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# Rho-kinase inhibitor treatment prevents pulmonary inflammation and coagulation in lipopolysaccharide-induced lung injury☆

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ABSTRACT

*Introduction:* In the pathogenesis of sepsis-induced acute lung injury (ALI), the crosstalk between inflammation and coagulation plays a pivotal role. The aim of this study was to investigate the role of Rho kinase (ROCK) inhibitor in alleviating pulmonary inflammation and coagulation in lipopolysaccharide (LPS)-induced acute lung injury (ALI) models.

*Methods:* In the in vivo study, mice were randomized to four different groups: Control, Y-27632 (Y), LPS, and LPS + Y-27632 (LPS + Y). ALI was induced by intranasally administering LPS (10 μg in 50 μL PBS). Y-27632 (10 mg/kg body weight,) was injected intraperitoneally at 18 h and 1 h before LPS challenge. Mice were euthanized at 3 h or 8 h post LPS challenge (N = 8 per group). In the in vitro study, human pulmonary microvascular endothelial cells (HPMECs) were incubated with LPS alone (1 μg/mL) or in combination with 10 μM Y-27632 or 50 μM BAY11-7082. Cells were pretreated with the inhibitors 30 min before exposure to LPS. Three hours later, cells were isolated for subsequent analysis.

*Results:* The myeloperoxidase (MPO) activity and fibrinogen deposits in the lung tissue significantly decreased and the lung damage in ALI mouse was attenuated. Pretreatment with Y-27632 markedly reduced the LPS-induced expression of interleukins 1β and 6, and the activation of nuclear factor (NF)-κB. Furthermore, ROCK inhibitor treatment antagonized the expression of tissue factor (TF) and plasminogen activator inhibitor (PAI)-1 in lung tissue and HPMECs.

*Conclusions:* ROCK inhibition protects against the endotoxin-induced pulmonary inflammation and coagulation via NF-kappaB pathway modulation.



## 1. Introduction

Acute lung injury (ALI) induces death and disability in critically ill patients [1]. It may be triggered by sepsis, ischemia/reperfusion, or mechanical ventilation [2]. Despite the recent advances in antibiotic therapy and intensive care, the prognosis of ALI remains poor [3,4].

Inflammatory cell infiltration is a key event in the sepsis-induced ALI [5,6]. Inflammation triggers the synthesis of interleukins 1-beta (IL-1β) and 6 (IL-6), and tumor necrosis factor (TNF)-α [6,7], which activate the coagulation pathway via expression of tissue factor (TF) and suppression of fibrinolysis following the expression of plasminogen activator inhibitors (PAIs). Consequently, fibrin accumulates in the air cavities and lung microvasculature [8–11]. TF and other coagulation factors, including fibrin, Factor Xa, and thrombin, directly and indirectly, regulate the inflammatory response in ALI via altered cytokine expression, inflammatory cell migration and activation, surfactant activity, and cellular repair [8,12]. Analysis of the interaction between inflammation and coagulation pathways would provide targets for therapeutic intervention in patients with ALI.

ROCK inhibitors attenuate systemic inflammation, leukocyte migration, endothelial injury, and vascular hyperpermeability [13–15]. We previously reported that fasudil, a ROCK inhibitor, suppressed

*Abbreviations:* Acute lung injury, (ALI); Lipopolysaccharide, (LPS); Human pulmonary microvascular endothelial cells, (HPMECs); Myeloperoxidase, (MPO); Interleukins 1-beta, (IL-1β); Interluekin-6, (IL-6); Nuclear factor, ((NF)-κB.); Tumor necrosis factor, (TNF)-α; Tissue factor, (TF); Plasminogen activator inhibitors, (PAIs); Bronchoalveolar lavage fluid, (BALF); Enzyme-linked immunosorbent assay, (ELISA); Radioimmunoprecipitation assay, (RIPA); Real-time polymerase chain reaction, (qPCR); Dimethylarginine dimethylaminohydrolase, (DDAH); Asymmetric dimethylarginine, (ADMA).

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0049-3848/

**Table 1**
Oligonucleotide primer sets for real-time PCR.

| name | Sequence(5′–3′) | Length | size |
|---|---|---|---|
| IL-6 F | TCACCTCTTCAGAACGAATTGACA | 24 | 115 |
| IL-6 R | AGTGCCTCTTTGCTGCTTTCACAC | 24 | |
| IL-1β F | CCTGGACTTTCCTGTTGTCTACACC | 25 | 178 |
| IL-1β R | TCTGTCAGGCGGGCTTTAAGTGAG | 24 | |
| PAI-1 F | GCCCTTGAGTGCTTGTTAGAG | 21 | 115 |
| PAI-1 R | GTGGCTGGACTTCCTGAGATA | 21 | |
| TF F | GACCTCACCTGGGACAATC | 19 | 246 |
| TF R | GCCTGTGTAGCCGTAGTATC | 20 | |
| β-actin F | CTTAGTTGCGTTACACCCTTTCTTG | 25 | 156 |
| β-actin R | CTGTCACCTTCACCGTTCCAGTTT | 24 | |

F: Forward; R: Reverse.

inflammation and afforded protection against death in mice exposed to toxemia [16]. In the present study, we explored the role of ROCK inhibition in pulmonary inflammation and coagulation induced by lipopolysaccharide (LPS) in vivo and in vitro, and elucidated the mechanism of nuclear factor (NF)-κB signalling involved in the process.

## 2. Materials and methods

### 2.1. Animals

Eight- to ten-week-old male C57BL/6 mice, each weighing 20 to 25 g, were acquired from the Experimental Animal Centre of China Medical University. Mice were acclimated to a temperature of 20 °C ± 2 °C under equal duration of alternating daylight and darkness (12 h light/12 h dark) for at least 1 week. The animals were provided with water and standard chow, after overnight fasting. Experiments were conducted in compliance with the regulations of the University Experimental Animal Administration Committee.

### 2.2. Endothelial cells

Human pulmonary microvascular endothelial cells (HPMECs) were cultured from endothelial cells obtained from ScienCell Research Laboratories. The growth medium contained 5% foetal bovine serum, 1% growth supplements, and 1% penicillin/streptomycin. The cells were maintained and the study was conducted in a 5% $CO_2$ humidified incubator. HPMECs were seeded at a concentration of $2 \times 10^5$ cells per well.

### 2.3. Experimental protocols

#### 2.3.1. In vivo

For the in vivo experiments, mice were divided into four groups based on the treatment: Control, Y, LPS, and LPS + Y. The mice were anesthetized using diethyl ether, followed by intranasal administration of 50 μL PBS, either alone or in combination with 10 μg of LPS (*Escherichia coli* serotype 055:B5, Sigma, St. Louis, Mo) to induce lung injury. Control mice were exposed to PBS alone. Before exposure to LPS, Y-27632 (10 mg/kg body weight, LPS + Y) in a sterile saline solution (10 μL/g body weight) was injected intraperitoneally at 18 h and 1 h [16]. The mice (N = 8) were euthanized at 3 h or 8 h after LPS treatment. Bronchoalveolar lavage fluid (BALF) samples were collected in triplicate using a tracheal cannula containing 1.5 mL of autoclaved PBS. The lung tissues and BALF samples were used for subsequent analysis.

#### 2.3.2. In vitro

HPMECs were exposed to LPS (1 μg/mL) or LPS combined with 10 μM Y-27632 or 25 μM BAY11-7082 (NF-κB inhibitor). The dosages of Y-27632 and BAY11-7082 were chosen based on previous studies [17,18]. Untreated cells served as the experimental controls. Before LPS exposure, cells were incubated with the inhibitors for 30 min. The untreated cells were mixed with PBS. After incubation for 3 h, cells were used for subsequent analysis.

#### 2.3.3. Histology

The lung tissues were subjected to histopathological analysis, which included treatment with 10% buffered formalin for 24 h, dehydration in ethanol followed by paraffin embedding, and sectioning (5-μm). The 5-μm-thick lung sections were loaded on slides and visualized under a light microscope after staining with haematoxylin and eosin (H&E). A pathologist, who was blinded to the experimental design, analysed the samples. The levels of lung injury were determined as described previously [16].

#### 2.3.4. Enzyme-linked immunosorbent assay (ELISA)

The interleukin levels in the BALF and the myeloperoxidase (MPO) activity in the lung tissues were measured using ELISA (R&D).

#### 2.3.5. Western blot

Frozen lung tissues were homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer followed by centrifugation. The supernatants were stored at −80 °C for subsequent analysis. Specific antibodies against TF, PAI-1, fibrinogen, and β-actin were used for detection [19]. The protein bands were analysed using the Gel pro 3.0.

#### 2.3.6. NF-κB P65-binding assays

Nuclear extraction kits (Active Motif, Carlsbad, CA) and TransAM NF-κB kits (Active Motif, Carlsbad, CA) were used to extract and analyse the binding of p65 to NF-κB. The sensitivity of the TransAM NF-κB kit for the

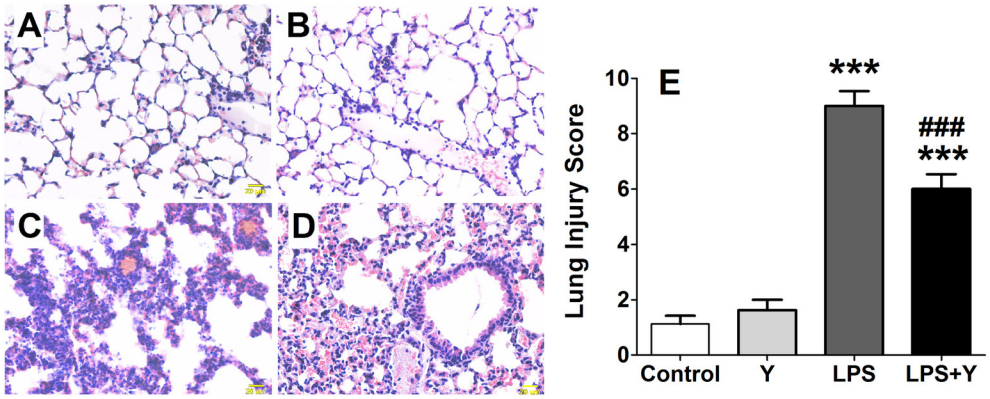


**Fig. 1.** ROCK inhibitor pretreatment attenuates the lipopolysaccharide (LPS)-induced lung injury. A–D: The lung samples from different groups at 8 h post LPS exposure were analysed histochemically. Representative pulmonary histology in the control (A), Y-27632 (B), LPS (C), and LPS + Y-27632 (D) group is shown (magnification, ×400). E: The lung injury score. *** $P < 0.001$, vs. control group; ## $P < 0.01$, vs. LPS group. Data represent the mean ± SEM (N = 8 per group).