IL-1ß Induces Lysozyme Overexpression through a Mechanism Involving ERK/p38 Mitogen activated Protein Kinase Activation in Human Airway Epithelial Cells

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The Master's Thesis submitted to the

Department of Medicine, the Graduate School of

Yonsei University in partial fulfillment of the

Requirement for the degree of Master of

Medical Science

Yoo Suk Kim

December 2006

Acknowledgements

When I started my master's thesis, it was very hard for me to continue the degree curriculum with my 1st year residency. But, now finally I am publishing my master's thesis with the great supports of many important persons around me.

First of all, I want to send my greatest respects to my parents. They guided the way how I should live my life and taught me most important things in life which I could not learn forever without them. Also I want to thank my thesis supervisior, Professor J.G. Lee , who watched over me, gave me close attention, and made me to understand the importance accomplishing this degree in my residency throughout the course. Because of his scrupulous interests to my course, I finished the degree with great pleasures.

I also wish to express my gratitudes to Professor J. H. Yoon, Professor S. J. Park . They taught me from the basis to the final revision of the report. For last, I want to send very special thanks my wife, who was my girlfriend when I started to write the master's thesis. She provided the greatest motivation for me to study and proceed my master's degree with all my efforts.

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Abstract

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Lysozyme is a major serous component of airway epithelial secretions, acts as cationic antimicrobial protein for innate immunity . Although lysozyme plays an important role in airway defense and is a key component of airway secretions under inflammatory conditions, little is understood about the regulation of its expression and the associated signaling pathway.

The object of this study was to investigate the regulation of lysozyme expresssion and the downstream signaling pathway of lysozyme expression and the related protein kinases under inflammatory conditions using IL-1ß, which acts as a significant cytokine in many airway inflammations.

IL-1ß treated normal human nasal epithelial cells overexpressed lysozyme compared to the control group, based on RT-PCR. Activated ERK/p38 kinase level showed marked increment by treating NHNE with IL-1ß,which was confirmed by Western blot analysis. Lysozyme expression and ERK/p38 kinase levels decreased when inhibitors of ERK/p38 MAP kinases were added to IL-1ß treated cells, as confirmed by Western blot analysis. Finally, expression of lysozyme and activated level of ERK/p38 MAP kinases decreased in a dominant-negative cell line even when treated with IL-1ß .

From these results, we concluded that IL-1ß induces overexpression of lysozyme via ERK/p38 MAP kinase signaling pathways in airway epithelial cells.

Key words : IL-1ß, Lysozyme, ERK, p38 MAP kinase, human nasal epithelial cells

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I. Introduction

Airway mucosal epithelium has many important functions. Among those functions, host defense by secretion is one of the most important. Components for secretion for defense are immunoglobulins, lactoferrin, mucus glycoprotein, secretory leukoprotease inhibitor(SLPI), lysozyme, uric acid, peroxidase, aminopeptidase and neutral endopeptidase. Lysozyme, first described by Alexander Fleming in 1922, is a single chain protein, composed of 129 amino acids. It acts as a cationic antimicrobial protein

by degrading the cell wall of microbial cells through hydrolysis of the linkage between N-acetylmuramic acid and N-acetylglucosamine. Lysozyme secretion during respiratory disease is known to be regulated by several factors, inlcuding IL-4, IL-9, IL-1β, TNF-α, neutrophil elastase.[1] Given that lysozyme is essential for the innate immunity of airway epithelium , understanding the signal transduction pathway for inflammatory cytokine-induced lysozyme expression would give us an important clues to the understanding of secretory defense function of airway epithelial cells. Mitogen-activated protein (MAP) kinase pathways are thought to be central in transmitting inflammatory signals from the cell surface to the nucleus.[2] After being initiated by several factors such as cytokines, a signal is delivered through a cascade including MAPKKK and MAPKK and to the MAP kinase. The MAP kinases play roles in cell proliferation, differentiation, apoptosis, and cell cycle. Expression of MUC5AC, major airway mucin, is documented to be regulated by ERK and p38 MAP kinases initiated by IL-1β and TNF-α.[3] Although lysozyme plays a key role in airway inflammatory, the mechanisms underlying lysozyme expression during airway inflammation and the

signaling pathway have not been elucidated.

We hypothesized that a major inflammatory cytokine IL-1β, known to regulate the production of many airway secretory components, upregulates lysozyme gene expression by activating specific signal transduction pathways in airway epithelial cells.

In this study, we showed that two different MAP kinases, ERK and p38 MAP kinases, are essential for IL-1β induced lysozyme gene expression in normal human nasal epithelial (NHNE) cells.

II . Material and Methods

1. Materials

PD98059, SB203580, and anti-α-tubulin antibody were purchased from Calbiochem.(Los Angeles,CA,USA) Anti-phospho-p44/42 MAP kinase (Thr^{202}/Tyr^{204}) antibody, anti-phospho-p38 MAP kinase (Thr^{180}/Tyr^{182}) antibody, anti-phospho-SAPK/JNK MAP kinase (Thr^{183}/Tyr^{185}) antibody were purchased from Cell Signaling (Beverly, MA,USA). Plasmid encoding kinase-deficient MEK1 mutant (*pcDNA5-MEK1DN*) and p38 mutant (*pcDNA3-p38AGF*) were kindly provided by Dr. Jian-Dong Li (House Ear Institute, Los Angeles, CA, USA) and Dr. Yoshiyuki Kuchino (National Cancer Center Research Institute, Saitama, Japan), respectively.

2. Cell Cultures

The culture system used for the normal human nasal epithelial (NHNE) cells has been described previously .[12] Briefly,the human lung mucoepidermoid carcinoma cell line (NCI-H292) was purchased from American Type Culture Collection (CRL-1848; Manassas, VA, USA) and cultured in RPMI 1640 (Invitrogen; Carlsbad, CA, USA) supplemented with 10% fetal bovine serum 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 and recultured in RPMI 1640 with

0.2% fetal bovine serum.

3. RT-PCR

Total RNA was isolated using TRIzol (Invitrogen) from NCI-H292 cells treated with IL-1β (10 ng/ml) . cDNA was synthesized with random hexamers (PerkinElmer Life Sciences and Roche Applied Science) using Moloney murine leukemia virus-reverse transcriptase (PerkinElmer Life Sciences). Oligonucleotide primers for PCR were designed based on the GenBank™ sequence of lysozyme (forward primer 5`TGCTGG AGACAGAAGCACTG 3` ; reverse primer 5` GGAGTTACACTCCACA ACT 3`) PCR conditions included 35cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and polymerization at 72 °C for 30 s. The oligonucleotide primers for β_2-microglobulin (used as a control gene for the RT-PCR) were designed based on its GenBank™ human sequence (GenBank™ accession number XM007650, forward primer 5` CTCGCGCTACTCTCTCTTTCTGG 3`; reverse primer 5` GCTTACATGTC TCGATCCCACTTAA 3`). PCR conditions included 23 cycles are the followings : denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and polymerization at 72 °C for 30 s. PCR products were run on a 1.5% agarose gel and visualized with ethidium bromide under a UV transilluminator.

4. Western Blot Analysis

NCI-H292 cells were grown to confluence in 6-well plates. After 15 or 45 min of treatment with IL-1β, the cells were lysed with 2x lysis buffer (250 mM Tris-Cl (pH 6.5), 2% SDS, 4% β-mercaptoethanol, 0.02% bromphenol blue and 10% glycerol). Equal amounts of whole cell lysates were resolved by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). 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 Tris-buffered saline). After washing with TTBS, the blot was further incubated for 45 min at room temperature with anti-rabbit or anti-mouse antibody (Cell Signaling) in TTBS and then visualized using the ECL system (Amersham Biosciences).

5. Preparation of Inducible Dominant Negative Mutant Stable Cell Lines

Plasmid encoding the kinase-deficient MEK1 mutant (*pcDNA5-MEK1DN*) was cut with *Bam*HI and ligated with *pBluescript* (Stratagene, La Jolla, CA, USA). This clone was cut with *Hin*dIII, filled in with Klenow, cut with *Sac*II (Promega), and then ligated with *pTRE* vector. Plasmid encoding kinase-inactive p38 mutant (*pcDNA3-p38*(*AGF*)) was cut with *Bam*HI, filled in with Klenow, cut with *Xba*I (Promega), and then ligated with *pTRE* vector (Clontech, Palo Alto, CA, USA). NCI-H292 cells were cotransfected with *pTet-off* (Clontech) regulation vector and *pTRE-p38DN* or *pTRE-MEK1DN* (1:20 ratio of regulation vector to expression vector) using FuGENE 6 transfection reagent

(Roche Applied Science), following the procedure. Stably transfected cell lines were selected with 200 μg/ml G418 (Calbiochem), and the medium was replaced with G418 and doxycycline every 3 days.

III. Results

IL-1β can induce the expression of lysozyme gene.

To determine whether IL-1β can induce lysozyme expression within cells, we carried out RT-PCR analysis on NHNE cells treated with IL-1β for 24hrs. The level of lysozyme mRNA was significantly higher after treatment with IL-1β in NHNE cells. But there was no significant change in the expression of the housekeeping gene β2-microglobulin (Fig.1A)

IL-1β induced lysozyme expression is mediated by ERK,p38 MAP kinases

To determine which MAP kinase signal pathway is activated within NHNE cells stimulated by IL-1β, we performed a Western blot analysis of these cells using phospho-specific antibodies. ERK and p38 MAP kinases were maximally activated at 15 min, and this effect decreased after 45min. However, no change was detected in the activation of JNK (A549 cells were used as a positive control for JNK activation). It thus appeared that stimulation by IL-1β induced the ERK and p38 MAP kinases pathways in NHNE cell (Fig.1B)

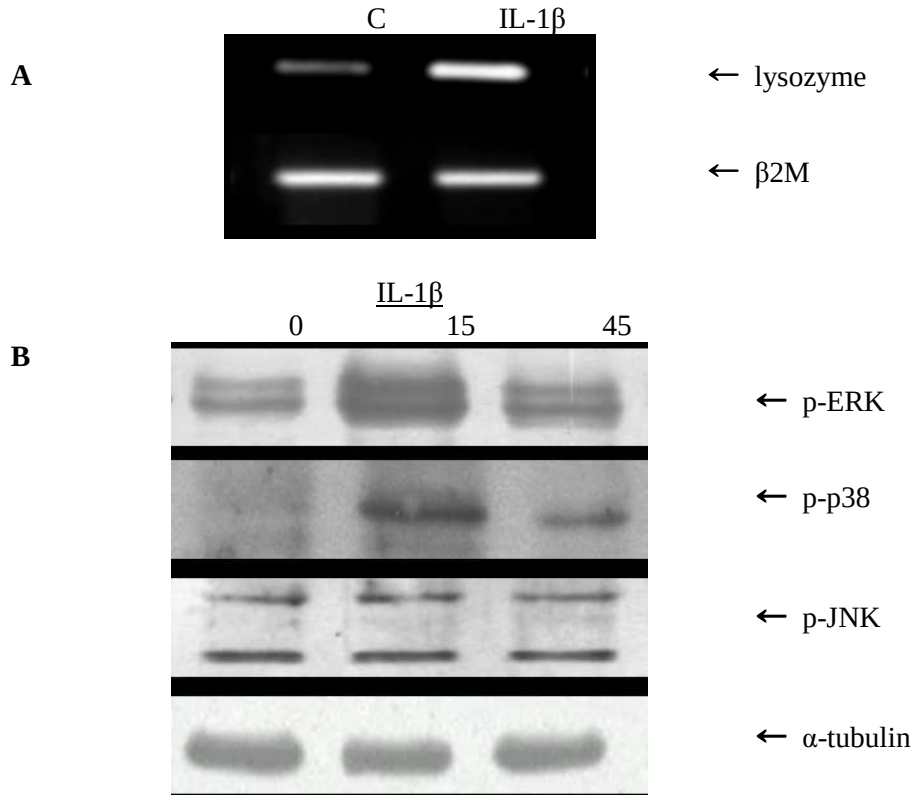
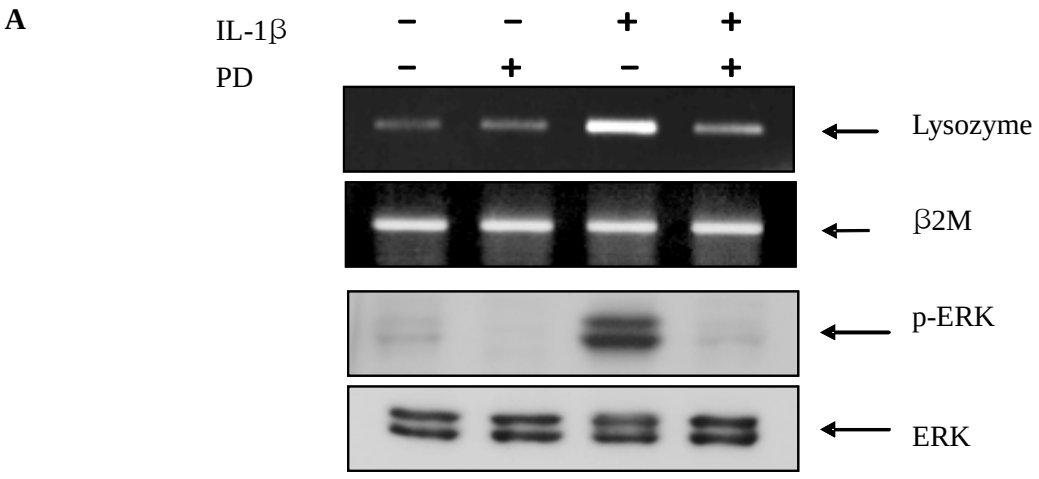


Fig 1. Effect of IL-1β on lysozyme gene expression in NHNE cells.

(A) Confluent cells were treated with IL-1β (10ng/ml) for 24hr, and cell lysates were harvested for RT-PCR . β2 microglobulin (β2M) was employed as an internal control(C). (B) Confluent cells were treated with IL-1β (10ng/ml) for 15 or 45min, and cell lysates were harvested for Western blot analysis. Representative Western blots using phospho-specific antibodies demonstrate transient activation of ERK and p38 but not of JNK, and maximum effect was observed at 15min.

In order to investigate the possible involvement of ERK and p38 MAP kinases in IL-1β induced lysozyme gene expression , NHNE cells were treated with 20 μM PD98059, specific MEK1/2 inhibitor, or 20 μM SB203580, p38 inhibitor, prior to addition of IL-1β. The Western blot showed that PD98059 and SB203580 clearly inhibited ERK and p38 MAP kinases respectively in NHNE cells (Fig.2A, 2B).



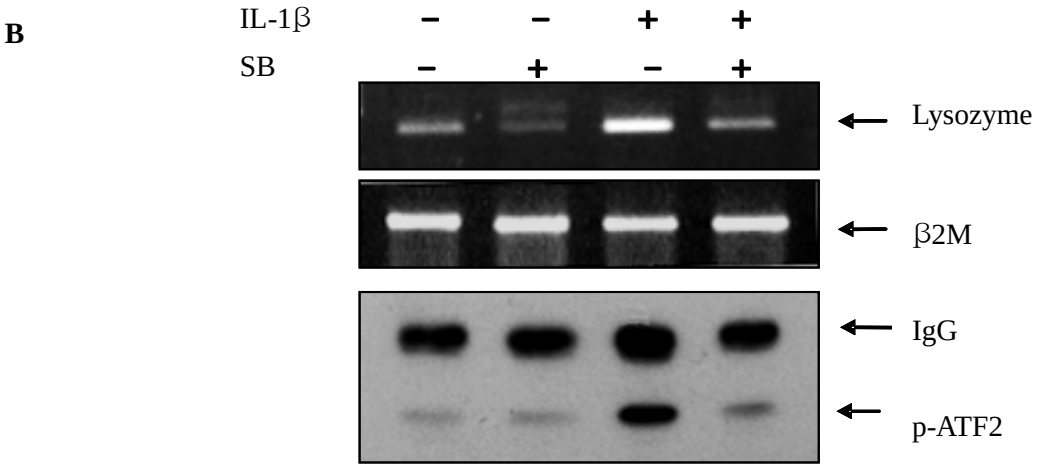


Fig 2. Effect of ERK and p38 MAP kinases on lysozyme gene expression in NHNE cells.

Confluent cells were pretreated for 1hr with 20 μM PD98059 or 20 μM SB203580 , stimulated for 15 min with IL-1β and harvested for Western blot analysis for p-ERK and p38 MAPK. Representative kinase assays show the phosphorylation of ERK by PD98059 (A) and ATF2 as an exogenous substrate (B) with p38 MAP kinase immunoprecipitated from IL-1β treated cells and inhibition of p38 MAP kinase activation by SB203580.

Both ERK and p38 MAP kinases are essential for IL-1β induced lysozyme gene expression

In order to confirm the significance of ERK and p38 kinases in lysozyme gene expression, we generated cells stably expressing dominant-negative (DN) mutant under the control of Tet-off system. The p38 DN mutant was generated by replacing Thr ^{180} and Tyr ^{182} by Ala and Phe, respectively. This inactive form of p38 MAP kinase binds endogenous substrates, thereby inhibiting signaling by the endogenous p38 MAP kinas pathway. After removing the doxycycline to induce the expression of MEK1DN, stimulation with IL-1β for 15 min decreased the phosphorylation of ERK (Fig.3A) In a similar way, we investigated the role of p38 MAP kinase on IL-1β induced lysozyme gene expression using p38DN. The result showed that the activation of p38 MAP

kinase in this mutant cell line was decreased following IL-1β stimulation. Consequently, the expression of lysozyme decreased 24 hr after treatment with IL-1β (Fig.3B) These results show that ERK and p38 MAP kinases are essential for IL-1β induced lysozyme gene expression .

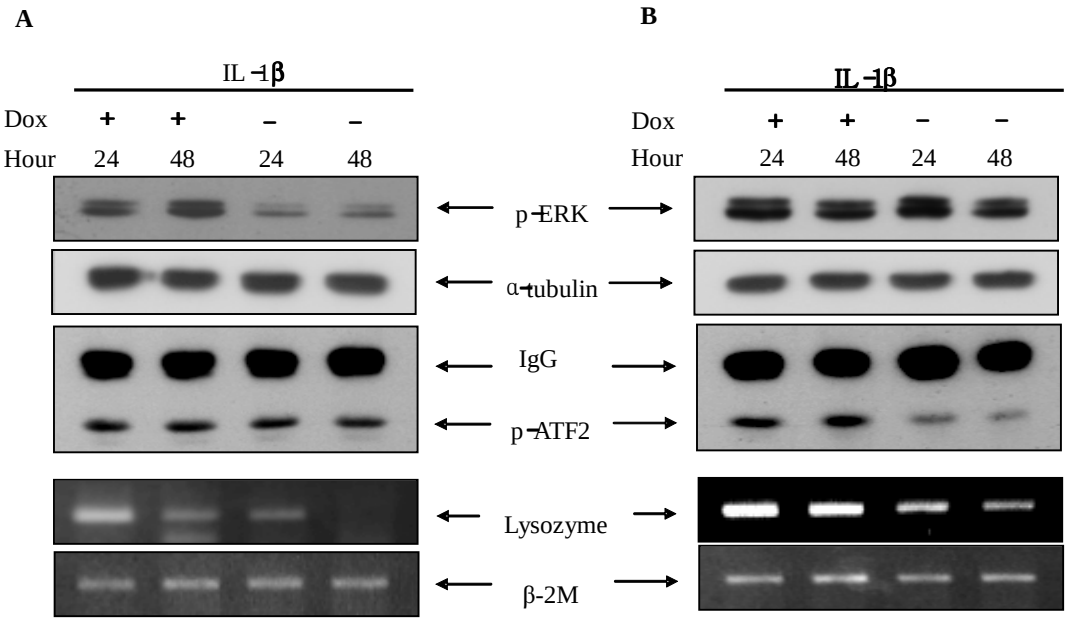


Fig 3. Human lysozyme gene expression in MEK1 (A) or p38 (B) dominant-negative stable cell lines.

Confluent, quiescent MEK1 (A) and p38 (B) dominant-negative mutant stable cells were preincubated for the indicated times to induce dominant-negative mutant protein

by removing doxycycline and then stimulated for 15min with IL-1β prior to Western blottng.

IV. Discussion

At present, considerable research is being carried out on the regulation of airway secretion. Although lysozyme is the major serous component of airway secretion, our understanding of its secretion and regulation of expression is poor. Expanding the knowledge and understanding about regulation of lysozyme expression may offer the new therapeutic strategies for the airway inflammatory diseases.

In the past studies, several factors have been suggested as regulatory factors of lysozyme expression and secretion.[4,5,6,9,12,13,14] In contrast to our observation, the major regulatory cytokine, IL-1β, TNF-a or both did not cause the up-regulation of lysozyme mRNA in NHNE cells in several reports. [4,5] However, the results may not be directly comparable because each project used slightly different cell lines and/or culture media. In a previous report, passage-2 NHNE cells cultured in a 1:1 mixture of BEGM : Dulbecco's modiefied Eagle's medium containing all supplements while in this report, human lung mucoepidermoid carcinoma cell line (NCI-H292) cultured in RPMI 1640 supplemented with bovine serum albumin.[4] One possible explanantion for

the different results is that according to the location of epithelial cells, upper or lower airway , expression levels of airway secretory proteins are known to be different. For example, MUC2, MUC5B mRNA expression level is low in human nasal epithelial cells while high in tracheal epithelial cells.[4,5] Another possible explanation is that culture media used can affect the cell homeostasis in different way, leading to the different effect on cells by IL-1β, finally resulting in the differential expression level of lysozyme mRNA. In the case of cultured human middle ear epithelial cells, omission of retinoic acid caused decrease in the secretion of mucin and lysozyme.[6] On the other hand,the other essential supplements for serum-free conditioned cell culture medium, triiodothyronine and hydrocortisone did not showed significant effect on lysozyme expression.[7,9] Extracellular uridine 5`-triphosphate (UTP) along with the P2Y purinergic receptor, which have various functions in airway epithelial cells such as chloride and fluid transport, mucociliary clearance and mucin secretion, also increased the lysozyme secretion. However, expression level of lysozyme as measured by mRNA level was not significantly affected by UTP treatment of the cells. That result showed

UTP acts as a secretogogue on lysozyme secretion.

Previous reports have also focused on lysozyme expression and secretion, rather than the mechanism behind the lysozyme regulation. Extracellular regulatory proteins bind to cell surface receptors to initiate cellular functions. Below the receptors, kinase pathways such as ERK/p38 and MSK1 transmit the extracellular signal to the nucleus. No signal transduction pathway has been discovered yet for lysozyme. Accordingly, we sought to determine the effect of IL-1β on lysozyme expression and to identify the intracellular signaling pathway responsible for lysozyme expression. We found that IL-1β upregulates the expression of lysozyme. We also demonstrated through the use of inhibitors and dominant negative mutants and the quantification of increased expression levels that the specific MAP kinases, p38 and ERK, are essentially involved in intracellular signaling for lysozyme expression.

This report has a great significance becuase it provides essential information on the downstream signaling pathway of lysozyme expression and related MAP kinases for the first time. Previous studies on the relationship between lysozyme expression and

IL-1β, suggested that IL-1β reported to has no definite regulatory function in lysozyme expression. Therefore this report corrects the misinformation about the IL-1β effect on lysozyme expression derived from the previous studies. Beginning with this study, further downstream level of signal transduction pathway, such as DNA binding proteins, transcriptional factor involved in the lysozyme expression should be studied. Identification and investigation of other signaling pathway that may intervene or cross-react with the reported lysozyme expression pathway will also be an important component of a comprehensive and complete understanding of lysozyme expression in human airway epithelial cells.

V. Conclusion

In summary, we established that IL-1β upregulates the expression of lysozyme through ERK and p38 MAP kinases, but not by JNK signaling. In addition, ERK and p38 MAP kinases are essential for IL-1β induced lysozyme expression. Further analysis of the downstream signaling pathway of lysozyme expression and other related signaling pathways will yield deeper insights into the signaling mechanism behind lysozyme expression.

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Abstract (in Korean)

사람 정상 기도 점막 상피 세포에서 IL-1ß에 의한 라이소자임 유전자 발현 증가 및 이에 관여하는 신호 전달 기전에 관한 연구

<지도 교수 이 정 권>

연세대학교 대학원 의학과
김 유 석

본 연구의 목적은 사람 정상 기도 상피 세포에서 주요 염증 상태에서 분비되어 선천적인 면역 기능을 담당하는 라이소자임의 유전자 발현 조절에 관여하는 인자와 세포 내에서의 신호 전달 기전에 대해 규명하고자 하는 것이었다. 첫번째로 사람 정상 코점막 상피 세포에 IL-1ß를 전처리 한 후 RT-PCR을 이용하여 Lysozyme의 유전자 발현이 대조군에 비하여 증가함을 확인하였다. 두번째로 같은 세포군에 IL-1ß를 전처리한 후 세포 내 신호전달에 관여하는 protein kinases 중 ERK/p38 MAP kinase의 활성화된 형태의 발현이 증가함을 Western blot 방법을 이용하여 확인하였다. 같은 방식으로 사람 정상 코점막 상피 세포에 IL-1ß와 ERK/p38 MAP kinase에 특이적으로 결합하는

억제인자를 전처치한 후 Western blot을 이용하여 라이소자임의 발현 및 ERK/p38 MAP kinase 활성화 형태의 발현이 감소함을 확인하였다. 마지막으로 비활성화 형태의 ERK/p38 MAP kinase를 지니고 있는 Dominant negative cell line에 IL-1ß를 처치할 경우 역시 라이소자임의 발현 및 ERK/p38 MAP kinase 활성화 형태의 발현이 감소함을 확인하였다. 따라서 본 연구에서는 사람 정상 기도 상피세포에서 IL-1ß 에 의해 라이소자임의 유전자 발현이 증가하며 그 과정은 필수적으로 ERK/p38 MAP kinase를 거쳐서 세포내로 신호 전달을 함으로써 발현이 증가함을 확인하였다.

핵심되는 말 : 라이소자임, IL-1ß, ERK/p38 MAP kinase, 사람 정상 코점막 상피세포