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Nesfatin-1 alleviates acute lung injury through reducing inflammation and oxidative stress *via* the regulation of HMGB1

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Abstract. – OBJECTIVE: Acute lung injury (ALI) is the most common complication of sepsis, with rapid onset and high mortality. There is currently no effective treatment for ALI. Therefore, we looked for a good method of treating ALI by studying the effect and mechanism of Nesfatin-1 on ALI.

MATERIALS AND METHODS: We used LPS to induce mouse and human alveolar epithelial cell line BEAS-2B to construct an ALI model. Recombinant Nesfatin-1 was administered subcutaneously to mice or used to stimulate BEAS-2B cells. We collected mouse bronchoalveolar lavage fluid and mouse lung tissue to detect changes in inflammatory factors and oxidative stress levels. In addition, we examined the expression changes of HMGB1 to study the mechanism of Nesfatin-1.

RESULTS: Exogenous Nesfatin-1 significantly attenuated LPS-induced ALI and reduced inflammation levels and oxidative stress levels in mouse lung tissue. In cell experiments, Nesfatin-1 also reduced inflammation levels and oxidative stress levels in BEAS-2B cells. In addition, Nesfatin-1 reduced the expression of HMGB1 in mouse lung tissues and BEAS-2B cells, and decreased the activity of p38MAPK and NF-κB signaling pathways in the inflammation-related pathway downstream of HMGB1. However, after overexpression of HMGB1, the therapeutic effect of Nesfatin-1 on ALI was attenuated.

CONCLUSIONS: Nesfatin-1 regulates the expression of HMGB1 in alveolar epithelial cells. By reducing the expression of HMGB1, Nesfatin-1 can reduce the inflammation-related signaling pathway downstream of HMGB1 to reduce the level of inflammation and oxidative stress in alveolar epithelial cells, thereby alleviating ALI.

Key Words:
Nesfatin-1, Acute lung injury, Inflammation, Oxidative stress, HMGB1.

Introduction

Sepsis is a complex systemic inflammatory response syndrome that can cause high mortality in critically ill patients and multiple organ failures, including the cardiovascular system, liver, kidneys, and lungs. Respiratory dysfunction is the most common complication of sepsis due to lung susceptibility. Therefore, acute lung injury (ALI) and its most severe form, acute respiratory distress syndrome, are the most serious consequences of sepsis and are the leading cause of death in ICU patients[1]. ALI is characterized by hypoxemia of respiratory failure, pulmonary hypertension, alveolar edema, decreased lung compliance, and weakened air exchange function. The pathophysiological process of ALI is recognized as an excessive, uncontrolled lung inflammatory response, which indicates that there are many types of inflammatory cell infiltration, alveolar capillary injury, abnormal ROS release characterized by excessive oxidative stress and alveolar epithelial cell apoptosis[2].

Nesfatin is a precursor neuropeptide derived from nucleobindin 2 (NUCB2) and is widely distributed in various tissues of mammals[3]. NUCB2 is encoded by the NEFA gene and can be cleaved into three fragments under the action of prohormone: Nesfatin-1, nesfatin-2, and nesfatin-3. Nesfatin-1 can induce dose-dependent feeding behavior inhibition and control weight gain through lateral ventricle injection. In addition to maintaining the feeding balance, it also has a variety of physiological effects. Shen et al[5] found that Nesfatin-1 inhibited apoptosis induced by renal ischemia-reperfusion injury after injection of 10 μg /(kg*d) Nesfatin-1 in rats, and decreased expression of MDA and MPO and increased SOD expression. It is suggested that Nesfatin-1 also

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has the effect of alleviating ischemia-reperfusion injury. Solmaz et al[6] found that 2 μg/(kg*d) Nesfatin-1 treatment of rats for 3 days can reduce apoptosis induced by oxidative stress in skin and that Nesfatin-1 reduces inflammatory factors IL-1β and IL-6, and increased the expression of TGF-β1. Therefore, Nesfatin-1 has shown potent anti-inflammatory, anti-apoptotic, and anti-oxidative stress capabilities. ALI, as a disease accompanied by a large amount of inflammation, reactive oxygen species and apoptosis, inhibition of inflammation, oxidative stress, and apoptosis is the key to treatment. However, there are still no relevant studies on the role of Nesfatin-1 in ALI.

Therefore, we raised C57/BL6 mice and used lipopolysaccharide (LPS) to construct a model of mouse sepsis to study the effect of Nesfatin-1 on ALI. In addition, the human alveolar epithelial cell line BEAS-2B was also used to explore the role and mechanism of Nesfatin-1. We hope that the study of Nesfatin-1 will help clinical ALI treatment.

Materials and Methods

Animals and Grouping

Eight-week-old C57/BL6 male mice (18-22 g) were purchased from Beijing Charles River Experimental Animal Technology Co., Ltd. We housed mice in SPF-class barriers and gave standard feed and drinking water. Mice were divided into 3 groups. The mice in Control group were routinely raised. ALI model was constructed in mice of ALI group and ALI+Nesfatin-1 (BioVision, Milpitas, CA, USA) group, while mice in ALI+Nesfatin-1 group were injected subcutaneously with 10 μg/(kg*d) Nesfatin-1 one week prior to the construction of the ALI model. This investigation was approved by the Animal Ethics Committee of Liaocheng People's Hospital Animal Center.

Operative Procedure of Mice ALI

After the mice were anesthetized with 5% chloral hydrate (10 μL/g), we fixed the mice in a supine position on the console. We use a hemostat to gently grip the mouse's tongue and fix it on one side. Then, we attached the scalp needle to the syringe and gently inserted the needle into the nose of the mouse. We gently injected the LPS into the nose of the mouse. Immediately after the injection, the mice were held in the hand, and the mice were gently shaken so that the LPS was evenly distributed in the lungs of the mice. Pronounced wet rales in the lungs of the mice meant that the LPS has successfully entered the lungs of the mice. After 24 h, we sacrificed the mice by cervical dislocation.

Wet/dry Weight Ratio (W/D) of Lung Tissue

At the end of the modeling, we took the intact lung tissue of the mouse, weighed it, and recorded it as wet weight. The mouse lung tissue was then placed in an incubator at 60°C for 48 h. Then, we took the lung tissue, weighed it, and recorded it as dry weight. The ratio of wet weight to dry weight is then calculated as W/D.

Myeloperoxidase (MPO), Malondialdehyde (MDA), Superoxide Dismutase (SOD) Glutathione Peroxidase (GSH-Px), and Activity Assay

After the mice were sacrificed, we took the mouse lung tissue and cleaned it with phosphate-buffered saline (PBS). We then ground the lung tissue into powder at low temperature and dissolved it in PBS. The expression levels of MPO, MDA, SOD, and GSH-Px in mouse lung tissue represent the level of oxidative stress in lung tissue. We used MPO kit (Lianke, Hangzhou, China), MDA kit (Lianke, Hangzhou, China), SOD kit (Lianke, Hangzhou, China), and GSH-Px kit (Lianke, Hangzhou, China) to detect the expression of relevant indicators.

Preparation of Bronchoalveolar Lavage Fluid (BALF)

After we removed the lung tissue of the mice, we washed the lung tissue 3 times with PBS and collected the PBS. Then, we centrifuged the collected BALF. The supernatant after centrifugation was used to measure the contents of IL-1β and TNF-α, and the sediment after centrifugation was redissolved in PBS and subjected to cell counting.

Enzyme Linked Immunosorbent Assay (ELISA)

We collected BALF from mice and analyzed the levels of IL-1β and TNF-α by ELISA. After collecting BALF, we removed the sediment by centrifugation and retained the supernatant, and then we measured the IL-1β and TNF-α levels in the supernatant using ELISA kits (Lianke, Hangzhou, China).

Hematoxylin-Eosin (HE) Staining

After the mice were sacrificed, we took the mouse lung tissue and injected 4% parafor-

maldehyde into the lungs from the lung main bronchus. Then, we soaked the lung tissue in 4% paraformaldehyde for 48 h. We then used PBS to clean the lung tissue and make paraffin and paraffin sections. We placed the paraffin sections in hematoxylin stain (Beyotime, Shanghai, China) for 5 min and then rinsed with water for 3 min. At the end of the rinse, we placed the paraffin sections in hydrochloric acid (Beyotime, Shanghai, China) for 3 s and then immediately rinsed the slices in running water for 3 min. The sections were then stained for 3 min in eosin solution (Beyotime, Shanghai, China). After staining, paraffin sections were dehydrated and sealed ,and the staining results were observed under a microscope.

Cell Culture and Treatment

BEAS-2B cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). We cultured BEAS-2B cells in Dulbecco's Modified Eagle's Medium (DMEM) medium (Gibco, Rockville, MD, USA) and complete medium contained 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin plus streptomycin (Gibco, Rockville, MD, USA). We used culture dish to culture cells and placed the cells in an incubator at 37°C and 5% CO_2. After the cell growth density reaches 60-70%, we use drugs to stimulate cells or perform cell transfection.

Cell Transfection

Lenti-NC and Lenti-HMGB1 were constructed at Shanghai Jima Biotechnology Co., Ltd. After cell growth density reached 60% in 6-well plates, we transfected Lenti-NC and Lenti-HMGB1 into BEAS-2B cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Lenti-NC was used as a negative control. Transfection efficiency was determined by Western blot and RT-PCR.

Western Blot

We took mouse lung tissue or BEAS-2B cells and used lysates to lyse tissues (Beyotime, Shanghai, China) or cells. After centrifuging the lysate, we took the supernatant and added 5 x loading buffer (Beyotime, Shanghai, China). An equal amount of protein was added to each well of the electrophoresis gel. After electrophoresis and membrane transfer, the protein is transferred to the polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After blocking with 5% skim milk, we used primary antibody dilution (IL-1β, 1:3000, Rabbit, Abcam, Cambridge, MA, USA; IL-6, 1:3000, Rabbit, Abcam, Cambridge, MA, USA; IL-8, 1:3000, Rabbit, Abcam, Cambridge, MA, USA; TNF-α, 1:3000, Rabbit, Abcam, Cambridge, MA, USA; SOD1, 1:3000, Rabbit, Abcam, Cambridge, MA, USA; SOD2, 1:3000, Rabbit, Abcam, Cambridge, MA, USA; HMGB1, 1:3000, Rabbit, Abcam, Cambridge, MA, USA; p38, 1:3000, Rabbit, Abcam, Cambridge, MA, USA; p-p38, 1:3000, Rabbit, Abcam, Cambridge, MA, USA; p65, 1:3000, Rabbit, Abcam, Cambridge, MA, USA; IKKα, 1:3000, Rabbit, Abcam, Cambridge, MA, USA; IκBα, 1:3000, Rabbit, Abcam, Cambridge, MA, USA; β-actin, 1:5000, Rabbit, Abcam, Cambridge, MA, USA) to incubate the PVDF membranes overnight at 4°C. We then washed the PVDF membrane with phosphate-buffered saline and tween-20 (PBST) and incubated the PVDF membranes with secondary antibody dilution (Goat anti-rabbit, 1:3000, Abcam, Cambridge, MA, USA) for 2 h. Finally, we used enhanced chemiluminescence (ECL) to observe protein bands.

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

After taking mouse lung tissue or BEAS-2B cells, we extracted total RNA using the TRIzol method (Invitrogen, Carlsbad, CA, USA). After detecting the RNA concentration using a spectrophotometer, we reverse-transcribed mRNA into complementary deoxyribose nucleic acid (cDNA) using a reverse transcription kit (Invitrogen, Carlsbad, CA, USA). We then used the designed primers to amplify the corresponding gene. The amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression in the cells was used as a control. The results of the detection are indicated by $2^{-\Delta\Delta CT}$. The primers were synthesized by Shanghai Jierui Biotech Co., Ltd. (Shanghai, China), shown in Table I.

Cell Counting Kit-8 (CCK8) Assay

We passaged BEAS-2B cells into 96-well plates. After the cell growth density reached 60%, we treated the cells and set up three replicate wells. We then added 10 μL of CCK8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan) to each well, and the cells were incubated in the incubator for 2 h. Then, we used a microplate reader to measure the absorbance of each well at 450 nm.

Table I. RT-PCR primers.

Name	Sense/anti-sense	Sequence (5′-3′)
Human		
IL-1β	Sense	AGCTATCGATCATGCTACGACG
	Anti-sense	ACGATCGACGTACGTACGTATCC
IL-6	Sense	ACTCACCTCTTCAGAACGAATTG
	Anti-sense	CCATCTTTGGAAGGTTCAGGTTG
IL-8	Sense	CAGTTGAAGTTGCCATCAGC
	Anti-sense	CAGTTGAAGTTACCATCAGC
TNF-α	Sense	CTACCATCACCGCACTGAGAT
	Anti-sense	GGTCACTTCACCATAGTGGACA
SOD1	Sense	GACTGAGTACCTGAACCGGCATC
	Anti-sense	CTGAGCAGCGTCTTCAGAGACA
SOD2	Sense	AGCTACACGACTGACACGTCATG
	Anti-sense	GCATCGTCAGTACGTACTGACGA
GPX1	Sense	GGTACTGACTGACACCCACAGCA
	Anti-sense	GTCGCATGCATGCCTATGCACA
CAT	Sense	AGCTACGACTGCTCATGCCA
	Anti-sense	GCATGCACGCCTAACGTA
HMGB1	Sense	CAGGTTCACCCAGTGACAACTCA
	Anti-sense	CACGAGACAGGTGGAAGAAGAGC
GAPDH	Sense	ACAACTTTGGTATCGTGGAAGG
	Anti-sense	GCCATCACGCCACAGTTTC
Mouse		
HMGB1	Sense	AGCTGCACGACTGACACGTA
	Anti-sense	GCTAGCTACACCGTACTGCA
GAPDH	Sense	GCATCACGTACTGACACCATG
	Anti-sense	CGACTGACTGACACTGCGA

Immunocytofluorescence (IF) Staining

We passaged BEAS-2B cells into 24-well plates. After the cell growth density reached 60%, we treated the cells differently. We then removed the 24-well plates and washed the cells with PBS. The cells were then sequentially soaked in 4% paraformaldehyde and 0.2% Triton-PBS for 15 min each. 5% BSA-PBS was used to block cells. We then incubated the cells with primary antibody dilution (IL-1β, 1:500, rabbit, Abcam, Cambridge, MA, USA; TNF-α, 1:500, rabbit, Abcam, Cambridge, MA, USA; SOD1, 1:500, rabbit, Abcam, Cambridge, MA, USA; SOD2, 1:500, rabbit, Abcam, Cambridge, MA, USA; HMGB1, 1:500, rabbit, Abcam, Cambridge, MA, USA) at 4 °C overnight. After washing the cells with PBS, we incubated the cells for 1 h using fluorescent secondary antibody (Goat anti-rabbit-FITC, 1:500, Abcam, Cambridge, MA, USA). 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA, USA) is used to stain the nucleus. After dyeing DAPI, we observed and recorded the staining results using a fluorescence microscope.

Reactive Oxygen Species (ROS) Level Detection

ROS levels in BEAS-2B cells were detected to determine changes in oxidative stress in the cells. We used 6-well plates to culture the cells and treat them differently. Then, we used the DCFH-DA kit (Keygen, Nanjing, China) to measure ROS levels according to the manufacturer's instructions.

Statistical Analysis

All experiments were repeated more than 3 times. Statistical Product and Service Solutions (SPSS) 21.0 (IBM, Armonk, NY, USA) and GraphPad prism 7.0 (La Jolla, CA, USA) were used to analyze experimental data for this study. All experimental data are represented by mean ± standard deviation. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

Exogenous Nesfatin-1 Attenuates LPS-Induced Mouse ALI and Reduces Inflammation and Oxidative Stress

To clarify the effect of Nesfatin-1 on sepsis-induced mouse ALI. We used LPS to induce sepsis in mice and induced ALI. The results of HE staining (Figure 1A) showed that the number of alveolar epithelial cells in LPS-induced mice decreased and edema occurred, and inflammatory cells infiltrated in the interstitial lung. The symptoms of alveolar epithelial cells in Nesfatin-1 treated mice were significantly improved and the inflammatory cells in the alveolar interstitial cells were reduced. In addition, Nesfatin-1

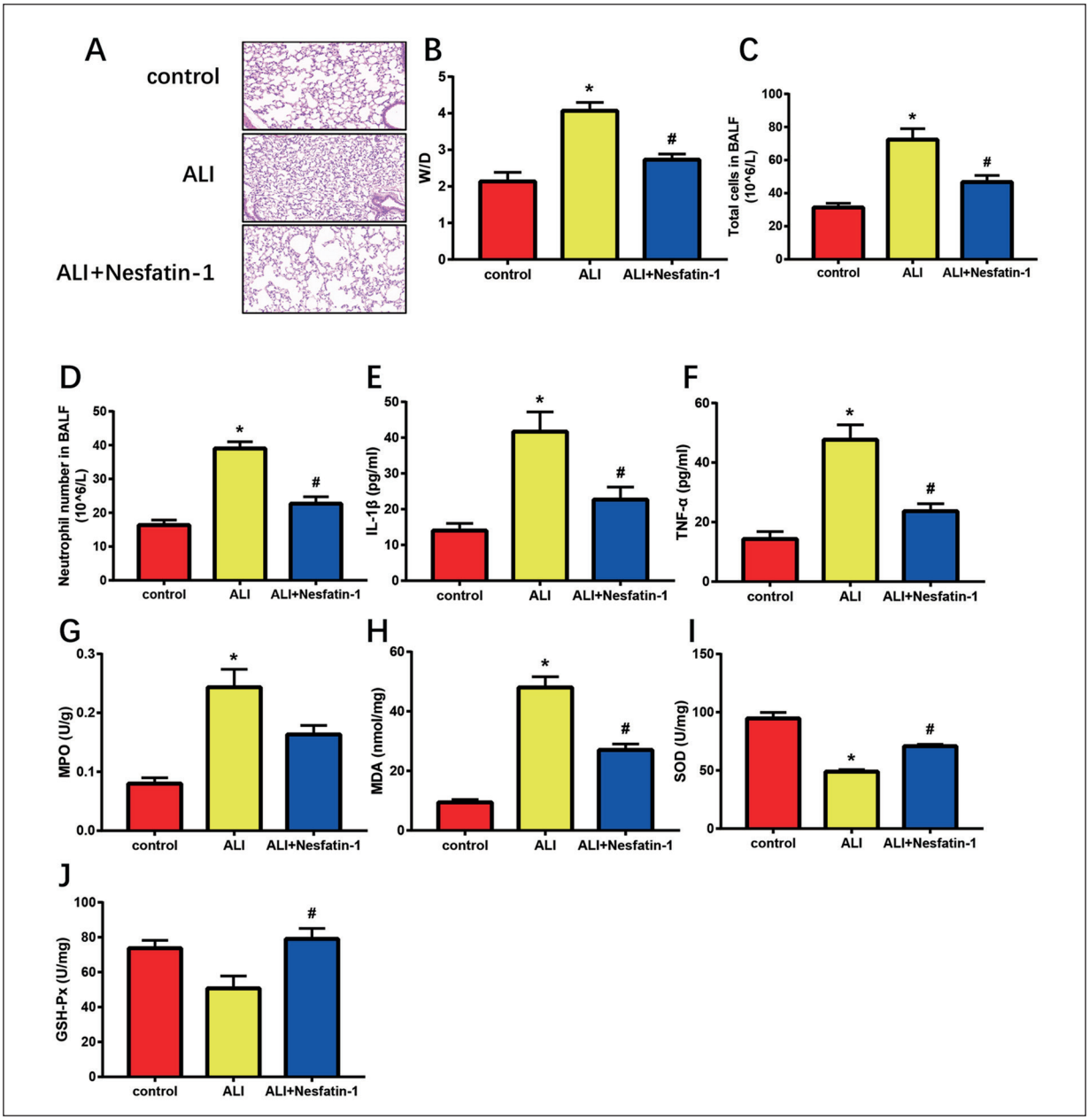

Figure 1. Exogenous Nesfatin-1 attenuates LPS-induced mouse ALI and reduces inflammation and oxidative stress. **A,** HE staining results of mice lung tissue (magnification: 400×); **B,** W/D of mice lung tissue; **C,** Total cell number in BALF; **D,** Neutrophil number in BALF, **E, F,** ELISA results of IL-1β and TNF-α in BALF; **G,** MPO activity in mice lung tissue; **H,** MDA activity in mice lung tissue; **I,** SOD activity in mice lung tissue; **J,** GSH-Px activity in mice lung tissue. ("*" means $p<0.05$ *vs.* the control group and "#" means $p<0.05$ *vs.* the ALI group).

also reduced W/D of lung tissue (Figure 1B). In BALF of mice, Nesfatin-1 reduced total cell number (Figure 1C) and neutrophil count (Figure 1D). The results of ELISA showed that Nesfatin-1 effectively reduced inflammatory factors (IL-1β and TNF-α) in BALF (Figure 1E-1F). In addition, LPS increased the level of oxidative stress in lung tissue, manifested by an increase in MPO and MDA and a decrease in SOD and GSH-Px. However, Nesfatin-1 reduced the oxidative stress level in mice (Figure 1G-1J). These results indicated that Nesfatin-1 protected mouse lung tissue from inflammation and oxidative stress.

Nesfatin-1 Reduces LPS-Induced Inflammation and Oxidative Stress in BEAS-2B Cells

We used LPS to induce damage to BEAS-2B cells to verify the effect of Nesfatin-1 on alveolar epithelial cells. CCK-8 detected the effect of 1, 2, 5, 10, and 20 nM Nesfatin-1 on the activity of BEAS-2B cells. The results showed that 10 Nm of Nesfatin-1 had the greatest effect on the activity of BEAS-2B cells (Figure 2A). In addition, 1 μg/ml of LPS can effectively inhibit the activity of BEAS-2B cells (Figure 2B). Therefore, 10 nM of Nesfatin-1 and 1 μg/mL of LPS were used to stimulate BEAS-2B cells.

First, we examined the effect of Nesfatin-1 on inflammation of BEAS-2B cells. The results of IF staining (Figure 2C, D) showed that Nesfatin-1 effectively reduced IL-β and TNF-α. The results of Western blot (Figure 2E) and RT-PCR (Figure 2F) also showed that Nesfatin-1 can decrease IL-1β, IL-6, IL-8, and TNF-α. In addition, Nesfatin-1 also significantly reduced the oxidative stress level in BEAS-2B cells. The results of IF staining (Figure 2G, 2H) and Western blot (Figure 2I) showed that Nesfatin-1 increased the expression of SOD1 and SOD2 in BEAS-2B cells. The results of RT-PCR (Figure 2J) also showed that BEAS-2B cells can increase the expression of SOD1, SOD2, GPX1, and CAT. Flow cytometry detected the level of ROS in the cells, and the results showed that Nesfatin-1 can reduce ROS levels in BEAS-2B cells (Figure 2K).

Nesfatin-1 Reduces the Expression of HMGB1 in Mouse Lung Tissue and BEAS-2B Cells

We examined changes in HMGB1 expression in mouse lung tissue and BEAS-2B cells, respectively. The results of animal experiments showed that the expression of HMGB1 in lung tissue of mice was significantly increased after LPS induced mouse ALI. The expression of HMGB1 was reduced after subcutaneous injection of Nesfatin-1 in mice (Figure 3A, 3B). In addition, in cell experiments, IF staining (Figure 3C) results indicate that Nesfatin-1 can reduce the expression of HMGB1 in BEAS-2B cells. The results of Western blot (Figure 3D) and RT-PCR (Figure 3E) were similar to those of IF staining.

Nesfatin-1 Reduces the Activity of p38MAPK and NF-κB Signaling Pathway by Decreasing HMGB1 Expression

To verify whether Nesfatin-1 reduces HMGB1 expression affects the inflammation-related signaling pathways downstream of HMGB1, we examined the activity of the p38MAPK and NF-κB signaling pathways. The results of Western blot (Figure 4A) showed that Nesfatin-1 had no significant effect on the expression of p38, but decreased the phosphorylation of p38. In addition, Nesfatin-1 decreased the expression of p65 and IKKα and increased the expression of IκBα. The results of RT-PCR (Figure 4B) were similar to those of Western blot. These results indicated that Nesfatin-1 can reduce the activity of inflammation-related signaling pathways downstream of HMGB1.

Lenti-HMGB1 Attenuates the Protective Effect of Nesfatin-1 on BEAS-2B Cells

To verify whether HMGB1 mediates the anti-inflammatory effects of Nesfatin-1 on BEAS-2B cells, we transfected BEAS-2B cells with Lenti-NC and Lenti-HMGB1. The results of Western blot (Figure 4C) and RT-PCR (Figure 4D) confirmed the transfection efficiency. The results of IF staining (Figure 4E, 4F) indicated that Lenti-HMGB1 transfected cells expressed higher IL-1β and SOD1. Western blot (Figure 4G) and RT-PCR (Figure 4H) also showed that overexpression of HMGB1 increased the expression of IL-1β and TNF-α and decreased the expression of SOD1 and SOD2. These results indicated that overexpression of HMGB1 attenuated the effect of Nesfatin-1 on BEAS-2B cells, suggesting a mediated role of HMGB1.

Discussion

Alveolar type 2 epithelial cells are one of the most important components of alveoli. At present, it is believed that different stem cells may

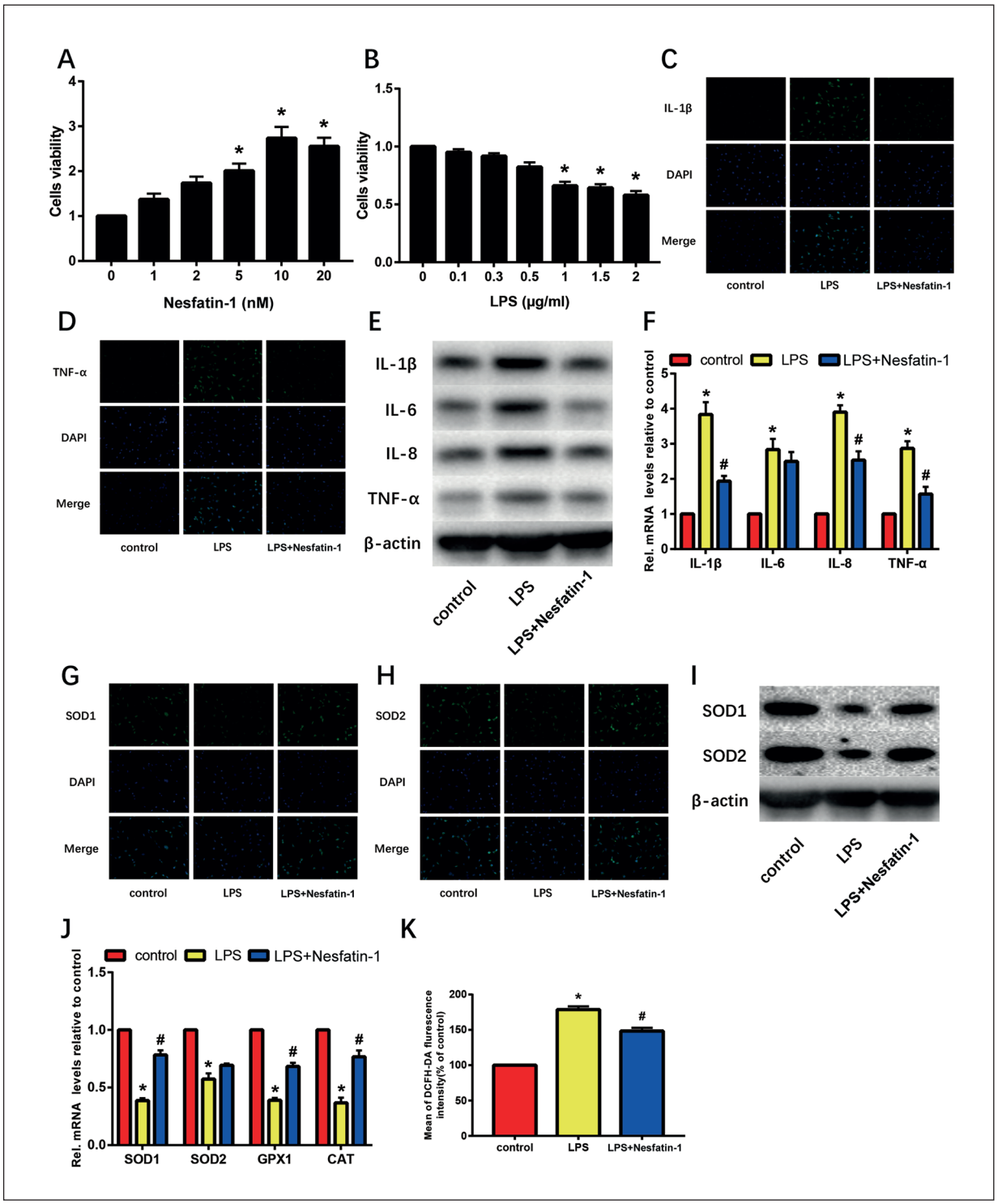

Figure 2. Nesfatin-1 reduces LPS-induced inflammation and oxidative stress in BEAS-2B cells. **A,** CCK8 assay of Nesfatin-1; **B,** CCK8 assay of LPS; **C, D,** IF staining results of IL-1β and TNF-α (magnification:400×); **E, F,** Western blot and RT-PCR results of IL-1β, IL-6, IL-8 and TNF-α; **G-I,** IF staining (magnification:400×) and Western blot results of SOD1 and SOD2; **J,** RT-PCR results of SOD1, SOD2, GPX1 and CAT; **K,** ROS level in BEAS-2B cells. ("*" means $p<0.05$ *vs.* the control group and "#" means $p<0.05$ *vs.* the LPS group).

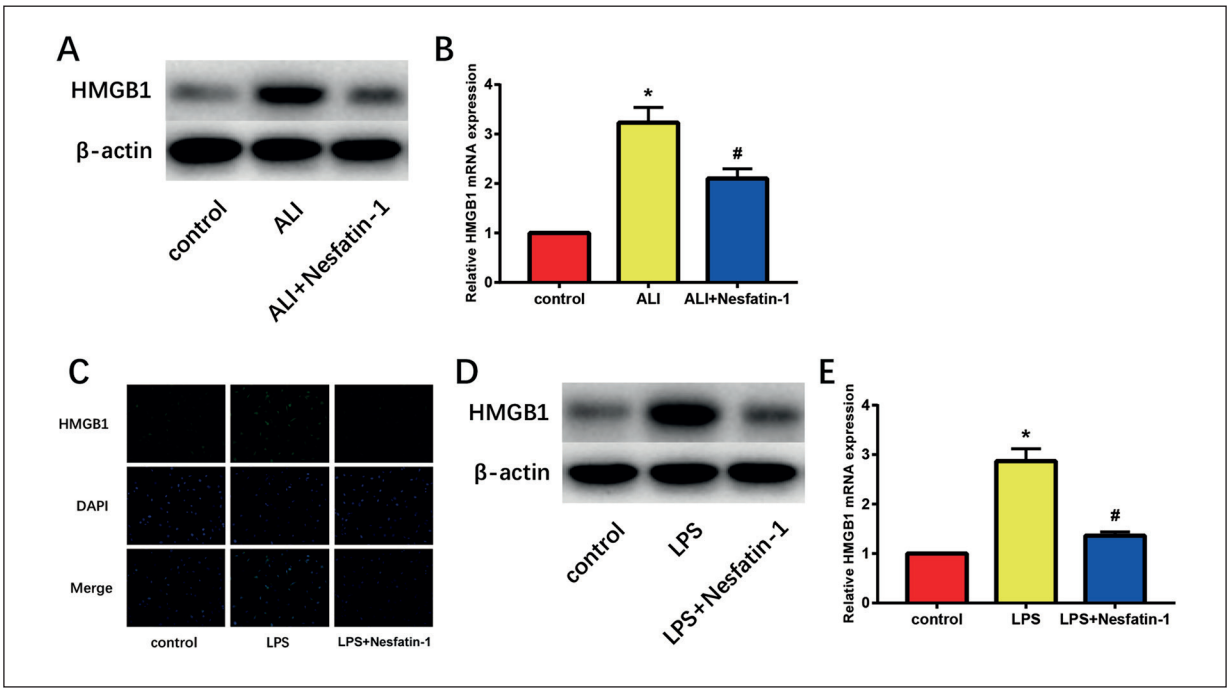

Figure 3. Nesfatin-1 reduces the expression of HMGB1 in mouse lung tissue and BEAS-2B cells. **A, B,** Western blot and RT-PCR results of HMGB1 in mice lung tissue; **C-E,** IF staining (magnification: 400×), Western blot and RT-PCR results of HMGB1 in BEAS-2B cells. ("*" means $p<0.05$ *vs.* the control group and "#" means $p<0.05$ *vs.* the LPS group).

exist in different parts of the respiratory tract[7]. AT II is considered to be a stem cell of the alveolar epithelium. There are only two types of epithelial cells in the alveolar epithelium, namely AT I and AT II. Most scholars believe that AT is the progenitor cells of these two types of alveolar epithelium[8]. In normal cell renewal and injury repair, it can differentiate into AT I, or it can produce progeny AT II through mitosis to maintain its own cell population. Functionally intact alveolar type 2 epithelial cells maintain the stability of the alveolar structure, reduce the entry of inflammatory cells and fluids into the alveoli, and produce large amounts of anti-inflammatory factors[9]. Li et al[10] found in the study that retinoic acid has a protective effect on hyperoxia-induced lung injury by reducing AT II apoptosis and necrosis. Hua et al[11] observed the ultrastructural changes of lung tissue at different time points in the ventilator-induced lung injury of newborn rabbits. It was found that AT II was converted to AT I under normal conditions to repair lung injury. These demonstrate that AT II proliferation and transformation are closely related to lung injury and its repair. However, in the lung injury of sepsis, alveolar type 2 epithelial cells undergo a large amount of apoptosis, and the function is impaired by inflammation[12]. Previous studies[13] have found that lung epithelial barrier function damage and pulmonary interstitial cell remodeling during ALI. In our study, LPS-induced lung tissue was significantly damaged, accompanied by tissue edema and inflammatory cell infiltration, and LPS-stimulated BEAS-2B cells also showed significant reduction in cell viability. After treatment of mice or BEAS-2B cells with Nesfatin-1, lung tissue or BEAS-2B was significantly improved, manifested by a decrease in inflammatory response and a decrease in oxygen free radicals.

Oxidative stress means that the body is stimulated by many factors, and the ROS in the body is too much, cannot be removed in time, leading to oxidation and anti-oxidation imbalance, resulting in oxidative damage of biological macromolecules such as proteins, lipids and nucleic acids, which interfere with normal life activities and formed a serious state of stress[14]. Sepsis-induced ALI is clearly associated with oxidative stress, and a large amount of ROS is found in BALF of ALI patients, which are mainly produced by alveolar macrophages, neutrophils, lung endothelial

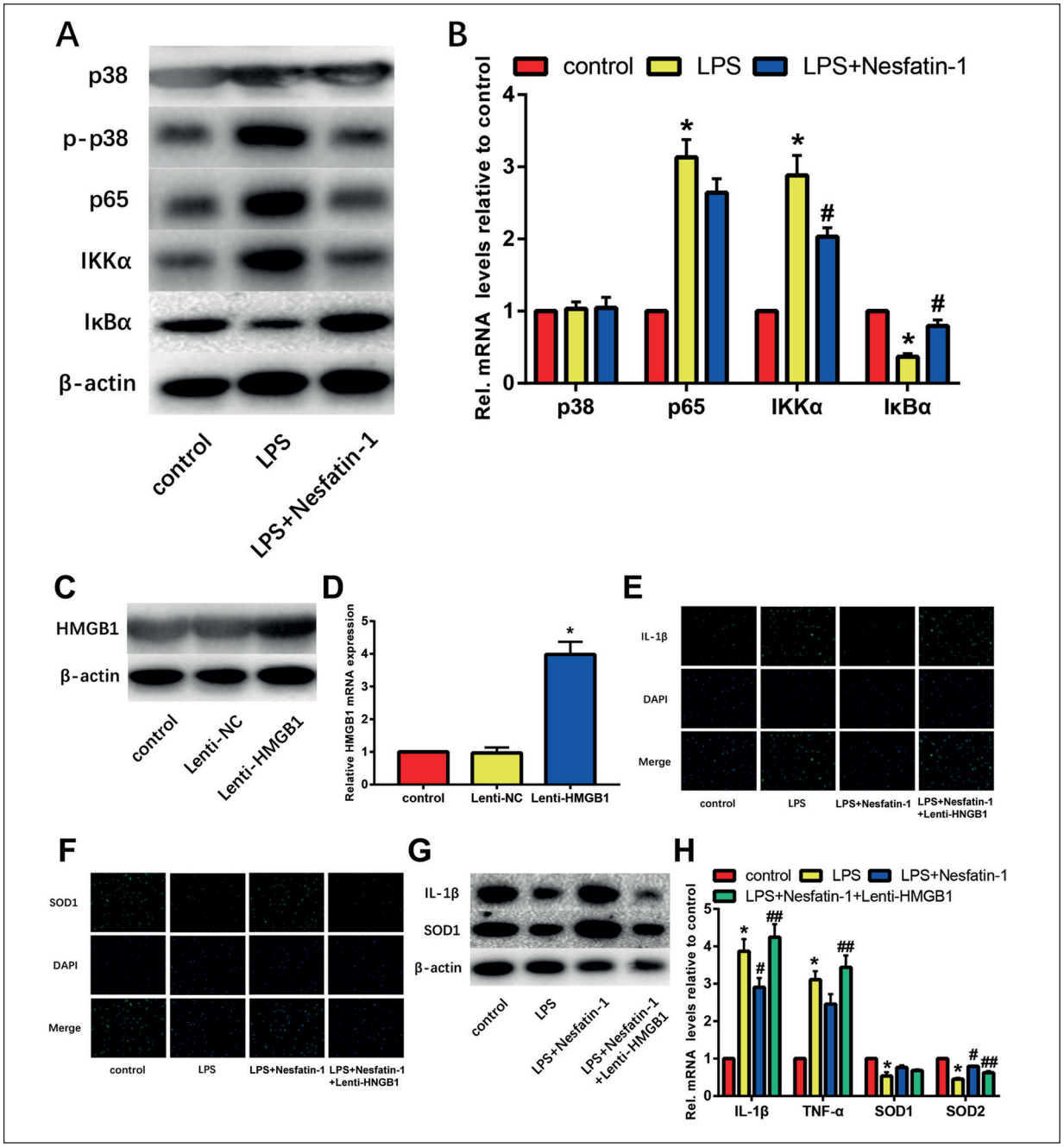

Figure 4. Nesfatin-1 reduces the activity of p38MAPK and NF-κB signaling pathway by decreasing HMGB1 expression. **A, B,** Western blot and RT-PCR results of p38, p-p38, p65, IKKα and IκBα. **C-H,** Lenti-HMGB1 attenuates the protective effect of Nesfatin-1 on BEAS-2B cells. **C-D,** Western blot and RT-PCR results of HMGB1; **E-G,** IF staining (magnification: 400×) and Western blot results of IL-1β and SOD1; **H,** RT-PCR results of IL-1β, TNF-α, SOD1 and SOD2. ("*" means $p<0.05$ *vs.* the control group, "#" means $p<0.05$ *vs.* the LPS group and "##" means $p<0.05$ *vs.* the LPS+ Nesfatin-1 group).

cells, and epithelial cells. About oxidative stress in ALI, there are mainly the following mechanisms: (1) regulation of apoptosis. Activation of the Caspase cascade is the most important part of apoptosis. ROS may promote cell apoptosis by increasing mitochondrial membrane permeability, mediating cytochrome c release, enhancing Caspase9 activation and apoptosis complex formation. (2) ROS affects nucleic acids and transcription factors. ROS can cause DNA damage and promote cell death by causing mitochondrial dysfunction, reducing cell proliferation, and in-

ducing ALI. ROS can also activate NF-κB, thereby enhancing the sensitivity of COX-2, TNF-α, and IL-6, and inducing cellular inflammation. (3) The anti-oxidant capacity is weakened. After[15] excessive ROS release, insufficient anti-oxidant capacity triggers the expression of pro-inflammatory mediators, leading to airway inflammation. Therefore, the elimination of oxygen free radicals in lung tissue or alveolar epithelial cells is the key to the treatment of ALI. In Nesfatin-1 treated mice, MPO and MDA were significantly decreased while SOD and GSH-Px were elevated, indicating that Nesfatin-1 reduced oxidative stress levels in lung tissue and increased the ability to resist oxidative stress.

Our study found that HMGB1 mediates the anti-inflammatory effects of Nesfatin-1 on ALI. HMGB1 is a DNA-binding protein widely found in the nucleus and cytoplasm of eukaryotic organisms. It binds to linear DNA to maintain its helical structure, regulates gene transcription, and regulates the activity of steroid hormone receptors[16]. In addition, intracellular HMGB1 also stabilizes nucleosomes and promotes DNA folding, replication and repair, and enhanced transcription. There are two main sources of extracellular HMGB1, namely active secretion and passive release of cells. When mononuclear/macrophages, histiocytes, neutrophils, dendritic cells, etc., are stimulated and activated, they can actively secrete HMGB1 to the outside of the cell. When the cells are affected by biological factors and cause cell damage, lysis or death, HMGB1 is passively released out of the cell. Active secretion and passive release to extracellular HMGB1 can induce local inflammation[17]. The role of HMGB1 in inflammatory response can be divided into the following points: (1) stimulating cell migration and promoting the localization of injury site by immune cells; (2) promoting the recognition of bacteria and its products by innate immune cells; (3) activating innate immunity cells, which produce pro-inflammatory factors and aggravate the inflammatory response; (4) inhibit neutrophil apoptosis, accumulate apoptotic neutrophils and then release cytokines again[18]. Therefore, the inhibition of HMGB1 by Nesfatin-1 is very beneficial for the treatment of ALI.

To sum up, Nesfatin-1 could ameliorate sepsis-induced ALI by reducing inflammation levels and oxidative stress levels in alveolar epithelial cells. We suggest that the results of our study could provide a new direction for the treatment of clinical ALI.

Conclusions

In summary, exogenous Nesfatin-1 significantly attenuated LPS-induced mouse ALI, manifested by a decrease in inflammation levels and oxidative stress levels. Similarly, Nesfatin-1 also reduced inflammation levels and oxidative stress levels in BEAS-2B cells. In addition, the therapeutic effect of Nesfatin-1 on ALI was achieved by the regulation of HMGB1. Nesfatin-1 attenuates ALI by decreasing the expression of HMGB1 to reduce the activity of downstream p38MAPK signaling pathway and NF-κB signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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