Tumor necrosis factor α-induced protein 8-like 2 contributes to penehyclidine hydrochloride pretreatment against lipopolysaccharide-induced acute lung injury in a mouse model

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Abstract. The aim of the present study was to investigate the effect of penehyclidine hydrochloride (PHC) pretreatment on mice with lipopolysaccharide (LPS)-induced acute lung injury (ALI) and its possible underlying mechanisms. Mice were randomly separated into six groups: i) Sham group; ii) LPS group; iii) LPS + PHC group; iv) tumor necrosis factor a-induced protein 8-like protein 2 (TIPE2) group; v) LPS + TIPE2 group; and vi) LPS + TIPE2 + PHC group. The ALI model was induced using LPS through intratracheal injection. The mice received adenovirus gene to induce the overexpression of TIPE2. After mice were sacrificed, lung injury indices were assessed, and arterial blood, bronchoalveolar lavage fluid and lung tissues were collected for subsequent assays. Expression levels of related proteins were detected by using western blotting. It was found that compared with the sham group, the mice treated with LPS showed increased lung injury and dysfunctions of gas exchange. However, these trends were significantly ameliorated in the LPS + PHC group. Evaluation of protein expression in lung tissues showed that the increased expression of nuclear NF-κB p65 and p-c-Jun N-terminal kinase (JNK) induced by LPS were suppressed in the LPS + PHC group and the expression of TIPE2 was increased. The mice that received adenovirus gene to induce TIPE2 overexpression could also showed protective effects compared with the mice in the LPS group. However, the expression of TIPE2 decreased rather than increased in LPS group. In the mice pretreated with PHC, the expression of TIPE2 increased in mice with LPS-induced ALI. To conclude, PHC pretreatment could inhibit the occurrence of inflammation and apoptosis in LPS-induced ALI. This process may be related to the activation of TIPE2 and the inhibition of NF-κB and JNK signaling pathway in the lungs of mice.

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Introduction

Acute lung injury (ALI) refers to acute hypoxic respiratory dysfunction induced by various direct or interconnecting injury factors, such as pneumonia, pyemia, reperfusion injury and shock (1). In severe cases, it can develop into acute respiratory distress syndrome (ARDS), which is associated with severe symptoms such as disruption of the alveolar epithelial barrier, proteinaceous pulmonary edema, acute inflammation and abnormalities in gas exchange (2). The pathophysiology of ALI is a complex process that includes the recruitment of neutrophils, increased numbers of inflammatory cytokines and the apoptosis of epithelial cells (3,4). In previous years, the morbidity and mortality rates of ALI/ARDS are still high despite improved knowledge on these conditions and treatment methods (5,6). In clinical settings, the occurrence of ALI is often unpredictable, therefore, exploring potential effective pretreatments to reduce the risk of ALI is necessary. Sepsis induced by lipopolysaccharide (LPS) release is one of the most common injury factors in the induction of ALI/ARDS (7,8). The inflammatory responses activated by LPS via binding to Toll-like receptors (TLRs) are the underlying mechanism of lung injury in patients with ALI (9-11). Therefore, finding more effective methods of targeting specific biomarkers are needed to reduce inflammatory responses and alleviate cell apoptosis.

Tumor necrosis factor (TNF) α-induced protein 8-like protein 2 (TNFAIP8/TIPE2) are a family of proteins that play essential roles as negative regulators of inflammation and immune homeostasis (12). As a member of the TNFAIP8 family, TIPE2 can also play an anti-inflammatory role by negatively regulating T cell receptor (TCR) and TLR signaling (13). Previous reports have stated that TIPE2 can

negatively regulate TCR and TLR signaling via binding to caspase-8 and inhibiting the activation of protein-1 (AP-1) and NF-κB (14,15). Furthermore, the depletion of TIPE2 can cause significantly increased levels of inflammatory mediators and severe inflammatory disease (16,17). Furthermore, binding of LPS to TLR4 has been found to lead to the excessive release of pro-inflammatory cytokines through promoting the activation of NF-κB and c-Jun N-terminal kinase (JNK) (18), the release of these inflammatory mediators can eventually cause serve lung injury in ALI (19). In our previous experiments, we observed the expression of TIPE2 in lung epithelial cells and macrophages. Immunohistochemical staining of TIPE2 protein expression in the lung showed that the expression of TIPE2 protein was significantly increased in the alveolar epithelium after adeno-associated virus-TIPE2 administration and adeno-associated virus-mediated TIPE2 overexpression remarkably inhibited inflammation and cell apoptosis induced by LPS (20). Thus, this evidence suggests that TIPE2 may have therapeutic implications in LPS-induced ALI.

Penehyclidine hydrochloride (PHC) is a common drug used in surgery that plays a significant role in inhibiting the secretion of salivary and airway glands through intravenous infusion prior to anesthesia (21). Several previous studies have confirmed that PHC exerts protective effects in the treatment of ALI. Treatment with PHC has been found to relieve LPS-induced pulmonary impairment and block LPS-mediated lung apoptosis (22), furthermore, it has been observed that ARDS rats administrated with PHC showed reduced inflammatory factor production and lipid peroxidation (23). Additionally, PHC also been shown to exert a notable effect on inhibiting the TLR4 signaling pathway in traumatic lung injury (24). In a previous study, we found that pulmonary microvascular endothelial apoptosis and lung damage induced by LPS could be inhibited through blocking the JNK and NF-κB signaling pathway (25,26), whereas TIPE2 and PHC could both be used to block p38 MAPK and NF-κB pathways in microglia (15,24,27). Therefore, we speculated that there is an association and underlying mechanism that exists between TIPE2 and PHC treatment in the prevention and treatment of ALI. Thus, the present study investigated the potential effect of TIPE2 in the treatment of ALI with PHC by measuring the degree of lung injury and related protein expression.

Materials and methods

Animal preparation. All animals received humane care in compliance with the Principles of Laboratory Animal Care (28). The experimental protocol was approved by the Animal Care and Use Committee of the Medical Ethics Committee of Renmin Hospital of Wuhan University (Wuhan, China).

Adult male BALB/c mice (age, 6-8 weeks old; weight, ~20±5 g) were housed in conditions with a controlled temperature (20-24˚C) and humidity (40-60%), with a 12-h light/dark cycle and access to food and water *ad libitum*. A total of 60 mice were randomly divided into the following six groups: i) Sham group; ii) LPS group (group LPS), the mice received an intraperitoneal injection with LPS to establish the ALI model at a dose of 5 mg/kg (9); iii) LPS + PHC group (group P+LPS), the mice received intraperitoneal injection with PHC at a dose of 2 mg/kg (24) before the ALI model was established; iv) TIPE2 group (group TIPE2), the mice received adenovirus gene to induce the overexpression of pulmonary TIPE2; v) LPS + TIPE2 group (group T+LPS); and vi) LPS + TIPE2 + PHC group (group T+LPS+P). LPS was purchased from Sigma-Aldrich (Merck KGaA) and PHC was from Chengdu List Pharmaceutical Co., Ltd.

Surgical procedure and animal treatment. All surgical procedures were performed under anesthesia using an intraperitoneal injection of pentobarbital sodium at a dose of 50 mg/kg (Sigma-Aldrich; Merck KGaA). Following anesthesia, the animals were injected with LPS (intratracheal). The sham group mice were intratracheally administered with sterile phosphate-buffered saline (PBS) as controls. For the last three groups, BALB/c mice were given recombinant adeno-associated virus of pAAV-FLAG-mTIPE2-IRES-ZsGREEN (Han Heng Biotechnology Co., Ltd.) via intratracheal administration to induce TIPE2 overexpression before LPS administration. The adeno-associated virus that expressed no transgene was used as a negative control. Sham group mice were treated with the control adeno-associated virus.

Histological examination. A total of 24 h after LPS treatment, the mice were euthanized with excessive pentobarbital sodium (100 mg/kg; intraperitoneal injection). Animal death was confirmed by observation of apnea and asystole. After mice were sacrificed, the lung tissues were collected to observe lung histopathology. The whole lung lobes were fixed with 4% (v/v) paraformaldehyde at 4˚C for 48 h. Then, the tissues were embedded in paraffin and sectioned into 4-mm thick slices. Hematoxylin and eosin (H&E)-stained slides were prepared with H&E solution (Sigma-Aldrich; Merck KGaA) for 3 min at room temperature. Subsequently, slides were observed under a light microscope (magnification, x200; BX51; Olympus Corporation) to observe morphological changes in the lung tissues. The degree of lung injury was scored from 0 to 4 according to the levels of inflammation in alveolar and peribronchial lesions (23).

Lung water content measurement. The right lung tissues were immediately removed after the chests of the mice were opened. The tissues were then blotted to remove any excess blood and weighed for the wet weight. Following dehydration at 65˚C in a drying oven for 48 h, tissues were reweighed to obtain the dry weight. The wet/dry ratio was calculated as an index for pulmonary edema.

Lung neutrophils, protein concentration and proinflammatory cytokines in bronchoalveolar lavage fluid (BALF). The lungs of mice were washed with ice-cold PBS to obtain the BALF. The BALF samples were centrifuged at 1,000 x g for 15 min at 4˚C for subsequent assays. A hemocytometer was used to count the total inflammatory cells in the BALF. To analyze differential cell counting, Wright's Stain solution (Sigma-Aldrich; Merck KGaA) was used to stain the cells for 2 min at room temperature, and the number of polymorphonuclear neutrophils (PMNs) was counted according to the staining results. Myeloperoxidase (MPO) activity was determined using the MPO Activity Assay kit (cat. no. A044-1-1; Nanjing

Jiancheng Bioengineering Institute) and the specific value was expressed as unit/mg tissue. The levels of proinflammatory cytokines, such as TNF-α, IL-6 and IL-1β, in the BALF were determined using commercially available enzyme-linked immunosorbent assay kits (IL-1β, cat. no. RLB00; IL-6, cat. no. R6000B; TNF-α, cat. no. RTA00; R&D Systems, Inc.).

Arterial blood gas analysis. At the end of the surgical procedure, appropriate amounts of blood samples were extracted from the arteries of mice in each group and these samples were injected into an ABL700 Radiometer (Radiometer America) to determine pH values, partial pressure of oxygen (PaO_2), PaO_2/fraction of inspired oxygen (FiO_2) and partial pressure of carbon dioxide ($PaCO_2$). These indicators were used to analyze the gas exchange function in the lungs of mice from different groups.

Western blotting. Lung tissues stored at -80˚C were crushed and mixed with RIPA lysate buffer (Sigma-Aldrich; Merck KGaA) to extract proteins. Equal amounts of proteins (30 μg) were separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then separated proteins were transferred to polyvinylidene difluoride membranes. Following which, membranes were blocked with 5% skimmed milk for 2 h at room temperature, and then incubated at 4˚C overnight with the following primary antibodies: Anti-cleaved caspase 9 (1:1,000; cat. no. 9507; Cell Signaling Technology, Inc.), anti-cleaved caspase 3 (1:1,000; cat. no. 9661; Cell Signaling Technology, Inc.), anti-Bax (1:2,000; cat. no. ab32503; Abcam), anti-Bcl-2 (1:1,000; cat. no. ab196495; Abcam), anti-phosphorylated (p)-JNK (1:1,000; cat. no. 4668; Cell Signaling Technology, Inc.), anti-JNK (1:1,000; cat. no. 9252; Cell Signaling Technology, Inc.), anti-TIPE2 (1:1,000; cat. no. DF3326; Affinity Biosciences), anti-β-actin (1:1,000; cat. no. 8457; Cell Signaling Technology, Inc.), anti-NF-κB p65 (1:1,000; cat. no. ab16502; Abcam) and anti-Lamin A (1:1,000; cat. no. ab26300; Abcam). The next day the membranes were incubated with a horseradish peroxidase-labeled secondary antibody (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 2 h at room temperature. Subsequently, the protein bands were visualized using an ECL detection kit (Thermo Fisher Scientific, Inc.) in the dark, according to the manufacturer's instructions. The expression levels of relevant proteins were analyzed by Image Lab software (version 5.2.1; Bio-Rad Laboratories, Inc.).

Statistical analysis. All experiments were repeated in triplicate. The data are presented as the mean ± standard deviation. Data analysis was performed using SPSS 17.0 software (SPSS, Inc.) with one-way ANOVA followed by the Tukey-Kramer post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PHC pretreatment and TIPE2 overexpression ameliorates lung injury in mice with ALI. The pathological changes in lung tissues, degree of pulmonary inflammation and the survival rate of mice in different groups were compared to evaluate the degree of lung injury. Pathological changes of lung tissues were examined by H&E staining. In the sham and TIPE2 groups, the structure of pulmonary alveoli was complete and the alveolar wall was smooth without obvious exudation of pulmonary stroma. Whereas, in the LPS group, there was an increased number of infiltrated neutrophils, the septum was wider and thicker, and alveolar wall edema was observed; however, alveolar wall edema and neutrophil infiltration were notably alleviated in the P+LPS and T+LPS groups (Fig. 1A). Overall, lung injury score in these groups were significantly decreased compared with the LPS group ($P<0.05$; Fig. 1B). In the T+LPS+P group, treatment with PHC and TIPE2 overexpression at the same time led to an increased protective effect compared with PHC or TIPE2 alone (P+LPS group vs. T+LPS+P group, $P<0.05$; T+LPS group vs. T+LPS+P group, $P<0.05$; Fig. 1A and B).

The ratio of PMNs to total cells in the BALF, BALF protein content, MPO activity and lung wet/dry ratio were analyzed at 24 h after LPS injection. Results of data analysis indicated that all these values were significantly increased in the LPS group compared with the sham group ($P<0.05$), but this trend was partially inhibited in the T+LPS, P+LPS and T+LPS+P groups ($P<0.05$; Fig. 1C-F).

PHC pretreatment and TIPE2 overexpression improves gas exchange in the lungs of LPS-induced mice. Gas analysis of arterial blood showed a significant decrease in PaO_2 and an increase in $PaCO_2$ in the LPS group ($P<0.05$; Fig. 2B and C). Similarly, the pH and PaO_2/FiO_2 ratio in arterial blood were decreased in the LPS group compared with the sham group ($P<0.05$; Fig. 2A and D). In the T+LPS, P+LPS and T+LPS+P groups, the dysfunction of gas exchange induced by LPS stimulation was partially reversed ($P<0.05$; Fig. 2A-D).

PHC pretreatment and TIPE2 overexpression attenuates the expression of pro-inflammatory cytokines. As one of the most important cytokines in the induction of ALI, TNF-α has a synergistic effect with IL-6 and IL-1β in ALI development. According to the present study, it was found that the expression levels of these cytokines were significantly increased in the LPS group. Furthermore, compared with the mice in the LPS group, the expression levels of these cytokines were significantly downregulated in the T+LPS, P+LPS and T+LPS+P groups ($P<0.05$; Fig. 3A-C).

Effects of PHC pretreatment and TIPE2 overexpression on the expression of different proteins in the lung tissues of mice. Mice were humanely sacrificed and the expression levels of related proteins were determined via western blotting (Fig. 4A). As shown in Fig. 4, the expression levels of pro-apoptotic proteins, such as Bax, cleaved caspase 3 and cleaved caspase 9, in mice lung tissue increased in the LPS group compared with the sham group ($P<0.05$; Fig. 4B, E and F), whereas the expression levels of anti-apoptotic Bcl-2 decreased compared with the sham group ($P<0.05$; Fig. 4C). In addition, the ratio of Bax/Bcl-2 increased in the LPS group compared with the sham group ($P<0.05$; Fig. 4I). These expression changes were partially reversed in the T+LPS and P+LPS groups, and most significantly reversed in the T+LPS+P group ($P<0.05$; Fig. 4B, C, E, F and I). In addition, the expression levels of NF-κB p65 and phosphorylated JNK increased ($P<0.05$;

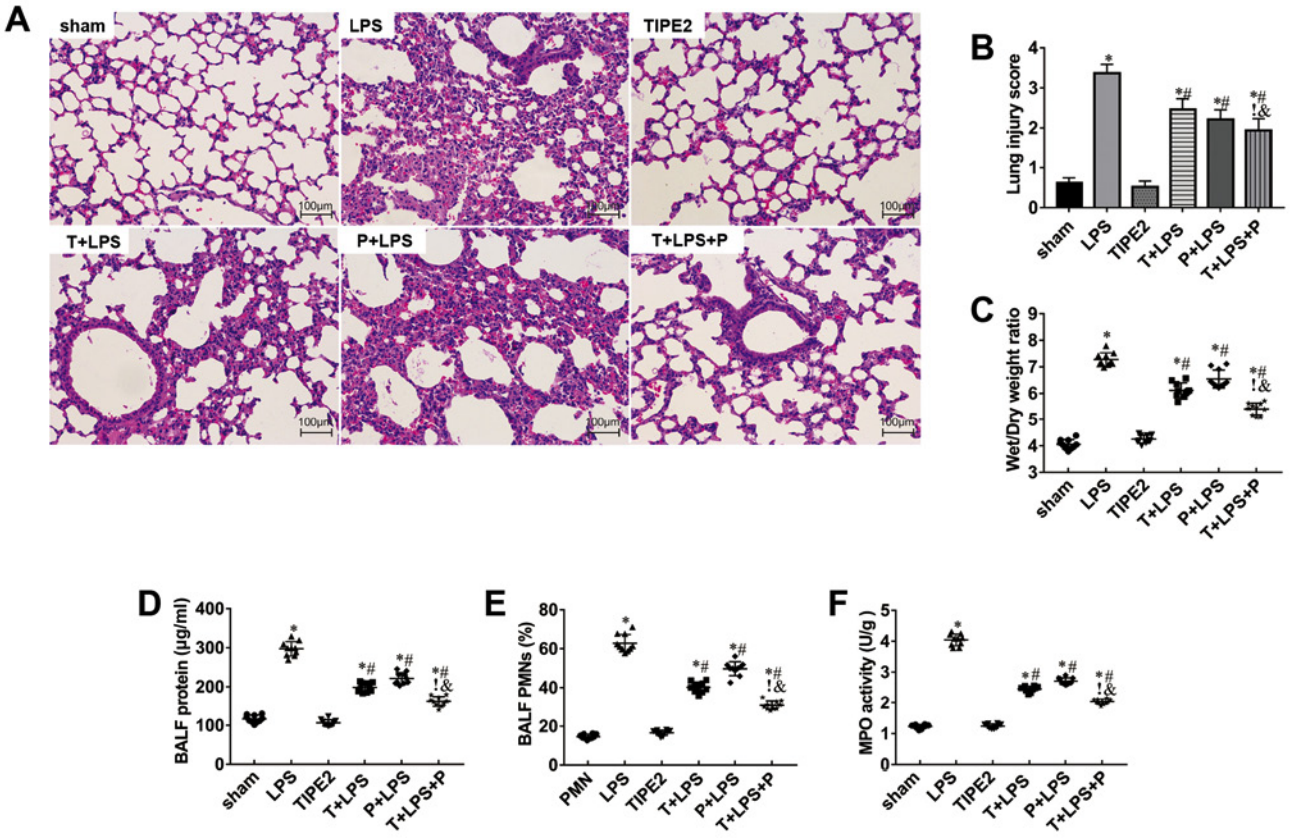

Figure 1. PHC pretreatment and TIPE2 overexpression ameliorates lung injury in mice with acute lung injury. (A) Histological assessments of lung tissues under light microscope. Scale bar, 100 μm. (B) The corresponding lung injury score. (C) The wet/dry weight ratio. (D) Protein concentration in the BALF. (E) PMNs/total cells in the BALF. (F) MPO activity. n=8/group. *P<0.05 vs. sham group; $^{\#}$P<0.05 vs. LPS group; $^{\&}$P<0.05 vs. T+LPS group; $^{!}$P<0.05 vs. P+LPS group. PHC/P, penehyclidine hydrochloride; TIPE2/T, tumor necrosis factor α-induced protein 8-like protein 2; BALF, bronchoalveolar lavage fluid; MPO, myeloperoxidase; PMNs, polymorphonuclear neutrophils; LPS, lipopolysaccharide.

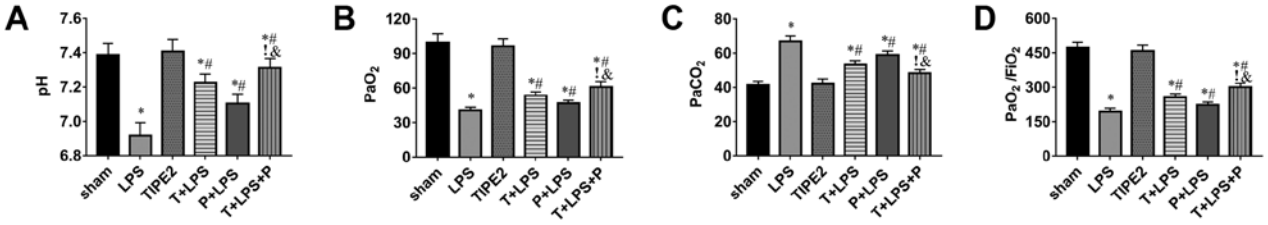

Figure 2. PHC pretreatment and TIPE2 overexpression improves gas exchange in the lungs of mice. Arterial blood gas analysis was performed to determine (A) pH, (B) PaO_2, (C) $PaCO_2$, (D) PaO_2/FiO_2. n=8/group. *P<0.05 vs. sham group; $^{\#}$P<0.05 vs. LPS group; $^{\&}$P<0.05 vs. T+LPS group; $^{!}$P<0.05 vs. P+LPS group. PHC/P, penehyclidine hydrochloride; TIPE2/T, tumor necrosis factor α-induced protein 8-like protein 2; LPS, lipopolysaccharide; PaO_2, partial pressure of oxygen; FiO_2, fraction of inspired oxygen; $PaCO_2$, partial pressure of carbon dioxide.

Fig. 4G and H), and the expression of TIPE2 decreased, in mice in the LPS group compared with the sham group (P<0.05; Fig. 4D). The expression levels of NF-κB p65 and p-JNK decreased (P<0.05; Fig. 4G and H), and the expression of TIPE2 increased (P<0.05; Fig. 4D), in mice in the T+LPS, P+LPS and T+LPS+P groups compared with the LPS group.

Discussion

The pathological changes of ALI are mainly associated with alveolar damage and the release of neutrophil and inflammatory cytokines (29). At present, no specific drugs are used for the treatment of ALI (1). In addition to controlling the primary disease, inhibiting systemic inflammation is an important measure to prevent and treat ALI (30). In the present study, it was found that LPS stimulation could worsen histological changes of lung tissues and dysfunctions of gas exchange.

PHC is a novel anticholinergic drug that can inhibit biomembrane lipid peroxidation and decrease the levels of cytokines and oxyradicals in patients with sepsis (31). Previous research has demonstrated that PHC plays a protective role against sepsis through inhibiting the expression of inflammatory factors and inducible nitric oxide synthase mRNA (32). Some previous studies (33,34) have shown that it plays a protective role in ischemia-reperfusion injury, but the role in transplant surgery has not yet been reported in the literature,

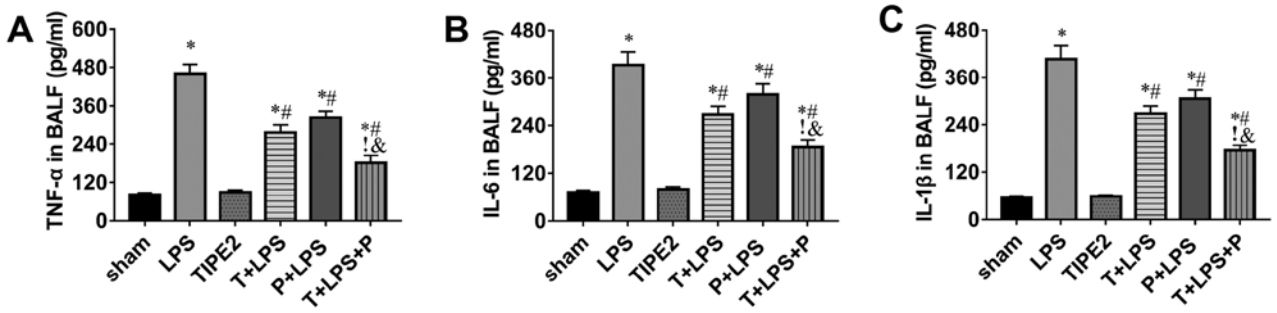

Figure 3. PHC pretreatment and TIPE2 overexpression attenuates the expression of pro-inflammatory cytokines. The BALF concentrations of (A) TNF-α, (B) IL-6 and (C) IL-1β. n=8/group. *P<0.05 vs. sham group; #P<0.05 vs. LPS group; &P<0.05 vs. T+LPS group; !P<0.05 vs. P+LPS group. PHC/P, penehyclidine hydrochloride; TIPE2/T, tumor necrosis factor α-induced protein 8-like protein 2; LPS, lipopolysaccharide; BALF, bronchoalveolar lavage fluid.

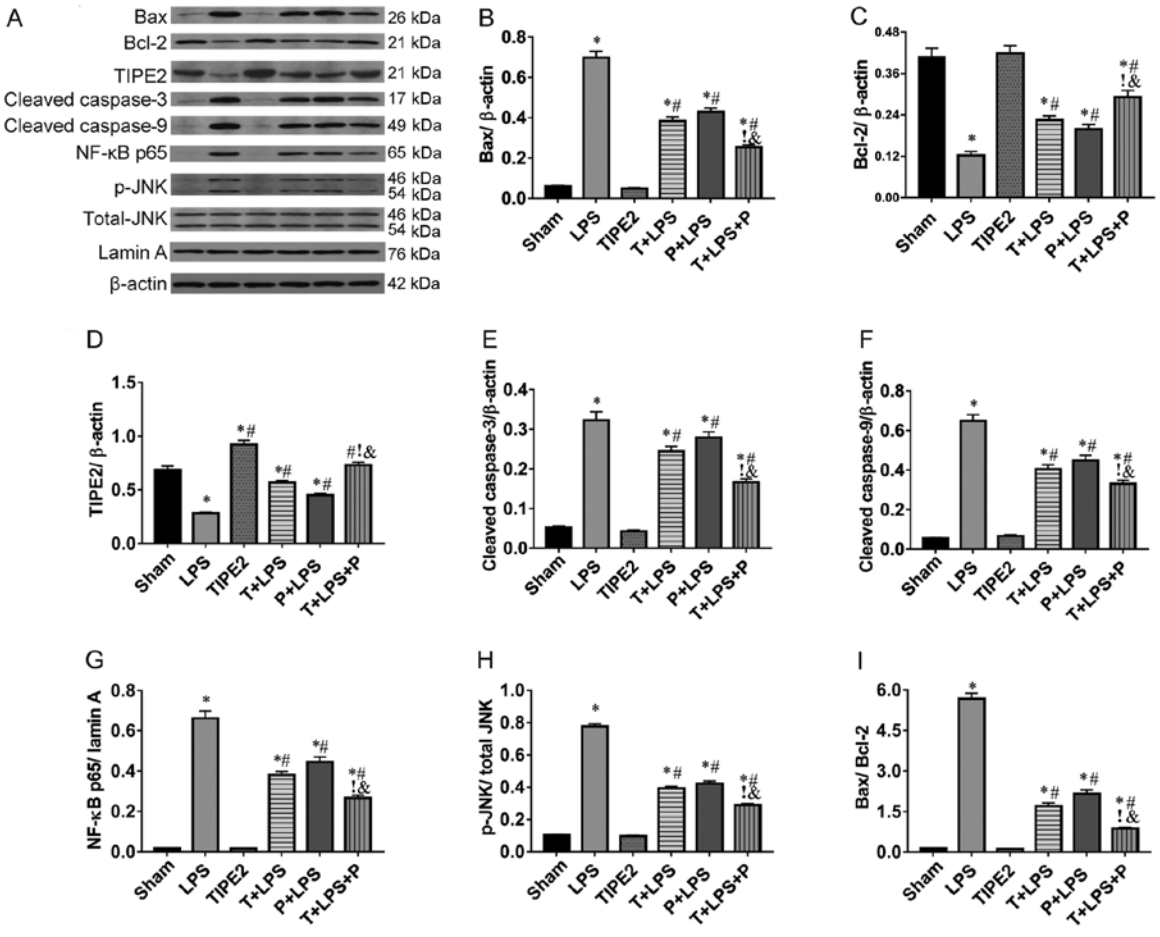

Figure 4. Effects of PHC pretreatment and TIPE2 overexpression on the expression of different proteins in the lung tissues of mice. (A) Western blotting analysis of protein expression levels. Semi-quantification of (B) Bax, (C) Bcl-2, (D) TIPE2, (E) cleaved caspase 3, (F) cleaved caspase 9, (G) NF-κB p65, (H) p-JNK/total JNK and (I) Bax/Bcl-2 ratio. n=8/group. *P<0.05 vs. sham group; #P<0.05 vs. LPS group; &P<0.05 vs. T+LPS group; !P<0.05 vs. P+LPS group. PHC/P, penehyclidine hydrochloride; TIPE2/T, tumor necrosis factor α-induced protein 8-like protein 2; LPS, lipopolysaccharide; p-, phosphorylated.

thus the related dosage and clinical effects need to be further investigated. A study reported that the protective effect of PHC may involve the inhibition of NF-κB activation via inhibition of the p38MAPK and ERK signaling pathways (35). In the present study, it was found that PHC pretreatment ameliorated pulmonary edema in mice with LPS-induced ALI, as evaluated by a significant decrease in the lung wet/dry ratio. At the same time, PMNs/total cells and total protein concentration in the BALF, which are the common indicators to detect pulmonary vascular permeability (36), were significantly increased in LPS group and decreased in the P+LPS group. PHC pretreatment also alleviated lung histological damage and improved gas exchange dysfunction induced by LPS. TNF-α is the earliest proinflammatory factor that plays a central role in stress response and is the initiator of the occurrence and development of ALI (37). The present results showed that PHC could significantly inhibit the expression of TNF-α, IL-6 and IL-1β, which indicated that PHC has anti-inflammatory properties.

At the same time, PHC pretreatment could decrease the Bax/Bcl-2 ratio, as well as the expression of cleaved caspase 3 and cleaved caspase 9 protein, indicating a potential antiapoptotic effect. Recent studies have also demonstrated that PHC can decrease the expression of phosphorylated JNK in mice and rats with cerebral ischemia-reperfusion and myocardial ischemia-reperfusion injury, and provided protective effects in the brain (38) and heart (39), respectively, which is consistent with the present experimental results in the lung tissues of mice.

TIPE2 is predominantly expressed in immune cells of the myeloid and lymphoid lineages (40), which provides TIPE2 with an important role in the maintenance of immune homeostasis (41). Therefore, the abnormal expression of TIPE2 can lead to diseases such as systemic autoimmunity, diabetic nephropathy and hepatitis B in humans (42). In the present study, it was demonstrated that TIPE2 overexpression could inhibit the expression of TNF-α, IL-6 and IL-1β compared with the LPS group, which was concomitant with a decrease in NF-κB activation. Some studies have found that TIPE2 is a negative regulator of MAPK and NF-κB signaling pathways (14,15), However, the underlying mechanisms are currently unknown. Thus, the present study explored the expression of related proteins and the underlying relationship between them. It was found that TIPE2 overexpression significantly reduced the expression of pro-apoptotic proteins such as Bax, cleaved caspase-9, cleaved caspase-3 and p-JNK, at the same time, the expression of anti-apoptotic protein Bcl-2 was increased. These data suggested that the effect of TIPE2 overexpression on cell apoptosis may be associated with suppression of JNK activation. Furthermore, treatment with PHC and TIPE2 overexpression at the same time led to a more obvious protective effect compared with PHC or TIPE2 alone. In our previous experiment, we found that TIPE2 overexpression markedly inhibited inflammation and cell apoptosis induced by LPS (20), and the present results suggested that the expression of TIPE2 was increased after the mice received intraperitoneal injection with PHC. These findings indicate that TIPE2 may be linked to the protective mechanism of PHC in mice with LPS-induced ALI.

In the present study, LPS-induced ALI in mice led to the increased expression of NF-κB and promoted the activation of JNK protein, subsequently causing the excessive release of pro-inflammatory cytokines and the activation of pro-apoptotic proteins, which was inhibited following PHC pretreatment-induced upregulation of TIPE2 expression. TIPE2 can negatively regulate TLR signaling, thereby inhibiting activation of NF-κB and JNK and reducing apoptosis (20). Thus, based on these findings, it was speculated that PHC inhibits these effects by upregulating the expression of TIPE2, thereby alleviating lung inflammation and apoptosis. The current study preliminarily investigated the effects of TIPE2 and PHC drug treatment on mice with LPS-induced ALI. However, the specific underlying mechanism of PHC treatment on TIPE2 expression also needs to be verified by knockout mice. At present, we have carried out the cultivation of gene knockout mice, but the number of mice was not enough to be included in the present study, so research on specific pathways will be investigated in future research.

In conclusion, the present study provided evidence that TIPE2 contributes to the protective effects of PHC pretreatment against LPS-induced ALI through decreasing the expression of NF-κB and inhibiting the activation of JNK in the lungs of mice. However, the potential underlying mechanisms will be further elucidated in depth in the future through the use of gene knockout mice.

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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

XMS and XJW participated in the design of the study and reviewed the manuscript. MY and QK carried out the experiments. GQJ and TQM performed the data analyses. MY wrote and revised the manuscript. XMS and XJW confirm the authenticity of all the raw data. All authors read and reviewed the final manuscript.

Ethics approval and consent to participate

Ethics approval was provided by the Medical Ethics Committee of Renmin Hospital of Wuhan University (Wuhan, China). All surgical procedures were performed in accordance with Wuhan University Animal Care and Use committee.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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