Protective effects of PNU-282987 on sepsis-induced acute lung injury in mice

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Abstract. The cholinergic anti-inflammatory pathway is considered an attractive approach for the alleviation of inflammatory diseases. Sepsis is characterized by systemic inflammation and widespread organ injury, especially that in the lung. In the present study, we explored the effects of an α7nAChR agonist, PNU-282987, on sepsis-induced lung injury and investigated the mechanisms of PNU-282987 in response to lipopolysaccharide (LPS) stimulation in peritoneal macrophages. Sepsis was induced in C57BL/6 mice via cecal ligation puncture (CLP). Fifty mice were randomly divided into five groups: The sham group treated with vehicle, the sham group treated with PNU-282987, the CLP group treated with PNU-282987 (1 mg/kg) 1 h before or 2 h after surgery. All mice were sacrificed at 12 or 24 h after CLP. Both pre- and post-CLP treatment with PNU-282987 significantly attenuated sepsis-induced lung injury and the release of IL-6 in the bronchoalveolar lavage fluid (BALF). Pre-treatment with PNU-282987 also inhibited sepsis-increased TNF-α and IL-6 production, while post-CLP treatment only inhibited IL-6 production in the lung tissue. Neither pre- nor post-CLP treatment with PNU-282987 affected IL-6 release in the serum. Furthermore, pretreatment with PNU-282987 resulted in reductions in TNF-α and IL-6 release in a dose- and time-dependent manner and decreased the phosphorylation levels of p38, JNK and ERK under LPS conditions in peritoneal macrophages. Our results demonstrate that activation of α7nAChR alleviates sepsis-induced lung injury; this effect is associated with the suppression of inflammatory responses via the MAPK pathway, suggesting that α7nAChR is a potential therapeutic target for the treatment of sepsis.

Introduction

Sepsis is the leading cause of death for patients in intensive care units (1). The pathogenesis of sepsis is generally believed to be caused by severe infection characterized by an overwhelming immune response. Sepsis is frequently associated with the dysfunction of vital organs, most commonly acute lung injury (ALI). ALI is associated with extreme morbidity and a high mortality rate in critically ill patients (2-4). The overactivation of inflammatory signaling pathways, such as mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB), leading to the excessive release of inflammatory mediators, including tumor necrosis factor α (TNF-α), interleukin-6 (IL-6) and high-mobility group box 1 protein (HMGB1), appear to contribute to organ dysfunction and mortality in sepsis (5-8). A variety of pharmacologic therapies have been evaluated, including the neutralization of cytokines and the activation of anti-inflammatory pathways. Despite decades of basic and clinical studies, there is no specific therapy available for this devastating disease. Therefore, the treatment of sepsis and related organ injury or dysfunction remains largely focused on supportive care (9,10).

Several lines of evidence have recently demonstrated that the exacerbated release of pro-inflammatory cytokines can be controlled by the cholinergic anti-inflammatory pathway via cholinergic mediators or by electrical stimulation of the vagus nerve in various experimental models, including lethal endotoxemia, hemorrhagic shock and ischemia-reperfusion injury (11-13). α7nAChR is an essential component of the cholinergic anti-inflammatory pathway (14). α7nAChR belongs to the family of acetylcholine-gated cation ion channels formed by five subunits; it exhibits distinct biophysical and pharmacological effects relative to other nAChR subclasses.
subtypes (15,16). A previous study has shown that α7nAChR presents on the reticuloendothelial system that targets foreign pathogens in the lung, liver, spleen and other organs (17).

Wang et al (13) found that nicotine decreased the level of HMGB1 in the serum and improved survival in a murine endotoxemia model. Although nicotine activates α7nAChR, it also interacts with α4β2 nAChRs; thus, it is unclear whether α4β2 properties contribute to or detract from the effects of nicotine. More recently, α7nAChR-selective ligands belonging to diverse chemotypes have been reported to demonstrate high affinity and efficacy, including PNU-282987 (18) and A585539 (19). PNU-282987 attenuates sterile inflammation, including in ischemia/reperfusion-induced brain or liver injury (20,21) and acid-induced ALI (22). Therefore, the aim of the present study was to investigate the effects of PNU-282987 on polymicrobial sepsis-induced ALI and examine its potential mechanism in LPS-stimulated peritoneal macrophages.

Materials and methods

Animals. Male pathogen-free C57BL/6 mice were obtained from the Laboratory Animal Research Center of Shanghai (SLAC, Shanghai, China). Each male pathogen-free C57BL/6 mouse (8-12 weeks of age and weighing approximately 25 g) were raised in cages in an air-conditioned room (20±1˚C) with controlled 12 h light/dark and maintained on standard laboratory food (Global Diet; Shanghai, China) and water ad libitum at the Laboratory Animal Research Center of Tongji (Shanghai, China). The total number of mice used in experiments was 40 (8 mice per group).

All animal studies were conducted in accordance with the National Institute of Health Guidelines on the use of laboratory animals and approved by the Ethics Committee of the University of Tongji (23).

Cell culture. Peritoneal macrophages were isolated from C57BL/6 mice as previously described (24). Peritoneal macrophages were treated with PNU-282987 (20-100 µM, P6499; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and agents were added 60 min before the challenge with lipopolysaccharides (LPS) (10 ng/ml, Escherichia coli 055:B5; List Biological Laboratories, Inc., Campbell, CA, USA).

Experimental design. Male C57BL/6 mice (6-8 weeks old) were randomly divided (n=8) into the sham group treated with vehicle (group control), the sham group treated with PNU-282987 (group PNU), the CLP group treated with vehicle (group CLP), and the group treated with PNU-282987 (1 mg/kg) administered 1 h before or 2 h after CLP (group CLP-Pre or CLP-Post). The surgical procedure to generate CLP-induced sepsis was performed as previously described (24). In brief, mice were anesthetized with sevoflurane, and a middle abdominal incision was made. The cecum was mobilized, ligated, and punctured with a 22-gauge needle. The bowel was repositioned and the abdomen was closed. The animals were resuscitated with sterile saline subcutaneously immediately after CLP surgery. The sham-operated control mice underwent the same procedure, without ligation or puncture of the cecum. All mice were sacrificed by cervical dislocation at 12 or 24 h after CLP. Blood samples were collected in tubes containing heparin. BALF was centrifuged immediately (at 4°C, 800 x g for 10 min) for harvesting of the cells and the supernatant. The supernatant was used to measure TNF-α and IL-6, and the deposits were collected for neutrophil and macrophage counting by Wright-Giemsa staining. Histopathological changes were examined in right lung tissues. Left lung tissues were collected for real-time reverse transcriptase polymerase chain reaction (RT-PCR).

Histopathological examination. For histological analyses, lung tissues were fixed in 4% paraformaldehyde phosphate-buffered saline (PBS) for 48 h at room temperature, embedded in paraffin, and sliced into 5-μm-thick sections using a machine. After deparaffinization, slides were stained with hematoxylin and eosin (H&E). Morphological alterations in the lungs were examined by a light microscopy (Leica DM6000 B, Leica, Wetzlar, Germany) and scored based on the extent of pathology on a scale of 0 to 4 by measuring interstitial edema, alveolar edema, hemorrhage and neutrophil infiltration (0, none, 4, severe). Composite lung injury scores represent the sum of the mean injury subtype scores for each condition on a scale of 0 to 16. All histological studies were performed in a blinded fashion.

MTT assay of cell viability. The effect of PNU-282987 on peritoneal viability was measured using the standard MTT assay as previously described by Wei et al (25). Briefly, cells were seeded in 96-well culture plates at a density of 2x10⁴ cells/well and allowed to attach overnight. Cells were washed twice with PBS and subsequently treated with various concentrations of PNU-282987 from 0.1 to 1 mM for 24 h. Then, 20 µl of MTT (Sigma-Aldrich; Merck KGaA) was added to each well and incubated for 4 h at 37°C. After removing the MTT solution, 200 µl of dimethyl sulfoxide (DMSO; Sigma-Aldrich; Sigma-Aldrich; Merck KGaA) was added to each well and incubated for 4 h at 37°C. After removing the MTT solution, 200 µl of dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) was added to each well. The absorbance was determined using a Synergy 2 Multiple ELISA (BioTek Instruments, Inc., Winooski, VT, USA) at a wavelength of 570 nm.

Enzyme linked immunosorbent assay. Levels of TNF-α and IL-6 were measured using commercially available ELISA assay kits (Bio-Ray, Laguna Hills, CA, USA) according to the manufacturer's instructions.

Western blot analysis. To detect the levels of p-P38MAPK (dilution 1:1,000; cat. no. 4511), p-JNK (dilution 1:800; cat. no. 4668) and p-ERK (dilution 1:200; cat. no. 4370; all from Cell Signaling Technology, Danvers, MA, USA) in peritoneal macrophages, immunoblotting was performed as previously described (21). Whole-cell lysates were prepared using RIPA (Beyotime Institute of Biotechnology, Haimen, China) containing protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and 10 µg/ml phenylmethylsulfonyl fluoride (PMSF). The protein concentration was determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts (20 µg) of the lysate were boiled for 8 min in equal volumes of 6X SDS buffer. The protein samples were subjected to electrophoresis on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide and transferred to a polyvinylidene difluoride (PVDF) membrane using a semi-dry transfer apparatus (Bio-Rad Laboratories). Non-specific
Results

PNU-282987 alleviates sepsis-induced acute lung injury. Among the vital organs in the body, the lung is particularly susceptible to acute injury in CLP-induced polymicrobial sepsis. Excessive inflammatory cell infiltration plays a key role in pulmonary failure during sepsis. To determine whether PNU-282987 could protect mice against sepsis-induced acute lung injury using histological sections by applying a semi-quantitative scale (described in detail in Materials and methods). As shown in Fig. 1C, the total injury score in lungs after CLP was significantly increased compared to that of the control group, while the score was significantly reduced when septic mice received PNU-282987.

PNU-282987 downregulates TNF-α and IL-6 levels in sepsis. The inflammatory response is induced in sepsis. Accordingly, we examined the levels of TNF-α and IL-6 in the serum and BALF of septic mice by ELISA at 12 and 24 h after CLP. The level of IL-6 was significantly increased in the CLP group compared to that in the control group. Neither PNU-282987 pre- nor post-CLP treatment significantly reduced IL-6 release in the serum (Fig. 2A). IL-6 levels in BALF were significantly decreased in the CLP-pre and CLP-post groups compared to those in the CLP group at 12 and 24 h (Fig. 2B). We did not observe significant differences in the TNF-α level among CLP and CLP with PNU-282987 administration groups in serum and BALF (Fig. 2C and D), except for the TNF-α level in the CLP group compared with the control which was significantly increased at 12 h. We also examined changes in the mRNA levels of TNF-α and IL-6 in the lung tissue by RT-PCR. In the lungs of septic mice, mRNA expression levels of TNF-α and IL-6 were significantly increased. The increases in the mRNA expression levels of IL-6 and TNF-α were significantly suppressed by PNU-282987 pretreatment. Post-treatment with PNU-282987 significantly inhibited IL-6 mRNA expression, but resulted in only slight decreases in TNF-α expression in the lung tissue (Fig. 2D and E). Our results suggest that PNU-282987 treatment inhibits the local inflammatory response in sepsis.

Effect of PNU-282987 on macrophage viability. Cell viability, essentially the mitochondrial activity of living cells, was measured by a quantitative colorimetric assay with MTT. In vitro, peritoneal macrophages were treated with PNU-282987 for 24 h and no significant differences in viability were found at concentrations ranging from 0.1 to 100 µM when compared with viability in the control cultures (Fig. 3).

PNU-282987 inhibits LPS-induced TNF-α and IL-6 release in peritoneal macrophages. To detect the effect of PNU-282987 on LPS-induced macrophage activation, the macrophage inflammatory cytokine production (TNF-α and IL-6) was examined after exposure to LPS (10 ng/ml) and PNU-282987 at various concentrations or time-points in vitro. The levels of TNF-α and IL-6 in the culture supernatant were significantly decreased by pretreatment with PNU-282987 in a dose-dependent manner at 8 h (Fig. 4A and B). Pretreatment with PNU-282987 at 100 µM inhibited LPS-induced TNF-α and IL-6 release in a time-dependent manner (Fig. 4C and D).
PNU-282987 pretreatment also significantly decreased TNF-α and IL-6 mRNA expression in macrophages at early times (4 and 8 h) (Fig. 4E and F). PNU-282987 had no inhibitory effect on the production of cytokines in resting cells.

**PNU-282987 inhibits LPS-activated MAPK signaling in macrophages.** The MAPK cascade is the key downstream pathway for LPS-stimulated signaling events (7,27). To further explore the intracellular mechanisms underlying the anti-inflammatory effects of PNU-282987, we investigated whether PNU-282987 could inhibit the LPS-induced activation of MAPK pathways. The MAPK family involves three major subgroups, including p38, ERK1/2 and JNK. The activation of p38, ERK1/2 and JNK were assessed by their phosphorylation levels. LPS strongly activated all three families of MAPKs in peritoneal macrophages in a time-dependent manner. PNU-282987 pretreatment significantly prevented LPS-induced increases in the levels of phosphorylated p38, ERK1/2 and JNK in a time- and dose-dependent manner (Fig. 5).

**Discussion**

Sepsis and subsequent multiple organ failure remain the leading cause of death in critically ill patients in intensive care units (1,3). Inflammatory mediators are markedly increased during the early phase. Recently, Huston et al described a cholinergic anti-inflammatory pathway based on the structure of the nervous system that restrains the production of pro-inflammatory cytokines by immune cells (17). α7nAChR plays a key role in the cholinergic anti-inflammatory pathway. In this study, the effects of PNU-282987, an α7nAChR-selective agonist, were examined in a highly clinically relevant mouse model of sepsis induced by cecal ligation puncture (CLP).

Previously, Wang et al (14) reported that nicotine inhibits high-mobility group box 1 protein (HMGB1) release induced by either LPS or TNF-α in human macrophages. They also indicated that treatment with nicotine attenuated the serum HMGB1 level and improved survival in experimental models of sepsis. Although nicotine activates α7nAChR, it also
interacts with α4β2 nAChRs; thus, it is unclear whether the properties of α4β2 contribute to or detract from the effects of nicotine. Su et al (22) demonstrated that pretreatment with PNU-282987, a highly specific α7nAChR agonist, attenuated acid-induced ALI in mice. Different from our model, Pinheiro et al (28) recently indicated that PNU-282987 treatment reduced ALI generated by intratracheal instillation of LPS via changes in the macrophage profile. He et al (29) found that α7nAChR activation attenuated intestine ischemia/reperfusion-induced lung injury in rats. In our previous study, it was found that PNU-282987 pretreatment alleviated ischemia-reperfusion-induced liver injury in mice (21). In this study, it was found that PNU-282987 significantly reduced inflammatory cell infiltration and lung injury, even when treatment was started 2 h after the onset of CLP. The pro-inflammatory cytokines TNF-α and IL-6

**Figure 2.** Effect of PNU-282987 on inflammatory cytokine production in sepsis. Mice were randomly divided into the sham group treated with vehicle (control), the sham group treated with PNU-282987 (PNU), the CLP group treated with vehicle (CLP) and the group treated with PNU-282987 (1 mg/kg) administered 1 h before or 2 h after CLP (CLP-Pre or CLP-Post, respectively). (A-D) IL-6 and TNF-α levels in serum and BALF were measured at 12 and 24 h by ELISA. (E and F) The relative expression levels of TNF-α and IL-6 in lung tissues were determined by RT-PCR. Data are presented as means ± SEM (n≥3). *P<0.05, the CLP group vs. the control group; **P<0.01, the CLP group vs. the control group; ***P<0.05, the CLP group vs. the CLP-Pre or CLP-Post group; $P<0.05, the CLP group vs. the vs. the CLP-Pre or CLP-Post group. BALF, bronchoalveolar lavage fluid; CLP, cecal ligation puncture.

**Figure 3.** Effects of PNU-282987 on cell viability in peritoneal macrophages. Peritoneal macrophages were cultured with different concentrations of PNU-282987 (0.1 to 1 mM) for 24 h. Cell viability was determined by MTT assays. Results are presented as means ± SEM of three independent experiments. *P<0.05, the PNU-282987 group vs. the control group.
have been implicated in the pathogenesis of inflammatory lung injury, particularly under conditions of sepsis (6). It was found that when PNU-282987 was administered to septic mice, elevated levels of genes encoding pro-inflammatory cytokines in the lungs and the secretion of IL-6 in BALF decreased substantially, implying that PNU administration can reduce local inflammation in septic mice.

The anti-inflammatory property of PNU-282987 was confirmed in vitro using peritoneal macrophages. PNU-282987 pretreatment markedly inhibited pro-inflammatory cytokine production in LPS-stimulated peritoneal macrophages. The mechanism of PNU-282987 was further examined in LPS-stimulated macrophages. The MAPK pathway is one of the most important signaling cascades that regulates the LPS-induced inflammatory response (30). MAPK activity results in the phosphorylation of substrates involved in inflammation. Acetylcholine represses hypoxia-induced TNF-α production via the regulation of MAPK phosphorylation in cardiomyocytes (31). According to a recent report, nicotine suppressed p38, Erk1/2 and JNK MAPK activation induced by MIA or IL-1β in chondrocytes (32). In the present study, the effects of PNU-282987 on MAPK signaling were investigated during LPS-stimulated peritoneal macrophages. As expected, LPS-induced MAPK phosphorylation was attenuated by
pretreatment with PNU-282987 before LPS stimulation, in macrophages in a time- and dose-dependent manner. These results suggest that PNU-282987 inhibits LPS-induced inflammatory responses partially via the blockade of the MAPK signaling pathways.

In conclusion, to the best of our knowledge, this is the first study to investigate the effect of PNU-282987 administration in sepsis-induced lung injury via cecal ligation puncture. A single dose of PNU-282987 administered by intraperitoneal injection before or even after CLP inhibited IL-6 release, and this inhibition consequently resulted in the alleviation of lung injury. Moreover, PNU-282987 inhibited LPS-induced pro-inflammatory cytokine release, partially via the blockade of MAPK signaling pathways in peritoneal macrophages.

Overall, these results suggest that PNU-282987 has potential preventive and therapeutic functions for protection against early inflammatory responses in sepsis-induced ALI.

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Availability of data and materials
The materials used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions
ZS and QL mainly performed the experiments and ZC performed the statistical analysis. SW helped to complete the additional experiment and revise the manuscript. ZC designed the study and wrote this manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate
All animal studies were conducted in accordance with the National Institute of Health Guidelines on the use of laboratory animals and approved by the Ethics Committee of the University of Tongji.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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