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Cooling and Rewarming-Induced IL-8 Expression in Human Bronchial Epithelial Cells through p38 MAP Kinase-Dependent Pathway

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p38 mitogen-activated protein kinase (MAP) kinase is activated by various stresses; however, little is known about cold stress which has been shown to cause various inflammatory diseases. In the present study, we examined the effect of cold stimulation on interleukin-8 (IL-8) expression and a role of p38 MAP kinase in IL-8expression in human bronchial epithelial cells (BEC) in order to clarify the mechanism in hypothermic temperature-induced inflammation. The results showed that cold stimulation induced tyrosine phosphorylation of p38 MAP kinase but not IL-8 expression. IL-8 expression in BEC was induced when the temperature of incubation changed from 1 °C to 37 °C (cooling and rewarming). The specific p38 MAP kinase inhibitor SB 203580 inhibited cooling and rewarming-induced IL-8 expression, indicating that cooling and rewarming-induced IL-8 expression in BEC was mediated through p38 MAP kinase-dependent pathway.

Key Words: **cold stress; p38 MAP kinase; bronchial epithelial cells.**

The mitogen-activated protein (MAP) kinases are important mediators of signal transduction from the cell membrane to the nucleus. Several subgroups of mammalian MAP kinases have been molecularly characterized: extracellular-signal regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK) and p38 MAP kinase (1). p38 MAP kinase which is mammalian homologue of the HOG-1 of Saccharomyces cerevisiae (1–3), is activated by proinflammatory cytokines and environmental stresses (1–5). As for environmental stresses, osmotic stress, heat shock and UV irradiation are known tocause the phosphorylation and activation of p38 MAP kinase; however,little is known about the effect of cold stress on p38 MAP kinase activation.

Cold stress causes various inflammatory processes. The hypothermictemperature of airway resulting from heat loss has a potential for contributing the feature of exercise-induced bronchoconstriction (EIB) (6, 7). The cold preservation and rewarming-induced injury often occurs after organ transplantation (8). Although the mechanismin the cold stimulation-mediated production of inflammation and injury has been extensively investigated, little is known about the cold stimulation-induced signal transduction pathway. In the presentstudy, we examined the effect of cold stimulation on the activation of p38 MAP kinase in bronchial epithelial cell (BEC) lines and the expression of interleukin-8 (IL-8) which is well known to be expressed in BEC and cause airway inflammation (9) in order to clarify the role of p38 MAP kinase in cold stimulation-induced production of inflammation in the lung.

MATERIALS AND METHODS

Cells and reagents. BEC lines, NCI-H_{292}, were obtained from American Type Culture Collection (Rockville, MD). NCI-H_{292} were grown in culture medium which is RPMI 1640 (Nissui Co. Ltd., Tokyo, Japan) supplemented with 10 % heat-inactivated fetal calf serum (FCS; Mitsubishikasei, Co. Ltd., Tokyo, Japan), streptomycin and penicillin (Meiji Pharmaceutical Co. Ltd., Tokyo, Japan). The pyridinyl imidazole SB 203580, the specific p38 MAP kinase inhibitor (10), was kindly provided by Smithkline Beecham and was dissolved indimethyl sulfoxide.

Cell culture. BEC were placed onto Falcon 1007 (Oxnard, CA) tissueculture plate for western blot analysis of p38 MAP kinase, 24-well flat bottomed tissue culture plate (Corning, Corning, NY) for IL-8 production and Falcon 1005 tissue culture plate for northern blot analysis of IL-8 mRNA expression using culture medium and cultured at 37 °C in humidified 5 % CO_2 atmosphere. When BEC were grown in confluent conditions, the cells were stimulated. For western bolt study, to examine the effect of cold stimulation on tyrosine phosphorylation of p38 MAP kinase, BEC were stimulated either with cold (1 °C) RPMI 1640 without FCS (medium) and incubated on ice or warmed medium (37 °C) and incubated at 37 °C for various times. After the end of stimulation, p38 MAP kinase was immunoblotted. For cytokine expression and the effect of SB 203580, in preliminary experiments, the cells were cultured either with cold medium on ice or warmed medium at 37 °C for 1, 2, 4, 8 and 24 hours and IL-8 concentrations in the supernatants were determined

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by ELISA. As the results, cold stimulation per se did not induced IL-8 production (data not shown). Consequently, BEC that had been cultured with coldmedium on ice were rewarmed with fresh warmed medium (cooling and rewarming) and cultured at 37 °C in humidified 5 % CO_2 atmosphere inorder to examine cold stimulation-induced IL-8 expression and the effect of SB 203580 on it. For the control, after stimulation with warmed medium, warmed medium was replaced with fresh warmed medium and cultured at 37 °C in humidified 5 % CO atmosphere. In order to examine the effect of SB 203580, BEC were preincubated with or without SB 203580 for 1 hour at 37 °C before stimulation with cold medium. For the determination of IL-8 protein production, the culture supernatants were harvested at 24 hours after stimulation either with cold or warmed medium, centrifuged and the supernatants were collected, filtrated with a milliporefilter and stored at −80 °C until assay. For the analysis of IL-8 mRNA expression, BEC were harvested at 6 hours after stimulation either with cold or warmed medium.

Western blot analysis of p38 MAP kinase. The tyrosine phosphorylation of 38 MAP kinase was analyzed by commercially available kits (PhosphoPlus p38 MAPK Antibody Kit,New England Biolabs, Inc., Beverly, MA). Analysis of tyrosine phosphorylation ofp38 MAP kinase was performed according to the manufacturer's instruction. Briefly, BEC that had been washed with cold Tris buffered saline were lysed in SDS buffer (62.5 mM Tris-HCl, pH 6.8, 2 % w/v SDS, 10 % glycerol, 50 mM DTT, 0.1 % w/v bromphenol blue) for 15 minutes on ice and sonicated for 2 seconds to shear DNA. The samples were heated in a boiling water bath for 5 minutes to fully denature the proteins prior to electrophoresis and then centrifuged at 12,000 × g for 5 minutes to remove insoluble debris. After separating proteins from cell lysate by 15 % SDS-PAGE, proteins wereelectrophoretically transferred to nitrocellulose membrane and then membrane was washed with 0.1 % Tween-20 supplemented with Tris-buffered saline (washing buffer). To block nonspecific protein binding, the membrane was incubated with 0.1 % Tween-20 supplementedwith Tris-buffered saline containing 5 % w/v nonfat dry milk for 3 hours at room temperature. It was then incubated with specific antibody to phosphorylated tyrosine of p38 MAP kinase (affinity purified rabbit polyclonal IgG) at 1:1000 dilution in 0.1 % Tween-20 supplemented with Tris buffered saline containing 5 % BSA at 4 °C overnight with gentle shaking. After washing with washing buffer three times, it was incubated with the horseradish peroxidase-conjugated anti-rabbit antibody (1:2000) and horseradish peroxidase-conjugated anti-biotin antibody (1:1000) to detect biotinylated protein markers for 1 hour with gentle shaking at room temperature and then washed three times with washing buffer. Blots were incubated with enhanced chemiluminescence solution (LumiGLO) for 1 minutes at room temperature and exposed on Kodak XAR film. In order to show the amounts of p38 MAP kinase preticipated, blots were stripped and reprobed using phosphorylation-state independent p38 MAP kinase-specific antibody to determine total p38 MAP kinase levels (affinity purified rabbit polyclonal IgG).

Measurement of IL-8. The concentration of IL-8 in the culture supernatants from BEC were measured by commercially available ELISA kits (Amersham). ELISA was performed according to the manufacturer's instruction. All samples were assayed in duplicate.

Northern blot analysis. Total RNA was prepared with an RNA extraction kit (RNA zol B. TM Cinna Scientific, Friedswods, TX) using the acid guanidine thiocyanate-phenol-choloroform extraction methods. Total RNA (10 μg) was denatured in a solution containing formaldehyde and folmamide, and electrophoresed in a 1 % agarose gel containing fromaldehyde (11). Then it was cappillary-transferredonto a nylon membrane (Hybond N, Amersham, UK). The membrane was prehybridized with rapid hybribuffer (Amersham) and then hybridized with [^{32}P]-labelled probes for 2 hours 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 (12) which was kindly provided by Dr. Koji Matsushima (Tokyo University School of Medicine, Departmentof Hygiene). After hybridization, the membrane were washed with SSC containing SDS and then autoradiographed with Kodax XAR film at −70 °C.

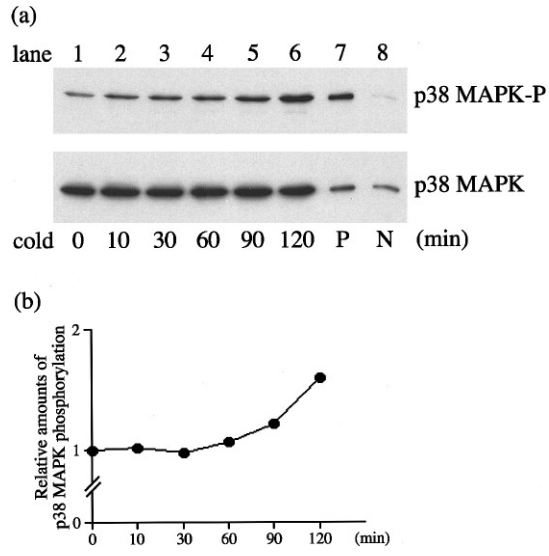

FIG. 1. Tyrosine phosphorylation of p38 MAP kinase in BEC stimulatedwith cold medium. BEC lines, NCI-H_{292}, were stimulated with cold RPMI 1640 without FCS (medium) and cultured on ice for various timesas indicated. The lysates from BEC were separated by a 10 % SDS-polyacrylamide gel, transferred to membranes, and blotted with a specific antibody to phosphorylated tyrosine of p38 MAP kinase (p38 MAPK-P; upper panel of Fig. 1a, lanes 1–8). Blots shown in upper panel of Fig. 1a were stripped and reprobed using a p38 MAP kinase-specific antibody to show the amounts of p38 MAP kinase blotted (p38 MAPK; lower panel of Fig. 1b). Lane 7 (P), positive protein prepared from C-6 glioma cells stimulated with anisomycin for phosphorylated tyrosine of p38 MAP kinase; Lane 8 (N), negative protein prepared from C-6 glioma cells unstimulated with anisomycin. Three identical experiments independently performed gave similar results.

Statistical analysis. Statistical significance was analyzed using the analysis of variance (ANOVA). P value less than 0.05 was considered significant.

RESULTS

Cold Stimulation Causes Tyrosine Phosphorylation of p38 MAP Kinase

To determine whether cold stimulation induces tyrosine phosphorylation of p38 MAP kinase, BEC were stimulated with cold medium for the desired times and p38 MAP kinase was immunoblotted. Immunoblot studies of lysate of BEC with a specific antibody to phosphorylated tyrosine of p38 MAP kinase showed that cold stimulation induced tyrosine phosphorylation of p38 MAP kinase. The maximal amounts of phosphorylated tyrosine of p38 MAP kinase proteinwas observed at 120 minutes (Fig. 1a, upper panel and Fig. 1b). Immunoblotting of lysate of cold medium-stimulated BEC with a p38 MAP kinase-specific antibody showed that equal amounts of p38 MAP kinase protein were immu-

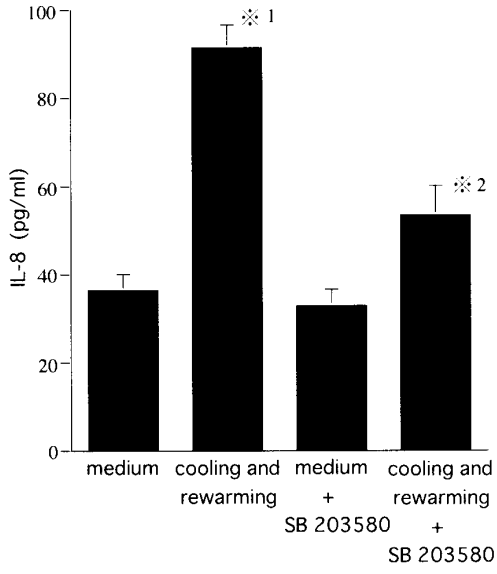

FIG. 2. Cooling and rewarming stimulation induces IL-8 production by BEC and SB 203580 inhibits it. BEC lines, NCI-H_{292} that had been preincubated with or without SB 203580 (10 μM) for 1 hour werecultured with cold medium on ice for 1 hour followed by warmed medium at 37 °C (cooling and rewarming) in humidified 5 % CO_2 atmosphere. For the control, BEC that had been preincubated with SB 203580 (10 μM) for 1 hour were cultured with warmed medium instead of cold medium for 1 hour followed by warmed medium (medium). The concentrations of IL-8 in the culture supernatants were determined at 24 hours after cultivation as described in Materials and Methods. The results are expressed as the mean ± s.d. in five different experiments. *1; $p<0.01$ compared with IL-8 concentrations in warmed medium culture (medium). *2; $p<0.01$ compared with IL-8 concentrations in cooling and rewarming culture in the absence of SB 203580. The concentration of dimethyl sulfoxideused in this study was 0.01%, which was without no effect.

noblotted at all times (Fig. 1b, lower panel), indicating that cold stimulation-induced increase in tyrosine phosphorylation of p38 MAP kinase occurred in the absence of changes in p38 MAP kinase protein levels. When BEC were stimulated with warmed medium instead of cold medium, tyrosine phosphorylation of p38 MAP kinase was not observed at any time of culture periods (data not shown).

Cooling and Rewarming-Stimulation Induces IL-8 Production and SB 203580 Inhibits It

Since cold stimulation per se did not induce IL-8 production, BEC that had been cultured either with cold medium on ice for 1 hour were rewarmed with fresh warmed medium (cooling and rewarming) and cultured at 37 °C. For the control, after cultivation of BEC with warmed medium instead of cold medium for 1 hour, the cells were cultured with warmed medium at 37 °C. As shown in Fig. 2, the concentrations of IL-8 in the culture supernatants from BEC cultured with cold medium followed by warmed medium (cooling and rewarming) were higher than those in the control culture (medium), indicating that cooling and rewarming induced IL-8 production. For analysis of the role of p38 MAP kinase in cooling and rewarming-induced IL-8 production, the effect of SB 203580 on cooling and rewarming-induced IL-8 production was examined. As shown in Fig. 2, SB 203580 inhibited cooling and rewarming-induced IL-8 production.

Cooling and Rewarming-Stimulation Induces IL-8 mRNA Expression and SB 203580 Inhibits It

In order to determine the levels at which SB 203580 inhibited IL-8 protein production by BEC, we performed northern blot analysis. As shown in upper panel of Fig. 3a, IL-8 mRNA expression was upregulated in BEC cultured with cold medium for 60 minutes followed by warmed medium (cooling and rewarming; lane 3) compared with the levels of IL-8 mRNA expression in the BEC cultured with warmed medium (lane 1). When BEC were cultured with cold medium for a shorter time (30 minutes, lane 2) followed by warmed medium or a longer time (90 minutes, lane 4) followed by warmed medium, the upregulation of IL-8 mRNA expression was not seen, indicating that atime for the stimulation of the cells with cold medium was critical for the induction of IL-8 mRNA expression. The levels of cooling andrewarming-induced IL-8 mRNA expression in SB 203580-pretreated BEC (lane 7) were apparently lower than those in SB 203580-untreated BEC (lane 3), indicating that SB 203580 inhibited cooling and rewarming-induced IL-8 mRNA expression. The levels of β-actin mRNA expressiondid not vary significantly with culture conditions (Fig. 3b, lower panel). The cell viability at the end of culture period of each

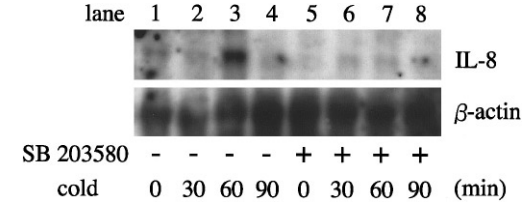

FIG. 3. Cooling and rewarming stimulation-induced IL-8 mRNA expression in BEC and SB 203580 inhibits it. BEC lines, NCI-H_{292} were cultured with cold medium on ice for 30, 60 or 90 minutes or warmed medium for 60 minutes in the presence or absence of SB 203580 (10 μM) and then cold medium and warmed medium were replaced with warmed medium and the cells were cultured for 6 hours, and the IL-8 mRNA expression (Fig. 3a) and the β-actin mRNA expression (Fig. 3b)were analyzed by northern blotting analysis as described in Materials and Methods. The cells were cultured in the absence of SB 203580 (lanes 1-4) or presence of SB 203580 (lanes 5-8). Lanes 1 and 5 represent the cells cultured with warmed medium for 60 minutesinstead of cold medium. Lanes 2 and 6, 3 and 7, and 4 and 8 represent the cells cultured with cold medium for 30, 60 or 90 minutes, respectively. The concentration of dimethyl sulfoxide used in this study was 0.01%, which was without no effect. Three identical experiments independently performed gave similar results.

experiment determined by trypan blue exclusion assay did not differ with culture conditions indicated in Figures 1 through 3, indicatingthat the cooling and rewarming-induced IL-8 expression and SB 203580-mediated inhibition of IL-8 expression did not result from inhibitor- and cooling and rewarming-induced cell cytotoxicity (data not shown).

DISCUSSION

In the present study, we examined the effect of cold stimulation on tyrosine phosphorylation of p38 MAP kinase and IL-8 production inhuman BEC. The results showed that cold stimulation induced tyrosinephosphorylation of p38 MAP kinase but not IL-8 expression. IL-8 expression in BEC was induced when the temperature of incubation changed from 1 °C to 37 °C (cooling and rewarming). SB 203580 as thespecific p38 MAP kinase inhibitor inhibited cooling and rewarming-induced IL-8 expression, indicating that cooling and rewarming-induced IL-8 expression in BEC was mediated through p38 MAP kinase-dependent pathway.

Cold stimulation *per se* induced tyrosine phosphorylation of p38 MAP kinase, but not IL-8 expression. However, cooling and rewarming induced IL-8 expression. These results indicated that a transit cold stimulation followed by thermal energy is absolutely necessary for the induction of IL-8 expression. It has been shown that cold stimulation induces the mRNA expression of several subgroups of MAP kinases in plant including ATMPK3 (*Arabidopsis thaliana* MAP kinase 3) which is structurally related to MAP kinase (13) and a transit cold stimulation followed by recovery at 37 °C induces heat shock protein expression in human lung fibroblasts (14). In the present study, we examined the effect of cold stimulation on tyrosine phosphorylation of p38 MAP kinase and the effect of a transit cold stimulation followed by recovery at 37 °C on IL-8 expression were examined using human bronchial epithelial cells, since it has been shown that the alternation of airway temperature, at least in part, contribute to the production of EIB (6, 7). The alternation of airway temperature resulting from heat loss and the change in airway-osmolarity resulting from water loss during exercise have been described to have a potential for contributing the feature of EIB. As for the alternation of airway temperature, breathing cold air perse increases the bronchial responsiveness to inhaled histamine in asthmatics and enhances EIB (15, 16). Alternatively, airway cooling alone is insufficient to produce airway obstruction, and airway cooling and rewarming which occurs in the immediate recovery period is necessary for the production of EIB (17, 18). EIB occurs typically 5 to 15 minutes after cessation of exercise. But in some instance, the late response occurs 3 to 13 hours after completing exercise (7). The pathophysiology of the late phase response has been investigated and the role of inflammatory mediators and cells have been described to contribute the development of the late phase response (7, 19). In the present study, we showed cooling and rewarming-induced IL-8 expression in BEC. IL-8 exhibit a variety of biological activity including chemotactic activity for neutrophils, T lymphocytes and eosinophils (20, 21). Taken together with the biological activity of IL-8 and our results, BEC may contribute to the development of EIB, especially the late phase response of EIB, through the recruitment of inflammatory cells. Finally, our results with cooling and rewarming-induced IL-8 expression through p38 MAP kinase-dependent pathway may prove, at least in part, the signal transduction pathway in the production of cold preservation and rewarming-induced organ injury.

From the results presented here, we conclude that cooling and rewarming stimulation induces IL-8 expression in BEC through p38 MAPkinase-dependent pathway. These results provide new evidences on themechanism in cooling and rewarming stimulation-induced airway inflammation and a strategy for treatment of airway inflammation with the specific p38 MAP kinase inhibitor.

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