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ORIGINAL RESEARCH

Heparin-Binding Protein Promotes Acute Lung Injury in Sepsis Mice by Blocking the Aryl Hydrocarbon Receptor Signaling Pathway

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Purpose: This study aimed to explore the therapeutic effect and potential mechanism of heparin-binding protein (HBP) reduction on sepsis-related acute lung injury.

Methods: We utilized a murine model of sepsis-induced by intraperitoneal injection of lipopolysaccharides (LPS) in C57BL/6J mice divided into four groups: Control, LPS, Anti-HBP, and ceftriaxone (CEF). Following sepsis induction, Anti-HBP or CEF treatments were administered, and survival rates were monitored for 48 h. We then used reverse-transcription quantitative PCR to analyze the expression levels of HBP in lung tissues, immunohistochemistry for protein localization, and Western blotting for protein quantification. Pulmonary inflammation was assessed using enzyme-linked immunosorbent assays of proinflammatory cytokines (tumor necrosis factor-α, interleukin [IL]-1β, IL-6, and interferon-γ). The activation state of the aryl hydrocarbon receptor (AhR) signaling pathway was determined via Western blotting, evaluating both cytoplasmic and nuclear localization of AhR and the expression of cytochrome P450 1A1 protein by its target gene.

Results: Anti-HBP specifically reduced HBP levels. The survival rate of mice in the Anti-HBP and CEF groups was much higher than that in the LPS group. The severity of lung injury and pulmonary inflammatory response in the Anti-HBP and CEF groups was significantly lower than that in the LPS group. AhR signaling pathway activation was observed in the Anti-HBP and CEF groups. Additionally, there was no significant difference in the above indices between the Anti-HBP and CEF groups.

Conclusion: HBP downregulation in lung tissues significantly improved LPS-induced lung injury and the pulmonary inflammatory response, thereby prolonging the survival of sepsis mice, suggesting activation of the AhR signaling pathway. Moreover, the effect of lowering the HBP level was equivalent to that of the classical antibiotic CEF.

Trial Registration: Not applicable.

Keywords: sepsis, acute lung injury, heparin-binding protein, aryl hydrocarbon receptor signaling pathway

Introduction

Sepsis is a leading cause of death in intensive care units of medical institutions. Worldwide, over 48 million new cases of sepsis occur annually, with approximately 11 million associated deaths.[1] The incidence of sepsis varies among countries, and it is an important cause of death in Europe, North America, and Australia.[2] Sepsis is defined as the systemic inflammatory response syndrome[3] induced by infection, including multiple organ dysfunction due to an overly strong, uncontrolled immune response.[4] Generally, sepsis involves multiple organs, of which the lung is one of the most easily and seriously affected by the pathological process of sepsis because of its unique sensitivity and fragility.[5] Patients with sepsis are prone to acute lung injury (ALI), resulting in respiratory dysfunction and eventual progression to respiratory failure and ultimately death.[6]

Unfortunately, the current clinical treatment approaches for sepsis-induced ALI have various shortcomings. The most fundamental component of the treatment plan is controlling the primary infection.[7] However, the conditions of patients

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with sepsis are often complicated, and it is challenging for clinicians to determine the source of infection within a short timeframe, which may delay treatment. Moreover, traditional anti-infection treatment is also limited by antibiotic resistance.[8] Low tidal volume ventilation can temporarily maintain the oxygen supply in patients with sepsis-induced ALI, but it does not address the source of the problem.[9] Extracorporeal membrane oxygenation provides patients with ALI with more effective and safer respiratory support than conventional mechanical ventilation, but it requires advanced equipment and is costly.[10] Therefore, the identification of intervention measures for the rapid, cheap, and effective treatment of sepsis-related ALI is of great significance in reducing the rate of sepsis-related deaths.

Heparin-binding protein (HBP; azurocidin, cationic antibiotic protein 37) is a member of the serine protease family and is mainly found in neutrophils. When an infection results in a systemic immune response, HBP is released into the bloodstream by mature neutrophils.[8] HBP is an important component of the immune and inflammatory responses. On one hand, HBP participates in the recruitment and localization of inflammatory cells by attracting and activating various white blood cells. On the other hand, it enhances endothelial cell permeability in many parts of the body, thereby assisting inflammatory cell migration to and aggregation at the infection source.[11] However, excessive HBP leads to vascular leakage, edema, and immune disorder in severe infections.[12,13] Increased levels of HBP in peripheral blood have been reported as a common feature of patients with sepsis. Thus, determination of the HBP level contributes to the early identification of sepsis, as well as evaluating severity and monitoring progression.[14,15] Although the value of HBP as a key diagnostic marker of sepsis is widely recognized, little studies have explored the possibility of treating sepsis with HBP as an intervention target.

Recent molecular biology advancements have highlighted the aryl hydrocarbon receptor (AhR) signaling pathway as a pivotal mediator of immune response modulation during sepsis and ALI.[16–18] AhR is a ligand-activated transcription factor primarily known for its role in xenobiotic metabolism. Notably, AhR activation in sepsis and ALI mitigates inflammatory damage via several mechanisms, including regulating cytokine production and inducing detoxifying enzymes, like cytochrome P450 1A1 (CYP1A1).[19] These actions help to alleviate the inflammatory onslaught characteristic of sepsis, potentially reducing lung injury and improving patient outcomes. Given its emerging significance, further exploration of the mechanism of the AhR pathway and its interaction with other molecular players in sepsis and ALI is warranted to fully harness its therapeutic potential.

Within the scope of this study, we hypothesized that HBP downregulation mitigated the severity of sepsis-induced ALI by blocking AhR signaling. To verify our hypothesis, we constructed a lipopolysaccharide (LPS)-induced sepsis mouse model to explore the antisepsis role and potential mechanism of HBP downregulation in improving lung injury, modulating inflammatory responses in lung tissue, and improving patient survival rate. Our findings provide a reference for developing new intervention treatment strategies for sepsis-related ALI.

Materials and Methods

Experimental Animals

We purchased 60 SPF male C57BL/6J mice (age, 6–8 weeks; weight, 20–26 g) from Vital River Laboratory Animal Technology Co., Ltd. (China) for use in our experiments. The mice were housed in the animal room of The Second Affiliated Hospital of Hainan Medical University under a standard 12 h light/12 h dark cycle at an indoor temperature of 22°C ± 2°C and a relative humidity of 55%–65%. All mice were given free access to food and water during the experiment.

This study was performed in strict accordance with the Guide for Care and Use of Laboratory Animals to ensure animal welfare. All experiments were reviewed and approved by the Laboratory Animal Ethical Review Committee of The Second Affiliated Hospital of Hainan Medical University (approval no.: LW2023158).

Reagents and Experimental Design

Lyophilized LPS was purchased from Beyotime Biotechnology (Shanghai, China) and dissolved in phosphate-buffered saline for subsequent use. Clinical-grade powdered ceftriaxone (CEF) was purchased from Solarbio Technology Co., Ltd. (China) and dissolved in sterile physiological saline for subsequent use.

To reduce HBP levels, multifunctional molecules specifically targeting HBP (hereafter called Anti-HBP) were synthesized by MedChemExpress Biotechnology Co., Ltd. (NJ, USA) using proteolysis targeting chimera technology.[20] Anti-HBP contains ligands of ubiquitin E3 and target protein HBP, which form ternary complexes with ubiquitin E3 and HBP to induce ubiquitination of the target protein HBP and degradation by the ubiquitin-proteasome system (UPS). Anti-HBP was dissolved in 0.1% DMSO and then diluted with sterile physiological saline for subsequent use.

Following a week of adaptive feeding, the mice were randomized into four groups as follows: (1) Control group, (2) LPS group, (3) Anti-HBP group, and (4) CEF group. A sepsis model was established for mice in the LPS, Anti-HBP, and CEF groups by intraperitoneal injection of 20 mg/kg LPS solution as per previous studies.[21] Mice in the Control group were injected intraperitoneally with an equal volume of normal saline. Following LPS injection, the gradual onset of symptoms, such as shortness of breath, lethargy, slow responsiveness, and abnormal behavior, were observed, indicating the successful establishment of the sepsis model.[22]

Two hours after LPS injection, subcutaneous injections of Anti-HBP (200 μg/kg), CEF solution (100 mg/kg), and the same volume of physiological saline were administered to mice in the Anti-HBP, CEF, and LPS groups, respectively. Trinh et al[23] and Dufour et al[24] reported that subcutaneous injection of 50 mg/kg or 75 mg/kg CEF was sufficient to effectively inhibit sepsis-induced by *Vibrio vulnificus* and acute pneumonia caused by *Escherichia coli* in mice. Because our study mainly focused on lung injury in sepsis mice, we referred to the dose and administration methods of CEF used in the above two studies. Thus, to enhance the therapeutic effect of CEF, we increased the dose to 100 mg/kg.

Survival Analysis

Following treatment, all mice were returned to their cage and observed continuously for 48 h. The number of surviving mice was recorded every 6 h. After completion of the experiment, taking time as the abscissa and survival rate as the ordinate, we used GraphPad Prism 7.0 software (GraphPad Software; USA) to construct a Kaplan–Meier survival curve to analyze the survival of mice in each group.

Terminal Mouse Treatment

If a mouse in one of the groups died of sepsis within 48 h, the chest skin and ribcage were immediately cut open using ophthalmic scissors and tweezers to expose the lung tissue. Then, the bilateral lung tissue was removed by gentle and careful separation from the surrounding blood vessels, muscles, and soft tissues and washed with normal saline to remove all traces of blood.

The mice in each group that were still alive 48 h after treatment were deeply anesthetized by isoflurane inhalation of (1%–2% volume) and then killed by cervical dislocation. The bilateral lung tissue of these mice was collected in the same manner as described above.

Reverse-Transcription Quantitative PCR (RT-qPCR)

The upper lobe tissue of the right lung was collected and homogenized with TRIzol reagent (Invitrogen, USA) to extract total RNA, followed by reverse-transcription into cDNA using a Hifair II 1st Strand cDNA Synthesis Kit (YEASEN, China). Subsequently, the level of HBP mRNA was quantitatively analyzed by RT-qPCR on a StepOnePlus real-time PCR system (Applied Biosystems, USA) using a Hifair III One-Step RT-qPCR SYBR Green Kit (YEASEN). All steps were performed in strict accordance with the manufacturer's instructions. The mRNA expression level of HBP was calibrated using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression level and quantified using the $2^{-\Delta\Delta ct}$ method. RT-qPCR assays were repeated in triplicate for each sample, and the mean was presented as the final result. The PCR primers are shown in Table 1.

Immunohistochemistry (IHC) Staining

The middle lobe tissue of the right lung was fixed in 4% paraformaldehyde overnight and then dehydrated through ascending concentrations of ethanol. Subsequently, the tissue was embedded in a wax block and cut into 5-μm thick sections using a microtome (Leica, Germany). Then, the paraffin sections were baked in a drying oven at 60°C for 2 h, dewaxed, hydrated, and boiled in 0.01 mol/L citrate buffer (pH 6.0). After cooling the sections naturally at room

Table 1 RT-qPCR Primers

mRNA	Sequences (5'–3')
HBP	5'-CAGATACGACCCCCAGGAGA-3' (forward)
	5'-CACCGTCACGTTCACAACTC-3' (reverse)
GAPDH	5'-CCTCGTCCCGTAGACAAAATG-3' (forward)
	5'-TGAGGTCAATGAAGGGGTCGT-3' (reverse)

Abbreviations: HBP, heparin-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

temperature, 50 μL 3% hydrogen peroxide solution was added and incubated at room temperature for 15 min to inhibit endogenous peroxidase activity. After blocking, each section was incubated overnight at 4°C with 50 μL Anti-HBP (1:100, #ab181989, Abcam, UK) as the primary antibody. The next day, HRP-labeled goat anti-rabbit IgG (1:1,000, #ab6721, Abcam) was added to the sections as the secondary antibody and incubated for 30 min at ambient temperature. After washing, the section was stained with DAB (Invitrogen) and counterstained with hematoxylin (Servicebio, China). Finally, the HBP-positive cells were observed under an optical microscope (Olympus, Japan) and photographed. Three fields of view were randomly selected from each section for observation, and the HBP-positive cells were quantitatively analyzed using ImageJ software (National Institute of Health, USA).

Measurement of Wet/Dry (W/D) Ratio of Lung Tissues

We measured the W/D ratio of lung tissues as previously reported.[25] Briefly, the left lung was immediately weighed upon removal using an electronic balance. Then it was dried at 65°C for 72 h and weighed again to obtain the dry weight. Finally, the W/D ratio, which reflects pulmonary edema severity, was calculated.

Hematoxylin-Eosin (H&E) Staining

The lower lobe tissue of the right lung was fixed in 4% paraformaldehyde overnight, dehydrated through ascending concentrations of ethanol, embedded in a wax block, and sliced into 5-μm thick sections using a microtome. The sections were soaked in 95% ethanol solution for 5 min to dewax, stained with hematoxylin (Servicebio) for 15 min, washed, and counterstained with eosin (Servicebio) for 2 min. Finally, they were observed under an optical microscope. Based on Mrozek et al,[26] we performed a semi-quantitative evaluation of the severity of lung injury using a scale of 0–4 points on the basis of the proportion of pathological changes (bleeding, edema, exudation, inflammatory cell infiltration, atelectasis, and hyaline membrane formation) and changes in morphological characteristics observed in the visual field. The scoring criteria were as follows: 0, absence of any pathological changes in the visual field; 1, pathological changes in <25% of the total visual field; 2, pathological changes in 25%–50% of the total visual field; 3, pathological changes in 51%–75% of the total visual field; and 4, pathological changes in >75% of the total visual field. Five randomly selected visual fields were selected and scored for each mouse, and the mean score was presented as the final result for that mouse.

Enzyme-Linked Immunosorbent Assay (ELISA)

An appropriate amount of tissue from part of the middle lobe of the right lung was collected and cut into small blocks with ophthalmic scissors. Then, an appropriate amount of pre-cooled normal saline was added, and the tissue was processed using an automatic tissue crushing homogenizer (RWD Life Science Co., Ltd., Shenzhen, China) to obtain lung tissue homogenate.

The levels of proinflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and interferon-γ (IFN-γ), were measured in the lung tissue homogenate using ELISA kits #ab208348, #ab197742, #ab222503, and #ab100689, respectively (Abcam) according to the specific kit instructions. Finally, the absorbance of each sample was measured at 450 nm using a multifunctional microplate reader (Thermo Fisher Scientific, USA) to determine the cytokine levels. Each experiment was repeated in triplicate for each sample, and the mean was presented as the final result.

Western Blotting

An appropriate amount of tissue from the middle lobe of the right lung was collected for use in Western blot experiments. The nuclear and cytoplasmic proteins were separated and extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (LMAI Bio, China), and the protein concentration was subsequently determined using a bicinchoninic acid assay kit (Thermo Fisher Scientific). After separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Beyotime), the protein samples were transferred onto polyvinylidene difluoride (PVDF) membranes (Abcam) and blocked with 5% skim milk for 1 h. Then, the membrane was incubated overnight at 4°C with the following primary antibodies purchased from Abcam: anti-HBP (1:2,000, #ab181989), anti-CYP1A1 (1:1,000, #ab124295), anti-AhR (1:500, #ab308215), anti-GAPDH (1:2,500, #ab9485), and anti-lamin B (1:1,000, #ab16048). On the next day, the PVDF membrane was washed with TBST solution and then incubated with HRP-labeled goat anti-rabbit IgG (1: 4000, #ab6721, Abcam) secondary antibody at room temperature for 1 h. Finally, the membrane was visualized using a Multi Gel Imaging Analysis System (Tanon, China), and the protein expression level was quantified using Image J software. The expression level of AhR protein in the nucleus was corrected using lamin B protein as an internal control, and the expression levels of HBP, CYP1A1, and AhR proteins in the cytoplasm were corrected using GAPDH protein as an internal control. The experiment was repeated in triplicate for each sample, and the mean was used as the final result.

Statistical Analysis

Shapiro–Wilk test was used to judge the normality of the data, revealing a relatively normal distribution for each mouse group. Therefore, one-way analysis of variance (ANOVA) was used to analyze differences among multiple groups. If the results were significant, Tukey's test was used to compare the differences between groups. All statistical analyses were performed using SPSS v25.0 software (IBM Corp., NY, USA). P values < 0.05 were considered statistically significant. Measurement data were expressed as the mean ± standard deviation.

Results

Anti-HBP Reduces HBP Levels in the Lung Tissue of Mice with LPS-Induced Sepsis

RT-qPCR and IHC experiments were undertaken to verify whether Anti-HBP degraded target protein HBP through the UPS. The RT-qPCR results showed that the level of HBP mRNA in the lung tissue of mice in the LPS group was significantly higher than in the lung tissue of mice in the Control group ($P < 0.01$). Compared with the LPS group, the level of HBP mRNA in lung tissue of mice in the Anti-HBP and CEF groups was significantly decreased ($P < 0.01$). Furthermore, the level of HBP mRNA in the Anti-HBP group was lower than the level of HBP mRNA in the CEF group, but the difference was not statistically significant ($P > 0.05$) (Figure 1A). IHC staining results revealed that compared with the Control group, the HBP level in lung tissue of mice in the LPS group was notably increased, and the percentage of HBP-positive cells was significantly increased ($P < 0.01$). Compared with the LPS group, HBP levels in lung tissue of mice in the Anti-HBP and CEF groups were significantly decreased, as was the percentage of HBP-positive cells ($P < 0.01$). The percentage of HBP-positive cells in the Anti-HBP group was slightly lower compared with the percentage of HBP-positive cells in the CEF group, but the difference was not statistically significant ($P > 0.05$) (Figure 1B and C). The above results confirmed the successful construction of a multifunctional Anti-HBP molecule that specifically reduced HBP levels.

HBP Degradation Prolongs the Survival of Sepsis Mice by Alleviating LPS-Induced Lung Injury

HBP is an important acute-phase reaction protein in sepsis that is crucial for the maintenance and progression of sepsis-induced inflammatory responses.[27] Our study is the first to explore the effects of HBP degradation on lung injury and survival in sepsis mice.

After 48 h of treatment, the survival rates of mice in the Control, LPS, Anti-HBP, and CEF groups were 100%, 0%, 67%, and 60%, respectively. Compared with the Control group, the survival rate of mice in the LPS group was significantly decreased ($P < 0.01$). Compared with the LPS group, the survival rate of mice in the Anti-HBP and CEF

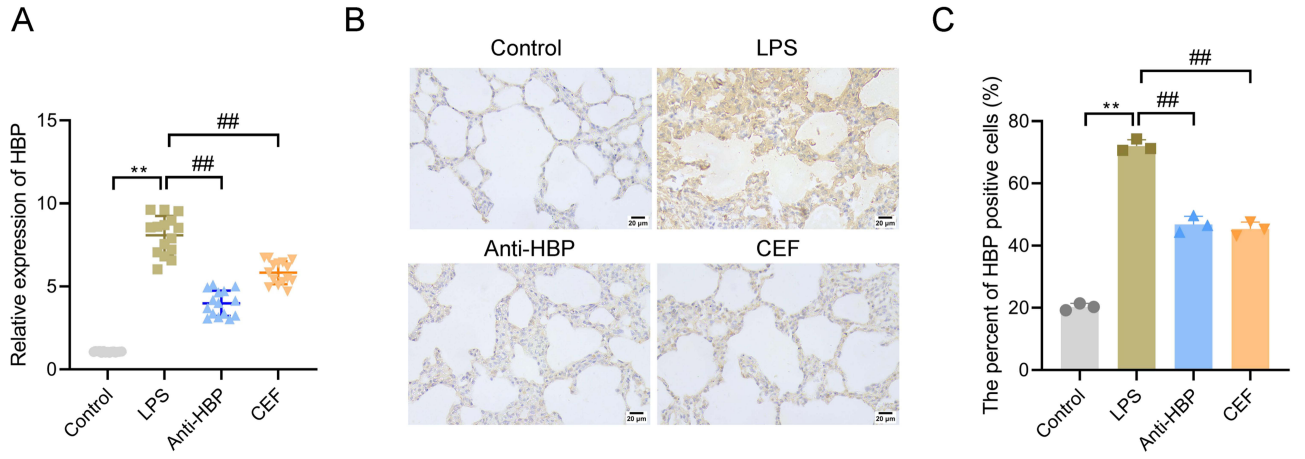

Figure 1 Anti-HBP reduces HBP levels in lung tissue of mice with LPS-induced sepsis. (**A**) RT-qPCR determination of the HBP mRNA levels in lung tissue of mice in the Control, LPS, Anti-HBP, and CEF groups; (**B**) Representative images of IHC determination of HBP levels in lung tissue of mice in each group, scale bar = 20 μm; (**C**) Statistical analysis of the percentage of HBP-positive cells. $^{**}P < 0.01$ vs Control group; $^{\#\#}P < 0.01$ vs LPS group.
Abbreviations: HBP, heparin-binding protein; IHC, immunohistochemistry.

groups was significantly increased ($P < 0.01$). Additionally, the survival rate of mice in the Anti-HBP group was slightly higher compared with the survival rate of mice in the CEF group, but the difference was not statistically significant ($P > 0.05$) (Figure 2A).

Measurement of the W/D ratio revealed that the water content in the lungs of mice in the LPS group was significantly increased compared with the water content in the lungs of mice in the Control group ($P < 0.01$). In contrast to the LPS group, the water content in the lungs of mice in the Anti-HBP and CEF groups was significantly decreased ($P < 0.01$). The water content in the lungs of mice in the Anti-HBP group was slightly lower compared with the water content in the lungs of mice in the CEF group, but the difference was not statistically significant ($P > 0.05$) (Figure 2B). H&E staining revealed that the alveolar structure of mice in the Control group was normal, with an intact alveolar wall and no edema in the cavity. In contrast, the lung tissue of mice in the LPS group showed serious damage, including thickened alveolar walls, collapsed alveolar cavities, infiltration of a large number of inflammatory cells, and pulmonary edema and bleeding. Relative to the LPS group, the lung injury of mice in the Anti-HBP and CEF groups was significantly improved, showing alleviation of alveolar wall injuries and infiltration of a small number of inflammatory cells, but there was no significant difference between the Anti-HBP and CEF groups. Compared with the Control group, where the severity of lung injury was scored as 0, the score of mice in the LPS group was significantly higher ($P < 0.01$). Compared with the LPS group, the scores of mice in the Anti-HBP and CEF groups were significantly decreased ($P < 0.01$), and there was no significant difference between the Anti-HBP and CEF groups ($P > 0.05$) (Figure 2C and D).

Collectively, our findings indicated that the specific degradation of HBP by the multifunctional molecule Anti-HBP effectively alleviated the pathological changes of lung injury induced by LPS, including pulmonary edema, thereby prolonging the survival time of sepsis mice. Moreover, the efficacy of Anti-HBP was similar to that of the classical antibiotic CEF regarding improvements in lung injury and the survival rate.

HBP Degradation Alleviates the Inflammatory Response in Lung Tissue of LPS-Induced Sepsis Mice

Because severe inflammatory response induced by sepsis is an important cause of lung injury,[28] we further investigated whether HBP degradation regulated the pulmonary inflammatory response. The ELISA results showed that the levels of pulmonary cytokines (TNF-α, IL-1β, IL-6, and IFN-γ) in lung homogenate of mice in the LPS group were significantly higher compared with those in the Control group ($P < 0.01$). Compared with the LPS group, the pulmonary cytokine levels in lung homogenate of mice in the Anti-HBP and CEF groups were significantly decreased ($P < 0.01$). The pulmonary cytokine levels in the Anti-HBP group were slightly lower than those in the CEF group, but the difference

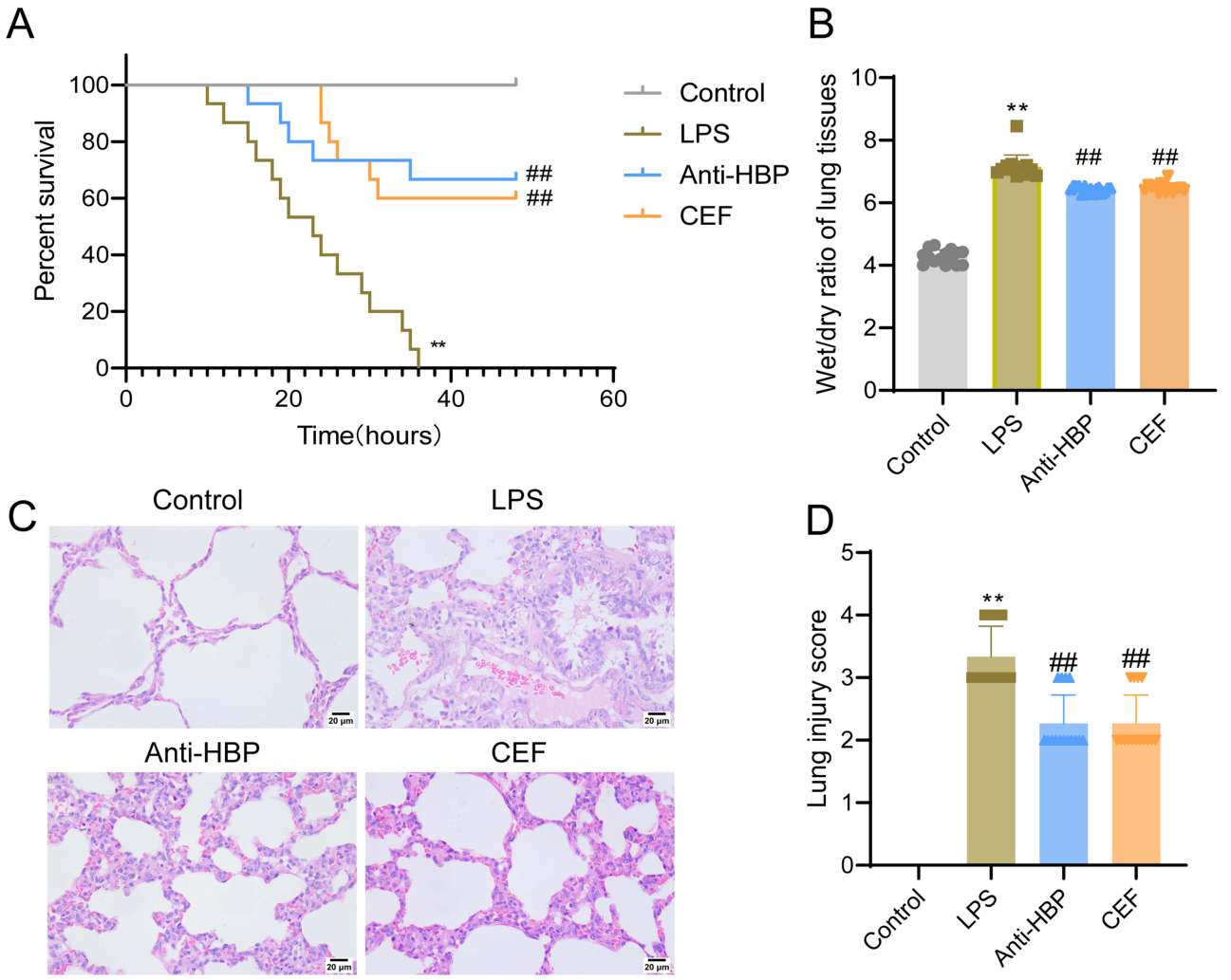

Figure 2 HBP degradation prolongs the survival of sepsis mice by alleviating LPS-induced lung injury. (**A**) HBP degradation improves the survival rate of LPS-induced sepsis mice; (**B**) Water content of lung tissue of mice in the Control, LPS, Anti-HBP, and CEF groups as determined using the W/D method; (**C**) Representative images of H&E staining results showing the pathological changes of lung tissue in each group, scale bar = 20 μm; (**D**) Statistical analysis of the lung injury severity scores in each group. $^{**}P < 0.01$ vs Control group; $^{\#\#}P < 0.01$ vs.

Abbreviations: LPS group. HBP, heparin-binding protein; LPS, lipopolysaccharides; W/D, wet/dry; H&E, hematoxylin-eosin.

was not statistically significant ($P > 0.05$) (Figure 3A–D). TNF-α, IL-1β, IL-6, and IFN-γ are established key proinflammatory cytokines of the immune response, and their levels are positively correlated with inflammatory response severity.[29] Therefore, the above outcomes demonstrated that the specific degradation of HBP by the multifunctional molecule Anti-HBP effectively inhibited the LPS-induced pulmonary inflammatory response. Moreover, the effect of Anti-HBP was similar to that of the classical antibiotic CEF in reducing the pulmonary inflammatory response.

HBP Degradation Activates the AhR Signaling Pathway in Lung Tissue of LPS-Induced Sepsis Mice

The AhR signaling pathway is crucial for maintaining immune system homeostasis, and its activation reduces the inflammatory response by inhibiting proinflammatory cytokine production.[30] Therefore, we further investigated whether the improvement in lung injury and alleviation of the pulmonary inflammatory response observed as a result of HBP degradation was related to the AhR signaling pathway. The Western blotting results showed that compared with the Control group, the levels of HBP and AhR (cytosol) proteins in lung tissue of mice in the LPS group were significantly increased ($P < 0.01$), while the levels of CYP1A1 and AhR (nuclear) proteins were significantly decreased ($P < 0.01$).

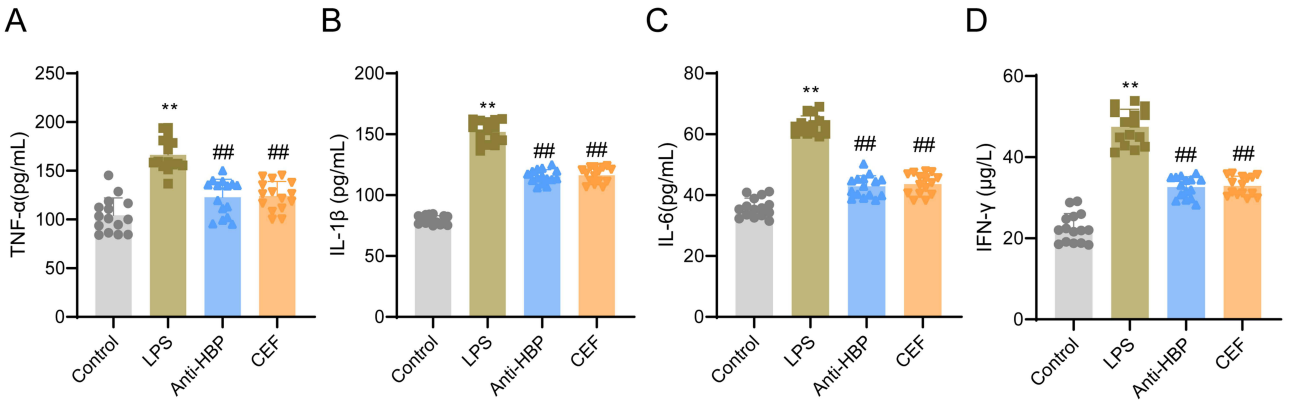

Figure 3 HBP degradation reduces the level of pulmonary proinflammatory cytokines in LPS-induced sepsis mice. A–D, ELISA was used to measure the levels of TNF-α (**A**), IL-1β (**B**), IL-6 (**C**), and IFN-γ (**D**) in lung homogenate of mice in the Control, LPS, Anti-HBP, and CEF groups. $^{**}P < 0.01$ vs Control group; $^{\#\#}P < 0.01$ vs LPS group.
Abbreviations: ELISA, enzyme-linked immunosorbent assay; TNF-α, tumor necrosis factor-α; IL, interleukin.

Moreover, compared with the LPS group, the levels of HBP and AhR (cytosol) proteins in lung tissue of mice in the Anti-HBP and CEF groups were significantly decreased ($P < 0.01$), while the levels of CYP1A1 and AhR (nuclear) proteins were significantly increased ($P < 0.01$). Furthermore, there was no significant difference in the levels of the above proteins between the Anti-HBP and CEF groups ($P > 0.05$) (Figure 4A–D). On one hand, such findings confirmed once again that Anti-HBP specifically degraded the target protein HBP; on the other hand, they demonstrated that Anti-HBP, a multifunctional molecule, effectively activated the AhR signaling pathway inhibited by LPS intervention by specifically degrading HBP. Additionally, the regulatory effect of Anti-HBP on the activity of the AhR signaling pathway was similar to that of the classical antibiotic CEF.

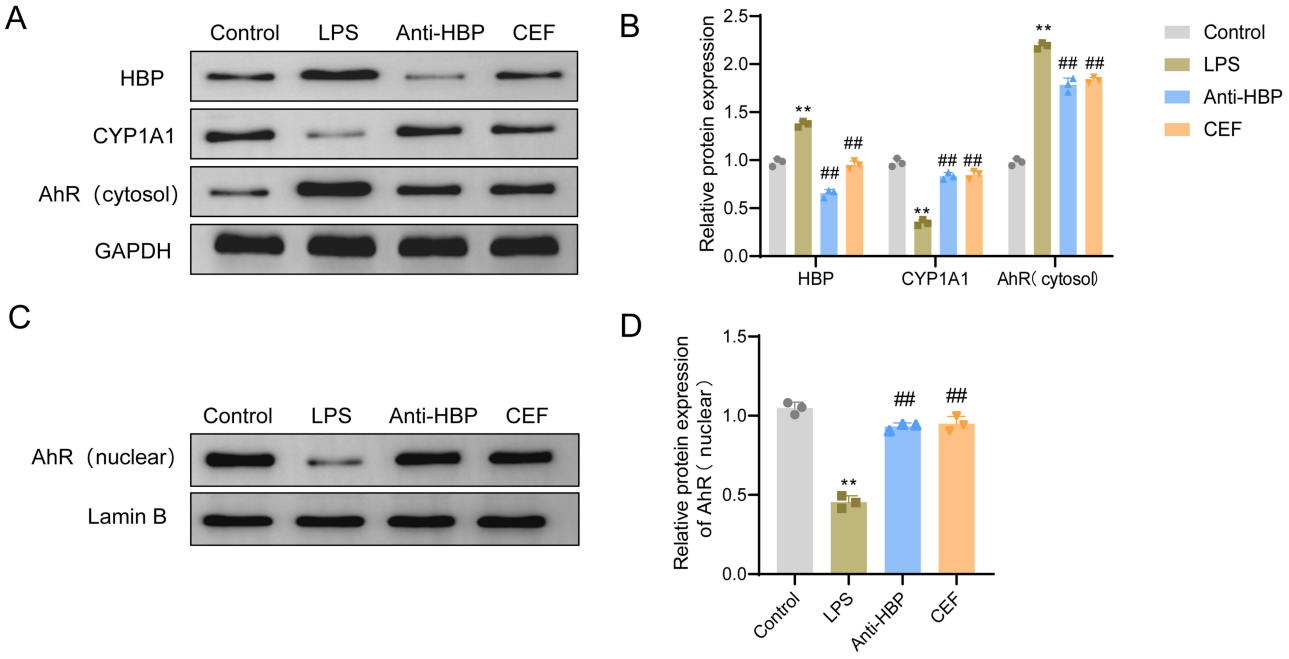

Figure 4 HBP degradation activates the AhR signaling pathway in lung tissue of LPS-induced sepsis mice. (**A**) Representative images of the levels of HBP, CYP1A1, and AhR proteins (cytosol) in mice of each group determined using Western blotting, with GAPDH used as the internal control; (**B**) Statistical analysis results of the levels of HBP, CYP1A1, and AhR proteins (cytosol); (**C**) Representative images of the level of AhR protein (nuclear) in lung tissue of mice in each group determined using Western blotting; (**D**) Statistical analysis results of AhR protein (nuclear) levels. $^{**}P < 0.01$ vs Control group; $^{\#\#}P < 0.01$ vs LPS group.
Abbreviations: HBP, heparin-binding protein; CYP1A1, cytochrome P450 1A1; AhR, aryl hydrocarbon receptor.

Discussion

We established a sepsis mouse model by intraperitoneal injection of LPS to evaluate the effect of HBP downregulation on ALI caused by sepsis. Our results demonstrated that the specific downregulation of HBP levels in lung tissue by the multifunctional molecule Anti-HBP significantly improved LPS-induced lung injury and the pulmonary inflammatory response, thereby prolonging the survival time of sepsis mice. Mechanistically, the anti-ALI effect of HBP degradation was likely realized by activating the AhR signaling pathway. To the best of our knowledge, this is the first study to reveal that HBP may be used as an intervention target to treat sepsis-related ALI.

LPS treatment and cecal ligation and puncture (CLP) are the most commonly used methods for sepsis modeling in experimental animals. Although CLP is widely used,[31–33] it is limited by shortcomings that cannot be resolved. First, it is difficult to accurately control the amount of intestinal leakage. Second, the number of punctures, suture position, and needle size all significantly affect the amount of proinflammatory cytokines released into the peritoneum and bloodstream. Therefore, the sepsis severity often varies greatly among experimental individuals,[34] and this heterogeneity is likely to negatively affect the accuracy and comparability of experimental results. In contrast, LPS treatment is not only technically simple but also shows minimal variation among individuals. Most experimental individuals release a large number of proinflammatory cytokines into the bloodstream shortly after LPS treatment, and this is reflected in increased serum cytokine concentrations.[34] Hence, there are many advantages to our selection of the LPS modeling method, and the homogeneity of its outcomes allows better comparison of the differences between groups.

The lung is an extremely susceptible organ to the pathological process of sepsis, and approximately 40% of ALI cases are attributed to sepsis.[35] This susceptibility is due to its unique characteristics. First, the lungs bear the key responsibility for blood oxygenation and are the sites of gaseous exchange. Pulmonary circulation allows the absorption of oxygen and the discharge of carbon dioxide, converting venous to arterial blood. However, such processes also enable pathogens in the circulatory system to reach the lungs quickly and cause lung infection. Moreover, the permeability of pulmonary capillaries is high to ensure the efficiency of gas exchange, and this also allows activated inflammatory cells to easily penetrate the vascular wall and accumulate in lung tissue when sepsis occurs, subsequently leading to pathological changes, such as pulmonary edema and fluid exudation.[36] Therefore, the alveolar-capillary barrier of the lung is often severely damaged by the pathological mechanism of sepsis, leading to the development of ALI characterized by pulmonary edema, a diffuse alveolar inflammatory response, and refractory hypoxemia in clinical patients.[37] Our comparison of mice in the Control group with mice in the LPS group revealed that sepsis modeling significantly worsened the pulmonary edema of mice and significantly increased the pulmonary pathological damage score. These pathological changes were consistent with the conclusions of clinical studies[38,39] focusing on the pulmonary characteristics of patients with sepsis.

HBP protein plays an important role in the pathological process of organ dysfunction induced by sepsis.[40] When sepsis involves the lungs, HBP interacts with glycosaminoglycans in the lungs. Such interaction activates the protein kinase C and Rho-kinase signaling pathways, resulting in an uncontrolled increase in pulmonary microvascular permeability, leading to a series of pulmonary dysfunction manifestations, such as pulmonary circulatory disorder and hypoxia.[41] Because increased HBP levels worsen the mechanism of sepsis-induced ALI, our study hypothesis that HBP downregulation may help alleviate sepsis-related ALI is supported by a sufficient theoretical base. In this study, we compared mice in the LPS and Anti-HBP groups, and the anti-ALI effect of downregulating HBP protein levels was explored from two aspects: the severity of pathological changes in the lung and the pulmonary inflammatory response. On one hand, HBP protein downregulation significantly reduced pulmonary edema, alveolar wall damage, local inflammatory cell infiltrates, and the lung injury score. Such outcomes intuitively suggested that HBP degradation improves pathological changes in the lungs. On the other hand, we observed that HBP protein downregulation significantly decreased the levels of TNF-α, IL-1β, IL-6, and IFN-γ in lung homogenate of mice. These four proinflammatory cytokines are produced and released by macrophages, lymphocytes, and other immune cells. The accumulation of these cytokines in the lung destroys alveolar epithelial cells, leading to the infiltration of activated inflammatory cells. Hence, these cytokines are key for the initiation and aggravation of the pulmonary inflammatory response.[42] The

levels of TNF-α, IL-1β, IL-6, and IFN-γ have been reported as positively correlated with ALI severity.[43] Therefore, HBP degradation may inhibit the pulmonary inflammatory response.

After discovering that HBP downregulation exerts a therapeutic effect on sepsis-related ALI, we further investigated its potential mechanism by focusing on the AhR signaling pathway. This pathway is involved in many physiological and pathological processes, such as substance metabolism and immune response and inflammation regulation, and is an important multifunctional signaling pathway in the body. Specifically, AhR signaling pathway activation effectively regulates pulmonary inflammation.[30] Al-Ghezi et al[44] and Guerrina et al[45] claimed AhR signaling pathway activation alleviated pulmonary inflammation caused by pertussis toxin and alleviated the local chronic inflammatory response mediated by chronic obstructive pulmonary disease. When the AhR signaling pathway is inactive, most AhR proteins are located in the cytoplasm. When the AhR signaling pathway is activated via external signaling, AhR enters the nucleus and binds to DNA to promote the transcription of target genes, such as *CYP1A1*, thereby upregulating the level of associated downstream proteins, such as CYP1A1.[17] Comparison of the results of the mice in the LPS and Anti-HBP groups revealed that reducing the HBP protein levels significantly lowered the level of AhR (cytosol) protein in mouse lung tissue while notably increasing the levels of AhR (nuclear) and CYP1A1 proteins, which strongly proved that HBP degradation activated the AhR signaling pathway. Based on the theory proposed in previous studies[17,44,45] and the results obtained in this research, we believe that AhR signaling pathway activation is important for downregulating HBP levels, thereby improving sepsis-related ALI.

A unique feature of this study is that we also set up a group of LPS-induced model mice that received CEF treatment alone. CEF is a third-generation cephalosporin with broad-spectrum antibacterial activity that causes bacterial death by inhibiting cell wall synthesis. Moreover, CEF also exerts an anti-inflammatory effect. Therefore, CEF is considered one of the first-line antibiotics for sepsis treatment.[46] Thus, in this study, the CEF group can be regarded as a positive control group, thanks to the grouping design of this study, we not only confirmed the anti-ALI effect of HBP degradation (there was a significant difference between the LPS group and Anti-HBP groups) but also proved that the therapeutic effect of HBP degradation was highly significant (there was no significant difference between the Anti-HBP and CEF groups).

This study has some shortcomings. First, sepsis is a serious disease involving multiple organs, but we only explored the protective effect of regulating HBP levels on the lungs. Therefore, future research must clarify whether HBP degradation protects the heart, liver, kidney, and other organs to fully evaluate the comprehensive effect of HBP regulation. Second, besides activating the AhR signaling pathway, HBP may also affect ALI via other pathways, so it is necessary to perform a more detailed investigation of the specific mechanism by which HBP regulation improves ALI in future studies.

Conclusion

In summary, our study revealed that lowering HBP levels in lung tissue significantly improved LPS-induced lung injury and the pulmonary inflammatory response by activating the AhR signaling pathway, thereby playing a role in prolonging the survival time of sepsis mice. Moreover, the therapeutic effect of lowering HBP protein levels is equivalent to that of the classical antibiotic CEF.

Abbreviations

ALI, Acute lung injury; HBP, Heparin-binding protein; LPS, Lipopolysaccharides; CEF, ceftriaxone; RT-qPCR, real-time quantitative PCR; IHC, immunohistochemistry; W/D, wet/dry; H&E, hematoxylin-eosin; ELISA, enzyme-linked immunosorbent assay; AhR, aryl hydrocarbon receptor; ICU, intensive care unit

Data Sharing Statement

Datasets used in this article are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

All experiments were reviewed and approved by the Laboratory Animal Ethical Review Committee of The Second Affiliated Hospital of Hainan Medical University (LW2023158).

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Disclosure

The authors declare that they have no competing interests in this work.

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