Berberine suppresses interleukin-1β-induced *MUC5AC* gene expression in human airway epithelial cells

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ABSTRACT

Berberine suppresses interleukin-1β-induced *MUC5AC* gene expression in human airway epithelial cells

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Berberine is a bitter-tasting, yellow plant alkaloid with a long history of medicinal use in Chinese and Ayurvedic medicine. There is some evidence to support its use in the treatment of heart failure, malaria, diabetes, glaucoma, hypercholesterolemia, diarrhea, trachoma, and leishmaniasis. Traditionally, it has been used as anti-fungal, anti-inflammatory, anti-viral agents, and also in respiratory disorders. However, there is no evidence that berberine can suppress mucin secretion in human airway. Therefore, the aim of this study was to investigate whether berberine suppresses IL-1β-induced *MUC5AC* gene expression in human airway epithelial cells and, if so, to examined which mitogen-activated protein kinases (MAPKs) were related to *MUC5AC* gene suppression. *MUC5AC* mRNA and protein were measured using reverse transcription-polymerase chain reaction (RT-PCR), real-time PCR, and western blot analysis in cultured NCI-H292 human airway epithelial cells. Extracellular signal-regulated kinase

(ERK) and p38 MAPK protein levels were analyzed by Western blot. When the cells were pretreated with 25 μM of berberine, expression of IL-1β-induced *MUC5AC* mRNA and protein was significantly suppressed compared to the control group. MAPKs proteins were determined by Western blot analysis after pretreatment with 25 μM berberine. Berberine suppressed phosphorylation of extracellular signal regulated kinase (ERK) and p38 MAPK, but there was no change in the expression of α-tubuline. Suppression of IL-1β-induced *MUC5AC* mRNA was also observed in cells pretreated with ERK- or p38 MAPK-specific inhibitors, suggesting that berberine suppression of IL-1β- induced *MUC5AC* mRNA operated via the ERK- and p38 MAPK-dependent pathways. Berberine suppresses IL-1β-induced *MUC5AC* gene expression in human airway epithelial cells via the ERK- and p38 MAPK-dependent pathways. Therefore, berberine may be considered a possible anti-hypersecretory agent.

Keywords : Anti-hypersecretory agent, Berberine, MUC5AC, Interleukin-1β, Human airway epithelial cell

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I. INTRODUCTION

Sinusitis, bronchitis, and bronchial asthma have a common characteristic, excessive secretion of mucus. The mechanism for mucus secretion is mediated by various cytokines, peptides, inflammatory mediators, and mucin genes such as up-regulated *MUC5AC* or *MUC8*.[1-2] According to studies on *MUC5AC* expression, overexpression of *MUC5AC* induced by interleukin(IL)-1β occurs in airway epithelial cells by activation of mitogen-activated protein kinases (MAPKs) and stress-activated protein kinase-1-cAMP response element-binding pathways in a sequential cascade. This finding suggests a treatment strategy for inflammatory airway diseases through the control of mucus hypersecretion.[3]

Berberine has been extensively studied for its multiple biological and pharmacological activities in various diseases, including respiratory diseases. Traditionally, it has been used as anti-fungal, anti-inflammatory, anti-viral agents, and also for respiratory disorders. There is some evidence to support its use in the treatment of heart failure, malaria, diabetes, glaucoma, hyper-

cholesterolemia, diarrhea, trachoma, and leishmaniasis.[4-6] However, there is no evidence that berberine can suppress mucin secretion in human airway epithelium. Berberine has similar pharmacological effects to clonidine, an α-adrenergic agonist, and interacts with an α-adrenergic receptor.[7]

Moreover, berberine inhibits release of TNF-α.[8] As *MUC5AC* expression is known to be up-regulated by TNF-α, we formulated a hypothesis that berberine suppresses *MUC5AC* gene expression in human airway epithelial cells.

Therefore, the aim of this study was to investigate whether berberine suppresses IL-1β-induced *MUC5AC* gene expression in human airway epithelial cells and, if so, to examine which MAPKs are involved in *MUC5AC* gene suppression.

II. MATERIALS AND METHODS

1. Cell culture

NCI-H292 cells were purchased from the American Type Culture Collection (Rockville, MD) and cultured in Roswell Park Memorial Institute (RPMI) 1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, 2 mM of 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. When cultures reached 70-80% confluency, cells were incubated in RPMI 1640 medium containing 0.5% fetal bovine serum for 24 hours.

2. Materials

Berberine was purchased from Sigma Co. (St. Louis, MO, USA). PD98059 (a specific inhibitor of ERK), and SB203580 (a specific inhibitor of p38 MAPK) were purchased from Calbiochem Biochemicals (San Diego, CA, USA). IL-1β was purchased from R&D Systems (Minneapolis, MN, USA) and anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibody, anti-phospho-p38 MAPK (Thr180/Tyr182) antibody, and anti-phospho-SAPK/c-Jun NH2-terminal kinase MAPK (Thr183/Tyr185) antibody were purchased from Cell Signaling (Beverly, MA, USA).

3. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) of *MUC5AC* mRNA

Total cellular RNA was isolated from cultured NCI-H292 cells treated with IL-1β under each condition using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA). Total RNA (1 μg/20μL) was reverse transcribed into

cDNA using random hexanucleotide primers and Moloney murine leukemia virus reverse transcriptase (Gibco BRL), and *MUC5AC* cDNA was amplified by PCR using a Perkin-Elmer Cetus DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA) using previously described methods.[3] A sequence of 5′ primer was CGACAACTACTTCTGCGGTGC, and that of 3′ primer was GCACTCATCC-TTCCTGTCGTT. Amplification of each target gene was confirmed by sequencing (dsDNA Cycle Sequencing System; Gibco BRL) of PCR fragments.

4. Real-time PCR of MUC5AC mRNA

Primers and probes were designed with PerkinElmer Life Sciences Prime Express software and purchased from PE Biosystems (Foster City, CA, USA). Commercial reagents (Taqman PCR Universal PCR Master Mix, PerkinElmer Life Sciences) and conditions according to the manufacturer's protocol were applied. An amount of 1 μg of cDNA (reverse transcription mixture) and oligonucleotides at a final concentration of 800 nM of primers and 200 nM of TaqMan hybridization probes were analyzed in a 25 μL volume. The real time-PCR probe was labeled with carboxyfluoroscein (FAM) at the 5' end and with a quencher carboxytetramethylrhodamine (TARMA) at the 3' end. MUC5AC, 2 M primers and TaqMan probe were designed as follows: MUC5AC (forward: 5'-CAGCCACGTCCCCTTCAATA-3' and reverse: 5'-AC CGCATTTGGGCATCC-3', TaqMan probe 6FAM-CCACCTCCGAG-CCCGT CACTGAG-TAMRA), 2 M (forward: 5'-CGCTCCGTGGCCTTAGC- 3' and reverse: 5'-GAGTACGCTGGATAGCCTCCA-3', and Taqman probe 6FAM-TGCTCGCGCTACTCTCTCTTTCTGGC-TAMRA). Real-time PCR was performed on a PerkinElmer Life Sciences ABI PRISM 7700 Sequence Detection System using previously described methods.[3] Data are expressed as mean ± SD. A minimum of at least three independent experiments were performed for each measurement. Differences between treatment groups were

assessed by ANOVA with post hoc test, and the statistical significance was accepted for p values <0.05.

5. Western Blot Analysis

Cells were scraped into 1 mL of radioimmunoprecipitation assay buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Protein concentration was measured by bicinchronic acid protein assay using bovine serum albumin. Cell lysates were centrifuged and supernatants were subjected to Western blot. Each lane was added with 30 g of protein, and electrophoresis was performed. Proteins were separated by 8% SDS-PAGE and transferred onto a nitrocellulose membrane. Blots were blocked with 10% non-fat dry milk in Tris-buffered saline with 0.05% Tween-20 (TBST) at 4°C for 12 hours and probed with anti-p-ERK (1:1,000), anti-p-p38 MAPK (1:1,000), and anti-p-JNK (1:1,000) antibody for 4 hours at room temperature. In addition, expression of MUC5AC protein was assessed with anti-MUC5AC antibody (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, blots were treated with horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ, USA) diluted to 1:5,000 in TBST for 1 hour and washed several times. Enhanced chemiluminescence and autoradiography were used for detection of bands. Blots were stripped and probed with α-tubulin antibody (Santa Cruz Biotechnology) to normalize for loading.

6. Cell Viability Assay

Cells were plated in 96-well plates at a density of 2,000 cells/well. After incubation for 16 hours, cells were washed with phosphate-buffered saline and incubated for 24 hours in serum-free medium containing varying concentrations of berberine. Cell viability was measured using a Cell Titer 96 Aqueous One

Solution Proliferation Assay Kit (Promega Inc., Madison, WI, USA) using as previously described.[3] The mean percentage of living cells was calculated as follows: [1 - (mean O.D. of experimental group / mean O.D. of control)] x 100. Experiments were performed more than 3 times, and statistical significance was accepted for p values <0.05 through the repeated measures ANOVA and multiple comparisons analysis.

III. RESULTS

1. Berberine suppresses IL-1β-induced *MUC5AC* mRNA expression

In a previous study, IL-1β maximally induced *MUC5AC* mRNA at a concentration of 10 ng/ml and incubation time of 24 hours.[3] Therefore, we used a 10 ng/ml concentration of IL-1β and 24 hours incubation time for experiments in this study. NCI-H292 cells were pretreated with berberine at concentrations of 0, 5, 25, and 50 μM 1 hour before adding 10 ng/mL of IL-1β. After adding IL-1β to the cells, RT-PCR and real-time PCR for *MUC5AC* mRNA expression was performed. In comparison with the control which was not treated with either IL-1β or berberine, the relative ratio of *MUC5AC* mRNA expression at 0, 5, 25, and 50 μM of berberine was 5.2 ± 0.6, 2.8 ± 0.5, 1.1 ± 0.3, 0.8 ± 0.3 respectively. From 25 μM of berberine, IL-1β-induced *MUC5AC* mRNA expression was significantly suppressed which was in a dose-dependent manner. ($p<0.05$) (Fig. 1A, B) To evaluate the cytotoxicity of berberine, cell viability was examined. Compared with the control, the percentage of living cells in 5, 25, and 50 μM of berberine was 93.6 ± 4.1 %, 86.0 ± 5.8%, 68.4 ± 7.3% respectively. In 25 μM berberine, there was no significant suppression by berberine on cell viability was observed compared to the control group. (Fig. 1C)

Therefore, we found that berberine directly suppressed IL-1β-induced *MUC5AC* mRNA expression, not by its cytotoxicity. We used a berberine concentration of 25 μM for other experiments.

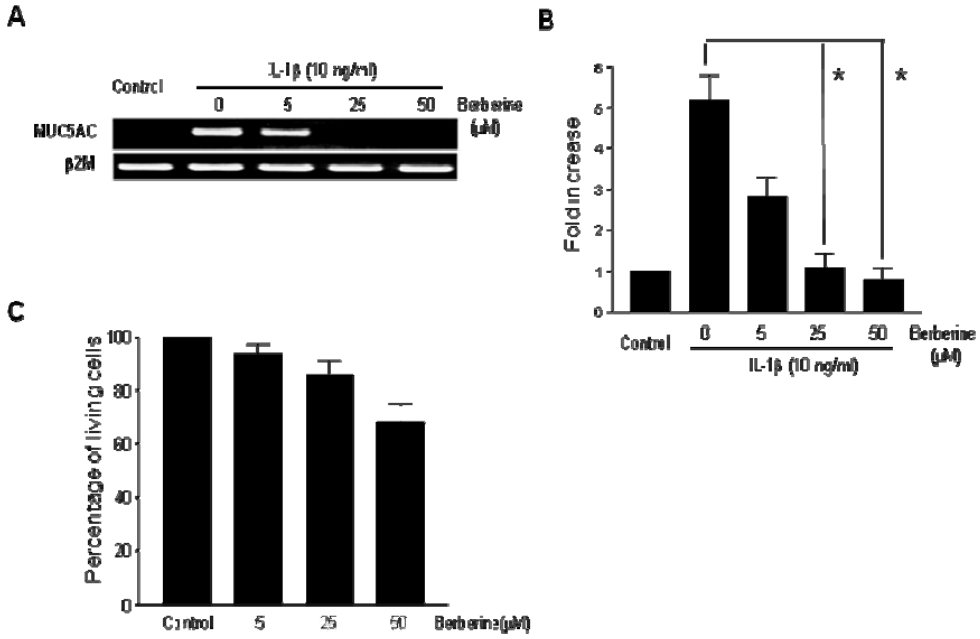

Figure 1. Suppression of IL-1β-induced *MUC5AC* gene expression by berberine. RT-PCR (A) and real-time PCR (B) for *MUC5AC* mRNA expression show that expression of *MUC5AC* mRNA is significantly suppressed from treatment with 25 μM of berberine in a dose-dependent manner. (C) Percentage of living cells determined by cell proliferation assay. *$p < 0.05$.

2. Suppression of IL-1β-induced *MUC5AC* gene and protein expression by berberine

NCI-H292 cells were pretreated with 25 μM berberine for 1 hour and then treated with 10 ng/mL of IL-1β. In comparison with the control that was not treated with either IL-1β or berberine, expression of *MUC5AC* mRNA in cells treated only with IL-1β increased by 4.8 ± 0.7-fold, but in cells pretreated with berberine, the expression was significantly decreased by 1.2 ± 0.2-fold ($p <$

0.05) (Fig. 2A). In Western blot analysis, IL-1β induced expression of MUC5AC protein, but pretreatment with berberine decreased expression of IL-1β-induced MUC5AC protein to the control level (Fig. 2B).

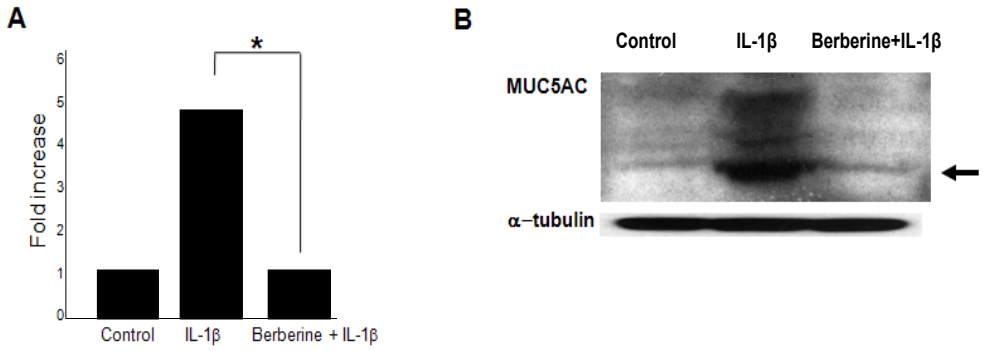

Figure 2. Suppression of IL-1β-induced *MUC5AC* gene and protein expression by berberine. Cells were pretreated with berberine at concentrations of 25 μM 1 hour before the addition of 10 ng/mL of IL-1β, and then real-time PCR (A) for *MUC5AC* mRNA expression and Western blot analysis (B) for *MUC5AC* protein, respectively, were performed. (A) Expression of *MUC5AC* mRNA increases in the presence of IL-1β, but this increase is significantly decreased to the control level by pretreatment with berberine (Berberine+IL-1β). (B) Western blot analysis for *MUC5AC* protein expression. Expression of MUC5AC protein (arrow, 213 kDa) induced by IL-1β is suppressed by pretreatment with berberine. *$p < 0.05$.

3. ERK- and p38 MAPK-dependent suppression of IL-1β-induced *MUC5AC* mRNA expression by berberine

To determine which MAPKs were activated in NCI-H292 cells stimulated by IL-1β, we performed Western blot analysis using phospho-specific antibodies.

ERK and p38 MAPKs were maximally activated 30 minutes after treatment with IL-1β, and this effect decreased after 60 minutes (Fig. 3A). Therefore, the incubation time to check changes in MAPK expression by berberine was determined as 30 minutes. NCI-H292 cells were pretreated with 25 μM of berberine for 1 hour, and then 10 ng/mL of IL-1β was added. After 30 minutes incubation, activation of p-ERK and p-p38 MAPK was significantly decreased to the control level in comparison with the IL-1β alone-treated group (Fig. 3B).

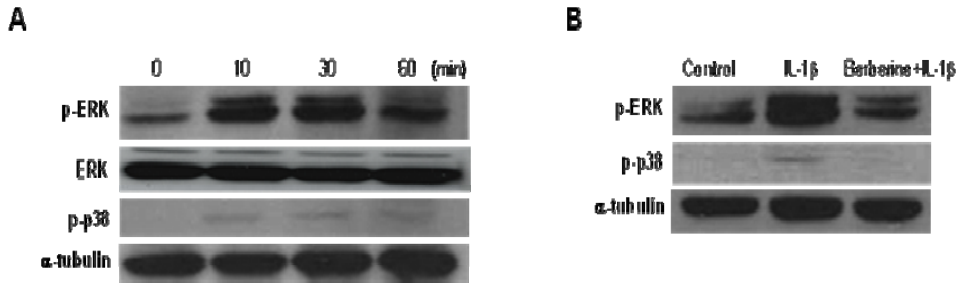

Figure 3. ERK and p38 mediate IL-1β-induced *MUC5AC* gene suppression by berberine. (A) Western blot analysis using phospho-specific antibodies. Phosphorylation of ERK and p38 MAPK is maximally activated at 30 minutes, and this effect decreases after 60 minutes. (B) Cells were pretreated with 25 μM of berberine for 1 hour, and then treated with 10 ng/mL of IL-1β (Berberine+IL-1β). After 30 minutes incubation, activation of p-ERK and p-p38 MAPK is strongly inhibited in berberine+IL-1β cells compared to IL-1β -only-treated cells.

To confirm the possible involvement of the ERK and p38 MAPK pathways in IL-1β-induced *MUC5AC* gene expression, 20 μM of PD98059 and 20 μM of

SB203580 were separately added to cells 1 hour before their treatment with IL-1β. Pretreatment with PD98059 suppressed expression of p-ERK; however, expressions of ERK and p-p38 remained unchanged. Conversely, pretreatment with SB203580 suppressed expression of p-p38, but p-ERK and ERK expressions did not change. These results showed that PD98059 and SB203580 clearly inhibited the ERK and p38 MAPK pathways, respectively (Fig. 4A). Under this experimental condition, we checked whether the ERK and p38 MAPK pathways were involved in the suppression of IL-1β-induced *MUC5AC* expression by berberine. When cells were treated with IL-1β only, the relative ratio of *MUC5AC* expression compared with the control was 5.0 ± 0.8. When specific inhibitors for ERK or p38 MAPK were added, the relative ratio of *MUC5AC* expression to the control was 1.3 ± 0.2 for cells pretreated with PD98059 and 0.9 ± 0.3 for cells pretreated with SB203580. Thus, *MUC5AC* expression induced by IL-1β was significantly suppressed by inhibiting the ERK or p38 MAPK signal transduction pathways ($p < 0.05$) (Fig. 4B).

These findings suggest that the ERK and p38 MAPK signaling pathways are involved in the suppression of MUC5AC expression by berberine.

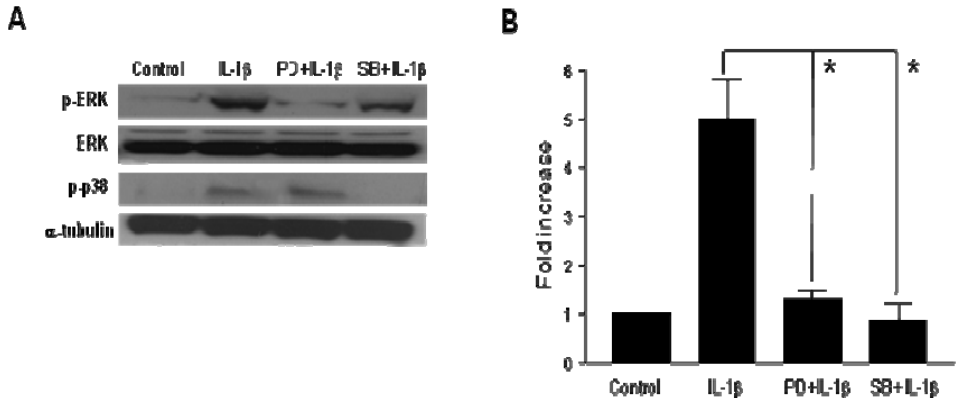

Figure 4. Inhibition of ERK or p38 MAPK suppresses IL-1β-induced *MUC5AC* gene expression. NCI-H292 cells were separately pretreated for 1 hour with 20 μM of PD98059 or 20 μM of SB203580, and then stimulated for 24 hours with 10ng/mL of IL-1β. (A) Western blot analysis. PD98059 (PD+IL-1β) and SB203580 (SB+IL-1β) clearly inhibited p-ERK and p-p38 MAPK, respectively. (B) Inhibition of either the ERK or p38 MAPK pathways with specific inhibitors significantly suppresses *MUC5AC* gene expression in real-time PCR. The figures shown are representative of three independent experiments. $*p < 0.05$.

IV. DISCUSSION

Berberine is a quaternary ammonium salt from the group of isoquinoline alkaloids and is found in plants such as Berberis, goldenseal (Hydrastis canadenis), and Coptis chinensis in the roots, rhizomes, and stem bark. It has been known to have many pharmacological activities, including anti-microbial and anti-inflammatory activities.[5-6] Kuo et al. found that berberine could inhibit cyclooxygenase-2 expression and prostaglandin E production by regulating transcription factor activator protein 1.[9] In addition, other studies showed that berberine induced IL-12 p40 production by activating p38 MAPK and α2-adrenergic receptor in mouse macrophages, and deviates $CD4^+$ T cell from a Th2 to a Th1 response.[10-11] Recently, some studies showed that berberine can inhibit lipopolysaccharide (LPS)-stimulated myocardial TNF-α secretion and improves LPS-induced contractile dysfunction in the intact heart.[9] As mentioned, berberine shows many pharmacologic activities, but there is no evidence that berberine can suppress mucin secretion in human airway epithelium.

Among 11 mucin genes expressed in the airways, *MUC5AC* has been reported to be associated with the pathogenesis of chronic inflammatory airway diseases.[12] Many cytokine mediators, including TNF-α, and bacterial products induce mucin gene expression in the airways. Since *MUC5AC* is up-regulated by TNF-α via NF-κB activation,[13] we thought that berberine could be somewhat involved in the down-regulation of *MUC5AC* expression, which we examined in this study.

In this study, when cells were pretreated with 25 μM of berberine, expressions of *MUC5AC* mRNA and protein were significantly suppressed. This *MUC5AC* suppression were also observed in studies on [6]-gingerol, quercetin, curcumin, and epigallocatechin gallate (EGCG).[3,14] Berberine has been reported to inhibit protein synthesis and cell cycle progression and to

induce apoptosis in different cancer cells.[15] In this study, berberine had no cytotoxic effect on NCI-H292 cells at 25 μM. These results indicate that berberine-induced suppression of *MUC5AC* expression was not the result of cytotoxic effects. Even though some polyphenols such as [6]-gingerol, quercetin, curcumin, and EGCG suppress *MUC5AC* expression induced by IL-1β, the minimal concentrations to suppress *MUC5AC* expression are different.[3,14] It may be due to the characteristic of the materials, and potency and toxicity of these polyphenols and berberine need to be examined before they could be clinically used.

To clarify the mechanism through which berberine suppresses IL-1β-induced *MUC5AC* gene expression, Western blot analysis on MAPKs was done. MAPKs are important enzymes in the production of mucin, and they also play a significant role in cell proliferation, differentiation, apoptosis, cytoskeletal remodeling, and cell cycle.[16] In the previous study on the activation of MAPKs by IL-1β, the involvement of ERK and p38 MAPK was revealed.[3] In this study, ERK and p38 MAPK which were activated by IL-1β treatment were suppressed by the pretreatment with 25 μM berberine. Therefore, we could infer that these MAPK are involved in the regulation of *MUC5AC* suppression by berberine. To further investigate the involvement of ERK and p38 MAPK, we performed the inhibitor study using specific inhibitors on ERK and p38 MAPK. As a result, either the ERK- or p38 MAPK-specific inhibitors suppressed IL-1β-induced *MUC5AC* gene expression. Thus, the ERK and p38 MAPK signal transduction pathways are involved in suppression of *MUC5AC* gene expression by berberine. In the study on [6]-gingerol, quercetin, and EGCG, *MUC5AC* expression was also suppressed.[3,14] Thus, this finding coincides with our result regarding berberine. However, there may be other pathways involved in the suppression of *MUC5AC* expression other than the ERK and p38 MAPK signal transduction pathways because MAPK is an early activated signal transduction pathway.[16] Thus, further studies are necessary to reveal the whole mechanisms.

V. CONCLUSION

Berberine, a natural alkaloid, significantly suppressed IL-1β-induced *MUC5AC* gene expression in human airway epithelial cells via the ERK- and p38 MAPK-dependent signal transduction pathways. Therefore, berberine may be considered as a possible anti-hypersecretory agent for inflammatory airway diseases.

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ABSTRACT(IN KOREAN)

사람 기도점액 상피세포에서 베르베린이 Interleukin-1β 로 인한 *MUC5AC* 유전자 발현에 미치는 영향

<지도교수 김 경 수>

연세대학교 대학원 의학과

김 경 록

베르베린(Berberine)은 노란색의 쓴맛의 식물 추출물로 중국과 대체 의학 분야에서는 이미 오래 전부터 약물로 이용하고 있었다. 전통적으로 심부전이나 말라리아, 당뇨, 녹내장, 고지혈증, 설사 및 감염증, 호흡기 질환에서 사용하였으며 일부 분야(심부전, 당뇨)에서는 과학적으로 효과가 입증되었지만 호흡기 질환에 관련하여 호흡상피세포에서 베르베린이 점액을 억제하는 기전은 정확히 밝혀진 바가 없었다.

이에 본 연구는 사람 호흡상피세포에서 IL-1β (Interleukin-1β)로 자극되었을 때 점액 분비에 중요한 역할을 하는 *MUC5AC* 유전자의 발현을 베르베린이 억제 할 것이라는 가설 하에 진행하였으며 더불어 MAPKs (Mitogen-activated protein kinases)를 통해 이루어지는 것을 밝혀 전통적으로 호흡기 질환에서 사용된 베르베린이 사람 호흡상피세포에서 점액의 분비를 억제하는 기전에 대해 과학적으로 알아보고자 하였다.

실험은 배양된 NCI-H292 사람 기도점액 상피세포에서 *MUC5AC* mRNA 와 단백질의 발현을 관찰하였으며 역전사 PCR (Reverse transcription-polymerase chain reaction) 과 실시간 PCR (real-time PCR), 그리고 Western blot 분석을 통해 이루어졌다. ERK (Extracellular signal-regulated kinase) 와 p38 MAPK 단백질도 Western blot 방법으로 분석하였다.

배양된 세포를 IL-1β 과 25 μM 의 베르베린으로 전 처치 하였을 때, IL-1β 로 인한 *MUC5AC* mRNA 와 단백질이 IL-1β 만 전 처치를 하였던 대조군에 비해 확연히 감소되었다. 같은 상황에서 MAPK 단백질을 Western blot 분석으로 보았을 때 베르베린이 ERK 와 p38 MAPK 의 인산화를 억제하였다. ERK 와 MAPK 가 *MUC5AC* mRNA 발현시키는 경로를 확인하기 위해 IL-1β 와 ERK-, P38 특이적 항체를 각각 전 처치하고 실험한 결과 대조군에 비해 *MUC5AC* mRNA 가 억제됨을 확인하였다.

그렇기에 베르베린이 IL-1β 으로 인한 *MUC5AC* 유전자 발현을 억제하며 이는 ERK 와 p38 MAPK 의 경로를 경유할 것이라고 판단하였다. 즉 베르베린은 호흡기 질환에 점액분비 억제제로 사용할 수 있을 것으로 생각된다.

핵심되는 말: 점액분비 억제제, 베르베린, *MUC5AC*, 사람 기도점액 상피세포, Intueleukin-1β