



N-acetylcysteine attenuates TNF- α -induced p38 MAP kinase activation and p38 MAP kinase-mediated IL-8 production by human pulmonary vascular endothelial cells

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1 We have previously shown that tumour necrosis factor- α (TNF- α) activates p38 mitogen-activated protein (MAP) kinase to produce interleukin-8 (IL-8) by human pulmonary vascular endothelial cells. Reactive oxygen species (ROS) including H₂O₂ generated by TNF- α can act as signalling intermediates for cytokine induction; therefore, scavenging ROS by anti-oxidants is important for the regulation of cytokine production. However, the effect of N-acetylcysteine (NAC), which acts as a precursor of glutathione (GSH) synthesis, on TNF- α -induced activation of p38 MAP kinase pathway and p38 MAP kinase-mediated IL-8 production by human pulmonary vascular endothelial cells has not been determined. To clarify these issues, we examined the effect of NAC on TNF- α -induced activation of p38 MAP kinase, MAP kinase kinase (MKK) 3 and MKK6 which are upstream regulators of p38 MAP kinase, and p38 MAP kinase-mediated IL-8 production.

2 Human pulmonary vascular endothelial cells that had been preincubated with NAC were stimulated with TNF- α and then the activation of p38 MAP kinase and MKK3/MKK6 in the cells and IL-8 concentrations in the culture supernatants were determined.

3 Intracellular GSH levels increased in NAC-treated cells.

4 NAC attenuated TNF- α -induced activation of p38 MAP kinase and MKK3/MKK6.

5 NAC attenuated p38 MAP kinase-mediated IL-8 production by TNF- α -stimulated cells.

6 These results indicate that the cellular reduction and oxidation (redox) regulated by intracellular GSH is critical for TNF- α -induced activation of p38 MAP kinase pathway and p38 MAP kinase-mediated IL-8 production by human pulmonary vascular endothelial cells, and we emphasize that anti-oxidant therapy is an important strategy for the treatment of acute lung injury.

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Abbreviations: ARDS, adult respiratory distress syndrome; EGF, epidermal growth factor; ERK, extracellular signal-related kinase; FBS, foetal bovine serum; FGF, fibroblast growth factor; GSH, glutathione; HPAEC, human pulmonary artery endothelial cell; IGF, insulin-like growth factor; IL, interleukin; IL-8, interleukin-8; JNK, c-Jun-NH₂-terminal kinase; MAP, mitogen-activated protein kinase; MKK, MAP kinase kinase; NAC, N-acetylcysteine; ROS, Reactive oxygen species; TNF- α , tumour necrosis factor- α ; VEGF, vascular endothelial growth factor

Introduction

Adult respiratory distress syndrome (ARDS), a form of acute lung injury, which is characterized by increased vascular permeability leading to pulmonary oedema and acute respiratory failure, is observed in severe insults such as bacteremia sepsis (Tate *et al.*, 1983; Nogare, 1989; Matthy, 1990; Hashimoto *et al.*, 1994). The pathogenesis of ARDS is complex and involves multiple inflammatory cells and mediators (Tate *et al.*, 1983; Nogare, 1989; Matthy, 1990; Hashimoto *et al.*, 1994). Neutrophils and their products including reactive oxygen species (ROS) have been suggested to play an important role in the production of acute lung injury (Chabot *et al.*, 1998). The extravasation and accumulation of neutrophils at the sites of injury depends upon adhesion to and migration through endothelial linings

(Hashimoto *et al.*, 1994). Interleukin-8 (IL-8) which displays chemotactic activity for neutrophils participates in sequestration of neutrophils into the sites of injury (Huber *et al.*, 1991). Vascular endothelial cells are well known to produce IL-8 (Mantovani *et al.*, 1989), therefore, it is an important issue to clarify the mechanism in the production of IL-8 by pulmonary vascular endothelial cells.

Many extracellular stimuli elicit the specific biological responses through the activation of mitogen-activated protein (MAP) kinase cascades (Davis, 1994). The activation of p38 MAP kinase that belongs to MAP kinase superfamily elicits a variety of biological responses including cytokine expression (Han *et al.*, 1994; Raingeaud *et al.*, 1995; Moriguchi *et al.*, 1996; Clerk *et al.*, 1998; Gon *et al.*, 1998; Matsumoto *et al.*, 1998a; Hashimoto *et al.*, 1999c). The mechanism of p38 MAP kinase activation has been extensively studied. p38 MAP kinase is activated by a variety of extracellular stresses. Among stresses, oxidative stresses can activate p38 MAP kinase cascade (Moriguchi *et al.*, 1996; Clerk *et al.*, 1998), and we

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have previously shown that (1) p38 MAP kinase regulates tumour necrosis factor- α (TNF- α)-induced IL-8 expression (Hashimoto *et al.*, 1999b); (2) N-acetylcysteine (NAC) inhibits IL-8 production (Matsumoto *et al.*, 1998b); and (3) a redox control protein, thioredoxin (TRX), negatively regulates p38 MAP kinase activation and p38 MAP kinase-mediated IL-6 expression (Hashimoto *et al.*, 1999a). These observations indicate that there may be a link between the cellular reduction/oxidation (redox) state and p38 MAP kinase-mediated cytokine expression. However, a role of cellular redox regulated by intracellular glutathione (GSH) in TNF- α -induced p38 MAP kinase activation and p38 MAP kinase-mediated IL-8 production by human pulmonary vascular endothelial cells have not been determined. In the present study, we examined the effect of NAC which acts as a precursor of GSH synthesis, thus scavenging ROS (Cotgreave *et al.*, 1991) on TNF- α -induced threonine and tyrosine phosphorylation of p38 MAP kinase and p38 MAP kinase-mediated IL-8 production by pulmonary vascular endothelial cells in order to clarify these issues. We simultaneously examined the effect of NAC on TNF- α -induced serine phosphorylation of MAP kinase kinase (MKK) 3 and MKK6 which are upstream regulators of p38 MAP kinase.

Methods

Reagents

NAC was obtained from Sigma Chemical Co. (St. Louis, MI, U.S.A.). The pyridinyl imidazole SB 203580, a specific inhibitor of p38 MAP kinase activity (Lee *et al.*, 1994), was obtained from Calbiochem-Novabiochem Corporation (La Jolla, CA, U.S.A.) and was dissolved in dimethyl sulphoxide. The final concentration of dimethyl sulphoxide used in this experiment was 0.01%. This had no effect on the results. Human recombinant TNF- α was kindly provided by Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan).

Cell culture

Human pulmonary arterial endothelial cells (HPAECs) derived from healthy normal subjects were used as pulmonary vascular endothelial cells, and were obtained from Clonetics (San Diego, CA, U.S.A.). The cells (1×10^4 cells ml^{-1}) were placed in a tissue culture dish (Falcon 1007; Falcon Labware, Oxnard, CA, U.S.A.) for Western blot analysis. Cells were placed onto 24-well flat-bottom tissue culture plate (Corning, Corning, NY, U.S.A.) for determination of cytokine production using vascular endothelial growth medium (EGM-2; Clonetics) containing 0.2% foetal bovine serum (FBS), gentamycin-amphotericin B, epidermal growth factor (EGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), ascorbic acid, heparin and hydrocortisone. Cells were incubated in humidified 5% CO_2 atmosphere at 37°C until subconfluence and the medium was replaced with EGM-2 medium without FBS, EGF, FGF, IGF, VEGF, ascorbic acid and hydrocortisone (growth factor free medium) for 16 h. To examine the effect of NAC on TNF- α -induced threonine and tyrosine phosphorylation of p38 MAP kinase, serine phosphorylation of MKK3/MKK6 and IL-8 produc-

tion, growth factor-starved HPAECs that had been preincubated with or without NAC for 1 h were stimulated with TNF- α , and cultured for the desired times as indicated. In order to examine the effect of SB 203580 on IL-8 production, growth factor-starved HPAECs that had been preincubated with or without SB 203580 for 1 h were stimulated with TNF- α , and cultured for 24 h in humidified 5% CO_2 atmosphere at 37°C. After 24 h of culture, the culture supernatants for determination of IL-8 protein were harvested, centrifuged and the supernatants retained, filtered through a Millipore filter (0.45 μm pore size; Millipore, Bedford, MA, U.S.A.) and stored at -80°C until assay.

Measurement of intracellular GSH and hydrogen peroxide

Intracellular GSH levels were measured using the methods described previously (Anderson, 1985). Briefly, the cells were washed with ice-cold PBS. After washing the cells, the cells were collected and suspended with ice-cold PBS, and then 10% trichloroacetic acid was added to the cell suspension. The mixture was centrifuged at 4°C, thereafter, the supernatant was extracted with diethylether. The resulting extract was incubated with 200 μM of NADPH (Sigma, St. Louis, MO, U.S.A.) and 1 unit ml^{-1} of GSH reductase (Sigma) for 10 min at 37°C, and then 1 mM of 5,5-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma) was added and the reaction was measured at 415 nm absorbance. Hydrogen peroxide (H_2O_2); intracellular H_2O_2 levels were measured using a fluorescent dye, 2',7'-dichlorofluorescein diacetate (DCFH-DA) as described previously (Ohba *et al.*, 1994). Briefly, the cells were stimulated with TNF- α for 5 min, and then medium was replaced with phosphate buffer saline (PBS) containing 5 μM of DCFH-DA. After 5 min of incubation with DCFH-DA, the fluorescence of intensity was measured by confocal laser microscopy (Olympus Kogyo Ltd., Tokyo, Japan). Relative fluorescence intensity was calculated using unstimulated control cells as standard.

Western blot analysis of p38 MAP kinase phosphorylation

Threonine and tyrosine phosphorylation of p38 MAP kinase was analysed by commercially available kits (PhosphoPlus p38 MAP kinase Antibody Kit, New England Biolabs, Inc., Beverly, MA, U.S.A.). The kit uses antiphospho-p38 MAP kinase that is specific for phosphorylated threonine and tyrosine kinase of p38 and does not cross-react with phosphorylated threonine and tyrosine of extracellular signal-regulated kinase (ERK) 1/2 or c-Jun-NH2-terminal kinase (JNK). 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. In order to show the amounts of p38 MAP kinase immunoblotted, blots were stripped and reprobed using phosphorylation-state independent p38 MAP kinase-specific antibody to determine total p38 MAP kinase levels.

Western blot analysis of MKK3/MKK6 phosphorylation

Serine phosphorylation of MKK3 and MKK6 kinase was analysed by commercially available kits (PhosphoPlus MKK3/MKK6 [Ser189/207] Antibody Kit, New England Biolabs). Phosphospecific MKK3/MKK6 antibody supplied in this kit selectively detects Ser189- and Ser207-phosphorylated MKK3 and MKK6, respectively. As the activation sites of both proteins are closely related, this antibody detects both phosphoproteins. Polyclonal antibodies were produced by immunizing rabbits with a synthetic phospho-Ser189 peptide (KLH coupled) corresponding with residues 185-194 of human MKK3. Analysis of serine phosphorylation of MKK3 and MKK6 was performed according to the manufacturer's instruction as described previously (Hashimoto *et al.*, 2000). Briefly, after separating proteins from the cell lysate by 15% SDS-polyacrylamide gel electrophoresis (PAGE), the cell lysate containing 10 μ g of protein was electrophoretically transferred to nitrocellulose membrane and the membrane was blotted with a specific antibody to phosphorylated MKK3/MKK6. In order to show the amounts of MKK3 immunoblotted, blots were stripped and reprobed using phosphorylation-state independent MKK3-specific antibody to determine total MKK3 levels.

Measurement of IL-8

The concentrations of IL-8 in the culture supernatants from HPAECs were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Amersham International, Aylesbury, U.K.). ELISA was performed according to the manufacturer's instruction. All samples were assayed in duplicate.

Statistical analysis

Statistical significance was analysed with ANOVA. *P* values <0.05 were considered significant. When statistical significance was reached, *post hoc* tests (Fischer's Protected Least Significant Difference, Scheff's *F*) were performed.

Results

Intracellular GSH and hydrogen peroxide levels

We measured the intracellular GSH levels and the intracellular H₂O₂ levels in order to confirm the intracellular GSH levels and the intracellular H₂O₂ levels in this study. When HPAECs were incubated with various concentrations of NAC for 1 h, the intracellular GSH increased in a concentration-dependent manner (Figure 1a). We also measured the intracellular H₂O₂ as measured by the oxidation of DCF and the pretreatment with NAC attenuated TNF- α -induced increases in the intracellular H₂O₂ (Figure 1b).

TNF- α activates p38 MAP kinase and NAC attenuates TNF- α -induced p38 MAP kinase activation

The increases in threonine- and tyrosine-phosphorylation of p38 MAP kinase reflect the activation state of p38 MAP

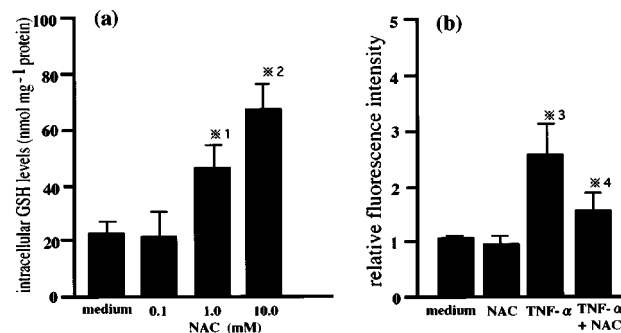


Figure 1 Cellular redox state. The intracellular GSH levels in HPAECs were measured at 1 h after incubation with various concentrations of NAC (a). The intracellular H₂O₂ levels in HPAECs that had been pretreated with or without 10 mM of NAC were measured at 5 min after TNF- α (10 ng ml⁻¹) stimulation (b). Relative fluorescence intensity was calculated using unstimulated control cells as standard. The results are expressed as mean \pm s.d. mean in three different experiments. *1 *P* < 0.05 compared with the intracellular GSH levels in the cells cultured with medium. *2 *P* < 0.01 compared with the intracellular GSH levels in the cells cultured with medium. *3 *P* < 0.01 compared with the intracellular H₂O₂ levels in the cells cultured with medium. *4 *P* < 0.05 compared with the intracellular H₂O₂ levels in the cells cultured with TNF- α .

kinase. Consequently, we examined the threonine- and tyrosine-phosphorylation of p38 MAP kinase. HPAECs were stimulated with TNF- α (10 ng ml⁻¹) for 0–60 min and p38 MAP kinase was immunoblotted. Amounts of phosphorylated threonine and tyrosine of p38 MAP kinase were increased at 5 min, these levels being sustained between 10 and 30 min; thereafter, they returned to near-basal levels at 60 min, indicating that threonine- and tyrosine-phosphorylation of p38 MAP kinase was transient (Figure 2, upper panel). In order to examine the effect of NAC on TNF- α -induced p38 MAP kinase activation, the cell that had been preincubated with various concentrations of NAC for 1 h were stimulated with TNF- α , and amounts of phosphorylated threonine and tyrosine of p38 MAP kinase were analysed at 5 min after stimulation. NAC attenuated TNF- α -induced p38 MAP kinase activation in a concentration-dependent manner. Amounts of phosphorylated threonine and tyrosine of p38 MAP kinase were lower in NAC-pretreated cells than those in NAC-untreated cells, indicating that the pretreatment with NAC attenuated TNF- α -induced p38 MAP kinase activation (Figure 3, upper panel). Lower panels of Figures 2 and 3 showed that equal amounts of p38 MAP kinase protein were immunoblotted using a phosphorylation state-independent p38 MAP kinase-specific antibody regardless of culture conditions. Ten mM of NAC also attenuated TNF- α -induced p38 MAP kinase activation at 15 and 30 min after stimulation (data not shown), indicating that the attenuation by NAC of TNF- α -induced p38 MAP kinase activation is not a delay.

TNF- α activates MKK3/MKK6 and NAC attenuates TNF- α -induced MKK3/MKK6 activation

In order to examine the effect of NAC on TNF- α -induced MKK3/MKK6 phosphorylation, the cells that had been

preincubated with 10 mM of NAC for 1 h were stimulated with TNF- α , and amounts of serine phosphorylation of

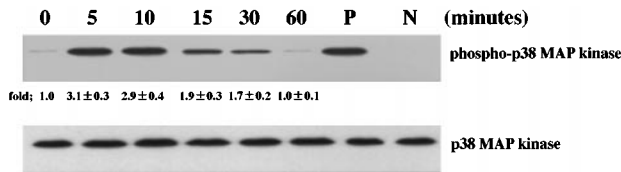


Figure 2 TNF- α activates p38 MAP kinase. HPAECs were stimulated with TNF- α (10 ng ml⁻¹) for the desired times as indicated. The HPAEC lysates were separated by a 15% SDS-PAGE, transferred to membranes, and probed with a specific antibody directed against the phosphorylated threonine and tyrosine of p38 MAP kinase (phospho-p38 MAP kinase; upper panel). These blots were then stripped and reprobed using a phosphorylation state-independent p38 MAP kinase-specific antibody to determine the amounts of p38 MAP kinase blotted (p38MAP kinase; lower panel). P: positive control, protein prepared from C-6 glioma cells stimulated with anisomycin to phosphorylate the threonine and tyrosine of p38 MAP kinase; N: negative control, protein prepared from C-6 glioma cells not stimulated with anisomycin. Blots are representative of three identical experiments independently performed. The amounts of phosphorylated p38 MAP kinase were quantitated by National Institutes of Health (NIH) image analyzer (National Institute of Health, Bethesda, MD, USA) and are presented as the amounts of phosphorylated p38 MAP kinase relative to control cells treated without agonist (1.0). Fold increase in amounts of phosphorylated p38 MAP kinase proteins as indicated as below are expressed as mean \pm s.d.mean in three different experiments.

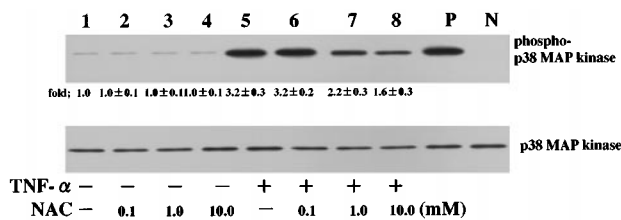


Figure 3 NAC attenuates TNF- α -induced p38 MAP kinase activation. HPAECs that had been pretreated either with medium or NAC (10 mM) for 1 h were stimulated with TNF- α (10 ng ml⁻¹) for 5 min. The HPAEC lysates were separated by a 15% SDS-PAGE, transferred to membranes, and probed with a specific antibody directed against the phosphorylated threonine and tyrosine of p38 MAP kinase (phospho-p38 MAP kinase; upper panel). These blots were then stripped and reprobed using a phosphorylation state-independent p38 MAP kinase-specific antibody to show the amounts of p38 MAP kinase blotted (p38MAP kinase; lower panels). The cells were cultured with medium (lane 1), NAC 0.1 mM (lane 2), NAC 1.0 mM (lane 3), NAC 10 mM (lane 4), TNF- α (lane 5), TNF- α + NAC 0.1 mM (lane 6), TNF- α + NAC 1.0 mM (lane 7), and TNF- α + NAC 10 mM (lane 8). Lane P, lane N and fold were described as Figure 2 legend. Blots are representative of three identical experiments independently performed. The amounts of phosphorylated p38 MAP kinase were quantitated by National Institutes of Health (NIH) image analyzer (National Institute of Health, Bethesda, MD, U.S.A.) and are presented as the amounts of phosphorylated p38 MAP kinase relative to control cells treated without agonist (1.0). Fold increase in amounts of phosphorylated p38 MAP kinase proteins as indicated as below are expressed as mean \pm s.d.mean in three different experiments. Amounts of phosphorylated threonine and tyrosine of p38MAP kinase were significantly lower in NAC-pretreated cells than those in NAC-untreated cells ($P < 0.01$).

MKK3/MKK6 were analysed at 5 min after stimulation. Amounts of phosphorylated serine of MKK3/MKK6 were lower in NAC-pretreated cells than those in NAC-untreated cells, indicating that the pretreatment with NAC attenuated TNF- α -induced MKK3/MKK6 phosphorylation (Figure 4, upper panel). Lower panels of Figure 4 showed that equal amounts of MKK3 protein were immunoblotted using a phosphorylation state-independent MKK3-specific antibody regardless of culture conditions.

NAC attenuates TNF- α -induced IL-8 production

Finally, we examined the effect of NAC on TNF- α -induced IL-8 production. Simultaneously, we examined the effect of SB 203580 on TNF- α -induced IL-8 production to confirm the role of p38 MAP kinase in IL-8 production. As shown in Figure 5, the pretreatment of the cells with NAC attenuated TNF- α -induced IL-8 production in a concentration-dependent manner. SB 203580 attenuated TNF- α -induced IL-8 production, indicating and confirming that p38 MAP kinase plays an important role in TNF- α -activated signalling pathway which regulates IL-8 production by HPAECs. These results indicated that cellular GSH increasing agent, NAC, negatively regulated TNF- α -induced p38 MAP kinase activation and p38 MAP kinase-mediated IL-8 production. The total number of cells and cell viability determined by means of the trypan blue exclusion dye assay, which was performed after 24 h of culture for the determination of IL-8 concentrations, did

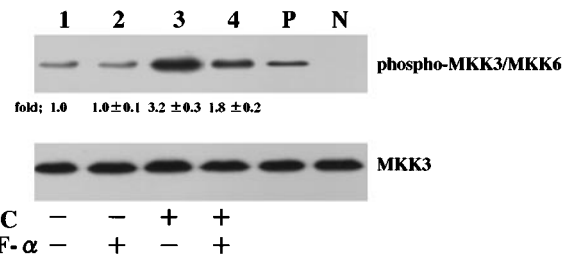


Figure 4 NAC attenuates TNF- α -induced MKK3 and MKK6 activation. HPAECs that had been pretreated either with medium or NAC (10 mM) for 1 h were stimulated with TNF- α (10 ng ml⁻¹) for 5 min. The HPAEC lysates were separated by a 15% SDS-PAGE, transferred to membranes, and probed with a specific antibody directed against the phosphorylated serine of MKK3 and MKK6 (phospho-MKK3/MKK6; upper panel). These blots were then stripped and reprobed using a phosphorylation-state independent MKK3-specific antibody to determine total MKK3 levels (MKK3; lower panels). The cells were cultured with medium (lane 1), NAC 10 mM (lane 2), TNF- α (lane 3), TNF- α + NAC 10 mM (lane 4). Lane P: positive protein prepared from NIH3T3 cells stimulated with UV treatment for phosphorylated serine of MKK3 and MKK6; Lane N: negative protein prepared from NIH3T3 cells without UV treatment. Blots are representative of three identical experiments independently performed. The amounts of phosphorylated MKK3/MKK6 were quantitated by National Institutes of Health (NIH) image analyzer (National Institute of Health, Bethesda, MD, U.S.A.) and are presented as the amounts of phosphorylated MKK3/MKK6 relative to control cells treated without agonist (1.0). Fold increase in amounts of phosphorylated MKK3/MKK6 proteins as indicated as below are expressed as mean \pm s.d.mean in three different experiments. Amounts of phosphorylated threonine and tyrosine of p38MAP kinase were significantly lower in NAC-pretreated cells than those in NAC-untreated cells ($P < 0.01$).

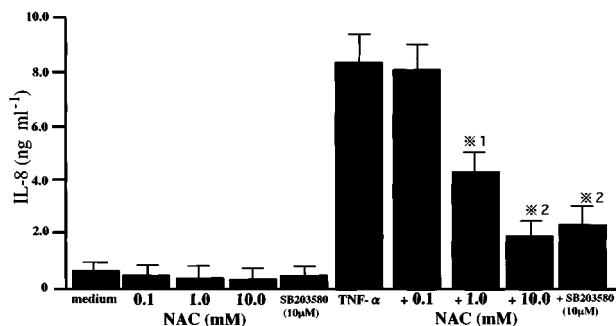


Figure 5 NAC attenuates TNF- α -induced IL-8 production. HPAECs that had been pretreated either with medium or various concentrations of NAC for 1 h were cultured with medium or TNF- α (10 ng ml⁻¹). Simultaneously, the cells that had been pretreated with SB 203580 (10 μ M) were stimulated with TNF- α (10 ng ml⁻¹) to examine the effect of SB 203580 on TNF- α -induced IL-8 production. The concentrations of IL-8 in the culture supernatants were determined after 24 h of culture. The results are expressed as mean \pm s.d. in five different experiments. *1 $P < 0.05$ compared with IL-8 concentrations in the cells cultured with TNF- α only. *2 $P < 0.01$ compared with IL-8 concentrations in the cells cultured with TNF- α only.

not differ with culture conditions (data not shown), suggesting that TNF- α -induced IL-8 production, its attenuation by NAC and SB 203580 did not result from cell cytotoxicity.

Discussion

In the present study, we examined the effect of NAC on TNF- α -induced p38 MAP kinase activation and p38 MAP kinase-mediated IL-8 production by HPAECs. The results showed that (1) Intracellular GSH levels increased in NAC-treated cells; (2) NAC attenuated TNF- α -induced activation of p38 MAP kinase and MKK3/MKK6; and (3) NAC attenuated p38 MAP kinase-mediated IL-8 production by TNF- α -stimulated cells. These results indicate that cellular redox regulated by intracellular GSH is critical for TNF- α -induced activation of p38 MAP kinase pathway and p38 MAP kinase-mediated IL-8 production by HPAECs.

The cellular redox changes have been implicated in the activation of MAP kinase superfamily (Guyton *et al.*, 1996; Moriguchi *et al.*, 1996; Laderoute *et al.*, 1997; Clerk *et al.*, 1998) and the induction of cytokine expression (Shreck *et al.*, 1991; 1992). ROS generated by TNF- α stimulation have been described to act as signalling intermediates for TNF- α -induced cytokine expression, since ROS are generated by TNF- α stimulation (Meier *et al.*, 1989; Shreck *et al.*, 1991; 1992) and antioxidants inhibit TNF- α -induced cytokine expression (Droge *et al.*, 1992; Talley *et al.*, 1995; Matsumoto *et al.*, 1998b). In addition, ROS *per se* can activate p38 MAP kinase (Moriguchi *et al.*, 1996; Clerk *et al.*, 1998). We have previously shown that p38 MAP kinase regulates TNF- α -induced IL-8 expression in human pulmonary vascular endothelial cells (Hashimoto *et al.*, 1999b). In addition, an increase in intracellular GSH levels has been shown to negatively regulate JNK activation (Wilhelm *et al.*, 1997). However, the role of cellular redox regulated by intracellular GSH in TNF- α -induced activation of p38 MAP kinase pathway and p38 MAP

kinase-mediated IL-8 production by human pulmonary vascular endothelial cells have not been determined. To clarify these issues, we examined the effect of NAC on TNF- α -induced activation of p38 MAP kinase and IL-8 production. In addition, we examined the effect of NAC on MKK3 and MKK6 activation in order to clarify the effect of NAC on the upstream kinase of p38 MAP kinase. The results showed that NAC attenuated TNF- α -induced activation of p38 MAP kinase activation and MKK3/MKK6 as well as p38 MAP kinase-mediated IL-8 production. These results indicate that TNF- α -induced activation of p38 MAP kinase pathway and p38 MAP kinase-mediated IL-8 production are negatively regulated by increasing intracellular GSH levels.

There are several possible mechanisms by which cellular redox regulated by intracellular GSH modulates TNF- α -induced p38 MAP kinase activation. p38 MAP kinase activation is mediated by dual phosphorylation of threonine and tyrosine by MKK3 and MKK6, which are upstream regulators of p38 MAP kinase (Clerk *et al.*, 1998). Recently, apoptosis signal-regulating kinase 1 (ASK1) was identified as a MAP kinase kinase kinase that activates p38 MAP kinase cascade (Ichijo *et al.*, 1997; Saitoh *et al.*, 1998). It has been shown that TNF- α -induced ASK1 activation is regulated by cellular redox state. TNF- α causes ASK1 activation through ROS-mediated dimerization of ASK1 and NAC reduces ASK1 activity (Gotoh *et al.*, 1998). Alternatively, it has been shown that thioredoxin (TRX) binds directly to the N-terminal portion of ASK1 and overexpression of TRX inhibits ASK1 activity (Saitoh *et al.*, 1998). TRX-mediated inhibition of ASK1 activity depends on their interaction and a reduced form of TRX is critical for the direct inhibition of ASK1 activity (Saitoh *et al.*, 1998). Conversely, when the cells are exposed to oxidative stress, the oxidation of TRX may disrupt their interaction and consequently activate ASK1. In the present study, we showed that TNF- α induced activation of p38 MAP kinase and MKK3/MKK6 in HPAECs, and that intracellular GSH modulated TNF- α -induced activation of p38 MAP kinase and MKK3/MKK6. Although we did not examine ASK1 activity, TNF- α -induced activation of p38 MAP kinase pathway might be mediated through ASK-1 activation by ROS-mediated ASK1 dimerization or dissociation of TRX from ASK1. The attenuation of TNF- α -induced activation of MKK3/MKK6 and p38 MAP kinase by NAC might result from the modulation of ASK1 activity by intracellular GSH. However, further study should be undertaken to clarify these issues.

The pathogenesis of acute lung injury is complex. The important role of the oxidant-antioxidant imbalance and inflammatory cytokines including TNF- α and IL-8 in the pathogenesis of acute lung injury has been described (Hyers *et al.*, 1991; Miller *et al.*, 1992). Oxidative stress-induced cellular damage participates in the production of acute lung injury (Chabot *et al.*, 1998). The present results may provide an alternative explanation for a role of oxidative stress in the production of acute lung injury. According to these observations, we emphasize that anti-oxidant therapy is an important strategy for the treatment of acute lung injury.

From the data presented here, we conclude that cellular redox regulated by intracellular GSH is critical for TNF- α -induced activation of p38 MAP kinase pathway and p38 MAP kinase-mediated IL-8 production by human pulmon-

ary vascular endothelial cells, and that anti-oxidant therapy is an important strategy for the treatment of acute lung injury.

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