Sphingosine kinase 1 regulates mucin production via ERK phosphorylation

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Dear Dr. Page,

Editor-in-Chief,

Pulmonary Pharmacology and Therapeutics

I am pleased to submit our revised manuscript by Kono et al. entitled “Sphingosine kinase 1 regulates mucin production via ERK phosphorylation” for your consideration. We have read carefully the comments by the reviewers, and several modifications were made to respond to their concerns. Each of the specific criticisms is addressed below, with our rationale and changes made. Especially, we quantified the degree of phosophorylated bands in Fig 4, and modified the figure and its legend according to the reviewers’ recommendations.

In keeping with these changes in the data, the results and discussion sections have been substantially modified. We hope that the manuscript is much improved through these changes.

Thank you for your consideration of our revised manuscript and we hope that you will now find the work suitable for publication in the Pulmonary Pharmacology and Therapeutics.

Sincerely,

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Reviewer #1:

We have read carefully the comments by the reviewer, and have modified the manuscript to address the concerns. Each of the specific criticisms is addressed below, with our rationale and changes to the original manuscript.

Minor Points:

Comment 1. *Page 13, lines 5, 6: Authors mentioned DMS did not inhibit IL-13-induced p38 MAPK phosphorylation. However, I can see that a band of phospho-p38 MAPK at 60 min of panel B in Fig. 4 is somewhat less dense than a control band. I would like to have a comment by authors on this point.*

Answer. We showed a representative figure from four independent experiments. We found that DMS did not change the band intensity of IL-13-induced p38 MAPK phosphorylation by semi-quantitative analysis of each western blotting band. To avoid any misunderstanding, we show the western blotting bands and their calculated quantitative data in the new Figure 4C. We have also modified the legend for Figure 4.

Comment 2. *Page 13, lines 8-10: Authors described that SB203580 did not affect IL-13-induced STAT6 phosphorylation, but a band of phosphor-STAT6 at 20 min of panel C in Fig. 4 gives me an impression that it is less dense than control. I desire authors to comment on the reproducibility of the effect of SB203580 and so on.*

Answer. We showed a representative figure from four independent experiments. We found that SB203580 did not change the band intensity of IL-13-induced STAT6 phosphorylation by semi-quantitative analysis of each western blotting band. To avoid any misunderstanding, we show the western blotting bands and their calculated quantitative values in the new Figure 4D.

Comment 3. *Page 14-15: I consider that "Discussion" is generally good since only essential points are compactly organized. However, a couple of obscure points on the discussion of SphK seem to be included, because descriptions of "SphK" and "SphK1" are intermingled. I recommend authors to distinguish clearly SphK or SphK1 and/or mention on SphK2.*

Answer. We did not mention the role of SphK2 in the Discussion section. We have added a sentence on Page 15, line 5-6. We have specified SphK1 where it is discussed.

Reviewer #3:

We have read carefully the comments by the reviewer, and have modified the manuscript to address the concerns. Each of the specific criticisms is addressed below, with our rationale and changes to the original manuscript.

Some minor concerns:

Comment 1. Mouse asthmatic model was used in the present study. There was no data regarding the model including pathology, bronchial hyperresponsiveness and other inflammatory markers. The correlation of SphK expression with airway pathology is not defined.

Answer. In a previous article, we have demonstrated that inhalation of sphingosine inhibitor reduces bronchial hyperresponsiveness, airway inflammation, and the release of Th2 cytokines (ref.15, Nishiuma et al. Am J Physiol Lung Cell Mol Physiol 2008, 284, L1085-1093). Although we used this mouse model here, we showed the citation for this article only in the Introduction section (Page 4, line 5). Now we have add the citation to ref.15 in the Method (Page 5, line 21) and Result (Page 11, line 4) sections, and modified the sentence in the Introduction section (Page4, line 5).

Comment 2. How to demonstrate goblet cell differentiation when treated with IL-13.

Answer. We cited two previous articles (ref. 17 and 18) describing the method to show how the cells were differentiated. We also confirmed the cell differentiation by detecting mucin production using periodic acid-Schiff (PAS) solution. We have added this sentence in the Method section (Page 6, line 19-20).

Comment 3. Fig4, only representative western blot was showed, semiquantitive analysis should be done.

Answer. As recommended, we have quantified each band's intensity from western blotting data and modified the Fig.4. We have also modified the legend for Fig.4 in the Result section (Page 22, line 19-21).

Sphingosine kinase 1 regulates mucin production via ERK phosphorylation

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Abstract

Our previous report showed that inhibition of sphingosine kinase (SphK) ameliorates eosinophilic inflammation and mucin production in a mouse asthmatic model. To clarify the role of SphK in airway mucin production, we utilized the mouse asthmatic model and found that both SphK and MUC5AC expression were increased and co-localized in airway epithelium. Next we cultured normal human bronchial epithelial cells in an air liquid interface and treated with IL-13 to induce their differentiation into goblet cells. We found that SphK1 and MUC5AC expression was increased by IL-13 treatment at both protein and mRNA levels, whereas SphK2 expression was not changed. N,N-dimethylsphingosine (DMS), a potent SphK inhibitor, decreased MUC5AC expression up-regulated by IL-13 treatment. Furthermore, DMS inhibited IL-13-induced ERK1/2 phosphorylation but neither p38 MAPK nor STAT6 phosphorylation. These results suggest that SphK1 is involved in MUC5AC production induced by IL-13 upstream of ERK1/2 phosphorylation, and independent of STAT6 phosphorylation.

Keywords

sphingosine 1-phosphate, sphingosine kinase, airway epithelium, IL-13, ERK

Abbreviations

SphK, sphingosine kinase; S1P, sphingosine 1-phosphate; IL, interleukin; DMS, N,N-dimethylsphingosine; MAPK, mitogen-activated kinase; ERK, extracellular signal-regulated kinase; STAT6, signal transducer and activator of transcription 6.

1. Introduction

Mucus is a critical component of the innate host defense system in the respiratory tract. On the other hand, mucus hypersecretion is a major cause of airway obstruction in asthma and is sometimes associated with increased morbidity or mortality. Excessive mucus in asthmatic patients reflects over production of mucin due to goblet cell hyperplasia (GCH), that is a major pathological feature of asthma [1]. Airway GCH is especially marked in patients who die from asthmatic disease, with a 30-fold increase in the percentage of goblet cells compared with patients dying from non-asthmatic respiratory disease [2].

MUC5AC protein is a major component of airway mucus and is expressed in goblet cells of upper and lower respiratory tracts. Various stimuli such as lipopolysaccharide (LPS) [3], TNFα [4], and Th2 lymphocyte-derived cytokines, IL-4 [5] and IL-13 [6, 7] have been reported to induce MUC5AC both *in vitro* and *in vivo*. In mouse asthmatic models IL-13 is a central mediator of mucin production [8, 9], and mucin production induced by other Th2 cytokines is stimulated through IL-13 and the IL-13 receptor-mediated signals [10]. Moreover, it has been described that the downstream cascade of IL-13 involves signaling molecules such as signal transducer and activator of transcription 6 (STAT6), and mitogen-activated kinases (MAPKs) through epidermal growth factor receptor (EGFR) in airway epithelial cells [11, 12].

Sphingosine 1-phosphate (S1P) is a bioactive lipid mediator of cellular functions such as proliferation, differentiation, apoptosis, tumor cell invasion, cell migration, and angiogenesis. Recently S1P has been shown to mediate asthmatic pathogenesis *in vivo* and *in vitro*. S1P levels are increased in bronchoalveolar lavage (BAL) fluid of asthmatics after challenge with antigen and correlated with eosinophil numbers in the BAL fluid of asthmatic subjects [13]. S1P secreted

by activated mast cells can promote allergic reactions by activating many types of immune cells including eosinophils, Th2 lymphocytes and neutrophils [14].

In a previous study, we demonstrated that inhibition of sphingosine kinase (SphK), a key enzyme that phosphorylates sphingosine to generate S1P, prevented airway mucin production and eosinophil inflammation [15]. We hypothesized that S1P produced by SphK may mediate airway mucin production in asthmatic pathogenesis. There is no information about the role of sphingosine metabolism in mucin production. In this study, we have clarified how SphK/S1P activation can affect the mucin production in human bronchial epithelial cells.

2. Materials and Methods

2.1. Materials

Recombinant human IL-13 was purchased from Strathmann Biotec (Hamburg, Germany). PD98059 and rabbit polyclonal antibodies against ERK1/2, phospho-ERK1/2, p38 MAPK, and phoshpo-p38 MAPK were from Cell Signaling Technology (Beverly, MA, USA). Anti-MUC5AC mouse monoclonal antibody was from Lab Vision (Fremont, CA, USA). N',N'-dimethylsphingosine (DMS) and anti-β-actin mouse monoclonal antibody were from Sigma-Aldrich (St. Louis, MO, USA). Anti-STAT6 mouse monoclonal and anti-phospho-STAT6 rabbit polyclonal antibodies were from R&D systems Inc. (Minneapolis, MN, USA). Anti-human SphK1 goat polyclonal antibody was from Abcam (Cambridge, UK). SB203580 was from Calbiochem (San Diego, CA, USA).

A rabbit polyclonal anti-mouse SphK1 antibody was raised against the synthetic peptide GSRDAPSGRDSRRGPPPEEP (amino acid residues 362-381) conjugated to glutathione S-transferase. The antibody was affinity-purified by using the immunogen-immobilized Sepharose 4B [16].

2.2 Mouse asthmatic model

Six- to 8-week-old C57BL/6 mice (Nippon CLEA, Tokyo, Japan) were sensitized with an intraperitoneal injection of 10 μg ovalbumin (OVA, Grade V; Sigma-Aldrich, St. Louis, MO, USA) adsorbed in 2 mg of aluminum hydroxide (Sigma-Aldrich) in 0.5 mL sterile phosphate-buffered saline (PBS). The mice were subsequently boosted intraperitoneally with the same mixture 14 days later. On day 28 after the initial sensitization, mice were exposed to aerosolized

1.0% OVA in sterile PBS for 30 min, and the same protocol was repeated on three consecutive days. The aerosolized OVA was generated using an ultrasonic nebulizer (NE-U12; Omron, Tokyo, Japan). Negative controls were injected and exposed to PBS. Our research was approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimental Regulations.

2.3. Cell culture

Normal human bronchial epithelial (NHBE) cells were purchased from Lonza Walkersville Inc. (Walkersville, MD, USA). Cells were seeded on plastic dishes coated with human placental collagen (Sigma-Aldrich) and grown in bronchial epithelial cell growth medium with supplements (Cambrex, Walkersville, MD, USA). At 3 to 7 passages, 80 to 90% confluent cells were seeded at a density of 4 x 10^4 cells/cm^2 onto 12 mm or 24 mm diameter Corning Costar Transwell^{TM} -Clear inserts with 0.4 μm pore size. After 7 days under immersed conditions, the apical medium was removed to establish an air-liquid interface that was maintained for the remainder of the cell culture period. Medium was changed every other day and apical surface of the cells was rinsed with PBS to remove any debris.

For goblet cell differentiation, cells were treated with recombinant human IL-13 as reported previously [17, 18]. IL-13 was added to the basal medium and cells cultured for 14 days. IL-13 containing medium was replaced every other day. Cell differentiation was confirmed by detecting mucin production using periodic acid-Schiff (PAS) staining. Inhibitors were added to the basal medium at the same time as IL-13 administration.

2.4. MAPKs phosphorylation by western blot analysis

After 14-days of culture without any stimuli at air-liquid interface (ALI), cells were treated with IL-13 (20 ng/ml) in the presence of complete culture medium for indicated times. Cells were then harvested and lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 15 mM NaF, 1% Triton X-100, 5 mM EDTA, 1 mM sodium orthovanadate, and protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were prepared in SDS-sample buffer and subjected to SDS-PAGE. Proteins were transferred onto nitrocellulose membrane and immunostained using antibodies against ERK and p38-MAPK. Positive protein bands were visualized by the enhanced chemiluminescence method and their intensities were quantified by MultiGauge^{TM} software (Fujifilm, Tokyo, Japan).

2.5. RNA isolation and real-time quantitative reverse transcription-PCR analysis

Total RNA was extracted using ISOGEN^{TM} reagent (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized from 1 μg of total RNA by using ExScript RT^{TM} reagent kits (Takara, Otsu, Japan) and random hexamer primers. Quantitative PCR was performed using real-time SYBR Green PCR technology and an ABI PRISM 7500 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). The primers for human SphK1, SphK2, and GAPDH were described previously [16]. The primers for human MUC5AC: forward primer, 5'-TGTGGGCTATGGGTCACCTG-3', and reverse primer, 5'-GATCACCATGTCCAAGCGTCA-3', were purchased from Takara. Amplification reactions were performed in duplicate with SYBR Premix Ex Taq^{TM} (Takara), and the thermal cycling conditions were as follows: 10 s at 95°C, 40 cycles of 5 s at 95°C, and 34 s at 60°C. The expression of each mRNA was normalized

to GAPDH mRNA expression.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Production of MUC5AC in cell lysates and cell culture supernatants was measured by ELISA using a MUC5AC antibody, as previously described [11]. The amount of MUC5AC in each sample was normalized to total protein in cell lysates and expressed as percent of control.

2.7. The measurement of SphK activity

NHBE cells cultured in ALI for 14 days were stimulated with 10 ng/ml IL-13 for 20 min. SphK activity in cell lysates was determined in the reaction mixture (50 μl) containing 10 μM sphingosine, 1 mM [γ-^{32}P]ATP (8 Ci/mol), and 10 mM $MgCl_2$ essentially as described previously [19]. [^{32}P]S1P was separated by TLC on Silica Gel 60 plates (Merck & Co., NJ, USA) with 1-butanol / acetic acid / water (3:1:1, by vol.) and quantified using a Fujix Bio-Imaging Analyzer BAS 2000 (Fuji Photo Film Co. Ltd., Tokyo, Japan).

2.8. Immunocytochemistry

After stimulation, cells were washed with PBS, fixed for 20 min at room temperature with acetone-methanol (1:1), and permeabilized with 0.1% Triton X-100 in PBS for 15 min. They were blocked with 3% bovine serum albumin for 1 hour, incubated with anti-MUC5AC antibody (1:100) for 1 hour, and then stained using VECTASTAIN^{TM} ABC kit (Vector Laboratories, Burlingame, CA, USA). The mean percentages of MUC5AC-positive cells were calculated from at least 4 different bright fields.

2.9. Immunohistochemistry

Mouse lungs were infused at a pressure of 20 cmH_2O with 10% buffered formalin for 24 hours, embedded in paraffin, and sectioned at 5-µm thickness. After deparaffinization, tissue sections were pretreated with 3% hydrogen peroxidase for 10 min and blocked with normal goat serum for 30 min. Then they were incubated for 1 hour with rabbit anti-SphK1 (1:100) or mouse anti-MUC5AC (1:100) antibody. Normal mouse or rabbit serum was used for negative controls. After washing with PBS, sections were incubated with biotinylated secondary antibodies, washed with PBS, and incubated with VECTASTAIN^{TM} ABC Reagent for 30 min. When mouse monoclonal antibody was used, the Vector M.O.M.^{TM} Immunodetection Kit (Vector Laboratories) was used according to the manufacturer's protocol. Diaminobenzidine was used as a substrate for the immunoperoxidase reaction. Sections were lightly counterstained with hematoxylin, and analyzed by bright field microscopy.

For immunofluorescent reactions, sections were incubated with rabbit anti-SphK1 (1:400) or anti-MUC5AC (1:400) antibody for 1 hour, followed by the incubation with anti-rabbit Alexa 594 (Sigma Aldrich) or FITC-conjugated anti-mouse antibody respectively for 1 hour. Samples were observed and imaged using confocal laser scanning microscope LSM510 (Carl Zeiss, Jena, Germany).

2.10. Statistical Analysis

Results are expressed as mean ± SE. Statistical significance was assessed by using paired Student's t-test or 1-way ANOVA or Kruskal Wallis test with appropriate post-hoc

analysis (Sheffe) to exclude possible interaction between various variables within subgroups using Statcel software (OMS Publishing Inc., Saitama, Japan). A value of $P<0.05$ was considered to be statistically significant.

3. Results

3.1. SphK1 expression in mouse asthmatic epithelium

We have previously demonstrated that SphK1 expression was increased in the bronchial epithelial walls of OVA-challenged asthmatic mice. Therefore, we hypothesized that SphK/S1P signaling may be involved in the molecular mechanisms underlying the etiology of airway mucin production in bronchial asthma.

To test this hypothesis, we first examined the localization of SphK and mucin proteins in mouse asthmatic models. After OVA inhalation for three consecutive days, C57BL/6 mice were sacrificed and paraffin-embedded lung sections were examined. As shown in Fig. 1A, SphK1 was expressed strongly in OVA-treated mouse airway epithelium compared with PBS-treated mice. MUC5AC expression was also induced in OVA-treated mice, but not in PBS-treated mice. Immunofluorescent microscopy revealed that SphK1 (red) and MUC5AC (green) were co-localizad in mouse asthmatic airway (Fig. 1B). These findings suggest that co-localization of SphK1 and MUC5AC may play some role in goblet cell formation in mouse asthmatic model.

3.2. SphK1 is up-regulated by IL-13 in normal human bronchial epithelial cells

To elucidate the role of SphK in goblet cell formation, we cultured NHBE cells at ALI and examined the expressions of SphK and MUC5AC. IL-13 stimulation induced MUC5AC expression at both protein (Fig. 2A) and mRNA (Fig. 2B) levels in a dose-dependent manner. Similarly, SphK1 expression analysis revealed a pronounced increase at both protein (Fig. 2C) and mRNA (Fig. 2D) levels in a dose-dependent manner. On the other hand, SphK2 mRNA expression was not changed (Fig. 2E). These data suggest that SphK1 has some role in IL-13-

induced transdifferentiation signals.

Next, we examined whether S1P produced by SphK could induce goblet cell differentiation in NHBE cells. When S1P was added exogenously, MUC5AC expression did not change at either the protein or the mRNA levels (data not shown). These results suggested that S1P produced by SphK may act as an intracellular messenger, but not as an exogenous ligand.

3.3. SphK inhibition decreases IL-13-induced MUC5AC expression

To clarify the contribution of SphK to IL-13-induced mucin production, we treated the NHBE cells with SphK inhibitor, DMS. As shown in Fig. 3A and 3B, DMS inhibited MUC5AC protein expression as well as MUC5AC mRNA levels in a dose-dependent manner. By immunocytochemical analyses, MUC5AC expressing cells were increased after IL-13 stimulation and this expression was reduced by DMS treatment (Fig. 3C). It has been reported that IL-13 treatment increases goblet cell density through MAP kinase signaling as well as phosphatidylinositol 3-kinase and Stat6 in ALI cultured cells [20]. We show that both MEK1/2 inhibitor PD-98059, and p38 MAPK inhibitor SB203580, reduced MUC5AC protein (Fig. 3A), mRNA (Fig. 3B) expression, and the number of MU5AC expressing cells (Fig. 3C). We also treated cells with pertussis toxin (PTX), a Gi-coupled receptor inhibitor, but PTX had no effect on MUC5AC expression (Fig 3A-C). There were no differences in total cell numbers among all groups (data not shown).

3.4. DMS decreases ERK1/2 phosphorylation induced by IL-13 stimulation

Since it is known that IL-13-induced MUC5AC production is mediated via MAPK

phosphorylation in human bronchial epithelial cells, we assessed the role of SphK in the downstream signaling of IL-13. As shown in Fig. 4A, SphK activity was up-regulated by IL-13 stimulation in a dose-dependent manner. This increase was attenuated by DMS pretreatment. Moreover, IL-13 induced both ERK1/2 (Fig. 4B) and p38 MAPK (Fig. 4C) phosphorylation at 0, 20, and 60min. DMS treatment inhibited IL-13-induced ERK 1/2 phosphorylation, but not p38 MAPK phosphorylation. These results suggest that SphK1 affects IL-13- induced MUC5AC expression through ERK 1/2 activation.

Next, we examined whether SphK could modify IL-13-STAT6 signals. DMS did not affect IL-13-induced STAT6 phosphorylation just as PD98059 and SB203580 also had no such effect (Fig. 4D). These data suggest that the involvement of SphK1 activation in mucin production is independent of STAT6 phosphorylation.

4. Discussion

Previous studies have demonstrated that SphK1 is expressed in airway epithelium, type II alveolar cells, serous glands, and endothelial cells of vessels in normal human lung tissue [15, 21]. We found that SphK1 was strongly stained in mouse airway epithelium treated with OVA and that SphK1 was co-localized with MUC5AC. On the other hand, SphK2 expression was not induced by IL-13 stimulation in bronchial epithelial cells. This observation is a novel finding to the best of our knowledge and these results imply roles of SphK1 in goblet cell formation in mouse asthmatic epithelium.

It has been reported that STAT6 is a key regulator in IL-13-mediated MUC5AC production [22]. Alternatively, recent data have implicated pivotal roles of p38 MAPK, ERK1/2 and phosphatidylinositol 3-kinase in the IL-13 signaling pathway [20]. We observed that IL-13 induced p38 MAPK and ERK1/2 phosphorylation and that their respective inhibitors reduced MUC5AC expression induced by IL-13. Fujisawa et al reported that both p38 MAPK and ERK1/2 inhibitors attenuated MUC5AC expression by IL-13 treatment in mouse tracheal epithelial cells [23]. However, ERK1/2 was constitutively phosphorylated and unrelated to IL-13 stimulation, whereas p38 MAPK was phosphorylated by IL-13. They concluded that p38 MAPK pathway was activated in an IL-13-STAT6 dependent manner, whereas the ERK1/2 pathway was independent of IL-13 signaling pathway. Since DMS did not alter IL-13-induced STAT6 activation in our study, these data suggest that SphK1 can control ERK1/2 activation induced by IL-13 treatment, independent of IL-13-STAT6-p38 MAPK signals.

S1P produced by SphKs can work both intracellularly as a second messenger and extracellularly as a ligand via the $S1P_{1-5}$ G-protein coupled receptors and both of these S1P

actions have been reported to activate MAPK signals. For example, Monick et al reported that RS virus stimulated SphK activity and increased S1P production, leading to ERK activation via an intracellular pathway and to Akt activation via extracellular activation of the $S1P_1$ receptor [24]. Shu et al also demonstrated that VEGF induced SphK activation resulted in ERK activation through intracellular S1P production [25]. PTX did not affect VEGF-induced ERK activation, therefore, VEGF signal triggers SphK activation and ERK activation independently of S1P receptors. Similarly, PTX did not change IL-13-induced ERK1/2 phosphoryration in our study. SphK1 up-regulated by IL-13 treatment activated ERK/1/2 via PTX-independent pathway and these results suggest that SphK1 may transactivate ERK1/2 activation through PTX-independent SIP receptor activation or intracellular S1P signals.

Although we have clarified that SphK1 expression and activity are increased under asthmatic conditions, it is important to know how S1P produced by SphK1 affects mucin production in NHBE cells. We therefore stimulated NHBE cells with S1P under various conditions. MUC5AC expression, however, was not changed either at the protein or the mRNA levels. This result implied that exogenous S1P has no effect on airway mucin production and it is suggested that S1P release followed by IL-13 treatment works as an intracellular second messenger.

5. Conclusions

In summary we have demonstrated that SphK1 plays important roles in the regulation of IL-13 induced MUC5A production via ERK1/2 signaling pathway. These data also provide insights into novel mechanisms with potential therapeutic implications for mucin hypersecretion in asthmatic patients.

Acknowledgements

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Figure legends

Fig. 1. MUC5AC and sphingosine kinase 1 (SphK1) are colocalized in airway epithelium of OVA-treated mouse lungs. (A) Lung sections from C57BL/6 mice treated with OVA (c, d, e and f) or PBS (a and b) were immunostained using antibodies to MUC5AC (a, c and e) and SphK1 (b, d and f). Original magnification: ×200 (a, b, c, and d) and ×400 (e and f). (B) Immunofluorescent microscopic images show OVA-treated lung sections stained with FITC-conjugated anti-MUC5AC (a, green) and Alexa 594-conjugated anti-SphK1 (b, red) antibodies. Colocalization of MUC5AC and SphK1 is shown as yellow color (c).

Fig. 2. IL-13 up-regulated SphK1 and MUC5AC expression in NHBE cells. Cells were cultured in air liquid interface (ALI) and stimulated with IL-13 for 14 days. MUC5AC protein (A) expression was analyzed using ELISA. MUC5AC (B), SphK1 (D), and SphK2 (E) mRNA expressions were analyzed by quantitative real-time RT-PCR. (C) SphK1 and β-actin protein

expressions were analyzed by western blot analysis. Data are mean ± SE of 4 independent experiments. *P<0.05 vs. control, **P<0.01 vs. control.

Fig. 3. Decrease in IL-13-induced MUC5AC expression by SphK inhibitor DMS. NHBE cells were treated with IL-13 (10 ng/ml) with or without various inhibitors (0.1-10 μM DMS, 20 μM PD98059, or 1 μM SB203580) for 14 days and MUC5AC expression was analyzed by ELISA (A) and quantitative real-time RT-PCR (B). NHBE cells were fixed and immunostained with MUC5AC antibodies. Representative photomicrographs of cells are shown (C). Positively stained cells as a % of total cells are quantified. Data are mean ± SE of 4 independent experiments. *P<0.05 vs. IL-13 group, **P<0.01 vs. IL-13 group.

Fig. 4. The effect of DMS on IL-13-induced phosphorylation of MAPKs and STAT6. A. NHBE cells in ALI were treated with IL-13 at indicated concentrations for 20 minutes and SphK activity was measured as described in methods. DMS (5 μM) was pretreated before IL-13 stimulation. Data are mean ± SE of 4 independent experiments. *P<0.05 vs. control. Then cells were stimulated with IL-13 (20 ng/ml) with or without inhibitors (5 μM DMS, 20 μM PD98059, or 1 μM SB203580) for indicated times. Phosphorylation of ERK1/2 (B), p38-MAPK (C), and STAT6 (D) was evaluated by western blot analysis. These blots are representative images from 4 independent experiments. The intensity of each band detected with anti-phosphorylated protein antibody was normalized to that with anti-nonphosphorylated protein antibody. Data are mean ± SE of 4 independent experiments. *P<0.05 vs. control

Sphingosine kinase 1 regulates mucin production via ERK phosphorylation

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Abstract

Our previous report showed that inhibition of sphingosine kinase (SphK) ameliorates eosinophilic inflammation and mucin production in a mouse asthmatic model. To clarify the role of SphK in airway mucin production, we utilized the mouse asthmatic model and found that both SphK and MUC5AC expression were increased and co-localized in airway epithelium. Next we cultured normal human bronchial epithelial cells in an air liquid interface and treated with IL-13 to induce their differentiation into goblet cells. We found that SphK1 and MUC5AC expression was increased by IL-13 treatment at both protein and mRNA levels, whereas SphK2 expression was not changed. N,N-dimethylsphingosine (DMS), a potent SphK inhibitor, decreased MUC5AC expression up-regulated by IL-13 treatment. Furthermore, DMS inhibited IL-13-induced ERK1/2 phosphorylation but neither p38 MAPK nor STAT6 phosphorylation. These results suggest that SphK1 is involved in MUC5AC production induced by IL-13 upstream of ERK1/2 phosphorylation, and independent of STAT6 phosphorylation.

Keywords

sphingosine 1-phosphate, sphingosine kinase, airway epithelium, IL-13, ERK

Abbreviations

SphK, sphingosine kinase; S1P, sphingosine 1-phosphate; IL, interleukin; DMS, N,N-dimethylsphingosine; MAPK, mitogen-activated kinase; ERK, extracellular signal-regulated kinase; STAT6, signal transducer and activator of transcription 6.

1. Introduction

Mucus is a critical component of the innate host defense system in the respiratory tract. On the other hand, mucus hypersecretion is a major cause of airway obstruction in asthma and is sometimes associated with increased morbidity or mortality. Excessive mucus in asthmatic patients reflects over production of mucin due to goblet cell hyperplasia (GCH), that is a major pathological feature of asthma [1]. Airway GCH is especially marked in patients who die from asthmatic disease, with a 30-fold increase in the percentage of goblet cells compared with patients dying from non-asthmatic respiratory disease [2].

MUC5AC protein is a major component of airway mucus and is expressed in goblet cells of upper and lower respiratory tracts. Various stimuli such as lipopolysaccharide (LPS) [3], TNFα [4], and Th2 lymphocyte-derived cytokines, IL-4 [5] and IL-13 [6, 7] have been reported to induce MUC5AC both *in vitro* and *in vivo*. In mouse asthmatic models IL-13 is a central mediator of mucin production [8, 9], and mucin production induced by other Th2 cytokines is stimulated through IL-13 and the IL-13 receptor-mediated signals [10]. Moreover, it has been described that the downstream cascade of IL-13 involves signaling molecules such as signal transducer and activator of transcription 6 (STAT6), and mitogen-activated kinases (MAPKs) through epidermal growth factor receptor (EGFR) in airway epithelial cells [11, 12].

Sphingosine 1-phosphate (S1P) is a bioactive lipid mediator of cellular functions such as proliferation, differentiation, apoptosis, tumor cell invasion, cell migration, and angiogenesis. Recently S1P has been shown to mediate asthmatic pathogenesis *in vivo* and *in vitro*. S1P levels are increased in bronchoalveolar lavage (BAL) fluid of asthmatics after challenge with antigen and correlated with eosinophil numbers in the BAL fluid of asthmatic subjects [13]. S1P secreted

by activated mast cells can promote allergic reactions by activating many types of immune cells including eosinophils, Th2 lymphocytes and neutrophils [14].

In a previous study, we demonstrated that inhibition of sphingosine kinase (SphK), a key enzyme that phosphorylates sphingosine to generate S1P, prevented airway mucin production and eosinophil inflammation [15]. We hypothesized that S1P produced by SphK may mediate airway mucin production in asthmatic pathogenesis. There is no information about the role of sphingosine metabolism in mucin production. In this study, we have clarified how SphK/S1P activation can affect the mucin production in human bronchial epithelial cells.

2. Materials and Methods

2.1. Materials

Recombinant human IL-13 was purchased from Strathmann Biotec (Hamburg, Germany). PD98059 and rabbit polyclonal antibodies against ERK1/2, phospho-ERK1/2, p38 MAPK, and phoshpo-p38 MAPK were from Cell Signaling Technology (Beverly, MA, USA). Anti-MUC5AC mouse monoclonal antibody was from Lab Vision (Fremont, CA, USA). N',N'-dimethylsphingosine (DMS) and anti-β-actin mouse monoclonal antibody were from Sigma-Aldrich (St. Louis, MO, USA). Anti-STAT6 mouse monoclonal and anti-phospho-STAT6 rabbit polyclonal antibodies were from R&D systems Inc. (Minneapolis, MN, USA). Anti-human SphK1 goat polyclonal antibody was from Abcam (Cambridge, UK). SB203580 was from Calbiochem (San Diego, CA, USA).

A rabbit polyclonal anti-mouse SphK1 antibody was raised against the synthetic peptide GSRDAPSGRDSRRGPPPEEP (amino acid residues 362-381) conjugated to glutathione S-transferase. The antibody was affinity-purified by using the immunogen-immobilized Sepharose 4B [16].

2.2 Mouse asthmatic model

Six- to 8-week-old C57BL/6 mice (Nippon CLEA, Tokyo, Japan) were sensitized with an intraperitoneal injection of 10 μg ovalbumin (OVA, Grade V; Sigma-Aldrich, St. Louis, MO, USA) adsorbed in 2 mg of aluminum hydroxide (Sigma-Aldrich) in 0.5 mL sterile phosphate-buffered saline (PBS). The mice were subsequently boosted intraperitoneally with the same mixture 14 days later. On day 28 after the initial sensitization, mice were exposed to aerosolized

1.0% OVA in sterile PBS for 30 min, and the same protocol was repeated on three consecutive days. The aerosolized OVA was generated using an ultrasonic nebulizer (NE-U12; Omron, Tokyo, Japan). Negative controls were injected and exposed to PBS. Our research was approved by the Institutional Animal Care and Use Committee and carried out according to the Kobe University Animal Experimental Regulations.

2.3. Cell culture

Normal human bronchial epithelial (NHBE) cells were purchased from Lonza Walkersville Inc. (Walkersville, MD, USA). Cells were seeded on plastic dishes coated with human placental collagen (Sigma-Aldrich) and grown in bronchial epithelial cell growth medium with supplements (Cambrex, Walkersville, MD, USA). At 3 to 7 passages, 80 to 90% confluent cells were seeded at a density of 4 x 10^4 cells/cm^2 onto 12 mm or 24 mm diameter Corning Costar Transwell^{TM} -Clear inserts with 0.4 μm pore size. After 7 days under immersed conditions, the apical medium was removed to establish an air-liquid interface that was maintained for the remainder of the cell culture period. Medium was changed every other day and apical surface of the cells was rinsed with PBS to remove any debris.

For goblet cell differentiation, cells were treated with recombinant human IL-13 as reported previously [17, 18]. IL-13 was added to the basal medium and cells cultured for 14 days. IL-13 containing medium was replaced every other day. Cell differentiation was confirmed by detecting mucin production using periodic acid-Schiff (PAS) staining. Inhibitors were added to the basal medium at the same time as IL-13 administration.

2.4. MAPKs phosphorylation by western blot analysis

After 14-days of culture without any stimuli at air-liquid interface (ALI), cells were treated with IL-13 (20 ng/ml) in the presence of complete culture medium for indicated times. Cells were then harvested and lysed in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 15 mM NaF, 1% Triton X-100, 5 mM EDTA, 1 mM sodium orthovanadate, and protease inhibitor cocktail (Sigma-Aldrich). Cell lysates were prepared in SDS-sample buffer and subjected to SDS-PAGE. Proteins were transferred onto nitrocellulose membrane and immunostained using antibodies against ERK and p38-MAPK. Positive protein bands were visualized by the enhanced chemiluminescence method and their intensities were quantified by MultiGauge^{TM} software (Fujifilm, Tokyo, Japan).

2.5. RNA isolation and real-time quantitative reverse transcription-PCR analysis

Total RNA was extracted using ISOGEN^{TM} reagent (Nippon Gene, Tokyo, Japan). First-strand cDNA was synthesized from 1 μg of total RNA by using ExScript RT^{TM} reagent kits (Takara, Otsu, Japan) and random hexamer primers. Quantitative PCR was performed using real-time SYBR Green PCR technology and an ABI PRISM 7500 Sequence Detection system (Applied Biosystems, Foster City, CA, USA). The primers for human SphK1, SphK2, and GAPDH were described previously [16]. The primers for human MUC5AC: forward primer, 5'-TGTGGGCTATGGGTCACCTG-3', and reverse primer, 5'-GATCACCATGTCCAAGCGTCA-3', were purchased from Takara. Amplification reactions were performed in duplicate with SYBR Premix Ex Taq^{TM} (Takara), and the thermal cycling conditions were as follows: 10 s at 95°C, 40 cycles of 5 s at 95°C, and 34 s at 60°C. The expression of each mRNA was normalized

to GAPDH mRNA expression.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Production of MUC5AC in cell lysates and cell culture supernatants was measured by ELISA using a MUC5AC antibody, as previously described [11]. The amount of MUC5AC in each sample was normalized to total protein in cell lysates and expressed as percent of control.

2.7. The measurement of SphK activity

NHBE cells cultured in ALI for 14 days were stimulated with 10 ng/ml IL-13 for 20 min. SphK activity in cell lysates was determined in the reaction mixture (50 μl) containing 10 μM sphingosine, 1 mM [γ-^{32}P]ATP (8 Ci/mol), and 10 mM $MgCl_2$ essentially as described previously [19]. [^{32}P]S1P was separated by TLC on Silica Gel 60 plates (Merck & Co., NJ, USA) with 1-butanol / acetic acid / water (3:1:1, by vol.) and quantified using a Fujix Bio-Imaging Analyzer BAS 2000 (Fuji Photo Film Co. Ltd., Tokyo, Japan).

2.8. Immunocytochemistry

After stimulation, cells were washed with PBS, fixed for 20 min at room temperature with acetone-methanol (1:1), and permeabilized with 0.1% Triton X-100 in PBS for 15 min. They were blocked with 3% bovine serum albumin for 1 hour, incubated with anti-MUC5AC antibody (1:100) for 1 hour, and then stained using VECTASTAIN^{TM} ABC kit (Vector Laboratories, Burlingame, CA, USA). The mean percentages of MUC5AC-positive cells were calculated from at least 4 different bright fields.

2.9. Immunohistochemistry

Mouse lungs were infused at a pressure of 20 cmH_2O with 10% buffered formalin for 24 hours, embedded in paraffin, and sectioned at 5-µm thickness. After deparaffinization, tissue sections were pretreated with 3% hydrogen peroxidase for 10 min and blocked with normal goat serum for 30 min. Then they were incubated for 1 hour with rabbit anti-SphK1 (1:100) or mouse anti-MUC5AC (1:100) antibody. Normal mouse or rabbit serum was used for negative controls. After washing with PBS, sections were incubated with biotinylated secondary antibodies, washed with PBS, and incubated with VECTASTAIN^{TM} ABC Reagent for 30 min. When mouse monoclonal antibody was used, the Vector M.O.M.^{TM} Immunodetection Kit (Vector Laboratories) was used according to the manufacturer's protocol. Diaminobenzidine was used as a substrate for the immunoperoxidase reaction. Sections were lightly counterstained with hematoxylin, and analyzed by bright field microscopy.

For immunofluorescent reactions, sections were incubated with rabbit anti-SphK1 (1:400) or anti-MUC5AC (1:400) antibody for 1 hour, followed by the incubation with anti-rabbit Alexa 594 (Sigma Aldrich) or FITC-conjugated anti-mouse antibody respectively for 1 hour. Samples were observed and imaged using confocal laser scanning microscope LSM510 (Carl Zeiss, Jena, Germany).

2.10. Statistical Analysis

Results are expressed as mean ± SE. Statistical significance was assessed by using paired Student's t-test or 1-way ANOVA or Kruskal Wallis test with appropriate post-hoc

analysis (Sheffe) to exclude possible interaction between various variables within subgroups using Statcel software (OMS Publishing Inc., Saitama, Japan). A value of $P<0.05$ was considered to be statistically significant.

3. Results

3.1. SphK1 expression in mouse asthmatic epithelium

We have previously demonstrated that SphK1 expression was increased in the bronchial epithelial walls of OVA-challenged asthmatic mice. Therefore, we hypothesized that SphK/S1P signaling may be involved in the molecular mechanisms underlying the etiology of airway mucin production in bronchial asthma.

To test this hypothesis, we first examined the localization of SphK and mucin proteins in mouse asthmatic models. After OVA inhalation for three consecutive days, C57BL/6 mice were sacrificed and paraffin-embedded lung sections were examined. As shown in Fig. 1A, SphK1 was expressed strongly in OVA-treated mouse airway epithelium compared with PBS-treated mice. MUC5AC expression was also induced in OVA-treated mice, but not in PBS-treated mice. Immunofluorescent microscopy revealed that SphK1 (red) and MUC5AC (green) were co-localizad in mouse asthmatic airway (Fig. 1B). These findings suggest that co-localization of SphK1 and MUC5AC may play some role in goblet cell formation in mouse asthmatic model.

3.2. SphK1 is up-regulated by IL-13 in normal human bronchial epithelial cells

To elucidate the role of SphK in goblet cell formation, we cultured NHBE cells at ALI and examined the expressions of SphK and MUC5AC. IL-13 stimulation induced MUC5AC expression at both protein (Fig. 2A) and mRNA (Fig. 2B) levels in a dose-dependent manner. Similarly, SphK1 expression analysis revealed a pronounced increase at both protein (Fig. 2C) and mRNA (Fig. 2D) levels in a dose-dependent manner. On the other hand, SphK2 mRNA expression was not changed (Fig. 2E). These data suggest that SphK1 has some role in IL-13-

induced transdifferentiation signals.

Next, we examined whether S1P produced by SphK could induce goblet cell differentiation in NHBE cells. When S1P was added exogenously, MUC5AC expression did not change at either the protein or the mRNA levels (data not shown). These results suggested that S1P produced by SphK may act as an intracellular messenger, but not as an exogenous ligand.

3.3. SphK inhibition decreases IL-13-induced MUC5AC expression

To clarify the contribution of SphK to IL-13-induced mucin production, we treated the NHBE cells with SphK inhibitor, DMS. As shown in Fig. 3A and 3B, DMS inhibited MUC5AC protein expression as well as MUC5AC mRNA levels in a dose-dependent manner. By immunocytochemical analyses, MUC5AC expressing cells were increased after IL-13 stimulation and this expression was reduced by DMS treatment (Fig. 3C). It has been reported that IL-13 treatment increases goblet cell density through MAP kinase signaling as well as phosphatidylinositol 3-kinase and Stat6 in ALI cultured cells [20]. We show that both MEK1/2 inhibitor PD-98059, and p38 MAPK inhibitor SB203580, reduced MUC5AC protein (Fig. 3A), mRNA (Fig. 3B) expression, and the number of MU5AC expressing cells (Fig. 3C). We also treated cells with pertussis toxin (PTX), a Gi-coupled receptor inhibitor, but PTX had no effect on MUC5AC expression (Fig 3A-C). There were no differences in total cell numbers among all groups (data not shown).

3.4. DMS decreases ERK1/2 phosphorylation induced by IL-13 stimulation

Since it is known that IL-13-induced MUC5AC production is mediated via MAPK

phosphorylation in human bronchial epithelial cells, we assessed the role of SphK in the downstream signaling of IL-13. As shown in Fig. 4A, SphK activity was up-regulated by IL-13 stimulation in a dose-dependent manner. This increase was attenuated by DMS pretreatment. Moreover, IL-13 induced both ERK1/2 (Fig. 4B) and p38 MAPK (Fig. 4C) phosphorylation at 0, 20, and 60min. DMS treatment inhibited IL-13-induced ERK 1/2 phosphorylation, but not p38 MAPK phosphorylation. These results suggest that SphK1 affects IL-13- induced MUC5AC expression through ERK 1/2 activation.

Next, we examined whether SphK could modify IL-13-STAT6 signals. DMS did not affect IL-13-induced STAT6 phosphorylation just as PD98059 and SB203580 also had no such effect (Fig. 4D). These data suggest that the involvement of SphK1 activation in mucin production is independent of STAT6 phosphorylation.

4. Discussion

Previous studies have demonstrated that SphK1 is expressed in airway epithelium, type II alveolar cells, serous glands, and endothelial cells of vessels in normal human lung tissue [15, 21]. We found that SphK1 was strongly stained in mouse airway epithelium treated with OVA and that SphK1 was co-localized with MUC5AC. On the other hand, SphK2 expression was not induced by IL-13 stimulation in bronchial epithelial cells. This observation is a novel finding to the best of our knowledge and these results imply roles of SphK1 in goblet cell formation in mouse asthmatic epithelium.

It has been reported that STAT6 is a key regulator in IL-13-mediated MUC5AC production [22]. Alternatively, recent data have implicated pivotal roles of p38 MAPK, ERK1/2 and phosphatidylinositol 3-kinase in the IL-13 signaling pathway [20]. We observed that IL-13 induced p38 MAPK and ERK1/2 phosphorylation and that their respective inhibitors reduced MUC5AC expression induced by IL-13. Fujisawa et al reported that both p38 MAPK and ERK1/2 inhibitors attenuated MUC5AC expression by IL-13 treatment in mouse tracheal epithelial cells [23]. However, ERK1/2 was constitutively phosphorylated and unrelated to IL-13 stimulation, whereas p38 MAPK was phosphorylated by IL-13. They concluded that p38 MAPK pathway was activated in an IL-13-STAT6 dependent manner, whereas the ERK1/2 pathway was independent of IL-13 signaling pathway. Since DMS did not alter IL-13-induced STAT6 activation in our study, these data suggest that SphK1 can control ERK1/2 activation induced by IL-13 treatment, independent of IL-13-STAT6-p38 MAPK signals.

S1P produced by SphKs can work both intracellularly as a second messenger and extracellularly as a ligand via the $S1P_{1-5}$ G-protein coupled receptors and both of these S1P

actions have been reported to activate MAPK signals. For example, Monick et al reported that RS virus stimulated SphK activity and increased S1P production, leading to ERK activation via an intracellular pathway and to Akt activation via extracellular activation of the $S1P_1$ receptor [24]. Shu et al also demonstrated that VEGF induced SphK activation resulted in ERK activation through intracellular S1P production [25]. PTX did not affect VEGF-induced ERK activation, therefore, VEGF signal triggers SphK activation and ERK activation independently of S1P receptors. Similarly, PTX did not change IL-13-induced ERK1/2 phosphoryration in our study. SphK1 up-regulated by IL-13 treatment activated ERK/1/2 via PTX-independent pathway and these results suggest that SphK1 may transactivate ERK1/2 activation through PTX-independent SIP receptor activation or intracellular S1P signals.

Although we have clarified that SphK1 expression and activity are increased under asthmatic conditions, it is important to know how S1P produced by SphK1 affects mucin production in NHBE cells. We therefore stimulated NHBE cells with S1P under various conditions. MUC5AC expression, however, was not changed either at the protein or the mRNA levels. This result implied that exogenous S1P has no effect on airway mucin production and it is suggested that S1P release followed by IL-13 treatment works as an intracellular second messenger.

5. Conclusions

In summary we have demonstrated that SphK1 plays important roles in the regulation of IL-13 induced MUC5A production via ERK1/2 signaling pathway. These data also provide insights into novel mechanisms with potential therapeutic implications for mucin hypersecretion in asthmatic patients.

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Figure legends

Fig. 1. MUC5AC and sphingosine kinase 1 (SphK1) are colocalized in airway epithelium of OVA-treated mouse lungs. (A) Lung sections from C57BL/6 mice treated with OVA (c, d, e and f) or PBS (a and b) were immunostained using antibodies to MUC5AC (a, c and e) and SphK1 (b, d and f). Original magnification: ×200 (a, b, c, and d) and ×400 (e and f). (B) Immunofluorescent microscopic images show OVA-treated lung sections stained with FITC-conjugated anti-MUC5AC (a, green) and Alexa 594-conjugated anti-SphK1 (b, red) antibodies. Colocalization of MUC5AC and SphK1 is shown as yellow color (c).

Fig. 2. IL-13 up-regulated SphK1 and MUC5AC expression in NHBE cells. Cells were cultured in air liquid interface (ALI) and stimulated with IL-13 for 14 days. MUC5AC protein (A) expression was analyzed using ELISA. MUC5AC (B), SphK1 (D), and SphK2 (E) mRNA expressions were analyzed by quantitative real-time RT-PCR. (C) SphK1 and β-actin protein

expressions were analyzed by western blot analysis. Data are mean ± SE of 4 independent experiments. *P<0.05 vs. control, **P<0.01 vs. control.

Fig. 3. Decrease in IL-13-induced MUC5AC expression by SphK inhibitor DMS. NHBE cells were treated with IL-13 (10 ng/ml) with or without various inhibitors (0.1-10 μM DMS, 20 μM PD98059, or 1 μM SB203580) for 14 days and MUC5AC expression was analyzed by ELISA (A) and quantitative real-time RT-PCR (B). NHBE cells were fixed and immunostained with MUC5AC antibodies. Representative photomicrographs of cells are shown (C). Positively stained cells as a % of total cells are quantified. Data are mean ± SE of 4 independent experiments. *P<0.05 vs. IL-13 group, **P<0.01 vs. IL-13 group.

Fig. 4. The effect of DMS on IL-13-induced phosphorylation of MAPKs and STAT6. A. NHBE cells in ALI were treated with IL-13 at indicated concentrations for 20 minutes and SphK activity was measured as described in methods. DMS (5 μM) was pretreated before IL-13 stimulation. Data are mean ± SE of 4 independent experiments. *P<0.05 vs. control. Then cells were stimulated with IL-13 (20 ng/ml) with or without inhibitors (5 μM DMS, 20 μM PD98059, or 1 μM SB203580) for indicated times. Phosphorylation of ERK1/2 (B), p38-MAPK (C), and STAT6 (D) was evaluated by western blot analysis. These blots are representative images from 4 independent experiments. The intensity of each band detected with anti-phosphorylated protein antibody was normalized to that with anti-nonphosphorylated protein antibody. Data are mean ± SE of 4 independent experiments. *P<0.05 vs. control

Figure 1

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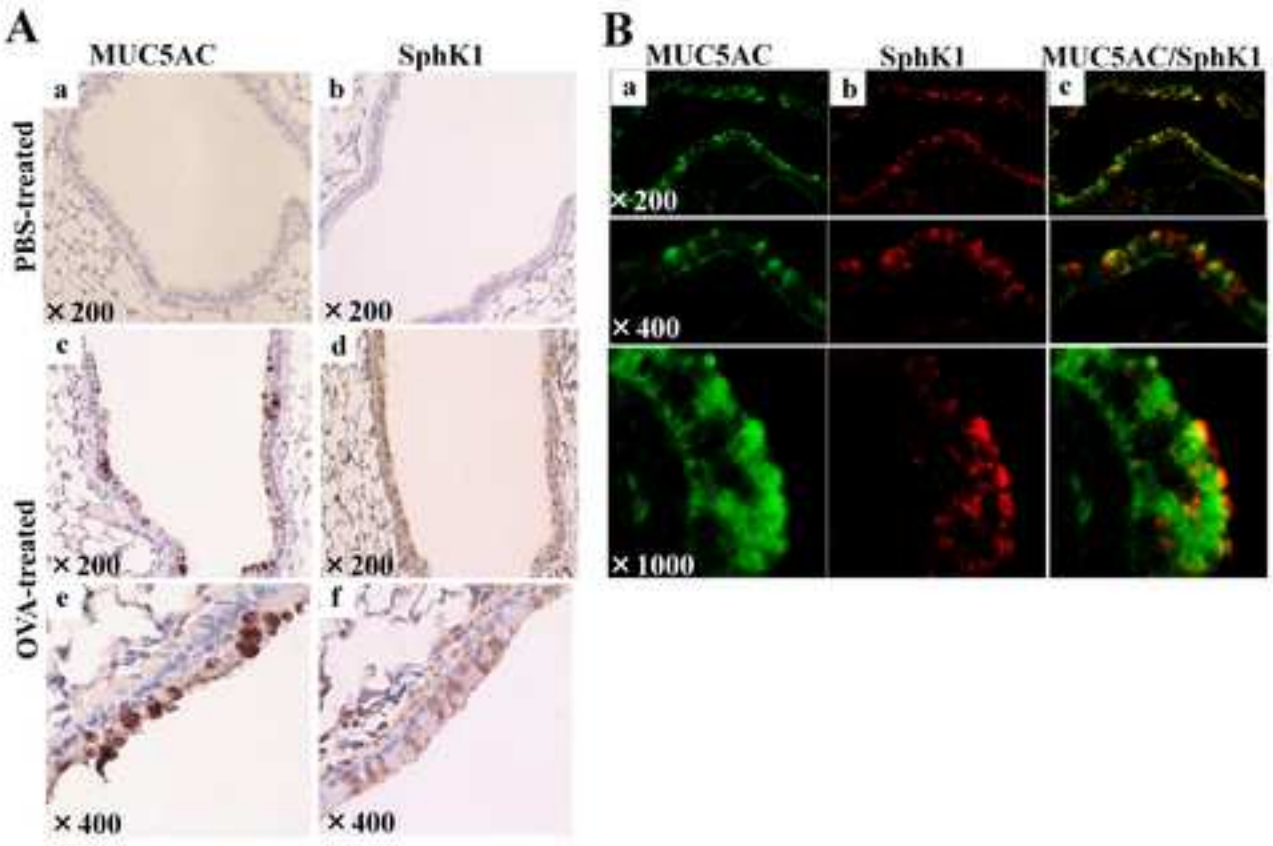

Figure 2

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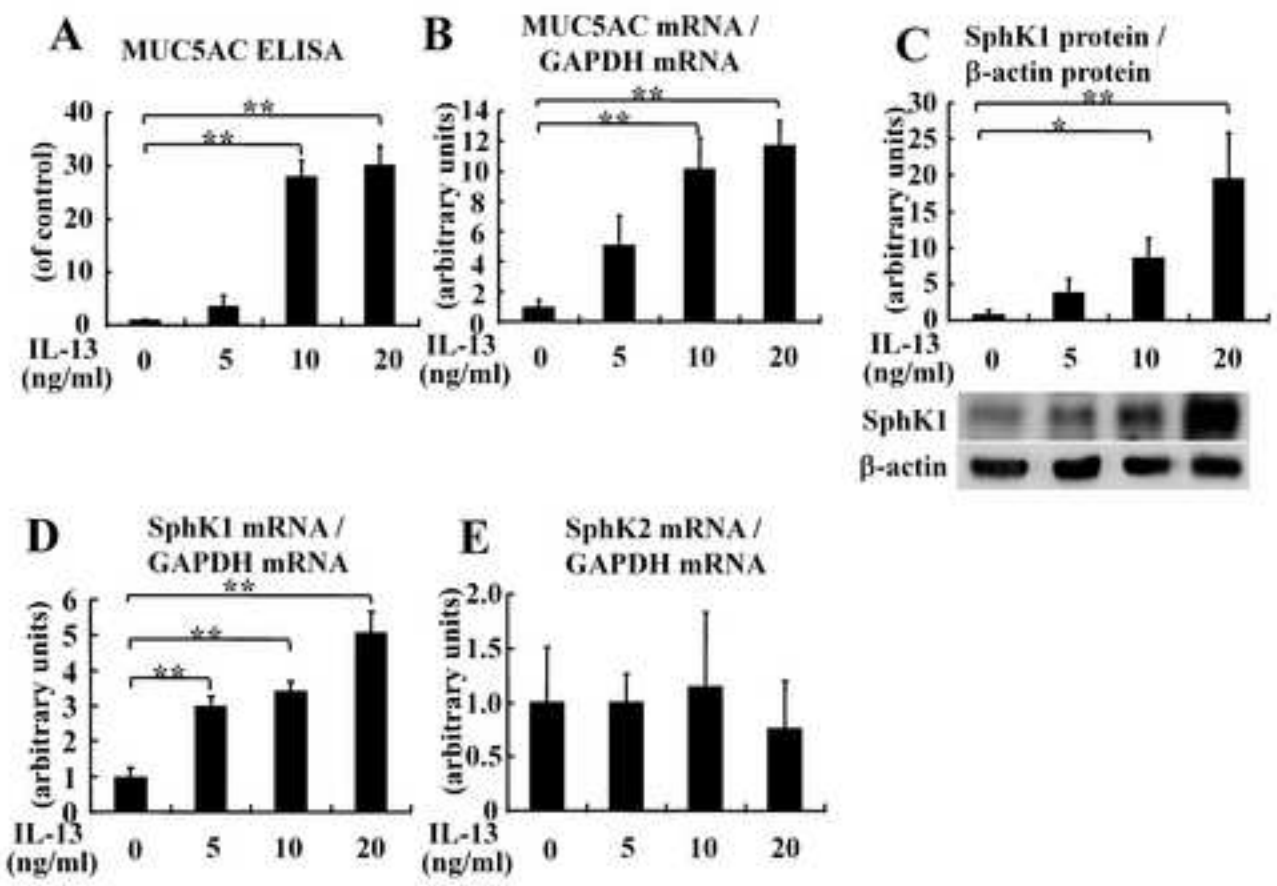

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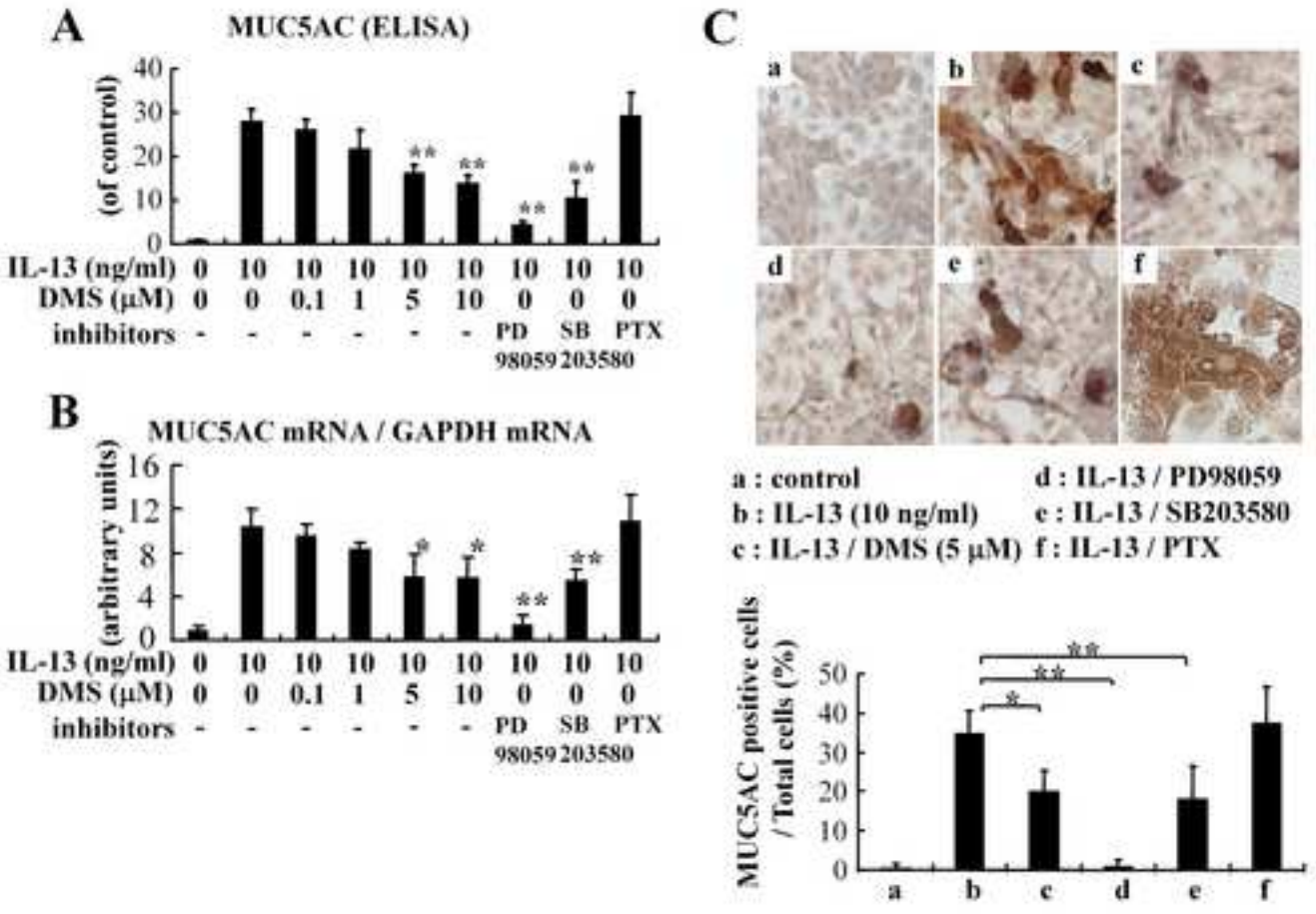

Figure 4

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