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IGFBP7 aggravates sepsis-induced acute lung injury by activating the ERK1/2 pathway

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Abstract

Introduction. Sepsis is characterized by an infection-caused acute inflammatory response, which is usually accompanied by multiple organ failure, especially lung injury. During sepsis, a large number of endotoxins such as lipopolysaccharides (LPSs) are secreted from Gram-negative bacteria. However, the mechanisms underlying acute lung dysfunction caused by sepsis have not yet been well defined.

Material and methods. To identify the mechanism of insulin-like growth factor binding protein 7 (IGFBP7) in acute lung injury during sepsis, the effects of IGFBP7 shRNA were evaluated in a model of cecal ligation puncture (CLP)-induced sepsis in mice. Histologic evaluation of the effects of IGFBP7 on CLP-induced acute lung injury was performed by H&E staining. Murine pulmonary microvascular endothelial cells (MPVECs) were transfected with shIGFBP7 or shNC before treatment with LPS to mimic the sepsis-induced lung dysfunction. The effects of CLP or LPS on IGFBP7 expression and the activation of ERK1/2 pathway were analyzed by western blot. MTT and LDH assays were used to measure the viability of MPVECs under different treatment regimes. The apoptosis rate of MPVECs in different groups was detected by flow-cytometry analysis.

Results. IGFBP7 was strongly up-regulated in sepsis-induced acute lung injury in mice. IGFBP7 silencing attenuated sepsis-induced apoptosis and cytotoxicity in MPVECs. Furthermore, the activation of ERK1/2 pathway was regulated by IGFBP7 during sepsis-induced inflammation. IGFBP7 inhibition by RNA interference in MPVECs attenuated CLP-induced morphological features of lung dysfunction. The knockdown of IGFBP7 attenuated LPS-induced MPVECs' apoptosis by the suppression of the ERK1/2 pathway.

Conclusions. We demonstrated for the first time that IGFBP7 is involved in the pathogenesis of sepsis-induced acute lung injury and may serve as a therapeutic target in sepsis-induced acute lung injury. *(Folia Histochemica et Cytobiologica 2020, Vol. 58, No. 4, 247–254)*

Key words: mouse; CLP sepsis; acute lung injury; MPVECs; IGFBP7; ERK1/2; siRNA; apoptosis

Introduction

Sepsis is characterized by an infection-caused acute inflammatory response to tissue injury and is often caused by bacterial, fungal, and viral infections [1–3]. Sepsis is a clinical disorder with a high mortality rate and is companied with numerous health problems worldwide [4, 5]. If the inflammatory response is particularly severe, the homeostasis of multiple organ system would be disrupted [6]. Severe sepsis is usually accompanied by multiple organ failure, and in the process of organ dysfunction, the lung is frequently the first to fail [7, 8]. The pathogenesis of sepsis-induced acute lung injury was related to the overexpression of cytokine-mediated inflammation [9]. Within sepsis, a large number of endotoxins such as lipopolysaccharides (LPSs) are commonly secreted from Gram-negative bacteria [10]. LPS activates Toll-like-receptor-4 and co-receptor CD14, thereby

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triggering the mitogen-activated protein kinase signaling pathways, including the p38, extracellular signal-related kinase pathway (ERK), and c-Jun N-terminal kinase pathways [11, 12]. However, the mechanisms underlying acute lung dysfunction caused by sepsis have not yet been well defined.

Insulin-like growth factor (IGF) signaling contributes to cell growth and differentiation and continues its role throughout life by triggering cell proliferation and inhibiting cell apoptosis [13]. Some studies have indicated that IGF-binding proteins (IGFBPs) acts as a part of transporters of IGFs, extending their half-life, and regulating their access to their receptors [14, 15]. IGFBP7 is a secreted protein with IGF-1, insulin and activin A binding properties that possess IGF-independent activity [16, 17]. IGFBP7 is a potential tumor endothelial cell marker that is expressed at higher levels in tumor-associated endothelium than in normal endothelial cells. Tumorigenesis [18], the cellular senescence and apoptosis are also regulated by IGFBP7 through the ERK signaling pathway [19–21]. Recently, multiple studies have revealed that the ERK pathway plays an important role in the pathogenesis of LPS-induced lung injury [22, 23]. However, the effect of IGFBP7 in sepsis-induced acute lung injury remains unclear. In the present study, we designed and performed experiments to demonstrate the potential mechanism of IGFBP7 and ERK signaling pathway in sepsis-induced acute lung injury in MPVECs and mice. In this study we provide evidence that knockdown of IGFBP7 suppressed sepsis-induced acute lung injury through ERK1/2 signaling inhibition.

Methods

Animal model. Wild type C57BL/6J male mice (8–10 weeks of age, weight 18 ± 2 g) were obtained from Weitong Lihua Biology Company (Beijing, China). All animal experiments were approved by the Experimental Animal Welfare Ethics Committee of Zhejiang Academy of Traditional Chinese Medicine and conducted in accordance with the guidelines of the Animal Care. Twenty-four mice were randomly divided into four experimental groups: (1) sham group, (2) cecal ligation puncture (CLP) group, (3) CLP + Ad-shNC group, and (4) CLP + Ad-shIGFBP7 group. Before the surgery, all animals were anesthetized by intraperitoneal administration of pentobarbital (50 mg/kg). The sham group without ligating or puncturing the cecum served as sham-operated. The CLP group was subjected to cecal ligation and puncture (CLP) surgery [24]. The animals received injection of negative control (NC) Ad-shNC or Ad-shIGFBP7 (5' GGA-CAUCUGGAACGUCACUTT 3') before the CLP surgery. To arise the knockdown efficiency, a second administration

of the adenovirus was performed 72 hours later. Two weeks after the first injection, the mice were ready for use in the experiments. One week later, mice were anesthetized with pentobarbital sodium and both lungs were harvested and kept frozen at −80°C until analysis.

Hematoxylin and eosin (H&E) staining. The lung tissues from the mice were fixed with 10% buffered paraformaldehyde for 24 hours, then embedded in paraffin and sectioned at a thickness of 5 μm. After hematoxylin and eosin (H&E) staining, the pathological changes of lung tissues were graded using the double-blind method.

Cell culture. The MPVECs were isolated from C57BL/6 mice. Shortly, mice were anesthetized with pentobarbital sodium and lungs were isolated from the thoracic cavity and perfused with phosphate-buffered saline (PBS) containing heparin. The lateral lobe was cut into small pieces under sterile conditions, placed in a dish which coated with 0.1% gelatin and cultured in DMEM (Gibco, Invitrogen, USA) with 20% FBS. The expression of cluster of differentiation 31 (CD31) was used for the identification of the characterization of primary cultured MPVECs [25]. Cells were transfected with shIGFBP7 or shNC lentiviral particles with 10 μg/ml Polybrene (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) before co-treatment with LPS for 48 hours. After incubation with lentiviral particles for 24 h, transduced cells were selected with puromycin (Sigma-Aldrich, Milwaukee, WI, USA).

Western blot analysis. The total protein was extracted from the lung tissue of C57BL/6 mice and MPVECs. The protein concentrations in the lung tissue homogenate or lysate of MPVECs were detected by BCA Protein Assay Kit (23227, Thermo Fisher Scientific, San Jose, CA, USA). The protein samples were separated on 10% SDS-Polyacrylamide gel and transferred onto nitrocellulose membrane. After blocking with 5% nonfat milk, the membrane was incubated with specific primary antibodies purchased from Abcam (Abcam, Cambridge, MA, USA), including IGFBP7 (ab74169), ERK1/2 (ab17942), p-ERK1/2 (ab214362) and actin (ab179467), Abcam) at 4° C overnight, followed with incubation with secondary antibodies (ab131368, Abcam) at room temperature. The signals were measured by Image J software (US National Institutes of Health, Bethesda, MD, USA).

Cell viability assay. The viability of MPVECs was assessed by the MTT assay kit (ab211091, Abcam) according to the manufacturer's instructions. Cells were seeded into 96-well plates at a density of 5×10^4/well. After experiment, MTT solution was added into each well and incubated at 37 for 5 hours. Then, the medium was removed and DMSO was added into each well. The absorbance of the developed color
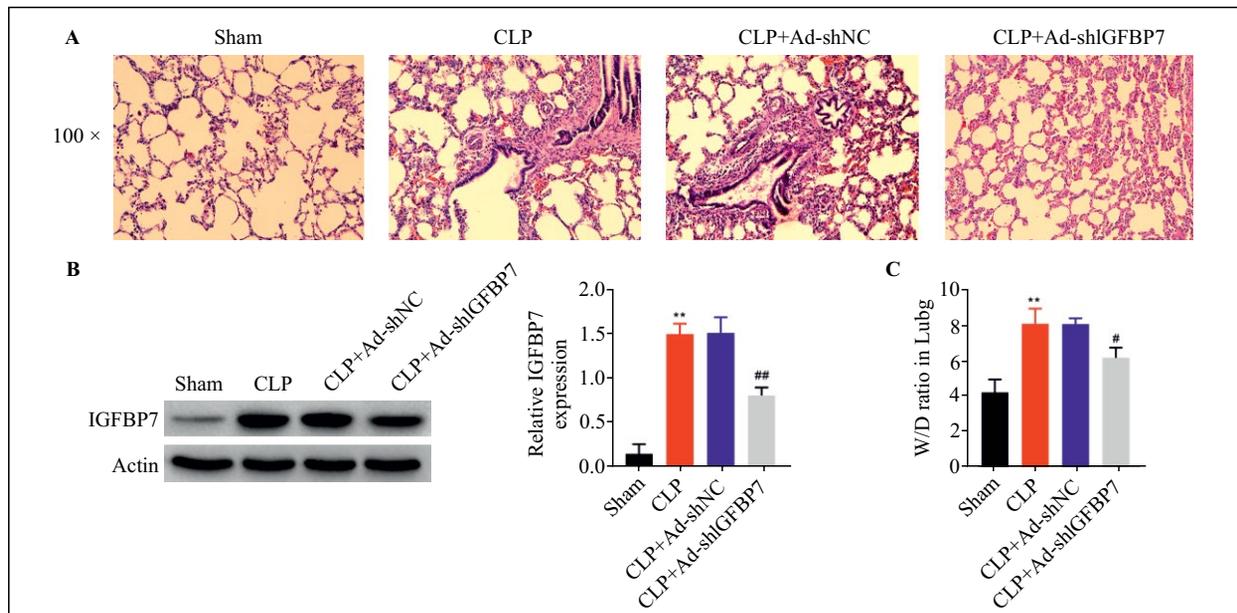
Figure 1. Effects of IGFBP7 in cecal ligation and puncture-induced acute lung injury. **A.** The lung tissue sections were staining by hematoxylin and eosin. Representative morphological changes of the lung tissues obtained from mice of different groups. **B.** The expression levels of IGFBP7 were assessed by western blot. **C.** The lung W/D (wet-to-dry) ratio from different groups. **p < 0.001 compared with Sham; ##p < 0.001 compared with CLP.

(590 nm) was detected by a microplate reader (Bio-Rad, Hercules, CA, USA).

Lactate Dehydrogenase (LDH) release assay. To determine the activity of cytoplasmic enzyme released by damaged MPVECs, LDH release was measured by LDH kit (ab65393, Abcam) according to the manufacturer's instructions. The intensity of released LDH in the culture was measured at 490 nm in a microplate reader. The cell viability was reflected by the rate of LDH activity released into the medium to total cellular LDH activity.

Cell apoptosis analysis. After being transfected with or without shIGFBP7 and treated with LPS, MPVECs were harvested and the apoptosis rate was analyzed by Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (ab14085, Abcam) according to the manufacturer's instruction. Cells were resuspended to 0.5×10^6 cells/mL in Annexin V binding buffer containing propidium iodide (PI) and Annexin V-FITC, and incubated for 15 min at room temperature in the dark. Cell apoptosis in MPVECs was measured with flow cytometry (BD Biosciences, San Diego, CA, USA) using the 488 nm detector for Annexin V-PI.

Statistical analysis. Biochemical values are expressed as mean ± standard deviation (SD). Statistical differences for multiple groups and the significance were evaluated using one-way analysis of variance followed by Student Newman-Keuls test. *P* values less than 0.05 were considered significant.

Results

Knockdown of IGFBP7 attenuated cecal ligation and puncture-induced acute lung injury in mice

To identify the mechanism of IGFBP7 action in acute lung injury during sepsis, the protective effect of IGFBP7 shRNA was evaluated in CLP-induced sepsis in mice. Male C57BL/6 mice were randomly divided into four groups: sham, CLP model, CLP + shNC, and CLP + shIGFBP7. Two weeks after the first shIGFBP7 or shNC injection, the mice were used for the experiments.

The effects of IGFBP7 on CLP-induced acute lung injury were histologically evaluated. Compared with the sham group, the lung tissues isolated from the CLP and CLP + shNC groups showed abnormal histological features, including acute alveolar damage, congestion, and thickened alveolar walls (Fig. 1A). However, injected with IGFBP7 shRNA after CLP modeling significantly prevented CLP-induced acute lung-tissue damage.

The effect of CLP on IGFBP7 expression was analyzed after CLP modeling by western blot. Compared with the sham group, the expression levels of IGF-BP7 in lung tissue were significantly increased after CLP modeling. IGFBP7 shRNA treatment reduced IGFBP7 expression compared with that of the CLP group (Fig. 1B). The lung wet-to-dry (W/D) weight ratio also significantly increased after CLP-induced sepsis while significantly decreased in the CLP +
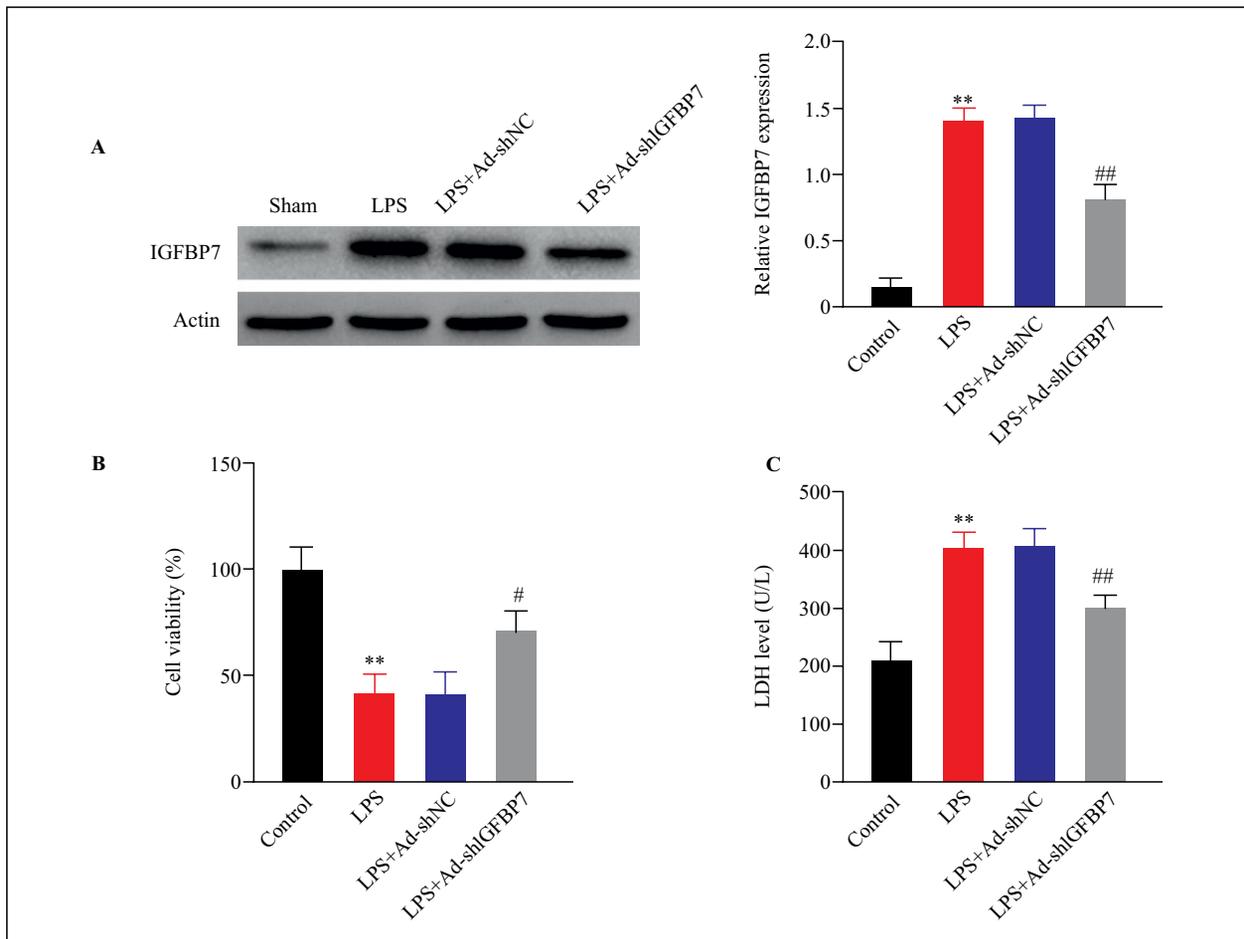
Figure 2. IGFBP7 knockdown attenuates LPS-induced acute injury in MPVECs. MPVECs were transfected with shIGF-
-BP7 or shNC and treated with or without LPS (2 μg/mL) for 48 h. **A.** IGFBP7 expression was measured by western blot.
B. MPVECs were treated with LPS and cell viability was measured by MTT assay. **C.** The cytotoxicity level in different groups
was measured by the LDH assay. **p < 0.001 compared with the control group; ##p < 0.001 compared with LPS group.

shIGFBP7 group (Fig. 1C). These results suggested
that inhibition of IGFBP7 attenuated CLP-induced
lung changes and thus may be critical in regulating
lung function during sepsis.

Knockdown of IGFBP7 attenuated LPS-induced acute damage of MPVECs

To investigate the role of IGFBP7 in sepsis-induced
acute lung injury, LPS induction of MPVECs was
used to mimic the sepsis-induced lung dysfunction.
MPVECs were transfected with shIGFBP7 or shNC
before treatment with LPS (5 μg/mL) for 48 h (the
dose of LPS and duration of treatment were based
on previous study [26]). LPS treatment was found to
significantly up-regulate the levels of IGFBP7 com-
pared with the control group (Fig. 2A). Conversely,
shIGFBP7 transfection significantly decreased the
LPS-induced IGFBP7 expression compared with
MPVECs without transfection.

MTT assay showed that the viability of MPVECs in
the LPS treatment group was significantly lower than
in the control group, but not in the group transfected
with shIGFBP7 (Fig. 2B). To explore the protective
mechanism of IGFBP7 inhibition on LPS-induced
cellular injury, cytotoxicity was measured by LDH
assay. As expected, LPS treatment significantly in-
duced MPVECs' damage by increasing LDH release,
whereas the shGFBP7 transfection significantly atten-
uated the LPS-induced LDH release from MPVECs
(Fig. 2C). These results indicated that knockdown of
IGFBP7 attenuated LPS-induced negative changes
in MPVECs.

Knockdown of IGFBP7 suppressed LPS-induced apoptosis of MPVECs

To investigate the involvement of IGFBP7 in the reg-
ulation of LPS-induced apoptosis, the apoptosis rate
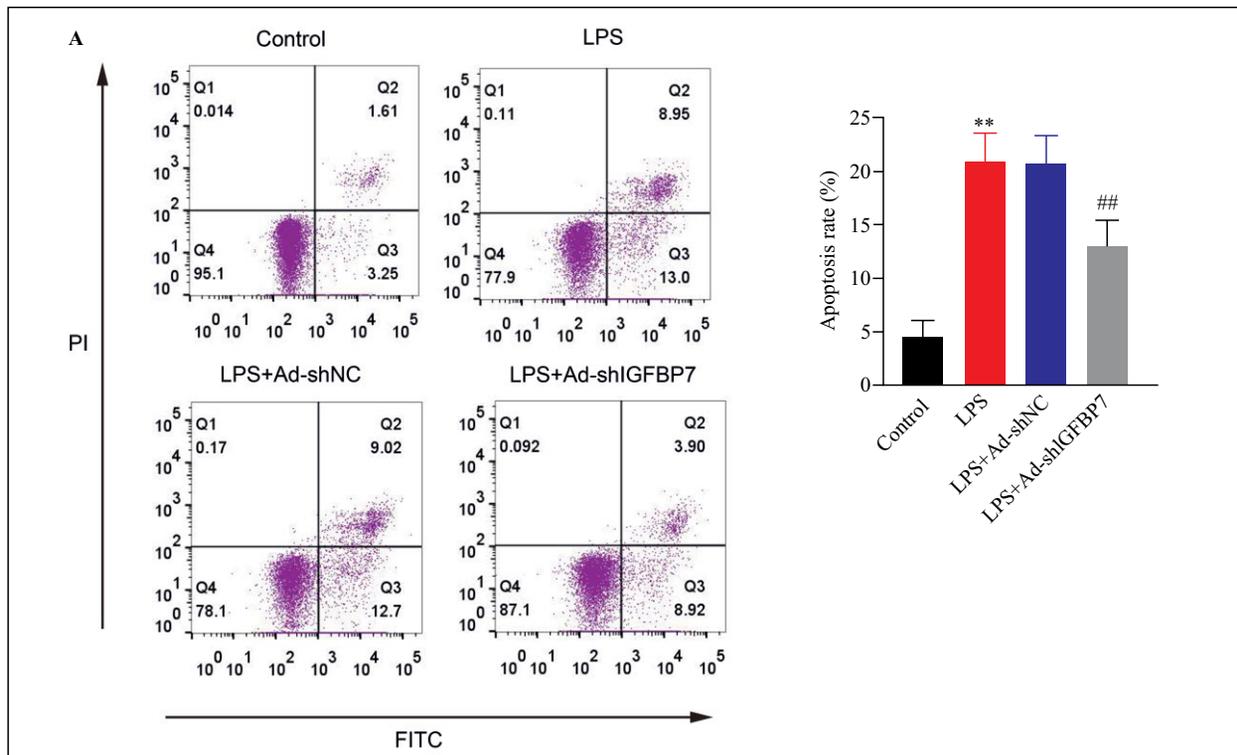in different groups was determined by flow-cytometry

Figure 3. IGFBP7 knockdown suppresses LPS-induced cellular apoptosis in MPVECs. MPVECs were transfected with shIGFBP7 or shNC and treated with or without LPS ($2\,\mu g/mL$) for 48 h. The apoptosis rate in different groups was measured by flow cytometry. **p < 0.001 compared with the control group; ##p < 0.001 compared with LPS group.

analysis. As shown in Figure 3, the apoptosis rate of MPVECs was significantly higher in the LPS-treated cells than in the control group. Furthermore, when shIGFBP7 treatment was conducted in the LPS-induced MPVECs the cellular apoptosis rate was significantly suppressed. These results indicated that the knockdown of IGFBP7, contributed to the reduction of LPS-induced apoptosis of MPVECs.

Knockdown of IGFBP7 inhibited the LPS-induced activation of the ERK1/2 pathway in MPVECs

To clarify the potential mechanism by which IGFBP7 regulated LPS-induced MPVECs' damage, activation of ERK1/2 pathway was measured by western blot. LPS induction significantly enhanced the expression of p-ERK1/2 but not that of total ERK1/2. By contrast, treatment of LPS-treated cells with shIGFBP7 significantly decreased the expression levels of p-ERK1/2 in MPVECs (Fig. 4). Therefore, knockdown of IGF-BP7 may alleviate the LPS-induced activation of the ERK1/2 pathway in MPVECs.

Discussion

Sepsis is a common and serious condition in surgical patients. It can be caused by a serious bacterial infection in the abdominal cavity and is accompanied by fever with or without hypotension [27]. A critical characteristics of abdominal sepsis is the release of endotoxins inducing the overexpression of the innate immune system and inflammatory responses that lead to tissue injury in the lungs and other organs [28, 29]. Acute lung injury is the primary complication in sepsis during the continuous development of multiple organ dysfunction [30, 31]. Lung edema, inflammatory cell infiltration, and intrapul-monary hemorrhage have been identified as the typical pathological manifestations of sepsis-induced acute lung injury [32]. The change of the alveolar capillary membrane permeability, inflammatory cell aggregation by production of inflammatory chemokines and release of other inflammatory mediators have been identified as characteristics of acute lung injury.

In the present study, we examined the essential mechanisms of IGFBP7 influence on CLP-induced acute lung injury in mice. Our findings showed that the expression level of IGFBP7 in lung tissue was significantly increased after CLP modeling, and knockdown of IGFBP7 suppressed pro-inflammatory response in lung tissue, such as alveolar-wall thickening, and acute alveolar damage. In addition, IGFBP7 silencing in MPVECs markedly suppressed cell apoptosis and ERK1/2 activation in LPS-stimulated cells. These
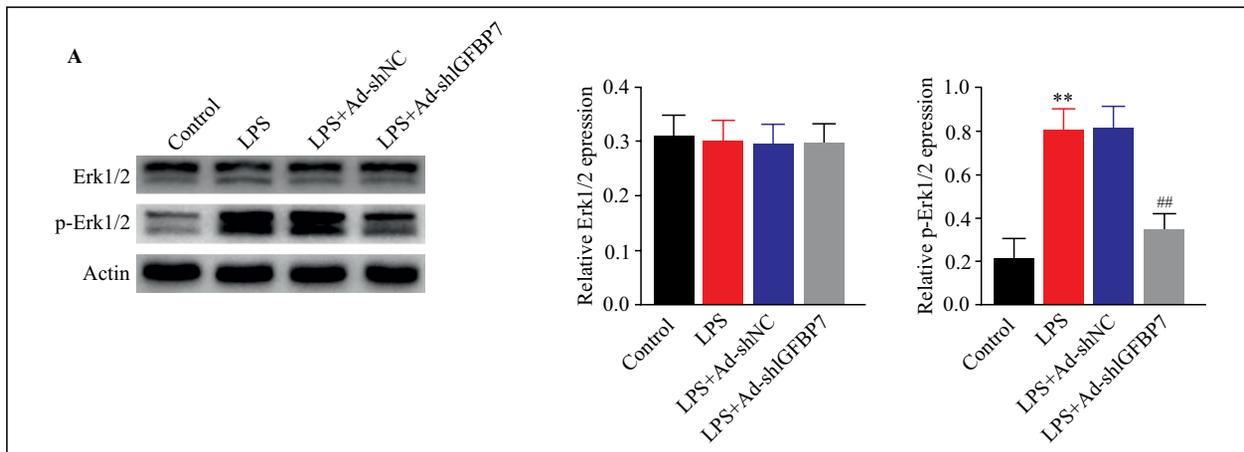
Figure 4. Effects of IGFBP7 on LPS-induced ERK1/2 activation in MPVECs. MPVECs were transfected with shIGFBP7 or shNC and treated with or without LPS (2 μg/mL) for 48 h. The expression of ERK1/2 and p-ERK1/2 was measured by western blot. **p < 0.001 compared with the control group; ##p < 0.001 compared with LPS group.

changes in lung-tissue pathophysiology were related to the altered viability of MPVECs.

Several molecules are involved in the pathogenesis of sepsis-induced injury, which is usually accompanied by the robust activation of complement system. The pathogen is removed; however, inflammation and organ damage still ensue. Extensive evidence suggests that IGFBP7 could regulate cell proliferation, adhesion, senescence, migration, apoptosis, and angiogenesis in breast, lung, and colorectal cancers [16]. Previous studies have reported that IGFBP7 induces cell-cycle arrest at G1 phase by directly enhancing the expression of p21 and p53, which could function as a biomarker of sepsis-induced acute kidney injury [26, 33]. In the present study, the expression levels of IGFBP7 in lung tissue were significantly increased after sepsis-induced acute lung injury, and the inhibition of IGFBP7 attenuated sepsis-induced lung dysfunction. These results indicated that IGFBP7 was involved in sepsis-induced lung tissue dysfunction. Vizioli *et al.* reported that IGFBP7 expression markedly enhances the activation of the mitogen-activated protein kinase ERK1/2, and ultimately results in the secretion of pro-inflammatory cytokines [34]. The ERK1/2 pathway also plays a critical role in IL-13-induced lung inflammatory response and LPS-induced acute lung injury [35]. Similar results were obtained in the present study, *i.e.*, knockdown of IGFBP7 alleviated the LPS-induced activation of the ERK1/2 pathway in MPVECs. Other investigators received similar results regarding IGFBP7-dependent ERK1/2 activation in sepsis-induced acute kidney injury [26]. This finding corroborated our results regarding sepsis-induced acute lung injury and indicated that the IGFBP7 regulation of lung-tissue damage was ERK dependent.

In conclusion, this research demonstrated for the first time that IGFBP7 was strongly up-regulated in sepsis-induced acute lung injury, thereby aggravating the lung inflammatory response and organ dysfunction. IGFBP7 silencing attenuated sepsis-induced apoptosis and cytotoxicity in MPVECs. Furthermore, the activation of ERK1/2 was regulated by IGFBP7 through sepsis-induced inflammation. Overall, IGFBP7 may serve as a diagnostic marker and therapeutic target in sepsis-induced acute lung injury; however, further molecular and clinical studies are still needed.

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Competing interests

The authors state that there are no conflicts of interest to disclose.

Ethics approval

Ethical approval was obtained from the Experimental Animal Welfare Ethics Committee of Zhejiang Academy of Traditional Chinese Medicine.

Statement of Informed Consent

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

Qiaolian Xu and Jun Wang designed the study, supervised the data collection, analyzed the data, interpreted the data, prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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