p38 MAPK Inhibitor Administration Provides Protection Against Acute Lung Injury in Rats with Intestinal Ischemia Reperfusion via Downregulating Interlukin-6 Expression

Liu-Lin Xiong, Yun-Xia Zuo, Chao-Zhi Luo, Jie Yu, Qing-Jie Xia, Fei-Fei Xu, Jin Liu, and Ting-Hua Wang.

ABSTRACT

Background: Intestinal ischemia-reperfusion (II/R) can lead to indirect lung injury. Previous studies have shown that p38 kinase may be involved in acute lung injury (ALI). However, the exact role of p38 mitogen-activated protein kinase (MAPK) and possible mechanisms in lungs are unknown and no specific treatment exists for II/R-induced ALI. The purpose of this study was to investigate the protective role of p38 MAPK inhibition in the regulation of the inflammatory response to ALI induced by II/R in rats.

Methods: II/R was induced by occlusion of the superior mesenteric artery (SMA) and coeliac artery (CA) for 40 minutes and subsequent reperfusion for 0, 8, 16, 24 hours. SB239063, a specific inhibitor of p38 MAPK, was injected (10 mg/kg) intraperitoneally 60 minutes before the operation. The severity of ALI was measured by histology analysis (hematoxylin-eosin [HE] staining and ALI scoring) and lung edema (lung wet/dry weight ratio). Quantitative polymerase chain reaction (qPCR), western blot (WB), and immunofluorescent staining were employed to assess expression and location of interleukin-6 (IL-6) and p38 MAPK, respectively.

Results: ALI including lung edema, alveolar collapse, hemorrhage, exudation and infiltration of inflammatory cells in the lungs was induced by II/R and expression levels of IL-6 and p38 MAPK protein in the lung tissues were significantly increased at 8 and 16 hours after reperfusion, respectively compared with that in the sham group. SB239063 administration significantly down-regulated p38 MAPK and IL-6 concentration in the lung specimens, therefore, protected effectively lung tissues from injury after II/R.

Conclusions: These findings indicated that p38 MAPK inhibition may downregulate the expression of IL-6 to protect lung from ALI induced by II/R, which could be used as a potential target for the prevention or treatment of ALI caused by II/R.
Intestinal ischemia-reperfusion (II/R) is the common pathophysiology basis for numerous clinical diseases (1-3). Currently, it has been well known that II/R is a complicated pathophysiology process which is associated with morbidity and mortality in patients (4). II/R has been shown not only causes local damage to the bowel but also releases numerous mediators in the circulation that can cause acute lung injury (ALI) (5-7). ALI induced by II/R is triggered by the release of proinflammatory cytokines and bacteria-derived endotoxins from the reperfused ischemic gut tissue (8-10). Animal models and clinical data support that intra-alveolar inflammation is mediated in part by pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, which are known to play a significant role in the progression of ALI (7). It had been shown that high circulating level of IL-6 has been considered as a marker of severity of inflammatory responses, which would react to the mitogen-activated protein kinases (MAPKs) signal pathway (11-13). Research also revealed that pretreatment with dexmedetomidine hydrochloride was a useful method of reducing the lung damage caused by II/R in regards to the decreased TNF-β, IL-6, Toll-like receptor protein 4 (TLR4) and myeloid differentiation factor 88 (MyD88) level (14). These suggested that high IL-6 levels may play a significant role during inflammatory reactions in ALI after II/R (15).

p38 signaling pathway can strongly activated by environmental stresses and inflammatory cytokines, oxidative stress, and growth factors (16-21). The p38 MAPK signaling pathway also interacts with other signaling pathways to modulate inflammation and cell proliferation (17, 18). Previous studies have demonstrated that inflammation, p38 MAPK and nuclear factor (NF)-κB may be involved in TLR4 signaling-mediated pathogenesis of II/R-induced ALI (18, 20, 22, 23). Additionally, p38 MAPK activation is correlated with the degree of lung function impairment and alveolar wall inflammation (4). Some researchers have reported that patients with chronic obstructive pulmonary disease (COPD) have increased p38 MAPK activation in both macrophages and other cells within the alveolar wall when compared to nonsmokers or smokers without COPD (24, 25). In addition, inhibition of p38 MAPK pathway reduced lipopolysaccharide-(LPS) induced prostaglandin E2 (PGE2), IL-6, and keratinocyte-derived chemokines (KC) (26). And p38 MAPK inhibitor SB203580 could relieve lung injury induced by parquat or swine influenza virus (SIV) in rats and mice and cause lower increases in inflammatory cytokine, such as IL-1β, IL-6 and TNF-alpha in bronchoalveolar lavage fluid (BALF) and lung tissues (19, 27). Together, it seems that p38 MAPK may be a crucial signal that coordinates various cellular stress responses that have been shown to participate in the pathogenesis of II/R-induced lung injury. Whereas, the precise role of p38 MAPK and its relations with IL-6 in lungs are still unclear and limited pharmacological treatment options exist for ALI following II/R (28). There is an urgent need for an effective approach for treatment of II/R-induced ALI.

Therefore, the present study was undertaken to establish the II/R model for determining the effect of p38 MAPK inhibitor SB239063 on the expression of IL-6, to explore the role of p38 MAPK in the occurrence and development of ALI caused by II/R.

MATERIALS AND METHODS

Animals and Ethics
Adult healthy male Sprague-Dawley (SD) rats (weighing 200-220 g), provided by the Experimental Animal Center of Sichuan University were employed in this study. Guidelines for laboratory animal care and safety from National Institute of Health (NIH) have been followed. All animals were housed in individual cages in a temperature (21-25°C) and humidity (45-50%)-controlled room, and were fasted with no restriction of water for 12 hours before experiment. Animal care and all experimental protocols were approved by the guidelines of the Institutional Medical Experimental Animal Care Committee of Sichuan University, West China Hospital, China.

Experimental Groups and II/R Model
Rats were randomly assigned to one of the following groups: sham, II/R, II/R + normal saline (control), II/R + SB239063 (Inhibitor group, 10...
mg/kg). Sample distribution was as described in table 1 and table 2.

II/R was induced by superior mesenteric artery (SMA) and coeliac artery (CA) occlusion as previously described (29). Briefly, the rats were anesthetized intraperitoneally (i.p.) with 3.6% chloralhydrate (1 ml/100 g) and placed in a supine position. The SMA and CA were exposed and isolated through performing midline laparotomy with a 3-4 cm incision and clamped with an atraumatic microvascular clip for 40 minutes. After 40 minutes ischemia, the artery clamps were removed and intestinal reperfusion was sustained for 0, 8, 16, 24 hours, respectively. The rats in II/R + SB239063 group were injected with SB239063 into the enterocoeilia 1 hour before the operation, and then they suffered from intestinal ischemia for 40 minutes and reperfusion for 16 hours. The rats in II/R + normal saline group were injected with equal normal saline as control. Rats of the sham group underwent the same anesthesia as II/R rats and experienced exploratory laparotomy but were not clamped in the SMA and CA.

**Tissue Harvest**

Lung tissues were derived at the end of experiment time. Experimental and sham animals were anaesthetized i.p. with 3.6% chloralhydrate (1 ml/100 g), and then the whole lungs were removed immediately for further analysis.

**Lung Edema**

Lung wet/dry weight ratio was used as a marker for lung edema. At the end time points of the experiments, the left lungs were immediately removed, and weighed to obtain the wet weight. Then, the tissues were dried in an oven at 90°C for 24 hours to obtain the dry weight. Therefore, lung edema was calculated as lung wet/dry weight ratio as described previously (27).

**Hematoxylin and Eosin Staining and Lung Injury Examination**

Histological analysis in the lungs was performed by hematoxylin-eosin (HE) staining. Briefly, tissue samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin after dehydration. Then, tissue blocks were sectioned at a thickness of 5 μm, and stained with HE to examine the morphological changes of the lung. Finally, these sections were observed under a light microscope. The number of red cells exudated in pulmonary alveoli from four fields of each section (three sections each animal and six animals/group) was prepared and subjected to be quantitatively analyzed. Three independent experiments were performed. Moreover, the lung injury was scored for edema, neutrophil infiltration, hemorrhage, bronchiole epithelial desquamation, and hyaline membrane formation as previously described (30). Lung injury at 0, 8, 16, 24 hours post reperfusion were confirmed and assessed by three blenders to the identity of the groups using a 4-point lung injury score, which represented the severity: 0, no or very minor; 1, modest and limited; 2, intermediate; 3, widespread or prominent; and 4, widespread and most prominent.

**Quantitative Polymerase Chain Reaction (qPCR)**

To explore the expressions of IL-6 after II/R, qPCR was employed. Briefly, total RNA from lung tissues of each group was extracted by Trizol reagent (Takara Bio Inc., Otsu, Japan) using the manufacturer’s protocol and transcribed to cDNA reversely with the Revert Aid TM First Strand cDNA Synthesis kit (Takara Bio Inc., Otsu, Japan). In order to remove any contaminating genomic DNA, samples were incubated at 37°C for 40 minutes with DNase I, followed by DNase inactivation with a DNase inactivation reagent. The primers of IL-6 and β-actin (as an internal control) were designed with Primer Premier (PREMIER Biosoft International, Canada), and the sequences were as follows: IL-6: forward, AGAGGAAGAGGGCAGATTTT; reverse, GAGAAAAGAGTTGTGCAATG; annealing tem-

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**Table 1. Animal Model Preparation and Sample Distribution.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Lung edema</th>
<th>HE</th>
<th>IF</th>
<th>WB</th>
</tr>
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<tbody>
<tr>
<td>Sham</td>
<td>Sham</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>0 hour</td>
<td>II/R</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>8 hours</td>
<td>II/R</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>16 hours</td>
<td>II/R</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>24 hours</td>
<td>II/R</td>
<td>8</td>
<td>8</td>
<td>4</td>
<td>8</td>
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</table>

**Table 2. Animal Model Preparation and SB239063 Applying.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>HE</th>
<th>qPCR/WB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>II/R+normal saline</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Inhibitor</td>
<td>II/R+SB239063</td>
<td>8</td>
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perature, 52°C; β-actin: forward, GAAGATCAAGATCATGCTCCT; reverse, TACTCCTGCTTGCTGATCCA; annealing temperature, 52°C. PCR reactive system was then established and reacted as the following condition: initial denaturation (1 cycle, 95°C for 2 minutes), denaturation (95°C for 15 seconds), amplification (53°C for 20 seconds, 60°C for 30 seconds), totally 40 cycles. β-actin was used as endogenous control. Afterwards, the threshold cycle (Ct) of each sample was acquired, and relative abundance of mRNA from corresponding genes was calculated after normalization to β-actin RNA and was calculated by the 2−ΔΔCt method (31).

Immunofluorescent Staining
At 0, 8, 16, 24 hours after reperfusion, IL-6 and p38 MAPK were detected by immunofluorescent method. After routinely deparaffinized and rehydrated, histological sections (5 μm) were permeated in PBS containing 3% goat serum for 30 minutes at 37°C, and incubated overnight at 4°C in primary antibodies of IL-6 (rabbit, Abcam Company, 1:500) and p38 MAPK (rabbit, Abcam Company, 1:100). Negative control was replaced the primary antibody with PBS. Then, sections were incubated with fluorescence-labeled secondary antibody, cy3 (anti-rabbit, Jackson, 1:200), in the dark condition for 30 minutes at 37°C. 4,6-diamino-2-phenyl indole (DAPI) was used to stain the nuclei. Pictures were captured with a fluorescent microscope (Leica, Germany).

Western Blot (WB)
At 0, 16, 24 hours post reperfusion, the ischemic/reperfused lung tissues were lysed and homogenized in radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime). Protein samples (100 μg) was resolved in 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA) at 350 mA for 4 hours. Then, the membranes were blocked with tris-buffered saline and tween 20 (TBST), containing 5% nonfat milk for 1 hour at room temperature, and incubated with the primary antibodies of IL-6 (rabbit anti-rat, 1:800, Ab6672) and p38 MAPK (rabbit anti-rat, 1:200, SC-7149) in TBS overnight at 4°C. β-actin was internal control. Subsequently, the membranes were incubated in the secondary antibody (goat anti-rabbit IgG, 1:5000, ZSGB-BIO, China) for 1.5 hours. Finally, the membranes were developed in Alpha Innotech (BIO-RAD) with enhanced chemiluminescence (ECL). Three independent experiments were performed to ensure reliability of the protein expressions.

Statistics
Statistical analysis was conducted using SPSS 18.0 software (IBM Corporation, NY, USA). Continuous data were expressed as mean ± standard deviation (SD) and were subjected to statistical analysis using Student’s t test with two-tailed distribution and one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test. Mann-Whitney and Kruskal-Wallis test were used for non-parametric data. P value < 0.05 was considered statistically significant.

RESULTS
II/R Induced Lung Edema and Damaged Morphology in Lung Tissues
At 0, 8, 16, 24 hours following reperfusion, the situation of lung edema and morphological change were detected by lung wet/dry weight ratio and HE staining. As shown in Figure 1A, the lung wet/dry weight ratio in sham and II/R group exhibited uptrend, with the highest level at 24 hours post reperfusion (P<0.05). HE staining revealed that sections in sham group exhibited normal staining with clear morphology of epithelium and only a few blood cells could be seen in cavity of pulmonary alveoli. Comparatively, an increased number in blood cells and significant congestion, neutrophil invasion and interstitial edema were found in alveolar space in II/R group (Figure 1B). Moreover, lung injury scores were significantly increased in the II/R group as compared with that in the sham one at 16 and 24 hours post reperfusion (P<0.05) (Figure 1C).

II/R Increased Expression of p38 MAPK and IL-6 in Lung Tissues
To detect expression of p38 MAPK and IL-6 in sham and II/R group (0, 16, 24 hours after reperfusion), WB was employed. II/R markedly increased IL-6 protein level comparable to the
sham group, with 8 hours post reperfusion exhibiting the most remarkable level (P<0.05) (Figure 2A). Meanwhile, as shown in figure 2B, p38 level in lung tissue in the II/R group was significantly increased at 16 hours after reperfusion than that in the sham group (P<0.05). To locate p38 MAPK and IL-6, immunofluorescence staining was performed. Results showed that the immunostaining intensity of p38 MAPK and IL-6 in the lung cells exhibited the same trend as their protein levels in both sham and II/R group. From morphology, stained cells should involve in epithelium and neutrophil and macrophage which invade into lung after injury (Figure 2C, D).

Effect of p38 MAPK Inhibition in ALI and Lung Tissue IL-6 after II/R.
p38 MAPK inhibitor SB239063, designed and purchased from company (GlaxoSmithKline Plc, London, UK), was applied for interfering the protein expression of p38 MAPK. The results showed that, in rats injected with SB239063 (Inhibitor group), low expression level of p38 MAPK could be detected, when compared with those of no injection one (control group) (Figure 3A). As a result, the mRNA expression for IL-6 was correspondingly decreased in rats subjected to p38 MAPK inhibitor (Inhibitor group), when compared with control group (Figure 3B). HE staining was performed to detect the number of red cells in alveoli. The results demonstrated that the number of red cells in injury group in alveoli were significantly increased as compared with the sham group, but in rats injected by p38 MAPK inhibitor (Inhibitor group), decreased red cells were exhibited in alveolar cavity when compared with those of no injection one (II/R group) , P<0.05 (Figure 3C, D). As shown in figure 3F, lung injury scores were worsened in the II/R group, while improved in inhibitor group (P<0.05), which were evaluated by HE staining (Figure 3E).

**DISCUSSION**

In this study, we got two important fruits. Firstly, we found that the rats suffered from II/R exhibited obvious evidence of ALI, including adherence and emigration of neutrophils, as well as the formation of interstitial edema, which were together with increased expression of IL-6 and p38 MAPK in lung tissues. Secondly, the severity of lung injury could be attenuated by administration of p38 MAPK inhibitor SB239063 via downregulating IL-6 and p38 MAPK expression levels.

**Lung Injury and Alterations in Cytokines Induced by II/R**
In the present study, histological analysis showed that II/R induced lung edema and increased lung injury scores. Meanwhile, expression of lung tissue IL-6 and p38 MAPK was markedly upregulated. Previous studies have reported that II/R could stimulate the activity of extracellular signal-regulated kinase (ERK), p38 MAPK and c-Jun N-terminal kinase (JNK) (21), and activated neutrophils as well as excessive elevation of proinflammatory cytokines were considered to be important factors in the occur-
rence and development of ALI caused by II/R (32, 33). Research also revealed that the levels of TNF-β, IL-6, TLR4 and MyD88 were increased in relation to the lung injury caused by II/R (34). Moreover, other studies have also suggested that IL-6 is directly involved in the modulation of inflammation (15). In addition, a recent study found that IL-6 also modulated intestinal epithelial tight junction permeability. Additionally, various studies have shown that decreased levels of IL-6 were related to the attenuation of the inflammatory responses in ALI (14, 20, 35). Our data therefore closely correlated with theirs and demonstrated the exact lung injury caused by II/R, which confirmed the success of the model.

Role of p38 MAPK Interference in the Lung Injury Following II/R
In order to investigate the role of p38 MAPK in II/R-caused ALI and in regulation of IL-6, SB239063 was employed to inhibit p38 MAPK. Results revealed that lung injury induced by II/R was alleviated in rats with administration of p38 MAPK inhibitor, the protective role was associated with the downregulation of p38 MAPK and IL-6 in the lung tissues. The data suggested that the p38 MAPK signaling pathway has an important role in regulating the production of IL-6 in II/R induced lung injury in rats.
For treatment of ALI after II/R, previous studies have demonstrated the osthole, valproic acid (VPA) treatment or pretreatment with glutamine could attenuate the lung injury induced by II/R in rats, at least in part, by inhibiting inflammatory response and oxidative stress (30, 36). As for the applying of p38 MAPK inhibitor and the relationship with other factors in humans and animal, various studies were involved in the lung injury caused by virus and some toxic substances or some inflammatory diseases. For example, previous researches have revealed that p38 MAPK inhibitor SB239063 inhibited LPS induced numbers in neutrophils and IL-6 levels in the skin in guinea pigs (37). Moreover, inhibition of p38 MAPK pathway attenuated LPS-dependent PGE2, IL-6, and KC production, thereby inhibited LPS-induced innate immune responses in murine intestinal myofibroblasts (26). Currently, p38 MAPK inhibitors are also considered to target inflammation in other diseases such as hyperlipidemia and rheumatoid arthritis (38, 39). Trials of p38 MAPK inhibitors in humans have also demonstrated that p38 MAPK inhibitors decreases serum levels of both TNF-α and IL-6 after LPS administration as well as acute phase reactants associated with inflammation, serum erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) (40). However, still no specific treatment exists for II/R-induced ALI, and few researches put emphasis on the effect of p38 MAPK inhibition on lung injury induced by II/R and the involved inflammatory factors regulated by p38 MAPK. So, to our knowledge, the present study is the first time to show a protective effect of p38 MAPK inhibition on ALI induced by II/R via down-regulating IL-6 and p38 MAPK (Figure 4).

In summary, our findings supported the hypothesis that inhibition of p38 MAPK may downregulate the expression of IL-6 to protect lung from acute injury in II/R, which could be used as a novel therapeutic strategy for the treatment of II/R-induced lung injury in the future clinical trial.

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All authors have no conflicts of interest for this work.

CONCLUSIONS

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