Signal transduction pathway of PGE_2-induced *MUC8* gene expression in human airway epithelial cells

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The Master's Thesis

submitted to the Department of Medical Science,

the Graduate School of Yonsei University

in partial fulfillment of the requirements for

the degree of Master of Medical Science

Ki Han Yoo

December 2003

This certifies that the Master's Thesis of Ki Han Yoo is approved.

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December 2003

Acknowledgements

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2003년 12월

유 기 한

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ABSTRACT

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Mucins are the major components of the mucus layer that covers and protects the respiratory, digestive, and reproductive tracts. Our previous studies showed that *MUC8* gene expression was overexpressed in *in vivo* polyp epithelium in chronic sinusitis

and was also increased by treatment with inflammatory mediators in an *in vitro* culture condition. However, the mechanisms by which the inflammatory mediators-induced *MUC8* gene expression in normal nasal epithelial cells evolved remain unclear. We examined the mechanism by which the important proinflammatory mediator, PGE_2, increases *MUC8* gene expression levels. We found that pharmacologic and genetic inhibition of ERK MAP kinase pathway abolished PGE_2-induced *MUC8* gene expression in normal human nasal epithelial cells. Moreover, the overexpression of the dominant negative mutant of p90 ribosomal S6 protein kinase 1 (RSK1) suppressed the PGE_2-induced *MUC8* gene expression. RSK1 was found to directly phosphorylate cAMP response element-binding protein (CREB), and this event led to the stimulation of subsequent CRE-mediated gene transcription. In conclusion, PGE_2 was found to induce *MUC8* gene expression via a sequential ERK/ RSK1/ CREB pathway in human airway epithelial cells.

Key Words : *MUC8*, PGE_2, ERK MAP kinase, RSK1, CREB

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I. INTRODUCTION

Mucin hypersecretion is commonly observed in many respiratory diseases, such as, rhinitis, sinusitis, otitis media, nasal allergy, chronic bronchitis and cystic fibrosis[1-4]. Nineteen types of

mucin genes have been discovered to date: *MUC1* [5], *MUC2* [6], *MUC3* [7], *MUC4* [8], *MUC5AC* [9], *MUC5B* [10], *MUC6* [11], *MUC7* [12], *MUC8* [13], *MUC9* [14], *MUC10* [15], *MUC11* [16], *MUC12* [16], *MUC13* [17], *MUC15* [18], *MUC16* [19], *MUC17* [20], *MUC18* [21], and *MUC19* [22]. Of these, *MUC5AC* and *MUC5B* are known to be major gel-foaming mucins secreted in the human airway. Accordingly, most studies on mucin genes have been focused on these two mucins. However, although *MUC5AC* is known to be expressed by most surface goblet cells, we found that only a portion of the goblet cells [23] expressed *MUC5AC* mRNA. This suggests that other mucin genes in addition to *MUC5AC* might be important for mucus hypersecretion. We have previously investigated the expressions of other *mucin* genes using various inflammatory tissues and cell lysates [24]. Interestingly, *in vivo*, we found that *MUC8* mRNA levels are clearly up-regulated in the polyp epithelium, which is invariably stimulated by inflammatory mediators [24]. In addition, *in vitro*, interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and a cocktail of inflammatory mediators were found to up-regulate *MUC8* mRNA and to down-regulate *MUC5AC* mRNA [25, 26]. These results showed that *MUC8* mRNA is increased both *in vivo* and *in vitro* during inflammatory

conditions. However, the mechanisms of *MUC8* gene expression during inflammation in normal airway epithelial cells and the signal molecules involved have not been elucidated.

Prostaglandins (PGs) are arachidonic acid metabolites with a wide range of biological actions. The PGs have many inflammatory effects [27], and it is known that cyclooxygenase (COX) converts arachidonic acid to prostaglandin H_2, which is further metabolized to various PGs and thromboxanes [28]. They are produced by cyclooxygenases (COX-1 and COX-2) in a wide variety of tissues and function as lipid mediators. Although PGE_2 is one of the major metabolites of arachidonic acid in human tissue, its function is not clear. Some evidence suggest that the PGs may stimulate mucin secretion [29, 30], and because mucin hypersecretion is a hallmark of airway inflammation, it is possible that PGs and its metabolites may regulate airway mucin secretion.

It is well documented that mitogen-activated protein (MAP) kinase pathways are thought to be most important in transmitting inflammatory signals from the cell surface to the nucleus [31]. On being triggered by growth factors, cytokines, UV rays, or other stress-inducing agents, a signal is delivered down the MAPKKK →

MAPKK (in the cases of ERK, JNK, and p38, the signal is delivered through MEK1/2, MKK4/7, and MKK3/6, respectively) to the MAP kinase cascade. The MAP kinases play a role in cell proliferation, differentiation, apoptosis, cytoskeletal remodeling, and the cell cycle [32-37]. The stimulation of ERK initiates a cascade of activating events, including the phosphorylation of p90 ribosomal S6 protein kinase 1 (RSK1) and its translocation to the nucleus, where RSK1 phosphorylates nuclear substrates [38]. Moreover, the phosphorylation of RSK1 could lead to the phosphorylation and activation of several transcription factors, like cAMP response element-binding protein (CREB) and activating transcription factor 1 (ATF1) [39].

We examined the mechanism by which the important inflammatory mediator, PGE_2, increases *MUC8* gene expression levels. Here we show that ERK MAP kinase is essential for PGE_2-induced *MUC8* gene expression in normal human nasal epithelial (NHNE) cells. We also show that RSK1 mediates the PGE_2-induced phosphorylation of CREB and CRE-mediated transcription. Molecular cloning of the *MUC8* promoter regulated by various stimuli may yield a deeper insight into cellular function.

II. MATERIALS AND METHODS

1. Materials

Prostaglandin E_2 was purchased from Cayman Chemical (Ann Arbor, MI, USA). PD98059 and anti-α-tubulin antibody were purchased from Calbiochem (San Diego, CA, USA). Anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody, anti-phospho-p38 MAP kinase (Thr180/Tyr182) antibody, anti-phospho-SAPK/JNK MAP kinase (Thr183/Tyr185) antibody, anti-phospho-RSK1 (Ser380) antibody and anti-phospho-CREB (Ser133) antibody were purchased from Cell Signaling (Beverly, MA, USA). Plasmid encoding kinase-deficient MEK1 mutant (pcDNA5-MEK1DN) was kindly provided by Dr. Jian-Dong Li (House Ear Institute, LA, CA, USA).

2. Cell Cultures

The culture system used for the normal human nasal epithelial (NHNE) cells was as described previously [40]. The human lung mucoepidermoid carcinoma cell line (NCI-H292) was purchased from the American Type Culture Collection (CRL-1848; Manassas, VA, USA) and cultured in RPMI-1640 (GIBCO BRL, Rockville,

MD, USA) supplemented with 10% fetal bovine serum (FBS) in the presence of penicillin-streptomycin at 37°C in a humidified chamber with 5% CO_2. For serum deprivation, confluent cells were washed twice with phosphate-buffered saline (PBS) and recultured in RPMI-1640 with 0.2% FBS.

3. RNA isolation and RT-PCR

Total RNA was isolated using TRIzol (GIBCO BRL) from NHNE and NCI-H292 cells treated with PGE2. cDNA was prepared by incubating 3 μg of total RNA with random hexamers (Perkin Elmer, Roche, Branchburg, NJ, USA) using MMLV reverse transcriptase (Perkin Elmer). Oligonucleotide primers for PCR were designed based on the Genbank sequence of *MUC8* (Genbank accession No. U14383, 5' primer ACAGGGTTTCTCCTCATTG; 3' primer CGTTTATTCCAGCACTGTTC). The PCR amplification was performed for 35 cycles with denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and polymerization at 72°C for 30 sec. The oligonucleotide primers for β_2-microglobulin (β_2M, used as a control gene for the RT-PCR) were designed based on the Genbank human sequence (Genbank accession No. XM007650, 5' primer

CTCGCGCTACTCTCTCTTTCTGG; 3' primer GCTTACATGT-CTCGATCCCACTTAA). The PCR reactions were performed for 23 cycles with denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and polymerization at 72°C for 30 sec. The PCR products were run in 1.5% agarose gel and visualized with ethidium bromide under a transilluminator

4. Western Blot Analysis

NCI-H292 cells were grown to confluence in 6 well plates. After treatment with PGE_2, the cells were lysed with 2x lysis buffer [250 mM Tris-Cl (pH 6.5), 2% SDS, 4% β-mercaptoethanol, 0.02% BPB, 10% glycerol]. Equal amounts of whole cell lysates were resolved by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF ; Millipore, Bedford, MA, USA). Membranes were blocked with 5% skim milk in Tris-buffered saline [50 mM Tris-Cl (pH 7.5), 150 mM NaCl] for 2 hr at room temperature. This blot was then incubated overnight with primary antibody in TTBS (0.5% Tween 20 in TBS). After washing with TTBS, the blot was further incubated for 1hr at room temperature with anti-rabbit or anti-mouse antibody (Cell Signaling) in TTBS, and then visualized

by using the ECL system (Amersham-Pharmacia, Piscataway, NJ, USA).

5. Transient Transfection and Luciferase Assay.

The NCI-H292 cells were plated at a density of 1 x 10^6 cells per well in a 6-well plate. When the cell confluency was 70~80%, the cells were transfected with plasmid constructs. Luciferase reporter construct (*pCRE-luc*) and plasmid expressing mutant CREB (*pCREB S133A*, the 133th serine residue was replaced by alanine) vector, were transiently transfected by using a FuGENE6 transfection reagent (Roche Applied Science), according to the manufacturer's instructions. Cells were incubated for 48 hr, harvested, and assayed for luciferase activity, using a luciferase assay system (Promega), according to the manufacturer's instructions. β-Galactosidase activity was also assayed to standardize the transfection efficiency of each sample.

III. RESULTS

1. PGE2 can induce the gene expression of *MUC8* through ERK MAP kinase signaling in NHNE and NCI-H292 cells.

To determine whether PGE2 can induce *MUC8* gene expression within NHNE cells, we carried out RT-PCR after treatment with varying concentrations of PGE2. As the dose of PGE2 was increased from 1 to 1000 nM, there was a gradual increase in *MUC8* gene expression from 10 nM. As shown in Fig. 1A, 10 nM PGE2 significantly induce *MUC8* gene expression. No corresponding change was found in the expression of internal control, β_2-microglobulin.

In order to determine whether PGE2 induced *MUC8* gene expression in a time-dependent manner, we examined the expression level of *MUC8* after various lengths of exposure to PGE2 (Fig. 1B). *MUC8* gene expression was significantly increased at 24 hr of exposure to PGE2. The results showed that the *MUC8* gene expression was significantly increased after treatment with PGE2 in NHNE cells. We used 10 nM PGE2 for all the subsequent

experiments. As a next step, to investigate which MAP kinase signal pathway is activated within NHNE cells stimulated by PGE_2, we performed a Western blot analysis using phospho-specific antibodies. ERK MAP kinase was maximally activated at 5 min, and this effect decreased after 20 min (Fig. 2). However, no change was detected in the activation of p38 and JNK. NCI-H292 cells and A549 cells treated IL-1β were used as positive controls for p38 and JNK activation, respectively. It thus appeared that stimulation by PGE_2 activates the ERK MAP kinase pathway in NHNE cells. Because PGE_2 significantly increased both *MUC8* gene expression and ERK MAP kinase activity, we wanted to determine whether PGE_2-induced *MUC8* gene expression involves the ERK MAP kinase pathway. As a next step, 20 μM PD98059, specific MEK1/2 inhibitor, was applied before treatment with PGE_2. Pretreatment of NCI-H292 cells with PD98059 for 1hr clearly inhibited ERK MAP kinase and significantly suppressed PGE_2-induced *MUC8* gene expression in NHNE cells (Fig. 3A). When the same experiments (Figs. 1, 2, and 3A) were performed using NCI-H292 cells, human lung mucoepidermoid carcinoma cell line, the results were the same in the NCI-H292 cells as in the normal cells (data not shown). To

further confirm whether ERK MAP kinase was involved in PGE_2-induced *MUC8* gene expression, cells were transiently transfected with DNA expression constructs encoding MEK1 dominant-negative (DN). Overexpression of MEK1 DN suppressed the PGE_2-induced ERK MAP kinase activity. Consistently, PGE_2-induced *MUC8* gene expression was significantly suppressed by MEK1 DN (Fig. 3B). These results show that the activation of ERK MAP kinase via MEK1 was essential for PGE_2-induced *MUC8* gene expression in NCI-H292 cells.

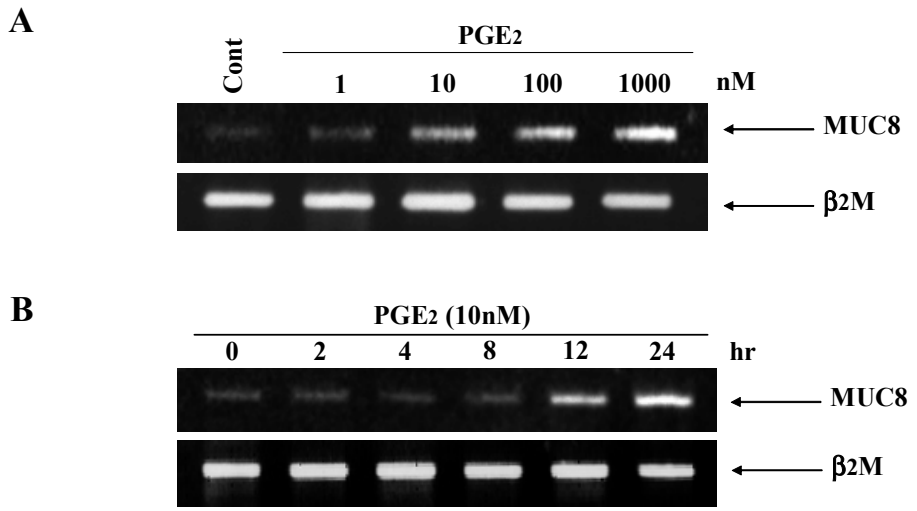

Fig. 1. Effect of PGE2 on *MUC8* gene expression in NHNE cells. Confluent cells were treated with the indicated concentration of PGE2 for 24 hr (***A***) and with 10 nM PGE2 in time-depandent manner (***B***). Cell lysates were harvested for RT-PCR. 3 μg of total RNA extracted from treated and non-treated cells were reverse-transcribed. The fragment was amplified by specific primer for *MUC8* or β2-Microglobulin (*β2M*). β2M was employed as an internal control. The amplified products were visualized on 1.5% agarose gel.

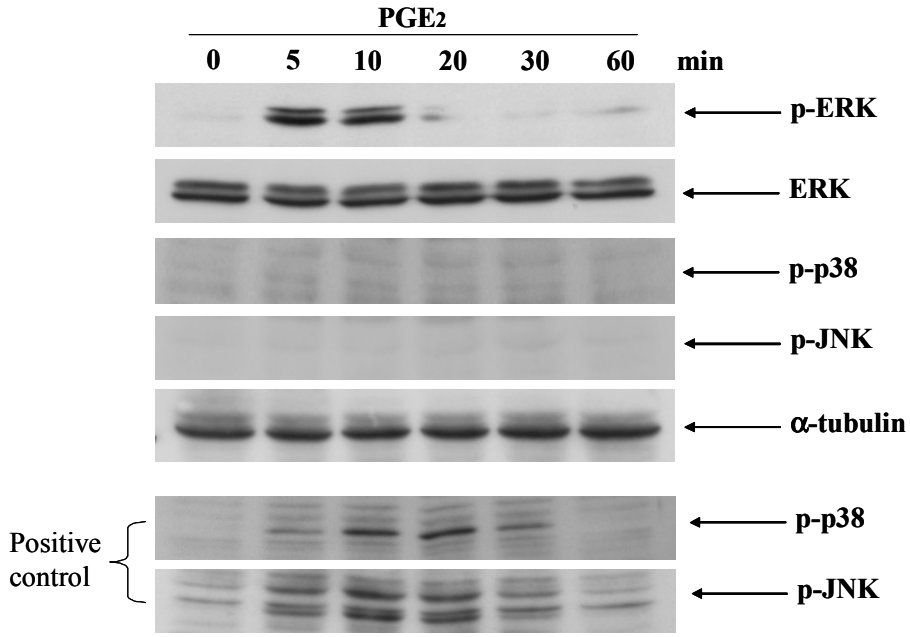

Fig. 2. Effect of PGE2 on MAPK pathways in NHNE cells. Confluent cells were treated with PGE2 (10 nM) for varying times (0-60 min), and cell lysates were harvested for western blot analysis. Representative Western blots using phospho-specific antibodies showed transient activation of ERK and the maximum effect is at 5 min. The figures shown are representative of three independent experiments.

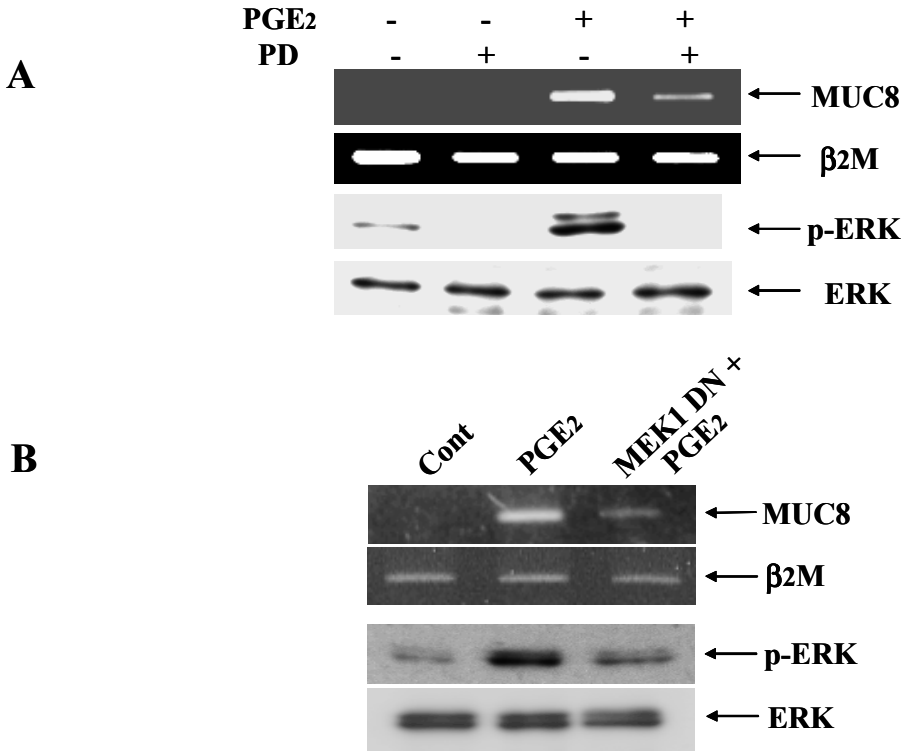

Fig. 3. Effects of PD98059 and MEK1 DN on PGE2-induced *MUC8* gene expression. Confluent, quiescent cells were pretreated for 1hr with 20 μM PD98059 (***A***) or transiently transfected with mutant MEK1 DN construct (***B***), then stimulated for 5 min or 24 hr with PGE2 prior to collection of total protein for Western blots and of total RNA for RT-PCR, respectively. Representative Western blots showing the phosphorylation of ERK and RT-PCR showing expression of *MUC8* mRNA by PD98059 (PD). The figure shown are representative of three independent experiments.

2. Effect of RSK1 on PGE_2-induced *MUC8* gene expression.

To determine which molecules are involved in the downstream signaling of ERK MAP kinase in PGE_2-induced *MUC8* gene expression, we investigated RSK1. RSK1 has been reported to be activated by ERK MAP kinase [41-43]. The phosphorylation of RSK1 by PGE_2 peaked at 5 min and then decreased at 20 min after PGE_2 stimulation (Fig. 4A). To determine whether RSK1 plays an important role in PGE_2-induced *MUC8* gene expression, an RSK1 mutant study was performed. RSK1 is a currently candidate for the mediation of cytokine-induced CREB phosphorylation at Ser^{133} [41, 44, 45]. Overexpression of RSK1 DN (D205N, A dominant-negative construct encoding RSK1 protein with Asp^{205} phosphorylation site mutated to Asn^{205}) suppressed the PGE_2-induced CREB and RSK1 phosphorylation. Consistently, PGE_2-induced *MUC8* gene expression was significantly suppressed by RSK1 DN (D205N) (Fig. 4B). These results show that RSK1 is required for PGE_2-induced *MUC8* gene expression.

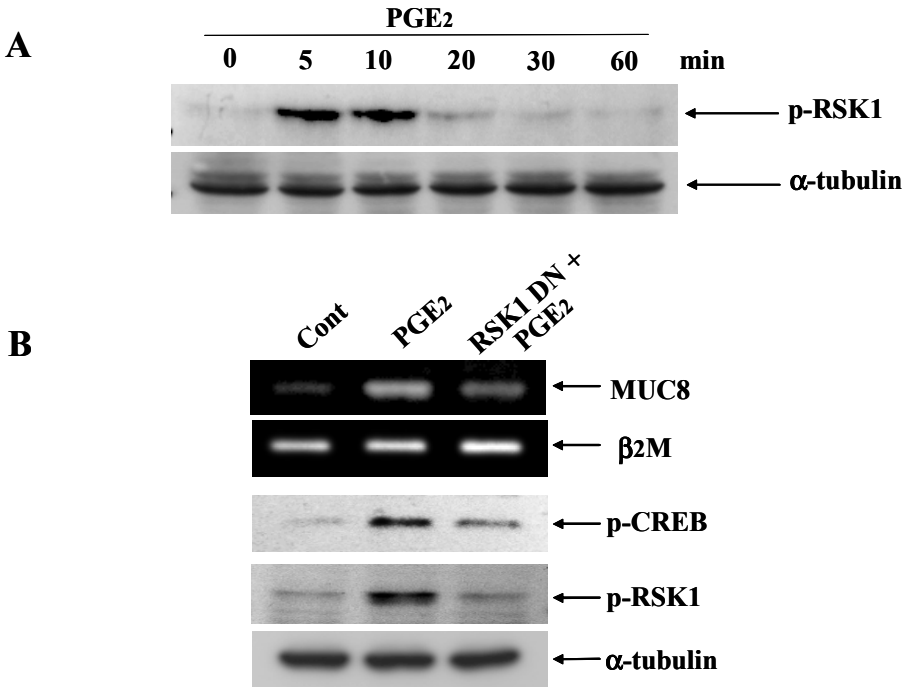

Fig. 4. Effect of RSK1 on PGE2-induced *MUC8* gene expression. Confluent, quiescent cells were stimulated for the indicated times with PGE2 and then total proteins were collected for Western blot analysis using phospho-RSK1 antibody (***A***). In other experiments, the cells were transiently transfected with mutant RSK1 D205N construct and stimulated with PGE2 for 5 min prior to Western blots analysis and for 24 hr prior to RT-PCR (***B***). The figures shown are representative of three independent experiments.

3. Effect of CREB on PGE2-induced *MUC8* gene expression.

To determine whether CREB plays a role in *MUC8* gene expression, we performed a Western blot analysis using phospho-CREB antibody. The phosphorylation of CREB by PGE2 peaked at 10 min and then decreased at 60 min after PGE2 stimulation (Fig. 5A). Furthermore, PGE2-induced *MUC8* gene expression was significantly suppressed in cells transfected with plasmid encoding CREB DN (S133A, A dominant-negative construct encoding CREB protein with Ser^{133} phosphorylation site mutated to Ala^{133}) (Fig. 5B). These findings suggest that the activation of CREB is essential for PGE2-induced *MUC8* gene expression via ERK MAP kinase and RSK1.

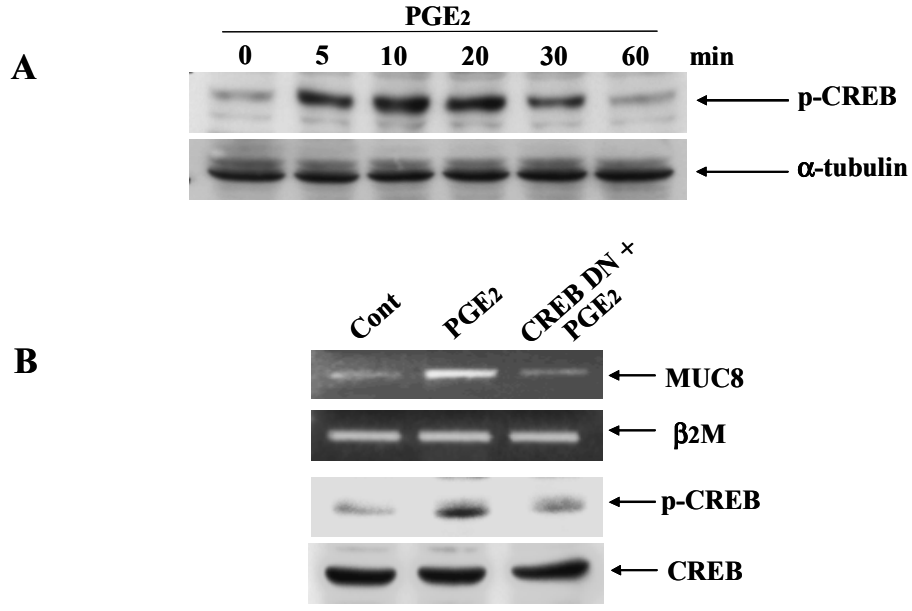

Fig. 5. Effects of CREB on PGE2-induced *MUC8* gene expression. Confluent, quiescent cells were stimulated for the indicated times with PGE2 and then total proteins were collected for Western blot analysis using phospho-CREB antibody (***A***). In other experiments, the cells were transiently transfected with mutant CREB S133A construct and stimulated with PGE2 for 10 min prior to Western blots analysis and for 24 hr prior to RT-PCR (***B***). The figures shown are representative of three independent experiments.

4. CREB activates CRE-mediated gene transcription in response to PGE_2.

To assess whether PGE_2 exerts its stimulatory effect on the activation of transcription factor CREB and subsequent CRE-mediated gene transcription, we assayed the gene expression of CRE-containing promoter-reporter construct. To investigate the role of CREB phosphorylation on CRE-mediated gene transcription, cells were transfected transiently with *pCRE-luc* vector only or both *pCRE-luc* vector and plasmid encoding CREB DN (S133A). Treatment of transfected NCI-H292 cells with PGE_2 resulted in increased CRE-mediated gene transcription in a time-dependent manner (Fig. 6). The expression of CREB containing a mutation of the critical regulatory Ser133 residue was found to significantly inhibit the luciferase activity induced by PGE_2. Mock transfection, used as a negative control, showed no significant induction of CRE-mediated reporter transcription. These results suggest that PGE_2-activated CREB triggers CRE-mediated transcription by binding to CRE.

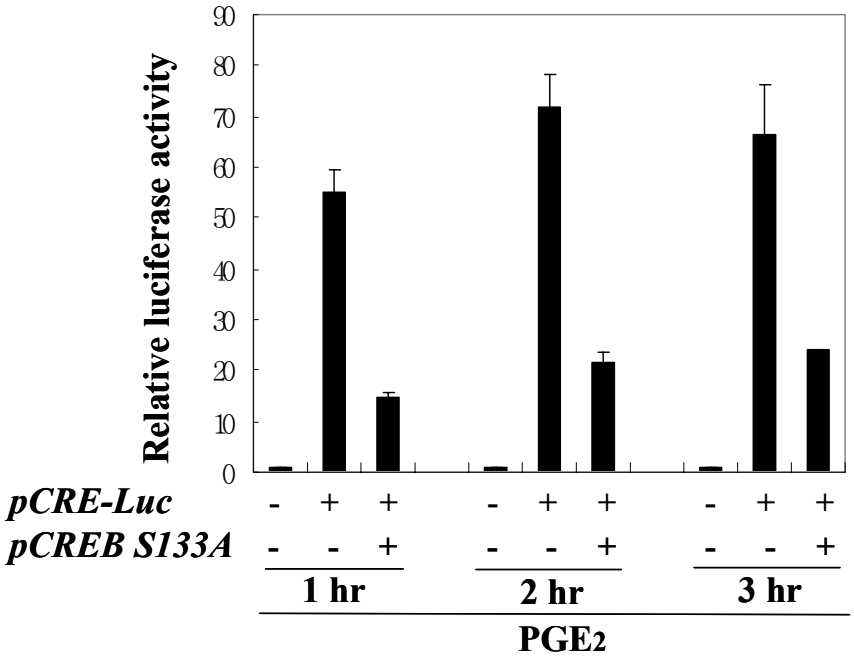

Fig. 6. PGE2-induced activation of CRE-mediated gene transcription via the *cis*-acting regulatory CRE motif. When the cells were 70% confluent, *pCRE-luc*, or both *pCRE-luc* and CREB DN (*pCREB S133A*) plasmid, and empty parental control vector (mock) as a control, were transiently transfected into the NCI-H292 cells. Confluent cells were then stimulated with PGE2 for the indicated times, and the luciferase activity of the reporter plasmid was measured. Luciferase activities were determined after correcting for transfection efficiency *versus* the β-galactosidase activity of the cell lysates. The values shown are means ± S.D. of experiments performed in triplicate.

IV. DISCUSSION

Mucociliary clearance is an important function of the airway epithelium. Human beings inhale noxious gases, air pollutants, bacteria, and viruses through the nose, and these are usually trapped by mucus and removed by ciliary beating toward the nasopharynx. Increased mucus secretion during inflammation may represent a defensive mechanism, and inflammatory mediators increase the ciliary beating of respiratory epithelial cells to promote mucociliary clearance [46–48].

The molecular mechanism by which *MUC8* gene expression is up-regulated by PGE_2 remains poorly understood. In the present study, we investigated the mechanisms by which *MUC8* gene expression is up-regulated by PGE_2 in normal human nasal epithelial cells. Our results show that only the activation of ERK MAPK was required for PGE_2-induced *MUC8* gene expression (Fig. 3A), although several reports have concluded that more than one MAPK might be necessary for the signal transduction of various inflammatory mediators [49–51]. Of these pathways, the activation of ERK MAP kinase is known to be mainly mediated by MEK1 [33].

In this study, we investigated whether PGE2-induced activation of ERK MAP kinase is MEK1- dependent or independent. The overexpression of MEK1 DN mutant significantly decreased the PGE2-induced phosphorylation of ERK MAP kinase and *MUC8* gene expression (Fig. 3B). These results show that activation of ERK MAP kinase by PGE2 occur via a MEK1-dependent pathway to induce *MUC8* gene expression in human airway epithelial cells.

To date, the signal molecules involved in the downstream signaling of ERK MAP kinase, for PGE2-induced *MUC8* gene expression, have not been yet identified. The role of RSK1 and CREB in the downstream signaling of ERK MAP kinase to induce *MUC8* gene expression is a major finding of the present study. The substrates of ERK MAP kinase are known to be the MSK and RSK family members [52]. Our results show that RSK1 might be essential for PGE2-induced *MUC8* gene expression. However, MSK1 did not affect the PGE2-induced *MUC8* gene expression (data not shown). RSK1 phosphorylates several transcription factors, including: CREB [53], c-Fos [54], CCAAT/enhancer binding protein [55], nuclear factor-κB [42], and the estrogen receptor [56], and interacts with transcriptional coactivator CREB-binding protein (also known as

p300) [57]. Many studies have shown that RSK1 phosphorylates Ser^{133} of CREB [58-61]. Although CREB activation by RSK1 has been established by previous studies in other cells, and CREB is a potent regulator of mucin (*MUC2, MUC5AC, MUC5B* and *MUC6*) gene expression in the p15 arm of chromosome 11 (11p15) [39], its role remains unclear in airway epithelial cells. In addition, little is known about the involvement of CREB in *MUC8* gene expression. In the present study, the activation of CREB was found, at least in part, essential for PGE2-induced *MUC8* gene expression via ERK MAP kinase and RSK1. Interestingly, *MUC8* gene expression was inhibited in CREB DN (S133A) transfected cells treated with PGE2.

We examined whether PGE2-induced *MUC8* gene expression in human airway epithelial cells is a CRE-mediated transcription. We found that PGE2-induced CRE activation increased in a time-dependent manner, whereas the overexpression of CREB DN mutant led to a ~90% decrease in the response of the CRE minimal promoter to PGE2 (Fig. 6). These results showed that the activation of the *cis*-element, CRE, appeared closely related to PGE2-induced *MUC8* gene expression in human airway epithelial cells. These results indicate that the *MUC8* promoter might have CRE site(s)

and the CRE might be an important transcription factor of the *MUC8* promoter, like the *mucins* of 11p15 chromosome [39]. However, unfortunately, the promoter and cDNA sequences of the *MUC8* gene have not yet been fully identified. Thus, further studies upon the *MUC8* promoter seem warranted.

In summary, PGE_2 was found to induce *MUC8* gene expression via the MEK1/ERK pathway. Furthermore, the activations of RSK1 and CREB are a crucial aspect of the intracellular mechanisms that mediate *MUC8* gene expression in human airway epithelial cells. Molecular cloning of the *MUC8* promoter regulated by various stimuli may yield a deeper insight into ciliated cell differentiation or function.

V. CONCLUSION

In this study, because *MUC8* hypersecretion during inflammation plays an important role in the pathogenesis of airway diseases, we examined that important proinflammatory mediator, PGE_2, up-regulate *MUC8* gene expression by activating specific signal transduction pathways in airway epithelial cells. Using pharmacologic or genetic inhibition of ERK MAP kinase pathway, we showed that ERK MAP kinase is essential for PGE_2-induced *MUC8* gene expression. In addition, the activation of RSK1 and CREB is a crucial aspect of the intracellular mechanisms that mediate *MUC8* gene expression, indicating that CREB activation is involved in the downstream signaling of MAP kinases and RSK1 for PGE_2-induced *MUC8* gene expression. According to Fig. 6, PGE_2-activated CREB bound to a *cis*-acting element, CRE minimal promoter. Taken together, these studies give additional insights into the molecular mechanism of PGE_2-induced *MUC8* gene expression and enhance our understanding on mucin hypersecretion during inflammation.

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국문요약

인체 호흡기 상피세포에서 PGE2에 의한 *MUC8* 점액유전자 발현의 신호전달경로

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점액은 호흡기계, 소화기계 및 생식기계를 덮고 보호하는 점막의 주요 구성 성분이다. 우리는 이전 연구에서 *MUC8* 유전자가 만성부비동염의 비용 상피세포에서 과발현하며 배양된 조건에서는 염증 매개체에 의해서 증가한다는 것을 보여주었다. 그러나 정상 코점막 상피세포에서 염증 매개체에

의한 *MUC8* 유전자 발현기전은 아직까지 정확히 밝혀지지 않았다. 본 연구에서는 중요한 염증유발 매개체인 PGE_2에 의해 *MUC8* 유전자의 발현이 증가되는 기전을 알아보고자 한다. 우리는 ERK MAP Kinase 신호전달 단백질의 생화학적, 유전학적 저해가 인체 정상 코점막 상피세포에서 PGE_2에 의해 유도되는 *MUC8* 유전자 발현을 억제시킨다는 것을 알았다. 게다가 RSK1 DN의 발현은 PGE_2에 의한 *MUC8* 유전자 발현을 억제하며, RSK1이 CREB을 활성화 시키고 활성화된 CREB은 CRE에 의해 매개되는 유전자의 전사를 일으킨다. 이러한 결과들에 의해서 인체 호흡기 상피세포에서 PGE_2에 의한 *MUC8* 유전자의 발현 기전은 순차적으로 ERK/ RSK1/ CREB에 의한 기전임을 증명하였다.

핵심되는 말 : ***MUC8*, PGE2, ERK MAP kinase, RSK1, CREB**