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Tamibarotene targets heparin-binding protein for attenuating lung injury in sepsis

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Abstract
Background: Excessively active pulmonary inflammation is a hallmark of sepsis-induced lung damage. A synthetic retinoid drug called tamibarotene reduces inflammation in a variety of conditions, including acute promyelocytic leukemia (APL), renal fibrosis, and neuroinflammation. Its effect on sepsis-related lung injury, however, has not been explained.
Purpose: The purpose of the study was to investigate how tamibarotene affected lung damage induced by cecal ligation and puncture (CLP) procedure.
Methods: A CLP sepsis mouse model was developed, and tamibarotene was pretreated to determine whether it improved lung injury and survival. The degree of lung injury was evaluated using the Hematoxylin and eosin staining and lung injury score. In order to determine pulmonary vascular permeability, measurements were taken for total protein and cell content of bronchoalveolar lavage fluid (BALF), wet/dry ratio of the lung, and Evans blue stain. The BALF inflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-1β, and IL-17A were discovered by enzyme-linked immunosorbent serologic assay (ELISA). Then, the levels of heparin-binding protein (HBP), and phospho-nuclear factor kappa-B (p-NF-κB) P65, and NF-κB P65 were determined using ELISA and Western blot analysis, respectively.
Results: Tamibarotene considerably increases survival and lessens lung damage stimulated by sepsis. Specifically, tamibarotene significantly relieves pulmonary vascular permeability and inhibits inflammation response in sepsis. Moreover, we further confirmed that these ameliorating effects of tamibarotene on sepsis may be exerted by targeting HBP and regulating the activation of NF-κB signaling pathway.
Conclusion: These findings demonstrated that tamibarotene lessens sepsis-induced lung injury, and the effect could be exerted by targeting HBP and thereby deregulating the NF-κB signaling pathway.

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Introduction

Sepsis is a system disease with life-threatening organ dysfunction, and lung injury is one of the most prevalent complications of sepsis.[1,2] Epidemiological investigations have demonstrated that sepsis-induced acute lung injury (ALI) is a clinical condition with a high incidence of morbidity and mortality.[2] However, the pathogenesis of sepsis-associated ALI has not been explained, and effective therapeutic approaches are lacking. Considering that sepsis is characterized by an out-of-control inflammatory response and many life-threatening organ dysfunctions, immunological homeostasis is undeniably crucial to the onset and development of sepsis-induced ALI.

Heparin-binding protein (HBP), referred to as Azurocidin or Cationic antimicrobial protein 37 (CAP37), is a protein primarily produced from neutrophils and found in the secretory and azurophilic granules of neutrophils.[3] Clinical studies have shown that patients with sepsis have dramatically raised plasma HBP levels, suggesting that HBP could be a pathogenic marker and therapeutic target for the disease.[4-6] Additionally, similar research has shown that HBP may control macrophage activity, stimulate macrophage activation, and cause the release of important inflammatory components.[7] HBP has also been reported to increase lactate-mediated nuclear factor kappa-B (NF-κB) pathway-mediated inflammatory gene transcription in M1 macrophages.[8] Consequently, we hypothesized that HBP plays a significant role in the regulation of inflammation and lung injury induced by sepsis.

Tamibarotene, a synthetic retinoid drug, is an activator of retinoic acid receptors (RARα receptor) having greater stability and potentially fewer adverse effects.[9] Tamibarotene could modulate immune and inflammation homeostasis to improve multiple diseases, including acute promyelocytic leukemia (APL),[9] renal fibrosis,[10] dermal fibrosis,[11] and neuroinflammation.[12] Specifically, tamibarotene has been reported to attenuate renal fibrosis by inhibiting fibroblast aggregation through interleukin(IL)-17A.[10] Tamibarotene can also modulate the *Mafb*/*Msr1*/PI3K-Akt/NF-κB pathway by over-promotion of the M1/M2 phenotypic polarization of microglia, and thus attenuate neuroinflammation after subarachnoid hemorrhage (SAH).[13]

Furthermore, through the networkanalyst website (https://www.networkanalyst.ca/), we predicted the potential compounds targeting *HBP*. Interestingly, we found that *HBP* is the target gene of tamibarotene. Additionally, in this study, we confirmed that tamibarotene targets *HBP* and inhibits the NF-κB pathway to improve pulmonary vascular permeability and inflammatory response, thereby improving sepsis-induced lung injury.

Method

Animals

In all, 25 Balb/c mice aged 6–8 weeks were provided by Zunyi Medical University Experimental Animal Center (Zunyi, China). The mice were housed in a standard environment for 7 days before experiments. This procedure was approved by the Ethics Committee of the Third Affiliated Hospital of Zunyi Medical University (The First People's Hospital of Zunyi) (Approval No. (2022) -1-256).

Cecal ligation and puncture (CLP) model

An identical CLP procedure protocol was used on the mice.[14] Briefly, mice were made unconscious, and a midline incision was created. The cecum was ligated and pierced with 22-gauge needles; then a small quantity of defecation was extracted and returned to its normal location, and the incision was closed. The sham group received the identical care, but the ligation and puncture procedures were omitted. The bronchoalveolar lavage fluid (BALF) and lung tissues of mice treated with CLP, or a sham procedure were examined 24 h after surgery.

Administration of tamibarotene

Tamibarotene (T3205) was purchased from Sigma-Aldrich (St. Louis, MO). The animals were randomly separated into four groups: sham group, CLP group, CLP+5-mg/kg tamibarotene, CLP+10-mg/kg tamibarotene, and CLP+20-mg/kg tamibarotene, with each group consisting of five mice. The CLP+tamibarotene-treated groups received different concentrations of intraperitoneal (i.p.) injection of tamibarotene after CLP procedure. The sham group received an equivalent volume of phosphate-buffered saline (PBS) solution intraperitoneally.

Lung histological test and lung injury scores

Lung tissue was embedded in paraffin and fixed with 4% paraformaldehyde before being sectioned. Tissue sections were stained with hematoxylin and eosin (H&E) and observed under a microscope. Mikawa technique was used to assess lung damage scores,[15] complying with the following four 0–4 graded objectives: respiratory congestion, hemorrhage, neutrophils infiltration, and alveolar wall thickening or a transparent film formation. Each item was scored on a 5-point scale as follows: 0 = minimal damage, 1+ = mild damage, 2+ = moderate damage, 3+ = severe damage, and 4+ = maximal damage.

Wet-dry weight ratio of lung tissue

Assessing pulmonary edema based on the ratio of wet to dry lung tissue. The lung tissue was separated from the upper left lung lobe. After extracting water from the tissues, they were weighed twice before being dehydrated at 80°C for 24 h. The results were determined by dividing moist mass by dry weight.[16]

Protein levels and cell counts in bronchoalveolar lavage fluid

Pentobarbitone (50 mg/kg) was used to anesthetize mice before tracheal intubations were carried out. (PBS) was

gently aspirated, pooled, and re-aspirated into the lungs before BALF was withdrawn through the tube. Centrifuging was done on lavaged samples at 1500 g for 10 min at 4°C. A complete blood count was performed on cell pellets after they were dissolved in PBS. A bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich) was used to measure total protein concentration in BALF.

Vascular permeability

In order to calculate lung vascular permeability, the amount of Evans blue stain that had accumulated in tissues was quantified. Evans blue (25 mg/kg; Sigma-Aldrich) was injected into the tail vein 2 h prior to the harvesting of the lungs. Evans blue was removed from the lungs by incubating them in 1 mL of formamide for 18 h at 60°C, then perfusing with 5 mL of PBS, homogenizing with 1 mL of PBS, and finally washing them twice. The supernatant was separated by centrifugation for 30 min at 5000 ×g. The quantity of Evans blue was determined using 620- and 740-nm dual-wavelength spectrophotometry, and the concentration was calculated using the following formula: E620 (Evans blue) = E620 − (1.426 E740 + 0.03).[17]

Enzyme-Linked Immunosorbent Serological Assay (ELISA)

BALF samples of the animals were taken and stored at −80°C. Then, using Invitrogen ELISA kits (Carlsbad, CA), cytokine detection containing TNF-α, IL-6, IL-1, and IL-17A as well as inflammatory regulator HBP was exercised.

Western blot analysis

Western blot analysis was used to examine phospho-nuclear factor kappa-B (p-NF-κB) P65, NF-κB P65, and β-actin expression in BALF. The upper left lung lobe was homogenized, and total proteins were extracted using a tissue protein extraction reagent. Subsequently, protein quantity was measured using a BCA kit (Beyotime, Shanghai, China). Equivalent amounts (10 μg) of denaturized proteins were separated electrophoretically based on molecular weight before being transferred to polyvinylidene fluoride (PVDF) membranes (LM1136; LMAI Bio). Membranes were blocked with 5% nonfat dry milk for 1 h at room temperature. Next, the membranes were treated with matching secondary antibodies for 1 h at 37°C after being incubated with primary antibodies overnight at 4°C, rinsed with tris buffered saline with tween (TBST). Then, particular protein bands were observed in membranes by using an enhanced chemiluminescence (ECL) detection technique (GE Healthcare, Piscataway, NJ).

Antibodies against p-NF-κB P65 (#ab, 1:1000), NF-κB P65 (#ab16502, 1:1000), and β-actin (#ab8226, 1:1000) were purchased from Abcam (Cambridge, UK). Anti-mouse (#4410, 1:10,000) and anti-rabbit (#4414, 1:10,000) peroxidase-conjugated secondary antibodies were obtained from Cell Signaling Technology (CST; Danvers, MA).

Statistical Analysis

All data were statistically examined by SPSS 22.0 (IBM, Armonk, NY) and presented as mean ± standard deviation (SD). Histologic scores were analyzed using Kruskal-Wallis analysis. Survival rates were analyzed by using Fisher's exact probability test. Student's *t*-test and one-way ANOVA were used to identify differences between two or more groups; $P \leq 0.05$ was considered as statistically significant.

Results

Sepsis survival and lung damage are extended and alleviated by tamibarotene treatment

Cecal ligation and puncture procedure was used to induce sepsis in mice and to administer different doses of tamibarotene (5, 10, or 20 mg/kg). Strong sepsis was observed in CLP, and the survival rate was as low as 80% in the initial 2 days, 40% in 3 days, and 0% in 4 days, compared to the 100% survival rate in the sham group (Figure 1A). Interestingly, significant decrease in the survival rate of mice caused by CLP procedure could be improved with concentration-dependent tamibarotene treatment (Figure 1A). Furthermore, lung tissues exhibited extensive histopathological damage, including alveolar wall thickening, interstitial edema, and pulmonary congestion in the CLP group, compared with the sham group, while the administration of concentration-dependent tamibarotene significantly reduced the severity of CLP-induced lung damage (Figure 1B). Specifically, quantitative analysis using the lung injury score showed that damage to CLP group was significantly higher than the sham group ($P < 0.001$, Figure 1C). However, compared to the CLP group, decrease in the severity and score of lung injury was observed in a drug concentration-dependent manner in tamibarotene-treated groups ($P < 0.001$, Figure 1C). Together, these results revealed that tamibarotene treatment could significantly improve survival rate and reduce lung injury in septic mice.

Tamibarotene alleviates pulmonary vascular leakage caused by sepsis

In order to evaluate the possible protective role of tamibarotene in lung injury induced by sepsis, some indicators of lung injury, including total proteins and cells in BALF, lung weight/dry ratio, and Evans blue, were observed (Figures 2A–D). Compared to the sham group, CLP procedure induced significant increase in the total proteins and total cells of BALF, while the administration of tamibarotene significantly reduced these in a concentration-dependent manner ($P < 0.001$, Figures 2A and B). Additionally, the wet/dry weight ratio of the lung was used to evaluate the degree of pulmonary edema, indicating that the wet/dry weight ratio was dependently reduced in the tamibarotene-treated groups than in the CLP group ($P < 0.001$, Figure 2C). Similarly, pulmonary vascular leakage, as indicated by Evans blue, was lessened after tamibarotene treatment, compared to CLP procedure ($P < 0.001$, Figure 2D). These results

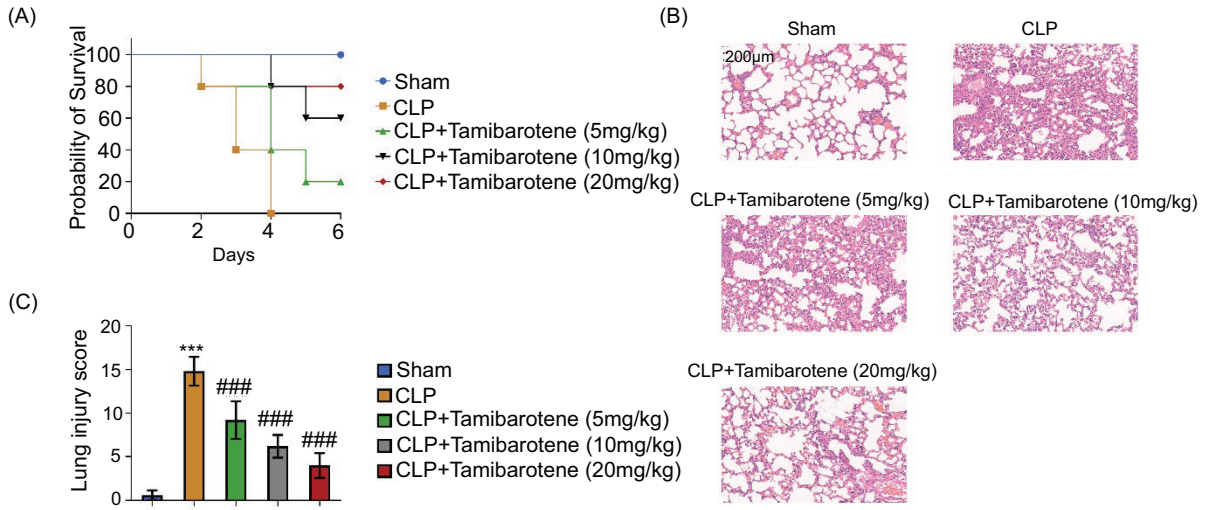

Figure 1 Sepsis survival and lung damage are respectively extended and attenuated by tamibarotene treatment. (A) Different concentrations of tamibarotene (5, 10, or 20 mg/kg) treatment injected intraperitoneally at 1 h after CLP procedure. The probability of survival was expressed as survival percentage. (B) Lung tissues from each experimental group were processed for histological evaluation on the day after CLP procedure. Representative images of lung sections stained with H&E (histological/microscopic images, 200× magnification). (C) The lung injury scores were estimated by Mikawa technique. Data are presented as mean ± SD. ***P < 0.001 vs the sham group. ###P < 0.001 vs the CLP group.

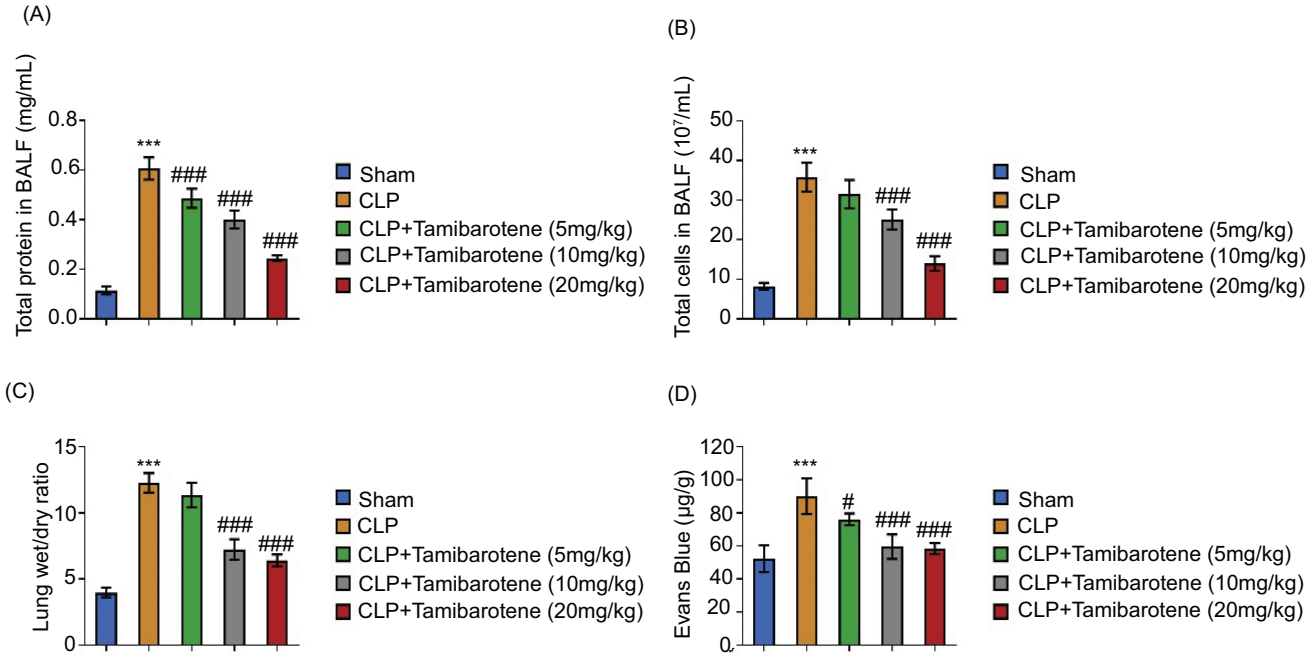

Figure 2 Tamibarotene treatment lessens pulmonary vascular leakage caused by sepsis. (A) Total proteins in BALF were assessed by BCA protein assay kit. (B) Total cells in BALF were assessed by complete blood count. (C) Lung tissue edema was measured by the lung wet/dry weight ratio. (D) Pulmonary permeability was measured by Evans blue stain. Data are presented as mean ± SD. ***P < 0.001 vs the sham group. #P < 0.05 and ###P < 0.001 vs the CLP group.

showed that tamibarotene pretreatment could lessen pulmonary vascular leakage in septic mice.

Tamibarotene reduces inflammatory responses in septic mice

Considering excessive inflammation induced by CLP in septic mice, the contents of inflammatory mediators in BALF, including TNF-α, IL-6, IL-1β, and IL-17A, were detected by ELISA. The result implied that CLP induced an increased level of relevant inflammatory mediators, compared to the sham group ($P < 0.001$, Figures 3A-D), while tamibarotene treatment could decrease the levels of TNF-α, IL-6, IL-1β, and IL-17A in BALF than that in the CLP group ($P < 0.001$, Figures 3A-D).

Tamibarotene inhibits the expression of heparin-binding protein and NF-κB pathway

Studies have shown that HBP, an inflammatory regulator, was crucial in sepsis-induced inflammation and elevated in sepsis patients.[4-6] In addition, we predicted potential compounds targeting HBP and found that tamibarotene could target HBP. Therefore, we hypothesized that tamibarotene exerted anti-inflammatory effect and weakened lung injury effects by regulating HBP. As mentioned above, the expression of HBP in BALF was up-regulated in the CLP group and tamibarotene ameliorated the increased trend in a concentration-dependent manner ($P < 0.001$, Figure 4A). Furthermore, the protein levels of p-NF-κB P65 and NF-κB P65 were assessed by Western blot analysis, suggesting that its relative level was significantly increased in septic mice stimulated by CLP, while a decreased trend was observed by tamibarotene treatment ($P < 0.001$, Figures 4B and C). These results revealed that tamibarotene attenuated sepsis-associated lung injury by targeting HBP and inhibiting the NF-κB signaling pathway.

Discussion

The present study showed that tamibarotene attenuated sepsis-induced lung injury. The pulmonary vascular permeability in CLP mice was inhibited, and the excessive release of inflammatory cytokines was suppressed by tamibarotene administration. Finally, at the mechanism level, we demonstrated that the inhibitory effect of tamibarotene on sepsis-induced lung injury was exerted by targeting HBP and deregulating the NF-κB signaling pathway.

Sepsis-induced lung dysfunction manifested high mortality and damage to the lung, which included severe alveolar wall thickening, interstitial edema, and congestion, with increase in inflammatory reactions.[1] In this study, we successfully established a sepsis model of mice by CLP, and the CLP-induced mice showed decreased survival rate and severe lung histopathological damage. Interestingly, tamibarotene treatment lessened CLP-related lung injury and extended survival of sepsis mice.

Markedly increased alveolar-capillary permeability, increased lung weight, and other severe pulmonary damages were typically induced by sepsis.[18,19] Specifically, research has shown that miR-144 acts as a lung protector

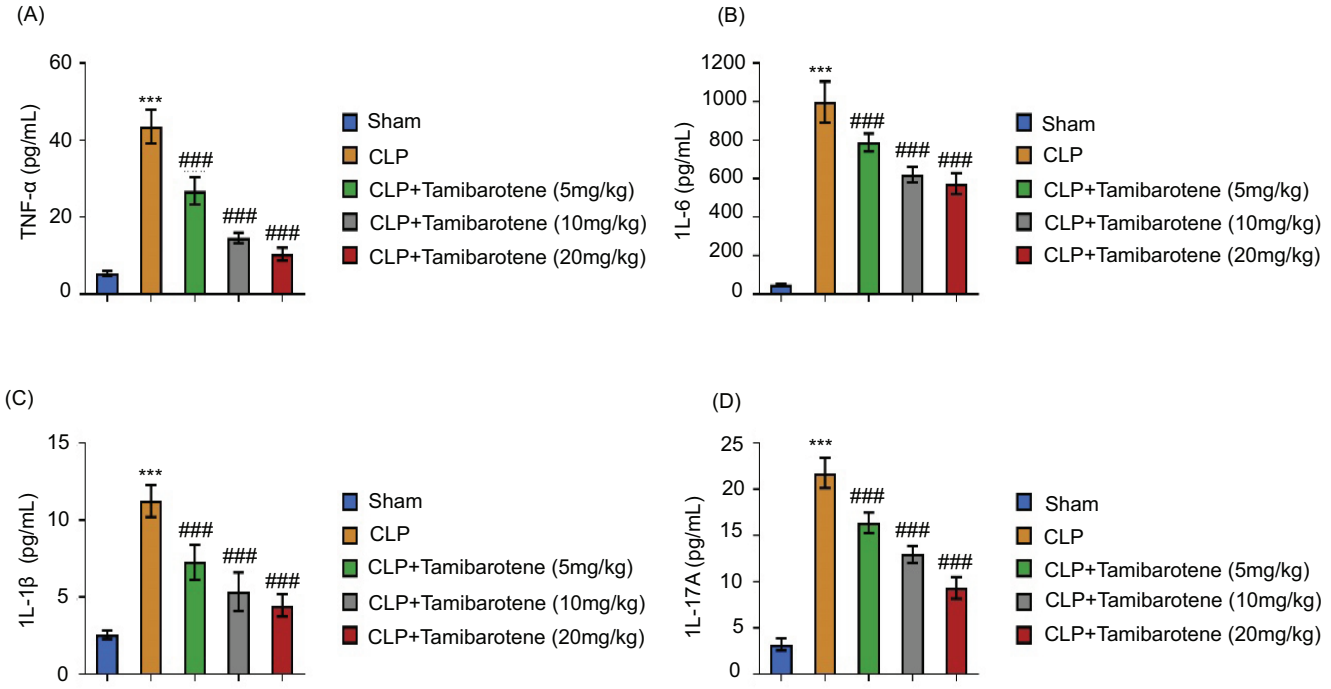

Figure 3 Tamibarotene treatment reduces inflammatory responses in sepsis mice. (A-D) The expression of inflammatory factors in BALF, such as TNF-α, IL-6, IL-1β, and IL-17A, were measured by ELISA. Data are presented as mean ± SD. ***P < 0.001 vs the sham group. ###P < 0.001 vs the CLP group.

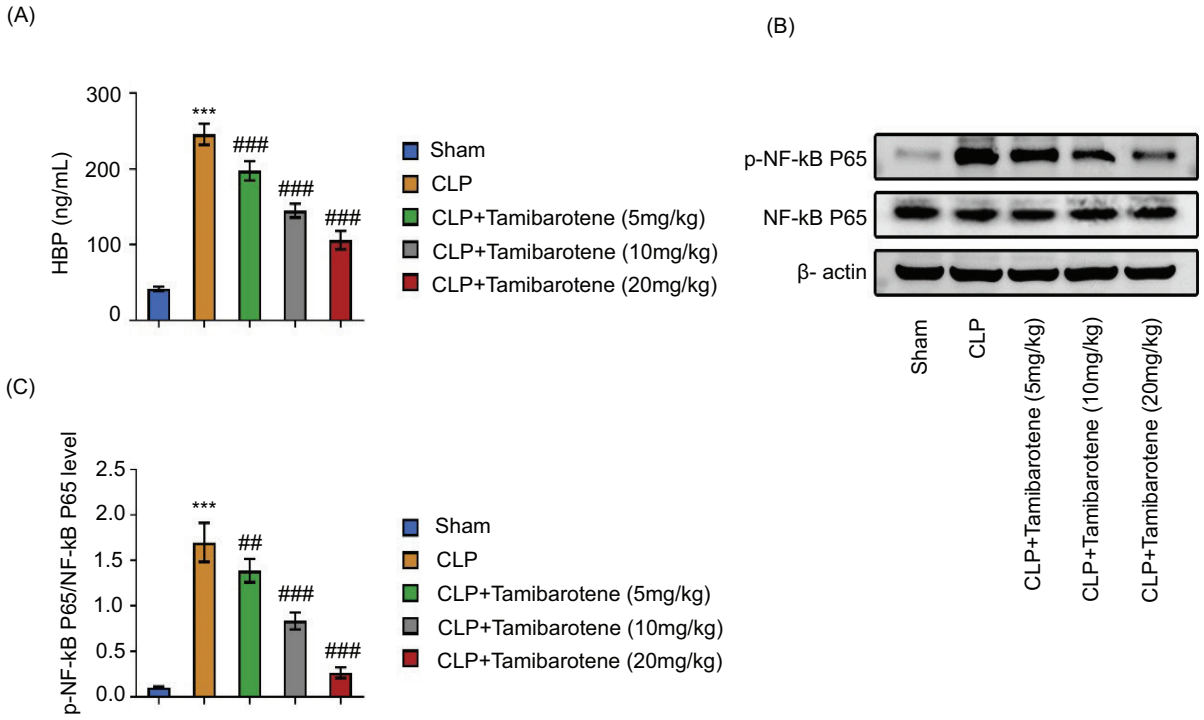

Figure 4 Tamibarotene treatment inhibits the expression of HBP and NF-κB pathway. (A) The expression of HBP in BALF was detected by ELISA. (B and C) The expression levels of of p-NF-κB P65 and NF-κB P65 were assessed by Western blot analysis. Histograms show the relative gray value of proteins evaluated by Image Lab. Data are presented as mean ± SD. $^{***}P < 0.001$ vs the sham group. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs the CLP group.

by mediating the recovery of endothelial cell function and decreasing lung vascular permeability.[20] Sepsis typically induced endothelial adhesion junction dissociation and disrupted alveolar vascular integrity and increased infiltrated cells, thereby increasing vascular permeability. Li et al. also discovered that bone marrow kinase on X chromosome (BMX) alleviated lung injury and inflammation in early sepsis by repressing pulmonary vascular endothelial permeability and reducing vascular leakage in CLP-induced sepsis mice.[21] Polydatin ameliorated severe acute lung damage induced by traumatic brain injury by promoting lung vascular permeability recovery, inhibiting the secretion of inflammatory factors (IL-6, IL-1β, TNF-α, and MCP-1) and alleviating oxidative stress response.[22] Similarly, the present study showed that CLP-induced sepsis mice had increased total proteins and total cells in BALF, and the over-activated release of inflammatory cytokines, such as TNF-α, IL-6, IL-1ß, and IL-17A, compared to the control. However, all these effects were dramatically reduced by tamibarotene treatment, which indicated that tamibarotene relieved lung damage by suppressing excessive inflammation and pulmonary vascular infiltration.

Heparin-binding protein, an inflammatory regulator derived from neutrophil secretory granules, is elevated in the serum of sepsis patients and acts as a crucial player in the pathophysiology of sepsis-induced inflammation.[5] Interestingly, in the previous study, we predicted the potential target genes of tamibarotene and found that it could target *HBP* gene. Furthermore, as expected, the expression of HBP, known as a new sepsis-associated biomarker, was increased in the CLP group but could be depressed by tamibarotene treatment. These findings indicated that tamibarotene could attenuate sepsis-induced lung damage by deregulating the levels of HBP.

In addition, studies have revealed that HBP is affected by the NF-κB signaling pathway. To further elucidate the mechanism of tamibarotene protection against sepsis-induced lung injury, the protein levels of p-NF-κB P65 and NF-κB P65 were assessed by Western blot analysis. The results showed that its relative level was significantly increased in septic mice stimulated with CLP, while a decreased trend was observed by tamibarotene treatment, which demonstrated that tamibarotene lessened lung injury in sepsis by targeting HBP and thereby inhibiting activation of the NF-κB signaling pathway.

Conclusion

This study establishes that tamibarotene attenuates lung injury caused by sepsis. This therapeutic effect is considered to specifically target HBP, which subsequently leads to the deregulation of the NF-κB signaling pathway, inhibiting the inflammation levels induced by sepsis. These findings provide a new perspective for tamibarotene treatment and prevention in developing sepsis-induced lung injury. Furthermore, regarding the characteristics of systemic toxicity caused by sepsis, the protective effects of tamibarotene must be determined in other sepsis-affected organs and tissues, such as the gastrointestinal tract and kidneys.

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