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Original Article

The effects of BML-111 and MAPK signaling pathway on the expression of AQP1 in rat lung tissue of acute lung injury caused by endotoxic shock

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Abstract: Objective: This study aims to explore the effects of BML-111 and MAPK signaling pathway on the expression of AQP1 in rat lung tissue of acute lung injury caused by endotoxic shock. Methods: Acute lung injury caused by LPS mouse model was established. They were divided into normal group, ALI model group, BML group, SB group and SP group. Wet and dry weight of the right lung was detected, number of neutrophils and pulmonary permeability index were determined. Pathological morphology of lung tissues was determined by HE staining. The levels of TNF-α, IL-1β and IL-6 were determined by ELISA. The levels of AQP1, p38 and JNK were determined by western-blotting method. Results: There was pulmonary interstitial edema and thickening alveolar wall in ALI model group, the ration of W/D, PPI, the number of neutrophils and the levels of TNF-α, IL-1β, IL-6, p38 MAPK and JNK phosphorylation increased, while the levels of AQP1 decreased when compared with normal group (P<0.01). However, W/D, PPI, the levels of TNF-α, IL-1β, IL-6 and the phosphorylation levels of p38 MAPK and JNK in BML group, SB group and SP group were significantly lower than that of model group (P<0.01), while the levels of AQP1 increased in BML group, SB group and SP group (P<0.01). Conclusions: BML-111, P38 MAPK inhibitor and JNK inhibitor could significantly inhibit the lung injury in ALI rats induced by LPS and increased the levels of AQP1, which may be through the inactivation of MAPK p38 and JNK signaling pathway.

Keywords: BML-111, mitogen activated protein kinase (MAPK), signaling pathway, lipopolysaccharide (LPS), acute lung injury (ALI), aquaporin 1 (AQP1)

Introduction

Endotoxin shock is a common complication in clinical burn, trauma and surgery and easily leads to multiple organ dysfunction or even failure. Lung is one of the most vulnerable organ and acute lung injury (ALI) easily occurs in the process of endotoxin shock. The mechanism of ALI is complex, the imbalance between inflammatory response and compensatory anti inflammatory response make the lung environment abnormal and pulmonary edema play an important role [1, 2]. Therefore, it will have a promoting role in treating ALI if the inflammatory response of ALI can be inhibited and make the alveolar dry.

Lipoxin is a kind of endogenous anti-inflammatory and pro-remission lipid medium. BML-111 is an agonist of the A4 lipoxin receptor, which can inhibit neutrophil chemotaxis and alleviate tissue damage. It was reported that BML-111 could reduce the hemorrhagic lung injury and the lung injury caused by mechanical ventilation, and reduce the levels of IL-1β, IL-6, TNF-α and BALF [3, 4].

Many kinds of signaling pathways are involved in the process of ALI. Mitogen activated protein kinase (MAPK) signaling pathway has the function of the sensor and regulates the expression of various inflammatory factors in ALI [5]. Previous study suggested that the development of ALI was significantly limited by making the MAPK signaling pathway inactivation or treatment of BML-111 [6].

The aquaporin (AQP) is closely related to the pulmonary edema induced by ALI. AQPs are cell membrane transport proteins which are related

Table 1. The results of W/D, number of neutrophils and PPI in different groups ($\bar{x}\pm s$)

Group	W/D	number of neutrophils (*10^3/ml)	PPI (10^{-3})
Normal	4.48±0.18	0.67±0.06	4.73±0.49
Model	5.91±0.29**	6.79±0.64**	11.98±1.00**
BML	4.87±0.32##	3.65±0.35##	5.73±0.54##
SB	4.88±0.41##	3.70±0.34##	5.74±0.58##
SP	5.10±0.50##	3.94±0.36##	5.99±0.60##

Compared with normal group, **P<0.01; Compared with model group, ##P<0.01.

to water permeability, and 6 kinds of AQPs have been found in the lung. AQP1 is mainly distributed in the pulmonary microvascular endothelial cells. It was confirmed that the expression of AQP1 significantly decreased in the process of ALI [7-10]. The pulmonary edema could be improved if the expression of AQP1 increased in the process of ALI [11]. The expression of AQP1 could be increased in animal models when they were treated by p38 MAPK inhibitor SB203580 [12], which suggested that MAPK signaling pathway was closely related to AQP1. BML-111 could reduce the lung injury in hemorrhagic shock by MAPK/AP-1 signaling pathway [13]. Therefore, we explored the effects of BML-111 and MAPK signaling pathway on the expression of AQP1 in ALI rats induced by LPS.

Materials and methods

Experimental animals

A total of 60 male SD rats weight 200±20 g were obtained from Shanghai Slac Laboratory Animal Co. Ltd. These rats were pre-feeding for 7 days with free access to food and water to adapt to the environment. Feeding room ventilation is good with natural lighting. The ALI model was induced by LPS. Animals were anesthetized with 10% chloral hydrate (400 mg/kg) and fixed onto the platform in a prone position. A longitudinal incision in the neck was performed. The internal carotid artery was exposed and ligated at the distal end, detecting instrument of the artery was connected to observe the changes of blood pressure in rats. The changes of blood pressure were recorded after the treatment of LPS (10 mg/kg) for 30 min, 60 min, 120 min and 240 min. The model was believed to be successfully established if the decrease in blood pressure is greater than 25% of the basal blood pressure after the treatment of LPS for 2 h. The animals were divided into normal group, ALI model group, BML (intraperitoneal injection of 1 mg/kg BML-111) group, SB (intraperitoneal injection of 0.5 mg/kg SB-203580) group and SP (intraperitoneal injection of 15 mg/kg SP600125) group randomly.

Determination of lung wet/dry weight (W/D), number of neutrophils and pulmonary permeability index (PPI)

Rats were executed after inducing by LPS for 6 h. Aortic blood and middle lobe of right lung were taken out. The lung was washed with PBS and the surface was dried. Its wet weight was determined (W) and dry weight was determined (D) after drying at 80°C for 48 h. The ratio of W/D was calculated. The number of neutrophils was counted under microscope. PPI was calculated by Lowry method. PPI = protein content of bronchoalveolar lavage fluid (BALF)/protein content of serum.

H&E staining

The lung tissues were fixed in 10% formalin and embedded in paraffin routinely. The paraffin blocks of specimen were cut into continuous sections with 5 μm respectively. The sections were dewaxed with xylene and washed with ethanol and water. They were stained with Hematoxylin after that and then differentiated, washed and stained with eosin, then dehydrated, hyalinized and finally mounted on slides and observed under microscope, pictures were taken.

Detection of IL-1β, IL-6 and TNF-α with ELISA

20-50 mg middle lobe of right lung was taken and homogenized and the supernatant were collected. The levels of IL-1β, IL-6 and TNF-α in lung tissues were detected with ELISA kit according to the manual. OD values at 450 nm were determined by ELISA detector.

RNA extraction and real-time PCR

Total RNA was extracted from lung tissue using trizol Kit according to the manufacturer's protocol. Their concentration and purity were detected with Qubit Fluorometer. 1 μg RNA was subjected to reverse transcription using reverse

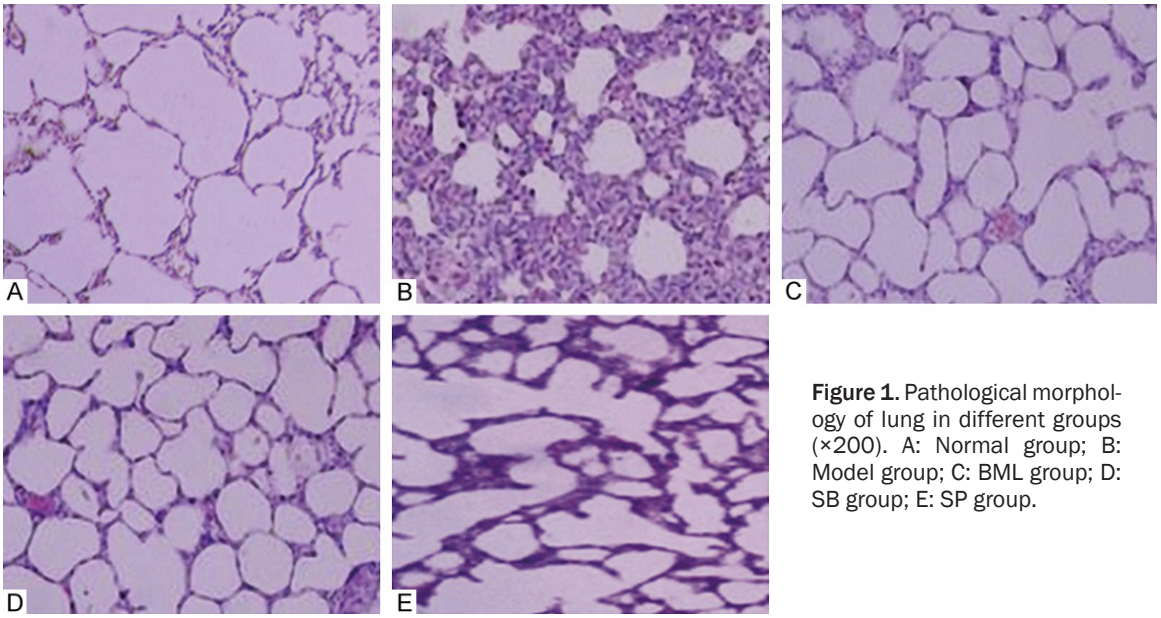

Figure 1. Pathological morphology of lung in different groups (×200). A: Normal group; B: Model group; C: BML group; D: SB group; E: SP group.

transcription kit (Promega). Real-time PCR were performed using SYNBR Green PCR Master Mix (Qigen). The primers used in this study were as follows: AQP1 forward: 5'-CTGGCCTTTGGTTTGAGCAT-3'; reverse: 5'-CCACACACTGGGCGATGAT-3'. GAPDH gene was used as an internal control for normalization of RNA quantity and quality differences in all samples. GAPDH forward: 5'-AGCCACATCGCTCAGACA-3'; reverse: 5'-TGGACTCCACGACGTACT-3'. Reaction parameters were degeneration at 95°C for 30 sec, annealing at 59°C for 45 sec and extension at 72°C for 60 sec with 40 cycles.

Western blotting detection

The lung tissues were lysed with RIPA lysis buffer and total proteins were extracted and analyzed with SDS-PAGE electrophoresis. Then it was electrotransferred to the PVDF membrane. After the transmembrane, PVDF membrane was rinsed with TBS for 10 to 15 min, placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and shook at room temperature for one hour. It was incubated at room temperature for two hours after added with appropriate dilution degree of primary antibody (diluted with TBST containing 1% (w/v) skimmed milk powder). Then the membrane was rinsed with TBST for three times (5 to 10 minutes one time). The membrane was incubated at room temperature for one hour with HRP labeled secondary antibody (1:10000) diluted with TBST containing 0.05% (w/v) skimmed milk powder and rinsed for three times with TBST (5 to 10 minutes at a time). The protein bands were scanned and gray values was determined using "Quantity One" software.

Statistical analysis

The results are expressed as mean ± SD and analyzed with SPSS 17.0 software. Student t-test were used to evaluate the differences between groups. A value of $P<0.05$ and $P<0.01$ were taken to denote statistical significance.

Results

The results of W/D, number of neutrophils and PPI in different groups

The results of W/D, number of neutrophils and PPI in different groups were shown in **Table 1**. It showed that the ratio of W/D, the number of neutrophils and PPI significantly increased in model group when compared with normal group ($P<0.01$); while they significantly decreased in BML group, SB group and SP group when compared with model group ($P<0.01$).

Pathological morphology of lung in different groups

As shown in **Figure 1**, the lung tissue in normal group was complete without infiltration of neutrophils. There was pulmonary interstitial ede-

Table 2. The levels of TNF-α, IL-1β and IL-6 in different groups ($\bar{x}\pm s$)

Group	TNF-α (ng/ml)	IL-1β (ng/ml)	IL-6 (pg/ml)
Normal	12.54±1.12	7.66±0.71	15.69±1.51
Model	40.84±4.23**	18.22±1.59**	39.31±3.64**
BML	17.72±1.29##	9.58±0.84##	19.62±2.00##
SB	17.70±1.55##	9.61±0.89##	19.60±1.93##
SP	18.14±1.26##	9.83±0.91##	19.86±1.94##

Compared with normal group, **P<0.01; Compared with model group, ##P<0.01.

ma, thickening alveolar wall and infiltration of neutrophils in model group. There was mild pulmonary edema and infiltration of neutrophils in BML, SB and SP groups.

The levels of TNF-α, IL-1β and IL-6 in different groups

The levels of TNF-α, IL-1β and IL-6 in different groups were shown in **Table 2**. It showed that the levels of TNF-α, IL-1β and IL-6 in model group were significantly higher than that of normal group (P<0.01). However, the levels of TNF-α, IL-1β and IL-6 in BML group, SB group and SP group were significantly lower than that of model group (P<0.01).

Phosphorylation levels of p38 MAPK and JNK in different groups

Western blotting results of p38 MAPK, JNK and their phosphorylation levels were shown in **Figure 2**. It showed that the phosphorylation levels of p38 MAPK and JNK in model group were significantly higher than that of normal group (P<0.01). However, the phosphorylation levels of p38 MAPK and JNK in BML group, SB group and SP group were significantly lower than that of model group (P<0.01).

The level of AQP1 in different groups

Western blotting results of AQP1 levels were shown in **Figure 3**. It showed that the level of AQP1 in model group was significantly lower than that of normal group (P<0.01). However, it was significantly higher in BML group, SB group and SP group than that of model group (P<0.01).

Discussion

ALI is a clinical syndrome with characteristic pathological changes in the lung, it could cause acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS) with its exacerbations. Lipoxin was thought to be a brake signal for inflammatory response. BML-111 is a newly synthesized agonist of lipoxin receptor. SB203580 (p38 MAPK inhibitor) could reduce the levels of TNF-α, IL-1β, IL-6 and IL-10a and was used to treat ALI. SP600125 (JNK inhibitor) could inhibit the expression of IL-2, IFN-γ and TNF-α [13]. In this study we found that compared with model group, in BML-111 group, SB203580 group and SP600125 group, the ratio of W/D, the number of neutrophils and the levels of TNF-α, IL-1β and IL-6 decreased, degree of infiltration was reduced. These results suggested that BML-111, SB203580 and SP600125 could attenuate the ALI of rat by inhibiting the inflammatory response. We also found that in BML-111 group, SB203580 group and SP600125 group, the phosphorylation levels of p38 MAPK and JNK decreased, which suggested that BML-111 could reduce the expression of inflammatory factors through inactivating the MAPK p38 and JNK signaling pathway, and then to resist inflammation of ALI rats.

ALI could be inhibited by anti-inflammatory reaction through p38 MAPK signaling pathway, pulmonary edema induced by ALI also could be alleviated by regulation of AQP1 through p38 MAPK signaling pathway [13]. AQP1 could promote the migration of non arterial smooth muscle cells through p38-MAPK signaling pathway [14]. AQP1 could be down-regulated by peptidoglycan via p38 MAPK pathways and could be down-regulated by LPS via p38 MAPK, JNK and ERK pathways [15, 16]. These results suggested that p38 MAPK and JNK pathways could also play an important role in pulmonary edema induced by ALI and were closely related to the expression of AQP1.

AQP1 and AQP5 participated in the abnormal transport of liquid in the pulmonary edema. Previous study found that water permeability decreased 10 times in AQP-5 knockout mice, while it decreased 25-30 times in AQP-1 and AQP-5 knockout mice [17]. ALI induced by LPS in rats could get the best treatment effect with up regulation of AQP1 and AQP5 [18]. In this study, we found that the AQP1 levels significantly decreased in ALI model group, while it increased after the treatment of BML-111, SB203580 and SP600125. These results sug-

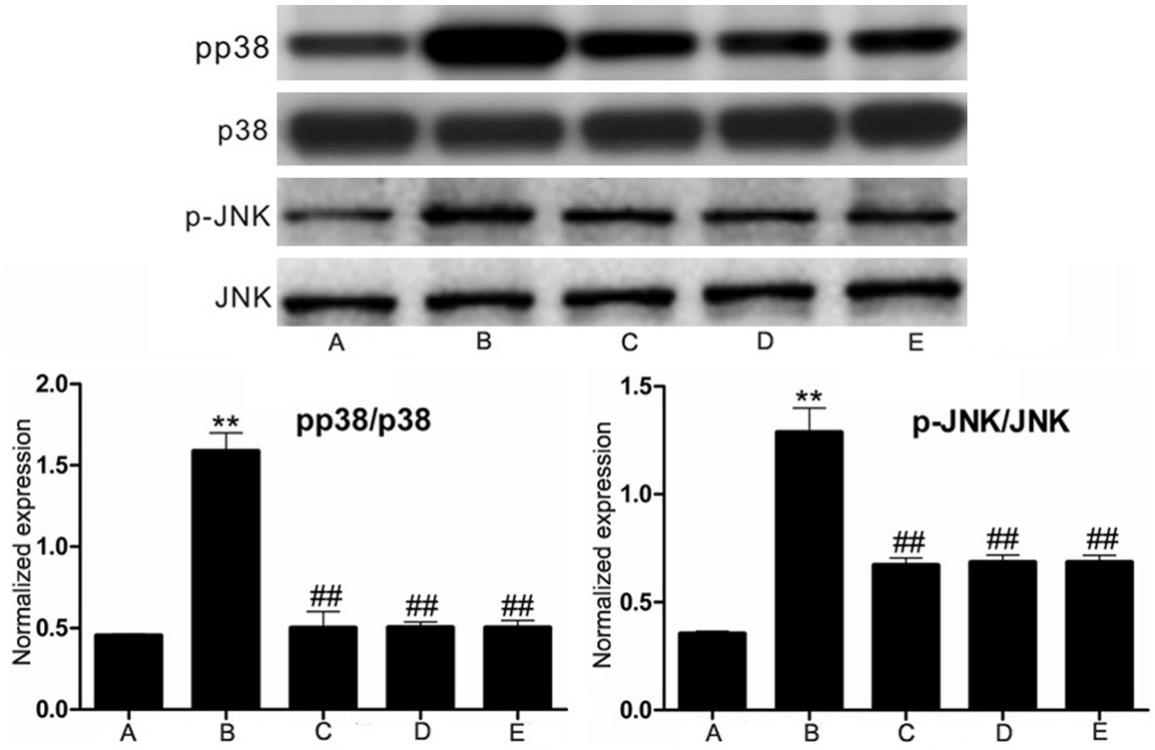

Figure 2. Western blotting results of Phosphorylation levels of p38 MAPK and JNK in different groups. A: Normal group; B: Model group; C: BML group; D: SB group; E: SP group. Compared with normal group, $^{**}P<0.01$; Compared with model group, $^{\#\#}P<0.01$.

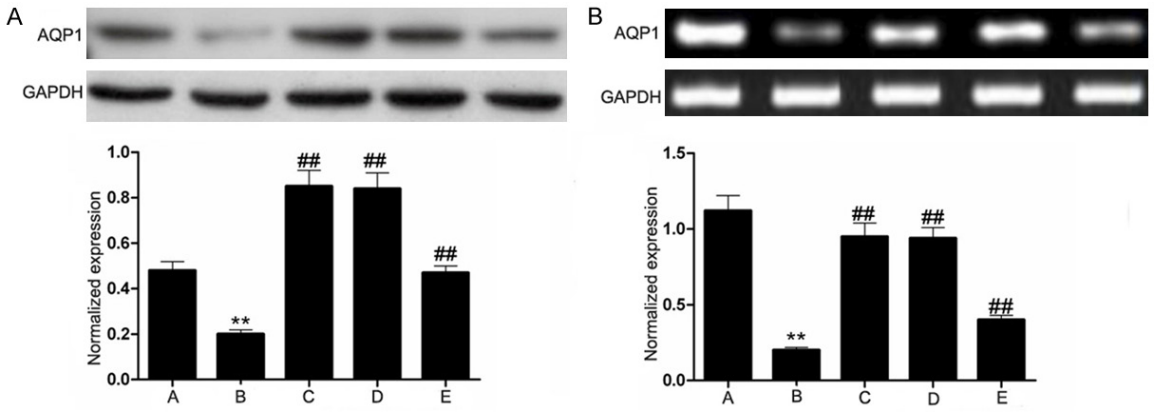

Figure 3. The levels of AQP1 in different groups. A: Normal group; B: Model group; C: BML group; D: SB group; E: SP group; A: Western-blotting results; B: RT-PCR results. Compared with normal group, $^{**}P<0.01$; Compared with model group, $^{\#\#}P<0.01$.

gested that BML-111, SB203580 and SP-600125 could alleviate pulmonary edema by up-regulation of AQP1.

In a word, BML-111 could significantly inhibit the lung injury in ALI rats induced by LPS and increased the levels of AQP1, which may be through the inactivation of MAPK p38 and JNK signaling pathway. It has important theoretical significance for the clinical application of BML-111.

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Disclosure of conflict of interest

None.

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