International Immunopharmacology 29 (2015) 560–567

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

# Ulinastatin inhibits the inflammation of LPS-induced acute lung injury in mice via regulation of AMPK/NF-κB pathway

Wuquan Li [a,1], Xiaochen Qiu [b,1], He Jiang [a,1], Yan Zhi [a], Jinfeng Fu [a], Jun Liu [a,*]

[a] Burn Center of Yunnan Province, Second Affiliated Hospital of Kunming Medical University, Kunming, China
[b] Department of General Surgery, The 309th Hospital of PLA, Beijing, China

ARTICLE INFO

*Article history:*
Received 1 September 2015
Received in revised form 21 September 2015
Accepted 29 September 2015
Available online 21 October 2015

*Keywords:*
Ulinastatin
Acute lung injury (ALI)
Mice
AMPK/NF-κB pathway

ABSTRACT

*Background:* Ulinastatin (ULI), a serine protease inhibitor, had been widely used as a drug for patients with acute inflammatory disorders. However, evidence regarding the anti-inflammatory effect of ulinastatin was still lacking. In this study, we investigated the protective mechanisms of ULI in LPS-induced acute lung injury (ALI).
*Methods:* ALI was induced in mice by intratracheal instillation of LPS. The cells in the bronchoalveolar lavage fluid (BALF) were counted. The degree of animal lung edema was evaluated by measuring the wet/dry weight ratio and oxygenation index. The levels of inflammatory mediators, tumor necrosis factor-α, interleukin-1β, and interleukin-6, were assayed by enzyme-linked immunosorbent assay. Pathological changes of lung tissues were observed by HE staining. The levels of NF-κB p65, AMPK, p-AMPK and IκBα expression were detected by Western blotting. Then, selective AMPK inhibitor Compound C was used to test whether AMPK activation was critical in protection process of ULI against LPS-induced ALI.
*Results:* Ulinastatin pretreatment at doses of 15, 30 and 45 mg/kg decreased LPS-induced evident lung histopathological changes, lung wet-to-dry weight ratio, and oxygenation index. Expression of IL-6, IL-1β, and TNF-α was suppressed by ULI at protein level in BALF. Additionally, the attenuation of inflammatory responses by ULI was closely associated with AMPK/NF-κB pathway and this effect was significantly inhibited by treatment with the AMPK inhibitor, Compound C.
*Conclusions:* The results presented here indicated that ULI has a protective effect against LPS-induced ALI and this effect may be attributed partly to decreased production of proinflammatory cytokines through the regulation of AMPK/NF-κB signaling pathway.



## 1. Introduction

Acute lung injury (ALI), was a frequent complication following sepsis in critically ill patients, and was a major clinical problem that has a high mortality rate of 30 to 40% despite significant advances in antimicrobial therapy and supportive care made in the past few decades [1–3]. There were still few effective measures or specific medicines to treat it [4]. Therefore, it was significant that anti-inflammatory drugs were used to reduce lung injury at early stage of ALI.

Increasing evidence suggested that nuclear factor-κB (NF-κB) was a central transcriptional factor in the regulation of inflammatory factors [5–8]. Chen et al. had reported that NF-κB plays an important role in the pathogenesis of lung diseases [9] NF-κB was known to the one of the factors required for maximal transcription of a wide array crucial transcription of pro-inflammatory molecules, including TNF-α, IL-1β and other mediators. Recent studies showed that blocking NF-κB activity could provide protection against LPS-induced acute lung injury in mice [10]. AMP-activated protein kinase (AMPK) was also a key regulator in inflammatory processes. Recent evidences showed that activation of AMPK was associated with diminished nuclear translocation of NF-κB in TNF-α-stimulated endothelial cells [11]. Many in vitro experiments also had demonstrated that the lipopolysaccharide (LPS)-induced inflammatory response could be inhibited by activating AMPK with AICAR [12–14]. Although activation of AMPK had been shown to have anti-inflammatory effects, there was little information concerning the role that AMPK may play in modulating neutrophil function and neutrophil-dependent inflammatory events, such as acute lung injury.

Ulinastatin, known as human urinary trypsin inhibitor, can be found in urine, plasma and all organs [15]. Apart from blocking the protease pathway, ULI had anti-inflammatory properties in vitro [16]. In recent years, ulinastatin was widely used in the treatment of a variety of severe diseases [17]. It has been reported that early application of ulinastatin can reduce the symptoms of ALI [18], but the mechanism was not fully elucidated. Whether ulinastatin exerted its effects on LPS-induced acute lung injury by suppressing NF-κB and activating AMPK required further elucidation. Therefore, we aimed to investigate whether ulinastatin can assist the recovery of lipopolysaccharide (LPS)-induced

* Corresponding author at: Burn Center of Yunnan Province, Second Affiliated Hospital of Kunming Medical University, Mayuan No. 1, Xishan District, Kunming 650101, China.
*E-mail address:* junliu2015@163.com (J. Liu).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.intimp.2015.09.028
1567-5769/

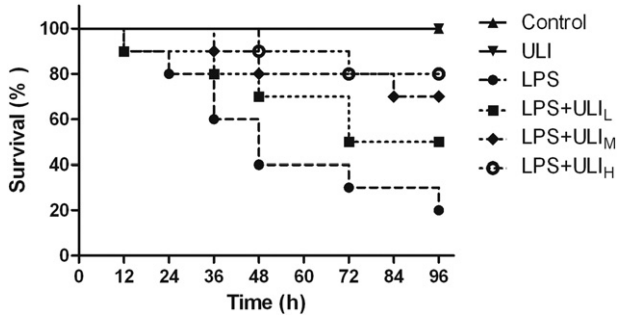


**Fig. 1.** The protective effect of Ulinastatin (ULI) on survival of LPS-treated mice. The mice were challenged by LPS (20 mg/kg) with or without ULI pretreatment. The survival rates were observed during 96 h after exposure to LPS. Survival analysis was by Log-rank (Mantel–Cox) test, $\chi^2 = 26.12$, $P < 0.01$.

ALI in a mouse model. In addition, the possible mechanisms of protection were also explored in the present study.

## 2. Materials and methods

### 2.1. Antibodies and reagents

Monoclonal antibodies against NF-κB p65, IκBα, AMPK, p-AMPK, and β-actin were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Ulinastatin, Compound C (Com C), and LPS (Escherichia coli055:B5) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The ELISA kits for TNF-α, IL-1β, and IL-6 were purchased from Nanjing KeyGen Biotech. Co., Ltd. (Nanjing, China).

### 2.2. Animals

BALB/c mice (male, 8–10 weeks old, 18–20 g each) were purchased from the Center of Experimental Animals of China Medical University. They were kept in plastic cages at 22 ± 2 °C with free access to pellet food and water on a 12 h light/dark cycle. Mice were allowed to acclimatize to the laboratory for at least 7 days under-climate controlled conditions. Animal welfare and experimental procedures were strictly in accordance with the Guide for the Care and Use of Laboratory Animals (US National Research Council, 1996, Jan. 12).

### 2.3. Experimental protocol for acute lung injury model

The intratracheal instillation was done as previously described with slight modifications [19]. Male BALB/c mice were randomly divided into six groups with 10 mice in each group: (1) control group (saline), (2) ULI (ULI, 45 mg/kg) group, (3) LPS group (50 mg/kg), (4) LPS + Ulinastatin (LPS + ULI$_L$, 15 mg/kg), (5) LPS + ULI$_M$ (ULI, 30 mg/kg), and (6) LPS + ULI$_H$ (ULI, 45 mg/kg). The animals were administered with saline or drugs intragastrically. After 30 min of treatment, mice were anesthetized with chloraldurate (3%), and 20 mg/kg of LPS in 50 μL phosphate buffered saline (PBS) was administered intratracheally to induce acute lung injury. Control group mice were given 50 μL sterile saline instead.

### 2.4. Bronchoalveolar lavage fluid (BALF) collection

BALF collection was performed using the method of Shin et al. [11]. To obtain the BALF, ice-cold PBS (0.7 mL) was infused into the lungs two times and withdrawn each time using a tracheal cannula (a total volume of 1.4 mL). The total leukocyte count was determined using a hemocytometer. BAL fluid (BALF) samples were centrifuged at 1500 rpm for 10 min at 4 °C, the supernatants were stored in −80 °C for analysis of cytokine concentrations.

### 2.5. Cytokine assay

Levels of TNF-α, IL-1β, and IL-6 in BALF were determined by ELISA kits according to the instructions recommended by the manufacturers. The optical density of each well was read at 450 nm.

### 2.6. Measurement of wet-to-dry ratio of the lungs

At 6 h after treatment with LPS, mice were euthanized, the right lung was then removed and the wet weight was determined. Subsequently,

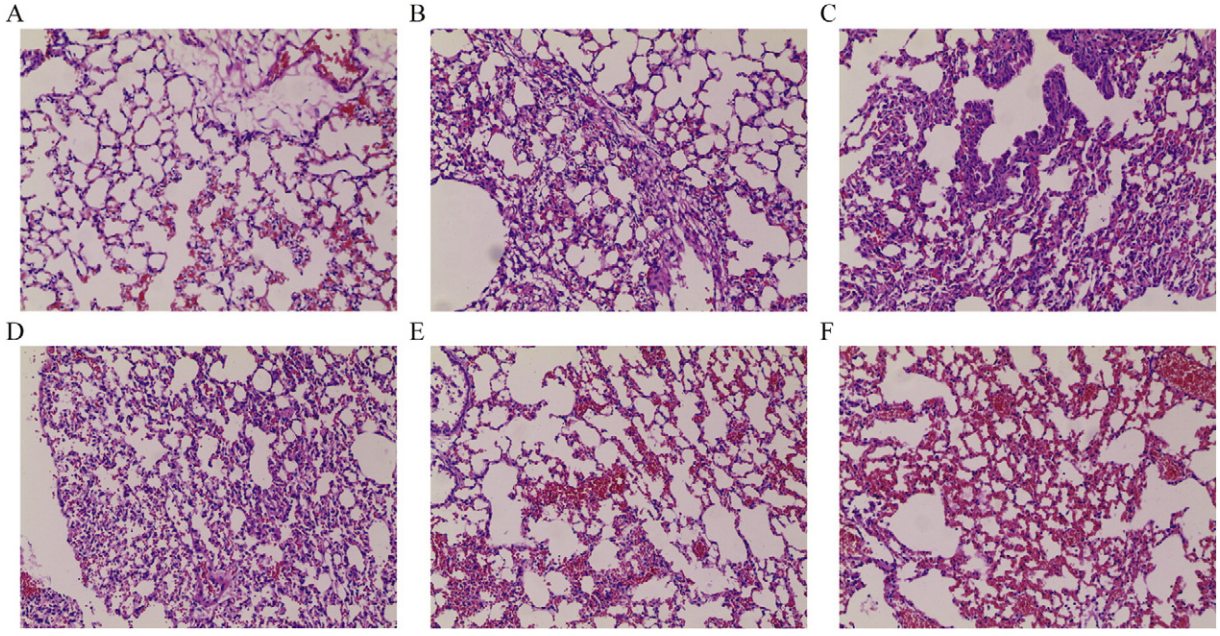


**Fig. 2.** Histologic assessment of the effect of ULI on LPS-induced ALI (200× magnification). Comparison of histopathological changes using HE staining: after LPS stimulation, lung in the LPS group showed a thickened alveolar wall, edema, less alveolar space and obvious inflammatory cell infiltration. Medium and large dose of ULI significantly prevented the histopathological changes caused by LPS. A, Control group, B, ULI (45 mg/kg) group, C, LPS group, D, LPS + ULI$_L$ (15 mg/kg) group, E, LPS + ULI$_M$ (30 mg/kg) group, F. LPS + ULI$_H$ (45 mg/kg) group.