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Insulin-like growth factor-1 induces MUC8 and MUC5B expression via ERK1 and p38 MAPK in human airway epithelial cells

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ABSTRACT

The biologic actions of insulin-like growth factor-1(IGF-1) are associated with cell growth, differentiation, migration, and survival. IGF-1 constitutes the pathogenic factor in formation of nasal polyps and the regulatory factor in expression of mucins. However, the effect of IGF-1 on MUC8 and MUC5B expression has not been reported. Therefore, in this study, the effect and brief signaling pathway of IGF-1 on MUC8 and MUC5B expression were investigated in human airway epithelial cells. In mucin-producing human NCI-H292 airway epithelial cells and the primary cultures of normal human nasal epithelial cells, the effect and signaling pathway of IGF-1 on MUC8 and MUC5B expression were investigated using reverse transcriptase-polymerase chain reaction (RT-PCR), real-time PCR, enzyme immunoassay, and immunoblot analysis with specific inhibitors and small interfering RNA (siRNA) for mitogen-activated protein kinase (MAPK). IGF-1 induced MUC8 and MUC5B expression, and activated phosphorylation of ERK1/2 and p38 MAPK. U0126 (ERK1/2 inhibitor) and SB203580 (p38 MAPK inhibitor) inhibited IGF-1 induced MUC8 and MUC5B mRNA expression. In addition, the knockdown of ERK1 and p38 MAPK by siRNA significantly blocked IGF-1 induced MUC8 and MUC5B mRNA expression; the knockdown of ERK2 MAPK by siRNA did not. These results demonstrate for the first time that IGF-1 induced MUC8 and MUC5B expression is regulated by activation of the ERK1 and p38 MAPK signaling pathway in human airway epithelial cells.

1. Introduction

Human airway epithelial cells secrete mucus which plays an important role in protecting the human airway from the external environment by entrapping inhaled foreign particles and bacteria, and clearing them from the airways by ciliary movement [1]. Mucins are glycoproteins that constitute a family of macromolecules that impart physical and biological properties to mucus [2]. There are two types of mucins in human airway epithelial cells are secreted and membrane-bound. The major secretory mucins are MUC5AC, MUC5B, and MUC8, whereas the major membrane-bound mucin is MUC4 [3]. These mucins are regulated by many pathophysiological mediators, such as bacterial proteinases and endotoxins, adenine and guanine nucleotides, cytokines, inflammatory mediators, eicosanoids, and insulin-like growth factor-1 (IGF-1) [4–8].

Among the pathophysiological mediators of mucin secretion, IGF-1 is a peptide that is structurally similar to proinsulin. It is the principal mediator of growth hormone activity in humans: it has mitogenic, metabolic, and growth-stimulating activities in many cells and tissues [9]. Recent studies have demonstrated an associated of IGF-1 with formation of nasal polyps [10]. In addition, involvement of IGF-1 in regulation of mucin secretion has been reported [7,8]. However, the effect of IGF-1 on secretion of major mucins in human airway epithelial cells has not been reported in the English-language literature. Therefore, the goal of this study was to determine whether IGF-1 might play an important role in regulation of the major mucins secretion in human airway epithelial cells. The effects and brief signaling pathway of IGF-1 associated with MUC8 and MUC5B expression in human airway epithelial cells were investigated.

2. Materials and methods

2.1. Materials

IGF-1 was obtained from R&D Systems (Minneapolis, MN, USA). The mucin-producing NCI-H292 human pulmonary mucoepidermoid carcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). RPMI 1640 medium, Trizol, predesigned siRNA targeting extracellular signal related kinase (ERK) 1, ERK2 and p38 mitogen-activated protein kinase (MAPK),

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and negative control siRNA were purchased from Invitrogen Corporation (Carlsbad, CA, USA). Reverse transcriptase-polymerase chain reaction (RT-PCR) kits were obtained from Applied Biosystems (Branchburg, NJ, USA). LC Fast Start DNA Master SYBR Green kit for use as a Real-time PCR kit was obtained from Roche Applied Science (Mannheim, Germany). Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT, USA), and an enhanced chemiluminescence reagent (ECL) kit was purchased from Perkin Elmer Life Sciences (Boston, MA, USA). The primary antibody for MUC5B or MUC8, and anti-goat or anti-mouse horseradish peroxidase (HRP) conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ERK1/2, phospho-ERK1/2, p38, and phospho-p38 antibodies were purchased from Cell Signaling (Danvers, MA, USA). Specific inhibitor, U0126 was purchased from Calbiochem (San Diego, CA, USA), and SB203580 was purchased from BIOMOL (Plymouth Meeting, PA, USA). Predesigned siRNA targeting ERK1, ERK2, and p38 mitogen-activated protein kinase (MAPK) and negative control siRNA were purchased from Invitrogen Corporation (Carlsbad, CA, USA).

2.2. Cell culture and treatment

Normal inferior turbinate was obtained from 10 cases of augmentation rhinoplasty (five men and five women, mean age: 33.6 years). The patients and control subjects had no personal or family history of allergy and had negative results on skin prick tests to 20 common airborne allergens and on multiple simultaneous allergen tests. All subjects discontinued oral and topical corticosteroid and antibiotic therapy for at least four weeks before surgery. The study protocol was approved by the institutional review board for human studies at Yeungnam University Medical Center and written informed consent was obtained from each patient.

For the primary culture of normal nasal epithelial cells, the inferior turbinate mucosa was washed with phosphate-buffered saline (PBS) and immersed in dispase (Boehringer Mannheim, Germany) for 90 min. After the tissue was scraped off the surface of the inferior turbinate mucosa using a scalpel, it was added to 1% PBS and filtered through a mesh. The cells were seeded in a 24-well plate at 2.5×10^5 cells/well, followed by incubation with EpiLife Medium (Cascade Biologics, Portland, OR, USA) and Human Keratinocyte Growth Supplement (5 mL/500 mL of medium, Cascade Biologics). When the cultures had become confluent, the primary cultures of normal nasal epithelial cells were exposed to the indicated concentrations of IGF-1 (R&D Systems, Minneapolis, MN, USA).

Mucin-producing human NCI-H292 airway epithelial cells (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% FBS. The cells were grown at 37 °C in 5% CO_2 fully humidified air and were subcultured twice weekly. When confluent, the cells were incubated in RPMI 1640 medium containing 0.5% FBS for 24 h. The cells were then rinsed with serum-free RPMI 1640 medium and exposed to the indicated concentrations of IGF-1.

In order to investigate the signaling pathway of IGF-1 induced MUC8 and MUC5B expression, each U0126, and/or SB2035, as a specific inhibitor, was used in pretreatment of the cultured cells for 1 h before exposure to the indicated concentrations of IGF-1. For the controls, the cultured cells were incubated with medium alone for the same amount of time.

2.3. Reverse tanscription-polymerase chain reaction (RT-PCR) and real-time PCR analysis of MUC4, MUC5AC, MUC5B, MUC8, and MUC16 mRNA expression

Isolation of total RNA from the cultured cells was performed according to the manufacturer's instructions (Applied Biosystems). Each sample was reverse transcribed into cDNA using the GeneAmp RNA PCR Core Kit (Applied Biosystems). The primer sequences and conditions were used according to previously published protocols for MUC4, MUC5AC, MUC5B, MUC8, and MUC16 [11,12]. RNA integrity and the success of the RT reaction were monitored by PCR amplification of the transcripts for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene [13]. The PCR products were electrophoresed on a 2% agarose gel, followed by staining with ethidium bromide, and visualization by UV fluorescence. Semiquantitative analysis of the RT-PCR product was performed on the scanned gel images, and the intensity of the PCR product was measured using commercially available imaging software (Scion, Frederick, MD, USA). The relative intensity of the individual bands on the gel image was determined as the ratio of the intensities of MUC4, MUC5AC, MUC5B, MUC8, and MUC16 to the intensity of GAPDH.

Real-time PCR was performed using the LC Fast Start DNA Master SYBR Green kit using 0.5 μL of cDNA, corresponding to 25 ng of total RNA in a 10 μL final volume, 2.5 mM $MgCl_2$ and 0.5 μM of each primer (final concentration). Quantitative PCR was performed using a LightCycler (Roche Applied Science) for 45 cycles at 95 °C for 10 s, specific annealing temperature for 5 s and 72 °C for 10 s. Data were normalized to GAPDH. Melting curve was used for evaluation of amplification specificity, following the manufacturer's instructions (Roche Applied Science).

2.4. Enzyme-linked immunosorbent assay (ELISA) analysis of MUC8 and MUC5B proteins

Protein levels for MUC8 and MUC5B were determined using an ELISA. Samples of supernatant or cell lysates from NCI-H292 cells were prepared in PBS at several dilutions, and each sample was incubated at 40 °C in a 96-well plate until dry. The plates were then washed three times with PBS, blocked with 2% bovine serum albumin for 1 h at room temperature, washed again three times with PBS, and incubated with primary antibody for MUC8 or MUC5B diluted at 1:200 with PBS containing 0.05% Tween 20 for 1 h. The wells were then washed three times with PBS, followed by dispensation of HRP-conjugated secondary antibody for MUC8 and MUC5B into each well. After 4 h, color was developed using 3,3′,5,5′-tetramethylbenzidine peroxidase solution and stopped with 2 N-H_2SO_4. Optical density measurements were obtained using an ELISA reader (EL800; BIO-TEK Instruments, Winooski, VT, USA) at 450 nm.

2.5. Western blot analysis of ERK1/2 and p38 MAPK phosphorylation

NCI-H292 airway epithelial cells were seeded in a 6-well plate and treated with IGF-1 for the indicated times. The cells were exposed to trypsin, and formed into pellets at 700g at 4 °C. The pellets were resuspended in lysis buffer. 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 sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. The membrane was then blocked with 5% nonfat dry milk, followed by incubation with the indicated primary antibody for ERK1/2 or p38 MAPK for 4 h. Subsequently, the membrane was washed and incubated with secondary antibody for ERK1/2 or p38 MAPK conjugated to HRP for 1 h, and developed using an enhanced chemiluminescence reagent kit, followed by exposure to X-ray film for 10 s.

2.6. Cell transfection with small interfering RNA (siRNA) for ERK1, ERK2, and p38 MAPK

Sequences of each siRNA were as follows: ERK1 MAPK; forward: UUG AUG AGC AGG UUG GAG GGC UUU A and reverse UAA AGC CCU CCA ACC UGC UCA UCA A, ERK2 MAPK; forward: AUG UCG

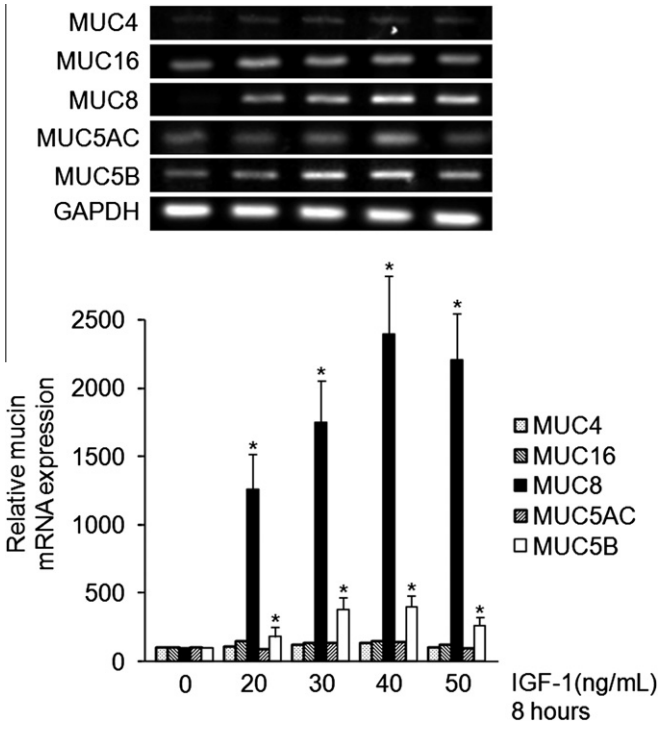

Fig. 1. The effects of IGF-1 on MUC4, MUC5AC, MUC5B, MUC8, and MUC16 mRNA expression in NCI-H292 airway epithelial cells. Results of RT-PCR showed that IGF-1 induced remarkable MUC8 and MUC5B mRNA expression: IGF-1 induced MUC8 mRNA expression was significantly stronger than IGF-1 induced MUC5B mRNA expression. However, MUC4, MUC5AC, and MUC16 mRNA expression were definitely not induced by IGF-1. Images are representative of three separate experiments performed in triplicate. Bars represent the averages ± SD of three independent experiments in triplicate. $^{*}p < 0.05$ compared with zero value.

AAC UUG AAU GGU GCU UCG G and reverse CCG AAG CAC CAU UCA AGU UCG ACA U, and p38 MAPK; forward: AUG AAU GAU GGA CUG AAA UGG UCU G and reverse: CAG ACC AUU UCA GUC CAU CAU UCA U. The transfection rate of each siRNA was verified to be over 90% in human NCI-H292 airway epithelial cells. Transfection was performed according to the manufacturer's protocol (Invitrogen). Briefly, human NCI-H292 airway epithelial cells were seeded in wells of a 6-well plate at 1×10^5 cells/well and incubated in RPMI 1640 medium. When the cells were confluent, OPTI-MEM I Reduced Serum Medium was added. Then, p38 MAPK siRNA and Lipofectamine 2000 were incubated together in OPTI-MEM I Reduced Serum Medium to form a p38 MAPK siRNA-Lipofectamine complex. The p38 MAPK siRNA-Lipofectamine complex-containing medium was added to each well containing the cells to a final p38 MAPK siRNA concentration of 20 nM. After 24 h of transfection with p38 MAPK siRNA, the cells were exposed to the indicated concentrations of IGF-1 and then harvested for RT-PCR analysis of MUC5B mRNA expression. The same procedure was performed with ERK1 and ERK2 MAPK siRNA, and negative control siRNA: the cells transfected with Lipofectamine 2000 only in negative control siRNA.

2.7. Statistical analysis

Commercially available software (SPSS, version 10.0; SPSS Inc, Chicago, IL, USA) was used in performance of statistical analysis. The mean for each of the obtained quantitative values was calculated. Comparisons were made using the Student's *t*-test. For all tests, a p value of less than 0.05 was considered statistically significant.

3. Results

3.1. IGF-1 induced MUC8 and MUC5B expression in NCI-H292 airway epithelial cells

Human NCI-H292 cells were incubated with IGF-1 (0, 20, 30, 40, and 50 ng/mL) for 8 h and RT-PCR was performed for analysis of MUC4, MUC5AC, MUC5B, MUC8, or MUC16 expression. The results showed remarkable IGF-1 induced MUC8 and MUC5B mRNA expression: IGF-1 induced MUC8 mRNA expression was significantly stronger than IGF-1 induced MUC5B mRNA expression.

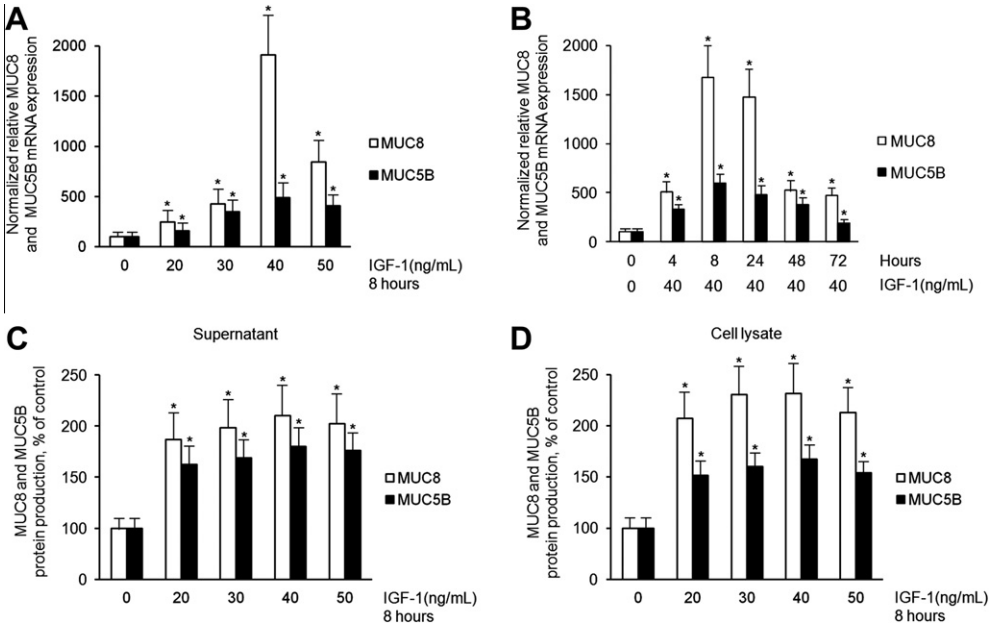

Fig. 2. The effects of IGF-1 on MUC8 and MUC5B expression in NCI-H292 airway epithelial cells. (A and B) Result of real-time PCR and (C and D) ELISA analysis showed that MUC8 and MUC5B expression were significantly increased by treatment with all dosages of IGF-1: the expression peaked at 8 h after treatment with IGF-1 (40 ng/mL). Images are representative of three separate experiments performed in triplicate. Bars represent the averages ± SD of three independent experiments in triplicate. $^{*}p < 0.05$ compared with zero value.

However, MUC4, MUC5AC, and MUC16 mRNA expression were definitely not induced by IGF-1 (Fig. 1, $p < 0.05$). Real-time RT-PCR and ELISA analysis were performed to investigate the effect of IGF-1 on MUC8 and MUC16 expression in a dose- and time-dependent manner. Human NCI-H292 airway epithelial cells were treated with different doses of IGF-1 (0, 20, 30, 40, and 50 ng/mL) for 8 h or IGF-1 (40 ng/mL) for variable times (0, 4, 8, 24, 48, and 72 h). The results also showed that IGF-1 did not induce MUC8 and MUC5B expression in a dose- and time-dependent manner. However, MUC8 and MUC5B expression showed a significant increase with treatment of all dosages of IGF-1: IGF-1 induced MUC8 mRNA expression was significantly stronger than IGF-1 induced MUC5B mRNA expression, and MUC8 and MUC5B expression peaked at 8 h after treatment with IGF-1 (40 ng/mL) (Fig. 2, $p < 0.05$).

3.2. IGF-1 activated phosphorylation of ERK1 and p38 MAPK in MUC8 and MUC5B expression in NCI-H292 airway epithelial cells

To evaluate the brief intracellular mechanisms of IGF-1 induced MUC8 and MUC5B expression, ERK1/2 and p38 MAPK signaling pathways were investigated in order to determine whether they were capable of activating MUC8 and MUC5B expression. Human NCI-H292 airway epithelial cells were stimulated with IGF-1 (0 and 40 ng/mL), and Western blot analysis was performed for detection of phosphorylation of ERK1/2 and p38 MAPK. The maximum levels of phosphorylation of ERK1/2 and p38 MAPK were determined at different times (0, 3, 5, 10, 15, and 20 min). IGF-1 activated the phosphorylation of ERK1/2 and p38 MAPK (Fig. 3A, $p < 0.05$). To confirm the phosphorylation of ERK1/2 and p38 MAPK in IGF-1 induced expression of MUC8 and MUC5B, the cells were stimulated with U0126 as a specific ERK1/2 inhibitor (0 and 2 μM) or SB203580 as a p38 MAPK inhibitor (0 and 20 μM) for 1 h before exposure to IFG-1 (0 and 40 ng/mL) for 8 h. And RT-PCR and ELISA were then performed for analysis of MUC8 and MUC5B expression. U0126 and SB203580 inhibited IGF-1 induced MUC8 and MUC5B expression (Fig. 3B, $p < 0.05$). In addition, cell transfection with siRNA was performed in order to confirm whether phosphorylation of ERK1, ERK2, and p38 MAPK was associated with IGF-1 induced MUC8 and MUC5B mRNA expression. After 24 h of transfection with ERK1, ERK2, and p38 MAPK siRNA (each 20 nM), the cells were exposed to IGF-1 (0 and 40 ng/mL) for 8 h, followed by performance of RT-PCR for analysis of MUC8 and MUC5B mRNA expression. The knockdown of ERK1 and p38 MAPK by siRNA significantly blocked IGF-1 induced MUC8 and MUC5B mRNA expression (Fig. 3C, $p < 0.05$); however, the knockdown of ERK2 MAPK by siRNA did not.

3.3. IGF-1-induced MUC8 and MUC5B mRNA expression via ERK1/2 and p38 MAPK in primary cultures of normal nasal epithelial cells

To investigate the effect of IGF-1 on MUC8 and MUC5B expression in primary cultures of normal nasal epithelial cells, the cells were incubated with IGF-1 (0, 20, 30, 40, or 50 ng/mL) for 8 h. And RT-PCR was then performed for analysis of MUC8 and MUC5B expression. The results revealed a significant increase of MUC8 and MUC5B mRNA expression by treatment with IGF-1 (0, 20, 30, 40, and 50 ng/mL); the expression peaked at 40 ng/mL of IGF-1 (Fig. 4A, $p < 0.05$). And IGF-1 induced MUC8 and MUC5B mRNA expression were significantly attenuated by pretreatment with U0126 as a specific ERK1/2 inhibitor and SB203580 as a p38 MAPK inhibitor (Fig. 4B, $p < 0.05$).

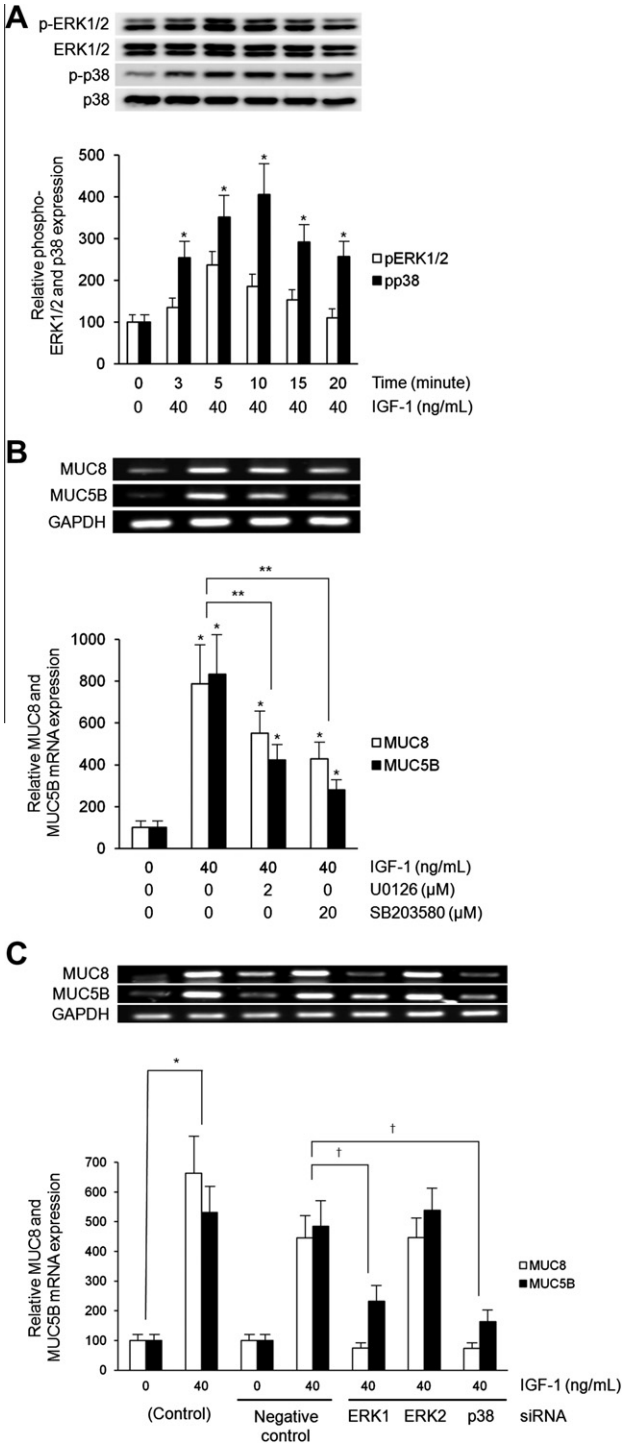

Fig. 3. The effect of IGF-1 on phosphorylations of ERK1/2 and p38 MAPK in NCI-H292 airway epithelial cells. (A) Results of Western blot analysis showed that IGF-1 activated phosphorylation of ERK1/2 and p38 MAPK. (B) Results of RT-PCR showed that U0126 and SB203580 inhibited IGF-1 induced MUC8 and MUC5B mRNA expression. (C) Results of RT-PCR showed that the knockdown of ERK1 and p38 MAPK by siRNA significantly blocked IGF-1 induced MUC8 and MUC5B mRNA expression; however, the knockdown of ERK2 MAPK by siRNA did not. Images are representative of three separate experiments performed in triplicate. Bars represent the averages ± SD of three independent experiments in triplicate. $^{*}p < 0.05$ compared with zero value, $^{**}p < 0.05$ compared with IGF-1 alone, $^{*}p < 0.05$ compared with negative control.

4. Discussion

IGF-1 is a heat-labile and acid-stable single chain non-glycosylated polypeptide with a molecular weight of 7.7-kDa [14]. IGF-1 is the product of the IGF-1 gene, which has been mapped to chromosome 12 in human. IGF-1 is an important mediator of cell growth, differentiation, migration, inhibition of apoptosis, and regulation of

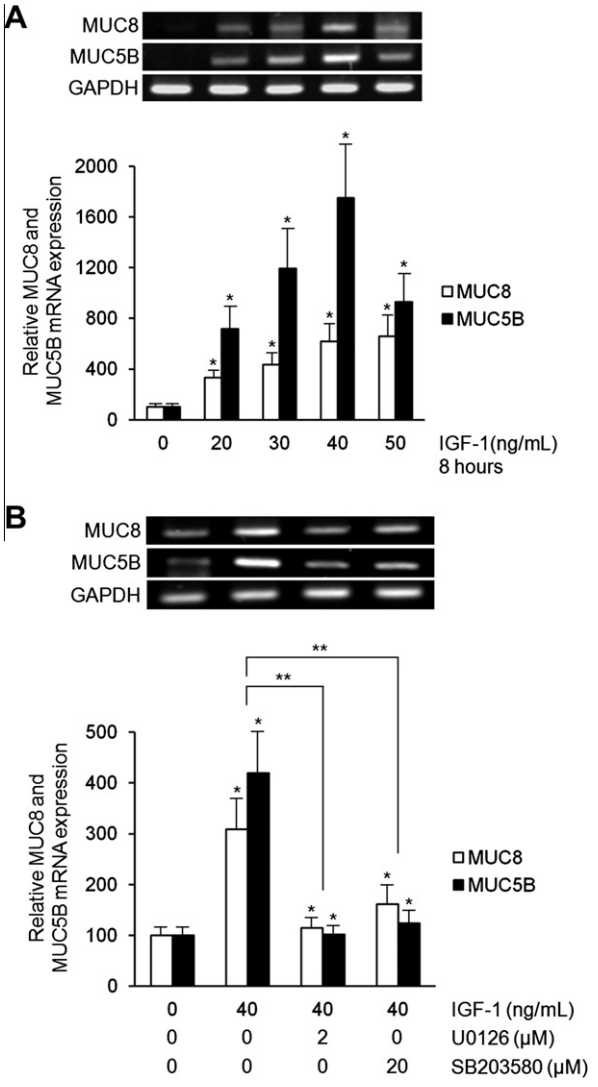

Fig. 4. The effects of IGF-1 and MAPKs inhibitors on MUC8 and MUC5B expression in primary cultures of normal nasal epithelial cells. (A) Results of RT-PCR showed that MUC8 and MUC5B mRNA expression were significantly increased by treatment with all dosages of IGF-1: the expression peaked at 40 ng/mL of IGF-1. (B) Results of RT-PCR showed that IGF-1 induced MUC8 and MUC5B mRNA expression were significantly attenuated by pretreatment with U0126 and SB203580. Images are representative of three separate experiments performed in triplicate. Bars represent the averages ± SD of three independent experiments in triplicate. $^{*}p < 0.05$ compared with zero value, $^{**}p < 0.05$ compared with IGF-1 alone.

gene transcription. The actions of IGF-1 occur primarily via the IGF-1 receptor, and are modulated by multiple IGF-1 binding proteins [15,16]. Expression of IGF-1 occurs in most tissues of the body. IGF-1 has characteristics of both a circulating hormone and a tissue growth factor with autocrine or paracrine properties. Circulating IGF-1 is synthesized primarily by the liver, and is regulated by growth hormone, insulin, and nutrients. In extrahepatic tissues, IGF-1 expression is regulated by growth hormone, prostaglandin E_2, parathyroid hormone, estradiol, thyroid-stimulating hormone, and estrogen [17].

Several studies using human airway epithelial cells have reported that a strong immunoreactivity for IGF-1 is detected in nasal polyps; IGF-1 is associated with formation of nasal polyps [10]. And IGF-1 is expressed in the lung and cultured human bronchial epithelial cells [14,18–20]. As a pathophysiological mediator of mucin secretion, only a few studies have reported that IGF-1 regulates sialomucin complex/rat MUC4 expression at the transcript level in isolated mammary epithelial cells, and IGF-1 receptor is activated by MUC1 expression in human breast cancer cell lines [7,8]. Therefore, this study was conducted in order to determine whether IGF-1 might regulate expression of the major secretory airway mucin genes in NCI-H292 airway epithelial cells and primary cultures of normal nasal epithelial cells. Findings of the current study demonstrated that IGF-1 induced MUC8 and MUC5B expression, compared with MUC4, MUC5AC, and MUC16 expression: IGF-1 induced MUC8 expression was significantly stronger than IGF-1 induced MUC5B expression in both types of cells.

Within the signaling pathway, MUC8 or MUC5B expression is induced in response to a wide variety of stimuli, including nerve activation and inflammatory cytokines, such as interleukin (IL)-1β, IL-6, IL-9, IL-13, tumor necrosis factor-α, and prostaglandin E_2 through a process involving MAPK activation [3,6,11,12]. As a mediator of mucin secretion, IGF-1 is associated with regulation of MUC1 and sialomucin complex/rat MUC4 expression by activation of the ERK MAPK signaling pathway [7,8]. Therefore, this study focused on IGF-1 induced MUC8 and MUC5B expression via the MAPK signaling pathway. Findings of the current study demonstrated that IGF-1 activated phosphorylation of ERK1/2 and p38 MAPK. U0126 (ERK1/2 inhibitor) and SB203580 (p38 MAPK inhibitor) inhibited IGF-1 induced MUC8 and MUC5B mRNA expression. In addition, the knockdown of ERK1 and p38 MAPK by siRNA significantly blocked IGF-1 induced MUC8 and MUC5B mRNA expression; however, the knockdown of ERK2 MAPK by siRNA did not. These results suggested that the signaling pathway of IGF-1 induced MUC8 and MUC5B expression in human airway epithelial cells might be the ERK1 MAPK and p38 MAPK pathways.

In conclusion, the results of this study demonstrate for first time that IGF-1 induced MUC8 and MUC5B expression appears to be regulated by activation of ERK1 MAPK and p38 MAPK signaling pathway in human airway epithelial cells. Although the effects of IGF-1 on mucins and the precise signal pathways for regulation of the mucin gene by IGF-1 in airway epithelial cells require further confirmation, these results provide important information indicating that IGF-1 may play a role in control of mucus hypersecretion through MAPK signaling pathways in human airway epithelial cells.

Acknowledgments

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