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Interleukin-1β Induces *MUC2* Gene Expression and Mucin Secretion via Activation of PKC-MEK/ERK, and PI3K in Human Airway Epithelial Cells

Interleukin 1β (IL-1β), a proinflammatory cytokine, is related with inflammatory diseases and it up-regulates *MUC2* gene expression and mucin secretion. This study was designed to investigate the signal transduction pathway of the IL-1β-mediated *MUC2* gene expression and mucin secretion in human airway epithelial cells. In cultured human airway NCI-H292 epithelial cells, the steady state of the mRNA level of *MUC2* gene expression and mucin secretion induced by IL-1β were determined by reverse transcriptase-polymerase chain reaction (RT-PCR), enzyme immunoassay, and immunoblot analysis. To observe the signal pathway of the IL-1β-mediated *MUC2* gene expression and mucin secretion, we used several specific inhibitors. PD98059 (MEK/ERK inhibitor) suppressed IL-1β-mediated *MUC2* gene expression and mucin secretion, while SB203580 (p38 inhibitor) did not. Ro31-8220 (PKC inhibitor) inhibited IL-1β-mediated *MUC2* gene expression and mucin secretion. It inhibited ERK phosphorylation, but did not inhibit p38 phosphorylation. LY294002 (PI3K inhibitor) also suppressed MUC2 expression, but did not inhibit any MAPKs phosphorylation. These results suggest that the IL-1β-mediated *MUC2* gene expression and mucin secretion in NCI-H292 cells are regulated through activation of the PKC-MEK/ERK pathway, and that PI3K is also involved in the IL-1β-mediated *MUC2* gene expression and mucin secretion.

Key Words : *Mucins; Interleukin-1; Cytokines; Epithelial Cells; Signal Transduction*

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INTRODUCTION

Mucin is a macromolecular glycoprotein produced in secretory epithelial cells of the respiratory, gastrointestinal, and the reproductive tract (1). Mucin is an important component of airway mucus secretion. Several mucin genes regulate mucin secretion in human body. Mucus hypersecretion is a major problem of the inflammatory airway diseases such as chronic bronchitis, asthma, cystic fibrosis, and chronic rhinosinusitis (1). Various cytokines and inflammatory mediators stimulate mucus hypersecretion, directly or indirectly (2, 3). Among these cytokines and inflammatory mediators, tumor necrosis factor-α (TNF-α) (4, 5), interleukin-1β (IL-1β) (6), and lipopolysaccharide (LPS) (7) activate mucin secretion by up-regulating expression of the mucin genes (2, 3, 7-9). Thirteen mucin genes have been identified. Among these mucin genes, the respiratory mucin genes are *MUC2, MUC4, MUC5AC, MUC5B, MUC6, MUC7,* and *MUC8* (10-14). The *MUC5AC* gene is one of the most important genes in the respiratory tract. The *MUC2* gene is expressed at a low level in a normal condition, but the expression is increased in chronic bronchitis and cystic fibrosis (15).

Among the cytokines that have various inflammatory functions in acute and chronic upper respiratory tract infection, IL-1β is related with the pathogenesis of respiratory tract infection in upper airway diseases such as asthma and bronchitis (5, 16, 17). Our recent study (6) demonstrated that IL-1β stimulates the expression of *MUC2* gene and mucin secretion by transcriptional regulation. However, we could not delineate the specific signal pathway involved in the mediation of mucin secretion in that study.

Since it was reported that *Pseudomonas aeruginosa*-induced mucin overproduction requires nuclear factor κB (NF-κB) activation in epithelial cells (3), some researchers have studied to elucidate the signal transduction pathway involved in the expression of the mucin gene (3, 4, 7, 9, 12). Although a previous study (18) showed that IL-1β activates mitogen-activated protein kinase (MAPK) subgroups c-Jun NH_2 terminal

kinase (JNK) and extracellular signal-regulated kinase (ERK) in human articular chondrocyte, the signal pathway involved in the IL-1β-mediated *MUC2* gene expression and mucin secretion has not yet been understood clearly.

The present study was undertaken to investigate the signal transduction pathway involved in the IL-1β-mediated *MUC2* gene expression and mucin secretion. We observed whether protein kinase C (PKC), MAPKs (ERK, p38 and JNK), and phosphatidylinositol 3-kinase (PI3K) are involved in the IL-1β-mediated *MUC2* gene expression and mucin secretion in cultured human airway NCI-H292 epithelial cells.

MATERIALS AND METHODS

Cell Culture

The NCI-H292 airway epithelial cells (human pulmonary mucoepidermoid carcinoma cell line, American Type Culture Collection, Rockville, MD) were seeded at a density of 1×10^6 cells into 6 well plates. Cultures were maintained in the RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco BRL), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.When the cultures were confluent, the cells were incubated with RPMI 1640 medium containing 0.5% fetal calf serum for 24 hr. They were rinsed with phosphate buffered saline (PBS) and exposed to human recombinant IL-lβ (R&D Systems Inc., Minneapolis, MN) treatment. To study which signal transduction pathway is related to the MUC2 expression, some cultures were pretreated with specific inhibitors such as PD98059 (MEK/ERK inhibitor, Biomol Research Laboratories, Inc., Plymouth Meeting, PA), SB203580 (p38 inhibitor, Biomol Research Laboratories), Ro31-8220 (protein kinase C inhibitor, Biomol Research Laboratories), and LY294002 (PI3K inhibitor, Biomol Research Laboratories) at 1 hr before exposed to IL-1β and then incubated for 8 hr after treatment of IL-1β. Control cultures remained untreated. Total cellular RNA was extracted using a Tri-Reagent (Molecular Research Center, Cincinnati, OH). Cell lysates were prepared in PBS.

IL-1β was dissolved with PBS containing 0.1% bovine serum albumin, and each specific inhibitor was dissolved in dimethyl sulfoxide (DMSO) prior to addition to cell cultures. The final concentrations of DMSO or other vehicle solvents in the medium were less than 0.1%.

RT-PCR Analysis of the *MUC2* Gene

The method used to detect and quantify the *MUC2* mRNA level employed a modified technique of RT-PCR and has been described previously in detail (6). Briefly, total RNA was reverse transcribed into cDNA using random hexanucleotide primers and MULV reverse transcriptase (Perkin-Elmer, Morrisville, NC). The oligonucleotide primers for the PCR part of the procedure were designed on the basis of the published sequences of human *MUC2* (GenBank Accession No. L21998, 5′ primer: TGC CTG GCC CTG TCT TTG: 3′ primer: CAG CTC CAG CAT GAG TGC). The PCR consisted of 35 cycles of denaturation (95°C, 1 min), annealing (60°C, 1 min), and extension (72°C, 1 min) in the presence of 2.5 mM $MgC1_2$, and a final extension at 72°C for 20 min. The *MUC2* DNA fragment generated was 440-bp in size as expected. The oligonucleotide primers for the β2 microglobulin (β2M, used as a control gene for the RT-PCR) were purchased from Clontech (Palo Alto, CA) and generated 335-bp PCR fragment. Specific amplification of *MUC2* was confirmed by sequencing (dsDNA Cycle Sequencing System, Gibco BRL). PCR products were separated by electrophoresis through a 2% agarose gel in 1% Tris-boric acid-EDTA (TBE) buffer containing 50 ng/mL of ethidium bromide and photographed using a Polaroid type 55 film. The intensity of the bands was analyzed with a densitometer.

Immunoassay of the MUC2 Mucin

Cell lysates were prepared in PBS at multiple dilutions, and 50 μL of each sample was incubated at 40°C in a 96-well plate until dry. Plates were then washed three times with PBS, blocked with 2% bovine serum albumin for 1 hr at room temperature, washed again three times with PBS, and then incubated with a MUC2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) that has been diluted (1:100) with PBS containing 0.05% Tween-20. After 1 hr, the wells were washed three times with PBS, and then horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) was dispensed into each well, and after 4 hr, the plates were washed three times with PBS. Color was developed with a 3,3′,5,5′-tetramethylbenzidine peroxidase solution and stopped with $2N\text{-}H_2SO_4$. Absorbance was read at 450 nm. The amount of estimated MUC2 mucin was represented as % above control, which indicates the excess proportion over control.

Immunoblot Analysis for MAPKs and phospho-MAPKs

For analysis of the MAPKs and their activated form, phospho-MAPKs, the treated cells were washed with cold-PBS, scraped off, pelleted at 700 × g at 4°C, and the cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 nM NaCl, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail). The preparation was then clarified by centrifugation, and the supernatant was saved as a whole-cell lysate. Proteins (50 μg) were separated using 10% reducing SDS-polyacrylamide gel and electroblotted in 20% methanol, 25 mM Tris, and 192 mM glycine onto a nitrocellulose membrane. The membrane was then blocked with 5% non-fat dry milk in TTBS (25 mM Tris-HCl, 150 mM NaCl, and 0.2% Tween-20)

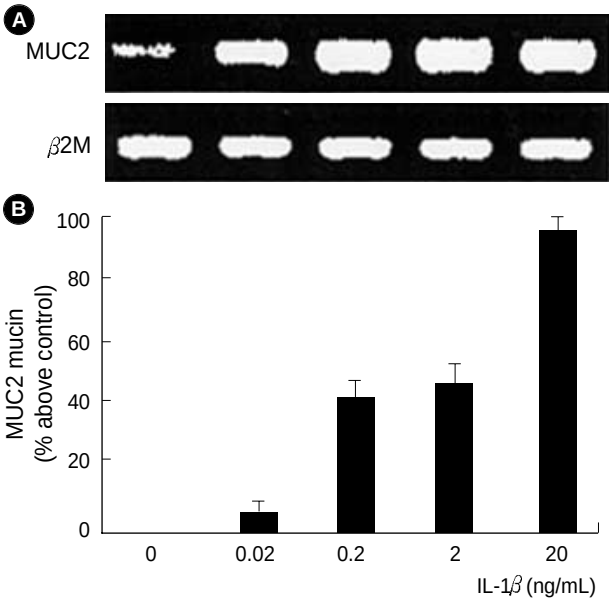

Fig. 1. Induction of the IL-1β-mediated *MUC2* gene expression and mucin secretion. (A) The NCI-H292 cells were treated with various concentrations of IL-1β (0.02, 0.2, 2, 20 ng/mL). The mRNA levels of *MUC2* and β2M were determined by RT-PCR. β2M is a positive internal control. The amounts of the RT-PCR products of *MUC2* were quantified using densitometry and expressed relative to the densities of β2M by the ratio of *MUC2*/β2M DNA bands. The data is representative of three independent experiments. (B) The *MUC2* mucin was determined by immunoassay. The data represent average values of three independent experiments plus standard deviation.

and then incubated with the indicated antibodies for 4 hr. ERK, phospho-ERK antibodies and p38, phospho-p38 antibodies were purchased from New England BioLabs (Beverly, MA). Subsequently, the membrane was washed and incubated for 1 hr with secondary antibodies conjugated to HRP, rewashed, and developed using an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech, Inc., Buckinghamshire, England).

RESULTS

Induction of the *MUC2* Gene Expression and Mucin Secretion by IL-1β

When the cultures were confluent, the cultured cells were incubated with IL-1β in the range of 0.02-20 ng/mL at 37°C for 8 hr. The total mRNA was extracted and subjected to RT-PCR analysis. As the dose of IL-1β was increased from 0.02 to 20 ng/mL, there was a parallel increase in the *MUC2* mRNA level. RT-PCR products of β2M mRNA were used as internal control (Fig. 1A). To define the regulation of mucin secretion by IL-1β at the protein level, we used immunoassay with a MUC2 monoclonal antibody. MUC2 mucin secretion was also increased in a dose-dependent manner, and to a maximal level at 20 ng/mL of IL-1β (Fig. 1B). These results of mucin secretion were consistent with gene expression data. These data were similar to those of our previous study (6).

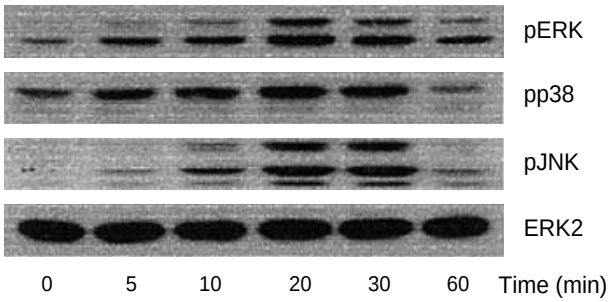

Fig. 2. Phosphorylation of three MAPKs (pERK, pp38, pJNK) according to time of IL-1β treatment. NCI-H292 cells were treated with 20 ng/mL of IL-1β for 5, 10, 20, 30, 60 min. Analysis of phosphorylation of each protein was done by immunoblot. ERK2 is a positive internal control.

Effect of MAPKs on the IL-1β-mediated *MUC2* Gene Expression and Mucin Secretion

To examine whether IL-1β activates MAPKs (ERK1/2, p38, JNK), the phosphorylation level of MAPKs was estimated by examining their phosphorylation in immunoblot with phospho-specific antibodies. All the three MAPKs phosphorylation by IL-1β were similar in their extent (Fig. 2). The maximum levels of phosphorylation induced by IL-1β, as indicated by immunoblotting, were observed at 20-30 min for three MAPKs. These results show that IL-1β activates ERK, p38, and JNK.

We evaluated whether the specific inhibitors of MAPKs appropriately blocked the phosphorylation of different MAPKs and which MAPK is involved in the *MUC2* gene expression and mucin secretion induced by IL-1β in NCI-H292 cells. After the cultured cells were pretreated with PD98059 (50 μM) or SB203580 (10 μM) for 1 hr, they were stimulated with IL-1β (20 ng/mL) for 8 hr. PD98059 completely inhibited IL-1β-stimulated ERK1/2 phosphorylation (Fig. 3A). Also, pretreatment of PD98059 inhibited the *MUC2* gene expression (Fig. 3B) and mucin secretion (Fig. 3C). SB203580 inhibited p38 phosphorylation (Fig. 4A), but did not suppress the IL-1β-mediated *MUC2* gene expression (Fig. 4B) or mucin secretion (Fig. 4C).

Effect of PKC on the IL-1β-mediated *MUC2* Gene Expression and Mucin Secretion

To observe whether PKC activation is related with the signal pathway of the IL-1β-induced *MUC2* gene expression and mucin secretion, Ro31-8220 (PKC inhibitor) was pretreated.

Pretreatment with Ro31-8220 (20 μM) for 1 hr inhibited the IL-1β-mediated *MUC2* gene expression and mucin secretion. To further determine whether PKC acts at upstream or downstream molecule of MAPKs, we tested the effect of PKC inhibitor on the IL-1β-activated MAPKs phosphorylation. When cells were pretreated with Ro31-8220 (20 μM) for 1 hr, only ERK1/2 phosphorylation induced by IL-1β (20 ng/mL) was attenuated, but p38 and JNK phosphorylation was not suppressed (Fig. 5B, C).

Effect of PI3K on the IL-1β-mediated *MUC2* Gene Expression and Mucin Secretion

To determine whether PI3K activation is involved in the IL-1β-induced *MUC2* gene expression and mucin secretion, the cells were pretreated with LY294002 (a PI3K inhibitor)

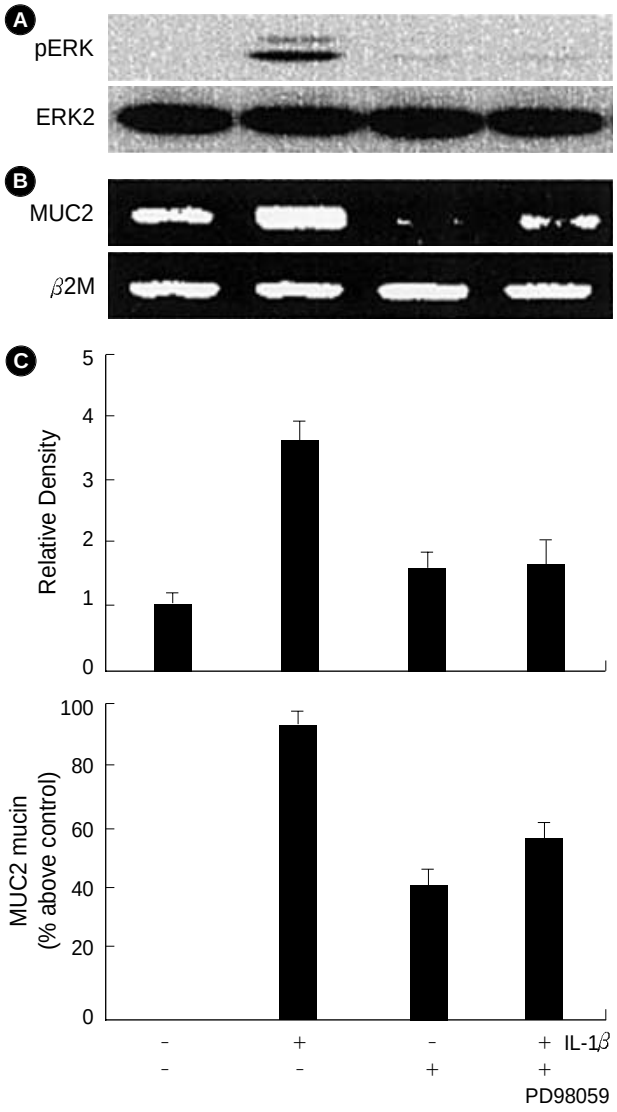

Fig. 3. Effects of MEK/ERK inhibitor, PD98059 on the expression of the IL-1β-mediated ERK phosphorylation (pERK) (A), *MUC2* gene expression (B), and mucin secretion (C). Analysis of ERK phosphorylation was done by immunoblot, *MUC2* gene by RT-PCR, and mucin by immunoassay. ERK2 is a positive internal control. The amounts of the RT-PCR products of *MUC2* gene were quantified using densitometry and expressed relative to the density of β2M by the ratio of the MUC2/β2M DNA bands. β2M is a positive internal control. The data A and B are acquired from three independent experiments. The data C represents average value of three independent experiments and standard deviation.

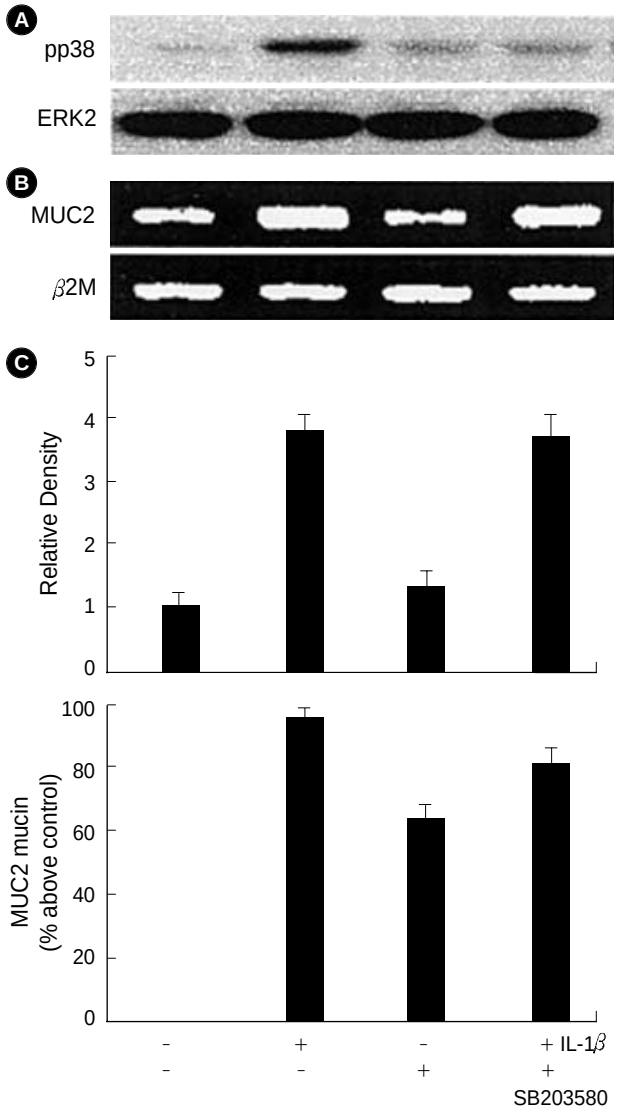

Fig. 4. Effects of p38 inhibitor, SB203580 on the expression of the IL-1β-mediated p38 phosphorylation (pp38) (A), *MUC2* gene expression (B) and mucin secretion (C). Analysis of p38 phosphorylation was done by immunoblot, *MUC2* gene by RT-PCR and mucin by immunoassay. ERK2 is a positive internal control. The amounts of the RT-PCR products of *MUC2* gene were quantified using densitometry and expressed relative to the density of β2M by the ratio of the MUC2/β2M DNA bands. β2M is a positive internal control. The data A and B are acquired from three independent experiments. The data C represents average value of three independent experiments and standard deviation.

for 1 hr before treatment of IL-1β. In addition, to investigate its relation with MAPKs, we observed the effect of the PI3K inhibitor on MAPKs phosphorylation induced by IL-1β. When the cells were pretreated with the LY294002 (25 μM) for 1 hr, LY294002 attenuated the IL-1β-induced *MUC2* gene expression and mucin secretion (Fig. 6B), but did not inhibit

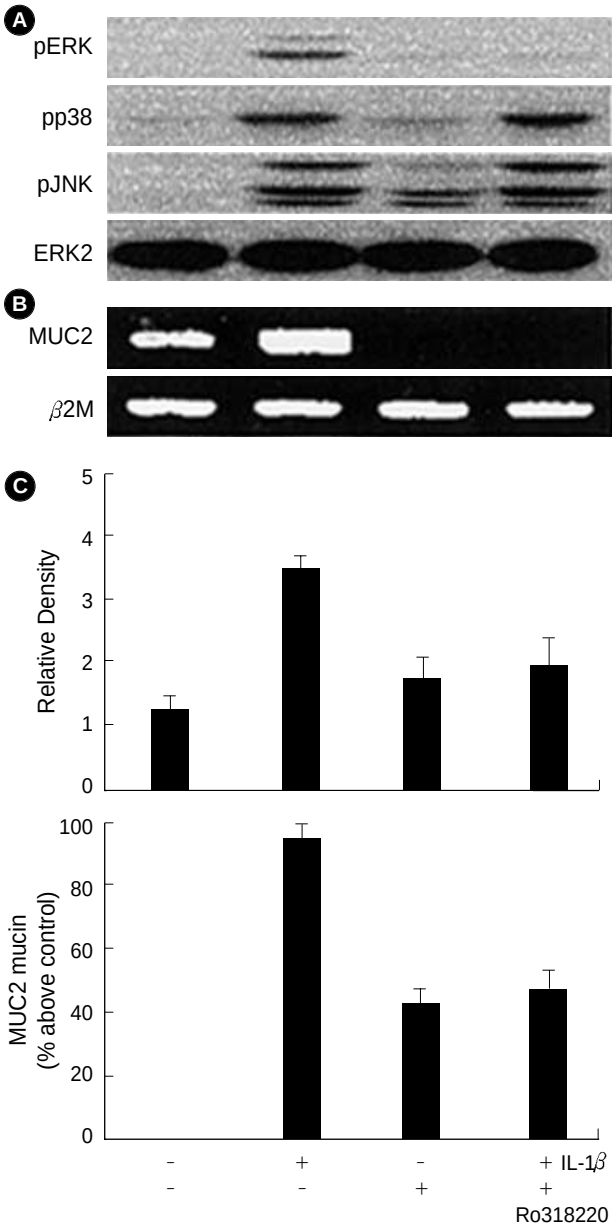

Fig. 5. Effects of PKC inhibitor, Ro31-8220 on the expression of the IL-1β-mediated MAPKs phosphorylation (pERK, pp38, pJNK) (A), *MUC2* gene expression (B) and mucin secretion (C). Analysis of ERK, p38, JNK phosphorylation was done by immunoblot, *MUC2* gene by RT-PCR and mucin by immunoassay. ERK2 is a positive internal control. The amounts of the RT-PCR products of *MUC2* gene were quantified using densitometry and expressed relative to the density of β2M by the ratio of the MUC2/β2M DNA bands. β2M is a positive internal control. The data A and B are acquired from three independent experiments. The data C represents average value of three independent experiments and standard deviation.

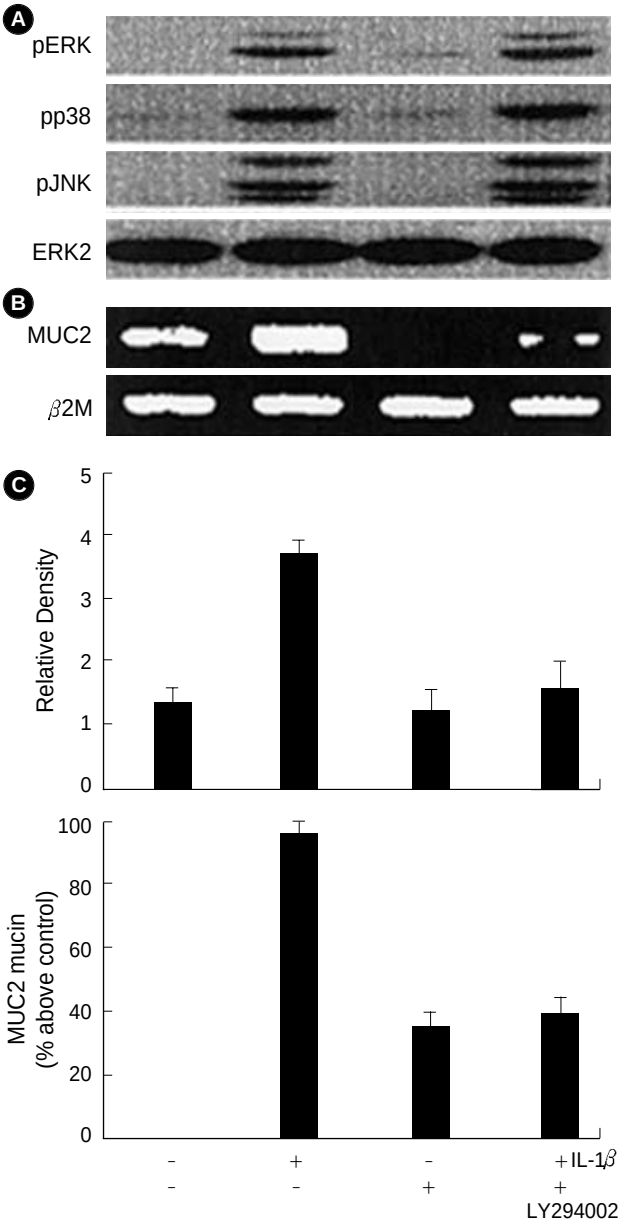

Fig. 6. Effects of PI3K inhibitor, LY294002 on the expression of the IL-1β-mediated MAPKs phosphorylations (pERK, pp38, pJNK) (A), *MUC2* gene expression (B), and mucin secretion (C). Analysis of ERK, p38, and JNK phosphorylation were done by immunoblot, *MUC2* gene by RT-PCR, and mucin by immunoassay. ERK2 is a positive internal control. The amounts of the RT-PCR products of *MUC2* gene were quantified using densitometry and expressed relative to the density of β2M by the ratio of the MUC2/β2M DNA bands. β2M is a positive internal positive control. The data A and B are acquired from three independent experiments. The data C represents average value of three independent experiments and standard deviation.

any MAPKs phosphorylation (Fig. 6A).

DISCUSSION

In this study, we observed whether IL-1β could induce *MUC2* gene expression and mucin synthesis through activation of PKC, MAPKs, or PI3K in cultured human airway NCI-H292 epithelial cell. Our results show that the MEK/ERK inhibitor, PD98059, inhibited the IL-1β-mediated *MUC2* gene expression and mucin secretion, but a p38 inhibitor, SB203580, did not. A PKC inhibitor, Ro31-8220, inhibited the IL-1β-mediated *MUC2* gene expression and mucin secretion. Moreover, Ro31-8220 attenuated ERK1/2 phosphorylation induced by IL-1β. These results show that PKC and MEK/ERK are related with the IL-1β-induced *MUC2* gene expression and mucin secretion, and that IL-1β activates PKC at upstream of ERK. Also, a PI3K inhibitor, LY294002, attenuated the IL-1β-induced *MUC2* gene expression and mucin secretion, but did not inhibit any MAPKs phosphorylation. These findings provide evidence that IL-1β induces *MUC2* gene expression and mucin secretion through activation of PKC-MEK/ERK-dependent pathway, and through activation of PI3K.

Since it has been reported that TNF-α is related with mucin secretion in 1995 (4), there were several reports about the signal pathway of mucin secretion. Li et al. (3) described that *Pseudomonas aeruginosa* induced *MUC2* gene expression by NF-κB activation via Src-dependent Ras-MAPK-pp90rsk pathway in epithelial cells. In addition, Basbaum et al. (18) suggested that the various mucin secretion in epithelial cells is commonly dependent upon c-Src, MAP kinase kinase MEK1/2, and NF-κB. Recently, Takeyama et al. (19) showed that the oxidative stress induces mucin synthesis in airway epithelial cells via epidermal growth factor receptor (EGFR), which leads to activation of the ERK signal transduction pathway. However, none of these studies found a direct link between PKC, ERK, or p38 in the IL-1β-mediated mucin gene expression and mucin secretion. Although we previously reported that IL-1β up-regulates *MUC2* gene expression and mucin secretion through transcriptional activation (6), the specific signal transduction pathways involved in the IL-1β-mediated mucin gene expression and mucin secretion were not shown.

In our experiments, to determine the signal transduction pathway involved in the IL-1β-mediated *MUC2* gene expression and mucin secretion, we observed the pattern of *MUC2* gene expression and mucin secretion using several specific inhibitors for regulatory molecules in signal transduction. The IL-1β-induced *MUC2* gene expression and mucin secretion are strongly attenuated by an MEK/ERK inhibitor, PD98059. We could conclude that MEK/ERK is necessary in the IL-1β-mediated *MUC2* gene expression and mucin secretion. This result is similar to that *Staphylococcus aureus* activates the transcription of the *MUC2* gene through the ERK activation (18) and the transcriptional activation of the *MUC2* gene induced by *Pseudomonas aeruginosa* requires MAPK (3, 7).

PKC is related with the MUC2 expression activated by *Pseudomonas aeruginosa* (18). The PKC family consists of at least 12 isoforms that possess distinct differences in structure, substrate requirment, expression, and localization (20). To evaluate the effect of PKC in the IL-1β-induced MUC2 expression, we used PKC inhibitor, Ro31-8220. While the straurosporine analogue Ro31-8220 inhibit PKCα, PKCβ I, PKCβII, PKCγ, and PKCε isoforms (20), it was found to be an equally potent inhibitor of mitogen- and stress-activated protein kinase-1(MSK1) activity in vitro (21). MSK1 plays a role in integrating the effects of different extracellular signals, and is located in the nucleus of unstimulated or stimulated cells (21). The substrates of MSK1 are the transcription factor CREB and ATF1, and MSK1 mediates the activation of CREB and ATF1 by either growth factors and stress signals (21). Therefore, there is a possibility that inhibitory effect of Ro31-8220 on IL-1β-mediated MUC2 expression was through inhibition of PKC in cytoplasm or inhibition of MSK1 in nucleus. In this study, the IL-1β-induced *MUC2* gene expression and mucin secretion were inhibited by pretreatment of a PKC inhibitor (Ro31-8220). This result suggests that PKC is involved in the signal transduction pathway of the IL-1β-mediated *MUC2* gene expression and mucin secretion. Furthermore, considering the result in which the phosphorylation of ERK was attenuated by Ro31-8220 at this time, we could conclude that PKC acts at upstream of ERK.

PI3K is an important molecule in mitogenic signaling and cell survival, cytoskeletal remodeling, metabolic control, and vesicular trafficking (22). And it has been known that PKC or MAPKs cascades require PI3K activation in several signal transduction pathway (23-27). In this study, LY294002, an inhibitor of PI3K, suppressed the IL-1β-induced *MUC2* gene expression and mucin secretion, but not the phosphorylation of ERK. This result suggests that PI3K also has an important role in up-regulation of the *MUC2* gene expression and mucin secretion induced by IL-1β. However, PI3K is involved independently of PKC-ERK or is located at downstream of MEK /ERK in the IL-1β-induced *MUC2* gene expression and mucin secretion because LY294002 did not inhibit phosphorylation of ERK.

In conclusion, we consider that activation of PKC-ERK and PI3K is required for the IL-1β-mediated *MUC2* gene expression and mucin secretion in human airway NCI-H292 epithelial cells. However, because specific inhibitors could not completely inhibit *MUC2* gene expression and mucin secretion induced by IL-1β, other signal pathways must be also involved in the IL-1β-mediated *MUC2* gene expression and mucin secretion. Furthermore, further studies with various epithelial cells including normal respiratory epithelium are needed to clarify the signal transduction pathway involved in the IL-1β-mediated mucin gene expression and mucin secretion.

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