Recombinant human brain natriuretic peptides exert protective effects against lipopolysaccharide-induced acute respiratory distress syndrome in rats by inhibiting angiotensin-converting enzyme/angiotensin II pathways

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Abstract: Angiotensin-converting enzyme (ACE)/angiotensin II (Ang II) pathways have a close relationship with acute respiratory distress syndrome (ARDS). This study aimed to assess the protective effects of recombinant human brain natriuretic peptides (rhBNP) against lipopolysaccharide (LPS)-induced ARDS in rats, investigating its effects on ACE/Ang II pathways. Sprague-Dawley rats were randomly divided into 4 groups: normal control group, model group, low-dosage rhBNP group, and high-dosage rhBNP group. The current study investigated the protective effects of rhBNP by measuring lung wet-dry ratios. This study employed histological analysis using hematoxylin-eosin (H&E) staining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. Ang II, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) levels in lung tissue homogenates were detected by ELISA. ACE levels in the lung tissues were measured by Western blotting. Compared with the model group, wet-dry ratios, histological scores, and pneumocyte apoptosis were significantly reduced in both the low-dosage and high-dosage rhBNP groups. Furthermore, compared with the model group, ACE expression in lung tissues and levels of Ang II, IL-1β, and TNF-α in lung tissue homogenates were significantly reduced in low-dosage and high-dosage rhBNP groups. Present results suggest that rhBNP may exert protective effects against LPS-induced ARDS by inhibiting ACE/Ang II pathways.

Keywords: Recombinant human brain natriuretic peptide, acute respiratory distress syndrome, angiotensin-converting enzyme, angiotensin II, lipopolysaccharide, inflammation, apoptosis
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Elevated Ang II ultimately exacerbates LPS-induced endothelial injuries [9]. Angiotensin-converting enzyme (ACE) is a key enzyme that catalyzes the conversion of Angiotensin II to Ang II. ACE/Ang II plays an important role in the development of pulmonary edema in ARDS by promoting inflammation [10, 11] and cell apoptosis [12]. It has been reported that captopril, an inhibitor of ACE, exerts protective effects on LPS-induced lung injuries, at least in part by regulating ACE expression [13]. A previous study also showed that losartan, an antagonist of the type 1 receptor for Ang II (AT-1R), attenuated LPS-induced acute lung injuries in rats [14]. Therefore, inhibiting ACE/Ang II pathways may help attenuate ARDS.

Brain natriuretic peptides (BNP), an antagonist to RAS, are mainly produced by ventricular myocytes [15, 16]. Recombinant human brain natriuretic peptide (rhBNP) is a synthetic polypeptide compound with the same biological activity as BNP. RhBNP has been widely used, clinically, for treatment of heart failure [17, 18]. It has been reported that rhBNP attenuates trauma-/hemorrhagic shock-induced acute lung injury by inhibiting oxidative stress and NF-κB-dependent inflammatory/MMP-9 pathways [19]. In another study, it was seen that rhBNP ameliorated trauma-induced acute lung injuries by inhibiting Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathways in rats [20]. However, there are no reports regarding the protective effects of rhBNP against LPS-induced ARDS or the underlying mechanisms associated with ACE/Ang II pathways. The current study tested the effects of rhBNP on LPS-induced ARDS in rat models. It was hypothesized that rhBNP can attenuate pulmonary edema of LPS-induced ARDS by inhibiting ACE/Ang II pathways.

Materials and methods

Animals

Adult male sprague-dawley rats (180-220 g) were obtained from the Laboratory Animal Center of Guangxi Medical University (Nanning, China). The rats were housed in air-filtered and temperature-controlled units with free access to food and water. All animal care and experimental protocols were approved by the Ethical Principles in Animal Research adopted by the Guangxi Medical University for Animal Experimentation. All experiments followed the Guide for the Care and Use of Laboratory Animals.

Experimental protocols

The rats were randomly divided into four groups: normal control group, model group, low-dosage rhBNP group, and high-dosage rhBNP group (n = 6 for each group). Moreover, rhBNP was purchased from Nuodikang Biological Pharmaceutical Company Ltd. (Chengdu, China). It was injected intravenously through the tail veins. Rats in the low-dosage rhBNP group received rhBNP (10 µg/kg), while rats in the high-dosage rhBNP group received rhBNP (20 µg/kg). After 30 minutes, 10 mg/kg of LPS (Sigma-Aldrich, St. Louis, MO, USA) was injected intraperitoneally in the model group, low-dosage rhBNP group, and high-dosage rhBNP group. Normal saline (NS) was injected in the control group. After six hours, all rats in each group were sacrificed. Left lung samples were used for determining wet-dry ratios. Right lung samples were used for hematoxylin-eosin (H&E) staining, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), enzyme-linked immunosorbent assay (ELISA), and Western blotting analysis.

Lung wet-dry ratios

Wet-dry ratios of the lung samples taken from the left lungs were calculated. Lung samples were obtained after the rats were sacrificed. They were weighed immediately after removal. The samples were placed in an oven at 70°C for 48 hours to obtain dry weights.

Lung histological analysis

The lower lobes of the right lung were fixed in 4% paraformaldehyde immediately after removal. They were then embedded in paraffin, cut into 4 µm sections, and stained with H&E. H&E staining was performed using standard protocol. Grading of the severity of lung injuries was performed by a blinded pathologist based on the degree of inflammatory cell infiltration, alveolar wall thickness, alveolar hemorrhage, and microvascular congestion, using the following scale: 0, normal; 1, mild; 2, moderate; 3, severe; and 4, extremely severe injury, with a maximum possible score of 16.
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Quantification of pneumocyte apoptosis

TUNEL staining was carried out to observe apoptosis. The procedure was carried out according to manufacturer instructions using an In Situ cell death detection kit, POD (Roche, Mannheim, Germany). Expression of positive cells was observed using a computer-based pathological image analysis system under high magnification (10 × 40). Results are expressed as the apoptotic index, defined as the number of TUNEL positive cells/total cells × 100%.

Measurement of cytokines

Ang II, IL-1β, and TNF-α levels in lung tissue homogenates were detected using ELISA. Upper lobes of the right lungs were homogenized in phosphate-buffered saline (PBS) solution (pH, 6.0). Tissues (100 mg) were rinsed with 1 × PBS, homogenized in 1 mL of 1 × PBS, and stored overnight at -20°C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 × g, 4°C. The supernatant was then removed and stored at -80°C until subsequent analysis. The samples were centrifuged again after thawing before the assay. Rat Ang II, IL-1β, and TNF-α ELISA kits were purchased from CUSABIO Company Ltd. (Wuhan, China). Cytokine concentrations of Ang II, IL-1β, and TNF-α in lung tissue homogenates were determined using commercially available ELISA kits, according to the manufacturer instructions. Absorbance was measured at 450 nm using 96-well plates.

Western blotting analysis of ACE expression

Expression of ACE protein in the lung tissues of rats was detected by Western blotting analysis. Tissues from the middle lobes of the right lungs were homogenized with a radioimmuno-precipitation assay buffer (Beyotime, Shanghai, China). Samples were centrifuged at 12,000 rpm for 15 minutes at 4°C and the supernatant was collected. Proteins in the supernatant were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, then transferred to polyvinylidene fluoride membranes (0.45 mm; Millipore Corp., Burlington, MA, USA). The membranes were blocked for 1 hour with tris-buffered saline-tween (TBST) (TBST containing 0.05% tween and 5% powdered milk) and probed overnight at 4°C with the primary antibody, rabbit anti-rat ACE antibody (1:400 dilution; ABBIOTEC Company Ltd., San Diego, CA, USA). After washing with TBST again, the membranes were incubated for 1 hour with the secondary antibody, fluorophore-labeled goat anti-rabbit antibody (1: 800 dilution; EarthOx, LLC, San Francisco, CA, USA). A sweep membrane apparatus (LI-COR, Inc., NE, USA) was used to detect signals from each sample.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS 19.0 statistical software. Multiple groups were compared using one-way analysis of variance (ANOVA). P-values < 0.05 indicate statistical significance.

Results

Effects of rhBNP pretreatment on lung edema in LPS-induced ARDS

Analysis of lung wet-dry ratios to investigate the effects of rhBNP on lung edema showed that, compared with the control group, lung wet-dry ratios were significantly increased in the model group (P < 0.01). Compared with the model group, both low-dosage and high-dosage rhBNP groups showed markedly decreased lung wet-dry ratios (P < 0.01) (Figure 1).

Effects of rhBNP pretreatment on LPS-induced histological changes in the lungs

Analysis of the pathological changes in the lungs in each group, observed using H&E stain-
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In the control group (A), the lung morphology was normal. In the model group (B), alveolar walls were destroyed. Alveolar spaces were reduced. There was edema and hemorrhaging, as well as a significant number of inflammatory cells infiltrating into the interstitial and alveolar spaces. In the low-dosage (C) and high-dosage rhBNP groups (D), lung injury was significantly alleviated compared with the model group (H&E staining, original magnification ×200). Effects of rhBNP pretreatment on pulmonary histological scores of LPS-induced ARDS rats (E). Values are expressed as mean ± SD (n = 6 in each group). *P < 0.01, versus the model group.

Effects of rhBNP on angiotensin II levels in lung tissue homogenates

Compared with the control group, Ang II levels in lung tissue were significantly increased in the model group (P < 0.01). Although differences in Ang II levels in lung tissue homogenates between the model group and low-dosage rhBNP group were not significant (P > 0.05), Ang II levels in lung tissue homogenates were significantly reduced in the high-dosage rhBNP group. TUNEL staining was performed to measure pneumocyte apoptosis. TUNEL-positive cells were the cells with brown positive nuclei. Positive cells had a scattered distribution. Representative sections from the lungs of the rats in each group are shown in Figure 3A-D. The control group had minimal apoptotic cells (Figure 3A). Apoptotic cells in the model group were significantly increased (Figure 3B), compared with those in the control group (P < 0.01). Further, compared with those in the model group, apoptotic cells in low-dosage and high-dosage rhBNP groups were significantly reduced (P < 0.01, P < 0.01) (Figure 3C, 3D). The ratio of TUNEL-positive cells to total cells is shown in Figure 3E.
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Effects of rhBNP pretreatment on IL-1β and TNF-α levels in lung tissue homogenates

Compared with the control group, IL-1β and TNF-α levels in lung tissue homogenates were significantly increased in the model group ($P < 0.01$, $P < 0.01$). However, compared with the model group, both low-dosage and high-dosage rhBNP groups showed significantly reduced IL-1β ($P < 0.01$, $P < 0.01$) and TNF-α ($P < 0.05$, $P < 0.01$) levels (Figure 5).  

Effects of rhBNP pretreatment on ACE expression in the lungs

Results of Western blotting analysis showed that, compared with that in the control group, ACE protein expression in the lungs was significantly increased in the model group ($P < 0.01$). Low-dosage and high-dosage rhBNP groups showed significantly reduced ACE expression, compared with the model group ($P < 0.01$) (Figure 6).  

Discussion

The current study assessed the protective effects of rhBNP against LPS-induced ARDS in rats, investigating its effects on ACE/Ang II pathways. It was found that rhBNP pretreatment improved wet-dry ratios and pathological changes in the lungs. It also reduced ACE/Ang II protein levels in the lungs, as well as levels of inflammatory factors, such as IL-1β and TNF-α.

The pathophysiology of ARDS is characterized by aggressive inflammation, resulting in increased alveolar epithelial and pulmonary microvascular endothelial permeability, pulmonary edema, and fibrosis [21]. The current study prepared rat models of ARDS using intraperitoneal injections of LPS. H&E staining results demonstrated serious pathological changes in the lungs of the rat models. There were very few TUNEL-positive cells in the control group, whereas alveolar epithelial cell apoptosis was significantly increased in the rat models. Thus, present results indicate that the ARDS models were...
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Successfully prepared. Furthermore, it was found that wet-dry ratios of the lungs and levels of ACE/Ang II and inflammatory factors such as IL-1β and TNF-α proteins, in the lung tissues were significantly increased in the rat models of ARDS, compared with the control group.

An important active component of RAS, Ang II induces activation of an intracellular signaling cascade by binding to the AT-1R, leading to upregulation of proinflammatory genes and a subsequent inflammatory response [10, 22]. Furthermore, Ang II can induce alveolar epithelial cell apoptosis, which damages alveolar epithelial barrier function and increases permeability [23]. It has been reported that Fas-induced apoptosis of alveolar epithelial cells requires Ang II generation and receptor interaction [24]. ACE inhibitors have been shown to be specific anti-inflammatory therapeutic agents that can attenuate lung injuries caused by LPS and ventilator-induced lung injuries [7, 25-27]. Therefore, regulating ACE/Ang II may improve inflammatory injuries and pneumocyte apoptosis. An earlier study also reported that vitamin D may attenuate LPS-induced acute lung injuries (ALI), at least in part, by inhibiting the ACE/Ang II/AT1-R cascade [28].

ARDS can cause an increase in BNP and N-terminal pro-BNP (NT-proBNP) levels [29-32]. It was reported that, in ICU patients with hypoxic respiratory failure, BNP appeared useful in excluding cardiogenic pulmonary edema and identifying patients with a high probability of ARDS. It was associated with mortality in patients with ARDS [33]. RhBNP has a chemical structure and biological function like that of BNP. One previous study suggested that rhBNP treatment may exert protective effects in dogs with LPS-induced ARDS and may be associated with adjusting endogenous antioxidant enzymes [34]. The current study observed that rhBNP pretreatment improved pulmonary edema and pathological changes in the lungs and reduced pneumocyte apoptosis in rat models, indicating that rhBNP may exert protective effects against LPS-induced ARDS. In addition, it was found that not only did rhBNP pretreatment significantly reduce inflammatory factors, such as IL-1β and TNF-α protein levels, but also reduced ACE/Ang II protein levels in lung tissues. Thus, the protective effects of rhBNP against LPS-induced ARDS may be associated with inhibition of ACE/Ang II pathways.

The current study showed that high-dosage rhBNP probably has better effects than low-dosage rhBNP in decreasing Ang II levels in lung tissues. However, both low-dosage and high-dosage rhBNP exerted similar protective effects in terms of ameliorating lung edema and pathological changes. A limitation of the current study is that it only showed the effects of rhBNP pretreatment on LPS-induced ARDS. Further research is necessary to evaluate the effects of rhBNP treatment at different time points of the disease.

Conclusion

In conclusion, the current study demonstrates that rhBNP may exert protective effects against LPS-induced ARDS by inhibiting ACE/Ang II pathways. Present results offer evidence for clinical treatment of ARDS with rhBNP. However, the specific pathways by which rhBNP causes inhibition of ACE/Ang II require further research.

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Figure 5. Changes in IL-1β and TNF-α levels in lung tissues. Compared with those in the control group, IL-1β (A) and TNF-α (B) levels in the model group were markedly increased. Compared with the model group, both low-dosage and high-dosage rhBNP groups showed significantly reduced IL-1β (A) and TNF-α (B) levels. Data are expressed as mean ± SD (n = 6 for each group). #P < 0.01, *P < 0.05, versus model group.

Disclosure of conflict of interest

None.

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