Protective effect of electrical stimulation of the vagus nerve in lipopolysaccharide-induced acute lung injury in rats

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Abstract. The present study investigated the role of electrical stimulation of the vagus nerve in the acute lung injury (ALI) inflammatory response induced by lipopolysaccharide (LPS) in rats. A rat model of ALI was established using LPS and by connecting an electrode to the left vagus nerve proximal to the heart in order to provide continuous electrical stimulation (1 mA; 1 msec; 10 Hz). After 120 min, the rat lung tissue was stained with hematoxylin and eosin and the expression of inflammatory factors was evaluated by reverse transcription-quantitative PCR and western blot analysis. The change in apoptosis rate in cells from bronchoalveolar lavage fluid (BALF) was analyzed using flow cytometry. The results of the present study demonstrated that inflammatory cell infiltration, alveolar wall and interstitial thickening, and lung hyperemia in rats with LPS-induced ALI were decreased following electrical stimulation of the vagus nerve. Electrical stimulation inhibited the expression levels of IL-1, IL-6, IL-10, IL-8 and TNF-α at both the mRNA and protein levels and decreased early and late apoptosis rates in inflammatory cells from BALF. The results indicated that vagus nerve stimulation can reverse the inflammatory response in lung injury, thereby exerting a pulmonary protective effect.

Introduction

Acute lung injury (ALI) is a pulmonary inflammatory response produced by inflammatory mediators and effector cells. It is characterized by decreased lung capacity and an imbalance in ventilatory flow (1). The inflammatory response in ALI results from a signaling cascade with amplified secondary damage, which may be accompanied by systemic immune inflammatory disorders. The onset and progression of ALI are rapid, the prognosis is extremely poor, and the mortality rate may be >30% (2).

The primary pathological features of ALI include the destruction of microvascular endothelial cells and alveolar epithelial cells, increased permeability of the alveolar-capillary barrier and infusion of protein-rich exudate into the alveoli, leading to pulmonary edema and hyaline membrane formation. The loss of control of the intrapulmonary inflammatory response mediated by inflammatory cells, such as polymorphonuclear (PMN) leukocytes and macrophages, is one of the primary pathogenic processes of ALI. When stimulated by non-cardiac factors, such as sepsis, trauma, shock and disseminated intravascular coagulation, the monocyte/macrophage system can become activated and release large numbers of inflammatory cytokines, including IL-1, IL-8, IL-6, IL-10 and TNF-α; the majority of which are pro-inflammatory cytokines. Pro-inflammatory cytokines further activate PMN leukocytes and macrophages, resulting in a severe imbalance between pro- and anti-inflammatory cytokines. Studies have reported that the upregulation of IL-6, IL-8 and TNF-α is positively correlated with mortality in ALI (3,4). In addition, the expression levels of cell adhesion molecules, such as cell adhesion molecule-1, P-selectin and E-selectin, on the surface of PMN leukocytes is upregulated, which may further aggravate pulmonary tissue inflammatory responses (5-7). The pathophysiological mechanisms of ALI are not only associated with inflammatory responses, but also with the activation of lung epithelial cells and inflammatory cell apoptosis (8,9). Albertine *et al* (10) demonstrated that the expression of Fas and FasL was positively associated with clinical prognosis in patients with ALI/acute respiratory distress syndrome. Compared with autologous serum, the expression levels of Fas and FasL in bronchoalveolar lavage fluid (BALF) were increased, indicating that apoptosis primarily occurs in lung tissues.

The immune system is not isolated and it interacts with the nervous system. When the central nervous system is stimulated by certain forms of immune stimulation, it can activate efferent vagus nerve fibers, leading to the release of

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acetylcholine (Ach) from peripheral nerve endings and the inhibition of the release of pro-inflammatory factors through intracellular signal transduction, thereby mitigating an excessive systemic inflammatory response (11-13). In sepsis, stimulation of the vagus nerve inhibits PMN activation and recruitment, decreases inflammation and improves survival rate (14). Guarini *et al* (15) demonstrated that stimulating the vagus nerve could attenuate the activity of NF-κB and inhibit the expression levels of MIP-2 and TNF-α, while simultaneously inhibiting the inflammatory cascade triggered by these factors and alleviating lung tissue damage. This protective effect was reversed following the administration of nicotinic receptor antagonists. Other studies have demonstrated that the cholinergic anti-inflammatory pathway associated with the suppression of inflammation is activated by the binding of acetylcholine released from vagus nerve endings to α-7 nicotinic acetylcholine receptors (α-7nACHR), which are found primarily on the surface of alveolar macrophages and alveolar epithelial cells (16,17). In a model of high tidal volume-induced lung injury (18), electrical stimulation of the vagus nerve was shown to decrease pulmonary edema and central granulocyte infiltration, thereby inhibiting lung tissue inflammation.

In summary, vagus nerve stimulation can decrease the release of inflammatory factors and affect the balance of pro- and anti-inflammatory cytokines, thus affecting the progress of ALI. However, the specific mechanism of these effects remains to be clarified. The present study investigated the role of the vagus nerve in ALI by establishing a model of LPS-induced lung injury, electrically stimulating the vagus nerve, measuring the levels of key inflammatory factors and determining the number of apoptotic cells in BALF. The present study aimed to provide a new theoretical basis for the diagnosis and treatment of ALI.

Materials and methods

Experimental animals. A total of 60 healthy and clean adult male (n=30) and female (n=30) Sprague-Dawley rats weighing 250±20 g were provided by the Animal Science Research Department of Nanchang University (Nanchang, China). Prior to the experiment, the animals were caged under normal conditions [normal atmospheric pressure, circadian rhythm (12-h light/dark cycle) and 40% humidity] and the room temperature was maintained at 22-24˚C. Stimulation with strong light and loud noises was avoided and normal circadian rhythm was maintained. Food and water were provided ad libitum. The present study was approved by the Medical Research Ethics Committee of The Second Affiliated Hospital of Nanchang University (http://www.chinalaw.gov.cn/news/node_search.html?q=%E5%8C%BB%E5%AD%A6%E4%BC%A6%E7%90%86&order=releasetime).

Animal treatment and experimental grouping. The rats were anesthetized with intraperitoneal injections of 40 mg/kg pentobarbital (Sinopharm Shanghai Shyndec Pharmaceutical Co., Ltd.; batch no. F20151216). The skin was shaved around the windpipe, the trachea opened and rats were mechanically ventilated. Ventilator parameters were: Tidal volume 2 ml/100 g; respiratory rate 60 beats/min; breathing ratio 1:1. The rats were randomly divided into four groups: Normal saline group (NS group), lipopolysaccharide group (LPS group), vagotomy group (LPS+VNB group) and vagus nerve stimulation group (LPS+VNS group; Fig. 1A). Each group included 15 rats. In the NS group, 5 mg/kg normal saline (Beijing Solarbio Science & Technology Co., Ltd.) was injected into the tail vein, the bilateral carotid sheaths were incised, and the bilateral vagus nerves were separated. In the LPS group, 5 mg/kg of 1% LPS (cat. no. L2880; Sigma-Aldrich; Merck KGaA) was injected into the tail vein, the bilateral carotid sheaths were incised, and the bilateral vagus nerves were separated. In the LPS+VNB group, 5 mg/kg of 1% LPS was injected into the tail vein, the bilateral carotid sheaths were opened, the bilateral vagus nerve was separated, the bilateral vagus nerve was cut and the stimulation electrode was connected to the distal end of the left vagus nerve proximal to the heart for continuous electrical stimulation (1 mA; 1 msec; 10 Hz). In the LPS+VNS group, 5 mg/kg of 1% LPS was injected into the tail vein, the bilateral carotid sheaths were opened, the bilateral vagus nerves were separated, stimulating electrodes were connected to the distal end of the left vagus nerve and continuous electrical stimulation (1 mA; 1 msec; 10 Hz) was applied. After 120 min, the ventilator was disconnected and the rats were euthanized by cervical dislocation under 40 mg/kg pentobarbital anesthesia.

HE staining. Rat lung tissue was taken for hematoxylin and eosin (HE) staining. Stains, Harris hematoxylin (Anatech Ltd. cat. no. 842), 1X stock for 2 min at 25˚C. Eosin (Anatech cat. no. 837), 1X stock for 15 sec at 25˚C. The stained sections were observed under an inverted light microscope (magnification x100 and x40; Olympus Corporation) and images were captured.

Flow cytometry analysis of the inflammatory cell apoptosis rate. BALF from the rats was collected in a 1.5 ml centrifuge tube 120 min after the ventilator was disconnected and the rats were euthanized by cervical dislocation under 40 mg/kg pentobarbital (Sigma-Aldrich; Merck KGaA, cat. no. P0900000-1EA) anesthesia, centrifuged at 1,006.2 x g for 20 min at 4˚C (Eppendorf), and the cell supernatant was discarded. 5 μl FITC-conjugated Annexin V and 10 μl propidium iodide (PI) was added to the cell pellet, incubated at 4˚C for 20 min, and the inflammatory cell apoptosis rate was measured by a flow cytometer (BD Biosciences, US6510007B1). A minimum of 10,000 events were acquired for each sample. The cytometer's inbuilt EXPO32 ADC software (Beckman Coulter, Inc.) was used for analysis.

RNA extraction. Total RNA was extracted from lung tissues using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA quality was ensured by distinct 18S, 28S and total RNA bands separated by electrophoresis in 1% agarose gel, The quantity of RNA was determined by A260 measurement (Carestream Gel Logic 2200 Pro imaging system, Carestream Molecular Imaging).

Protein extraction. Total proteins were extracted from the lung tissues by radio immunoprecipitation assay cell lysis reagent (C3702-120 ml; Beyotime Institute of Technology) containing proteinase and phosphatase inhibitors (Beijing Solarbio Science & Technology Co., Ltd.) at 4˚C for 30 min.

Table I. Primer sequences for reverse transcription-quantitative PCR.

Gene	Sequence
IL-1	(F) 5'-AGAAGCTTCCACCAATACTC-3'
	(R) 5'-AGAAGCTTCCACCAATACTC-3'
IL-8	(F) 5'-ATTTCAGCAGCTCTGTGTGAA-3'
	(R) 5'-TGAATTCTCAGCCCTCTTCAA-3'
IL-10	(F) 5'-AGGGCACCCAGTCTGAGAACA-3'
	(R) 5'-CGGCCTTGCTCTTGTTTTCAC-3'
IL-6	(F) 5'-AACTCCTTCTCCACAAGCG-3'
	(R) 5'-TGGACTGCAGGAACTCCTT-3'
β-actin	(F) 5'-CATGTACGTTGCTATCCAGGC-3'
	(R) 5'-CTCCTTAATGTCACGCACGAT-3'
TNF-α	(F) 5'-ATGAGCACTGAAAGCATGATC-3'
	(R)5'-TCACAGGGCAATGATCCCAAAGTAGACCTGCCC-3'

IL, interleukin; F, forward; R, reverse.

The cell extracts were centrifuged at 12,000 x g for 20 min at 4˚C. The supernatants containing total proteins were mixed with an equal volume of 5x sodium dodecyl sulfate (SDS) loading buffer (P0015L; Beyotime Institute of Technology) and the samples were heated to 95˚C for 5 min. All procedures were performed according to the manufacturers' instructions.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA (1 μg) extracted from lung tissues was used for RT-qPCR using the Reverse Transcription system (Takara Bio, Inc.) according to the manufacturer's protocol. RT-qPCR was performed using SYBR® Premix Ex Taq II (TliRNase H Plus; Takara Bio, Inc.) in the ABI PRISM® 7500 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with β-actin as an internal control. Thermocycling was performed in a final volume of 20 μl consisting of 40 cycles at 95˚C for 5 sec then 55˚C for 30 sec, following an initial denaturation step at 95˚C for 10 sec. The sequences of the PCR primers of IL-1, IL-6, IL-10, TNF-α and IL-8 are listed in Table I. The results were analyzed by the $2^{-\Delta\Delta Cq}$ method (19).

Western blot analysis. Protein samples were denatured in SDS sample buffer (125 mmol/l Tris-HCl; pH 6.8; 50% glycerol; 2% SDS; 5% mercaptoethanol; 0.01% bromophenol blue) and subjected to SDS-PAGE (5% concentration and 12% separation) and blotted onto Immobilon-FL transfer membranes (EMD Millipore). The blotted membranes were blocked at 4˚C with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (Beijing Solarbio Science & Technology Co., Ltd.) for 2 h and were subsequently incubated with primary antibodies against IL-1 (1:500, cat. no. 50794), IL-6 (1:400, cat. no. 12912), IL-10 (1:400, cat. no. 12163), IL-8 (1:100, ab110727 Abcam) and TNF-α (1:100, cat. no. 8184) proteins (all from Cell Signaling Technology, Inc. unless indicated otherwise) overnight at 4˚C. After three washes in Tris-buffered saline containing 0.1% Tween-20, the PVDF membranes were incubated with anti-mouse IgG (dilution 1:5,000, cat. no. SW1030, Beijing Solarbio Science & Technology Co., Ltd.) for 2 h at 25˚C. Then, the PVDF membranes were soaked in chemiluminescence reagents (cat. no. SW2030, Beijing Solarbio Science & Technology Co., Ltd.). Quantification of the western blotting was performed using a Bio-Rad imaging system (ChemiDoc MP v721BR06186; Bio-Rad Laboratories, Inc.).

Statistical analysis. The data were analyzed using SPSS 21.0 statistical software (IBM Corp.). Each experiment was performed in triplicate. The measurement data are expressed as the mean ± standard deviation. Statistical analysis was performed to determine the significance of the difference between groups using ANOVA with Tukey's honestly significant difference test used as the post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HE staining to observe the extent of lung tissue damage in each group. In the LPS group, the normal structure of the alveoli disappeared and notable amounts of inflammatory cell infiltration, pulmonary tissue congestion and edema were observed. In the LPS+VNB group, the alveolar structure was destroyed, inflammatory cell infiltration was observed, the lung tissue was congested and the alveolar wall and the interstitial space exhibited edema and increased thickness. In the LPS+VNS group, the alveolar structure was clearly distinguishable by different degrees of inflammatory cell infiltration and alveolar structure damage), a small amount of inflammatory cell infiltration was observed, there was slight thickening of the alveolar wall and lung interstitial space and a small amount of congestion in the lung tissue (Fig. 1B).

Detection of inflammatory cell apoptosis rates in BALF by flow cytometry. Compared with the NS group, the early and late inflammatory cell apoptosis rates in BALF from the LPS, LPS+VNB and LPS+VNS groups were significantly increased ($P<0.05$). The early and late inflammatory cell apoptosis rates in BALF from the LPS+VNS group were significantly lower

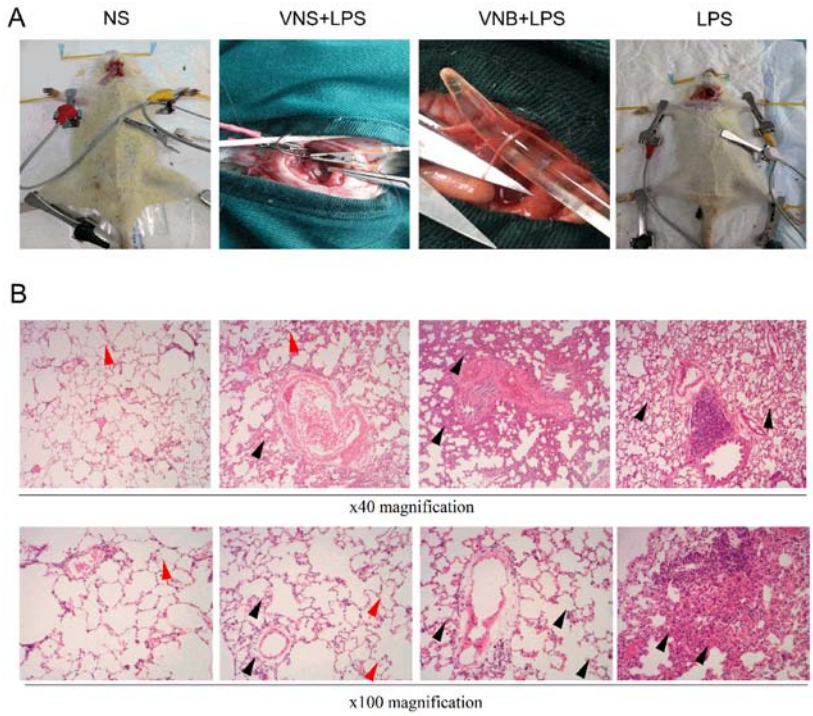

Figure 1. Lung operations and histology. (A) Operations in each group and (B) hematoxylin and eosin staining of lung tissue in each group. NS, normal saline; VNS, vagus nerve stimulation; LPS, lipopolysaccharide; VNB, vagotomy. Black arrow, inflammatory cell infiltration. Red arrow, alveolar structure.

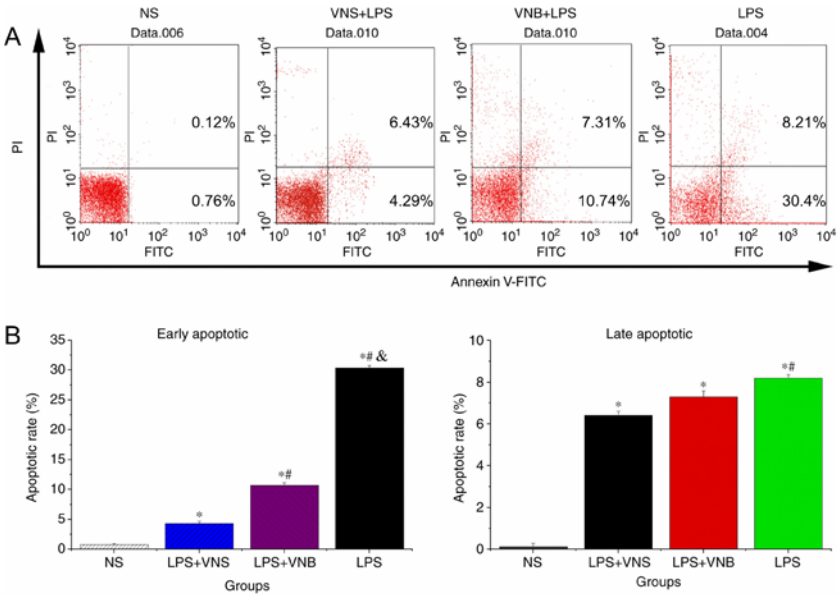

Figure 2. Flow cytometric analysis of apoptosis in bronchoalveolar lavage fluid inflammatory cells. (A) AnnexinV-FITC/PI double-staining fluorescence two-dimensional chart. (B) Bar graph analysis of inflammatory cell apoptosis. *P<0.05 vs. NS group, #P<0.05 vs. LPS+VNS group, &P<0.05 vs. LPS+VNB group. PI, propidium iodide; NS, normal saline; LPS, lipopolysaccharide; VNS, vagus nerve stimulation; VNB, vagotomy.

than those of the LPS group (P<0.05). Compared with the LPS group, the early apoptosis rate in BALF from the LPS+VNB group was significantly lower (P<0.05) and the late apoptosis rate was also lower, but the difference was not statistically significant (P>0.05; Fig. 2).

Detection of changes in IL-1, IL-6, IL-10, TNF-α and IL-8 in lung tissue mRNA levels by RT-qPCR. The expression levels of IL-6, IL-10, IL-8 and TNF-α mRNA in lung tissue from the LPS group were significantly higher than that of the NS group (P<0.05). In the LPS+VNS group, the expression levels of IL-1, IL-6, IL-8 and TNF-α mRNA were significantly lower than those of the LPS group (P<0.05) and the mRNA expression level of IL-10 was decreased in the LPS+VNS group compared with the LPS group, although the difference was not statistically significant (P>0.05). The expression levels of IL-6 and TNF-α mRNA in the LPS+VNB group were significantly lower than those in the LPS group (P<0.05) and the mRNA

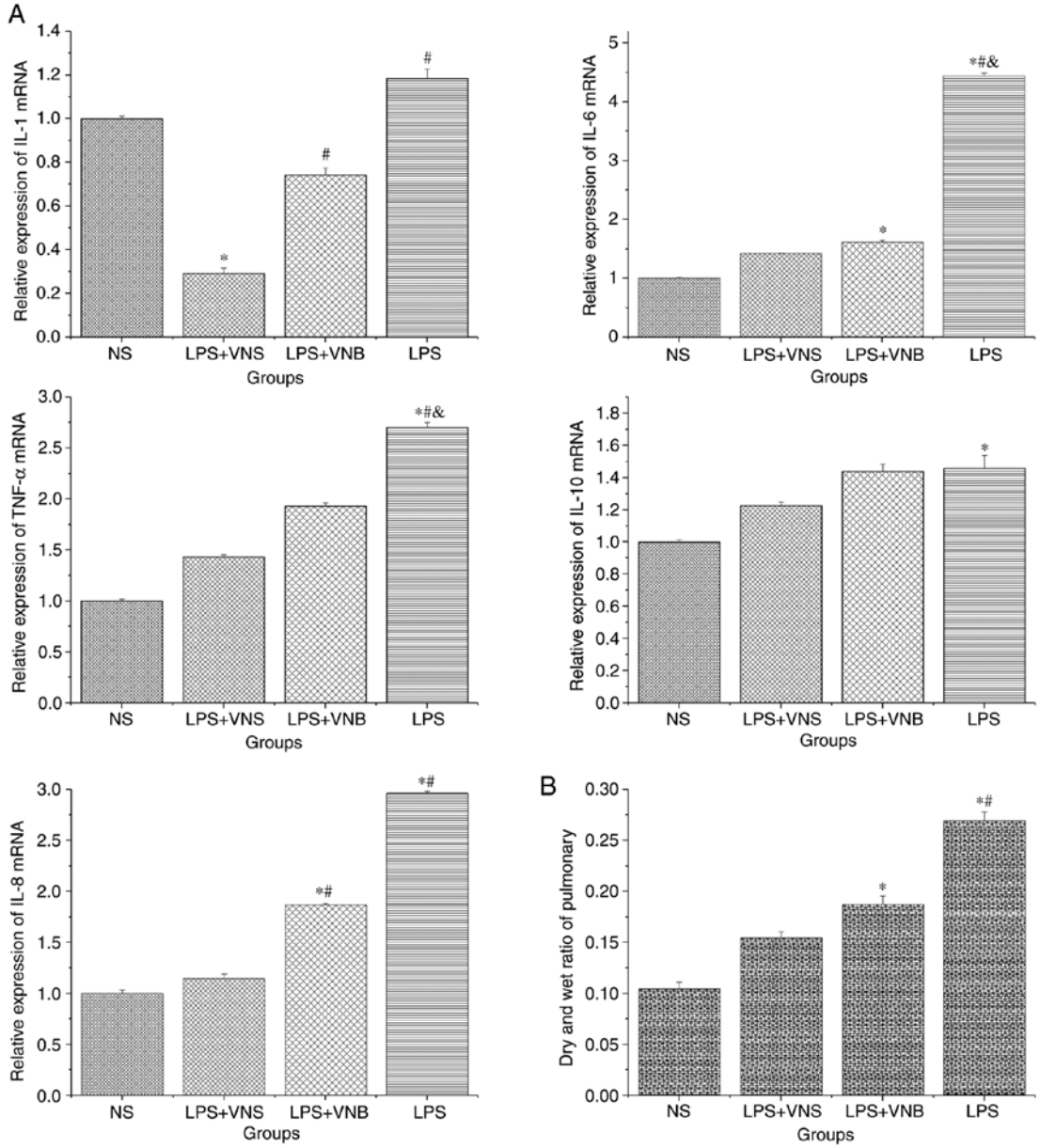

Figure 3. mRNA in lung tissues. (A) Detection of IL-1, IL-6, TNF-α, IL-10 and IL-8 mRNA in lung tissues by reverse transcription-quantitative PCR. (B) Ratio of dry-to-wet weight. *P<0.05 vs. NS group, #P<0.05 vs. LPS+VNS group, &P<0.05 vs. LPS+VNB group. NS, normal saline; LPS, lipopolysaccharide; VNS, vagus nerve stimulation; VNB, vagotomy.

expression levels of IL-1, IL-8 and IL-10 in the LPS+VNS group compared with were lower than those in the LPS group, although the difference was not statistically significant (P>0.05). The expression levels of IL-1 and IL-8 mRNA were significantly higher in the LPS+VNB group compared with the LPS+VNS group (P<0.05). The expression levels of IL-6, IL-10 and TNF-α mRNA were comparatively higher in the LPS+VNB group compared with the LPS+VNS group, but the difference was not statistically significant (P>0.05; Fig. 3A).

Western blot analysis of protein expression levels of IL-1, IL-6, IL-10, IL-8 and TNF-α in lung tissue. The expression levels of IL-1, IL-6, IL-10, IL-8 and TNF-α in lung tissue from the LPS group were significantly increased compared with those of the NS group (P<0.05). In contrast, in the LPS+VNS group, the expression levels of IL-1, IL-6, IL-10, IL-8 and TNF-α protein in lung tissue were significantly lower than those in the LPS group (P<0.05). In the LPS+VNB group, the expression levels of IL-1, IL-6, IL-10, IL-8 and TNF-α in lung tissue were significantly increased compared with those of the LPS+VNS group (P<0.05), whereas the expression levels of IL-1 and IL-10 protein were significantly decreased compared with those of the LPS group (P<0.05). The expression levels of TNF-α and IL-8 protein in the LPS+VNB group were not significantly different from those of the LPS group (P>0.05), whereas the expression level of IL-6 protein was significantly increased compared with the LPS group (P<0.05; Fig. 4).

Wet/dry lung weight ratio determination. The wet/dry lung weight ratio of the LPS group was significantly higher than that of the NS group (P<0.05), whereas the wet/dry lung ratio of the LPS+VNS group was significantly lower than that of the LPS group (P<0.05).

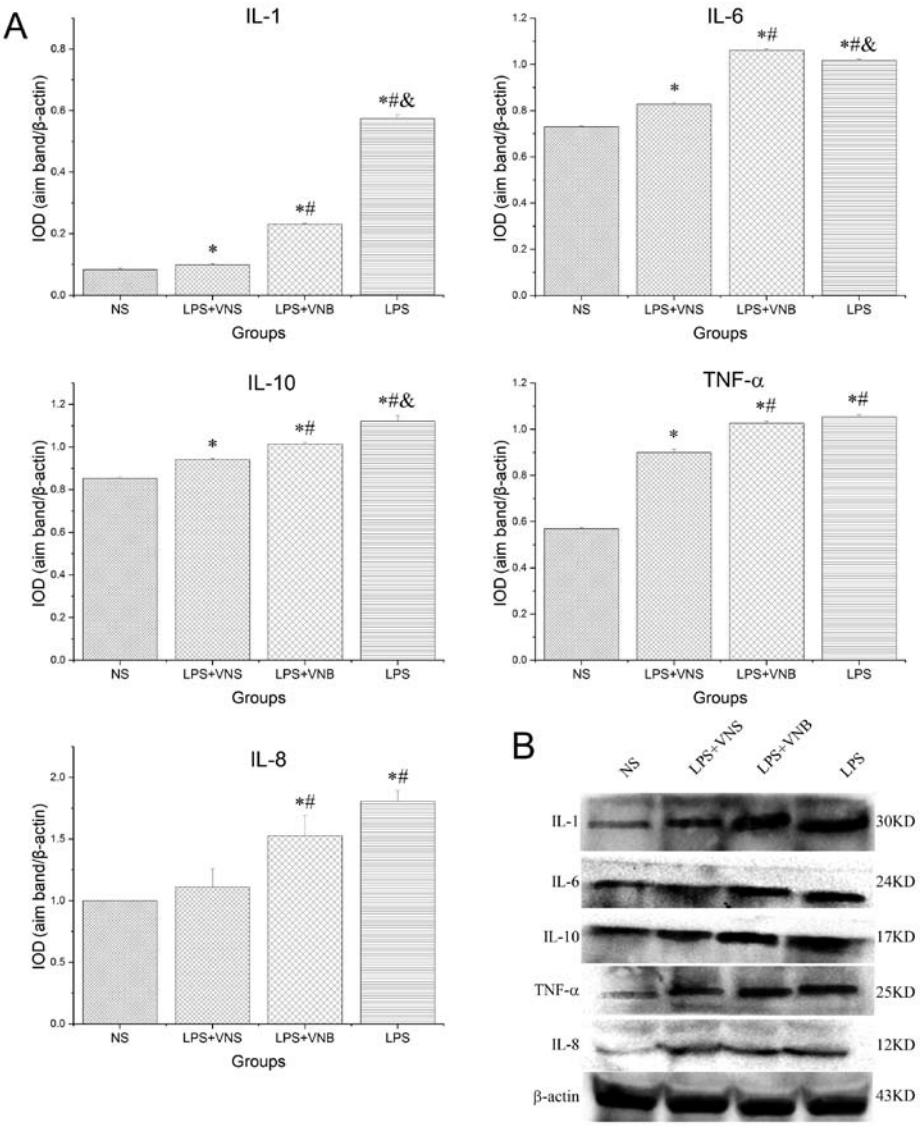

Figure 4. (A) Detection of IL-1, IL-6, IL-10, TNF-α and IL-8 protein expression levels in lung tissue by (B) western blot. *P<0.05 vs. NS group, #P<0.05 vs. LPS+VNS group, &P<0.05 vs. LPS+VNB group. NS, normal saline; LPS, lipopolysaccharide; VNS, vagus nerve stimulation; VNB, vagotomy; IOD, integral optical density.

The wet/dry lung ratio of the LPS+VNB group was compared with that of the LPS group and showed a decrease, but the difference was not statistically significant (P>0.05; Fig. 3B).

Discussion

Since Borovikova *et al* (20) proposed the concept of a cholinergic anti-inflammatory pathway (CAP) in 2000, the role of CAP in regulating inflammation has become a topic of interest. Situated between the immune system and the nervous system, CAP consists of the vagus nerve and releases Ach, which suggests a new approach to the treatment of critical illnesses caused by excessive inflammation. The molecular basis of the close association between the immune system and the nervous system is α7nACHR, which is expressed on the surface of alveolar macrophages and alveolar epithelial cells. After receiving immune stimulation, the central nervous system activates the vagus nerve to send out nerve fibers, releasing Ach and α7nACHR, and activating the anti-inflammatory pathway, decreasing the recruitment and infiltration of inflammatory cells and decreasing the inflammatory response in lung tissue, thereby exerting a pulmonary protective effect (21-23).

A number of studies have demonstrated that CAP may inhibit lung tissue inflammation caused by LPS and decrease damage to microvascular endothelial cells and alveolar epithelial cells, thus contributing to lung protection (24-26). Reports have confirmed that stimulation of the vagus nerve (via electrical stimulation or drug agonist) could significantly improve the prognosis of ALI caused by burns, multiple injuries, hemorrhagic shock and severe infection (27-30). These studies demonstrate that vagus nerve stimulation may be an important breakthrough in treatment strategies for ALI.

Su *et al* (17) reported that α7nACHR agonists inhibited NF-κB activity and the production of pro-inflammatory factors (such as MIP-2 and TNF-α) in alveolar macrophages, and decreased the migration and accumulation of neutrophils and lung edema. dos Santos *et al* (18) used the model of ventilator-induced lung injury caused by high tidal volume

to demonstrate that vagus nerve separation could aggravate pulmonary edema and neutrophil infiltration, accelerate epithelial cell apoptosis and increase IL-6 expression levels, but that electrical stimulation of the vagus nerve decreased inflammation in the lung tissue, apoptosis of epithelial cells and the expression levels of IL-6, and alleviated the symptoms of lung injury. In vivo experiments with septic shock caused by endotoxins demonstrated that vagal nerve stimulation with drugs (such as CNI-1943) or electrophysiological methods inhibited the release of TNF-α and decreased the systemic inflammatory response (18). However, in the vagus nerve separation group, treatment of inflammation was far less effective than in the vagus nerve stimulation group (31).

TNF-α is the first cytokine to be released during the inflammatory response. It can directly stimulate alveolar endothelial cells or indirectly stimulate macrophages and granulocytes to promote their migration, recruitment and infiltration, further stimulating the release of IL-1 and IL-6, and induce lung injury (6). IL-1 and IL-6 are cytokines with roles in cell proliferation and differentiation in inflammatory and immune responses and can promote the migration and recruitment of inflammatory cells (32,33). IL-10 originates from Th2 cells and certain regulatory T-cells and inhibits the antigen presentation and cytokine synthesis functions of macrophages. It has a dual pro-inflammatory and anti-inflammatory role and its expression level is significantly increased in the early stage of ALI, in which the pro-inflammatory effect is predominant (34).

The present study demonstrated that LPS+VNS significantly decreased the inflammatory response of ALI, the damage to lung tissues, the infiltration of inflammatory cells, edema in the alveolar wall, alveolar expansion and the apoptosis rate in BALF cells. Elevated IL-1, IL-6, IL-10 and TNF-α protein expression levels were inhibited and lung protection was achieved. After the vagus nerve was separated in the present study, it was electrically stimulated, and it was demonstrated that the inflammatory reaction of the lung tissues was inhibited. IL-1 and IL-10 protein expression levels were significantly decreased and the expression level of IL-6 protein was significantly increased, whereas the RT-qPCR assay showed that IL-6 mRNA expression levels were significantly lower in the LPS+VNS group than in the LPS group. IL-6 is a B-cell growth factor produced by activated macrophages and is considered to be a cytokine produced early in the inflammatory response (35). A previous study revealed that elevated levels of IL-6 were closely associated with the severity of lung injury caused by acute pancreatitis (36). The results of the present study suggest that it is possible that protein expression is affected by the regulation of a certain step in the mRNA translation pathway.

Compared with the LPS+VNB group, the VNS+LPS group had a more pronounced effect on preventing the inflammatory response. This statistically significant difference suggests that when the integrity of the vagus nerve was destroyed, electrical stimulation of the vagus nerve could not achieve pulmonary protection, and that intactness of the vagus nerve was an important factor in ensuring that electrical stimulation of the vagus nerve could regulate inflammation in the pulmonary protection mechanism.

Compared with the LPS groups, levels of inflammatory cytokines in the LPS+VNB group were significantly decreased. To the best of our knowledge, this phenomenon has not previously been reported. Studies suggest that following vagotomy, inhibition of vagal nerve activity on the heart is decreased, resulting in the acceleration of heart rate (37), the acceleration of cardiopulmonary blood flow (38), the shortening of the stay time of inflammatory cells in the pulmonary capillaries and the relative decrease in the exudation of inflammatory cells.

That the death rate was not compared with longevity and the lack of an explanation for why the inflammation cytokines in the LPS+VNB group were significantly decreased compared with to LPS group were limitations of the present study; the BALF may have also contained non-inflammatory cells, such as alveolar epithelial cells. This will be considered in future research.

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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

YZ and LW made substantial contributions to the conception or design of the work. WH and YW analyzed or interpreted data. WH, drafted the manuscript or revised it critically for important intellectual content. YW, BC and LX interpreted data and agreed to be accountable for the work and ensuring that questions related to the integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The use of animals in this study was approved by the animal research committee in the Second Affiliated Hospital of Nanchang University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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