Protective Effect of Nicotine on Lipopolysaccharide-Induced Acute Lung Injury in Mice

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Key Words

Lipopolysaccharide • Acute lung injury • Nicotine • Tumor necrosis factor- α • Interleukin-1 β • High mobility group box 1

Abstract

Background: Recently, nicotine administration has been shown to be a potent inhibitor of a variety of innate immune responses, including endotoxin-induced sepsis. **Objective:** It was the aim of this study to evaluate the effect of nicotine on attenuating lung injury and improving the survival in mice with lipopolysaccharide (LPS)-induced acute lung injury (ALI). **Methods:** ALI was induced in mice by intratracheal instillation of LPS (3 mg/ml). The mice received intratracheal instillation of nicotine (50, 250 and 500 μ g/kg) before or after LPS administration. Pulmonary histological changes were evaluated by hematoxylin-eosin stain, and lung wet/dry weight ratios were observed. Concentrations of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β and high mobility group box (HMGB)-1, as well as myeloperoxidase (MPO) activity were measured by enzyme-linked immunosorbent assay. The mortality rate was recorded and analyzed by the Kaplan-Meier method. **Results:** Nicotine pretreatment significantly attenuated the severity of lung injury and inhibit-

ed the production of TNF- α , IL-1 β and HMGB-1 in mice with ALI. After LPS administration, the lung wet/dry weight ratios, as an index of lung edema, and MPO activity were also markedly reduced by nicotine pretreatment. Early treatment with a high dose of nicotine (500 μ g/kg) after LPS administration decreased the mortality in mice with ALI, even when treatment was started 24 h after LPS administration. **Conclusion:** Nicotine attenuated the lung injury and reduced mortality in mice with LPS-induced ALI.

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Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are still major causes of mortality in intensive care units even though the mortality rates decreased from 66 to 34% due to changes in the method of mechanical ventilation and improvement in the supportive care of critically ill patients [1]. It is considered

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that the network of inflammatory cytokines and chemokines plays a major role in mediating, amplifying and perpetuating the lung injury process [2]. The proinflammatory cytokines, tumor necrosis factor (TNF)- α and interleukin (IL)-1 β , can stimulate the production of a host of other cytokines and eventually result in lung injury. The reduction in the mortality rate in animal sepsis models has been observed in experimental therapies against TNF- α and IL-1 β [3, 4]. However, these therapies have proved difficult clinically, in part, because TNF- α and IL-1 β are released within minutes after bacterial challenge [5, 6]. Therefore, we need to search for late proinflammatory cytokines that may offer a wider therapeutic window.

High mobility group box (HMGB)-1, a nuclear non-histone DNA-binding protein, has been identified as a 'late' mediator of endotoxin lethality [7]. HMGB-1 is detected in the serum of patients with sepsis, and serum HMGB-1 levels are significantly increased in patients with poor prognosis [7]. In addition, high levels of HMGB-1 were associated with neutrophil-mediated diseases including lung inflammation and ALI [7, 8]. Unlike the 'early' cytokines, such as TNF- α and IL-1 β , HMGB-1 is released days after lipopolysaccharide (LPS) exposure that provides a wider time frame for clinical intervention against progressive inflammatory disorders [9]. Therefore, HMGB-1 is a potential novel therapeutic target for inflammatory diseases.

Nicotine, a small organic alkaloid, is a major component of cigarette smoke and acts as an agonist on the nicotinic acetylcholine receptors (nAChRs) found mainly in the central and peripheral nervous systems and on many other tissue cells throughout the body, including immune cells [10, 11]. Recently, a study showed that nicotine acted on the $\alpha 7$ nAChR, inhibited the nuclear factor- κ B pathway and suppressed HMGB-1 release from human macrophages; in vivo, treatment with nicotine attenuated serum HMGB-1 levels and improved survival in 'established' sepsis, even when treatment was initiated after the onset of the disease [12]. In addition, nicotine protected the kidney from renal ischemia/perfusion injury by preventing neutrophil infiltration and decreasing productions of keratinocyte-derived chemokine, TNF- α and HMGB-1 [13]. Although the anti-inflammatory effects of nicotine have been showed in different animal models, the protective effect of nicotine on ALI is controversial. Therefore, the present study was designed to investigate whether nicotine could be beneficial in mice with LPS-induced ALI.

Materials and Methods

Animals and Reagents

One hundred and eighty male BALB/C mice weighing 20–25 g were purchased from the Animal Center of the Fourth Military Medical University (Xi'an, China). All animals were allowed to take food and tap water ad libitum. All procedures were in accordance with the Declaration of Helsinki of the World Medical Association. The protocols were also approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University Tangdu Hospital. LPS (*Escherichia coli* lipopolysaccharide, 055:B5) and nicotine were obtained from Sigma Chemical Company, St. Louis, Mo., USA. Enzyme-linked immunosorbent assay (ELISA) kits of TNF- α , IL-1 β , myeloperoxidase (MPO) and HMGB-1 were purchased from the Wuhan USCN Sciences Co., Ltd., Wuhan, China.

Animal Model of ALI

Before the induction of ALI, mice were fasted overnight but allowed water ad libitum. Animals were anesthetized with intraperitoneal pentobarbital (50 mg/kg). LPS (3 mg/kg) was instilled intratracheally to induce ALI. Sham-operated mice underwent the same procedure with intratracheal instillation of saline.

Experimental Protocol

In the first set of experiments, animals were randomly divided into 4 groups ($n = 15$ for each group). Group 1 (control group) received an intratracheal instillation of saline, group 2 (nicotine group) received an intranasal instillation of nicotine (500 μ g/kg), group 3 (LPS group) received an intratracheal instillation of LPS (3 mg/kg), and group 4 (LPS + nicotine group) received an intranasal instillation of nicotine (500 μ g/kg) 30 min before LPS administration. Twelve hours after LPS administration, animals were anesthetized and serum was collected. Bronchoalveolar lavage (BAL) was performed through the left lung. The superior lobe of the right lung was excised for histopathologic examination. The middle lobe was excised for analysis of the lung wet/dry weight ratio. The lower lobe was homogenized and frozen in a cold phosphate solution at -80°C for MPO analysis.

The second set of experiments was designed to evaluate the effect on mice survival of treatment with variable doses of nicotine (50, 250 and 500 μ g/kg). Nicotine treatment was started 30 min before LPS administration, and thereafter, nicotine was administered twice a day for 3 days. Animals were randomly divided into 4 groups ($n = 20$ for each group). The mice in these groups were treated with nicotine at 50, 250 and 500 μ g/kg intranasally, and the control mice received the same volume of saline. Mortality was recorded up to a week after the procedure.

The third set of experiments was designed to investigate whether nicotine could improve survival in 'established' ALI. Mice were randomly divided into 2 groups ($n = 20$ for each group). Delayed nicotine treatment (500 μ g/kg) started 24 h later, and thereafter, was administered twice a day for 3 days intranasally. Control mice received the same volume of saline. Mortality was recorded up to a week after LPS administration.

Bronchoalveolar Lavage

Animals were anesthetized with intraperitoneal pentobarbital (50 mg/kg). A median sternotomy allowed for exposure of both of the lungs. The trachea was exposed and inserted with an intrave-

Fig. 1. Effect of nicotine on the concentrations of TNF- α and IL-1 β in BALF and HMGB-1 in the serum of mice with ALI. **a** Concentrations of TNF- α in BALF 12 h after LPS administration and the effect of nicotine pretreatment. **b** Concentrations of IL-1 β in BALF 12 h after LPS administration and the effect of nicotine pretreatment. **c** Concentrations of HMGB-1 in serum 12 h after LPS administration and the effect of nicotine pretreatment. Data are expressed as the mean $\pm$ SEM. ^a $p < 0.05$ versus the control and nicotine groups; ^b $p < 0.05$ versus the LPS group.

nous infusion needle. After ligating the hilum of the right lung, the left lung was lavaged 5 times with 0.5 ml ice-cold phosphate-buffered saline. The recovery ratio of the fluid was about 90%. The BAL fluid (BALF) was immediately centrifuged at 500 g for 10 min at 4°C, and the cell-free supernatant was stored at -80°C for analysis of cytokines.

Cytokine Measurement

Concentrations of TNF- α and IL-1 β in BALF, and concentrations of HMGB-1 in serum were measured by using ELISA kits. All procedures were done in accordance with the manufacturer's instructions.

MPO Assays

To carry out the assays, tissue samples were subjected to 3 further freeze-thaw cycles and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was assayed for MPO activity with ELISA kits. All procedures were done in accordance with the manufacturer's instructions.

Lung Wet/Dry Weight Ratio

As an index of lung edema, the amount of extravascular lung water was calculated. The middle lobe of the right lung was excised and the wet weight was recorded. The lobe was then placed in an incubator at 80°C for 24 h to obtain the dry weight. And the wet/dry weight ratios were calculated by dividing the wet weight by the dry weight.

Pulmonary Histopathology

The superior lobe of the right lung was harvested 12 h after LPS administration and fixed with an intratracheal instillation of 1 ml buffered formalin (10%, pH 7.2). The lobe was further fixed in 10% neutral buffered formalin for 24 h at 4°C. The tissues were embedded in paraffin and cut into 5- μ m sections. Hematoxylin-eosin stains were performed using the standard protocol.

Statistical Analyses

Data were entered into a database and analyzed using SPSS software and are expressed as means $\pm$ SD. On a preliminary

Fig. 2. Effect of nicotine on MPO activity in lung tissues of mice with ALI. MPO activity in lung tissues 12 h after LPS administration and the effect of nicotine pretreatment. Data are expressed as the mean $\pm$ SEM. ^a $p < 0.05$ versus the control and nicotine groups; ^b $p < 0.05$ versus the LPS group.

Fig. 3. Effect of nicotine on lung edema of mice with ALI. After LPS administration, pretreatment of nicotine markedly decreased lung wet/dry ratios. Data are expressed as the mean $\pm$ SEM. ^a $p < 0.05$ versus the control and nicotine groups; ^b $p < 0.05$ versus the LPS group.

analysis, the Kolmogorov-Smirnov test found that the raw pooled data followed a Gaussian distribution. Thus, statistically significant differences between groups were determined by ANOVA followed by Tukey's test. In the mortality study, time to survival data were analyzed by the Kaplan-Meier method and compared with the log-rank test. Significance was accepted when $p < 0.05$.

Results

Effect of Nicotine on the Concentrations of Cytokines in BALF or Serum of Mice with ALI

A significant elevation of TNF- α and IL-1 β concentrations was observed in BALF of the LPS group, when compared with the control and nicotine groups. Pretreatment with nicotine prevented prominent elevation in TNF- α and IL-1 β concentrations (fig. 1a, b). The concentrations of HMGB-1 in the serum of the LPS group were significantly higher than those of the control and nicotine groups, whereas nicotine pretreatment significantly prevented this change (fig. 1c).

Effect of Nicotine on MPO Activity in Lung Tissues of Mice with ALI

The MPO activity in lung tissues increased significantly in the LPS group compared with the control and nicotine groups. This elevation in MPO activity was

found to be markedly inhibited in the LPS + nicotine group (fig. 2).

Effect of Nicotine on Lung Edema of Mice with ALI

Compared with the control and nicotine groups, the lung wet/dry weight ratios were significantly increased in the LPS group. The increase in lung wet/dry weight ratios was significantly reduced by nicotine pretreatment (fig. 3).

Effect of Nicotine on Pulmonary Histopathological Changes of Mice with ALI

Lung tissues from the control and nicotine groups showed a normal structure and no histopathological changes under a light microscope (fig. 4a, b). In the LPS group, the lungs stained with hematoxylin-eosin indicated widespread alveolar wall thickness caused by edema, severe hemorrhage in the alveolus, alveolus collapse and obvious inflammatory cell infiltration (fig. 4c). In the LPS + nicotine group, the histopathological changes of the lung were minor compared with those in the LPS group, especially in inflammatory cell infiltration (fig. 4d).

Effect of Nicotine on Mortality of Mice with ALI

Nicotine treatment at a high dose (500 μ g/kg) significantly improved survival in mice with ALI (nicotine-

Fig. 4. Effect of nicotine on the pulmonary histopathological changes of mice with ALI. Lung sections stained with hematoxylin-eosin 12 h after LPS administration revealed pulmonary histopathological changes. $\times 200$. **a** Control group: normal struc-

ture. **b** Nicotine group: same as control group. **c** LPS group: alveolar wall thickness, hemorrhage, alveolus collapse and obvious inflammatory cell infiltration. **d** LPS + nicotine group: minor histopathological changes compared with the LPS group.

treated group survival 80%, 16 of 20 mice; saline-treated group survival 25%, 5 of 20 mice; $p < 0.05$). This protective effect was dose dependent, and the low dose of nicotine (50 and 250 $\mu\text{g}/\text{kg}$) failed to significantly improve survival (fig. 5a). In addition, to investigate whether nicotine could improve survival in 'established' ALI, we treated mice with nicotine (500 $\mu\text{g}/\text{kg}$) 24 h after LPS administration. The results showed that delayed nicotine treatment markedly improved survival in mice with 'established' ALI (delayed nicotine-treated group survival 70%, 14 of 20 mice; saline-treated group survival 25%, 5 of 20 mice; $p < 0.05$) (fig. 5b).

Discussion

Neutrophils are an important component of the inflammatory response that characterizes ALI and are considered to be the final effector cell responsible for lung injury, due to their ability to express multiple cytotoxic products [14, 15]. In endotoxemia-induced ALI, the neutrophils accumulated in the lungs, expressed proinflammatory cytokines, such as IL-1 β and TNF- α , and finally lead to pulmonary injury [16]. MPO is a major constituent of neutrophil cytoplasmic granules. Therefore, the total activity of MPO in a tissue is a direct measure of neu-

Fig. 5. Effect of nicotine on mortality of mice with ALI. **a** Mortality of mice with ALI pretreated with variable doses of nicotine (50, 250 and 500 µg/kg). **b** Mortality of mice with 'established' ALI with delayed nicotine treatment (500 µg/kg). Data are expressed as the mean $\pm$ SEM. * $p < 0.05$ versus the control group.

trophil sequestration in that tissue [17]. In a recent study, nicotine treatment reduced massive neutrophil infiltration in the kidney after renal ischemia/perfusion [13]. Therefore, we tested the effect of nicotine on MPO activity in lungs after LPS administration. As expected, pretreatment of nicotine significantly decreased MPO activity in the lungs. In addition, a histopathological study also indicated that nicotine pretreatment markedly attenuated neutrophil infiltration in the lungs. The mechanism of neutrophil recruitment inhibition by nicotine might be related to the nicotine-mediated suppression of cytokine and chemokine production.

The intrapulmonary inflammatory cytokines are closely related to various pulmonary diseases [18–22]. Especially, proinflammatory cytokines play a key role in the pathogenesis and progression of ALI. Among these proinflammatory cytokines, TNF- α and IL-1 β , released within minutes after endotoxin exposure, are the most important early response cytokines. The concentrations of TNF- α and IL-1 β in BALF from patients at risk for ARDS and with established ARDS were elevated above those measured in BALF from normal volunteers [23]. At the onset of ARDS, nonsurvivors had significantly higher BAL levels of TNF- α and IL-1 β , which remained persistently elevated over time [24]. These suggest a critical role of TNF- α and IL-1 β in mortality. In addition, in re-

cent years, the importance of HMGB-1, a late mediator of sepsis, in ALI is gaining recognition [8]. Intratracheal administration of HMGB-1 produced acute inflammatory injury to the lungs, with neutrophil accumulation, the development of lung edema and increased pulmonary production of IL-1 β , TNF- α and macrophage-inflammatory protein-2 [8]. HMGB-1 was first detectable in the circulation 8 h after the onset of lethal endotoxemia and sepsis, subsequently increasing to plateau levels from 16 to 32 h [7, 25]. This late appearance of circulating HMGB-1 distinguished HMGB-1 from TNF- α and other early proinflammatory cytokines [26]. In a widely used animal model of LPS-induced ALI, administration of anti-HMGB-1 either before or after LPS treatment significantly decreased LPS-induced neutrophil accumulation into the lungs and attenuated the severity of lung edema produced by intratracheal administration of LPS [7].

In this study, the concentrations of TNF- α , IL-1 β and HMGB-1 in BALF or serum increased significantly after LPS administration. But these changes were significantly inhibited by nicotine pretreatment. Moreover, nicotine pretreatment significantly improved pulmonary histopathological changes and attenuated the severity of lung edema. These effects are in agreement with the study indicating that nicotine treatment decreased the production of cytokines, such as TNF- α and IL-1 β , and chemo-

kines in peritoneal lavage fluid and prevented liver damage during septic peritonitis [27]. Likewise, in a renal ischemia/reperfusion model, nicotine administration reduced the production of inflammatory cytokines, such as TNF- α and HMGB-1, and decreased tubular epithelial cell apoptosis and proliferation [13]. In addition, Su et al. [28] have shown that nicotine protected against acid-induced ALI as reflected by a marked reduction in excess lung water, lung vascular permeability, proinflammatory cytokines and protein in the BAL, neutrophil infiltration and epithelial cell injury.

Our results also showed that high-dose nicotine pretreatment significantly improved survival in ALI. Most importantly, in 'established' ALI, the delayed nicotine treatment still significantly improved survival. However, the effect of nicotine on ALI observed in a recent study from Boland et al. [29] is in contradiction to our study and the study from Su et al. [28]. Boland et al. [29] have shown that intraperitoneal injection of nicotine increased lung neutrophil infiltration and mortality in peritonitis-induced ALI. In our study and the study from Su et al. [28], the animal models of ALI induced by LPS and acid were both a sterile inflammation process. Conversely, the living bacteria play a key role in peritonitis-induced ALI.

Furthermore, a previous study has shown that because of a decrease in bacterial clearance and enhanced dissemination of bacteria, pretreatment with nicotine increased lethality in septic peritonitis [27]. Therefore, the different ALI models used in our and above studies may be the primary reason to explain why the findings of Boland et al. [29] were different from that of Su et al. [28] and ours.

Although delayed high-dose nicotine treatment was effective in this study, further studies are needed to explore the optimal therapeutic window and dose of nicotine before clinical application. Besides, the other effects of nicotine, especially in the central nervous system, should not be overlooked when nicotine is used to treat ALI.

In conclusion, our results showed that in the LPS-induced ALI model, the histological degree of lung injury, the severity of lung edema and the level of inflammatory cytokines were significantly reduced by nicotine pretreatment. Furthermore, nicotine treatment markedly improved survival in mice undergoing ALI, even in 'established' ALI. Still, further comprehensive studies are needed to investigate whether nicotine could be developed as a novel therapeutic adjunct in the treatment of ALI or other inflammatory diseases.

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