Ghrelin Protects Alveolar Macrophages Against Lipopolysaccharide-Induced Apoptosis Through Growth Hormone Secretagogue Receptor 1a-Dependent c-Jun N-Terminal Kinase and Wnt/β-Catenin Signaling and Suppresses Lung Inflammation

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Alveolar macrophages (AMs) undergo increased apoptosis during sepsis-induced acute respiratory distress syndrome (ARDS). Ghrelin exhibits an antiapoptotic effect in several cell types and protects against sepsis-induced ARDS in rats; however, the molecular mechanisms underlying this antiapoptotic effect remain poorly understood. In this study, we first examined the antiapoptotic effect of ghrelin on lipopolysaccharide (LPS)-stimulated AMs in vitro. In AMs, GH secretagogue receptor-1a (GHSR-1a), the ghrelin receptor, was expressed, and treatment of AMs with ghrelin markedly reduced LPS-induced apoptosis, mitochondrial transmembrane potential decrease, and cytochrome c release. These effects of ghrelin were mediated by GHSR-1a because a GHSR-1a-targeting small interfering RNA abolished the antiapoptotic action of ghrelin. LPS treatment activated the c-Jun N-terminal kinase (JNK) signaling pathway but inhibited the Wnt/β-catenin pathway. Interestingly, combined LPS-ghrelin treatment reduced JNK activation and increased Wnt/β-catenin activation. Furthermore, like ghrelin treatment, the addition of the JNK inhibitor SP600125 or the glycogen synthase kinase-3β (GSK-3β) inhibitor SB216763 rescued AMs from apoptosis. We also demonstrated that ghrelin altered the balance of Bcl-2-family proteins and inhibited caspase-3 activity.

Next, we investigated whether ghrelin protected against septic ARDS in vivo. Sepsis was induced in male rats by performing cecal ligation and puncture; administration of ghrelin reduced sepsis-induced AMs apoptosis, pulmonary injury, protein concentrations in the bronchoalveolar lavage fluid, the lung neutrophil infiltration, and wet to dry weight ratio. However, administration of a specific ghrelin-receptor antagonist, [D-Lys-3]-GH-releasing peptide-6, abolished the beneficial effects of ghrelin. Collectively our results suggest that ghrelin exerts an antiapoptotic effect on AMs at least partly by inhibiting JNK and activating the Wnt/β-catenin pathway and thereby helps alleviate septic ARDS in rats. (Endocrinology 156: 203–217, 2015)

Ghrelin is a unique, acylated 28-amino acid peptide that is produced primarily in the stomach and secreted into the systemic circulation. Ghrelin was identified as an endogenous ligand for GH secretagogue receptor-1a (GHSR-1a) (1). Ghrelin-producing cells and GHSR-1a expression have been detected in various tissues including...
The hypothalamus, pituitary, stomach, heart, lung, pancreas, intestine, kidney, testis, and ovary (2). The wide distribution of ghrelin and its receptor suggests that ghrelin can potentially exhibit multiple biological activities (2, 3). In addition to strongly stimulating GH release through GHSR-1a in humans and rodents, ghrelin serves multiple regulatory functions; for example, it stimulates food intake, promotes adipogenesis, lowers energy metabolism, improves cardiovascular function, and stimulates prolactin and cortisol release (4). Recently ghrelin was reported to inhibit apoptosis in several cell types, such as cardiomyocytes, endothelial cells, adipocytes, adrenal zona glomerulosa cells, pancreatic β-cells, osteoblastic MC3T3-E1 cells, intestinal epithelial cells, and hypothalamic neurons (5–12).

Sepsis is a potentially life-threatening medical condition that remains one of the most frequent causes of morbidity and mortality in patients admitted to intensive care units. Acute respiratory distress syndrome (ARDS) is closely related to sepsis, either as an initiating factor or as a severe complication (13). The pathophysiology of sepsis-induced ARDS is characterized by complex mechanisms that involve cell inflammation, cytokines, and chemokines as well as abnormal apoptosis (14, 15). Immune paralysis occurs during sepsis, but a robust immune response to microbial infection is central to bacterial clearance and recovery. A major demonstration of immune paralysis is the occurrence of alveolar macrophage (AMs) apoptosis and dysfunction late in sepsis, which is implicated in increased mortality (16). Accumulating evidence indicates a consistent association between AMs and ARDS in humans and animal models (17–20) because AMs are recognized as the first line of defense against inhaled substances and play a critical role in maintaining lung homeostasis. However, little is known about the intracellular signaling pathways that regulate the fate of AMs in ARDS, and efforts directed toward understanding the key mechanisms to improve ARDS detection and treatment are urgently required.

Recently both systemic and tissue-associated expression of ghrelin and GHSR-1a were shown to be regulated during states of acute and chronic inflammation (21, 22). In vitro and in vivo data have indicated that ghrelin and its target receptors are localized in neutrophils, lymphocytes, and macrophages, which suggests that ghrelin can potentially influence the immune system and that the administering exogenous ghrelin might ameliorate pathological inflammatory conditions (23). Supporting this possibility, ghrelin protected against bleomycin-induced ARDS by reducing the apoptosis of alveolar epithelial cells and regulating lung inflammation (24). Moreover, ghrelin and its receptor were markedly down-regulated in lung tissue during sepsis, but the administration of ghrelin reduced lung injury, increased pulmonary blood flow, down-regulated proinflammatory cytokines, and improved survival in sepsis (25), suggesting that ghrelin might function as an antiinflammatory peptide during sepsis. However, the cellular targets of ghrelin and the molecular mechanisms underlying the protective effects of ghrelin in sepsis remain poorly understood. Previously, ghrelin was shown to be expressed in peritoneal macrophages, and small interfering RNA (siRNA)-mediated the suppression of ghrelin expression increased the TNF-α level and the lipopolysaccharide (LPS)-stimulated nuclear factor-κB activation in RAW264 cells, a macrophage cell line (26). Thus, the protective effect exerted by ghrelin on AMs might represent one of the mechanisms by which ghrelin relieves sepsis-induced ARDS. However, how ghrelin affects AMs apoptosis and whether the inhibition of AMs apoptosis by ghrelin is related to its ability to alleviate pulmonary injury are aspects that remain unclear.

The c-Jun N-terminal kinase (JNK) and Wnt/β-catenin pathways have been implicated in the regulation of apoptosis in distinct cell types (27, 28). However, little is known about the intracellular signaling pathways that regulate AMs apoptosis in ARDS. In this study, we investigated whether ghrelin rescues AMs from apoptosis and whether this protective effect contributes to ARDS remission.

Materials and Methods

Cell line

The rat AM line NR8383 was obtained from American Type Culture Collection. Cells were maintained in Ham’s F-12K (Kaighn’s) medium (Gibco) supplemented with 15% fetal bovine serum (Gibco), penicillin (100 U/mL), and streptomycin (100 mg/mL) and cultured in a humidified incubator at 37°C with 5% CO₂.

Silencing of GHSR-1a by using RNA interference

RNA interference was used to silence GHSR-1a expression in AMs. GHSR-1a-siRNA and a scrambled siRNA were synthesized by RiboBioCo. Cells were plated in six-well plates and cultured for 24 hours in media lacking antibiotics, after which GHSR-1a-siRNA or the scrambled siRNA was transfected at a final oligonucleotide concentration of 100 nM using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions and cultured for another 48 hours. The effectiveness of the siRNA was determined by analyzing protein expression by means of Western blotting.

Cell-viability assay

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories), in which colored formazan is formed in viable cells in response to cellular dehydrogenase activity. Briefly, cells (2 × 10⁴ per 100 μL/well) were grown in 96-well plates and exposed to LPS (Sigma; 50, 100, 150, 200, 250, or 300 μg/mL) for 8, 24, and 48 hours or pre-
treated with ghrelin (0.01, 0.1, 1, 10, 100, or 1000 nM) for 30 minutes and exposed to 250 μg/mL LPS for 24 hours. Each dose of LPS and ghrelin was tested on three wells. At the indicated time points, 10 μL of the CCK-8 solution was added to each well, and the plates were incubated at 37°C for 2 hours, after which the OD of each well was measured at 450 nm on a microplate reader (BioTek). The results are presented here as the percentage of viable cells relative to untreated controls.

**Flow cytometry**

Membrane and nuclear events indicating apoptosis were tracked using flow cytometry. The assays involved a two-color analysis of a fluorescein isothiocyanate (FITC)-labeled binding and propidium iodide (PI) uptake and were performed by using the Annexin V-FITC apoptosis detection kit (Becton-Dickinson Biosciences) according to the manufacturer’s instructions. Briefly, cells were harvested and washed with ice-cold PBS, and the collected cells were resuspended in 200 μL of binding buffer and then incubated with 5 μL each of Annexin V-FITC and PI for 15 minutes in the dark at room temperature (RT). Lastly, the fluorescence of at least 10 000 events/sample was analyzed directly on a FACSClone flow cytometer (Becton-Dickinson).

**Terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick-end labeling (TUNEL) assay**

The TUNEL assay was performed on cells plated on glass coverslips by using an in situ apoptosis detection kit (Roche Molecular Biology) according to the manufacturer’s instructions. Briefly, cells were washed three times with PBS, fixed in 4% paraformaldehyde for 1 hour, washed three times with PBS, and then incubated with a permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) on ice for 3 minutes. After three washes, the liquid around the samples was drained, and the samples were incubated with the TUNEL reagent at 37°C for 1 hour in a dark humidified chamber. After three washes with PBS, the samples were examined and photographed under a fluorescence microscope (Carl Zeiss, Inc). The cells in the viewing field were identified by staining with 4’, 6-diamidino-2-phenylindole (DAPI; Invitrogen), and the percentage of apoptotic cells was determined by counting the TUNEL-positive cells and dividing the number by the total number of cells; the values obtained by counting five fields were averaged.

**Mitochondrial transmembrane potential (ΔΨm)**

Mitochondrial depolarization was evaluated by measuring the incorporation of rhodamine 123 (Sigma), a cell-permeable, noncytotoxic, cationic fluorescent dye that is readily sequestered by active mitochondria; mitochondrial uptake of rhodamine 123 is directly proportional to the mitochondrial membrane potential. Cells were washed twice and resuspended in PBS at a concentration of 1 × 10^6 cells/mL, and 500 μL of this suspension was transferred to a 5-mL culture tube and the rhodamine 123 solution was added and then incubated with 5 μL each of Annexin V-FITC and PI for 15 minutes in the dark at room temperature (RT). Lastly, the mitochondrial transmembrane potential was calculated.

**Cytochrome c release**

Cells were treated with reagents, and then subcellular fractionation was performed using a mitochondrial/cytosolic fractionation kit (BioVision). Briefly, cells (1 × 10^6) were harvested and washed twice with cold PBS and then disrupted using a Dounce homogenizer (30 strokes) on ice, and the lysate was centrifuged at 10 000 × g for 30 minutes at 4°C. The supernatant (cytosolic fraction) was collected and the pellet was reconstituted in 100 μL of the mitochondrial extraction buffer. Cytochrome c was quantified in both the cytosolic and the mitochondrial fractions by using a cytochrome c ELISA kit (R&D Systems). Briefly, the cytosolic and mitochondrial fractions were added to 96-well plates precoated with a rat monoclonal antibody specific for cytochrome c, and the OD of each well was measured at 450 nm using a microplate reader (BioTek); the data are expressed as the mean OD of the samples normalized as a percentage of the control value.

**Western blotting**

After various treatments, cells were harvested in ice-cold lysis buffer and lysates were clarified by centrifuging them at 13 000 × g for 15 minutes. The protein concentration of each supernatant was determined using the bicinchoninic acid assay kit (Pierce). The total proteins extracted from AMs were separated using SDS-PAGE (8% or 10% gels) and then transferred to polyvinylidene difluoride membranes. The membranes were blocked for 2 hours at RT with 5% nonfat dry milk in a buffer of Tris-buffered saline containing 20 mM NaF, 2 mM EDTA, and 0.2% Tween 20 and then incubated overnight at 4°C with the following primary antibodies: anti-GHSR-1α (1:1200; Santa Cruz Biotechnology, Inc); anti-JNK1/2, anti-phosphorylated JNK1/2 (Thr183/Tyr185), anti-Bcl-2, anti-Bcl-2-associated X protein (Bax), anti-glycogen synthase kinase (GSK)-3β, and anti-phosphorylated GSK-3β (all 1:1000; Cell Signaling Technology); and anti-β-catenin and anticleaved caspase-3 (both 1:200; Millipore). Next, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:1000; Cell Signaling Technology) for 1 hour at RT, and then the detected bands were visualized using the Immobilon Western chemiluminescent horseradish peroxidase substrate (Millipore). Equal protein loading was confirmed by staining the membranes with an antibody against glyceraldehyde-3-phosphate dehydrogenase (1:1000; Cell Signaling Technology), and the protein levels were estimated semiquantitatively using Image Pro Plus 6.0 software (Media Cybernetics).

**Immunofluorescence staining**

AMs were plated on glass coverslips placed in 24-well plates. After various in vitro treatments, the cells were fixed with 4% paraformaldehyde for 15 minutes, washed three times with PBS, permeabilized with 0.3% Triton X-100 for 30 minutes, and then blocked with 5% normal fetal bovine serum for 1 hour at RT. Next, the cells were exposed to anticleaved caspase-3 (1:20; Millipore) or anti-β-catenin (1:50; Millipore) primary antibodies overnight at 4°C, washed three times with PBS, and then incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) and Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen) for 1 hour. The coverslips were mounted on slides by using mounting medium containing DAPI (Invitrogen), and the cells were visualized using a fluorescence microscope (Carl Zeiss, Inc).
**Construction of an ARDS model**

Sprague Dawley rats (6–7 wk old; weighing 300 ± 20 g) were obtained from the Experimental Animal Center of Sun Yat-Sen University (Guangzhou, China). All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University and were performed in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health (Bethesda, Maryland). We randomly divided 40 male Sprague Dawley rats into 5 groups: the sham operation, sham operation plus ghrelin, cecal ligation and puncture (CLP), CLP plus ghrelin, and CLP plus ghrelin and [D-Lys-3]-GHRP-6 peptide (GHRP)-6 groups. CLP was performed according to the procedure used by Hubbard et al (29). Briefly, after animals were anesthetized using 2% pentobarbital in saline (40 mg/kg, ip injection; Sigma), a 2-cm midline abdominal incision was made to expose the cecum, which was isolated carefully and ligated at approximately 20% of the total length just below the ileocecal valve to prevent bowel obstruction. The cecum was punctured twice on the antimesenteric side by using a sterile 20-gauge needle and then gently squeezed to extrude the fecal material into the peritoneal cavity, after which the cecum was repositioned in the abdomen and the incision was closed in two layers by using sutures. Lastly, warm sterile saline (30 mL/kg) was sc administered for fluid resuscitation.

The sham-operated animals underwent the same procedure, except that the cecum was neither ligated nor punctured. Rats were killed under anesthesia and the lungs were harvested 20 hours after the CLP or sham operation. The doses of ghrelin and [D-Lys-3]-GHRP-6 were 500 μg/kg, except that the cecum was neither ligated nor punctured. Rats were killed under anesthesia and the lungs were harvested 20 hours after the CLP or sham operation. The doses of ghrelin and [D-Lys-3]-GHRP-6 were 500 μg/kg, 15 μg/kg, or vehicle alone, followed by ghrelin administration. Each rat was injected with 45 nmol/kg ghrelin (normal saline) or vehicle (30 mL/kg) for fluid resuscitation. Five hours after the CLP, the rats were administered a slow iv bolus injection of 2 nmol ghrelin (~6.6 μg) or 200 μL of vehicle, after which the minipump was connected to a jugular venous catheter and implanted sc. The rats received an ip injection of [D-Lys-3]-GHRP-6 (3 mg/kg) dissolved in 1 mL saline or vehicle alone, followed by ghrelin administration. Each rat received a total ghrelin dose of approximately 45 nmol/kg (~15.4 μg per 100 g) over the entire treatment period.

**Assessment of pulmonary inflammation**

**Total protein assay and AM apoptosis assay on bronchoalveolar lavage fluid (BALF)**

AMs were obtained by repeatedly performing bronchoalveolar lavage with PBS at RT for a total of eight washes. The BALF was immediately placed on ice and then centrifuged at 300 × g for 10 minutes; the obtained pellets were washed twice with cold PBS. The supernatant from the first 1 mL of the BALF was used for measuring protein concentrations (bicinchoninic acid protein assay). AMs were isolated by means of adherence in six-well plates (3 × 10^6 cells/well; 3 h at 37°C in 5% CO₂), and nonadherent cells were removed by washing twice with PBS. AMs were stained with Giemsa (Sigma) to examine the cellular morphology, and apoptosis was assessed using flow cytometry.

**Lung wet to dry weight ratio and myeloperoxidase (MPO) levels**

To use as an index of lung edema, we calculated the amount of extravascular lung water. A 1 × 1-cm section of the right upper lung was collected and its wet weight was recorded. The tissue was then placed in an incubator at 80°C for 48 hours, and its dry weight was measured. The wet to dry weight ratio was calculated by dividing the wet weight by the dry weight. The middle lobe of the right lung was removed, and the MPO activity was measured as an index of tissue neutrophil sequestration. Briefly, lung tissue was homogenized in 50 mM potassium phosphate buffer (pH 6.0), centrifuged at 300 × g for 15 minutes, and the pellet was resuspended in 50 mM potassium phosphate buffer with 50 mM hexadecyltrimethylammonium bromide (Sigma). After three cycles of snap freezing in liquid nitrogen, thawing, and sonication for 20 seconds, samples were centrifuged at 300 × g for 10 minutes. Aliquots of supernatant were added to potassium phosphate buffer containing 0.167 mg/mL o-dianisidine dihydrochloride (Sigma) and 0.0005% hydrogen peroxide. Absorbance was read at 460 nm using a 96-well plate reader.

**Hematoxylin-eosin staining and assessment of histopathological changes**

Lung tissues were excised from the rats in each group and immediately fixed in 10% formaldehyde at RT; the tissues were embedded in paraffin, sectioned (thickness 4 μm), and stained with hematoxylin-eosin for use in histological analysis. Lung injury was measured based on the findings in 10 randomly selected high-power fields (×200) for each tissue slide. Edema, alveolar and interstitial inflammation and hemorrhage, atelectasis, necrosis, and hyaline membrane formation were separately scored on a 0- to 4-point scale: 0, no injury; 1, injury in 25% of the field; 2, injury in 50% of the field; 3, injury in 75% of the field; and 4, injury throughout the field (33); the final lung injury score was obtained by adding these scores.

**Statistics**

All data are expressed as means ± SD. Various treatment groups were compared by using ANOVA followed by the least significant differences Student’s t test for differences. The data on the percentage changes were analyzed using the Kruskal-Wallis test. P < .05 was considered statistically significant. All the experiments were repeated at least three times.

**Results**

**Ghrelin protects AMs against LPS-induced apoptosis by acting through GHSR-1a**

To initiate AMs apoptosis, we used LPS, which is recognized as a principal pathogenic component in ARDS. The cytotoxicity of LPS was evaluated by exposing cells to LPS (50, 100, 150, 200, 250, and 300 μg/mL) and determining cell viability at 8, 24, and 48 hours by using the CCK-8 assay. LPS treatment induced a time- and dose-dependent reduction in cell viability (Figure 1A) as re-
ported earlier (31). By contrast, the addition of ghrelin (0.01, 0.1, 1, 10, 100, and 1000 nM) dose dependently increased AM survival after LPS treatment in a statistically significant manner (Figure 1B). Based on these results, 250 µg/mL LPS and 100 nM ghrelin were used in all experiments.

**Figure 1.** Effect of ghrelin on the viability of LPS-treated AMs. A, AMs were exposed to various concentrations of LPS (50, 100, 150, 200, 250, 300 µg/mL) for 8, 24, and 48 hours, and then cell viability was measured using the CCK-8 assay. Cell survival is expressed as a percentage relative to that in the control group. CON, control. B, AMs were exposed to various concentrations of ghrelin (0.01, 0.1, 1, 10, 100, 1000 nM) for 30 minutes and then treated with 250 µg/mL LPS. AMs survival at 24 hours after LPS treatment was measured using the CCK-8 assay, and the survival has been expressed as a percentage relative to that in the control group. C, GHSR-1a protein expression in AMs is silenced after siRNA treatment. Lane 1, AMs lysate; lane 2, lysate of AMs treated with a scrambled siRNA; and lane 3, lysate of AMs treated with a GHSR-1a-specific siRNA. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. D, The protective effect of ghrelin against LPS-induced apoptosis in AMs is mediated by GHSR-1a. AMs treated or not treated with the GHSR-1a-specific siRNA were treated with vehicle or ghrelin for 30 minutes and then exposed to LPS for 24 hours. The expression of Bcl-2, Bax, and cleaved caspase-3 was examined by performing Western blotting analysis, and then the band intensities were quantified. E, Annexin V-FITC and PI staining and flow cytometry were used for measuring LPS-induced AMs apoptosis in the presence or absence of ghrelin. All data represent the mean ± SD of three independent experiments. *, P < .05 compared with control; #, P < .05 compared with LPS treated alone; **, P < .05 compared with LPS-plus-ghrelin treatment.
In accord with previous findings (33, 34), GHSR-1a was readily detected in AMs, and targeting the canonical GHSR-1a by using a specific siRNA sequence diminished the expression of GHSR-1a (Figure 1C). Moreover, treatment with the GHSR-1a-specific siRNA reversed the ghrelin-dependent reduction in Bax expression and increase in Bcl-2 (Figure 1D). Next, flow cytometry results (Figure 1E) revealed that ghrelin treatment prevented an LPS-mediated increase in the proportion of early and late apoptotic AMs, which led to an increase in the proportion of vital cells. A statistically significant difference was observed between the LPS-plus-ghrelin group and the LPS-plus-ghrelin and GHSR-1a-targeting siRNA group, which indicated that the GHSR-1a siRNA abrogated the protective effect exerted by ghrelin on AMs survival after LPS treatment. Thus, ghrelin protects AMs against LPS-induced apoptosis by acting through the recognized ghrelin receptor, GHSR-1a.

To detect DNA fragmentation after treatment with 250 μg/mL LPS, we performed TUNEL assays. Quantification revealed that the addition of ghrelin (100 nM) before LPS treatment decreased the number of TUNEL-positive AMs in a statistically significant manner; as expected, this antiapoptotic effect of ghrelin was abolished by the GHSR-1a siRNA (Figure 2, A and B). The δψm is an indicator of cells undergoing the terminal phase of mitochondrion-dependent apoptosis. LPS treatment drastically lowered the δψm in a time-dependent manner, but this decrease could be partly blocked by ghrelin (Figure 2C). In mitochondrion-dependent apoptosis, the release of cytochrome c from mitochondria into the cytosol is critical for the activation of caspases and the ensuing cell death (32). After LPS treatment, cytochrome c release from AMs at 24 hours was increased, but this release was potently inhibited after exposure to ghrelin (Figure 2D).

**Ghrelin affects Bcl-2 and Bax expression and caspase-3 activity in AMs**

To investigate the molecular mechanism by which ghrelin protects AMs against LPS-induced apoptosis, we performed Western blotting to measure the levels of Bcl-2, Bax, and cleaved caspase-3. LPS induced a dose- and time-dependent reduction in Bax expression and an increase in Bcl-2 expression (Figure 1D). Moreover, treatment with the GHSR-1a-specific siRNA reversed the ghrelin-dependent reduction in Bax expression and increase in Bcl-2 (Figure 1D).

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**Figure 2.** Protective effect of ghrelin against LPS-induced apoptosis in AMs. A, Representative photographs of TUNEL staining in various groups. Scale bar, 50 μm. CON, control. B, Quantification of TUNEL-positive apoptotic cells; six fields were analyzed in each cell population. The bar graph shows the percentage of TUNEL-positive nuclei relative to DAPI-positive total nuclei. C, Effect of ghrelin on δψm in AMs. Cells were either incubated or not incubated with ghrelin before LPS treatment. At the end of the treatment period, cells were loaded with 10 μM rhodamine 123 for 30 minutes, after which the fluorescence intensity was measured using flow cytometry. D, The levels of cytochrome c released into the cytoplasm in AMs treated with or without ghrelin were determined using a cytochrome c ELISA kit; the absorbance was measured by using a microplate reader at 450 nm wavelengths. All data are expressed as means ± SD obtained from triplicate experiments. *, P < .05 compared with control; #, P < .05 compared with LPS treated alone.
dependent change in the expression of Bcl-2, Bax, and cleaved caspase-3 in AMs (Figure 3, A and B): the levels of Bax and cleaved caspase-3 elevated, whereas the expression of Bcl-2 was markedly decreased as the concentration or treatment time of LPS on AMs was increased. By contrast, at 24 hours after ghrelin plus LPS treatment, statistically significant decreases in both Bax and cleaved caspase-3 and an increase in Bcl-2 were observed (Figure 3C). Moreover, the results of immunofluorescence assays performed in parallel revealed that LPS treatment increased the red color, indicating cleaved caspase-3 in AMs and that ghrelin addition lowered the level of cleaved caspase-3 when compared with the level in LPS-treated AMs (Figure 3D).

The protective effect of ghrelin is mediated by a reduction in JNK activation and an increase in Wnt/β-catenin activation

To gain further insight into the mechanism by which ghrelin inhibits apoptosis in AMs, we investigated

Figure 3. Effects of LPS and ghrelin on the expression of Bax, Bcl-2, and cleaved caspase-3 in AMs. A and B, Changes in apoptosis-associated protein levels after LPS treatment. Levels of Bax, Bcl-2, and cleaved caspase-3 were determined by performing Western blotting on protein extracts of AMs challenged with various doses of LPS (100, 150, 200, 250, 300 µg/mL) for 24 hours or with 250 µg/mL LPS for 8, 24, and 48 hours. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. C, Ghrelin prevents LPS-induced changes in the expression of apoptosis-associated proteins. Cultured AMs were pretreated with or without ghrelin for 30 minutes and then incubated with or without LPS for 24 hours. The expression of Bax, Bcl-2, and cleaved caspase-3 was examined using Western blotting analysis, and band intensities were quantified using densitometry. All results are expressed as means ± SD obtained from three independent experiments. *P < .05 compared with control; #, P < .05 compared with LPS treated alone. D, Immunofluorescence detection of cleaved caspase-3 during LPS-induced apoptosis. Cells were pretreated with or without ghrelin for 30 minutes and then exposed to LPS for 24 hours. Cells were fixed and stained with an antibody that recognizes cleaved caspase-3 (red) and then stained with DAPI (blue) to visualize nuclei. Scale bar, 50 µm.
whether JNK and Wnt/β-catenin signaling pathways, which are recognized to be associated with cell death or survival in vitro and in vivo, are involved in mediating the antiapoptotic effect of ghrelin on AMs. Western blotting analysis revealed that LPS treatment dose dependently increased the JNK phosphorylation but lowered the phosphorylated GSK-3β and β-catenin levels (Figure 4, A and B); LPS treatment did not alter the total JNK and GSK-3β levels. Next, we determined how ghrelin affected JNK, GSK-3β, and β-catenin activation after LPS treatment (Figure 4, C and D): pretreatment of AMs with ghrelin markedly reduced LPS-dependent JNK phosphorylation but elevated phosphorylated GSK-3β and β-catenin expression. We also investigated the effect of ghrelin on Wnt/β-catenin activation by evaluating the cellular localization of β-catenin; immunofluorescence labeling showed that LPS exposure suppressed the nuclear and prenuclear accumulation of β-catenin and that this was partly abrogated in ghrelin-treated AMs (Figure 4E). Moreover, we determined that silencing GHSR-1a expression potently reversed ghrelin-dependent JNK inhibition and Wnt/β-catenin activation (Figure 5A). These results collectively indicated that the antiapoptotic effect of ghrelin on LPS-treated AMs is exerted through the ghrelin receptor and is mediated at least partly through JNK and Wnt/β-catenin signaling.

When AMs were preincubated with the JNK-specific inhibitor SP600125, LPS-induced JNK phosphorylation was markedly diminished, as in the case of ghrelin treatment, and the levels of both phosphorylated GSK-3β and β-catenin were elevated, which indicated that the Wnt/β-catenin pathway might be inhibited as a result of JNK activation (Figure 5B). Moreover, like ghrelin treatment, the addition of SB216763, an inhibitor of GSK-3, stabilized phosphorylated GSK-3β and β-catenin levels (Figure 5C). In parallel flow cytometry assays (Figure 5D), we observed that SP600125 and SB216763 increased AMs survival by approximately 70% and 75%, respectively. However, no additive or synergistic effect on the cells was observed after treatment with ghrelin plus SP600125 or SB216763. Collectively our results suggest that ghrelin promotes AMs survival at least partly by suppressing JNK signaling and activating Wnt/β-catenin signaling.

### Effects of ghrelin administration on acute lung injury

Lastly, we used an ARDS model constructed by using CLP to investigate whether exogenous application of ghrelin can contribute to the remission of sepsis-induced ARDS in rats and whether this beneficial effect is associated with a delay in AMs apoptosis. The percentage of apoptosis in isolated AMs was markedly lower in rats treated with CLP plus ghrelin than in CLP-treated rats, but this antiapoptotic effect was absent in [D-Lys-3]-GHRP-6-treated rats (Figure 6A). The total protein concentration in BALF, a marker of pulmonary damage, increased after the CLP challenge, and this indicated vascular leakage and ARDS caused by sepsis. However, the addition of ghrelin protected the integrity of the microvascular barrier, which was indicated by the significantly lower BALF protein concentration in the CLP-plus-ghrelin group than in the CLP group (Figure 6B).

Neutrophils are considered to play a central role in the pathogenesis of most forms of ARDS (34), and the MPO activity in the lung indicates neutrophil accumulation. CLP elicited a marked up-regulation of MPO activity in the lung tissues, but after treatment with ghrelin, the CLP-induced increase in MPO activity was lower than that measured in the absence of ghrelin (Figure 6C). Moreover, the lung wet to dry weight ratio was significantly higher in the CLP group than in the sham group, but the lung water content decreased significantly in the septic animals treated with ghrelin (Figure 6D). These ameliorative inflammatory effects were abolished after treatment with [D-Lys-3]GHRP-6.

Histopathological changes in lung tissue were observed 20 hours after CLP administration (Figure 6E). In the sham and sham-plus-ghrelin groups, fluid and protein accumulation and the infiltration of inflammatory cells and red blood cells was not prominent in the alveolar space. By contrast, lungs from the CLP-group rats exhibited increased alveolar septal thickening, interstitial edema, and vascular congestion as well as substantial neutrophil infiltration in the interstitium and diffuse alveolar hemorrhage and collapse. By comparison, the rats in the CLP-plus-ghrelin group showed less distortion of alveolar architecture, fewer thickened and disrupted septa, and less intraalveolar bleeding and neutrophil infiltration; this protective effect was again absent in animals treated with [D-Lys-3]GHRP-6. A semiquantitative analysis of the histopathological scores measured for rat lungs is presented in Figure 6F. Our results also showed that treatment with [D-Lys-3]GHRP-6 alone failed to affect the inflammation level in lung and the degree of lung injury in septic rats (date not shown).

### Discussion

The early recognition of and response to invading pathogens exhibited by AMs generates signaling cascades that constitute the initial immune response in the lung. For instance, AMs are responsible for efficiently clearing apoptotic neutrophils by means of phagocytosis, as a result
of which the AMs secrete antiinflammatory cytokines such as TGF-β and IL-10 to prevent further tissue injury and perpetuate inflammation (35–38). However, during sepsis, macrophages were previously shown enhanced apoptosis and being incapable of producing inflammatory mediators in response to stimulation (39, 40), which characterize sepsis-induced immunosuppression (39, 41). Lu et al (17) used a male Sprague Dawley rat sepsis model.

Figure 4. Effect of ghrelin on the activation of JNK and Wnt/β-catenin signaling in AMs. A, Concentration-dependent activation of JNK and inhibition of Wnt/β-catenin signaling by LPS in AMs. AMs were incubated for 8 hours with increasing concentrations of LPS, and then Western blotting was performed to measure the levels of phosphorylated (p)-JNK, JNK, p-GSK-3β, GSK-3β, and β-catenin. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B, Ghrelin inhibits JNK phosphorylation and activates the Wnt/β-catenin pathway. AMs were pretreated with various concentrations of ghrelin (0, 0.1, 1, 10, 100, 1000 nM) for 30 minutes and then incubated with LPS for 8 hours. Western blotting was performed to once again detect p-JNK, JNK, p-GSK-3β, GSK-3β, and β-catenin. Band intensities were quantified, and the values obtained are presented as means ± SD of three separate experiments. *, P < .05 compared with control. C, Ghrelin induces nuclear translocation of β-catenin in AMs. AMs were treated with LPS for 24 hours in the absence or presence of pretreatment with ghrelin for 30 minutes, and then immunofluorescence staining was performed to localize β-catenin (green); nuclei were stained with DAPI. The images show the relative nuclear and cytoplasmic distribution of β-catenin in the cells. Scale bar, 50 μm. CON, control.
Figure 5. Effects of GHSR-1a siRNA, SP600125, and SB216763 on the activation of JNK and Wnt/β-catenin signaling. A, Cells were transfected with the GHSR-1a-specific siRNA before ghrelin and LPS treatment. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B, AMs were pretreated with SP600125 (40 μM) and ghrelin for 30 minutes each sequentially and then stimulated with LPS for 8 hours. C, AMs were pretreated with SB216763 (25 μM) and ghrelin for 30 minutes each sequentially and then stimulated with LPS for 8 hours. Total cell lysates were prepared and the levels of p-GSK-3β, GSK-3β, and β-catenin were determined using Western blotting. D, Effects of SP600125 and SB216763 on LPS-induced apoptosis in AMs. AMs were pretreated with or without SP600125, SB216763, or ghrelin for 30 minutes and then stimulated with LPS for 24 hours. AMs apoptosis was measured using Annexin V-FITC/PI staining and flow cytometry. Values shown are means ± SD of three separate experiments. *, P < .05 compared with control; #, P < .05 compared with LPS treated alone; **, P < .05 compared with LPS-plus-ghrelin treatment.
Figure 6. Influence of ghrelin on the remission of CLP-induced ARDS. After CLP, rats were treated with vehicle, ghrelin (45 nmol/kg), or the GHSR-1a inhibitor [D-Lys-3]GHRP-6 (3 mg/kg), and the animals were killed 20 hours after CLP or sham operation. We determined the percentage of Annexin V-positive (apoptotic) AMs (A), BALF total protein concentration (B), and the lung MPO activity (C) and wet to dry weight ratio (D). E, Effects of ghrelin on histopathological changes during CLP-induced lung inflammation. Rats were anesthetized, and lung tissue samples were collected at 20 hours after CLP for use in histological analysis. The representative histological changes of the lung shown here were obtained from rats of distinct groups (hematoxylin and eosin staining, original magnification, ×200). F, Severity of lung injury expressed as injury scores. Values presented are means ± SD. *, P < .05 compared with sham group; #, P < .05 compared with CLP group.
constructed by performing CLP; they determined that the number of AMs in late-stage septic rats decreased substantially and that this was accompanied by 2.5- and 3.2-fold increases in the number of apoptotic AMs in early- and late-stage septic animals, respectively, when compared with the levels in sham-operated controls. Thus, accumulating evidence indicates a consistent association between AMs apoptosis and ARDS in humans and animal models (42, 43).

Ghrelin regulates various cellular functions and physiological processes such as apoptosis, vascular permeability, and innate and acquired immunity and contributes to the resolution of a variety of lung diseases such as pulmonary edema, emphysema, cystic fibrosis, and pneumonia (44, 45). In agreement with previous findings, our results demonstrated that the preincubation of AMs with ghrelin inhibited LPS-induced apoptosis in vitro. Moreover, the prosurvival effect of ghrelin on AMs is likely mediated by ghrelin receptors because in AMs transfected with a GHSR-1a-specific siRNA, ghrelin did not exert an antiapoptotic effect in vitro, and this was accompanied by a decreased ratio of Bcl-2 to Bax.

The Bcl-2 family of proteins plays a major role in intracellular apoptotic signal transduction, and cell survival is enhanced when Bcl-2 expression is high but Bax expression is low. Early studies showed that, after sepsis, the proapoptotic Bax was up-regulated and Bcl-2 was down-regulated (46, 47). This study has demonstrated that ghrelin reversed the LPS-induced increase in Bax and decrease in Bcl-2 levels in AMs, which agrees with previous findings (48–50). Moreover, Bcl-2-family proteins might regulate the 3Δm and the release of mitochondrial cytochrome c during oxidative stress in cells. When the Bcl-2 to Bax ratio elevates, the mitochondrial transmembrane potential increases, resulting in suppression of release of cytochrome c from mitochondria and prevention of caspase-3 activation. In this study, we determined that ghrelin treatment blocked LPS-dependent reduction in the 3Δm and the elevation of cytochrome c release; this suggests that ghrelin likely exerts its antiapoptotic effect by mitigating LPS-induced mitochondrial dysfunction. Moreover, caspases function downstream from proteins of the Bcl-2 family in the apoptotic cascade, which activates a deoxyribonuclease that fragments oligonucleosomal DNA; our results showed that caspase-3 was clearly activated in AMs after LPS treatment and that this activation was diminished following ghrelin pretreatment.

To uncover the molecular underpinnings of a sepsis-induced increase in AMs apoptosis and to gain insights into the mechanism by which ghrelin inhibits apoptosis in AMs, we investigated two intracellular signaling pathways, the JNK and Wnt/β-catenin pathways, which can be influenced by various extracellular signals and regulate fundamental cellular processes such as cell proliferation, differentiation, and apoptosis (51–54). JNK, a member of the MAPK family and a critical mediator of cell survival, became activated and subsequently phosphorylated numerous downstream proteins including the c-Jun and Bcl-2 families and induced the mitochondrial release of cytochrome c, which led to the activation of death signals (27). JNK activation is required for LPS-induced apoptosis in macrophages during sepsis (56, 57). GSK-3β is a proapoptotic protein, and GSK-3β inhibitors have been shown to reduce infarct sizes after focal cerebral ischemia in vivo and improve neuronal survival in vitro (58).

Several transcription factors such as cAMP-response element-binding protein, nuclear factor-κB, and β-catenin were reported to be regulated by GSK-3β as part of the cell
survival mechanism (59). Inactivation of GSK-3β by phosphorylation of Ser9 results in β-catenin translocation to the nucleus, which is required for the prosurvival effect of Wnt/β-catenin (53). Ghrelin was only recently reported to inhibit the phosphorylation of JNK (60, 61) and activate the Wnt/β-catenin pathway (62, 63). In our study, LPS treatment was followed by rapid JNK phosphorylation and a reduction in phosphorylated GSK-3β and β-catenin levels; conversely, pretreatment with ghrelin inhibited JNK phosphorylation and increased the expression of phosphorylated GSK-3β and β-catenin. Although no synergistic effect was detected when cells were pretreated with ghrelin plus SP600125 or SB216763, these pharmaceutical agents inhibited apoptosis similarly as ghrelin did, which indicates that inactivation of JNK or GSK-3β is at least partly responsible for the antiapoptotic effects of ghrelin, which agrees with previous reports (64, 65). Several mechanisms that mediate the cross talk between the JNK and Wnt/β-catenin pathways have been reported recently (55, 58, 66–69), and our current results have shown that JNK activation controls the phosphorylation of GSK-3β.

In our in vivo data agreed with our in vitro results. In accord with past studies, our results showed that ghrelin treatment substantially lowered these inflammatory parameters measured in the lung: BALF protein concentration, lung MPO activity, lung wet to dry weight ratio, and histopathological scores measured at 20 hours after CLP operation. Correspondingly, our in vivo results indicated that ghrelin protected cells by activating GHSR-1a because a GSHR-1a-specific antagonist suppressed the ability of ghrelin to protect cells against septic insult. Furthermore, AMs isolated from the rats in the CLP-plus-ghrelin group exhibited a low apoptosis percentage. Thus, we conclude that ghrelin’s protective effects against septic ARDS are related to ghrelin’s ability to modulate the expression of inflammatory mediators and the induction of apoptosis of AMs in the lung. However, several questions related to the mechanisms by which ghrelin exerts its effects remain unanswered, such as whether the systemic administration of ghrelin and [D-Lys-3]GHRP-6 affects not only AMs but also alveolar neutrophils and lung epithelial and endothelial cells and whether ghrelin is involved in inducing changes in cytokines and other inflammatory mediators from the perspective of the whole body. Therefore, future studies should investigate how ghrelin functions in maintaining homeostasis in the lung.

In conclusion, our findings suggest that GHSR-1a is expressed in AMs and that ghrelin protects AMs against apoptosis in vivo and vitro by activating GHSR-1a, which helps reduce the severity of sepsis-induced ARDS. We have also provided evidence that the antiapoptotic effect of ghrelin might at least partly involve the inhibition of JNK signaling and the activation of the Wnt/β-catenin pathway (Figure 7). Our results indicate that ghrelin is a promising candidate for use in a novel adjuvant therapy strategy designed for sepsis-induced ARDS.

Acknowledgments

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