

Research Article

Ghrelin Protects Lipopolysaccharide-Induced Acute Lung Injury Rats against Pulmonary Vascular Dysfunction by Inhibiting Inflammation

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Objective. To determine the effect and mechanism of the anti-inflammatory agent ghrelin on pulmonary vascular dysfunction (PVD) in lipopolysaccharide- (LPS-) induced acute lung injury (ALI) rat models. **Methods.** Thirty-two adult male Sprague Dawley rats ($n = 16/\text{group}$) were randomly divided into ghrelin and saline groups, wherein ghrelin (10 nmol/kg) or saline was subcutaneously administered. After 30 min, eight rats from each group were randomly selected, and LPS (5 mg/kg) or saline was administered by intratracheal instillation to induce ALI. Four hours after establishing the ALI rat model, the mean pulmonary arterial pressure (mPAP), mean right ventricular systolic pressure (RVSP), levels of proinflammatory cytokines tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) in the bronchoalveolar lavage fluid (BALF), BALF cell count, wet-to-dry (W/D) lung weight ratios, and myeloperoxidase (MPO) activity in lung tissue for all four groups (ghrelin, ghrelin + ALI, saline, and saline + ALI) were measured. Immunohistochemical staining to detect alpha-smooth muscle actin (α -SMA) and proliferating cell nuclear antigen (PCNA) expression was performed to assess the intrapulmonary arterial wall thickness and the proliferation of smooth muscle cells, respectively. **Results.** The ghrelin-pretreated ALI rats showed lower mPAP, RVSP, PCNA expression, MPO activity, W/D lung weight ratio, TNF- α and IL-6 levels, and BALF cell count than the saline-pretreated ALI rats, but ghrelin had no effect on the intrapulmonary arterial wall thickness of ALI rats. **Conclusion.** Our results confirmed the association between inflammation and PVD in ALI and suggested that the suppression of inflammation by ghrelin pretreatment could protect LPS-induced ALI rats against PVD.

1. Introduction

Acute lung injury (ALI) and its most severe form, acute respiratory distress syndrome (ARDS), are characterized by acute-onset hypoxia, which leads to diffuse alveolar damage, increased pulmonary vascular permeability, noncardiogenic pulmonary edema, and poor lung compliance [1]. Sepsis and lipopolysaccharide (LPS) are well-known factors that can induce ALI due to their proinflammatory effects. Notably, sepsis-induced ALI has a high mortality rate of 25–50% [2]. However, at present, there is no effective therapy for ALI/ARDS due to the lack of understanding of the disease etiology, despite significant efforts in investigating the mechanisms underlying the pathogenesis, treatment, and prevention of this disease.

Right ventricular dysfunction as well as persistent and severe hypoxemia due to pulmonary vascular dysfunction (PVD), which is common in ALI [3, 4], increases the morbidity and mortality rates of ALI [5]. Therefore, there is an urgent need to better understand PVD in order to clarify the etiology and pathogenesis of ALI. The reported pathophysiological changes occurring due to PVD in ALI include endothelial dysfunction, pulmonary vascular occlusion, increased vascular tone, extrinsic vessel occlusion, and vascular remodeling [6]. The excessive secretion of chemokines and cytokines during an inflammatory response in ALI reportedly results in PVD [7, 8]. Thus, this study aimed to determine the association between inflammation and PVD in ALI through the use of the anti-inflammatory agent ghrelin.

Ghrelin, a 28-amino-acid peptide produced by enteroendocrine cells of the gastrointestinal tract, is an endogenous ligand for the growth hormone secretagogue receptor [9]. It can stimulate the hypothalamus to enhance food intake by inducing pituitary-controlled growth hormone secretion. In addition, ghrelin has been shown to exert pleiotropic anti-inflammatory effects *in vitro* and *in vivo* [10–12], including increased pulmonary blood flow and decreased levels of proinflammatory cytokines, thereby alleviating ALI [13]. We have also shown in a previous study that the ghrelin agonist, GHRP-2, can minimize ventilator-induced lung injury and alleviate LPS-induced ALI [14, 15]. As the effect of ghrelin on PVD in ALI has not been characterized, this study was performed to elucidate the effect and mechanism of ghrelin on PVD using an LPS-induced ALI rat model.

2. Materials and Methods

2.1. Animal Care and Grouping. Thirty-two male Sprague Dawley rats (Experimental Animal Center of Wuhan University, China), weighing 180–200 g, were housed in a pathogen-free environment under a 12 h:12 h light-dark cycle. Food and water were available *ad libitum*. The study protocol was approved by the Ethics Review Committee for Animal Studies of our institution, and the experiments were performed according to animal welfare guidelines. The rats were randomly divided into four groups ($n=8/\text{group}$): ghrelin, ghrelin + ALI, saline, and saline + ALI. Specifically, the rats were first divided into two groups ($n=16/\text{group}$) for pretreatment with either ghrelin (10 nmol/kg; Shino-test, Sagami, Japan; $n=16$) or saline ($n=16$) via subcutaneous injection. After 30 min, 8 rats were randomly selected from each group to establish the ALI rat models, wherein LPS (5 mg/kg, O111:B4; Sigma, St. Louis, MO, USA) was administered by intratracheal instillation to induce ALI, while the remaining 16 non-ALI rats from the ghrelin and saline groups received normal saline by intratracheal instillation.

2.2. Mean Pulmonary Artery Pressure (mPAP) Measurement. The rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate, the limbs were fixed, a longitudinal skin incision in the neck was made, and the muscles were separated. The second and third anterior costal chest ribs were cut off on the left side of the sternum incision, and the trunk of the pulmonary artery was revealed by opening the pericardium. A silk suture and an 18 G polyethylene catheter were positioned under and along the pulmonary artery trunk, respectively. Right-heart catheterization was conducted at 4 h after the LPS challenge to measure mPAP and the right ventricular systolic pressure (RVSP), as described previously [16]. The rats were sacrificed by a pentobarbital injection (200 mg/kg) after obtaining the mPAP and RVSP measurements, and the data were immediately used to analyze other parameters.

2.3. Immunohistochemical Detection of Proliferating Cell Nuclear Antigen (PCNA) and Alpha-Smooth Muscle Actin (α -SMA). Following fixation in 4% paraformaldehyde at

4°C overnight, paraffin-embedded sections of the right upper lobe of the rat lung were stained by the streptavidin-biotin-peroxidase complex method to detect PCNA and α -SMC expression (the antibodies were purchased from Sigma, USA) to determine the effect of ghrelin on pulmonary vascular remodeling. The immunostained lung sections (six cross sections from each group) were visualized and imaged with a microscope equipped with a digital camera system (Nikon DS-Fi2, Japan) at 200x magnification. The cells (PCNA⁺ nuclei and the total number of nuclei in the neointima and media) were counted using images collected from three fields of view, with at least 250 cells per field of view, for each stained lung section. The nuclei of PCNA⁺ cells were stained brown, while those of PCNA⁻ cells appeared blue. ImageJ software (NIH, USA) was used for image analysis. The proportion of PCNA⁺ cells was derived using the following formula: % of PCNA⁺ cells = number of PCNA⁺ nuclei/total number of nuclei. The intima-media wall thickness of 50–100 μm -diameter arterioles in the lungs of six different rats from each group was assessed based on the α -SMC expression using the following formula: % of wall thickness = (intima-media thickness/external diameter) \times 100.

2.4. Measurement of the Levels of Proinflammatory Cytokines and the Total Cell Count in the Bronchoalveolar Lavage Fluid (BALF). After the right main bronchus ligation, the left lung was thoroughly rinsed three times with 5 mL of fresh saline at 4°C. Approximately 85–90% of the BALF was then recovered and was centrifuged at 1,500 rpm and 4°C for 10 min. The supernatant was immediately flash-frozen for storage at -80°C for subsequent measurement of the levels of the proinflammatory cytokines tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6). The cell pellets were coated on glass slides and were subjected to Giemsa staining for visualization under an optical microscope and counting of the total BALF cell count using a hemocytometer.

2.5. Calculation of the Wet-to-Dry (W/D) Lung Weight Ratio. To calculate the W/D lung weight ratio, the right lower lungs were briefly rinsed with phosphate-buffered saline and weighed immediately to obtain the wet weight and were weighed again after drying in an oven at 80°C for 48 h to obtain the dry weight.

2.6. Myeloperoxidase (MPO) Activity Measurement. Lung homogenates of frozen lung specimens in hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50 mM phosphate buffer, pH 6.0) were used for the measurement of MPO activity to determine the degree of oxidation of H_2O_2 -dependent *o*-dihydroindene hydrochloride. The frozen lung specimens were weighed before any experimental manipulations. Each sample was then sonicated and centrifuged at 40,000 g and 4°C for 15 min, and the MPO activity in the supernatant was measured at 460 nm using an assay kit (Jiancheng

Biotechnology Co., Ltd., Nanjing, China). The MPO activity per gram of lung weight was calculated for each sample.

2.7. Statistical Analysis. Statistical analyses were performed using SPSS v17.0 software (SPSS Inc., Chicago, IL, USA). All the data are expressed as the mean \pm standard deviation. Comparisons of parameters among the four groups were performed using one-way analysis of variance followed by the Newman-Keuls test. A p value <0.05 was considered statistically significant.

3. Results

3.1. Ghrelin Pretreatment Partially Reduces mPAP and RVSP of LPS-Induced ALI Rats. The saline + ALI group showed an elevated mPAP and RVSP after 4 h, compared with the non-ALI (saline and saline + ghrelin) groups. In addition, ghrelin pretreatment partially lowered the mPAP and RVSP (Figure 1; $p < 0.05$).

3.2. Ghrelin Reduces the Number of PCNA⁺ Cells in the Pulmonary Artery. Next, we examined the effect of ghrelin on cell proliferation and the pulmonary vasculature by detecting PCNA and α -SMA expression, respectively. PCNA⁺ cells (brown) were occasionally observed in the vascular adventitia, medial membrane, and intima in the saline and saline + ghrelin groups. In the saline + ALI group, high numbers of PCNA⁺ cells were observed in the vascular adventitia and neointima, especially at the luminal surface (Figure 2(a)), indicating the proliferation of smooth muscle cells in the pulmonary artery. Fewer and occasionally visible PCNA⁺ cells were observed in the vascular adventitia, medial membrane, intima, and neointima for the ghrelin + ALI group, compared with the ALI group. There was no significant difference in the thickness of the intrapulmonary intima-media arterial wall among all four groups (Figure 2(b)).

3.3. Ghrelin Reduces the Levels of Proinflammatory Cytokines and the Total Cell Count in the BALF of ALI Rats. Higher TNF- α and IL-6 levels (Figures 3(a) and 3(b); $p < 0.05$) and a higher BALF total cell count (Figures 3(c) and 3(d); $p < 0.05$) were observed for the ALI group, compared with the saline group. It is noteworthy that ghrelin pretreatment significantly decreased the levels of TNF- α and IL-6 (Figures 3(a) and 3(b); $p < 0.05$) as well as the BALF total cell count (Figures 3(c) and 3(d); $p < 0.05$) in the ALI rats.

3.4. Ghrelin Reduces Pulmonary Edema and MPO Activity in ALI Rats. A significantly higher W/D lung ratio was observed for the ALI group, compared with the saline group (Figure 4(a); $p < 0.05$), but this was reduced in the ghrelin + ALI group, suggesting that ghrelin likely suppressed the LPS-induced pulmonary edema. As MPO in the granules of neutrophils is secreted as part of the inflammatory response, MPO activity is a well-established biomarker of inflammation. The markedly higher MPO activity for the ALI

group, compared with the saline group (Figure 4(b); $p < 0.05$), suggests neutrophil infiltration into the lung parenchyma or alveolar spaces. Ghrelin pretreatment inhibited MPO activity (Figure 4(b); $p < 0.05$), suggesting that ghrelin could prevent the infiltration of neutrophils into the lung parenchyma or alveolar spaces in LPS-induced ALI rats.

4. Discussion

PVD has been shown to be an independent predictor of mortality in ALI/ARDS [5]. Since inflammation is reportedly associated with PVD in ALI/ARDS [7, 8], we conducted this study to evaluate the effect of the anti-inflammatory agent ghrelin on alleviating PVD in ALI. Intratracheal instillation of LPS, rather than intravenous injection, was employed to induce ALI in rats to avoid endotoxemia-induced lung injury and for a faster induction of ALI. We noted pulmonary edema, alveolar structural destruction, and prominent inflammatory cell infiltration, indicating the successful induction of ALI in rats by the intratracheal instillation of LPS. In addition, these findings were supported by elevated mPAP and RVSP, proliferation of PCNA⁺ smooth muscle cells, proinflammatory cytokine levels, BALF total cell count, W/D lung ratio, and MPO activity in the LPS-induced ALI rat models. Notably, ghrelin pretreatment reduced all of the aforementioned indicators of PVD, thus confirming the involvement of inflammation in PVD in ALI. More importantly, the results showed the efficacy of ghrelin in protecting against PVD and advocated its potential as a therapeutic agent for the clinical treatment of ALI.

Exposure to LPS stimulates the secretion of the cytokines TNF- α and IL-6, which are not only biomarkers of poor outcomes but also are pathogenic mediators of PVD in ALI [17]. Indeed, infusion of recombinant IL-6 protein in rats has been shown to induce pulmonary vascular remodeling as well as smooth muscle cell proliferation in the pulmonary artery [17]. Similarly, TNF- α -related apoptosis-inducing ligand has been demonstrated to promote the apoptosis of endothelial cells and the proliferation of smooth muscle cells in pulmonary hypertension animal models as well as to induce disease pathogenesis. Consistent with previous reports of pulmonary hypertension and elevated IL-6 levels in an inhaled LPS-induced ARDS rat model contributing to PVD [18], we also noted similar findings for the saline + ALI group, thereby confirming the role of inflammation in the pathogenesis of ALI. Moreover, the reduction of the proinflammatory cytokine levels in the BALF by ghrelin confirmed the role of the inflammatory response in ALI.

In the present study, we assessed the extent of pulmonary edema, a typical symptom of systemic and local inflammation, by evaluating the W/D lung ratio. In the ghrelin + ALI group, the reduction in the W/D lung ratio suggests a significant decrease in edema and congestion in the lungs. This finding indicates that ghrelin effectively decreased the lung vascular permeability and promoted the clearance of lung edema, thus confirming the protective effect of ghrelin against LPS-induced ALI. This result is consistent with the observed decrease of MPO activity in the ghrelin + ALI group, indicating that ghrelin inhibited

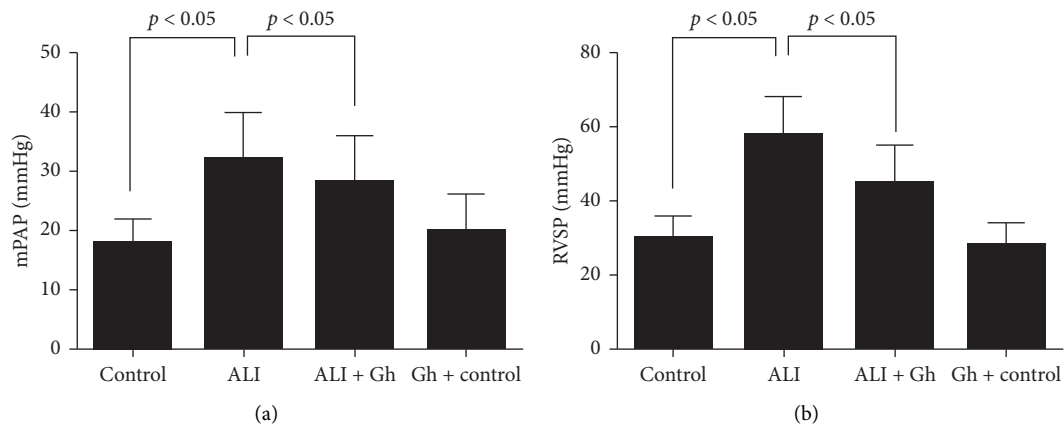


FIGURE 1: Ghrelin pretreatment reduced the mean pulmonary arterial pressure (mPAP) and right ventricular systolic pressure (RVSP) of LPS-induced ALI rats. (a) The mean mPAP and (b) RVSP were measured at 4 h after the LPS challenge (5 mg/kg). Data are presented as the mean \pm SD ($n = 4-6$).

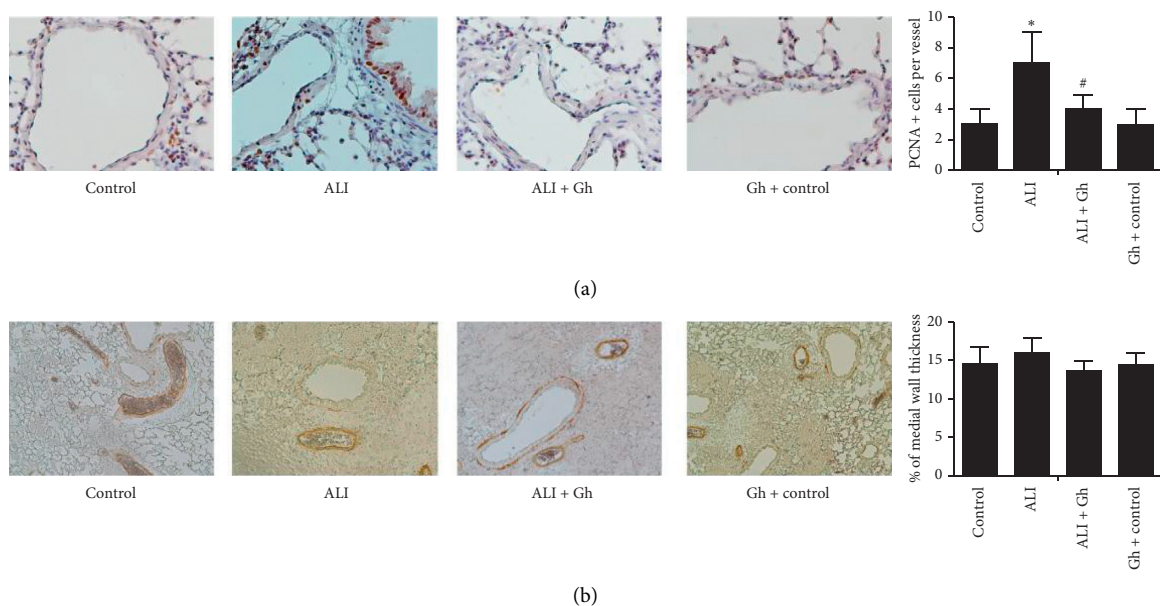
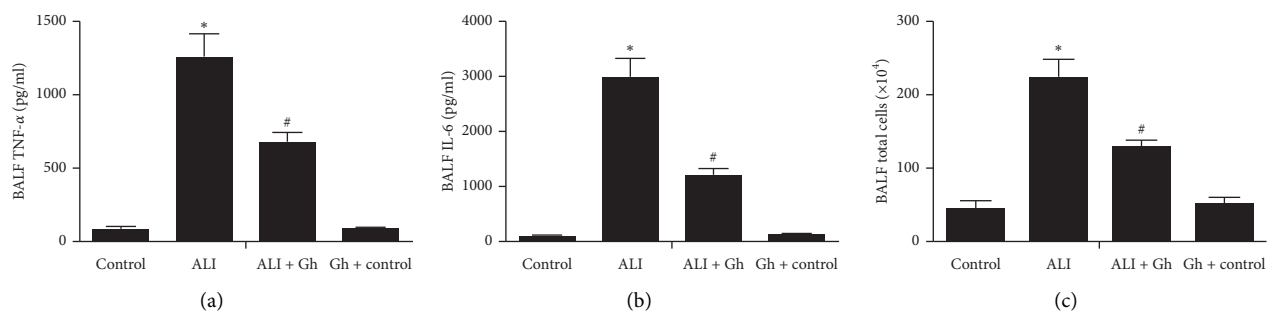


FIGURE 2: Ghrelin pretreatment reduced the number of PCNA⁺ cells but had no effect on the intima-media arterial wall thickness of LPS-induced ALI rats. (a) Representative images of PCNA⁺ smooth muscle cells of the four groups at 200x magnification and the corresponding histograms. The nuclei of PCNA⁺ cells were stained brown, while those of PCNA⁻ cells appeared blue. (b) Representative images of an anti- α -smooth muscle actin- (α -SMA-) stained pulmonary artery. The intima-media arterial wall thickness of the lungs of six different rats from each of the four groups was calculated using the following formula: % of wall thickness = (intima-media thickness/external diameter) \times 100. Data are presented as the mean \pm SD ($n = 6$).



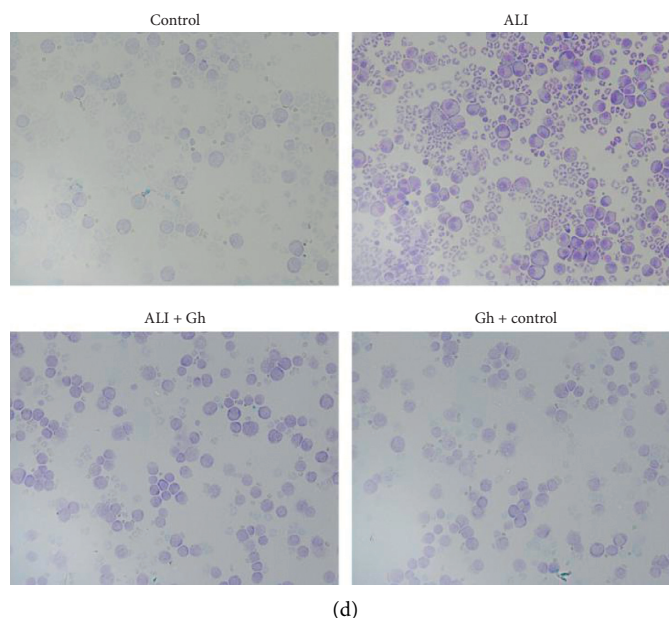


FIGURE 3: Ghrelin pretreatment reduced inflammation in the BALF of LPS-induced ALI rats. The levels of (a) $\text{TNF-}\alpha$ and (b) IL-6 as well as (c) the total cell count in the bronchoalveolar lavage fluid (BALF) of the four groups. (d) Giemsa staining of cytospin preparations of BALF for the four groups. * $p < 0.05$ vs. the saline group; # $p < 0.05$ vs. the ALI group. Data are presented as the mean \pm SD ($n = 6$).

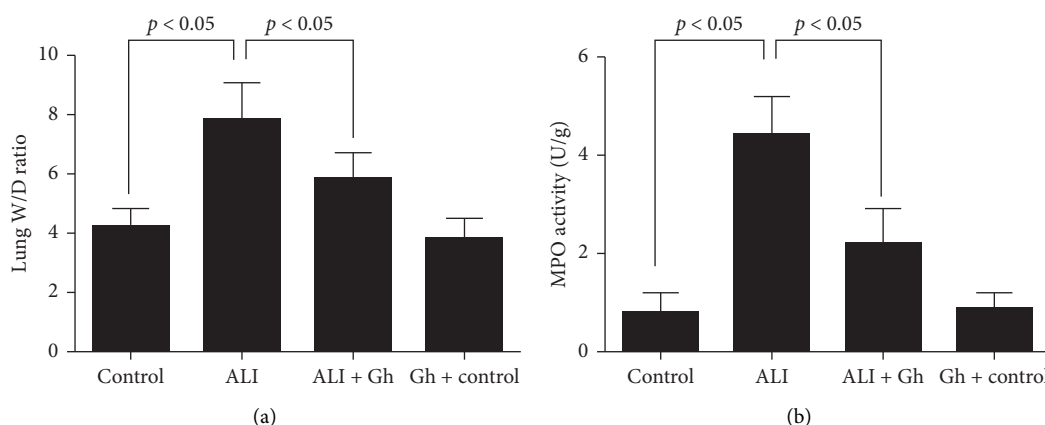


FIGURE 4: Ghrelin pretreatment reduced the wet-to-dry (W/D) lung weight ratio and the MPO activity in the LPS-induced ALI rats. (a) The W/D lung ratio and (b) the MPO activity were determined at 4 h after the LPS administration. Data are presented as the mean \pm SD ($n = 3$).

inflammatory cell sequestration and migration into the lung tissue. Taken together, these results suggest that ghrelin can protect LPS-induced ALI rats against PVD.

In conclusion, this study showed that ghrelin pretreatment can suppress pulmonary inflammation in ALI rats by reducing inflammatory cell infiltration and proinflammatory cytokine secretion in lung tissues. Our findings confirm the importance of inflammation in the pathogenesis of PVD in ALI and suggest the potential of ghrelin as an alternative adjuvant therapeutic agent in treating LPS-associated PVD.

Data Availability

The data used to support the findings of this study are included within the article.

Ethical Approval

The study protocol was approved by the Ethics Review Committee for Animal Studies of our institution, and experiments were performed in accordance with animal welfare guidelines.

Conflicts of Interest

The authors report no conflicts of interest.

Acknowledgments

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