Inhibition of *MUC5AC* gene expression by anethole in human airway epithelial cell via TAK1-MAPK-AP-1 and TAK1-IκB-NF-κB pathways

Eun Jung Lee

Department of Medicine

The Graduate School, Yonsei University

Inhibition of *MUC5AC* gene expression by anethole in human airway epithelial cell via TAK1-MAPK-AP-1 and TAK1-IκB-NF-κB pathways

Eun Jung Lee

Department of Medicine

The Graduate School, Yonsei University

Inhibition of *MUC5AC* gene expression by anethole in human airway epithelial cell via TAK1-MAPK-AP-1 and TAK1-IκB-NF-κB pathways

Directed by Professor Kyung-Su Kim

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctor of Philosophy

Eun Jung Lee

June 2016

This certifies that the Doctoral Dissertation of Eun Jung Lee is approved.

Thesis Supervisor : Kyung-Su Kim

Thesis Committee Member#1 : Chang-Hoon Kim

Thesis Committee Member#2 : Jin Kook Kim

Thesis Committee Member#3: Myung Hyun Sohn

Thesis Committee Member#4: Joo Young Kim

The Graduate School
Yonsei University

June 2016

ACKNOWLEDGEMENTS

First of all, I would like to express my deepest gratitude towards my mentor, Professor Chang-Hoon Kim, for his full of enthusiasm and passion. He has guided me all the way and taught me in every aspects of my life to become what I am now today.

Next, I am obliged to pass my most sincere appreciation to Professor Kyung-Su Kim, who has provided every help to put all this together. I thank him for his guidance, enthusiasm, incredible patience and kindness. He has been a tremendous positive influence throughout my time.

Truly thanks to Professors Jin Kook Kim, Myung Hyun Sohn, Joo Young Kim, and So Yeon Kim who have spared no pains in giving critical advice for improvement of the quality of this study, in spite of their busy schedule.

Many people have played important roles in my life, non-more so that my family. For that I thank my mum Mi Ryung, my dad Chong Kwon and my brother Jung Hoon. They, who have provided me with such an overwhelmingly strong foundation, such love and guidance to follow my dreams and fight for them. For without my family's understanding and unfathomed support, this Dissertation could never see the light of day.

<TABLE OF CONTENTS>

LIST OF FIGURES

ABSTRACT

Inhibition of *MUC5AC* gene expression by anethole in human airway epithelial cell via TAK1-MAPK-AP-1 and TAK1-IκB-NF-κB pathways

Eun Jung Lee

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Kyung-Su Kim)

Mucin overproduction is a hallmark of chronic rhinosinusitis. Natural compounds with mucin-suppressive property are attractive and effective as mucin regulatory agents in airway diseases such as chronic rhinosinusitis. The aim of this study was to investigate whether anethole suppresses interleukin (IL)-1β induced *MUC5AC* gene expression in human airway epithelial cells and whether this activity of anethole is related to TAK1-MAPK-AP-1 and TAK1-IκB-NF-κB signaling pathways. NCI-H292 cells were pretreated with 50 μM of anethole for 1 h, then 10 ng/mL of IL-1β was added for 24 h. *MUC5AC* mRNA expression was then measured by real-time PCR. The phosphorylation levels of proteins were assayed by western blot. The nuclear components of NF-κB and AP-1 were assayed using luciferase activity. And the nuclear translocation of NF-κB and AP-1 from cytosol was observed by the confocal laser scanning microscopy. Cell survival remained above 90% in the presence of anethole at a concentration of 50 μM. IL-1β induced *MUC5AC* mRNA expression was significantly decreased by 50 μM anethole, to the level of the

untreated control group, as opposed to an increase to 3.2 ± 0.5-fold for IL-1β alone, and this suppression of *MUC5AC* expression was dose-dependent. This decrease in *MUC5AC* expression by anethole was mediated via the MAPK pathway, such as phospho-p38 and phospho-ERK. And the suppression of *MUC5AC* by anethole was also mediated via the TAK1-MAPK-AP-1 and TAK1-IκB-NF-κB signaling pathways. In suppressing IL-1β-induced *MUC5AC* gene expression by anethole, NF-κB or AP-1 was important transcription factor. These results suggest that anethole suppresses IL-1β-induced *MUC5AC* gene expression in human airway epithelial cells via the TAK1-MAPK-AP-1 and TAK1-IκB-NF-κB signaling pathways, and may be considered an anti-hypersecretory agent.

Key words : anethole, cell membrane, mucin, MAP kinase, NF-κB, AP-1

Inhibition of *MUC5AC* gene expression by anethole in human airway epithelial cell via TAK1-MAPK-AP-1 and TAK1-IκB-NF-κB pathways

Eun Jung Lee

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Kyung-Su Kim)

I. INTRODUCTION

Chronic inflammatory upper airway diseases such as allergic rhinitis and chronic rhinosinusitis have the common pathologic findings of secretory cell hyperplasia and mucus hypersecretion. Mucin hypersecretion results in inflammatory airway diseases such as rhinitis, sinusitis, and bronchitis.[1] Various genes and cytokines are related to mucin secretion.[2-4] Until now, at least 21 human mucin genes have been distinguished and *MUC5AC* and *MUC5B* proteins among mucins are expressed in sinus mucosa causing goblet cell hyperplasia and metaplasia. Jung et al reported that *MUC4*, *MUC5AC*, *MUC5B*, and *MUC8* mRNA are main mucins in the ethmoid mucosa and are up-regulated by chronic inflammation.[5] When comparing the expression of *MUC5AC* and *MUC5B* mRNAs in normal and chronic sinus mucosa, in immunohistochemical analysis, more intense staining for *MUC5AC* and *MUC5B* proteins was displayed in the chronic sinusitis specimens than in the healthy controls.[6] In

other words, *MUC5AC* and *MUC5B* proteins are expressed in sinus mucosa, indicating increased number of *MUC5AC*- and *MUC5B*-positive staining cells due to goblet cell hyperplasia and metaplasia. Also, *MUC5AC* is hypersecreted in many respiratory diseases such as rhinitis, sinusitis, nasal allergy, and chronic bronchitis.[7] Therefore, *MUC5AC* is recognized as the major airway mucin gene in the airway epithelium. Hypersecretion of this mucin results in clinical problems such as rhinorrhea, nasal stuffiness, and sputum. Accordingly, regulation of *MUC5AC* can be a new strategy for treating respiratory diseases because it decreases mucus hypersecretion.

IL-1β is a key inflammatory cytokine of innate immunity.[8-9] The inflammasome is a cytosolic multiprotein complex that detects pathogens and danger signals and induces the activation of the proinflammatory cytokines such as IL-1β. Some studies suggest that IL-1β does not only induce urticarial rashes in autoinflammatory diseases but also plays a role in other allergy-related diseases such as bronchial asthma, contact hypersensitivity and atopic dermatitis.[10-11] Recently, it has been determined that IL-1β induces *MUC5AC* gene over-expression in NCI-H292 cells via extracellular signal regulated kinase (ERK)/p38 mitogen-activated protein kinase (MAPK) cascade.[12] Previous studies also showed the involvement of p38/ERK MAPK pathways in suppression of IL-1β-induced *MUC5AC* gene and protein expressions by natural compounds such as ginkgo biloba extract (EGb 761), [6]-gingerol, and berberine.[13-15]

Anethole [1-methoxy-4-(1-propenyl)benzene] occurs naturally as a major component of the essential oil of star anise (*Illicium verum* Hook.f., family Illiciaceae), comprising more than 90 % of its volatile components. *Illicium verum* Hook.f. (Illiciaceae), popularly known as star anise, is a plant native to southeastern China, Vietnam, India, and Japan. This plant's essential oil has anethole [1-methoxy-4-(1-propenyl) benzene] as the major component (about 90 %), as well as methyl chavicol and anisaldehyde, and the oil is widely used in the food, pharmaceutical, and cosmetic industries. Modern pharmacologic studies demonstrated that its crude extracts and active compounds possess wide pharmacological actions, especially in antimicrobial, antioxidant, insecticidal, analgesic, sedative, and anti-convulsive activities.[16-18] Recent study proved that anethole inhibits edematous effect in the acute inflammatory model of mouse paw.[19] Also, Kang showed that anethole prevents lipopolysaccarride-induced acute lung inflammation in mice, suggesting that anethole may be therapeutically effective in inflammatory conditions in humans.[20]

MUC5AC gene plays important roles in the pathogenesis of sinus hypersecretion in chronic rhinosinusitis. And, natural compounds such as anethole would have potential in regulating mucin secretion. Therefore, the aim of the present study is to elucidate the role of anethole, which has potent mucin regulation effect, in IL-1β-induced *MUC5AC* gene expression in NCI-H292 human airway epithelial cells. Also we investigated the signal transduction pathway involved in IL-1β-induced *MUC5AC* gene suppression by anethole.

II. MATERIALS AND METHODS

1. Materials

Anethole was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). IL-1β was obtained from R&D Systems. Antibodies against phosphorylated p38 MAPK (Thr180/Tyr182), phosphorylated p44/42 MAPK (Thr202/Tyr204), total p44/42 MAPK (Thr202/Tyr204), phosphorylated TAK1 (Ser 412), phosphorylated MEK1/2, phosphorylated NF-κB p65, total NF-κB p65, phosphorylated IκBα, phosphorylated c-Jun, and c-Fos were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody to Type I IL-1 receptor (phospho-Y496) was purchased from Abcam (Cambridge, MA, USA). The α-tubulin, goat anti-rabbit and goat anti-mouse antibodies conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology Lnc. (Santa Cruz, CA, USA).

2. Cell culture

NCI-H292 cells, a human pulmonary mucoepidermoid carcinoma cell lines, were purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI 1640 (Gibco BRL, Grand Island, NY, USA) and DMEM (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Cellgro, Hemdon, VA, USA) in the presence of 2 mM L-glutamine, penicillin (100 μg/mL), and streptomycin (100 μg/mL) at 37°C in

a humidified chamber with 95% air and 5% CO_2 incubator. For serum deprivation, cultures were washed with phosphate-buffered saline (PBS) and re-cultured in RPMI 1640 for 24 hr.

3. Cell viability by MTS assay

Cell viability was determined by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium, inner salt] and electron coupling reagent (phenazine ethosulfate) assays from Promega (Madison, WI, USA). The cells were seeded in 96 well plates at plating densities as 2,000 cells / well. After serum starvation for 24 hr, the cells were treated with anethole at various concentrations (0, 5, 25, 50 μM) for 24 hr. At the end of the exposure period, the media was removed, and cells were incubated in CellTiter96 AQueous One Solution Proliferation Assay reagent from Promega (Madison, WI, USA) for 1 hr at 37°C in 5% CO_2. The absorbance at 490 nm was recorded using a microplate reader. Cell viability was calculated as percentage of control (untreated cells).

4. Quantitative real-time polymerase chain reaction of *MUC5AC* mRNA

NCI-H292 cells were plated in 60-mm cell culture dishes, serum-starved, and pretreated with anethole for 1 hr. Subsequently they were stimulated with IL-1β for 24 hr. The total RNAs were isolated from cells under each condition using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Equal amounts of RNA (1 μg)

was reverse transcribed into cDNA using oligo dT primers and AMV reverse transcriptase XL (Takara BIO INC, Japan) and *MUC5AC* cDNA was amplified by PCR with a Perkin-Elmer Cetus DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT). The Thermocycler (ABI PRISM 7700 Sequence Detection System) parameters were 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. All reactions were performed in triplicate. Real-time PCR was performed with Commercial reagents (SYBR GreenER qPCR SuperMix for ABI PRISM, Invitrogen, USA) and conditions were applied in accordance with the manufacturer's protocol. One microgram of cDNA (reverse transcription mixture) and oligonucleotides at final concentrations of 800 nM for primers and 200 nM for TaqMan hybridization probes were analyzed in a 25 μL volume. The real time-polymerase chain reaction (PCR) probe was labeled with carboxyfluorescein (FAM) at the 5' end and with the quencher carboxytetramethylrhodamine (TARMA) at the 3' end. The following primer sequences were used: human *MUC5AC* (forward, 5'-CGA CAA CTA CTT CTG CGG TGC-3'; reverse, 5'-GCA CTC ATC CTT CCT GTC GTT-3') and β2-microglobulin (β2M) (forward, 5'-CTC GCG CTA CTC CTT TCT GG-3'; reverse, 5'- GCT TAC ATG TCT CGA TCC CAC TTA A-3'). The *MUC5AC* mRNA levels were measured using an Applied Biosystems 7300. Data were calculated on the basis of the threshold cycle (Ct) value. Relative quantities of *MUC5AC* mRNA were normalized using β2M as an endogenous control.

5. Western blot analysis

Control and treated cells were harvested in PBS. Cell lysates were prepared for 30 minutes in radioimmunoprecipitation assay buffer from Cell Signaling Technology (Danvers, MA, USA) and phosphatase inhibitor cocktail from Sigma-Aldrich Inc. (St. Louis, MO, USA). Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL 61105) using bovine serum albumin as a standard. Equal amounts (30 μg) of each protein lysate were separated by SDS/PAGE using electrophoresis. We used 4-12% gels (Invitrogen, Grand Island, NY, USA) and resolved proteins were transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Freiburg, Germany). The p-IL-1RI (1:500), p-extracellular signal-regulated kinases (ERK) (1:1,000), and p-p38 MAPK (1:1,000) antibodies were used. The p-TAK1, p-IκB, total IκB, p-NF-κB-p65, p-c-Jun, p-MEK1/2, total ERK1/2, and c-Fos antibodies were also used. The membranes were blocked with 5% bovine serum albumin at room temperature and then probed with primary antibodies and horseradish peroxidase–conjugated secondary antibodies. Protein bands were visualized by incubating membranes with Novex Chemiluminescent substrates (Invitrogen, Grand Island, NY) and exposed to x-ray film (Kodak, Wiesbaden, Germany). The intensity of each band was quantified using the NIH Image J software (National Institutes of Health, Bethesda, MD). The value of each band was marked as a ratio using their corresponding α-tubulin level.

6. Luciferase reporter assay

We used a Nano-Glo Dual Luciferase Reporter Assay System from Promega (Madison, WI, USA) to determine the effect of anethole on NF-κB and AP-1 transcriptional activity induced by IL-1β in NCI-H292 cells. Cells were transfected with NF-κB and AP-1 luciferase reporter plasmid using Lipofectamine 2000 (Invitrogen Carlsbad, CA, USA) according to the manufacturer's instructions. For NF-κB, the 20μg pNL3.2.NF-κB-RE[NlucP/NF-κB-RE/Hygro] and pGL4.53[luc2/PGK] vector was used. For AP-1, the pNL1.1PCK[Nluc/PGK] and pGL4.44[luc2P/AP1RE/Hygro] vector was used. Control plasmid was co-transfected as an internal control to evaluate transfection efficiency. Transiently transfected cells were treated with anethole for 24 hr before stimulated with IL-1β for 3 hr. Luminescence was quantitated with Nano-Glo Dual-Luciferase Reporter Assay System from Promega (Madison, WI, USA) according to the manufacturer's protocol. Luciferase activity was measured using the FLUO star OPTIMA microplate reader (BMG LABTECH, Offenburg, Germany). NF-κB and AP-1 transcriptional activity were expressed as relative luciferase units (fold) after normalization to control and carried out in triplicate.

7. Immunofluorescence staining

Fluorescence immunocytochemistry was used to examine the effect of anethole on the nuclear translocation of NF-κB p65, c-Jun, and c-Fos. NCI-H292 Cells were cultured on chamber microscope slides. Cells were pretreated with anethole for 24 hr and then stimulated with 10 ng/ml IL-1β for 3 hr. The cells were fixed in paraformaldehyde solution (4%) and incubated with NF-κB p65, c-Fos, and p-c-Jun antibodies. Primary antibody was detected with Texas Red-conjugated goat anti-rabbit IgG (red fluorescence) which was excited at 594 nm wavelength. Cell nuclei were stained with Hoechst (blue fluorescence) which was excited at 461 nm wavelength and analyzed under a Zeiss LSM 780 microscope (Zeiss, Jena, Germany).

8. Statistical analysis

Each experiment was performed at least three times. The mean value and the standard deviation were calculated and the parameters were expressed as the means ± SD. Student's t test and one way ANOVA were performed using SPSS 20.0 statistical software (SPSS, Chicago, IL, USA). P-values less than 0.05 were defined as significant (* $P<0.05$).

III. RESULTS

1. Cell viability by anethole

To evaluate the cytotoxicity of anethole, we treated cells with anethole at various concentrations (0, 5, 25, 50 μM) for 24 hr, and examined cell viability. The 5 μM anethole showed 99 ± 1 % cell viability compared with the control group (no treatment with anethole); 25 μM of anethole showed 99 ± 2 % and 50 μM of anethole showed 95 ± 4%. (Fig. 1) Cell viability by anethole was not affected by various concentrations.

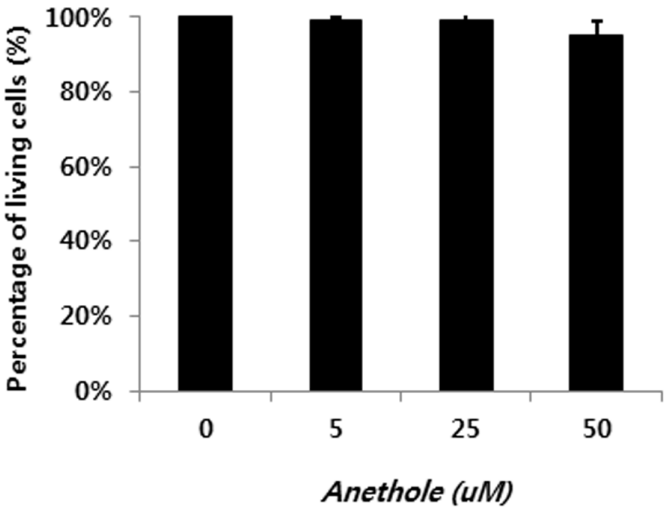

Fig. 1. Cell viability by anethole. Cell viability by anethole is not affected by all concentrations.

2. Changes in *MUC5AC* expression by anethole

Cultured NCI-H292 cells were pretreated with various concentrations of the anethole for 1 hr, and then they were treated with 10 ng/mL of IL-1β for 24 hr. *MUC5AC* expression in each anethole at various concentrations was found as follows: 1.4 ± 0.3 at 5 μM, 1.3 ± 0.2 at 25 μM, 1.5 ± 0.3 at 50 μM. IL-1β induced the *MUC5AC* expression, but this expression was significantly decreased by treatment with anethole 50 μM in real-time PCR (control : anethole 50 μM : IL-1β : IL-1β + anethole 50 μM = 1: 1.5 ± 0.3 : 3.2 ± 0.5 : 1.4 ± 0.3). (Fig. 2) (*: $p<0.05$) The suppression of IL-1β-induced *MUC5AC* expression by anethole appeared dose-dependent. These results indicated that anethole suppressed IL-1β-induced *MUC5AC* expression, which was dose-dependent, and 50 μM anethole significantly suppressed the IL-1β-induced *MUC5AC* expression. Therefore, we used 50 μM anethole in the following experiments.

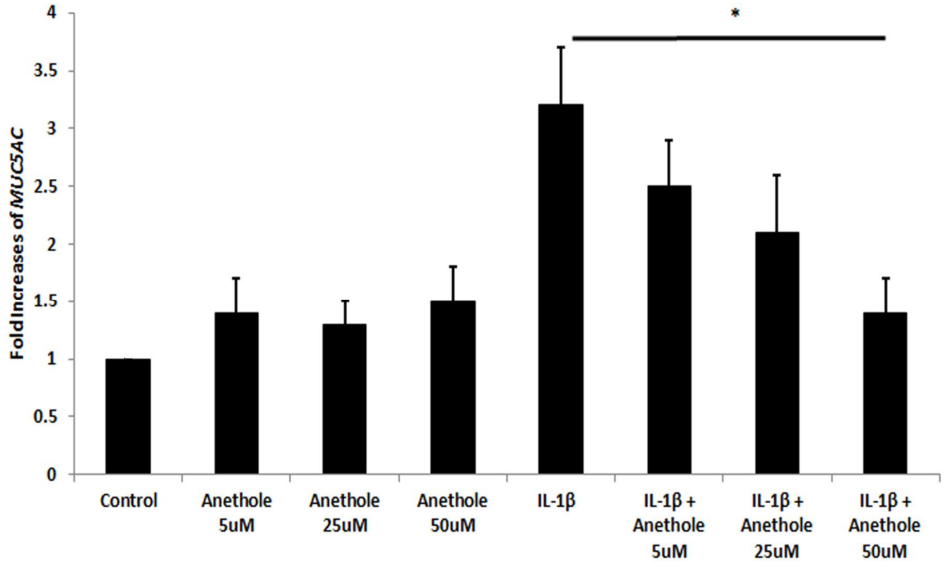

Fig. 2. Changes in *MUC5AC* gene expression by anethole. The suppression of IL-1β-induced *MUC5AC* expression by anethole is dose-dependent and 50 μM anethole significantly suppressed the IL-1β-induced *MUC5AC* expression. (*: $p<0.05$)

3. Changes of IL-1 receptor activity by anethole

To establish whether this decrease of *MUC5AC* expression induced by anethole was related to IL-1 receptor activity, we pretreated cells for 24 hr with 50 μM of anethole and then incubated them with IL-1β for 5 minutes. A group without exposure to either anethole or IL-1β was used as a negative control group and only IL-1β was administered in the positive control group. After each

experiment, Western blot analysis of p-IL-1RI was performed. Expression of p-IL-1RI was decreased in IL-1β-plus anethole-treated cells compared with IL-1β-alone-treated cells, but the decrease was not ststistically significant (control : anethole 50 μM : IL-1β : IL-1β + anethole 50 μM = 1 : 1.6 ± 0.3 : 3.2 ± 0.3 : 1.9 ± 0.2). (Fig. 3A, B)

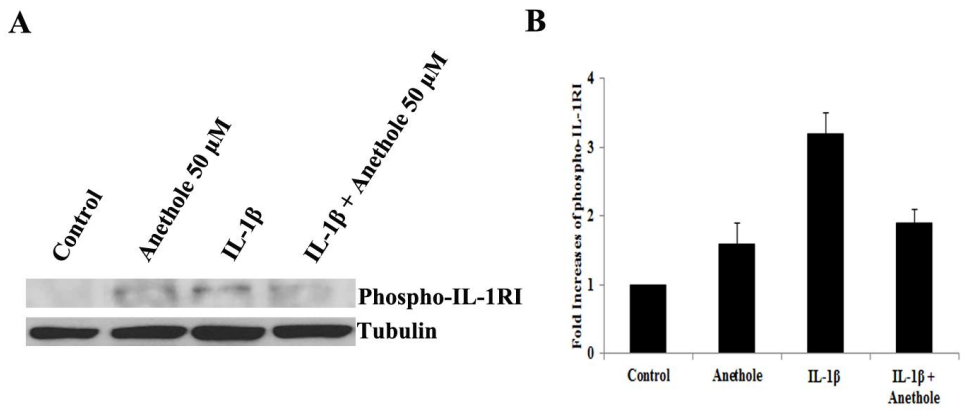

Fig. 3. Change of IL-1β-induced activation of p-IL-1RI by treatment with anethole in NCI-H292 cells. The expression of p-IL-1RI is not significantly decreased in IL-1β-plus anethole-treated cells compared with IL-1β-alone-treated cells.

4. Changes of MAPK phosphorylation by anethole

To establish whether this decrease in *MUC5AC* expression induced by anethole was related to MAPK activity, we pretreated cells for 24 hr with 50 μM of anethole and then incubated them with IL-1β for 5 minutes. A group without exposure to either anethole or IL-1β was used as a negative control group and only IL-1β was administered in the positive control group. After each experiment, protein was obtained and Western blot analysis of p-ERK and p-p38 MAPK was performed. A significant decrease in both p-p38 (Fig. 4A, B) and p-ERK (Fig. 4A, C) levels was noted in IL-1β-plus anethole-treated cells compared with IL-1β-alone-treated cells (for p-p38 expression, control : anethole 50 μM : IL-1β : IL-1β + anethole 50 μM = 1 : 0.8 ± 0.2 : 3.7 ± 0.4 : 1.1 ± 0.2; for p-ERK expression, control : anethole 50 μM : IL-1β : IL-1β + anethole 50 μM = 1 : 1.1 ± 0.2 : 4.5 ± 0.4 : 1.6 ± 0.3). (Fig. 4) (*: $p<0.05$)

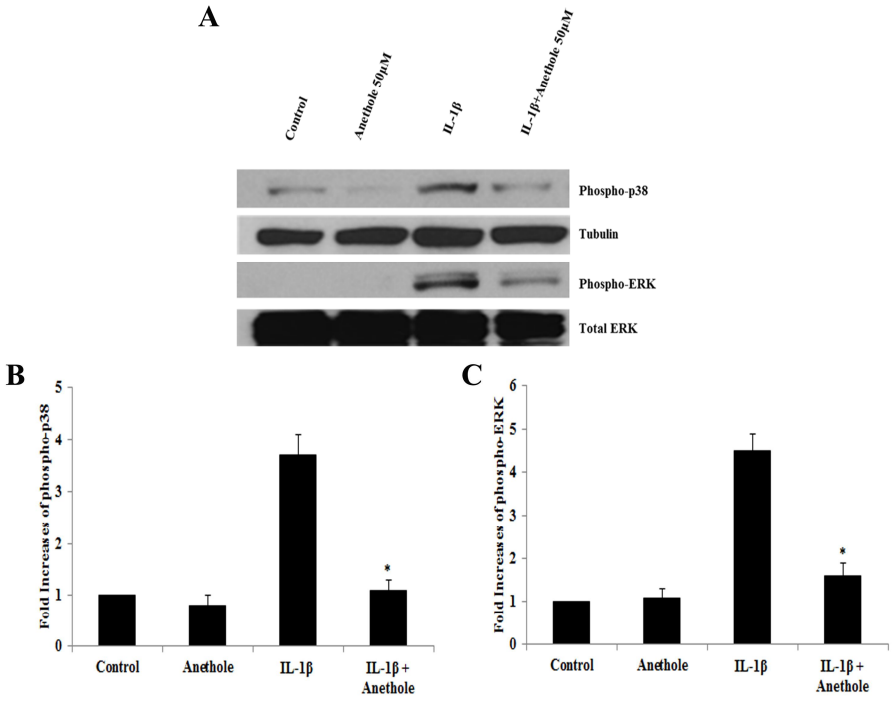

Fig. 4. Suppression of IL-1β-induced activation of MAPK by treatment with anethole in NCI-H292 cells. In MAPK signaling, a significant decrease in both p-p38 and p-ERK expression was noted in IL-1β-plus anethole-treated cells compared with IL-1β-alone-treated cells. (*: $p<0.05$)

5. Anethole suppresses IL-1β-induced *MUC5AC* expression via a TAK1-p38 MAPK-AP-1 signaling pathway

According to the previous studies, the activation of TAK1 leads to the p38 MAPK-AP-1 (c-Jun) signaling pathways.[29] To establish whether this decrease in *MUC5AC* expression induced by anethole was related to TAK1-p38 MAPK-AP-1 (c-Jun) signaling pathway, we pretreated cells for 24 hr with 50

μM of anethole and then incubated them with IL-1β for 5 minutes. A group without exposure to either anethole or IL-1β was used as a negative control group and only IL-1β was administered in the positive control group. After the experiment, protein was obtained and Western blot analysis of p-TAK1, p-p38, and p-c-Jun was performed. A significant decrease in p-TAK1, p-p38, and p-c-Jun levels was noted in IL-1β-plus anethole-treated cells compared with IL-1β-alone-treated cells. (Fig. 5) These results show that anethole suppressed IL-1β-induced *MUC5AC* expression via a TAK1-p38 MAPK-AP-1 (c-Jun) signaling pathway.

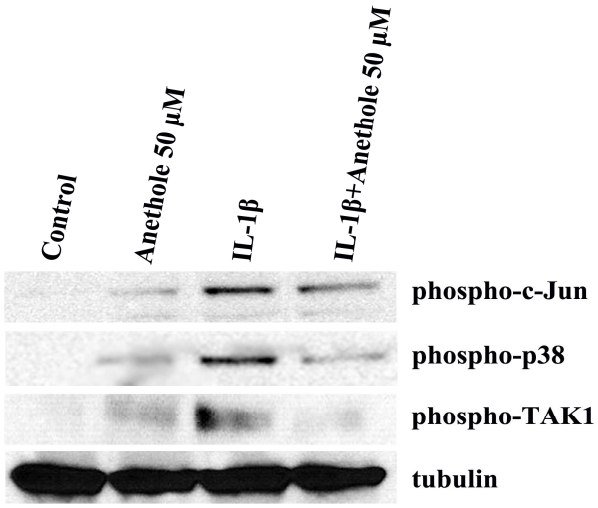

Fig. 5. The TAK1-p38 MAPK-AP-1-dependent suppression of IL-1β-induced *MUC5AC* expression by anethole in NCI-H292 cells. A significant decrease in p-TAK1, p-p38, and p-c-Jun was noted in IL-1β-plus anethole-treated cells compared with IL-1β-alone-treated cells.

6. Anethole suppresses IL-1β-induced *MUC5AC* expression via a TAK1-IκB-NFκB signaling pathway

According to the previous studies, the activation of TAK1 will in turn lead to the activation of IκB signaling pathways.[29] To determine the involvement of the TAK1-IκB-NF-κB signaling cascade in *MUC5AC* suppression by anethole, we pretreated cells for 24 hr with 50 μM of anethole and then incubated them with IL-1β. Considering the change of *MUC5AC* transcription according to the time, p-TAK1 and p-IκB were the most significantly increased at 5 minutes of IL-1β. The change of IκB according to time was continuously decreased, which indicated the IκB degradation. (Fig. 6A) After the experiment, protein was obtained and Western blot analysis of p-TAK1, p-IκB and p-NF-κB-p65 was performed at 5 minutes time point. Elevated expression of p-TAK1, p-IκB and p-NF-κB-p65 in the IL-1β-only-treated cells markedly decreased in the IL-1β-plus anethole–treated cells. (Fig. 6B) (*: $p<0.05$) From this result, we conclude that the TAK1-IκB-NFκB signaling cascade was involved in *MUC5AC* suppression by anethole.

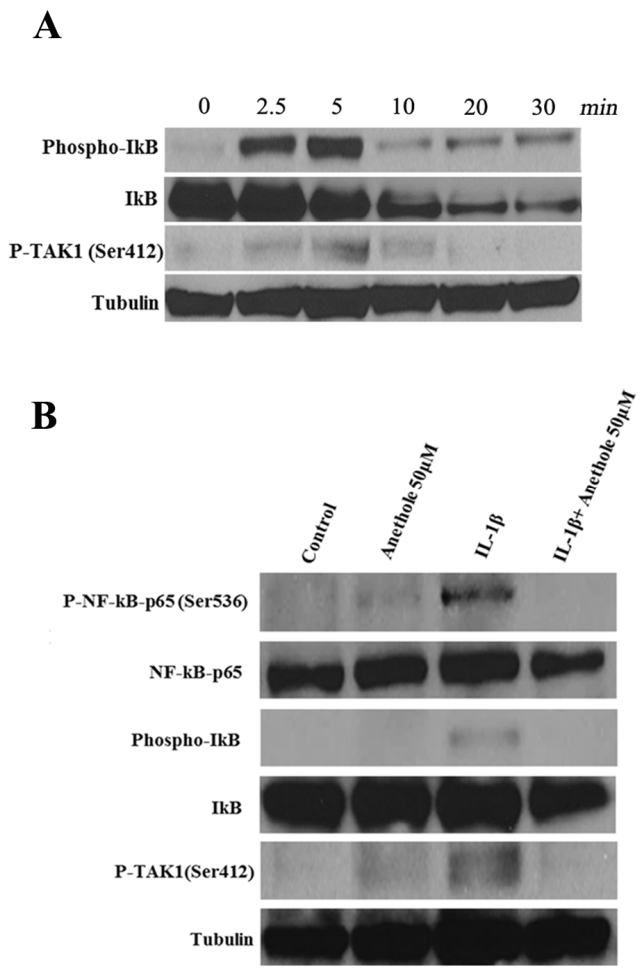

Fig. 6. The TAK1-IκB-NFκB-dependent suppression of IL-1β-induced *MUC5AC* expression by anethole in NCI-H292 cells. The expression of IκB continuously decreases with elapse of time, which indicates IκB degradation. (Fig. 6A) Elevated expression of p-TAK1, p-IκB and p-NF-κB-p65 in the IL-1β-only-treated cells markedly decreases in the IL-1β-plus anethole–treated cells. (Fig. 6B) (*: $p<0.05$)

7. Anethole suppresses IL-1β-induced *MUC5AC* expression via a MEK-ERK MAPK-AP-1 signaling pathway

Ikari A et al showed that the MEK/ERK/c-Fos pathway was involved in the secretion of epidermal growth factor in A549 cell and the activation of MEK1/2 could lead to the phosphorylation of ERK1/2.[30] To establish whether this decrease in *MUC5AC* expression induced by anethole is related to ERK signaling, especially for MEK1/2-ERK1/2-AP-1 (c-Fos) signaling pathway, we pretreated cells for 24 hr with 50 μM of anethole and then incubated them with IL-1β for 5 minutes. A group without exposure to either anethole or IL-1β was used as a negative control group and only IL-1β was administered in the positive control group. After the experiment, protein was obtained and Western blot analysis of p-MEK1/2, p-ERK1/2, and c-Fos was performed. Significant decrease in p-MEK1/2, p-ERK1/2, and c-Fos levels was noted in IL-1β-plus anethole-treated cells compared with IL-1β-alone-treated cells. (Fig. 7) These results show that anethole suppressed IL-1β-induced *MUC5AC* expression via a MEK-ERK MAPK-AP-1 (c-Fos) signaling pathway.

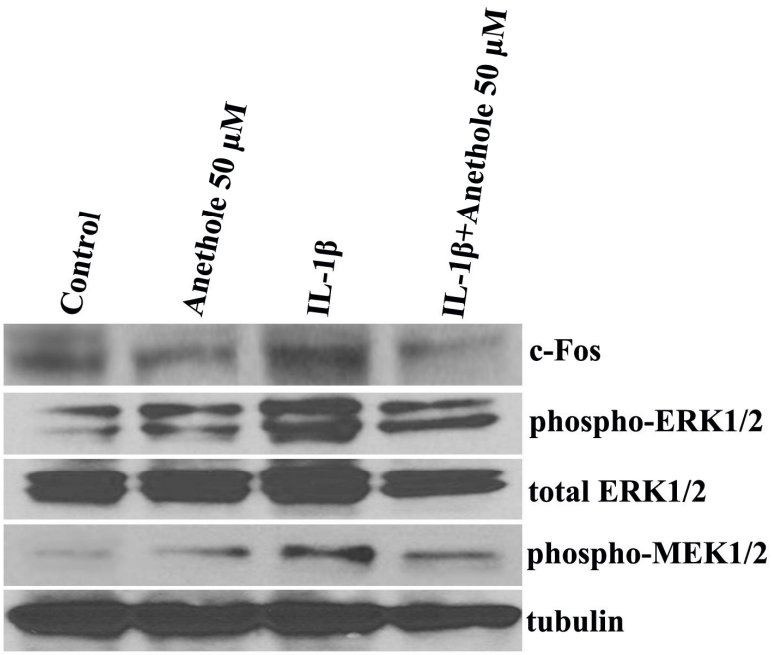

Fig. 7. The MEK-ERK MAPK-AP-1-dependent suppression of IL-1β-induced *MUC5AC* expression by anethole in NCI-H292 cells. A significant decrease in p-MEK1/2, p-ERK1/2, and c-Fos is noted in IL-1β-plus anethole-treated cells compared with IL-1β-alone-treated cells.

8. The inhibitory effect of anethole on IL-1β-induced activation of NF-κB and AP-1

To determine the involvement of the NF-κB and AP-1 in *MUC5AC* transcription, we assessed the change of NF-κB p65 and AP-1 activation. As to NF-κB, IL-1β-induced NF-κB activation was significantly decreased by treatment with

IL-1β-plus anethole 50 μM in luciferase assay (control : anethole 50 μM : IL-1β : IL-1β + anethole 50 μM = 1: 0.7 ± 0.3 : 3.1 ± 0.4 : 1.4 ± 0.3). (Fig. 8A) (*: $p<0.05$) Considering AP-1, IL-1β-induced AP-1 activation was also significantly decreased by treatment with IL-1β-plus anethole 50 μM in luciferase assay (control : anethole 50 μM : IL-1β : IL-1β + anethole 50 μM = 1: 0.9 ± 0.2 : 2.9 ± 0.3 : 1.2 ± 0.3). (Fig. 8B) (*: $p<0.05$)

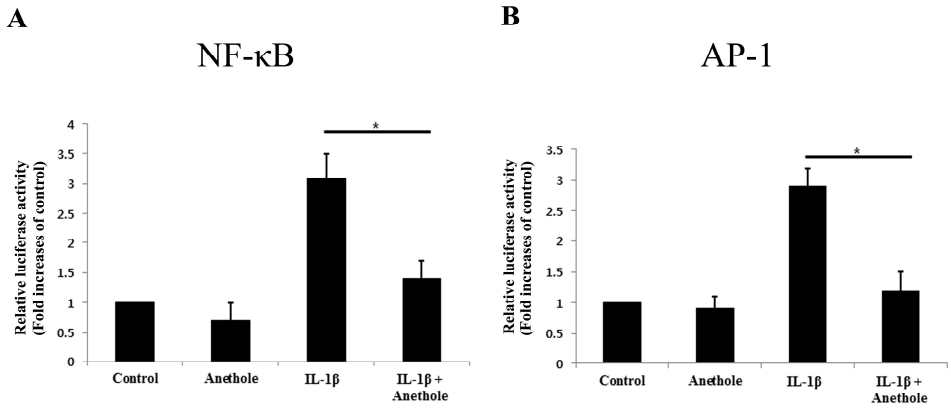

Fig. 8. The inhibitory effect of anethole on IL-1β-induced activation of NF-κB and AP-1. IL-1β-induced NF-κB and AP-1 activations are significantly decreased by treatment with IL-1β-plus anethole in luciferase assay. (*: $p<0.05$)

9. Confirmation of nuclear translocation of NF-κB and AP-1 by confocal laser scanning microscopy

To confirm the nuclear translocation of NF-κB and AP-1, confocal laser scanning microscopy was performed. The nuclear translocation of NF-κB was observed in IL-1β-induced activation of NF-κB, but was decreased by anethole treatment. The translocated NF-κB in nucleus is shown as pink color in IL-1β-induced activation of NF-κB. The response of IL-1β-induced activation of NF-κB by anethole treatment was characterized by re-compartmentalization of NF-κB resulting in a decrease in its nuclear fraction and increase in its cytosolic fraction as red color. (Fig. 9A) Also, the nuclear translocation of c-Jun and c-Fos which are the components of AP-1, was observed in IL-1β-induced activation of AP-1, but was decreased by anethole treatment. The translocated AP-1 in nucleus is shown as pink color in IL-1β-induced activation of AP-1. The response of IL-1β-induced activation of AP-1 by anethole treatment was characterized by re-compartmentalization of AP-1 resulting in a decrease in its nuclear fraction and increase in its cytosolic fraction as red color. (Fig. 9B, 9C) These results confirm that the NF-κB and AP-1 were involved in the suppression of IL-1β-induced *MUC5AC* expression by anethole.

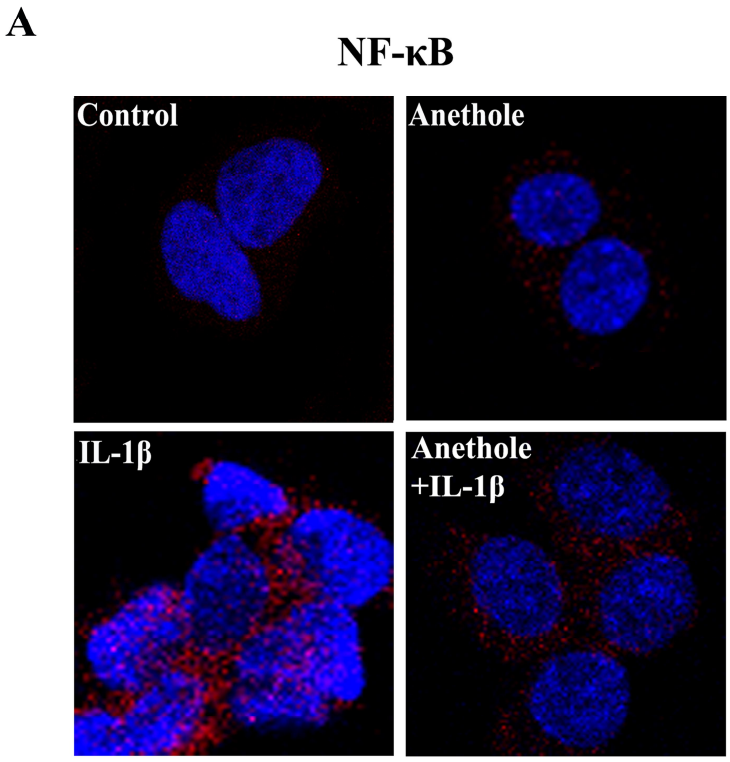

Fig. 9. Merged confocal immunofluorescence imaging of nuclear translocation of NF-κB and AP-1. The nuclear translocation of NF-κB from cytosol in IL-1β-alone condition is noted. In IL-1β-plus anethole condition, the response of IL-1β-induced activation of NF-κB is characterized by re-compartmentalization of NF-κB resulting in a decrease in nuclear fraction and increase in cytosolic fraction. (Fig. 9A) Nucleus is seen as blue color stained with Hoechst. Primary antibody, NF-κB, is revealed with secondary antibody which is shown as red color stained with Texas Red.

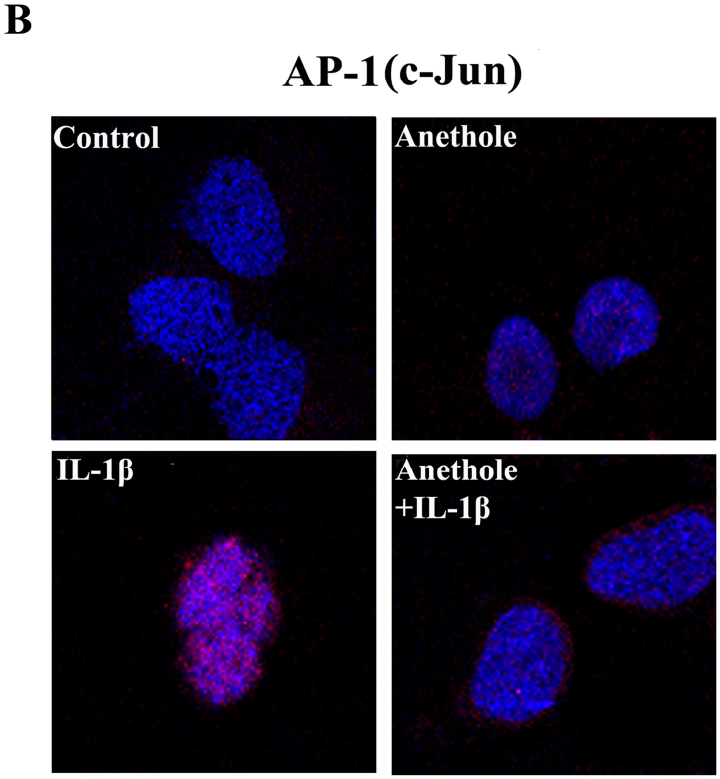

Fig. 9. Merged confocal immunofluorescence imaging of nuclear translocation of NF-κB and AP-1. The nuclear translocation of AP-1 from cytosol in IL-1β-alone condition is noted. In IL-1β-plus anethole condition, the response of IL-1β-induced activation of AP-1 is characterized by re-compartmentalization of AP-1 resulting in a decrease in nuclear fraction and increase in cytosolic fraction. (Fig. 9B) Nucleus is seen as blue color stained with Hoechst. Primary antibody, p-c-Jun, is revealed with secondary antibody which is shown as red color stained with Texas Red.

C

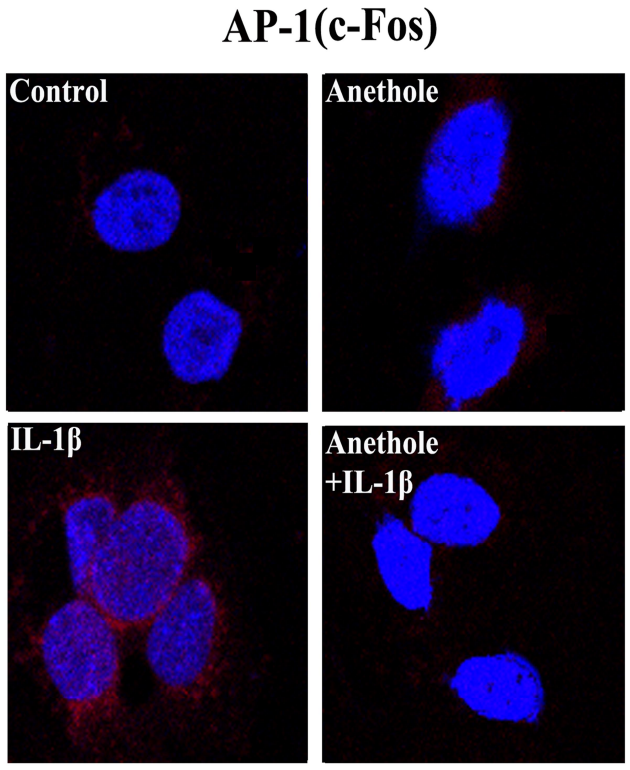

Fig. 9. Merged confocal immunofluorescence imaging of nuclear translocation of NF-κB and AP-1. The nuclear translocation of AP-1 from cytosol in IL-1β-alone condition is noted. In IL-1β-plus anethole condition, the response of IL-1β-induced activation of AP-1 is characterized by re-compartmentalization of AP-1 resulting in a decrease in nuclear fraction and increase in cytosolic fraction. (Fig. 9C) Nucleus is seen as blue color stained with Hoechst. Primary antibody, c-Fos, is revealed with secondary antibody which is shown as red color stained with Texas Red.

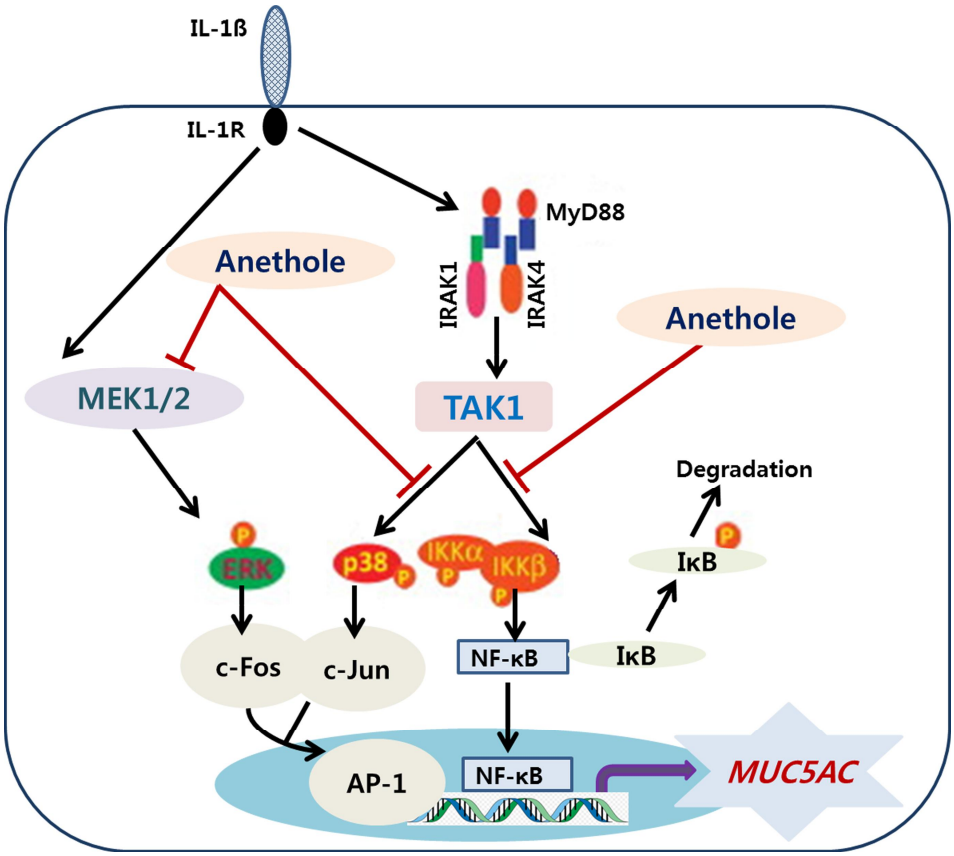

Fig. 10. Schematic representation of the signaling pathways involved in the suppression of IL-1β-induced *MUC5AC* gene expression by anethole. As indicated, anethole suppresses *MUC5AC* mucin gene transcription via TAK1-MAPK-AP-1 and TAK1-IκB-NF-κB signaling pathways in NCI-H292 cells.

IV. DISCUSSION

Mucin overproduction is a hallmark of bacterial infectious diseases such as chronic rhinosinusitis. Among many human mucin genes, *MUC5AC* is hypersecreted in many respiratory diseases such as rhinitis, sinusitis, nasal allergy, and chronic bronchitis.[5-7] Meanwhile, many studies have attempted to identify therapeutic natural materials from several plants to treat airway-related inflammatory disease.[21] These dietary agents are believed to suppress the inflammatory processes and ultimately suppress the mucin formation. Therefore, the regulation of *MUC5AC* by natural compounds can be a new therapeutic strategy for respiratory diseases because it decreases mucus hypersecretion. Here, this study was aim to demonstrate whether anethole decreases IL-1β-induced *MUC5AC* gene expression in NCI-H292 human airway epithelial cells. Moreover, we demonstrated which signal transduction pathway was involved in suppression of IL-1β-induced *MUC5AC* gene expression by anethole.

Anethole consists of methoxybenzene of methoxyphenol ring and a propenyl substitution and the conjugate double bonds in anethole are known to stabilize phenoloxy and benzyloxy reactivity. We initially checked the cell viability from anethole treatment because this chemical reactivity may influence the cell viability. The cell viability was not affected by anethole of varying concentrations. (Fig. 1) Chainy et al. proved the effect of anethole on both early and late cellular responses.[22] From Chainy's result, the concentration of the

anethole and the time of incubation had no effect on cell viability which was maintained greater than 97%.[22] Chainy's result coincides with this present study. Anethole acts as the precursor to illicit lots of drugs as well as the flavoring agent or essential oils in practice. Although large quantities of anethole can be mildly toxic, anethole has no safety concerns at current levels of intake when used as a flavoring agent according to the Joint FAO/WHO Expert Committee on Food Additives.[23] In this study, the various concentrations of anethole showed no toxicity to cell viability.

Since anethole exhibits anti-inflammatory properties, we postulated that the effect of anethole is involved in the suppression of IL-1β-induced *MUC5AC* gene expression. The results showed that the IL-1β-induced *MUC5AC* expression was dose-dependently suppressed by anethole. (Fig. 2) These results suggested that anethole suppressed IL-1β-induced *MUC5AC* expression in a dose-dependent manner, where 50 μM anethole significantly suppressed the IL-1β-induced *MUC5AC* expression. Therefore, we used 50 μM anethole for the following experiments. Kwon et al. showed at least 10 mM sodium salicylate inhibits expression of COX-2 through the suppression of MAPK and subsequent NF-κB activation.[24] The present study proved 50 μM anethole was sufficiently efficient for the suppression of IL-1β-induced *MUC5AC* expression and suggested anethole may be a more potent inhibitor for inflammation than sodium salicylate.

The MAP kinase is an important cell regulating protein kinase in the MAP3K

→ MAP2K → MAPK pathway which is involved in proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis.[25] MAPKs are involved in directing cellular responses to a diverse array of stimuli, such as mitogens, osmotic stress, heat shock protein and proinflammatory cytokines. IL-1β induces *MUC5AC* overexpression through a mechanism involving p38/ERK MAPK-activated protein kinase-MSK1-CREB activation in human airway epithelial cells.[12] Also, there has been many studies investigating the role of MAPK in the suppression of IL-1β-induced *MUC5AC* gene expression by natural compounds.[26-27] In our previous studies, we proved that natural compounds such as ginkgo biloba extract (EGb 761), [6]-gingerol, and berberine–mediated suppression of IL-1β-induced *MUC5AC* mRNA occurred specifically at the IL-1 receptor and operated via p38/ERK MAPK-dependent pathway.[13-15] Therefore, to reveal the involvement of IL-1 receptor and the MAPK pathway for the decrease of *MUC5AC* expression by anethole, we examined IL-1 receptor activity and the MAPK cascade, especially the p38 and ERK MAPK pathway. The p-IL-1RI expression by IL-1β stimulation showed no significant change by anethole. (Fig 3A, B) IL-1 receptor antagonist is a member of the IL-1 family and is a natural inhibitor of the pro-inflammatory effect of IL-1β. Although there were many studies about the action of IL-1 receptor antagonist, the receptor-specific effect of the natural compounds has not been studied until now. Thus, IL-1 receptor is not considered as a direct target of anethole in regulation of *MUC5AC* induced by IL-1β. Meanwhile, the

human airway epithelial cell membrane consists of a phospholipid bilayer with embedded proteins. Since the cell membrane is flexible and made of lipid molecules, it can be mixed with protein molecules such as natural compounds that act as channels through which natural compounds enter and act on the cell by diffusion, trans-membrane protein channels, endocytosis, and exocytosis. From these processes, the p-p38 MAPK induced by IL-1β stimulation was decreased by anethole treatment and this decrease in p-p38 MAPK was associated with a decrease in *MUC5AC* expression. (Fig 4A, B) The p-ERK MAPK was also decreased by anethole. (Fig 4A, C)

We have previously reported that some natural compounds potently down-regulate IL-1β-induced *MUC5AC* expression.[13-15] We could also prove that anethole could decrease IL-1β-induced *MUC5AC* expression via MAPK signaling. Here we further investigated the TAK1-related signaling. Farias R et al. showed the TAK1 → IKKβ → TPL2 → MKK1/MKK2 pathway regulates IL-33 expression following an infection with *Pseudomonas aeruginosa* in cystic fibrosis airway epithelial cells.[28] Furthermore, Chen et al. showed that MyD88–IRAK1–TRAF6–TAK1 signaling cascade is involved in P6-induced *MUC5AC* mucin transcription, and the activation of TAK1 will in turn lead to the activation of IκB-NF-κB and p38 MAPK signaling pathways.[29] Also, upon TAK1 activation, NF-κB or AP-1 is required for *MUC5AC* expression in normal human bronchial epithelial cells.[29,31] On the basis of these findings, it could be inferred that TAK1-p38 MAPK-AP-1 and TAK1-IκB-NF-κB signaling

pathways were responsible for the regulation of IL-1β-induced *MUC5AC* expression when human airway epithelial cells stimulated by IL-1β were treated with anethole. Therefore, we further elucidated the TAK1-p38 MAPK-AP-1 and TAK1-IκB-NF-κB signaling pathways.

For TAK1-p38 MAPK-AP-1 pathway, p-TAK1, p-p38, and p-c-Jun induced by IL-1β stimulation were decreased by anethole treatment indicating that the suppression of IL-1β-induced *MUC5AC* expression by anethole was done via TAK1-p38 MAPK-AP-1 signaling. (Fig 5) For TAK1-IκB-NF-κB signaling, the activation of p-TAK1 and p-IκB by IL-1β were shown and was maximally increased at 5 minutes of IL-1β stimulation accompanying with decrease in IκB. (Fig 6A) The expression of p-TAK1, p-IκB, and p-NF-κB-p65 was significantly reduced following anethole 50μM treatment. (Fig 6B) These indicate that anethole suppressed IL-1β-induced *MUC5AC* expression via TAK1-p38 MAPK-AP-1 and TAK1-IκB-NF-κB signaling pathways.

TAK1, a protein kinase of the MLK family, is activated by various cytokines, including IL-1. In response to IL-1, TAK1 forms a kinase complex including TRAF6 and TAK1 activation leads to the activation of IKK and JNK as well as p38. Activated IKK phosphorylated IκBα proteins, and phosphorylated IκBα proteins are degraded by the ubiquitin-mediated proteasome pathway. The degradation of IκBα proteins leads to NF-κB translocation into the nucleus and activation of NF-κB-dependent gene transcription in the nucleus. This is the classical TAK1-dependent pathway. There is also the TAK1-independent

pathway in which IKK phosphorylation and IKKα activation leads to NF-κB activation without IκB degradation.[32] In this study, we demonstrated the decrease of IκB through elapse of time and IκB degradation was proven during TAK1-IκB-NF-κB signaling pathway. (Fig 6A) Therefore, classical TAK1-IκB-NF-κB-dependent pathway is involved in the suppression of IL-1β-induced *MUC5AC* expression by anethole.

Although p-ERK MAPK was also decreased by anethole in this study, the ERK pathway has not been fully revealed in IL-1β-induced mucin regulation. Ikari A et al. showed that the MEK-ERK-c-Fos pathway was involved in the secretion of epidermal growth factor in airway epithelial cell.[30] Based on these findings, we investigated the MEK1/2-ERK1/2-AP-1 (c-Fos) signaling pathway and showed that anethole suppressed IL-1β-induced *MUC5AC* expression via the MEK1/2-ERK1/2 MAPK-AP-1 signaling pathway. (Fig 7) This result means that the ERK pathway is also involved in the mucin regulation such as IL-1β-induced *MUC5AC* expression.

Fujisawa et al. showed that NF-κB-based transcriptional mechanism is involved in *MUC5AC* regulation by IL-1β and IL-17A in the airway epithelium.[33] Also, Chen et al. proved activation of both NF-κB and AP-1 is required for P6-induced *MUC5AC* transcription.[29] Based on these findings, we investigated whether the activation of NF-κB or AP-1 is required for IL-1β-induced *MUC5AC* transcription by luciferase assay. The results showed IL-1β-induced NF-κB activation was markedly reduced by treatment with

anethole 50μM. (Fig. 8A) Also, IL-1β-induced AP-1 activation was also significantly decreased by treatment with anethole 50μM. (Fig. 8B) Thus, these results demonstrated that activation of NF-κB or AP-1 is required for IL-1β-induced *MUC5AC* transcription. The confocal laser scanning microscopy also proved the nuclear translocation of NF-κB and AP-1 from cytosol in IL-1β-alone-treated condition. And the nuclear translocation was significantly decreased in IL-1β-plus anethole-treated condition. (Fig. 9)

The main drawback of this study is the used cells. If we used normal bronchial or nasal cells, the result may have been different because of the culture method, drug delivery method, and nature of cells. Thus, future studies using these cells are needed. Second, although MEK-ERK-AP-1 pathway signaling was also decreased by anethole in this study, the detailed ERK pathway was not revealed in this study. Previous studies showed the ERK pathway are more involved in cancer-related mechanism such as cell proliferation and uncontrolled growth than inflammation.[34-35] Although these studies reflect the ERK pathway is also involved in IL-1β-induced *MUC5AC* suppression by anethole, additional mechanism studies for ERK pathway in mucin regulation will be required. Third, the effect of anethole on chronic rhinosinusitis animal model will be required. Recently, anethole and eugenol may effectively ameliorate the progression of asthma in animal models and could be used as a therapy for patients with allergic asthma.[36] Therefore, additional *in vivo* studies about the effect of anethole are needed.

In summary, this study demonstrates that TAK1-MAPK-AP-1 and TAK1-IκB-NF-κB signaling were involved in the suppression of IL-1β-induced *MUC5AC* expression by anethole, and the activation of NF-κB and AP-1 is also suppressed by anethole treatment. This study proved 50 μM anethole was sufficiently efficient for the suppression of IL-1β-induced *MUC5AC* expression indicating potent inhibitor for inflammation. (Fig. 10) Therefore, in CRS which shows hypersecretion of *MUC5AC* overexpression, the natural compounds such as anethole could be considered an effective anti-hypersecretory agent in airway disease such as chronic rhinosinusitis.

V. CONCLUSION

Anethole suppresses IL-1β-induced *MUC5AC* mRNA expression in human airway epithelial cells via the TAK1-MAPK-AP-1 and TAK1-IκB-NF-κB signaling pathways, and may be considered as an anti-hypersecretory agent.

REFERENCES

1. Voynow JA, Rubin BK. Mucins, mucus, and airway secretions. CHEST 2009;135:505–12.

2. Hovenberg HW, Davies JR, Herrmann A, Lindén CJ, Carlstedt I. *MUC5AC*, but not MUC2, is a prominent mucin in respiratory secretions. Glycoconj J 1996;13:839–47.

3. Thornton DJ, Howard M, Khan N, Sheehan JK. Identification of two glycoforms of the MUC5B mucin in human respiratory mucus. Evidence for a cysteine-rich sequence repeated within the molecule. J Biol Chem 1997;272:9561–6.

4. Wickström C, Davies JR, Eriksen GV, Veerman EC, Carlstedt I. MUC5B is a major gel-forming, oligomeric mucin from human salivary gland, respiratory tract and endocervix: identification of glycoforms and C-terminal cleavage. Biochem J 1998;334:685–93.

5. Jung HH, Lee JH, Kim YT, Lee SD, Park JH. Expression of mucin genes in chronic ethmoiditis. Am J Rhinol 2000;14:163-70.

6. Kim DH, Chu HS, Lee JY, Hwang SJ, Lee SH, Lee HM. Up-regulation of *MUC5AC* and MUC5B mucin genes in chronic rhinosinusitis. Arch Otolaryngol Head Neck Surg 2004;130:747-52.

7. Ali MS, Hutton DA, Wilson JA, Pearson JP. Major secretory mucin expression in chronic sinusitis. Otolaryngol Head Neck Surg 2005;133:423-8.

8. Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. Clin Microbiol Rev 2009;22:240-73.

9. Garlanda C, Dinarello CA, Mantovani A. The interleukin-1 family: back to the future. Immunity 2013;39:1003-18.

10. Goldbach-Mansky R, Kastner DL. Autoinflammation: the prominent role of IL-1 in monogenic autoinflammatory diseases and implications for common illnesses. J Allergy Clin Immunol 2009;124:1141-9.

11. Almeida de Jesus A, Goldbach-Mansky R. Monogenic autoinflammatory diseases: concept and clinical manifestations. Clin Immunol 2013;147:155-74.

12. Song KS, Lee WJ, Chung KC, Koo JS, Yang EJ, Choi JY, et al. Interleukin-1 beta and tumor necrosis factor-alpha induce *MUC5AC* overexpression through a mechanism involving ERK/p38 mitogen-activated protein kinases-MSK1-CREB activation in human airway epithelial cells. J Biol Chem 2003;278:23243-50.

13. Kwon SH, Nam JI, Kim SH, Kim JH, Yoon JH, Kim KS. Kaempferol and quercetin, essential ingredients in Ginkgo biloba extract, inhibit interleukin-1beta-induced *MUC5AC* gene expression in human airway epithelial cells. Phytother Res 2009;23:1708-12.

14. Kim JH, Chang JH, Yoon JH, Kwon SH, Bae JH, Kim KS. [6]-Gingerol suppresses interleukin-1 beta-induced *MUC5AC* gene expression in human airway epithelial cells. Am J Rhinol Allergy 2009;23:385-91.

15. Kim KR, Kim NH, Park Y, Kim JH, Lee EJ, Kim KS. Berberine Suppresses Interleukin-1β-Induced *MUC5AC* gene expression in human airway epithelial cells. J. Rhinol 2011;18:116-21.

16. Chang KS, Ahn YJ. Fumigant activity of (E)-anethole identified in Illicium verum fruit against Blattella germanica. Pest Manag Sci 2002;58:161-6.

17. Waumans D, Bruneel N, Tytgat J. Anise oil as para-methoxyamphetamine (PMA) precursor. Forensic Sci Int 2003;133:159-70.

18. Waumans D, Hermans B, Bruneel N, Tytgat J. A neolignan-type impurity arising from the peracid oxidation reaction of anethole in the surreptitious synthesis of 4-methoxyamphetamine (PMA). Forensic Sci Int 2004;143:133-9.

19. Domiciano TP, Dalalio MM, Silva EL, Ritter AM, Estevão-Silva CF, Ramos FS, et al. Inhibitory effect of anethole in nonimmune acute inflammation. Naunyn Schmiedebergs Arch Pharmacol 2013;386:331-8.

20. Kang P, Kim KY, Lee HS, Min SS, Seol GH. Anti-inflammatory effects of anethole in lipopolysaccharide-induced acute lung injury in mice. Life Sci 2013;93:955-61.

21. Schumacher M, Juncker T, Schnekenburger M, Gaascht F, Diederich M. Natural compounds as inflammation inhibitors. Genes Nutr 2011;6:89-92.

22. Chainy GB, Manna SK, Chaturvedi MM, Aggarwal BB. Anethole blocks both early and late cellular responses transduced by tumor necrosis factor: effect on NF-kappaB, AP-1, JNK, MAPKK and apoptosis. Oncogene

2000;19:2943-50.

23. International Program on Chemical Safety. Summary of evaluations performed by the joint FAO/WHO expert committee on food additives: Trans-anethole. Retrieved March 10, 2009.

24. Kwon KS, Chae HJ. Sodium salicylate inhibits expression of COX-2 through suppression of ERK and subsequent NF-kappaB activation in rat ventricular cardiomyocytes. Arch Pharm Res 2003;26:545-53.

25. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocrine Rev 2001;22:153–83.

26. Jiang DP, Perelman JM, Kolosov VP, Zhou XD. Effects of scutellarin on *MUC5AC* mucin production induced by human neutrophil elastase or interleukin 13 on airway epithelial cells. J Korean Med Sci 2011;26:778-84.

27. Shin HD, Lee HJ, Sikder MA, Park SH, Ryu J, Hong JH, et al. Effect of Chrysin on Gene Expression and Production of *MUC5AC* Mucin from Cultured Airway Epithelial Cells. Tuberc Respir Dis 2012;73:204-9.

28. Farias R, Rousseau S. The TAK1→IKKβ→TPL2→MKK1/MKK2 signaling cascade regulates IL-33 expression in cystic fibrosis airway epithelial cells following infection by Pseudomonas aeruginosa. Front Cell Dev Biol 2016;3:87.

29. Chen R, Lim JH, Jono H, Gu XX, Kim YS, Basbaum CB, et al.

Nontypeable Haemophilus influenzae lipoprotein P6 induces *MUC5AC* mucin transcription via TLR2-TAK1-dependent p38 MAPK-AP1 and IKKbeta-IkappaBalpha-NF-kappaB signaling pathways. Biochem Biophys Res Commun 2004;324:1087-94.

30. Ikari A, Sato T, Watanabe R, Yamazaki Y, Sugatani J. Increase in claudin-2 expression by an EGFR/MEK/ERK/c-Fos pathway in lung adenocarcinoma A549 cells. Biochim Biophys Acta 2012;1823:1110-8.
31. Fujisawa T, Velichko S, Thai P, Hung LY, Huang F, Wu R. Regulation of airway *MUC5AC* expression by IL-1beta and IL-17A; the NF-kappaB paradigm. J Immunol 2009;183:6236-43.
32. Shim JH, Xiao C, Paschal AE, Bailey ST, Rao P, Hayden MS, et al. TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. Genes Dev 2005;19:2668-81.
33. Fujisawa T, Chang MM, Velichko S, Thai P, Hung LY, Huang F, et al. NF-κB mediates IL-1β- and IL-17A-induced MUC5B expression in airway epithelial cells. Am J Respir CellMol Biol 2011;45:246-52.
34. McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EW, Chang F, et al. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. Biochim. Biophys. Acta 2007;1773: 1263–84.
35. Zheng B, Fiumara P, Li YV, Georgakis G, Snell V, Younes M, et al. MEK/ERK pathway is aberrantly active in Hodgkin disease: a signaling

pathway shared by CD30, CD40, and RANK that regulates cell proliferation and survival. Blood 2003;102:1019–27.

36. Estevão-Silva CF, Kummer R, Fachini-Queiroz FC, Grespan R, Nogueira de Melo GA, Baroni S, et al. Anethole and eugenol reduce in vitro and in vivo leukocyte migration induced by fMLP, LTB4, and carrageenan. J Nat Med 2014;68:567-75.

ABSTRACT (IN KOREAN)

사람 호흡기 상피세포에서 TAK1-MAPK-AP-1과 TAK1-IκB-NFκB 경로를 통한 anethole의 *MUC5AC* 유전자 발현 억제 작용

< 지도교수 김 경 수 >

연세대학교 대학원 의학과

이 은 정

사람 점액을 조절하는 MUC 유전자는 약 20개 정도가 밝혀져 있는데, 그 중 *MUC5AC* 과발현은 호흡기 질환에서 가장 흔히 보이는 현상으로 만성 비부비동염을 포함한 호흡기 질환에서 점액 과분비로 인한 다양한 임상 증상을 유발한다. 이에 *MUC5AC*의 조절은 호흡기 질환에서 치료 표적으로 연구되어 왔다. 6-gingerol, ginkgo biloba extract, berberine 등 다양한 천연물에 의해 IL-1β 유도 *MUC5AC* 유전자 발현이 조절되는 기전이 밝혀진 바 있고 이는 p38/ERK MAPK 경로를 통하여 이뤄진다는 기존의 연구가 있으나 이와 관련된 상세 경로는 아직 밝혀진 바가 없다. 이에 본 연구의 목적은 anethole에 의한 IL-1β 유도 *MUC5AC* 유전자 발현의 억제 여부를 확인한 후, 만약 억제가 된다면 해당하는 상세 경로를 밝혀 anethole이 *MUC5AC* 점액의 과분비를 조절하여 과분비로 인한 임상증상을 억제하는 데 사용될 수 있는 가능성을 보이고 호흡기 질환의 특정 표적 치료의 효과를 극대화하고자 함에 있다. Anethole의 세포 독성 실험을 시행하여 안전성 여부를 확인한 후, 다양한 농도의 anethole이 IL-1β 유도 *MUC5AC* 유전자 발현을 유의하게 억제하는지 여부를 확인하였다. 또한, 관련 경로인 TAK1-MAPK-AP-1과 TAK1-IκB-NF-κB 경로에 있어 anethole의 억제 효과를 살펴보았다. Anethole은 농도 변화에 따라 세포 독성을 보이지 않았고 IL-1β 유도 *MUC5AC* 유전자 발현을 유의하게

억제하였다. 또한, anethole의 IL-1β 유도 *MUC5AC* 유전자 발현 억제는 TAK1-MAPK-AP-1과 TAK1-IκB-NF-κB 경로를 경유함을 증명하였다. 이에 Anethole이 사람 호흡기 상피세포에서 TAK1-MAPK-AP-1과 TAK1-IκB-NF-κB 경로를 통해 IL-1β 유도 *MUC5AC* 발현을 억제하는 작용을 하고 있는 것을 확인할 수 있었으며 이는 향후 anethole이 과분비 조절이 필요한 호흡기 질환에 사용될 수 있음을 알 수 있었다.

핵심되는 말: Anethole, *MUC5AC*, 사람 호흡기상피세포, 신호전달

PUBLICATION LIST

1. Lee EJ, Song KJ, Kwon JH, Park AY, Jo KH, Kim KS. Chronic cholesterol depletion by lovastatin suppresses *MUC5AC* gene expression in human airway epithelial cells. Am J Rhinol Allergy 2014;28:e125-9.
2. Lee EJ, Kim JH, Suh YS, Choi BI, Jung CM, Kim KS. Analysis of prevalence and risk factors of chronic rhinosinusitis in hyperlipidemia patients. Korean J Otorhinolaryngol Head Neck Surg 2015;58:25-31.
3. Kim KR, Kim NH, Park Y, Kim JH, Lee EJ, Kim KS. Berberine Suppresses Interleukin-1β-Induced *MUC5AC* gene expression in human airway epithelial cells. J. Rhinol 2011;18:116-21.