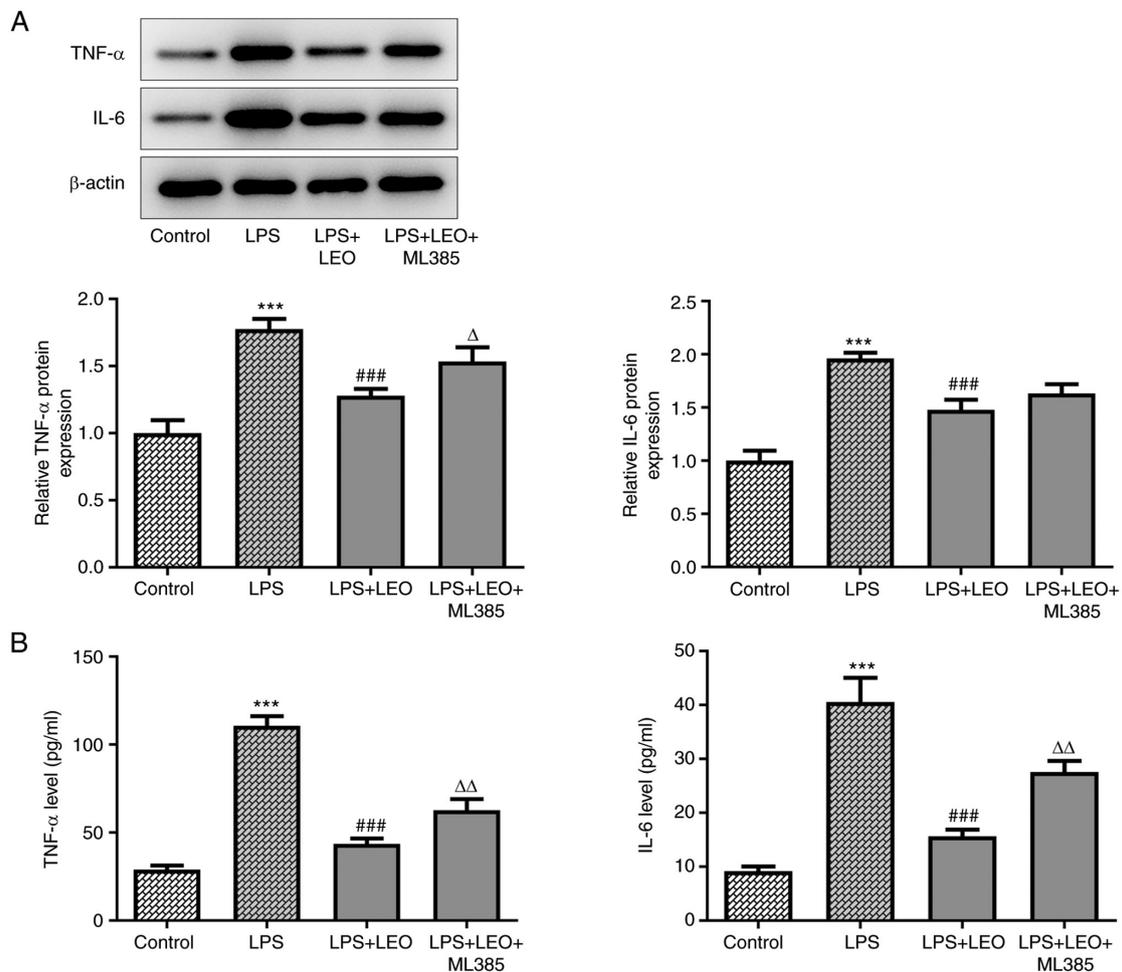


Figure 6. ML385 Nrf2 inhibitor treatment reversed the effects of LEO on the LPS-induced inflammatory response in BEAS-2B cells. (A) Western blot analysis was used to determine the expression levels of TNF- α and IL-6. (B) ELISA was used to quantify the concentrations of TNF- α and IL-6. Data are presented as the mean \pm SD from three independent experiments. *** P <0.001 vs. control; ### P <0.001 vs. LPS; Δ P <0.05, $\Delta\Delta$ P <0.01 vs. LPS + LEO. LEO, leonurine; LPS, lipopolysaccharide; Nrf2, nuclear factor erythroid 2-related factor 2.

the expression level of TNF- α in BEAS-2B cells was significantly enhanced by ML385 treatment, whereas that of IL-6 was only slightly increased compared with those in the LPS + LEO group (Fig. 6A and B). Therefore, it was concluded that Nrf2 inhibition abrogated the effects of LEO treatment on the secretion of oxidative markers and inflammatory cytokines.

Discussion

Sepsis is a common life-threatening organ dysfunction that is triggered by a dysregulated host immune response and remains one of the leading causes of mortality worldwide (1). Previous studies have demonstrated that sepsis-induced ALI is associated with high morbidity and mortality rates (19,20). Patients with ALI often present with hypoxemia, and oxygen therapy can be administered to improve hypoxemia, such as nasal catheterization. Oxygen inhalation by mask and mechanical ventilation should be provided at an early stage when necessary (2). Despite an improved understanding of the pathophysiology of this disease, current therapeutic strategies remain only partially effective (5). Therefore, the present study aimed to develop a novel promising agent for the prevention of ALI.

The findings of the present study suggested that LEO did not exert significant cytotoxic effects on BEAS-2B cell viability. Subsequently, it was found that LEO treatment enhanced the viability of LPS-stimulated BEAS-2B cells. BEAS-2B cells are pulmonary epithelial cells, which are the major cells that form the mechanical barrier in the lungs, and have a key protective function against environmental aggressors (21). The results of the present study suggested that LEO could protect against LPS-induced epithelial cell injury and increase cell viability. LEO was first discovered to have a protective effect on the survival of lung epithelial cells. Subsequently, to investigate the underlying mechanism related to the effects of LEO, the induction of oxidative stress and inflammation were analyzed by additional experiments. In a previous study, inflammation and oxidative stress were reported to serve critical roles in the development of ALI. Moreover, the inhibition of the induction of inflammation and oxidative stress was shown to reverse the effects of ALI induced by LPS (22). Increased ROS production and inflammatory cytokine hypersecretion have been implicated in the development of lung injury (23). It was reported that the levels of MDA and the expression levels of TNF- α were significantly increased, whereas SOD levels were significantly decreased in the lungs of ALI mice (24). Furthermore, IL-6 was found to be elevated in the alveolar lavage fluid of patients with ALI (25). In the present study, LEO treatment reduced the levels of ROS, LDH and MDA, and increased SOD activity and downregulated the expression levels of TNF- α and IL-6. These findings demonstrated that LEO inhibited the LPS-induced oxidative stress and inflammation in BEAS-2B cells. Subsequently, the associated signaling pathway was assessed to explore the molecular mechanism underlying the effects of LEO on oxidative stress and inflammation.

Nrf2 is a transcription factor that improves cytoprotective responses (26). The Nrf2 signaling pathway has been reported to play an important role in inhibiting multiple inflammatory and oxidative stress-associated diseases, including ALI (27). More importantly, it has been reported that LEO can induce

activation of the Nrf2 signaling pathway *in vivo*, which is consistent with the findings of the current *in vitro* study (16). In the present study, LEO treatment led to the activation of the Nrf2 signaling pathway in a dose-dependent manner. Of note, the Nrf2 inhibitor (ML385) abolished the effects of LEO treatment on cell viability, inflammatory cytokine secretion and oxidative stress, suggesting that LEO enhanced the viability and inhibited the LPS-induced oxidative stress and inflammatory response in BEAS-2B cells via activation of the Nrf2 signaling pathway. These findings suggested that LEO specifically regulates the Nrf2 pathway to alleviate ALI, further elucidating the pharmacological basis of its action. However, the present study was limited to BEAS-2B cells, and further research on additional cell lines, as well as *in vivo* studies, are required. Our future plan is to explore the effects of LEO on immune cells (such as macrophages) *in vitro* and establish ALI mouse models to study the role of LEO *in vivo*.

In summary, the present study demonstrated that LEO suppressed oxidative stress and inflammation in LPS-induced BEAS-2B cells via the Nrf2 signaling pathway, suggesting that LEO has the potential to prevent sepsis-induced ALI.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LW wrote manuscript and participated in the experimental design. GZ performed the experiments and analyzed the results. LW and GZ confirm the authenticity of all the raw data. Both authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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