Saudi Pharmaceutical Journal 25 (2017) 666–670

Contents lists available at ScienceDirect

Saudi Pharmaceutical Journal

journal homepage: www.sciencedirect.com

The alleviative effects of metformin for lipopolysaccharide-induced acute lung injury rat model and its underlying mechanism

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ARTICLE INFO

Article history:
Available online 10 May 2017

Keywords:
Metformin
Sepsis
Acute respiratory distress syndrome

ABSTRACT

For patients who have sepsis, acute lung injury (ALI) causes most of death. Metformin (Met) is an anti-hyperglycemic agent and it has extensive pharmacological properties. This study aimed to analyze the influence of Met on lipopolysaccharide (LPS) -induced ALI. Met (1, 2, and 4 mg/kg) were injected and LPS was injected 30 min later. The data suggested Met can reduce release of inflammatory cytokines and bronchoalveolar lavage fluid (BALF) protein expression, reduce lung wet/dry ratio, and significantly improve LPS-induced lung destruction during ALI. In addition, Met inhibits LPS-induced neutrophil and macrophage infiltration, reduces MPO activity, and promotes AMPK-α1 expression in lung tissues. Our data suggested that metformin alleviates capillary injury during ALI via AMPK-α1.

1. Introduction

For the acute respiratory distress syndrome (ARDS) involving ALI (Ranieri et al., 2012), the lung insult is one of the major causes. Changes in vascular functions participate in ALI occurrence and progression. The previous studies have demonstrated that the vascular bed of distal vessels was damaged during ALI (Matute-Bello et al., 2011). In addition, alveolar capillaries were injured, resulting in increased endothelial permeability (Matthay et al., 2003; Vadasz and Sznajder, 2011). Currently, therapeutic regimens have no effect for reversing endothelial cell dysfunction (Levitt and Matthay, 2012).

Met is an anti-hyperglycemic agent and it shows good oral bioavailability (50 ± 60%) and a favorable safety profile (Wilcock and Bailey, 1994; Rizos and Elisaf, 2013). Notably, this drug also has anti-proliferative properties on cancer cells, in both non-diabetic and diabetic patients (Hosono et al., 2010). Met effects include inhibition of ATP production, activation of AMPK, and consequent inhibition of TORC1 (Pernicova and Korbonits, 2014; Shaw, 2009). Recent researches showed that AMPK activation reduces the inflammatory response of various cells, regulates cardiovascular complications related to ischemia or diabetes, and reduces the proinflammatory effects of neutrophils and macrophages activation (Steinberg and Kemp, 2009).

2. Experimental animals and methodology

2.1. Experimental animals

Adult male SD rats weighting average 275–300 g were obtained from Sichuan University, which were stored in a room controlled by humidity and were free to eat granules.

2.2. Study design

Rats were classified into different groups randomly, such as control group, Met (4 mg/kg) group, LPS group (LPS group 5 mg/kg, iv), LPS + Met group 1, LPS + Met group 2, and LPS + Met group 3. Met was in intravenously injected at the dosage of 1, 2, and 4 mg/kg for LPS + Met group 1, LPS + Met group 2, and LPS + Met group 3, respectively. In order to induce ALI, 5 mg/kg LPS was intravenously injected (Shen et al., 2009). Before injection of LPS, Met was intravenously injected 30 min in advance (Ragelle et al., 2012; Goh et al., 2012).

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.jsps.2017.05.001

2.3. BALF protein measurement and cell counts

For the tracheal cannula, there was an injection with a total of 5 mL ice-cold phosphate-buffered saline (PBS). Then aspiration was performed to conduct bronchoalveolar lavage (BAL). BAL was centrifuged at 4 °C at 1200g for 10 min. Supernatants were collected to measure the total protein level and count the cytokines.

2.4. Lung wet/dry weight ratio

The lung wet/dry weight ratio was assessed to measure pulmonary edema. There were three steps to obtain the "wet" weight of lung: resection, rinse with PBS and weigh. Then, the lung was stored at 60 °C for 72 h, the "dry" weight was measured to calculate the wet/dry weight ratio.

2.5. TNF-α and IL-6 ELISA assay

Based on the protocol of manufacturer, ELISA was used to examined BALF TNF-α and IL-6.

2.6. MPO activity assay

The activity of MPO was used to measure the accumulation of neutrophil. Based on the protocol of manufacturer, the test kits were used to measure the MPO activity.

2.7. Histological evaluation

LPS was injected and the collection of lung tissues was carried 6 h later. Then it fixed at 4 °C for 48 h using 10% neutral phosphate buffered formalin. H&E was used to stain the lung tissue. The pathological changes of lung tissue were observed with light microscopy.

2.8. Western blot analysis

The final supernatant was obtained by centrifugation at 12,000g for 20 min. For the concentration of protein, bovine serum albumin (BSA) determined it and Thermo Fisher Scientific Protein Kit (Thermo Fisher Scientific, Inc.) was used to measure it. The same amount of total protein was subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. The membranes were then stored in 5% BSA and then immunoblotted using the following antibodies diluted 1: 1000: rabbit anti-AMPK antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.9. Analysis

IBM SPSS 17.0 (SPSS Inc, Chicago, IL, USA) was applied in this analysis. Data of this research was represented as mean ± standard deviation. Six groups are compared using unpaired student *t* test. The value of $P < 0.05$ was critical in statistic.

3. Results

3.1. Effects of met on the lung wet/dry weight ratio and the concentration of total protein in BALF

According to Figs. 1 and 2, the lung wet/dry ratio and total protein level of BALF in the LPS group was obviously higher than those in the control group.

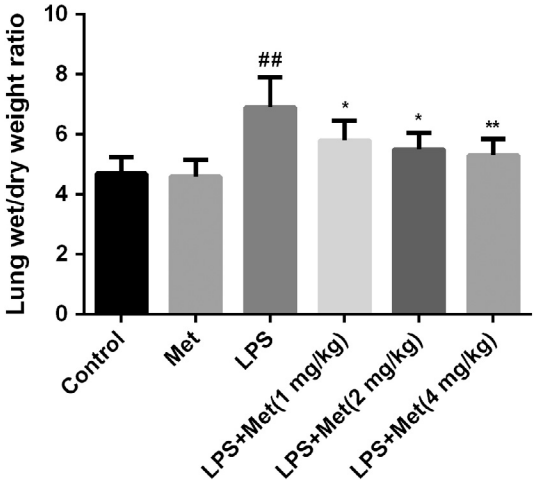

Fig. 1. The influence of Met on lung wet/dry ratio. Met (1, 2 and 4 mg/kg) were intravenously injected into rats and LPS. $^{\#\#}P < 0.01$ was injected 30 min later, compared with the control group; $^{*}P < 0.05$, compared with the LPS group; $^{**}P < 0.01$, compared with the LPS group.

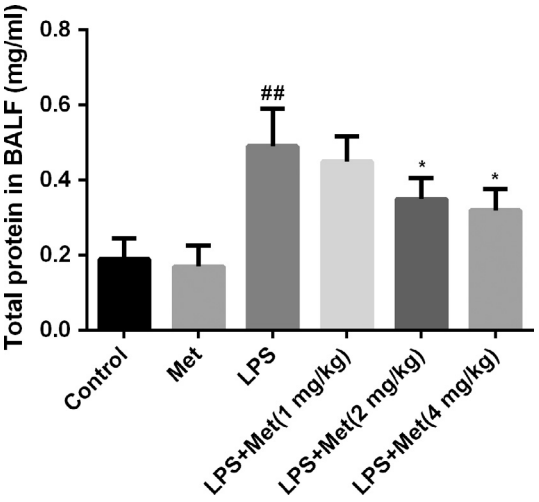

Fig. 2. Effects of Met on total protein concentration of BALF. Met were administered to rats at 30 min prior to administration of LPS. BALF was collected 6 h after LPS administration, and total protein concentration was analyzed. $^{\#\#}P < 0.01$, compared with the control group; $^{*}P < 0.05$, compared with the LPS group.

3.2. Influence of met on lps-mediated lung histopathologic changes

According to Fig. 3, the control group represented integral alveoli structure without edema (Fig. 3a). LPS was injected and lung destruction was significant 6 h later (Fig. 3c). Administration with Met alleviated lung destruction (Fig. 3d–f).

3.3. Influence of met on the inflammatory cell counts in BALF

According to Fig. 4, comparing with those of control group, the numbers of neutrophils, macrophages and total cells in BALF increased sharply in the LPS group. Met administration significantly reduced the numbers of neutrophils, macrophages and total cells.

3.4. Effects of met on MPO activity in lung tissues

MPO is produced by neutrophils and results in lung tissue damages. Therefore, MPO participates in ALI occurrence and

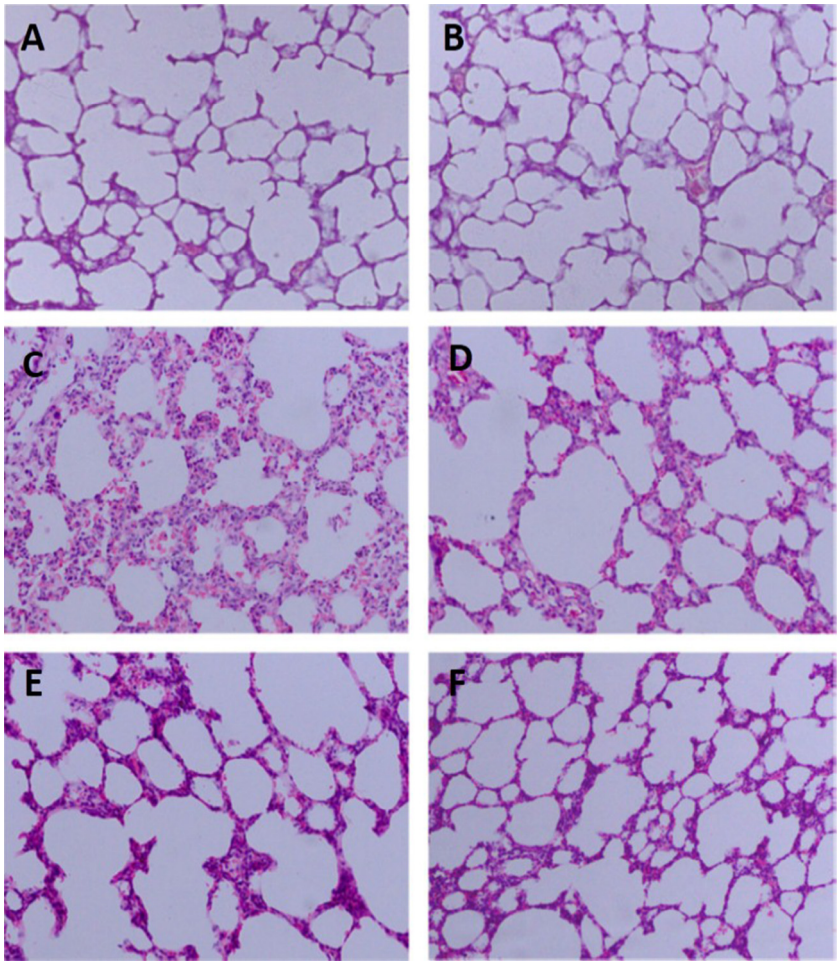

Fig. 3. Met were administered to rats at 30 min prior to administration of LPS. Lung histological evaluation (magnification 200×) from each experimental group was performed. Group A was the control group. Group B was Met (4 mg/kg) group. Group C was LPS group, Group D was LPS + Met group 1. Group E was LPS + Met group 2. Group F was LPS + Met group 3.

progression. In this research, the activity of MPO was detected six hours after LPS administration. According to Fig. 5, after injection of LPS, compared with the control group, MPO activity was significantly increased. Met administration significantly decreased MPO activity which was increased by LPS.

3.5. Effects of met on the concentrations of TNF-α and IL-6

ELISA was performed to measure TNF-α and IL-6 levels in BALF. After LPS injection, TNF-α and IL-6 levels in BALF were significantly increased. Met reduced the increase of TNF-α and IL-6 levels induced by LPS (see Fig. 6).

3.6. Influence of met on AMPK expression in lung tissues

According to Fig. 7, compared with the control group, the LPS group experienced a sharp decrease of AMPK expression in lung tissues. Met administration significantly increased the expression AMPK. These results indicated that Met may promote AMPK.

4. Discussion

In this research, we studied the anti-inflammatory effects of Met for ALI in rats and its possible underlying mechanisms. Our data showed that Met had alleviative effects on LPS-induced ALI. In addition, Met increased the AMPK expression. These results showed alleviative influence of Met may be via AMPK signaling pathway. Met could be a candidate of ALI treatment.

There were significant capillary congestion and interstitial edema in LPS control rats. After administration of Met, the LPS-caused damages to alveoli and respiratory bronchioles were repaired to some extent. Furthermore, because of the characteristics of ALI, pulmonary edema is a prognostic indicator of ALI/ARDS (Wilkins and Seahorn 2004). In this research, compared with the LPS group, the Met groups demonstrated a significantly alleviated interstitial edema.

One of the typical characteristic of LPS-induced ALI is infiltration of inflammatory cells into lung tissues (Matthay and Zemans 2011; Lucas et al., 2009). As an enzyme stayed in the primary granules of neutrophils, neutrophil infiltration can be indicated by MPO activity (Reumaux et al., 2003). In this research, the activity of MPO was significantly increased after the injection of LPS, and experienced a sharp reduction by Met administration, suggesting that Met inhibited neutrophil infiltration into lung tissues caused by LPS. In addition, protein extravasation is believed to indicate vascular leakage. Therefore, total protein level in BALF was measured. It was much lower in the Met-treatment groups than that of control group.

It is known that a very unresponsive early proinflammatory response occurs in the ALI and cause death (Zarember and Godowski 2002). TNF-alpha and IL-6 is crucial for ALI occurrence and progression. NF-κB was activated by TLR-4 after administration

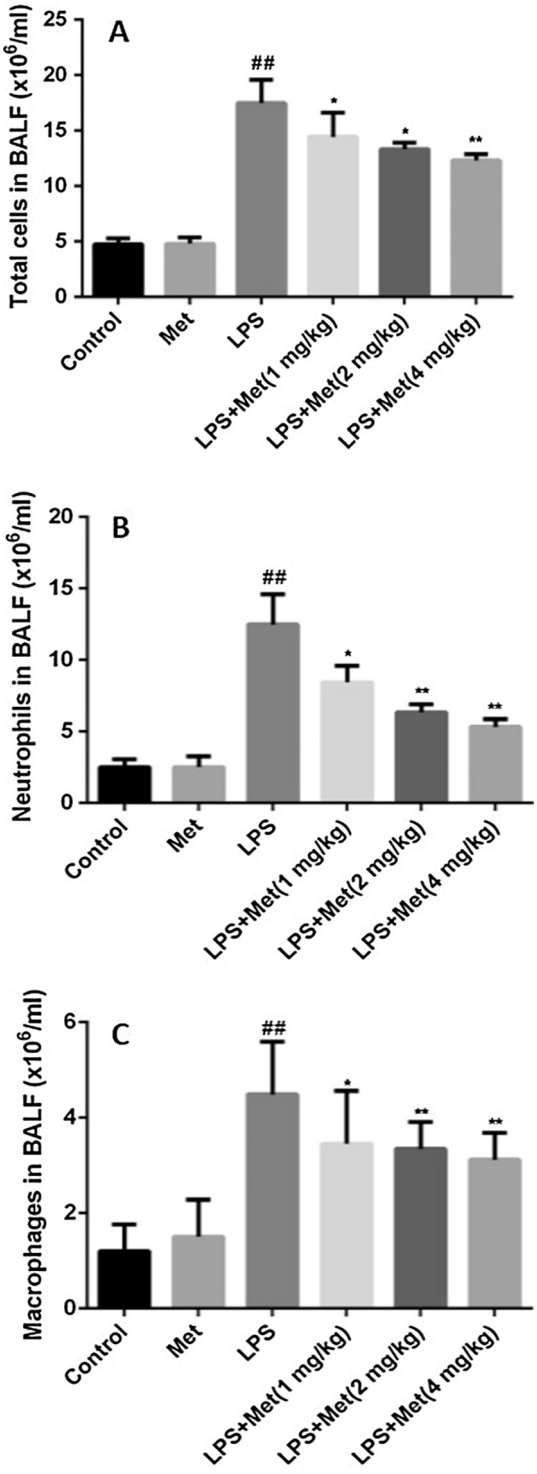

Fig. 4. Effects of Met on neutrophils, macrophages and total cells in BALF. Met (1, 2 and 4 mg/kg) were intravenously administered to rats and LPS was injected 30 min later. After 6 h, BALF was collected and the total number of cells (A), neutrophils (B) and macrophages (C) were measured. $^{\#\#}P < 0.01$ compared with the control group; $^{*}P < 0.05$, compared with the LPS group; $^{**}P < 0.01$, compared with the LPS group.

of LPS increased to increase TNF-alpha and IL-6 production, which activate NF-κB in return to form a vicious cycle. This vicious cycle can broaden the original immune responses. In this study, the early treatment with Met significantly reduced NF-κB, thereby terminating the vicious cycle and limiting the immune responses.

AMPK is extensively studied and it is crucial in regulating metabolism (Hardie et al., 2012). However, the knowledge regarding the

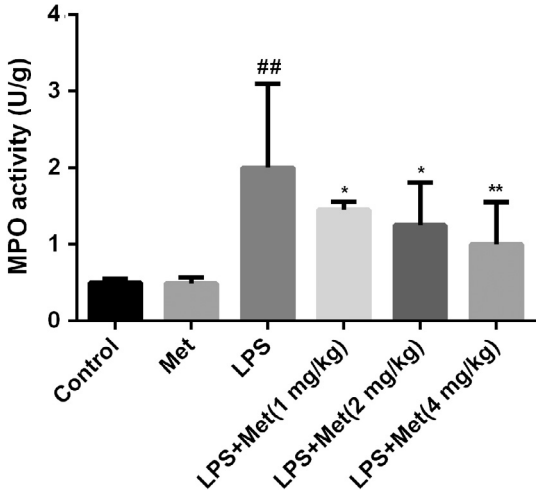

Fig. 5. Met inhibited MPO activity. Met (1, 2 and 4 mg/kg) was injected intravenously to rats and LPS was injected 30 min later. 6 h later, the activity of MPO was measured. $^{\#\#}P < 0.01$ compared with the control group; $^{*}P < 0.05$, compared with the LPS group; $^{**}P < 0.01$, compared with the LPS group.

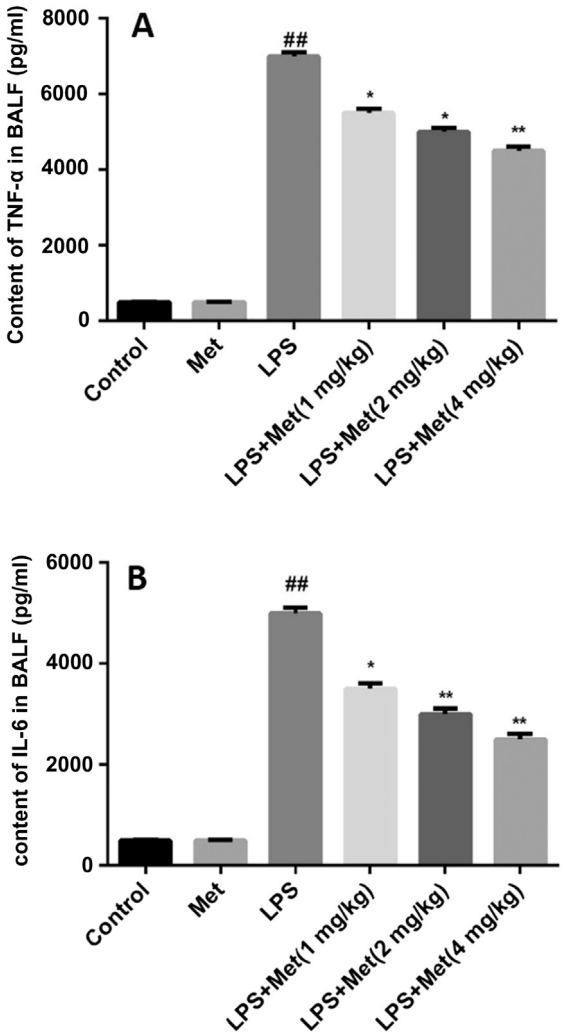

Fig. 6. LPS-induced changes in TNF-α and IL-6 in BALF and inhibition of Met. Met were intravenously administered to rats and LPS was injected 30 min later. 6 h later, BALF was collected, and inflammatory cytokines TNF-α (A) and IL-6 (B) were analyzed. $^{\#\#}P < 0.01$, compared with the control group; $^{*}P < 0.05$, compared with the LPS group; $^{**}P < 0.01$, compared with the LPS group.

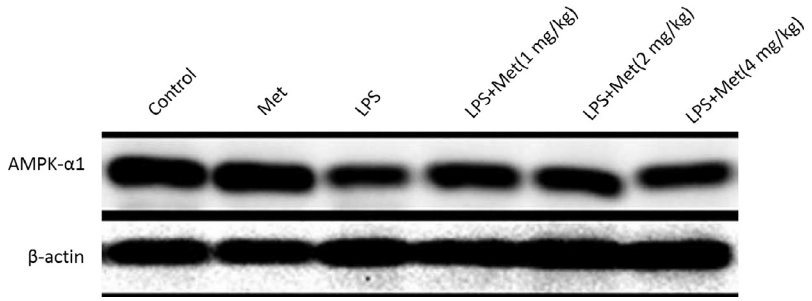

Fig. 7. Met increases the expression of AMPK expression in lung tissue. Met were injected into rats and LPS was injected 30 min later. The expression of AMPK was detected by Western blotting.

function of AMPK in the lungs is relatively limited. The molecular mechanism of metformin makes the subject debate. However, administration of metformin leads to activation of AMPK. The results of this research showed that Met administration sharply rised the expression AMPK in the lung tissues, indicating that Met may promote AMPK signaling pathway in LPS-induced ALI.

In summary, this research indicated that Met regulated the immune responses to cause alleviative effects for ALI, which may be due to its ability to promote the expression of AMPK.

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