Chronic cholesterol depletion by lovastatin suppresses *MUC5AC* gene expression in human airway epithelial cells

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ABSTRACT

Background: *We recently showed that acute cholesterol depletion in the plasma membrane of NCI-H292 cells by methyl-β-cyclodextrin suppressed IL-1beta–induced* MUC5AC *gene expression. Because cholesterol depletion is clinically used as an antihypersecretory method, chronic cholesterol depletion by lovastatin is more rational and safe than acute depletion. Therefore, we sought to investigate whether chronic cholesterol depletion by lovastatin is feasible and, if so, suppresses the expression of GMUC5AC in NCI-H292 cells. We also considered whether this alteration of* MUC5AC *expression is related to IL-1 receptor and mitogen-activated protein kinase (MAPK) activity.*

Methods: *After NCI-H292 cells were pretreated with 10 μM of lovastatin for 1 hour, 10 ng/mL of IL-1β was added and cotreated with lovastatin for 24 hours.* MUC5AC *mRNA expression was then determined by real-time polymerase chain reaction. Cholesterol depletion by lovastatin was measured by modified microenzymatic fluorescence assay and filipin staining. The phosphorylation of IL-1 receptor, ERK, and p38 MAPK was analyzed by Western blot.*

Results: *Cholesterol in the plasma membrane was significantly depleted by lovastatin treatment for 24 hours. IL-1beta0-induced* MUC5AC *mRNA expression was decreased by lovastatin and this decrease occurred IL-1 receptor specifically. Lovastatin suppressed the activation of p38 MAPK but not ERK1/2 in cells activated with IL-1beta. This result suggests that lovastatin-mediated suppression of IL-1beta–induced* MUC5AC *mRNA operated only via the p38 MAPK-dependent pathway.*

Conclusion: *Chronic cholesterol depletion in the plasma membrane of NCI-H292 cells may be considered an antihypersecretory method, because it effectively inhibits mucin gene expression of human airway epithelial cells.*

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Cholesterol is a lipid present in the plasma membranes of eukaryotes and circulates in the bloodstream. Eighty to 90% of total cellular cholesterol is present at the plasma membrane, whereas little cholesterol resides in the endoplasmic reticulum and inner mitochondrial membranes.[1,2] Cholesterol is essential for biological functions from intracellular membrane trafficking to signal transduction.[3] Statins are used as lipid-lowering agents that inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, an enzyme that catalyzes the conversion of HMG-CoA to mevalonate. For this action in cholesterol biosynthesis, lovastatin has been used as a medication against hypercholesterolemia since it was approved by the United States Food and Drug Administration in 1987.[4]

Our previous research revealed that acute cholesterol depletion in the plasma membrane of NCI-H292 cells by methyl-β-cyclodextrin (MβCD) suppressed IL-1β–induced *MUC5AC* gene expression, which regulates in IL-1β receptor–specific and mitogen-activated protein kinase (MAPK)–dependent pathway. Accordingly, cholesterol depletion by MβCD may be considered an antihypersecretory method and may be used in inflammatory airway diseases such as rhinitis, sinusitis, and bronchitis.[5] However, MβCD has not been clinically useful because its pharmacologic activity and cytotoxicity are still under evaluation.[6,7]

In the current study, we sought to investigate whether cholesterol depletion from the plasma membrane by lovastatin is feasible and whether it suppresses the expression of *MUC5AC* in IL-1β–treated NCI-H292 cells. We also considered whether this alteration of *MUC5AC* expression is related to IL-1 receptor and MAPK activity.

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MATERIALS AND METHODS

Cell Culture

Human pulmonary mucoepidermoid carcinoma cell lines (NCI-H292 cells) from American Type Culture Collection (Rockville, MD) were cultured in RPMI-1640 (Gibco BRL, Grand Island, NY) and DMEM (Gibco BRL) supplemented with 10% fetal bovine serum (Cellgro, Hemdon, VA) in the presence of 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 60–80% confluence, the cells were incubated in each medium containing 0.5% fetal bovine serum for 24 hours, after which they were rinsed with phosphate-buffered saline (PBS) and exposed to 10 ng/mL of human recombinant IL-1β (R & D Systems, Minneapolis, MN). Some cultures were pretreated with lovastatin (Sigma Chemical Co., St. Louis, MO) for 1 hour before exposure to IL-1β. IL-1β was dissolved with PBS containing 0.1% bovine serum albumin.

Materials

IL-1β was purchased from R & D Systems; anti-phospho-type I IL-1 receptor (phospho-Y496) antibody (IL-1RI) was purchased from Abcam Co. (Cambridge, MA); anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) antibodies and anti-phospho-p38 MAPK (Thr^{180}/Tyr^{182}) antibodies, goat anti-rabbit and goat anti-mouse antibodies conjugated to horseradish were purchased from Cell Signaling Co. (Beverly, MA). α-Tubulin was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Determination of Cell Viability (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. The cells were seeded on 96-well plates at 2000 cells/well. After serum starvation for 24 hours, the cells were treated with lovastatin at various concentrations (0, 5, 10, and 20 μM) for 24 hours. After exposure period, the media were removed. The media were then changed and incubated with reagent (CellTiter96 AQueous One So-

lution Proliferation Assay; Promega, Madison, WI) for 1 hour at 37°C, 5% CO_2. The optical density was measured by spectrophotometer at 492 nm.

Cellular Cholesterol Assays

Cells were rinsed twice with 1 mL of cold PBS and lipids were extracted with chloroform–methanol 2:1 (v/v).[8] Homogenized cell lysates were centrifuged for 10 minutes at 14,000 rpm, and the organic phase was transferred to a clean tube, dried under vacuum, and redissolved in 20 μL of 2-propanol and 10% Triton X-100. Cellular cholesterol assay was performed using a Cholesterol Fluorometric Assay kit (Cayman Chemical Co., Ann Arbor, MI) using a previously described method.[5] One microliter per assay was used, adjusting to 50 μL with cholesterol reaction buffer in the wells of a 96-well plate. Cholesterol levels were measured using a modified microenzymatic fluorescence assay. The results were described as the ratio of cholesterol/cell protein (μg/mg).

Filipin Staining

Cells, cultured in a polylysine-coated coverslip, were divided into two groups, a control group and an experimental group, and treated with lovastatin. They were subsequently rinsed with cold PBS and fixed on ice using 4% paraformaldehyde. The cells were again rinsed with cold PBS for 10 minutes and stained at room temperature with 100 μg/mL of filipin (Sigma Chemical Co.) for 2 hours. After being rinsed once with PBS, they were observed through a fluorescence microscope with a UV filter set (340–380 nm excitation, 40 diachronic, and 430-nm-long pass filter).

Real-Time Polymerase Chain Reaction

Primers and probes were designed with PerkinElmer Life Sciences Prime Express software and purchased from PE Biosystems (Foster City, CA). Commercial reagents (Taqman PCR Universal PCR Master Mix; PerkinElmer Life Sciences, Waltham, MA) under proper conditions were applied according to the manufacturer's protocol. One microgram 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-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 *MUC5AC*, β2M primers, and TaqMan probe were designed as follows: *MUC5AC* (forward, 5′-CAGCCACGTCCCCTTCAATA-3′, and reverse, 5′-ACCGCATTTGGGCATCC-3′; TaqMan probe, 6FAM-CCACCTCCGAGCCCGTCACTGAG-TAMRA), β2M (forward, 5′-CGCTCCGTGGCCTTAGC-3′, and reverse, 5′-GAGTACGCTGGATAGCCTCCA-3′; Taqman probe, 6FAM-TGCTCGCGCTACTCTCTCTTTCTGGC-TAMRA). Real-time PCR was performed on a PerkinElmer Life Sciences ABI PRISM 7700 Sequence Detection System. 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. Relative quantity of *MUC5AC* mRNA was obtained using a comparative cycle threshold method and was normalized using β2M as an endogenous control.

Western Blot Analysis

Control and treated cells were harvested in PBS and lysed for 30 minutes in RIPA buffer (Cell Signaling Co.) and phosphatase inhibitor cocktail (Sigma Chemical Co.). Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein (30 μg/lane) were separated by SDS/PAGE and transferred on to membranes (Amersham Pharmacia Biotech, Freiburg, Germany). p-IL-1RI (1:500), p-extracellular signal-regulated kinases (1:1,000), and p-p38 MAPK (1:1,000) antibodies were used.

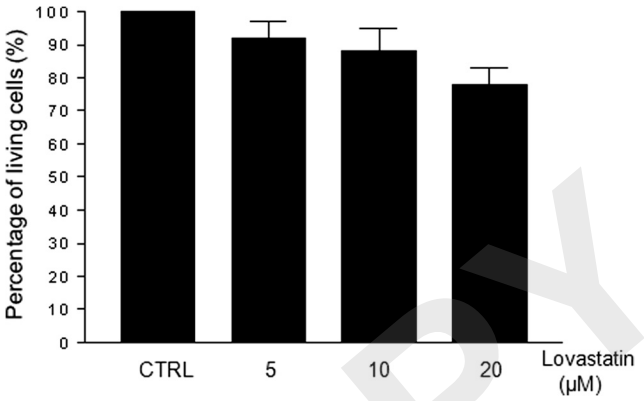

Figure 1. Cell viability after lovastatin treatment in NCI-H292 cells. Cell viability was examined after 24 hours of treatment with various concentrations of lovastatin, and the data were compared with that of the control group (CTRL). Cell survival was maintained above 80% with 10 μM of lovastatin treatment. Data are presented as mean ± SD of six replicates from three independent experiments.

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 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.

Statistics

The experiment was performed at least three times, and the mean value and the standard deviation were calculated. The Wilcoxon rank sum test and repeated measures ANOVA test were used to analyze differences, and multiple comparisons were added. A value of $p < 0.$ was defined as significant.

RESULTS

Cell Viability by Lovastatin Treatment in NCI-H292 Cells

We treated cells with lovastatin at various concentrations (0, 5, 10, and 20 μM) for 24 hours and examined cell viability. The 5-μM lovastatin showed 92 ± 5% cell viability compared with the control group (no treatment with lovastatin); 10 μM of lovastatin showed 88 ± 7% and 20 μM of lovastatin showed 78 ± 5% (Fig. 1). Cells exposed to 10 μM of lovastatin exhibited normal viability, but those exposed to 20 μM of lovastatin showed some loss of cell viability. Therefore, we used 10 μM of lovastatin in the following experiments.

Depletion of Cholesterol by Lovastatin Treatment

To verify whether cellular cholesterol was depleted by lovastatin, cellular cholesterol level analysis and filipin staining were performed. To measure the cellular cholesterol level, cells were treated with lovastatin for 24 hours. Then, the cellular cholesterol/protein ratio was calculated and the data for the experimental group were compared with that of the control group, which had not been treated with lovastatin. The relative ratio of cholesterol/protein after treatment with lovastatin was 0.41 ± 0.08 ($p < 0.05$). This result indicated that lovastatin significantly decreased the cellular cholesterol level (Fig. 2 *A*).

In the filipin staining analysis, filipin was observed to adhere specifically to cholesterol along the plasma membrane in the control

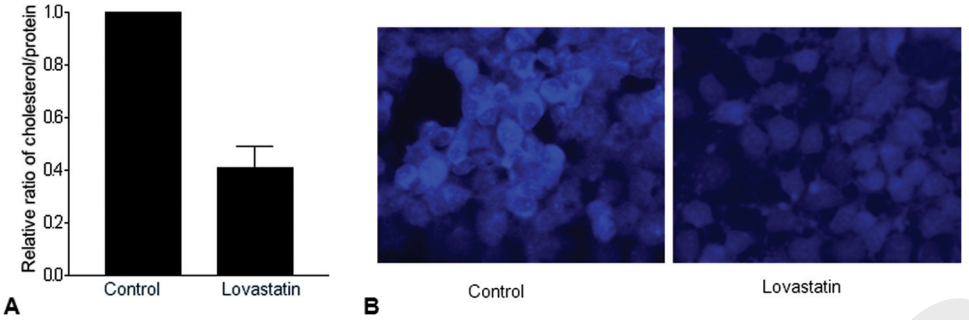

Figure 2. Determination of cellular cholesterol level by a (A) modified microenzymatic fluorescence assay and (B) filipin staining. (A) The cellular cholesterol level was determined using a modified microenzymatic fluorescence assay. The amount was expressed as a cholesterol/protein ratio, and the data of the experimental group were compared with that of the control group (no treatment of lovastatin). The cholesterol/protein ratio was significantly decreased by 10 μM of lovastatin treatment in NCI-H292 cells ($p < 0.05$). Data are presented as mean ± SD of three independent experiments. (B) Filipin staining in NCI-H292 cells after treatment with lovastatin. Filipin staining was performed after 24 hours of treatment with 10 μM od lovastatin and was observed with a fluorescence microscope. Compared with the control group, the staining intensity along the plasma membrane was significantly decreased.

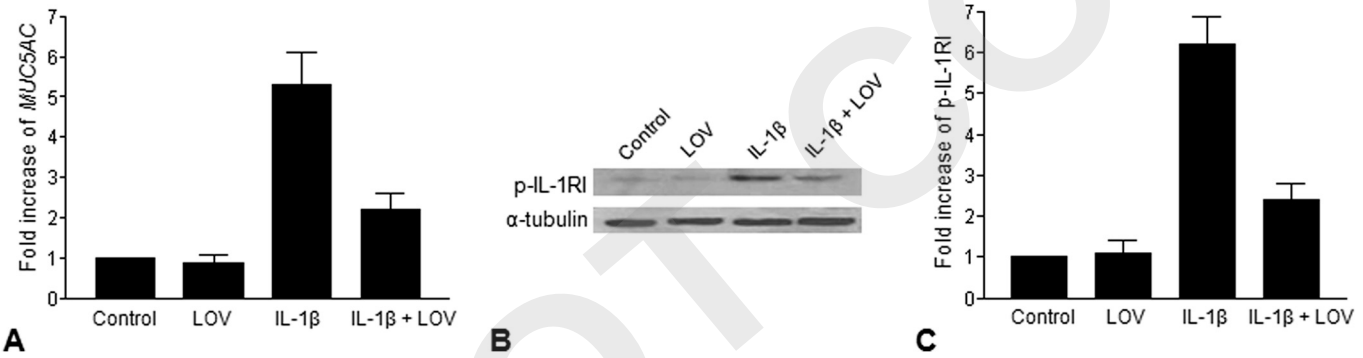

Figure 3. MUC5AC *gene expression after treatment with lovastatin. After NCI-H292 cells were pretreated with 10 μM of lovastatin for 1 hour, 10 ng/mL of IL-1β was added and cotreated with lovastatin for 24 hours.* MUC5AC *gene expression was examined by real-time polymerase chain reaction (PCR) analysis and β_2M served as the internal control. (A) The expression of p-IL-1RI was examined by Western analysis and α-tubulin served as the internal control. (B) IL-1β induced expression of* MUC5AC*, but this expression was significantly decreased by lovastatin treatment (IL-1β + LOV; $p < 0.05$). (C) Bar graph of p-IL-1RI expression. Data are presented as mean ± SD of three independent experiments.*

group. However, in the lovastatin treatment group, the intensity of fluorescence decreased significantly (Fig. 2 *B*).

Suppression of *MUC5AC* Expression after Lovastatin Treatment

After NCI-H292 cells were pretreated with 10 μM of lovastatin for 1 hour, 10 ng/mL of IL-1β was added and cotreated with lovastatin for 24 hours. We picked *MUC5AC* mRNA and examined its expression through real-time PCR. The expression of p-IL-1RI was examined by Western analysis. IL-1β induced expression of *MUC5AC*, but this expression was significantly decreased by treatment with lovastatin in real-time PCR analysis (control : lovastatin : IL-1β : IL-1β + lovastatin = 1 : 0.9 ± 0.2 : 5.3 ± 0.8 : 2.2 ± 0.4; $p < 0.05$; Fig. 3 *A*). The expression of p-IL-1RI was increased by IL-1β treatment, but this increase was significantly reduced after lovastatin treatment (control : lovastatin : IL-1β : IL-1β + lovastatin = 1 : 1.1 ± 0.3 : 6.2 ± 0.7 : 2.4 ± 0.4; $p < 0.05$; Fig. 3, *B* and *C*).

Changes of MAPK Phosphorylation by Lovastatin Treatment

To establish whether this decrease in *MUC5AC* expression induced by lovastatin treatment was dependent on the depression of MAPK activity, we pretreated cells for 24 hours with 10 μM of lovastatin and then incubated them with IL-1β for 20 minutes. A group without exposure to either lovastatin 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. Elevated expression of p-p38 MAPK in the IL-1β-only treatment cells decreased markedly in the combined IL-1β- and lovastatin–treated cells (control : lovastatin : IL-1β : IL-1β + lovastatin = 1 : 1.2 ± 0.2 : 3.2 ± 0.5 : 1.5 ± 0.4; $p < 0.05$; Fig. 4, *A* and *B*). However, expression of p-ERK MAPK showed no significant change in response to lovastatin treatment (control : lovastatin : IL-1β : IL-1β + lovastatin = 1 : 0.8 ± 0.3 : 2.7 ± 0.6 : 2.5 ± 0.4; $p > 0.05$; Fig. 4, *C* and *D*).

DISCUSSION

Lovastatin and MβCD act differently on cholesterol metabolism. MβCD is a compound that specifically combines with cholesterol and removes it. Thus, the role of MβCD in lowering cholesterol seems to be direct, and this can occur in as little as 1–2 hours after treatment.[6,9,10] In contrast, lovastatin is a specific inhibitor of HMG-CoA reductase and reduces *de novo* production of cholesterol. Therefore, the action of lovastatin seems to be somewhat indirect and can require 17 hours to 4 days after treatment and the terminology of "chronic" in contrast to "acute" is usually used.[4,11,12] Cholesterol depletion in the plasma membrane affects kinases of the plasma membrane receptors and may cause alterations to cell proliferation.[9,10] This means that lovastatin induces inhibition of cell proliferation according to cell type and concentration of lovastatin. Thus, we needed to determine the appropriate concentration of lovastatin. Our results showed that cell viability is above 80% when using 10 μM of lovastatin, and,

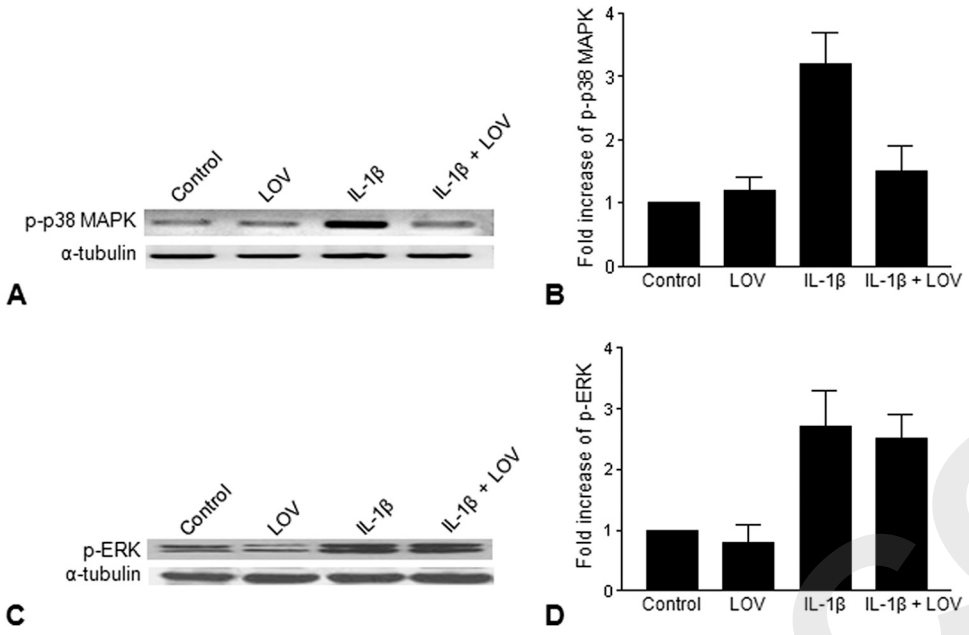

Figure 4. Suppression of IL-1β–induced activation of p38 mitogen-activated protein kinase (MAPK) by treatment with lovastatin. NCI-H292 cells were pretreated for 24 hours with 10 μM of lovastatin, and cells were then treated for 20 minutes with IL-1β. Western blot analysis was performed. Expression of both (A) p-p38 MAPK and (C) p-ERK was increased by IL-1β treatment (IL-1β), but this increased expression of p-p38 MAPK was reduced with lovastatin treatment (IL-1β + LOV; $p < 0.05$). The increased expression of p-ERK showed no significant change in response to lovastatin treatment. (B) Bar graph of p-p38 MAPK expression. (D) Bar graph of p-ERK expression. Data are from three independent experiments.

therefore, this concentration was considered to be safe in the experiments.

Although we found that lovastatin significantly lowered the cellular cholesterol level, we could not ascertain that cholesterol in the plasma membrane was depleted because the data gives only the relative ratio of cholesterol/protein.[13,14] Thus, we determined whether the cholesterol of the plasma membrane was depleted through staining with filipin, which selectively combines with cholesterol. We verified that filipin on the membrane was further decreased in lovastatin-treated cell lines compared with the control group. From these results, we confirmed that lovastatin selectively lowered cholesterol in the plasma membrane-like MβCD.

In NCI-H292 cells, *MUC5AC* expression is induced by proinflammatory cytokines such as IL-1β, TNF-α, and transforming growth factor β.[5,15] In this study, we pretreated lovastatin before IL-1β application. As a result, 10 μM of lovastatin significantly suppressed *MUC5AC* expression in real-time PCR. In our previous study on MβCD, it reduced IL-1β–induced *MUC5AC* expression to the negative control level.[5] However, in this study, the suppression of *MUC5AC* expression by lovastatin could not reach to the negative control level. This difference seems to be a result of the superiority of MβCD to lovastatin in lowering the cellular cholesterol/protein ratio. Also, in our previous study, we proved that MβCD-mediated suppression of IL-1β–induced *MUC5AC* mRNA occurred specifically at the IL-1 receptor and operated *via* the ERK- and p38 MAPK-dependent pathway.[5] To reveal the possible signal pathway for the decrease of *MUC5AC* by lovastatin, we examined the MAPK cascade. In the present study, the p-p38 MAPK induced by IL-1β treatment was decreased by lovastatin treatment and this decrease in p-p38 MAPK was associated with a decrease in *MUC5AC* expression. However, lovastatin did not suppress the activation of ERK1/2 induced by IL-1β. This result suggests that lovastatin-mediated suppression of IL-1β–induced *MUC5AC* mRNA operated only *via* the p38 MAPK-dependent pathway. Calleros *et al.* showed that low cell cholesterol levels in NIH3T3 cells increased NF-κB activity by increasing p38 activity.[16] Thus, with respect to p38 MAPK dependence, the study results by Calleros *et al.* coincide with our result. On the other hand, NF-κB pathway is also activated by IL-1β in NCI-H292 cells but the research on NF-κB pathway is beyond the scope of this study. Additional studies are needed to investigate the possible role of the NF-κB pathway.[17,18]

Hypersecretion of mucin results in clinical problems such as rhinorrhea, nasal stuffiness, and sputum and the regulation of *MUC5AC* or IL-1 receptor can be a new strategy for treating respiratory diseases.[5,19–23] Lovastatin is a good candidate with respect to safety, but the matter of dosage should be considered. The dose range used in hypercholesterolemia is 10–80 mg/day in a single dose or divided into two doses.[24] The dose of lovastatin used in this study was 10 μM or 0.41 μg/mL. In a single-dose study in four hypercholesterolemic patients, it was estimated that <5% of an oral dose of lovastatin reaches the general circulation.[25,26] If 40 mg of lovastatin is administered in a single oral dose to a 70-kg adult, the estimated plasma concentration will be 0.7 μg/mL. Thus, the dose of the present study is low and safe compared with the dose used in hypercholesterolemia, and using lovastatin in lowering *MUC5AC* secretion may be clinically promising.

The main drawback of this study is the cells that were used. In this study, we used human airway epithelial cells rather than normal human bronchial or nasal cells. If these normal cells had been used, the data may be different because the culture method, drug delivery method, and nature of cells differ. Future studies using these normal cells are needed.

In conclusion, chronic cholesterol depletion in the plasma membrane by lovastatin may be considered as an antihypersecretory method because it effectively inhibits *MUC5AC* expression in human airway epithelial cells.

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