Selective Inhibitor of p38 Mitogen-Activated Protein Kinase Inhibits Lipopolysaccharide-Induced Interleukin-8 Expression in Human Pulmonary Vascular Endothelial Cells

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ABSTRACT

Adult respiratory distress syndrome (ARDS) characterized by permeability edema is observed in severe insults such as bacteremia sepsis. Interleukin (IL)-8, which chemotactizes and activates neutrophils, has been suggested to play an important role in the production of ARDS. Therefore, the inhibition of IL-8 production is an important strategy for the treatment of ARDS. Recent studies have revealed the role of p38 mitogen-activated protein (MAP) kinase in cytokine expression and the inhibition by a selective inhibitor of p38 MAP kinase activity of cytokine expression in a variety of cell types. However, little is known about the role of p38 MAP kinase in lipopolysaccharide (LPS)-induced IL-8 expression in pulmonary vascular endothelial cells and the effect of a selective p38 MAP kinase inhibitor on it. In the present study, we therefore attempted to clarify these issues. The results showed that LPS induced p38 MAP kinase phosphorylation and activity, and SB 203580 as a selective inhibitor of p38 MAP kinase activity inhibited p38 MAP kinase activity and IL-8 expression in LPS-stimulated pulmonary vascular endothelial cells. These results indicate that p38 MAP kinase regulates LPS-induced IL-8 expression in pulmonary vascular endothelial cells. Although it is currently not known whether SB 203580 is capable of producing beneficial effects on ARDS, a strategy of inhibiting p38 MAP kinase activity by a selective p38 MAP kinase inhibitor may apply to the therapy for ARDS.

Adult respiratory distress syndrome (ARDS), a form of acute lung injury, which is characterized by permeability edema, is observed in severe insults such as bacteremia sepsis (Nogare, 1989; Matthay, 1990; Bernard et al., 1994; Hashimoto and Horie, 1994). Although understanding of the basic mechanisms in the production of ARDS has increased, little therapeutic progress has been made and mortality remains high. The pathogenesis of ARDS is complex and involves multiple inflammatory cells and mediators (Tate and Repine, 1983; Nogare, 1989; Matthay, 1990; Hashimoto and Horie, 1994). Although understanding of the basic mechanisms in the production of ARDS has increased, little therapeutic progress has been made and mortality remains high. The pathogenesis of ARDS is complex and involves multiple inflammatory cells and mediators (Tate and Repine, 1983; Nogare, 1989; Matthay, 1990; Hashimoto and Horie, 1994). Although ARDS occurs in neutropenic patients (Mauder et al., 1986; Ognibene et al., 1986), it has been shown that neutrophils play an important role in the production of acute lung injury (Tate and Repine, 1983; Weiland et al., 1986; Hogg, 1987; Nogare, 1989; Matthay, 1990; Hashimoto and Horie, 1994). The extravasation and accumulation of neutrophils at the sites of injury depend on adhesion to and migration through endothelial linings (Hashimoto and Horie, 1994). Interleukin (IL)-8 that displays a chemotactic activity for neutrophils participates in this process through the recruitment of neutrophils (Mantovani and Dejana, 1989; Huber et al., 1991; Baggioolini et al., 1994). IL-8 also activates neutrophils to generate several toxic products such as reactive oxygen species, proteases, and arachidonic acid metabolites (Mantovani and Dejana, 1989; Baggioolini et al., 1994), each of which may initiate endothelial cell damage leading to an increase in endothelial permeability (Zimmerman et al., 1983; Idell et al., 1985; Tagan et al., 1991). Several studies have shown that IL-8 plays an important role in the production of ARDS (Miller et al., 1992; Matsumoto et al., 1997; Mukaida et al., 1998). ARDS is often seen in bacteremia sepsis and bacterial endotoxin, a lipopolysaccharide (LPS), can stimulate vascular endothelial cells to produce IL-8 (Mantovani and Dejana, 1989; Strieter et al., 1989; Baggioolini et al., 1994). Therefore, it is important to clarify the mechanism of IL-8 expression in LPS-stimulated pulmonary vascular endothelial cells.

Recent studies, which explored the intracellular signal regulating cytokine expression, have shown that p38 mitogen-activated protein (MAP) kinase that belongs to MAP kinase superfamily (Davis, 1994) is involved in cytokine expression (Cuenda et al., 1995; Shapiro and Dinarello, 1995; Matsu-
moto et al., 1998; Hashimoto et al., 1999a,b). We have previously shown that p38 MAP kinase regulates tumor necrosis factor-α-induced IL-8 expression in human pulmonary vascular endothelial cells (Hashimoto et al., 1999a); however, the role of p38 MAP kinase in LPS-induced IL-8 expression in pulmonary vascular endothelial cells has not been determined. In the present study, therefore, we studied the role of p38 MAP kinase in IL-8 expression in pulmonary vascular endothelial cells and the production of ARDS more precisely by examining the role of p38 MAP kinase in LPS-stimulated IL-8 expression in these cells. To this end, we attempted to examine p38 MAP kinase phosphorylation and activation, and the effect of SB 203580 as the specific inhibitor of p38 MAP kinase activity on p38 MAP kinase activity and IL-8 expression in LPS-stimulated human pulmonary vascular endothelial cells.

Materials and Methods

Reagents. LPS was obtained from Sigma Chemical Co. (St. Louis, MO). The pyridinyl imidazole SB 203580, a selective inhibitor of p38 MAP kinase activity (Lee et al., 1994), was kindly provided by SmithKline Beecham (King of Prussia, PA) and was dissolved in dimethyl sulfoxide.

Cell Culture. Normal human pulmonary arterial endothelial cells (HPAECs) used as pulmonary vascular endothelial cells in this study were obtained from Clonetics (San Diego, CA). The cells (1 × 10^6 cells/ml) were plated onto a 24-well flat-bottom tissue culture plate (Corning, Corning, NY) for determination of cytokine production. Cells were plated in a tissue culture dish (Falcon 1007; Falcon Labware, Oxnard, CA) for Western blot analysis. For Northern blot analysis (Falcon 1005), cells were placed with vascular endothelial growth medium-2 (Clonetics) containing 0.2% fetal bovine serum, gentamicin-amphotericin B, epidermal growth factor, insulin-like growth factor, fibroblast growth factor, vascular endothelial growth factor, ascorbic acid, heparin, and hydrocortisone. The cells were grown until they were subconfluent and the medium was changed. To examine the effect of LPS on threonine and tyrosine phosphorylation of p38 MAP kinase in HPAECs, the cells that had been pretreated with endothelial growth medium-2 without fetal bovine serum, epidermal growth factor, fibroblast growth factor, insulin-like growth factor, vascular endothelial growth factor, ascorbic acid, and hydrocortisone (growth factor-free medium) for 16 h were stimulated with various concentrations of LPS and incubated for the desired times. To examine the effect of SB 203580 on p38 MAP kinase activity and IL-8 expression in HPAECs, the cells that had been preincubated with growth factor-free medium with or without SB 203580 for 1 h were stimulated with LPS and cultured for the desired times at 37°C in humidified 5% CO_2. The cells for analysis of p38 MAP kinase activity were lysed at 10 min after stimulation with LPS and the culture supernatants for determination of IL-8 protein were harvested at 24 h, centrifuged, and the culture supernatants were collected, filtrated with a Millipore filter, and stored at −80°C until assay. The cells for analysis of IL-8 mRNA expression were collected at 6 h and stored at −80°C until analysis of mRNA expression.

Measurement of IL-8. The concentrations of IL-8 in the culture supernatants from HPAECs were measured by commercially available enzyme-linked immunosorbent assay kits (Amersham International, Aylesbury, UK). Enzyme-linked immunosorbent assay was performed according to the manufacturer’s instructions. All samples were assayed in duplicate.

Western Blot Analysis of p38 MAP Kinase Phosphorylation. Threonine and tyrosine phosphorylation of p38 MAP kinase was analyzed by commercially available kits (PhosphoPlus p38 MAP kinase antibody kit; New England Biolabs, Inc., Beverly, MA). The kit uses antiphospho-p38 MAP kinase that is specific for phosphorylated threonine and tyrosine of p38 and does not cross-react with phosphorylated threonine and tyrosine of extracellular signal-regulated kinase-1 or -2 or c-Jun-NH2-terminal kinase. Analysis of threonine and tyrosine phosphorylation of p38 MAP kinase was performed according to the manufacturer's instructions. Briefly, after separating proteins from the cell lysate by 15% SDS-polyacrylamide gel electrophoresis (PAGE), the cell lysate containing 10 μg of protein was electrophoretically transferred to nitrocellulose membrane and the membrane was blotted with a specific antibody to phosphorylated threonine and tyrosine of p38 MAP kinase. To show the amounts of p38 MAP kinase immunoblotted, blots were stripped and reprobed with phosphorylation-state independent p38 MAP kinase-specific antibody to determine total p38 MAP kinase levels.

p38 MAP Kinase Assay. The activity of p38 MAP kinase was analyzed by commercially available kits (p38 MAP kinase assay kit; New England Biolabs Inc.). The kit uses two different antibodies, anti-p38 MAP kinase antibody that is specific for p38 MAP kinase and does not cross-react with extracellular signal-regulated kinase-1 or -2 or c-Jun-NH2-terminal kinase, and antiphospho-specific activating transcription factor (ATF)-2 antibody to detect p38 MAP kinase-induced phosphorylation of ATF-2. p38 MAP kinase activity was analyzed according to the manufacturer’s instructions. Briefly, the cell lysate containing 200 μg of protein was incubated with antiphospho-p38 MAP kinase antibody to selectively immunoprecipitate p38 MAP kinase from the cell lysates and the immunoprecipitates were incubated with ATF-2 fusion protein in the presence of ATP, which allowed immunoprecipitated active p38 MAP kinase to phosphorylate its substrate, ATF-2. The samples were separated by a 15% SDS-PAGE, transferred to membranes, and blotted with antiphospho-specific ATF-2 antibody.

Northern Blot Analysis. Total RNA was prepared with an RNA extraction kit (RNA zol B; Cinna, Friendswood, TX) with the aid of guanidine thiocyanate-phenol-chloroform extraction methods. Total RNA (10 μg) was denatured in a solution containing formaldehyde and formamide, and electrophoresed in a 1% agarose gel containing formaldehyde (Thomas, 1983). Then it was capillary-transferred onto a nylon membrane (Hybond N; Amersham International). The membrane was prehybridized with rapid hybribuffer (Amersham International) and then hybridized with 32P-labeled probes for 2 h at 65°C. The probe used in this study were the PstI-PstI fragments of cDNA for β-actin and the full length of cDNA for IL-8, which was kindly provided by Dr. Koji Matsushima (Tokyo University School of Medicine, Department of Hygiene; Mukaida et al., 1989). After hybridization, the membrane was washed with standard saline citrate containing SDS and then autoradiographed with Kodak XAR film at −70°C.

Statistical Analysis. Statistical significance was analyzed with ANOVA. P values <.05 were considered significant.

Results

LPS Induces IL-8 Production by Pulmonary Vascular Endothelial Cells. First, we examined a dose-dependent effect of LPS on IL-8 production by HPAECs. To this end, the culture supernatants from HPAECs stimulated with various concentrations of LPS were harvested at 24 h after cultivation (Fig. 1). The concentrations of IL-8 in the culture supernatants from LPS-stimulated culture increased in a dose-dependent manner.

LPS Induces p38 MAP Kinase Phosphorylation. To determine whether LPS could induce the threonine and tyrosine phosphorylation of p38 MAP kinase, HPAECs were stimulated for the desired times and p38 MAP kinase was immunoblotted. Immunoblot study showed that stimulation of the cells with LPS caused increases in the threonine and
tyrosine phosphorylation of p38 MAP kinase in a dose-dependent manner (Fig. 2a, top). To determine the time course of tyrosine phosphorylation of p38 MAP kinase, HPAECs were stimulated with 1000 ng/ml LPS for the desired times as indicated. Amounts of LPS-induced threonine and tyrosine phosphorylation of p38 MAP kinase increased at 5 min, sustained between 10 and 30 min, and returned to near basal levels at 60 min (Fig. 2b, top). Figure 2, a and b (bottom), showed that equal amounts of p38 MAP kinase protein were immunoblotted with phosphorylation-independent p38 MAP kinase-specific antibody regardless of dose of LPS and time of culture periods, indicating that LPS stimulation-induced increases in the threonine and tyrosine phosphorylation of p38 MAP kinase occurred in the absence of changes in p38 MAP kinase protein levels.

**SB 203580 Inhibits LPS-Induced p38 MAP Kinase Activity.** Activation of p38 MAP kinase is mediated by dual phosphorylation of the threonine residues and tyrosine residues of p38 MAP kinase (Raingeaud et al., 1995). In addition to analysis of phosphorylation of p38 MAP kinase, we examined whether LPS could induce p38 MAP kinase activity. p38 MAP kinase activity was analyzed by a specific immunoprecipitation with anti-p38 MAP kinase antibody after an in vitro kinase assay of its substrate ATF-2. As shown in Fig. 3, LPS induced p38 MAP kinase activity as demonstrated by the increased phosphorylation of ATF-2. SB 203580 inhibited LPS-induced increases in p38 MAP kinase activity.

**SB 203580 Inhibits LPS-Induced IL-8 Production.** LPS induced IL-8 production and SB 203580 inhibited LPS-induced p38 MAP kinase activity. These results suggested that LPS stimulation-induced IL-8 production might be mediated through p38 MAP kinase-dependent pathway. To test this possibility, HPAECs that had been preincubated with or without SB 203580 were stimulated with LPS, and the concentrations of IL-8 in the culture supernatants were determined at 24 h after cultivation. The concentrations of IL-8 in the culture supernatants from the cells cultured with LPS in the presence of SB 203580 were lower than those from the cells cultured with LPS in the absence of the SB 203580 (Fig. 4), indicating that SB 203580 inhibited LPS-induced IL-8 production.

**SB 203580 Inhibits LPS-Induced IL-8 mRNA Expression.** An inhibitory effect of SB 203580 on LPS-induced IL-8 protein production by HPAEC suggested that this action might have resulted from an inhibitory effect of this inhibitor on IL-8 gene expression. To test this possibility, HPAEC that had been preincubated with or without SB 203580 were stimulated with LPS and IL-8 mRNA expression was ana-

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**Fig. 1.** LPS induces IL-8 production. HPAECs were cultured either with medium or various concentrations of LPS, and the concentrations of IL-8 in the culture supernatants were determined at 24 h after cultivation. The data are expressed as the mean ± S.D. of five different experiments. *P < .01 compared with IL-8 concentrations in HPAECs cultured with medium.

**Fig. 2.** LPS induces the threonine and tyrosine phosphorylation of p38 MAP kinase. HPAECs were stimulated with various concentrations of LPS for 5 min (a) and were stimulated with LPS (1000 ng/ml) for the desired times as indicated (b). The lysates from HPAECs were separated by 15% SDS-PAGE, transferred to membranes, and blotted with a specific antibody to phosphorylated threonine and tyrosine of p38 MAP kinase (p38 MAPK-P; a and b, top). Blots shown in a and b (top) were stripped and reprobed with a phosphorylation-independent p38 MAP kinase-specific antibody to show the amounts of p38 MAP kinase blotted (p38 MAPK; a and b, bottom). Lane P, positive protein prepared from C-6 glioma cells stimulated with anisomycin for phosphorylated threonine and tyrosine of p38 MAP kinase; and lane N, negative protein prepared from C-6 glioma cells unstimulated with anisomycin. The amounts of p38 MAP kinase phosphorylation were quantified by NIH Image analyzer and are presented as the amounts of p38 MAP kinase phosphorylation relative to control cells treated without agonist (1.0; bottom graphs). Three identical experiments independently performed gave similar results.
lyzed at 6 h after cultivation (Fig. 5). IL-8 mRNA expression was up-regulated when HPAEC were stimulated with LPS (lane 3), whereas SB 203580 inhibited LPS-induced up-regulation of IL-8 mRNA expression (lane 4; LPS + SB 203580). The concentrations of dimethyl sulfoxide used in this study were 0.01%, which had no effect. Three identical experiments independently performed gave similar results.

Discussion

In the present study, we examined a role of p38 MAP kinase and the effect of SB 203580 as a selective inhibitor of p38 MAP kinase activity on LPS-induced IL-8 expression in HPAECs. The results showed that LPS induced IL-8 expression and the phosphorylation and activity of p38 MAP kinase in HPAECs. SB 203580 inhibited LPS-induced increases in p38 MAP kinase activity, IL-8 protein and mRNA expression in HPAECs. These results indicate that p38 MAP kinase plays an important role in LPS-activated signaling pathway that regulates IL-8 expression in HPAECs, and that SB 203580 might inhibit LPS-induced IL-8 expression at the level of transcription.

p38 MAP kinase is activated by various environmental stresses such as hyperosmotic shock, heat shock, cold shock, UV-irradiation, and inflammatory cytokines, and it plays an important role in apoptosis and cytokine expression. (Davis, 1994, Cuenda et al., 1995; Raingeaud et al., 1995; Shapiro and Dinarello, 1995; Matsumoto et al., 1998; Hashimoto et al., 1999a,b). In addition, LPS stimulates human peripheral blood monocytes (Manthey et al., 1998) and human vascular umbilical vein endothelial cells (Schumann et al., 1996) to activate p38 MAP kinase. In the present study, we showed that LPS induced the phosphorylation and activity of p38 MAP kinase in HPAECs. Collectively, these results indicated that p38 MAP kinase activation is a general event in LPS-signaling pathway in these cell types.

The specific inhibitor of p38 MAP kinase has been identified (Lee et al., 1994), providing effective tool for investigating the role of p38 MAP kinase in cellular signaling (Cuenda et al., 1995; Shapiro and Dinarello, 1995; Matsumoto et al., 1998; Hashimoto et al., 1999a,b). In the present study, SB 203580 was used as the specific inhibitor of p38 MAP kinase activity to elucidate the biologic function of p38 MAP kinase in LPS-induced IL-8 expression. SB 203580 inhibits p38 MAP kinase activity and IL-8 expression in LPS-stimulated cells, whereas SB 203580 did not affect p38 MAP kinase
activity and IL-8 production in unstimulated cells, showing that the lack of effect of SB 203580 on basal levels of p38 MAP kinase activity and IL-8 production. SB 203580 inhibits the catalytic activity of p38 MAP kinase by binding to the ATP site and subsequently phosphorylating its substrate (Young et al., 1997). SB 203580 might exert an inhibitory effect on the induced p38 MAP kinase activity and subsequent IL-8 production, but not on basal levels of p38 MAP kinase activity and IL-8 production under our experimental conditions. Alternatively, the lack of effect of this compound on basal levels of p38 MAP kinase activity and IL-8 production might result from the sensitivity of SB 203580 to inhibit p38 MAP kinase activity and subsequent IL-8 production. Regardless, these results may indicate a favored effect of SB 203580 on controlling p38 MAP kinase-mediated IL-8 production in inflammatory diseases.

ARDS is frequently seen in conjunction with Gram-negative bacteremia sepsis (Nogare, 1989). Several studies have suggested that IL-8 and neutrophils play an important role in the production and progression of ARDS and animal models of acute lung injury (Weiland et al., 1986; Hogg, 1987; Nogare, 1989; Matthay, 1990; Miller et al., 1992; Bernard et al., 1997; Hashimoto and Horie, 1994; Matsumoto et al., 1997; Mukaida et al., 1998). An anti-inflammatory treatment aimed at inhibiting and ameliorating the production of ARDS and animal models of acute lung injury have been studied, there is no established treatment to halt the production of ARDS (Metz and Sibbald, 1991; Meduri, 1996; Brett et al., 1998). The administration of a neutralizing antibody against IL-8 is effective in prevention of endotoxin-induced ARDS-like lung injury (Mukaida et al., 1998). Consequently, the inhibition of IL-8 production and the attenuation of neutrophil recruitment into the site of inflammation are important strategies for controlling acute lung injury. IL-8 produced by vascular endothelial cells has been suggested to contribute to the production of acute lung injury through recruitment of neutrophils into the site of inflammation. A selective inhibitor of p38 MAP kinase activity has been identified, providing effective tool for investigating the role of p38 MAP kinase in cytokine expression (Davis, 1994; Cuenda et al., 1995; Shapiro and Dinarello, 1995; Matsumoto et al., 1998; Hashimoto et al., 1999a,b). It has been reported that the administration of SB 203580 reduces mortality in murine model of LPS-induced endotoxin shock (Badger et al., 1996). In this study, we demonstrated that SB 203580 inhibited LPS-induced IL-8 expression in HPAECs. Thus, our results may indicate a favorable effect of SB 203580 on ARDS seen in Gram-negative bacteria sepsis. ARDS has high mortality and an effective treatment of ARDS has not been established (Metz and Sibbald, 1991; Meduri, 1996; Brett et al., 1998). In the present study, we shed light on elucidating the role of p38 MAP kinase in LPS-induced IL-8 expression in HPAECs, whereas a variety of cytokines have been suggested to be involved in the pathogenesis of ARDS (Hyers et al., 1991; Hashimoto and Horie, 1994; Meduri et al., 1995; Pugin et al., 1996; Mukaida et al., 1998). We showed that p38 MAP kinase regulates LPS-induced IL-8 expression and SB 203580 inhibited IL-8 expression in HPAECs. Although it is currently not known whether SB 203580 is capable of producing beneficial effects on ARDS, a strategy of inhibiting signal cascade, p38 MAP kinase, may apply to the therapy controlling ARDS. Further investigations are needed to clarify this point.

References


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