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p38MAPK inhibition attenuates LPS-induced acute lung injury involvement of NF-κB pathway

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Abstract

The pathogenesis of acute lung injury/acute respiratory distress syndrome (ARDS) is complex and involves multiple signal transduction processes. It is believed that p38MAPK (mitogen-activated protein kinase) is one of the most kinases in inflammatory signaling. At present study, we demonstrated the role of p38MAPK in lipopolysaccharide (LPS)-induced acute lung injury with pharmacologic p38MAPK inhibition by SB203580. SB203580, p38MAPK specific inhibitor, was injected (10 mg/kg, i.v.) 30 min before LPS administration (5 mg/kg, i.v.). The hematoxylin–eosin staining of lung tissues showed that p38MAPK inhibition significantly attenuated the pulmonary inflammatory responses induced by LPS. Moreover, SB203580 can also inhibit the inflammatory cytokine release, and reduce the mortality rate of LPS-induced acute lung injury. Further, western blot analysis that showed SB203580 administration can inhibit the activation of NF-κB, which was associated with the inhibition of IκBα degradation in cytoplasm. These data suggest that p38MAPK signaling may be involved in the activation of NF-κB, and activation of p38MAPK signaling may be one of the mechanisms of acute lung injury.

Keywords: p38MAPK; Lipopolysaccharide; Acute lung injury; NF-κB; TNF-α; IL-6

1. Introduction

Acute lung injury/acute respiratory distress syndrome (ARDS) occurs in the setting of an acute severe illness complicated by systemic inflammation. It represents a state of excess production of inflammatory mediators from immune cells, such as cytokines, chemokines, adhesion molecules and bioactive lipid products (Ware and Matthay, 2000). The most common condition precipitating ARDS is sepsis. Lipopolysaccharide (LPS) released during sepsis is a major stimulus for the release of cytokines (Pittet et al., 1997). When LPS is administered to experimental animals, it mimics the condition of an ongoing sepsis and concomitant ARDS-like lung injury including polymorphonuclear neutrophil sequestration and lung edema (Yoshinari et al., 2001). Unfortunately, despite marked efforts, little therapeutic progress has been made and the mortality rate of ARDS remains high (Stapleton et al., 2005). There remains a lack of understanding of the cellular and molecular mechanisms that underlie the development of acute lung injury.

Recently, a number of in vitro studies have shown that the production of inflammatory mediators is strongly affected by mitogen-activated protein kinases (MAPKs), such as p38MAPK, c-Jun NH_2-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) (Yoshinari et al., 2001; Li et al., 2004; Huang et al., 2004). Among these MAPK groups, p38MAPK is believed to be one of the more important kinases in stress signaling. It has been shown that p38MAPK is induced by LPS and critical for the LPS-induced cytokine release (Lee and Young, 1996; Carter et al., 1999a,b). There is

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also evidence suggested that p38MAPK mediated the bronchoconstriction and the neutrophil recruitment provoked by LPS (Candrian et al., 2005). Recent studies have shown that p38MAPK is involved in the development of acute lung injury/ARDS with different stimuli (Yang et al., 1999; Haddad et al., 2001; Nash and Heuertz, 2005). So a better understanding of the role of p38MAPK in LPS-induced acute lung injury is mandatory to clarify the mechanisms of present treatment and to develop a better therapeutic strategy in acute lung injury/ARDS.

Here we test the hypothesis that p38MAPK plays an essential role in LPS-induced acute lung injury. Pharmacologic p38MAPK inhibition by SB203580 [4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1*H*-imidazole], p38MAPK specific inhibitor, significantly attenuates the severity of lung injury, which may be associated with the inhibition of NF-κB activation.

2. Materials and methods

2.1. Animals

Adult male Sprague–Dawley rats weighing 200–250 g were purchased from Shanghai Experimental Animal Center of Chinese Academy of Science. The rats were housed in a temperature-controlled environment with 12-hour light–dark cycles and were fed standard laboratory diet and water *ad libitum*. All experiments were approved by the Animal Care and Use Committee at the Xuzhou Medical College and were in accordance with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Reagents

LPS (*Escherichia coli* lipopolysaccharide, 055:B5) was obtained from Sigma Chemical Company (St. Louis, MO), SB203580 from Biosource (Biosource International, Camarillo, CA), antibodies used in this study [anti-NF-κB p65 (sc-109), anti-IκBα (sc-1643), anti-p38 (sc-7972), and anti-phospho-p38 (sc-7973)] from Santa Cruz biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA), and TNF-α and IL-10 ELISA kits from R&D corporation (R&D Systems Inc. Minneapolis, MN, USA).

2.3. Model and grouping

The animals were randomly divided into 4 groups. Group 1 (control group, $n=6$) received an intravenous injection of 0.9% sodium chloride, group 2 (LPS group, $n=6$ each) received LPS injection (5 mg/kg, i.v.), group 3 (SB group, $n=6$ each) received SB203580 injection (10 mg/kg, i.v.), and group 4 (SB+LPS group, $n=6$ each) received SB203580 injection (10 mg/kg, i.v.) 30 min before LPS administration. The doses of these drugs we chose were on the basis of previous studies (Duma et al., 2004; Chen et al., 2005) and our preliminary experiments. All the animals were killed under anesthesia by intra-peritoneal injection of pentobarbital (30 mg/kg) at each time point.

2.4. Survival studies

To confirm the role of p38MAPK in this model, rats were injected with LPS (5 mg/kg, i.v.) with or without p38MAPK inhibitor — SB203580 pretreatment (10 mg/kg, i.v., 30 min before, $n=15$ each) and observed for 48 h. Experiments were performed with littermate rats.

2.5. Bronchoalveolar lavage

Animals were exsanguinated through the abdominal aorta under general anesthesia (pentobarbital, 30 mg/kg, i.p.) at each time point. A median sternotomy allowed for exposure of both of the lungs. After ligating the hilum of right lung, the left lung was lavaged three times with 5 mL ice-cold PBS (phosphate buffered saline). The recovery ratio of the fluid was about 90%. And then the bronchoalveolar lavage fluids were stored at −80 °C for cytokine analysis.

2.6. Cytokine measurements

Concentrations of TNF-α and IL-6 in bronchoalveolar lavage fluids were measured by using a "sandwich" enzyme linked immunosorbent assay with TNF-α and IL-6 ELISA kits for rats, according to the manufacturer's instructions. Samples with a concentration that exceeded the limits of the standard curve were repeated after dilution.

2.7. Pulmonary histopathology

The caudal lobes were harvested at 6 h after LPS administration and immediately put into 10% neutral formalin for fixation. The tissues were embedded in paraffin and cut into five-micrometer-thick slices. Conventional hematoxylin and eosin staining was performed.

2.8. Western blot analysis

The right middle and accessory lobes of the lungs were harvested separately at 1, 3, 6, and 12 h after LPS administration, cleared of non-pulmonary tissues, and frozen in liquid nitrogen immediately until homogenization. Tissue samples were homogenized in lysis buffer A (in mmol/L, pH 7.9): HEPES 10.0, Na_3VO_4 1.0, $MgCl_2$ 1.5, KCl 10.0, NaF 50.0, edetic acid (EDTA) 0.1, egtazic acid (EGTA) 0.1, phenylmethylsulfonyl fluoride (PMSF) 0.5, dithiothreitol (DDT) 1.0 and 0.02% protease inhibitor cocktail. After the addition of 90 μL NP-40 (10%), the homogenates (900 μL) were vortexed for 30 s and then centrifuged at 800 ×*g* for 15 min at 4 °C. The supernatants were used for Western blot analysis as cytosolic proteins. The nuclear pellets were resuspended in buffer B (in mmol/L, pH 7.9): HEPES 20.0, NaCl 420.0, $MgCl_2$ 1.5, EDTA1.0, EGTA 1.0, PMSF 0.5, DDT 1.0, 20% glycerol, and 0.02% protease inhibitor cocktail. The homogenates were

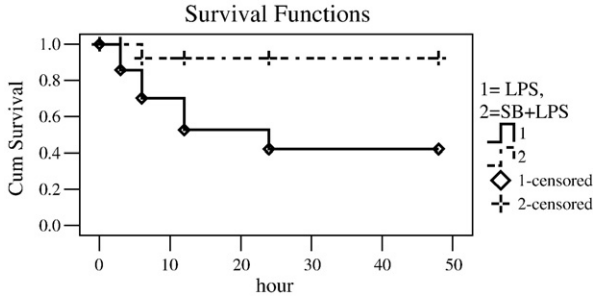

Fig. 1. SB203580, p38MAPK inhibitor, suppresses LPS-induced mortality. For the SB+LPS group, rats were injected with SB203580 (10 mg/kg, i.v.) 30 min before LPS (5 mg/kg, i.v.) treatment. $P<0.05$ for SB+LPS group ($n=15$) compared with LPS group ($n=15$).

incubated for 30 min in ice-cold water with constant agitation and then centrifuged at 13 000 ×*g* for 15 min at 4 °C to separate the nuclear proteins. Protein concentrations were determined using the Bradford method and the protein samples were stored at −80 °C.

Protein samples were dissolved in 4× sample buffer (in mmol/L, pH 6.8): Tris–HCl 250.0, sucrose 200.0, DDT 300.0, 0.01% Coomassie brilliant blue-G, and 8% sodium dodecyl sulfate (SDS), and denatured at 95 °C for 5 min, then the equivalent amounts of proteins were separated by using 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane. The membranes were incubated overnight at 4 °C with the following primary antibodies: mouse polyclonal anti-p38MAPK antibody (1:500), mouse polyclonal anti-phospho-p38MAPK antibody (1:400), anti-IκBα antibody (1:600), and rabbit polyclonal anti-NF-κBp65 antibody (1:600). The membranes were extensively washed with Tris-Buffered Saline Tween-20 (TBST) and incubated for 2 h with the secondary antibody conjugated with alkaline phosphatase (AP) at room temperature. The immune complexes were detected by using a NBT/BCIP assay kit (Promega, Shanghai, China). The scanned images were imported into an image analyzer (LabWorks Software, UVP Upland, CA, USA). Scanning densitometry was used for semiquantitative analysis of the data.

2.9. Lung wet/dry weight ratio

The water content of the lungs was determined by calculating the wet/dry weight ratio of lung tissues. The right cranial lobe was excised, rinsed briefly in PBS, blotted, and then weighed to obtain the "wet" weight. The lung was then dried at 80 °C for 72 h to obtain the "dry" weight. And the wet/dry ratio was calculated by dividing the wet weight by the dry weight.

2.10. Statistical analysis

All data are expressed as mean±S.D. Comparisons were performed by one-way analysis of variance (ANOVA) with multiple comparisons or Student's *t*-test. The survival studies were analyzed by Kaplan–Meier test. $P<0.05$ was considered statistically significant.

3. Results

3.1. The effect of SB203580 on LPS-induced lethality

To evaluate the effects of p38MAPK in LPS-induced lung injury, we compared the mortality rates. In LPS group, the mortality rate induced by LPS was high to 46.7%, however, in SB+LPS group, with p38MAPK inhibition, the mortality rate reduced to 6.7% ($P<0.05$) (Fig. 1). This indicated that p38MAPK inhibition was required to limit LPS-induced lethality.

3.2. The concentrations of TNF-α and IL-6 in bronchoalveolar lavage fluids

In LPS group, we found that both the concentrations of TNF-α and IL-6 in bronchoalveolar lavage fluids increased significantly in a time-dependent manner after LPS treatment (Fig. 2A and B). However, in SB+LPS group, SB203580 pretreatment effectively decreased the levels of TNF-α and IL-6 in bronchoalveolar lavage fluids by 2- to 3-folds (Fig. 2A and B).

3.3. Pulmonary protection of SB203580

Under light microscopy, the hematoxylin–eosin staining of lung tissues showed that after LPS administration, the structure

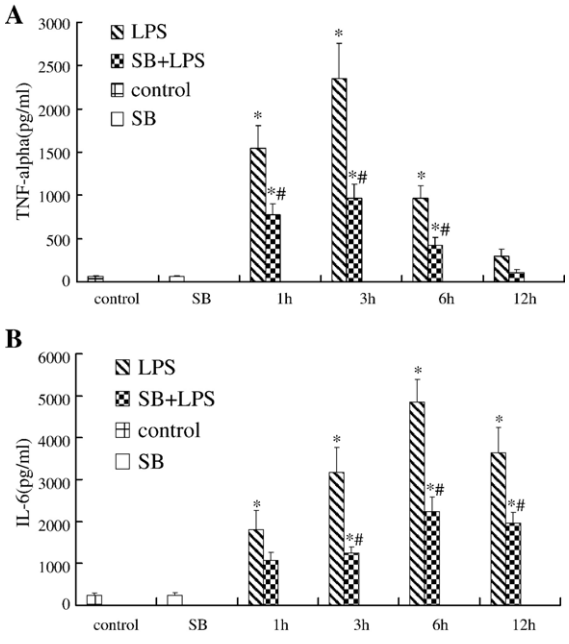

Fig. 2. LPS-induced alterations of TNF-α and IL-6 levels in bronchoalveolar lavage fluid (BALF) from rats and the suppression of SB203580. A. The concentrations of TNF-α in BALF at each time point after LPS administration and the suppression of SB203580 pretreatment. B. The concentrations of IL-6 in BALF at each time point after LPS administration and the suppression of SB203580 pretreatment. Data are expressed as mean±S.D., *$P<0.05$ vs. control group; $^{\#}P<0.05$ vs. the LPS group.

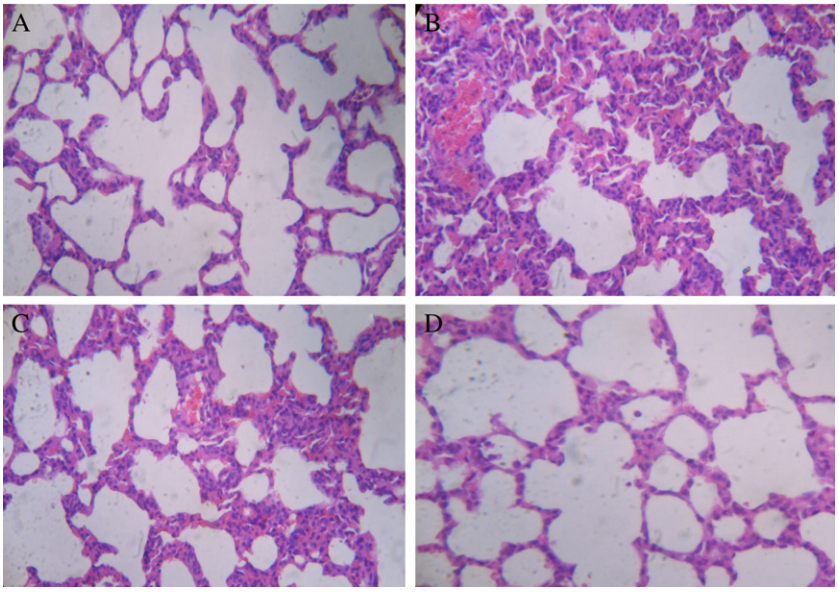

Fig. 3. Microscopic findings of lung tissues stained with hematoxylin–eosin (H.E.×400). Histopathologic examination of the lungs was performed at 6 h after LPS injection. A. Control group; B. LPS group: edematous changes of alveolar walls, swelling of alveolar epithelial cells, and massive polymorphonuclear infiltration were observed; C. SB+LPS group: less damage was observed compared with the LPS group. D. SB group: no differences were observed compared with control group.

of lung tissues destroyed obviously, including intra-alveolar hemorrhage, interstitial edema, alveolar collapse and massive inflammatory cells infiltration (Fig. 3B). However, with the pretreatment with SB203580 in SB+LPS group, the destruction of lung structure was significantly reduced, compared with LPS group (Fig. 3C). SB group, with SB203580 administration only, showed no changes of the structure of lung tissues, compared with control group (Fig. 3D). Fig. 3A shows the normal histologic features of lung tissues in control group.

3.4. Lung wet/dry weight ratio

Lung wet/dry weight ratios were evidently higher at 6 h after LPS administration in the LPS group compared with the control group (5.60±0.67 vs. 4.32±0.28, $P<0.05$). SB203580 pretreatment significantly reduced the lung wet/dry ratios at the same time point compared with LPS group (4.94±0.21 vs. 5.60±0.67, $P<0.05$).

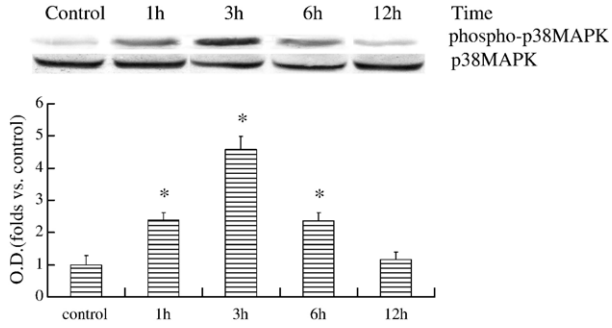

Fig. 4. LPS time-dependently induces p38MAPK activation in lung tissues. Representatives of 3 to 4 independent experiments. Data are expressed as mean±S.D., *$P<0.05$ vs. control group.

3.5. Activation of p38MAPK in lung tissue

Western blot analysis was employed to assess the expression of phospho-p38MAPK, which reflected the p38 activation in the lung tissues. The expression of phospho-p38MAPK was slight in unstimulated lungs (Fig. 4). After LPS injection,

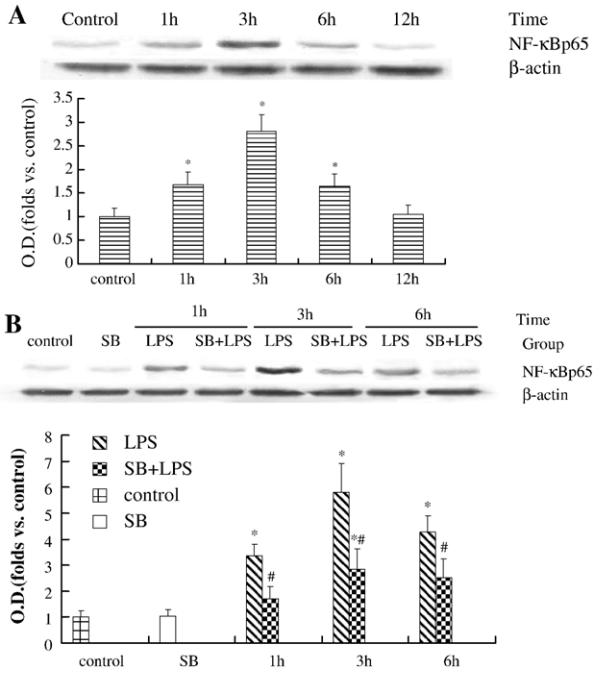

Fig. 5. The expression of NF-κBp65 in nucleus of lung tissues. A. LPS time-dependently induced the expression NF-κBp65 protein in nuclear extracts of lung tissues. B. SB203580 pretreatment inhibits LPS-induced NF-κBp65 nuclear translocation and activation at 1, 3, 6 h after LPS administration. Representatives of 3 to 4 independent experiments. Data are expressed as mean±S.D., *$P<0.05$ vs. control group; #$P<0.05$ vs. the LPS group.

phosphorylation of p38MAPK substantially increased and reached its peak level at 3 h, then decreased (Fig. 4).

3.6. Activation of NF-κB in lung tissue

Expression of NF-κBp65 in nucleus, which reflected the activation of NF-κB, extracted at each time point after LPS treatment as shown in Fig. 5A. The expression of NF-κBp65 increased and reached its peak level at 3 h after LPS administration, and then decreased. At 12 h after LPS treatment the expression of NF-κBp65 in nuclear extracts showed no differences from that of control group. Therefore, we chose the time points at 1 h, 3 h, and 6 h after LPS administration to examine the effect of SB203580 on NF-κB activation. Our data showed that pretreatment with SB203580 significantly suppressed LPS-induced activation of NF-κB at each time point (Fig. 5B).

3.7. Expression of IκBα in lung tissue

In order to investigate the possible mechanism underlying the activation of p38MAPK on LPS induction of pathways loading to NF-κB activation, the degradation of IκBα in lung tissues was analyzed by western blot. As shown in Fig. 6A, LPS treatment resulted in the reduction in IκBα protein content in lung tissue. After administration of LPS the distinct degradation of IκBα was observed at 1 h. By 3 h after LPS treatment, newly synthesized IκBα protein was apparent. And at 12 h after LPS administration, the protein content of IκBα in cytoplasm showed no differences than that in control group. Pretreatment with SB203580 significantly inhibited the degradation of IκBα in cytoplasm of lung tissues at 1, 3, 6 h after LPS treatment (Fig. 6B).

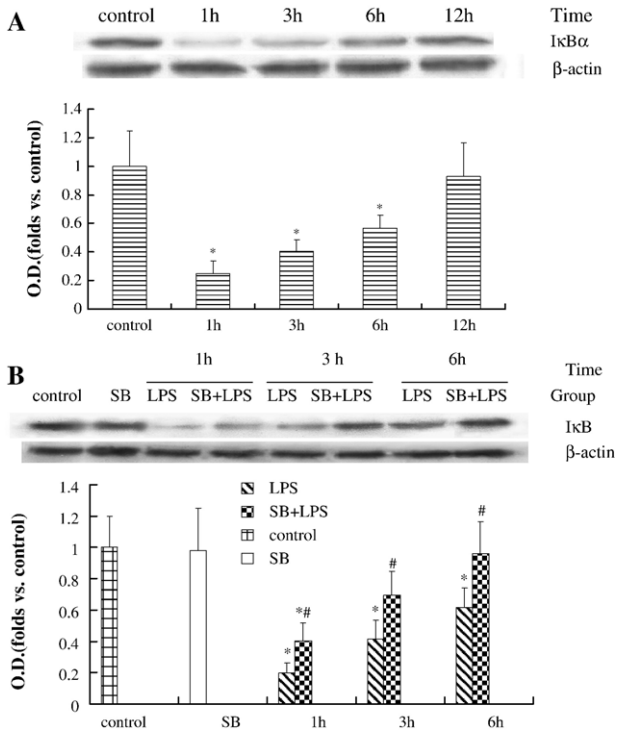

Fig. 6. Degradation of IκBα in cytoplasm of lung tissues with different treatments. A. The degradation of IκBα in cytoplastic extracts at each time point after LPS administration. B. SB203580 pretreatment inhibited the degradation of IκBα in cytoplasm. Representatives of 3 to 4 independent experiments. Data are expressed as mean ± S.D., *$P<0.05$ vs. control group; $^{\#}P<0.05$ vs. the LPS group.

4. Discussion

In the present study, with the in vivo administration of p38MAPK specific inhibitor SB203580 we found that p38MAPK plays an essential role in LPS-induced acute lung injury. p38MAPK inhibition not only reduced the inflammatory cytokine release in bronchoalveolar lavage fluids and improved the pulmonary histology, but also limited LPS-induced lethality. Further, SB203580 can significantly inhibit the activation of NF-κB in lung tissue, which was associated with the inhibition of IκBα degradation in cytoplasm. These results suggest that p38MAPK signaling may be involved in the activation of NF-κB in LPS-induced acute lung injury.

The pathogenesis of acute lung injury/ARDS is complex and involves multiple signal transduction processes. Previously, particular attention has been given to the NF-κB signaling, which is required for expression of many cytokines (Baeuerle and Henkel, 1994; Abraham, 2003). However, recently much attention has been given to the mitogen-activated protein kinase (MAPK) superfamily, especially the p38MAPK, due to its consistent activation by pro-inflammatory cytokines, and to its role in nuclear signaling. It has been reported that phospho-p38MAPK is induced by LPS and plays a key role in LPS-induced signal transduction pathways leading to cytokine synthesis (Lee and Young, 1996; Candrian et al., 2005; Haddad et al., 2001; Nick et al., 2000). Yoshinari et al. (2001) reported that p38MAPK was involved in the production of TNF-α and IL-1 in LPS-induced lung injury. There is evidence that inhibition of p38MAPK is associated with significant decreases in neutrophil adhesion (Nick et al., 1999) and attenuation of pulmonary injury with different stimuli (Yang et al., 1999; Haddad et al., 2001; Nash and Heuertz, 2005). Recently, it has been shown that macrophage glucocorticoid receptors regulate toll-like receptor 4-mediated inflammatory responses by selective inhibition of p38MAP kinase (Bhattacharyya et al., 2007). In our study, after injection of LPS, the phosphorylation of p38MAPK substantially increased, concomitant with the increase of cytokines and inflammatory cell infiltration in pulmonary tissue. Over release of inflammatory cytokines is the immediate cause of lung injury. Furthermore, the cytokines can induce the activation of p38MAPK in turn. This positive feedback could amplify the inflammatory response and form a vicious cycle, which leads to multiple organ dysfunction syndrome at last. At present study, in vivo administration of p38MAPK specific inhibitor SB203580 resulted in a decrease in TNF-α and IL-6 in bronchoalveolar lavage fluids, and reduced the severity of lung injury. These results suggest that p38MAPK may mediate the production of TNF-α and IL-6 in lung and contributed to LPS-induced pulmonary inflammation.

The importance of p38MAPK in LPS-induced lung injury was highlighted further by the survival of SB203580-treated rats exposed to LPS. However, it is reported that inhibition of p38MAPK did not decrease neutrophil accumulation in the lung and the increased production of pro-inflammatory cytokine, although p38MAPK was activated in lung neutrophils after hemorrhage or endotoxemia (Arcaroli et al., 2001). These conflicting results may possibly be explained by the species examined, the routes of LPS and SB203580 administration, and the period of observation.

NF-κB is a critical transcription factor required for maximal expression of many cytokines involved in the pathogenesis of ARDS (Abraham, 2003; Baeuerle and Henkel, 1994; Fan et al., 2001). It appears that activation of NF-κB is central to the development of pulmonary inflammation and acute lung injury. In our present study, we found that the expression of NF-κB in the nucleus was significantly increased after LPS administration. Although both p38MAPK and NF-κB are critical modulators of inflammation and they regulate the same cytokines expression, however the relationship between p38MAPK and NF-κB is still unclear.

At the present study, SB203580 pretreatment significantly inhibited the activation of NF-κB. The expression of NF-κBp65 in the nucleus was reduced evidently. These data demonstrate that p38MAPK may be involved in the activation of NF-κB in this model. Further, we found that SB203580 inhibited the degradation of IκBα in cytoplasm. IκB is an inhibitor protein of NF-κB. In unstimulated cells, NF-κB is retained as an inactive complex bound to the cytoplasmic inhibitor protein of IκBα. A key step in the activation of NF-κB involves phosphorylation and degradation of IκB. The degradation of IκBα permits NF-κB to translocate to the nucleus and affect gene transcription (De Bosscher et al., 2003). Data from the present study showed that SB203580 trapped NF-κB in cytoplasm by inhibition of degradation of IκBα, which confirmed the involvement of p38MAPK in NF-κB activation again. It is reported that p38MAPK regulated several transcription factors, including NF-κB (Raingeaud et al., 1996). Recent studies in vitro have suggested that p38MAPK is required for NF-κB-dependent gene expression (Campbell et al., 2004; Carter et al., 1999a,b). Campbell et al. (2004) confirmed that p38MAPK regulated TNF-α transcription and expression in LPS-activated human macrophages through NF-κB pathway. Kim et al. (2006) indicated that p38MAPK up-regulated LPS-induced NF-κB activation in RAW264.7 macrophages. However, data remains controversial (Alpert et al., 1999). Alpert et al. (1999) reported that p38MAPK inhibited the activation of NF-κB. Therefore, the exact relationship between p38MAPK and NF-κB, whether the relationship of the two is cell type- or stimuli-specific and whether p38MAPK signaling is the upstream of NF-κB needs to be further studied.

In conclusion, data from our present study suggest that p38MAPK plays an essential role in LPS-induced acute lung injury. Pharmacologic p38MAPK inhibition by SB203580 can significantly attenuate the lung injury, reduce LPS-mediated mortality rate and inhibit the release of cytokines in bronchoalveolar lavage fluids. The protection of SB203580 may involve the inhibition of NF-κB activation via inhibition of IκBα degradation in cytoplasm. Activation of p38MAPK signaling pathway may be one of the mechanisms of acute lung injury and inhibition of p38MAPK may be an effective therapeutic strategy against acute lung injury/ARDS.

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