**Original Article**

*Protective effects of neurotensins on lipopolysaccaride-induced acute lung injury by blocking tachykinin mediated pathway*

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**Abstract:** Neurotensin, a bioactive tridecapeptide, has been shown to regulate inflammatory process in lung tissues. However, the effect of neurotensin on LPS-induced lung injury and underlying detailed molecular mechanisms has not been studied. The aim of present study is to investigate the effect of neurotensin on LPS-induced acute lung injury in mice. Mice were treated with LPS intratracheally to induce acute lung injury. 1 hour after ALI induction, and then mice were treated with neurotensins (NTs) (20 mg/kg, 40 mg/kg, and 80 mg/kg) via tail vein injection. Next, the severity of lung injury, MPO activity, neutrophils infiltration, lung edema, protein and pro-inflammatory cytokines concentration in BALF were determined to evaluate the effect of Nts on ALI. Additionally, the expression of tachykinins receptors, including NK1, NK2, and NK3 and the production of IL-8, COX-2, and PGE₂ mediated by tachykinins-tachykinins receptors pathway were determined to investigate the blocking effect of Nts on tachykinins and its receptors pathway. Neurotensins treatment significantly decreased the lung edema and the infiltration of inflammatory cells into lung tissue caused by LPS induction. Meanwhile, the elevation of pro-inflammatory cytokines and chemokine in BALF was dramatically reduced by neurotensins treatment. Furthermore, neurotensins could interact with tachykinins receptors and block the inflammatory responses activated by tachykinins pathways. In summary, neurotensins has a potentially protective effect on LPS-induced acute lung injury through the interaction with tachykinins receptors and subsequently blocking the inflammatory responses induced by activation of tachykinins pathway.

**Keywords:** Neurotensin, inflammation, acute lung injury, tachykinins, COX-2, PGE₂

**Introduction**

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are devastating and life-threatening syndromes and a major cause of death in critically ill patients [1]. ALI is characterized by hypoxemia and respiratory failure due to exudation to alveolar spaces which impairs gas exchange. Histologically, ALI in humans is characterized by a severe acute inflammatory response in the lungs and neutrophilic alveolitis [2, 3]. The risk factors for developing this syndrome include pneumonia, gastric aspiration, sepsis, shock, and acute pancreatitis. A major cause for development of ALI is sepsis, wherein Gram-negative bacteria are a prominent cause. Lipopolysaccharides (LPSs) are the main components of the outer membrane of gram-negative bacteria and act as basic mediators that host the inflammatory sequelae after a gram-negative bacte-
lar permeability, stimulation of mast cells, B cells, T-lymphocytes, and macrophages, additionally, they also could induce the infiltration of neutrophils. Meanwhile, the release of SP and NKA also induces microvascular reactions such as vasodilatation and plasma extravasation which contribute to the edema formation of lung tissue [11, 12].

The effects of SP and NKA on inflammatory responses are mediated by the neurokinin 1 and 2 (NK1, NK2) receptors [13]. Both receptors are widely expressed in peripheral tissues. In addition, the expression of NK receptors could be induced by inflammatory stimulations. Accumulated data reported that the activation of NK1 and NK2 receptors could trigger a number of biological responses, including microvascular leakage [14], mucus secretion [15], and inflammatory cell response [16, 17]. Elevated levels of SP and NKA have been observed in the airways of patients with chronic obstructive pulmonary disease and asthma. Preclinical studies have suggested that the tachykinin NK1 and NK2 receptors play an important role in bronchoconstriction, airway hyperresponsiveness and airway inflammation caused by allergic and nonallergic stimuli [18]. Therefore, the antagonists that could block these tachykinin receptors hold promise for the treatment of airways diseases, like COPD or ALI.

Neurotensin (NT) is a bioactive tridecapeptide that is widely distributed through the brain and the gastrointestinal tract [19, 20]. It has been shown to regulate a wide range of biological functions like inflammatory process in the lung [21]. Moreover, NT could interact with a number of immune cells, including leukocytes, peritoneal mast cells, and dendritic cells [22, 23]. In particular, due to the direct interaction of NT with macrophages, NT are important in modulating macrophage function and inhibiting the production of proinflammatory cytokines, suggesting a protective effect in inflammatory conditions [24, 25].

Therefore, in present study, we aimed to investigate the role of NT during acute lung inflammation using the LPS-induced acute lung injury (ALI) model, a well accepted model for ARDS. Our results demonstrated that NT has a potentially protective effect on LPS-induced ALI through interacting and blocking tachykinin receptors in ALI mouse.

Materials and methods

Mice

100 specific-pathogen free C57BL/6 mice (20-25 g, 6-8 weeks, male =50, female =50) were obtained from the Shanghai Laboratory Animal Center (SLAC) (Shanghai, China) and housed in stainless-steel cages in a room maintained at 22 ± 1°C and a 12-hour light/dark cycle controlled environment with free access to food and water. Animal housing conditions and experimental procedures conformed to institutional regulations and were in accordance with the National Institute of Health guidelines for animal care, and all experiments were conducted were approved by the local Animal Care and Use Committee.

Induction of acute lung injury

Mice were anesthetized by an intraperitoneal injection of thiopental (37 mg/kg), and then were fixed on operation platforms. Next LPS (60 μg in 60 μl of PBS; Sigma-Aldrich, St. Louis, MO) was treated intratracheally. Neurotensins (Nts) treated started at 1 hour after ALI induction.

All animals were randomly divided into five groups: (1) LPS+ Vehicle group (n=20, male =10, female =10), mice were subjected to LPS-induced ALI as described above and received saline via tail vein injection 1 hour after the induction of ALI. (2) LPS+20 mg Nts group (n=20, male =10, female =10), mice were subjected to LPS-induced ALI and treated with 20 mg/kg Nts via tail vein injection at 1 hour after ALI induction. (3) LPS+40 mg Nts group (n=20, male =10, female =10), mice were subjected to LPS-induced ALI and treated with 40 mg/kg Nts via tail vein injection at 1 hour after ALI induction. (4) LPS+80 mg Nts group (n=20, male =10, female =10), mice were subjected to LPS-induced ALI and treated with 80 mg/kg Nts via tail vein injection at 1 hour after ALI induction. (5) Control group (n=20, male =10, female =10), identical to the LPS+ vehicle group, but mice received intra-tracheal administration of saline, instead of LPS.

Bronchoalveolar lavage (BAL) fluid collection

Twenty-four hours after ALI induction, the bronchoalveolar lavage (BAL) fluid was collected as previously described [26]. Briefly, Mice were euthanised and the tracheas were immediately
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cannulated with an I.V. polyethylene catheter equipped with a twenty-four-gauge needle on a 1-ml syringe. Next, lungs were lavaged once with 1.0 ml PBS for three times. The recovery percentage were >95%. Then the collected BALF were centrifuged at 1000 g for 5 min at 4°C, the supernatants were stored at -80°C for further experiments and the pelleted cells were resuspended with 0.5 ml PBS to determine the total BAL cells. The amount of proteins in the BALF was measured using a Bradford Protein assay kit (Beyotime Biotech Inc., Jiangsu, China).

**Measurement of myeloperoxidase (MPO) activity**

Myeloperoxidase activity was measured as described previously [27]. Briefly, the lungs were removed after BALF was collected and then stored at -80°C for further assay. For measurement, the lungs were homogenized 1 ml of 20 mM potassium phosphate buffer which containing 5% hexadecyl trimethyl ammonium bromide (PH 7.0) and centrifuged at 17,000 rpm for 30 min at 4°C. Next the supernatant was mixed with a solution of tetramethylbenzidine (1.6 mM) and 0.1 mM-H2O2 to react. The absorbance was measured spectrophotometrically at 650 nm by a spectrophotometer (DU 640B; Beckman Coulter, Inc.). Myeloperoxidase activity was calculated as the quantity of enzyme degrading 1 μm of peroxide per minute at 37°C and expressed in unit per gram weight of wet tissue.

**Determination of lung wet to dry weight (W/D) ratio of lung**

The measurement of lung edema was described previously [26]. In brief, the lung tissues were excised and rinsed using PBS solution, next the wet weight was measured immediately. Then the lungs were dried at 70°C for 4 days in an oven. Then the dry weight was measured. The wet-to-dry ratio was calculated through dividing the wet weight by the dry weight.

**Measurement of cytokines in BALF**

The levels of cytokines TNF-α, IL-6, IL-1β and MCP-1 in BALF were determined by ELISA kits purchased from R&D system (Minneapolis, MN, USA) according to the manufacturer’s protocols.

**Real-time PCR assay**

The lung tissues were removed and the total mRNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA). Next the cDNA was synthesized using a Prime-Script RT-PCR kit (Takara Bio Company, Shanghai, China), and the expressions of NK1, NK2, and NK3 were examined with the polymerase chain reaction (PCR) using an ABI7900fast qPCR detection system (Applied Bio System). Primer sequences as follows: NK1, Forward, 5’-TTCCCAAC-ACCTCCACAA-3’; Reverse, 5’-AGCCAGGACCC-AGATGACAA-3’; NK2, Forward, 5’-TGCTGTCA-TGCGTGCTAG-3’; Reverse, 5’-TCTTCCTCGGT-TGATTCC-3’; NK3, Forward, 5’-CATCTCAGT-GCATCACC-3’; Reverse, 5’-CTTTGCGGCC-GAAGCTT-3’.

**Western blotting detection**

The lung tissues were homogenated and the total proteins in the lung tissues were extracted with a protein extraction kit (Beyotime, Shanghai, China) according to the manufacturer’s instructions. A BCA Protein Assay Kit (Beyotime, Shanghai, China) was employed to determine the concentration of total proteins. Then the samples were boiled for 5 min with loading buffer. Next the western blot assay was performed as follows: Protein samples (50 μg) were separated by denaturing 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using standard protocols. Next the proteins were transferred to PVDF membranes. Then the membranes were incubated with indicated primary antibodies for 2 h at room temperature after blocking with 5% skimmed milk. Afterward, the membranes were further incubated with horseradish peroxidase-conjugated (HRP) secondary antibodies for 1 h at room temperature, and finally the results were visualized using enhanced chemiluminescence reagents (Bio-Rad Laboratories). The data was quantified with ImageJ 1.48v software.

**Measurement of COX-2 and PGE2 in BALF with ELISA assay**

The protein concentrations of COX-2 and PGE2 in BALF were determined with an ELISA assay. Briefly, the microtiter plates were pre-coated with indicated antibodies. And then the samples were added to the appreciated microtiter plate wells with a biotin-conjugated polyclonal
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Antibody. Next, the avidin conjugated horseradish peroxidase was added and incubated for appreciated time. Then a tetramethylbenzidine substrate solution was added to each well and the changes of substance color was measured at 450 nm with a spectrophotometer (DU 640B; Beckman Coulter, Inc.).

Survival rate

Mice were divided into four groups: (1) control group, in which the animals were not treated (Ctr group, n=20); (2) LPS group, in which the animals were treated with LPS only (LPS group, n=20); (3) 80 mg/kg Nts treatment group, in which the mice were treated with 80 mg/kg Nts followed by treatment with LPS (LPS+80 mg/kg group, n=20); (4) CP-96345 treatment group, in which the mice were treated with CP-96345 followed by ALI induction (LPS+ CP, n=20). Then the survival rate was evaluated 120 h following treatments.

Statistical analysis

Survival rates were compared by Kaplan-Meier log rank test. Data were expressed as mean ± standard deviation. All statistical analysis was performed with SPSS 17.0 software package (SPSS Inc, Chicago, IL). Statistically significant differences between groups were determined by ANOVA followed by Tukey’s test. Results were considered statistically significant if P values were <0.05.

Results

LPS stimulation enhanced the expression of tachykinins receptors in ALI mice

Previous study has been documented that tachykinins, such as substance P (SP) and neurokinin A (NKA), and their receptors, including NK1, NK2, and NK3, play a critical role in the process of inflammatory responses during acute lung injury induction. Therefore, the expression of the tachykinins receptors, including NK1, NK2, and NK3, were determined using Real-time PCR method and Western blot method. As shown in Figure 1, we observed that the mRNA levels (Figure 1A) and the protein levels (Figure 1B) of different tachykinins receptors was significantly increased in the lung tissues from LPS-induced ALI mice compared with those control group mice. These results were consistent with the previous studies.

Nts treatment prevent mice from LPS induced acute lung injury

As a tridecapeptide, previous study has been reported that neurotensin has a potential role on the downregulation of proinflammatory gene expression [23, 28]. Therefore, to investigate the effect of neurotensins on LPS-induced acute lung injury, we treated LPS-induced ALI mice with neurotensins in different dosages via tail vein injection 1 h after ALI induction. As shown in Figure 2, we observed that LPS treatment induced severe lung injury in mice compared with PBS treated control group mice (Figure 2A), including obvious hyperaemia and edema, necrosis and desquamation of epithe-
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Neurotensins treatment reduced the increasing of MPO activity, neutrophils infiltration, lung wet/dry ratio and protein concentration in BALF caused by LPS induction

To further evaluate whether neurotensins treatment could inhibit the infiltration of inflammatory cell into the lung tissue, we measured the MPO activity and the infiltrated neutrophils in BALF. As shown in Figure 3, we observed that the increasing of MPO activity in the lung tissues (Figure 3A) and the infiltration of neutrophils in BALF (Figure 3B) of ALI mice caused by LPS induction were dramatically inhibited by the treatment of neurotensins with different dosages.

Next, we examined the effect of neurotensins on the lung edema caused by LPS induction. We found that the elevation of lung tissue wet/dry ratio (Figure 3C) and protein concentration in BALF (Figure 3D) caused by LPS stimulation were significantly reduced by the administration of neurotensins compared with those no treated ALI control group mice. These results were consistent with the date about the lung injury index.

Neurotensins attenuate LPS-induced production of inflammatory cytokines and chemokine

A number of studies have reported that inflammatory response or inflammation play an important role in the pathogenesis of LPS-induced lung injury. Thus we detected the pro-

Figure 2. The effects of Nts on the lung histopathological changes. The lung tissues isolated from differentially treated ALI mice were stained with hematoxylin and eosin (×200). A. The lung section from of a control mice; B. The lung section from of LPS-induced ALI mice; C. The lung section from of 20 mg/kg Nts treated ALI mice; D. The lung section from 40 mg/kg Nts treated ALI mice; E. The lung section from 80 mg/kg Nts treated ALI mice. F. The lung injury index of differentially treated ALI mice. Results are expressed as mean ± SD. *P<0.05 compared with the control group; #P<0.05 compared with the LPS-induced ALI group.
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As shown in Figure 4, the production of inflammatory cytokines and chemokine, including TNF-α (Figure 4A), IL-6 (Figure 4B), IL-1β (Figure 4C), and MCP-1 (Figure 4D), were significantly increased by LPS induction compared with those from the control group. However, these increasing of inflammatory cytokines and chemokine such as TNF-α (Figure 4A), IL-6 (Figure 4B), IL-1β (Figure 4C), and MCP-1 (Figure 4D) caused by LPS induction was restored by neurotensins treatment in different dosages compared with the vehicle treated control group mice. These results indicated that neurotensins has a potentially anti-inflammatory activity.

Neurotensins blocked the expression of IL-8 mediated by NR1 in vitro

Accumulated evidences have been demonstrated that tachykinins and they mediated pathways play a key role in the process of airway diseases, like COPD. Moreover, the release of tachykinins from inflammatory cell, such as eosinophils, monocytes, macrophages, lymphocytes and dendritic cell might further activate and stimulate the inflammatory responses in an autocrine or paracrine fashion through activation of tachykinins-their receptors mediated pathway [30]. Therefore, tachykinin receptor antagonists were considered to be an effectively therapeutic strategy for airway diseases. Our above results also observed that neurotensins treatment could significantly protect mice form LPS-induced acute lung injury. Based on the above observations, we hypothesized that whether neurotensins could be used as a tachykinin receptor antagonist and block the tachykinin pathway mediated inflammatory responses in LPS-induced ALI mice. To examine the antagonistic effect of neurotensins on tachykinin receptor, NK1, the mice microphage cell line Raw264.7 cell was employed. As shown in Figure 5, compared with the control group, SP stimulations dramatically increased the expression (Figure 5A) and production (Figure 5B) of IL-8 in Raw264.7 cells, however, these elevations caused by SP stimulation were significantly restored by neurotensins treatment. Furthermore, we also observed that the antagonistic effect of neurotensins on SP trigged IL-8 production was similar to CP-96345 (Figure 5), a specific tachykinin NK1 receptor antagonist. These results suggested that neurotensins may attenuate LPS-induced inflammation in ALI mice through blocking the activation of tachykinin NK1 receptor.

Neurotensins attenuate LPS-induced acute lung injury by blocking the production of COX-2 and PGE₂ stimulated by substance P

Previous study has demonstrated that COX-2 and PGE₂ play an important role in the pathogenesis of ALI or ARDS [31]. Furthermore, researchers also reported that the activation of SP-NK1 pathway could promote the expression of Cyclooxygenase-2 and Prostaglandin
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E 2 in vitro [32]. To further investigate whether neurotensins attenuate LPS-induced ALI through blocking the activation of SP-NK1 pathway, we examined the production of COX-2 and PGE$_2$ in BALF of differentially treated ALI mice. We observed that the elevation of COX-2 (Figure 6A) and PGE$_2$ (Figure 6B) in BALF caused by LPS induction was dramatically reduced by neurotensins treatment in different dosages. Next, to further evaluate the antagonistic effect of neurotensins on SP-NK1 pathway activation in vitro, we measured the production of COX-2 and PGE$_2$ in Raw264.7 cells in the presence of neurotensins with different concentrations. As showed in Figure 6, we found that the addition of SP could significantly increase the production of COX-2 (Figure 6C) and PGE$_2$ (Figure 6D) compared with control group. However, the increasing of COX-2 and PGE$_2$ production caused by SP stimulation was dramatically decreased by the addition of neurotensins (Figure 6C and 6D). This antagonistic effect was similar to CP-96345.

Neurotensins improve the survival rate of LPS-induced ALI mice

To further assess the protective effect of neurotensins on LPS-induced lung injury, we determined the mortality of differentially treated ALI mice caused by LPS induction. As shown in Figure 7, compared with the LPS-induced ALI group, we observed that the administration of neurotensins significantly increased the survival rate of ALI mice; this was similar to the CP-96345.

Discussion

Previous study has been documented that the overwhelming lung inflammation was the major characters of acute lung injury and acute respiratory distress syndrome [33]. Due to the LPS administration could stimulate a series of

Figure 4. Effect of Nts on the production of inflammatory cytokines and chemokine in BALF from differentially treated ALI mice. A: The level of TNF-α in BALF from differentially treated ALI mice; B: The level of IL-6 in BALF from differentially treated ALI mice; C: The level of IL-1β in BALF from differentially treated ALI mice; D: The level of MCP-1 in BALF from differentially treated ALI mice. Results are expressed as mean ± SD. *P<0.05 compared with the control group; #P<0.05 compared with the LPS-induced ALI group.

Figure 5. Nts treatment blocks the expression of IL-8 mediated by NR1 in vitro. A: The mRNA level of IL-8 in differentially treated RAW264.7 cells; B: The protein level of IL-8 in differentially treated RAW264.7 cells. Results are expressed as mean ± SD. *P<0.05.
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In this study, we employed LPS-induced acute lung injury mouse model to investigate the therapeutic effect of neurotensins on acute lung injury. Accumulated evidences have been indicated that as a tredecapeptide, neurotensins could effectively regulate inflammatory responses in different diseases [23, 39, 40]. The experimental results also demonstrated that the administration of neurotensins could effectively attenuate LPS-induced acute lung injury, including decreasing the lung edema, MPO activity in lung tissues, and the infiltration of inflammatory cells into the lung tissue. In the process of the acute lung injury, inflammation plays an important role in the disruption of the alveolar space and epithelial endothelial barrier [37, 38]. Therefore, inhibition of inflammatory responses during acute lung injury is considered as a potential approach for ALI treatment.

The infiltration of inflammatory cells into lung tissues [41, 42] and the lung edema [43] are typical feathers of acute lung injury. To evaluate the effect of neurotensins on acute lung injury, we measured the MPO activity in lung tissues and the infiltrated inflammatory cell in BALF, we observed that neurotensins significantly reduced the elevation of MPO activity.
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(Figure 2A) and infiltrated inflammatory cells (Figure 2B) caused by LPS stimulation compared with those from LPS-induced ALI mice. Furthermore, we also evaluate the magnitude of lung edema by lung wet/dry ratio and protein content in BALF. The results showed that neurotensins treatment dramatically decreased the lung wet/dry ratio (Figure 2C) and protein concentration (Figure 2D) in BALF induced by LPS. These results were consistent with the data of histological analysis and indicated that neurotensins has a therapeutic effect on LPS-induced acute lung injury.

Pro-inflammatory mediators, such as TNF-α, IL-1β, IL-6, and MCP-1, have been considered to be the important pathogenesis of ALI [37, 44]. The excessively released inflammatory cytokines contributed to the severity of lung injury. Our results showed that LPS induction significantly increased the production of inflammatory cytokines in lung tissues compared with the no treated control mice, however, the increasing of inflammatory cytokines and chemokine, including TNF-α (Figure 3A), IL-1β (Figure 3B), IL-6 (Figure 3C), and MCP-1 (Figure 3D) caused by LPS induction were dramatically decreased by neurotensins treatment in different dosages.

The tachykinins are one of the most intensively studied neuropeptides. The tachykinins, particularly substance P and neurokinin A, are expressed in the airway never cells and some immune cells. Previous studies have been observed that the expression of tachykinins NK1 and NK2 receptors were upregulated by stimulation of the airway inflammations. Additionally, the release of SP and NKA also promotes the development of airway diseases. In our present study, we observed that the expression of IL-8 caused by the activation of SP and NK1 receptor mediated pathway was significantly inhibited by treatment of neurotensins with different concentrations in vitro (Figure 5). Previous study has been demonstrated that substance P could stimulate the expression cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) [32], additionally, COX-2 and PGE2 also play critical roles in the pathogenesis of ALI. In our study, we observed that neurotensins treatment significantly reduced the concentration of COX-2 (Figure 6A) and PGE2 (Figure 6B) in BALF, meanwhile, we also observed that the productions of COX-2 (Figure 6C) and PGE2 (Figure 6D) induced by substance P were dramatically inhibited by neurotensins treatment in vitro.

In summary, our study demonstrated that neurotensins treatment could effectively attenuate LPS-induced acute lung injury, including the changes of lung histopathology, the severity of lung edema, the infiltration of inflammatory cell and the production of inflammatory cytokines in vivo. Furthermore, we also found that neurosentin could interact with tachykinins receptors and block the inflammatory responses caused by substance P activation in lung tissues. Although our study found the therapeutic effect of neurotensins on LPS-induced acute lung injury, further and more comprehensive investigations are still needed before the application of neurotensins on ALI treatment.

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Disclosure of conflict of interest

None.

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