Growth Hormone Releasing Peptide-2, a Ghrelin Agonist, Attenuates Lipopolysaccharide-Induced Acute Lung Injury in Rats

Guang Li, Jianguo Li, Qing Zhou, Xuemin Song, Hui Liang and Lili Huang

Department of Anesthesia, Critical Care Medicine & Emergency Medicine Center, Zhongnan Hospital, Wuhan University, Wuhan, Hubei Province, PR China

Acute lung injury (ALI) and its severe form, acute respiratory distress syndrome (ARDS), are the most common complications of sepsis; despite extensive investigations into new strategies for treatment, the morbidity and mortality of sepsis-induced ALI in critically ill patients remains still unacceptably high (Ware and Matthay 2000). Lipopolysaccharide (LPS), a bacterial cell wall component, is known to induce the production of several inflammatory and chemotactic cytokines, which has been well recognized as a principal component in the causation of sepsis-induced ALI (Brigham and Meyrick 1986), and intratracheal instillation of LPS has gained wide acceptance as an experimental model of ALI (Matute-Bello et al. 2008). LPS exerts its toxic effects on the lungs through two mechanisms: direct injury to endothelial cells and indirect activation of neutrophils and macrophages. The latter mechanism may lead to the release of proinflammatory cytokines, such as tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and interleukin-6 (IL-6). The complex network of these proinflammatory cytokines initiates, amplifies, and perpetuates the inflammatory response, which is associated with severe alterations in gas exchange and refractory hypoxemia. Therefore, an anti-inflammatory therapeutic strategy may be important in the management of LPS-induced ALI.

Ghrelin, a novel endogenous ligand for the growth hormone secretagogue receptor subtype 1a (GHSR-1a), is a 28-amino-acid acylated peptide that is produced predominantly by the stomach (Kojima et al. 1999). Since food intake regulation, stimulation of gastric motility and secretion are among the most important biological effects of ghrelin (Kojima et al. 1999), ghrelin has also emerged as an effective anti-inflammatory and immunomodulatory agent in sepsis, endotoxemia-induced acute kidney injury, endotoxic shock and ALI in previous studies (Chang et al. 2003; Chen et al. 2008; Shah et al. 2009; Wang et al. 2009), and the underlying anti-inflammatory mechanism of ghrelin might
through inhibition of nuclear factor kappa B (NF-κB) activation. However, ghrelin is a small peptide with a relatively short half-life, Wu et al. (2007) indicated that the half-life of ghrelin is only 11 min in a normal rat and 17 min during the late stage of sepsis. Thus, a continuous ghrelin infusion is required to maintain an effective concentration. Growth hormone releasing peptide-2 (GHRP-2) is a hexapeptide and a potent ghrelin receptor agonist of the GHRP family, which can attenuate the liver inflammatory response in endotoxemia and symptoms of experimental arthritis (Granado et al. 2005, 2008). These findings indicate that GHRP-2 is a potential anti-inflammatory drug, however, whether GHRP-2 attenuates LPS-induced ALI remains unknown.

In the present study, we aimed to investigate the protective effects of GHRP-2 on ALI induced by LPS. To further elucidate the underlying anti-inflammatory effect of GHRP-2, we assessed the nuclear translocation of NF-κB in whole lung extracts after LPS exposure.

**Materials and Methods**

**Animals**

Male Sprague-Dawley rats (Wuhan University Experiment Animals Center) weighing 250-300 g were used for the experiments. All animals were allowed food and tap water ad libitum and exposed to a 12-h light/12-h dark cycle. All protocols approved by the Wuhan University of Science and Technology Animal Care and Use Committee, and the animals received humane care in compliance with the Principles of Laboratory Animal Care.

**Drug and reagents**

GHRP-2 were purchased from Kaijie Pharmaceutical Company (Chendu, China) and dissolved in 0.9% saline before used. LPS (Escherichia coli 0111:B4) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cytokine immunoassay kits were purchased from R&D systems (Minneapolis, MN, USA). Antibodies for Western blotting were purchased from Boster Biotechnology Co (Wuhan, China). Tissue protein extraction reagent was purchased from Pierce Biotechnology (Rockford, IL, USA). The kits used to determine MPO were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

**LPS induced Acute Lung Injury model**

The rats were anesthetized with sodium pentobarbital (40 mg/kg) and fixed on a board at an angle of 45° in a supine position. After sterilization, a mid-line incision was performed in the neck to isolate the trachea. Rats were intratracheally instilled with 2 mg/kg LPS which dissolved in 1 ml sterile saline with a 20 gauge-needle syringe as previously described (Kang et al. 2001). With this model, ALI, as characterized by neutrophil infiltration into the lung interstitium, development of interstitial edema, and increased proinflammatory cytokine production, occurs after injection of LPS, and appropriate for preliminary pharmacological studies because intratracheal instillation of LPS into rats can produce controlled ALI, without causing systemic inflammation and multiorgan failure (Asti et al. 2000; Rowe et al. 2002).

**Experiment protocol**

Animals were randomly assigned to one of the following four groups: the saline group, the LPS group, the GHRP-2 group and the GHRP-2+LPS group. In the LPS group, rats were intratracheally instilled with 2 mg/kg LPS dissolved in 1 ml sterile saline. In the saline group, animals were received saline instead of LPS in the same manner and served as control. In the GHRP-2+LPS group, GHRP-2 (100 μg/kg) was subcutaneous injected 0.5 h before LPS administration. In the GHRP-2 control group, rats were giving a subcutaneous injection of GHRP-2 (100 μg/kg) 0.5 h before saline intratracheally instilled. After surgical preparation, the blood pressure and gas exchanges were allowed to stabilize. Blood samples were collected every 2 h for gas exchange measurement, other measurements were made at 6 h after LPS or saline administration. The rats were anesthetized and paralyzed throughout the experiment.

**Blood gas analysis**

Blood gas levels were determined every 2 h after LPS intratracheal instilled. The rats (n = 6, respectively) were anesthetized with sodium pentobarbital (40 mg/kg), then left ventricular puncture was performed with a 22-gauge needle connected by a polyethylene tube (PE50) to a syringe containing heparinized saline (Shen et al. 2009), arterial blood PaO₂ and PaCO₂ were measured immediately by Blood Gas Analyzer (ABL 520, Radiometer, Copenhagen, Denmark).

**Determination of bronchoalveolar lavage protein, cell counts and proinflammatory cytokines**

The animals (n = 6, respectively) were sacrificed at 6 h after the LPS challenge. The chest was opened and the lungs were removed intact from the thoracic cavity with the endotracheal tube in place and lavaged with 5 ml of phosphate buffer saline (PBS). PBS was flushed in and out of the lungs three times for each animal. In each time, 90% (4.5 ml) of the total injected volume was consistently recovered. bronchoalveolar lavage fluid (BALF) sample was centrifuged at 500 × g for 10 min at 4°C and the sediment cells were washed and resuspended in PBS, the total BALF cells were counted double-blindly using a hemocytometer, the resulting supernatants were stored at −20°C until used for cytokines and protein concentrations assay. The concentrations of cytokine TNF-α and IL-6 in the supernatants of the BALF were measured by sandwich enzyme-linked immunosorbsent assay (ELISA) using commercially available reagents according to the manufacturer’s instructions, the concentrations of protein in the supernatants of the BALF were quantified according to the method of Hartree (1972), and using bovine serum albumin as the standard.

**Histological examination**

Histopathologic evaluation was performed on rats (n = 6, respectively) that were not subjected to BALF. The lungs were harvested and fixed in 10% buffered formalin for 24 h, embedded in paraffin. A series of microsections (4 μm) was stained with hematoxylin with hematoxylin-eosin (H&E) and observed by light microscopy.

**Wet-to-dry Weight Ratio**

The lung wet-to-dry weight ratio (W/D ratio) was used as a parameter of lung edema induced by LPS (Fehrenbach et al. 1998), and calculated at 6 h in the rats that were not subjected to BALF or histological examination (n = 6, respectively). After the rats were sac-
rifed using pentobarbital overdose, the chest was opened and the right ventricle was injected with 10 ml of PBS to wash blood cells from the pulmonary circulation. The lungs were removed, weighed, and then dried in an oven at 80°C for 48 h. The dried lungs were weighed again to calculate pulmonary W/D ratios.

MPO Activity in lung tissue
Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte accumulation, was measured using a modification of the method described in a previous study (Goldblum et al. 1985). In brief, lung tissue was homogenized in 0.5 ml of 0.5% hexadecyl trimethyl ammonium bromide in 50 mM potassium phosphate buffer (pH 6.0). The homogenate was centrifuged at 14,000 g for 30 min at 4°C, and the supernatant was collected for the assay of MPO activity. This was done by measuring the H₂O₂-dependent oxidation of o-dianisidine solution (potassium phosphate buffer, pH 6.0) at 450 nm.

Electrophoretic mobility shift assay for the NF-κB activities
Nuclear extracts were prepared and assayed by electrophoretic mobility shift assay (EMSA), as previously described (Lee et al. 2007). The oligonucleotide used as a probe for EMSA was a dsDNA fragment. Synthetic double-stranded sequences (with enhancer motifs underlined) were filled-in and labeled with [α-32P]dATP (Free Biotech, Beijing, China) using sequenase DNA polymerase as follows: B sequence, 5-GCCATGGGGGA TCCTCAAG-3.

Cold competition was performed by adding 100 ng unlabeled, double-stranded probe to the reaction mixture.

Statistical analysis
Statistical analyses were conducted using SPSS 16.0 software package. All data were expressed as mean ± s.d. and compared through one-way analysis of variance (ANOVA) and Student-Newman-Keuls method for multiple-group analysis or Student's t-test for two-group analysis. Differences in values were considered significant at P < 0.05.

Results
Effects of GHRP-2 on the blood gas analysis in LPS-induced ALI rats
As shown in Fig. 1, intratracheal instillation of LPS caused a significant decrease in the partial pressure of arterial blood oxygen (PaO₂) and a notable increase in the partial pressure of arterial blood carbon dioxide (PaCO₂). In contrast, administration of GHRP-2 markedly reversed the decrease in PaO₂ and cut down the increase in PaCO₂ induced by LPS.

Effects of GHRP-2 on the histological changes in LPS-induced ALI rats
As shown in Fig. 2B, intratracheal instillation of LPS caused severe infiltration of inflammatory cells into the lung interstitium and alveolar spaces, alveolar wall thickening and hemorrhage at 6 h after LPS exposure. However, GHRP-2 pretreatment significantly attenuated these histological changes (Fig. 2D). The lung tissue histopathological changes of the rats in the saline and GHRP-2 control groups displayed no pulmonary histological alterations under light microscopy (Fig. 2A and C).

Effects of GHRP-2 on the lung W/D weight ratio in LPS-induced ALI rats
To investigate the effect of GHRP-2 on LPS-induced lung edema, lung wet-to-dry ratio were observed (Table 1). Results demonstrate that there were no significant differences between saline and GHRP-2 control groups, which indicated that GHRP-2 had little effect on lung water content in normal rats. In LPS group, wet-to-dry ratio were markedly increased compared with the control group (P < 0.05). However, administration of GHRP-2 0.5 h prior to LPS challenge ameliorated LPS-induced lung edema (P < 0.01).

Effects of GHRP-2 on the MPO activity
To assess the neutrophil infiltration in the lung, MPO activity assay were performed, LPS administration markedly increased MPO activity in lungs compared with the saline control group (P < 0.05, Table 1), whereas the MPO activity was significantly decreased in the GHRP-2+LPS group (P < 0.01, Table 1).

Effects of GHRP-2 on proinflammatory cytokines in BALF
The concentration of TNF-α and IL-6 in BALF represents proinflammatory mediators, which play important

Fig. 1. Effect of GHRP-2 pretreatment on changes in arterial oxygen tension.
The parameters depicted are (A) PaO₂, (B) PaCO₂. The GHRP-2 treatment group is shown with square circle symbols, the saline control group is shown with triangles symbols, the LPS group is shown with circle symbols, and the GHRP-2 control group is shown with inverted triangles symbols. Data were expressed as mean ± s.d. (n = 6). *P < 0.05 vs. saline group. **P < 0.01 vs. LPS group.
As shown in Fig. 3, we found that TNF-α and IL-6 were only minimally expressed in the control group rats. Six hours after LPS administration, substantial increases in TNF-α and IL-6 levels were found in BALF. However, these levels were significantly decreased by pretreatment with GHRP-2.

Effects of GHRP-2 on the cell and protein exudation in BALF

Total cell count and protein content in BALF indicates the extent of lung leakage, which denotes the severity of lung injury. Fig. 3 illustrates that administration of LPS elicited a massive recruitment of total cell numbers and protein in BALF ($P < 0.05$). GHRP-2 pretreatment significantly reduce the total cell counts and proteins content in BALF.

Administration of GHRP-2 decreases LPS-induced NF-κB in the lungs

To investigate the possible molecular mechanism of GHRP-2 inhibition the expression of proinflammatory cytokines, the activation of NF-κB was measured by electrophoretic mobility shift assay (EMSA). The NF-κB nuclear translocation was obviously enhanced in the lung homogenate at 6 h after LPS instillation compare to the saline group (Fig. 4A). However, rats pretreated with GHRP-2 exhibited less NF-κB DNA binding activity. The specificity of the NF-κB bands was confirmed by cold competition analysis in the presence of excess unlabeled NF-κB consensus motif.

Degradation of IκBα in lung tissue

To examine the mechanism underlying the actions of GHRP-2 on the LPS induction of pathways leading to NF-κB activation, the degradation of IκBα in the lung tissues was analyzed by western blotting. As shown in Fig. 4B, a distinct degradation of IκBα was observed at 6 h after LPS
GHRP-2 Inhibits Lung Injury

Pretreatment with GHRP-2 significantly inhibited the degradation of IκBα in lung tissue cytoplasm at 6 h after LPS treatment. Pretreatment with GHRP-2 significantly inhibited the degradation of IκBα in lung tissue cytoplasm at 6 h after LPS treatment.

Discussion

In present study, we have demonstrated that GHRP-2 exerts potent anti-inflammatory and immunomodulatory effects in lungs exposed to LPS, as evidenced by the remarkable decreases in lung edema (W/D ratio), neutrophil infiltration (MPO activity), and pulmonary permeability (total cells and protein concentration in BALF). Meanwhile, GHRP-2 attenuated concentrations of proinflammatory cytokines such as TNF-α, and IL-6 in BALF. Furthermore, GHRP-2 effectively repressed the activation of transcription factor NF-κB, which was associated with the inhibition of IκBα degradation in cytoplasm, and this may be part of the mechanisms whereby GHRP-2 exerts its anti-inflammatory effects.

Excessive cytokine-mediated inflammation is likely to play a fundamental role in the pathogenesis of ALI/ARDS (Goodman et al. 2003). Previous studies shown that the increased levels of TNF-α, IL-1β, and IL-6 in BALF are noted in ARDS patients, and the persistent elevation of proinflammatory cytokines in humans with ALI or sepsis
has been associated with a worse outcome (Minamino and Komuro 2006). In the present study, we found that LPS instillation markedly increased the concentration of TNF-α and IL-6 in the BALF of LPS-induced ALI rats. In contrast, pretreatment with GHRP-2 significantly reduced the pro-inflammatory cytokines level in the BALF. This is consistent in part with a recent report that GHRP-2 decreased the TNF-α expression and exerted anti-inflammatory effect on liver inflammatory response (Granado et al. 2008). Therefore, GHRP-2 may protect against LPS-induced ALI by decreasing the production or activities of these pro-inflammatory cytokines.

Expression of proinflammatory genes is regulated by the transcriptional mechanism, and NF-κB is one of the critical transcription factors that regulate expression of a large number of inflammation-related genes, including TNF-α, IL-1β and IL-6, which are involved in the pathogenesis of ALI (Fan et al. 2001). In unstimulated cells, NF-κB is retained as an inactive complex bound to the cytoplasmic inhibitor protein of IκB. Once the cell is exposed to such activation signals as LPS, the IκB is degraded. Subsequently, NF-κB is dissociated from its inhibitor and is translocated to the nucleus, thereby enhancing the transcription of cytokines, which potentially intensifies the inflammatory process of ALI. In patients with septic lung injury, NF-κB activity in alveolar macrophages has been shown to be significantly increased (Schwartz et al. 1996). Conversely, the inhibition of NF-κB activation reduces the lung injury associated with LPS treatment (Blackwell et al. 1996; Liu et al. 1997).

To further explore the anti-inflammatory mechanisms of GHRP-2 that down-regulate proinflammatory cytokines expression, the NF-κB signaling pathway in the lung tissue was investigated following the LPS challenge in present study. Our results demonstrated that the DNA-binding activities of NF-κB in lung tissue were significantly elevated 6 h after LPS exposure. Consistent with the change of NF-κB activation, IκBα was degraded obviously. These results indicate that pretreatment with GHRP-2 obviously blunted the activation of NF-κB in lung tissue via inhibiting the degradation of IκBα at 6 h after LPS exposure, thus terminating cytokine transcription to limit the inflammatory response.

In conclusion, the present study demonstrates that GHRP-2 obviously attenuates the lung injury induced by LPS in rats via modulation of NF-κB signaling pathway, which subsequently leads to a remarkable reduction in pro-inflammatory cytokine expression. Therefore, these data suggest that the synthetic compound ghrelin agonist, GHRP-2, may be a potential treatment for lung injury in the early stages and represents a novel strategy for modulating inflammatory response.

Acknowledgments

This work was supported by a grant from the National Natural Science Foundation of China (No. 30671586).

References


