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Secretory Phospholipases A_2 Are Secreted From Ciliated Cells and Increase Mucin and Eicosanoid Secretion From Goblet Cells

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BACKGROUND: Secretory phospholipases A_2 ($sPLA_2$) initiate the biosynthesis of eicosanoids, are increased in the airways of people with severe asthma, and induce mucin hypersecretion. We used IL-13-transformed, highly enriched goblet cells and differentiated (ciliary cell-enriched) human bronchial epithelial cell culture to evaluate the relative contribution of ciliated and goblet cells to airway $sPLA_2$ generation and response. We wished to determine the primary source(s) of $sPLA_2$ and leukotrienes in human airway epithelial cells.

METHODS: Human bronchial epithelial cells from subjects without lung disease were differentiated to a ciliated-enriched or goblet-enriched cell phenotype. Synthesis of $sPLA_2$, cysteinyl leukotrienes (cysLTs), and airway mucin messenger RNA and protein was measured by real-time-polymerase chain reaction and an enzyme-linked immunosorbent assay, and the localization of mucin and $sPLA_2$ to specific cells types was confirmed by confocal microscopy.

RESULTS: $sPLA_2$ group IIa, V, and X messenger RNA expression was increased in ciliated-enriched cells ($P < .001$) but not in goblet-enriched cells. $sPLA_2$ were secreted from the apical (air) side of ciliated-enriched cells but not goblet-enriched cells ($P < .001$). Immunostaining of $sPLA_2$ V was strongly positive in ciliated-enriched cells but not in goblet-enriched cells. $sPLA_2$ released cysLTs from goblet-enriched cells but not from ciliated-enriched cells, and this result was greatest with $sPLA_2$ V ($P < .05$). $sPLA_2$ V increased goblet-enriched cell mucin secretion, which was inhibited by inhibitors of lipoxygenase or cyclooxygenase ($P < .02$).

CONCLUSIONS: $sPLA_2$ are secreted from ciliated cells and appear to induce mucin and cysLT secretion from goblet cells, strongly suggesting that airway goblet cells are proinflammatory effector cells.

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ABBREVIATIONS: 5-LO = 5-lipoxygenase; ALI = air-liquid interface; BALF = BAL fluid; cysLT = cysteinyl leukotriene; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HBE = human bronchial epithelial; LO = lipoxygenase; LTB_4 = leukotriene B_4; LTC_4 = leukotriene C_4; mRNA = messenger RNA; PAS = periodic acid-Schiff; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; PGE_2 = prostaglandin E_2; $sPLA_2$ = secretory phospholipases A_2

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Phospholipases A_2 are a group of enzymes that hydrolyze fatty acids, including arachidonic acid, from the sn-2 position of glycerophospholipids, providing the substrate for the synthesis of leukotrienes, prostaglandins, platelet-activating factor, and lysophospholipids.[1] More than one-third of the mammalian phospholipases A_2 enzymes belong to the secretory phospholipases A_2 ($sPLA_2$) family.[2] Human genes have been identified for 12 $sPLA_2$ enzymes, which are subdivided into conventional groups (I, II, V, X, and otoconin-90) and two atypical groups (III and XII).[3] $sPLA_2$ are released in the plasma and BAL fluid (BALF) of patients with asthma,[4] ARDS,[5] and pneumonia.[6] Of the three $sPLA_2$ expressed in human lungs, group IIa and X enzymes are the major $sPLA_2$ that are increased in the BALF from subjects with asthma.[7] Gene silencing of either group V or X $sPLA_2$ in mice with ovalbumin-induced asthma attenuates T-helper cell type 2 inflammation.[8-10] We have reported that $sPLA_2$ stimulate mucus hypersecretion in the ferret trachea through activation of the lipoxygenase (LO) pathway.[11]

Mucus obstruction of airways and goblet cell hyperplasia are characteristic of severe asthma.[12,13] IL-13 is increased in asthma and induces goblet cell hyperplasia with mucus hypersecretion in vivo and in vitro.[13-16] MUC5AC and MUC5B are the predominant gel-forming mucins in the human airway. MUC5AC is expressed primarily by goblet cells of the surface epithelium.[17] IL-13 increases MUC5AC-expressing goblet cells in the airways. MUC5AC secretion is also increased during asthmatic inflammation[18]; even in mild asthma, there are increases in airway goblet cell number and stored and secreted MUC5AC protein.[13]

Several $sPLA_2$ isoforms are expressed and released by inflammatory cells.[19] In addition to inflammatory cells, the epithelial cells lining the respiratory tract may be an important source of $sPLA_2$.[6,9,20-22] It has not been shown which airway epithelial cells produce $sPLA_2$, or which cells types respond to $sPLA_2$ by producing leukotrienes. We hypothesized that airway goblet cells act as immune effector cells and that inflammatory mediators released from goblet cells may act in an autocrine manner, which, in turn, contributes to the severity of asthma. Therefore, we studied the production and secretion of $sPLA_2$ isotypes from differentiated human bronchial epithelial (HBE) cells in ciliated cell-enriched culture or IL-13-differentiated goblet cell-enriched cultures. We also evaluated eicosanoid and mucin synthesis after exposure to specific $sPLA_2$ subtypes in these same HBE cell culture systems.

Materials and Methods

Reagents

Recombinant human IL-13 was obtained from R&D Systems; anti-MUC5AC antibody from Lab Vision Corporation; anti MUC5B antibody from Santa Cruz Biotechnology; anti-$sPLA_2$ group V from the Cayman Chemical Company; acetylated α-tubulin antibody from Cell Signaling Technology; recombinant human $sPLA_2$ group IIa, V, or X from BioVendor LLC; and MK-886 from Calbiochem. Indomethacin, quercetin, and other reagents were purchased from Sigma-Aldrich unless otherwise indicated.

Culture and Differentiation of HBE Cells

The cultivation of HBE cells and differentiation at an air-liquid interface (ALI) have been described previously.[12,23-25] HBE cells (Clonetics; Lonza Group Ltd) were plated at 3,500 cells/cm^2 in small-airway epithelial cell growth medium (Clonetics; Lonza Group Ltd) and cultured at 37°C in a 5% CO_2 incubator. Second-passage HBE cells were seeded at a density of 2.0×10^5/cm^2 onto polyester inserts (6.5-mm diameter, 0.4-μm pore size, and 10-μm thickness; Costar Transwell Clear), coated with type 1 rat tail collagen (Sigma), and then cultured in serum-free DMEM/F12 medium containing ITS-A (1.0%; Invitrogen Ltd), triiodothyronine (10 ng/mL; ICN Biochemicals), epidermal growth factor (recombinant human EGF, 0.5 ng/mL; Invitrogen Ltd), all-trans retinoic acid (10^{-7} M; Sigma), hydrocortisone (0.5 μg/mL; ICN Biochemicals), bovine serum albumin (2.0 μg/mL; Sigma), bovine pituitary extract (30 μg/mL; Invitrogen Ltd), and antibiotic-antimycotic (1.0%; Invitrogen Ltd). Culture medium was added to both the apical and the basolateral side of the inserts, and the cells were cultured at submerged condition in the medium. The culture medium was changed every 2 days. Confluence was reached within 5 days, and the cells were then cultured at ALI over 14 days after removing the apical medium and feeding from the basolateral side only. The culture medium was changed every 2 days, and the cells were maintained in an incubator under a humidified 5% CO_2 atmosphere at 37°C.

IL-13 Exposure

HBE cells were grown from day 0 with IL-13 at an ALI. The culture medium with IL-13 or phosphate-buffered saline (PBS) was changed every 48 h, and HBE cells were cultured for 14 days. To evaluate the effects of IL-13, after withdrawing IL-13 for 24 h on day 15, IL-13-transformed highly enriched goblet cells or ciliated-enriched cells were exposed to IL-13 or PBS for 24 h on day 16 (Fig 1B).

Real-Time-Polymerase Chain Reaction

To examine the messenger RNA (mRNA) expression of MUC5AC, MUC5B, or $sPLA_2$ group IIa, V, or X isotypes, RNA was extracted from the cells 5, 10, or 14 days after culturing with IL-13 or PBS.[12,23-25] The apical side of the cells was washed three times with PBS, and total RNA from the cells was extracted using the iScript RT-qPCR sample preparation reagent (Bio-Rad). The total RNA was then used to synthesize the first-strand complementary DNA using the iScript cDNA synthesis kit (Bio-Rad). Real-time-polymerase chain reaction (PCR) was performed on the C 1000 thermal cycler equipped with the CFX96 real-time PCR system (Bio-Rad). For the relative quantification of MUC5AC, MUC5B, or $sPLA_2$ group IIa, V, or X mRNA expression, the mRNA expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. EvaGreen was used as a DNA intercalator dye to monitor amplified DNA quantification, and real-time quantitative PCR curves were analyzed by CFX Manager software (Bio-Rad) to obtain threshold cycle values for each sample. The mRNA expression level was calculated based on a generated standard curve.

The following primers were used[6,12,23,26]:

- MUC5AC forward: 5′-TACTCCACAGACTGCACCAACTG-3′
- MUC5AC reverse: 5′-CGTGTATTGCTTCCCGTCAA-3′
- MUC5B forward: 5′-CACATCCACCCTTCCAAC-3;
- MUC5B reverse: 5′-GGCTCATTGTCGTCTCTG-3′

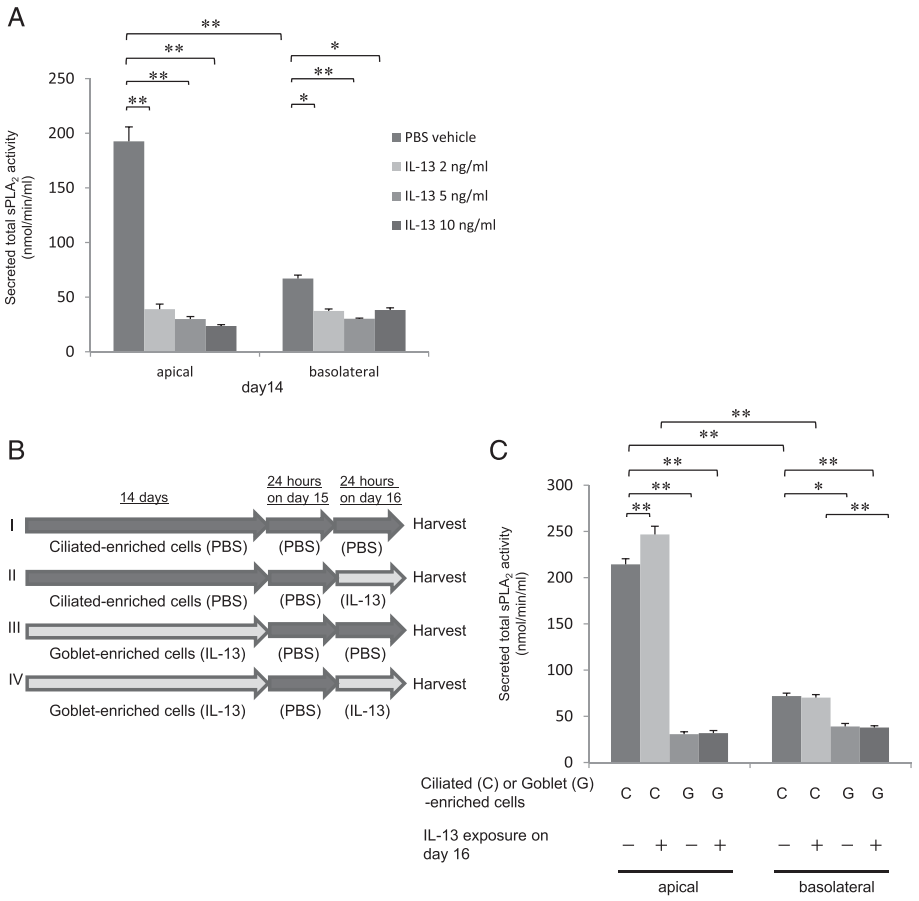

Figure 1 – *A, $sPLA_2$ are secreted primarily from ciliated cells. Secreted total $sPLA_2$ activity from apical supernatant and basolateral culture media in human bronchial epithelial (HBE) cells with basal exposure to IL-13 (0, 2, 5, or 10 ng/mL). B, HBE cells were cultured for 14 d with PBS to produce a ciliated-enriched phenotype (I, II) or with IL-13 to produce goblet-enriched cells (III, IV). After washing the cells and withdrawing IL-13 for 24 h on d 15 (I-IV), the cell cultures were then exposed to IL-13 at 0 (PBS; I, III) or 5 ng/mL (II, IV) for 24 h to evaluate the direct effects of IL-13 on d 16. We measured $sPLA_2$ activity in apical supernatant and basolateral culture media from these cells. C, IL-13 did not inhibit $sPLA_2$ secretion from either cell type. Secreted $sPLA_2$ activity was measured in apical supernatant and basolateral culture media from ciliated cell- or goblet cell-enriched culture after basal exposure to IL-13 at 0 or 5 ng/mL for 24 h. Data are presented as the mean ± SEM from four samples. Significant differences are indicated by **P < .001 and *P < .01. PBS = phosphate-buffered saline; $sPLA_2$ = secretory phospholipases A_2.*

- $sPLA_2$ subgroup IIa forward: 5′-ATGAAGACCCTCCTACTGTT-GGC-3′
- $sPLA_2$ subgroup IIa reverse: 5′-TCAGCAACGAGGGGTGCTCC-CTC-3′
- $sPLA_2$ subgroup V forward: 5′-ATGAAAGGCCTCCTCCCACT-GGC -3′
- $sPLA_2$ subgroup V reverse: 5′-GGCCTAGGAGCAGAGGATGT-TGG -3′
- $sPLA_2$ subgroup X forward: 5′-ATGGGGCCGCTACCTGTGT-GCC-3′
- $sPLA_2$ subgroup X reverse: 5′-TCAGTCACACTTGGGCGAG-TCC -3′
- GAPDH forward: 5′-TGAACGGGAAGCTCACTGG-3′
- GAPDH reverse: 5′-TCCACCACCCTGTTGCTGTA-3′

Histochemical Analysis

The cells on porous filters were fixed in 10% formalin neutral buffer, embedded in paraffin, and cut into 8-μm slices. To examine morphology, hematoxylin and eosin staining and periodic acid-Schiff (PAS) staining were performed as described previously.[12,23,25] Cell morphology was assessed with a light microscope (CKX41; Olympus) and photographed using a digital camera system (AxioCam ICc 1; ZEISS).

Immunohistochemical Analysis

MUC5AC, MUC5B, or $sPLA_2$ V expression was evaluated by immunohistochemistry. The sections were stained with anti-MUC5AC antibody, anti-MUC5B antibody, or anti-$sPLA_2$ group V antibody as the first antibody. EnVisionTM + Dual Link System-HRP (Dako) was added as the second antibody. Antigen-antibody complexes were visualized using the Liquid DAB + Substrate Chromogen System (Dako).[12,23,25]

Immunofluorescent Confocal Microscopy Analysis

Acetylated α-tubulin, MUC5AC, and $sPLA_2$ group V were identified in HBE cells using immunofluorescence techniques and confocal microscopy.[27,28] For the apical surface view, cultured HBE cell membranes were cut from the insert using a scalpel and were fixed in 3% formaldehyde. For the z-stack view, the cell membrane was embedded in Tissue-Tek OCT Compound (Sakura FineTechnical Co Ltd), frozen in liquid nitrogen, and stored at –80°C. The frozen samples were cut into 8-μm slices using a cryostat at –20°C, mounted on slides, and warmed to room temperature before fixation in 3% formaldehyde. The secondary antibodies were goat anti-mouse Alexa Fluor 488 (Invitrogen Ltd) for MUC5AC or $sPLA_2$ group V and goat anti-rabbit Alexa Fluor 568 (Invitrogen Ltd) for acetylated α-tubulin. After incubation with the primary and secondary antibodies, the slides were rinsed with PBS three times, and nuclei were stained with DAPI (Invitrogen Ltd) for 1 min in the dark. The slides were again rinsed three times with PBS and were immersed in VECTASHIELD Mounting Medium for fluorescence (Vector Laboratories). Coverslips were applied, and the slides were stored at 4°C in the dark. Within 24 h of staining, the slides were examined by confocal laser scanning microscopy (LSM 700; ZEISS). Immunostaining was visualized with a blue diode, argon ion, and helium neon laser using 405-, 488-, and 568-nm laser lines, respectively.

Enzyme-Linked Immunosorbent Assay for MUC5AC and MUC5B Mucins in Cell Supernatants

Apically secreted MUC5AC or MUC5B protein was measured using an enzyme-linked immunosorbent assay.[12,23,29] The cells were grown at ALI with either PBS or IL-13 for 5, 10, or 14 days. Culture supernatants were collected by adding Hanks' Balanced Salt Solution (Lonza Group Ltd) to the apical side of the inserts. The 96-well plates (Becton, Dickinson and

Company) were coated with cell supernatant samples (50 μL) and bicarbonate-carbonate buffer (50 μL) and were incubated at 37°C overnight until samples dried. After washing, 2% bovine serum albumin/PBS containing 0.05% Tween20 was added to each well and incubated at room temperature for 1 h as blocking. After washing with Tween-PBS, anti-MUC5AC antibody or anti-MUC5B antibody was added and the plate was incubated for 2 h at room temperature. After washing, anti-mouse immunoglobulin, horseradish peroxidase-linked whole antibody (GE Healthcare) for MUC5AC or anti-rabbit immunoglobulin HRP-linked antibody (Cell Signaling Technology) for MUC5B was added, and the plate was incubated for 1 h at room temperature. After washing, 3,3′,5,5′-tetramethylbenzine peroxidase solution was added, and the plate was incubated at room temperature. The reaction was stopped with 2 N H_3PO_4 and the absorbance was measured at 450 nm with the ELx808 Ultra Microplate Reader (Bio Tek Instruments, Inc). Data were expressed as the percentage above the PBS control.

Assay for sPLA$_2$, Leukotriene B$_4$, Leukotriene C$_4$, and Prostaglandin E$_2$

Differentiated cells were incubated with PBS or IL-13 for 5, 10, or 14 days at ALI. The culture supernatant from each well was collected by adding Hanks' Balanced Salt Solution to the apical side of the inserts, and the culture media were collected from the basolateral side of the inserts.

The activity of $sPLA_2$ and the concentrations of leukotriene B_4 (LTB_4), leukotriene C_4 (LTC_4), and prostaglandin E_2 (PGE_2) in the apical culture supernatant and the basal culture media were measured with an $sPLA_2$ assay kit and an LTB_4, LTC_4, or PGE_2 EIA kit (Cayman Chemical Company) using high performance liquid chromatography-grade water according to the manufacturer's instructions. The absorbance was measured with the ELx808 Ultra Microplate Reader (Bio Tek Instruments, Inc). The detection range of the $sPLA_2$ assay is approximately 20 to 200 nmol/min/mL. LTC_4, LTB_4, and PGE_2 EIA have detection limits of approximately 10, 13, and 15 pg/mL, respectively.

Evaluation of Secretory Pathways

Inhibitors were used to evaluate potential mechanisms for $sPLA_2$-induced MUC5AC and cysteinyl leukotriene (cysLT) secretion. Quercetin (a nonspecific LO inhibitor), indomethacin (a cyclooxygenase inhibitor), and MK-886 (a specific 5-LO [5-LO] inhibitor) at 0, 0.1, 1, or 10 μM were used. Cells were cultured for 14 days with 5 ng/mL of IL-13 at ALI, and on day 14 were first exposed to an inhibitor or vehicle for 30 min and then for 60 min or 24 h, adding recombinant $sPLA_2$ group V, 0.5 μg/mL, to the apical side for MUC5AC or cysLT measurement.

Statistical Analysis

Data are expressed as mean ± SEM. Comparisons were made by one-way analysis of variance, except for time-course experiments (Figs 2, 3, 4, 5B), which were analyzed by two-way repeated-measures analysis of variance. Conventionally, adjusted $P < .05$ was considered significant.

Results

IL-13-Induced Goblet Cell Differentiation

We have reported previously that IL-13-exposed HBE cells transformed to a highly enriched goblet cell epithelial culture with MUC5AC protein covering the apical surface but that cilia were not seen and that IL-13 increased the number of total cells, goblet cells, PAS-positive cells, and MUC5AC-positive cells.[12,29] IL-13 increased the total number of cells (hyperplasia) primarily by

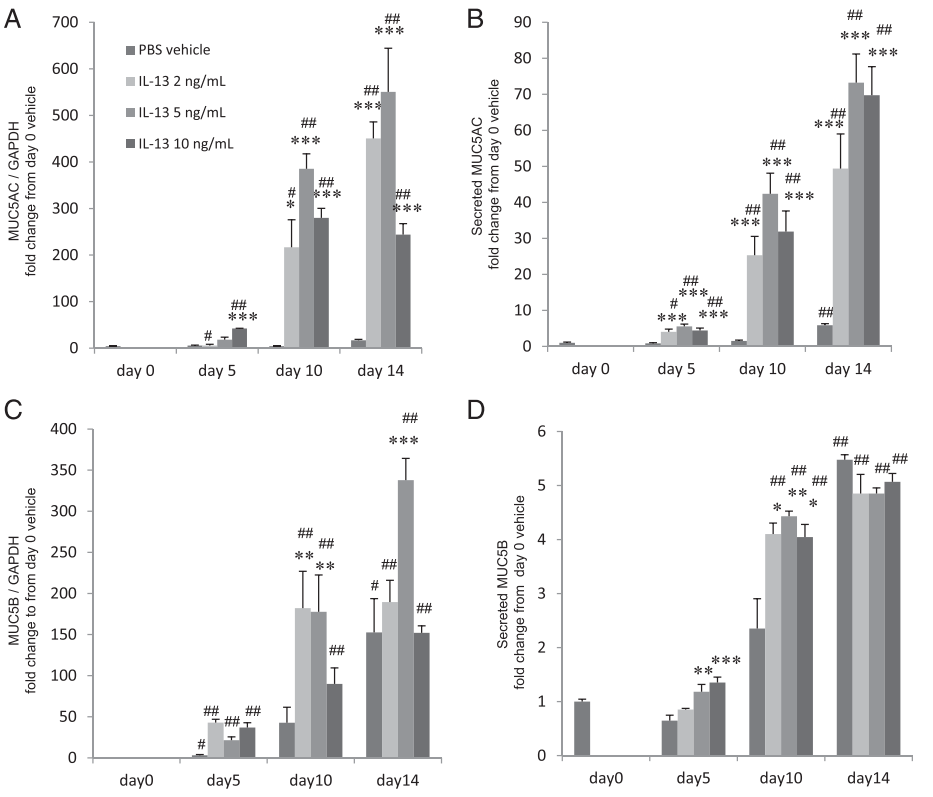

Figure 2 – *Mucins are secreted from IL-13-transformed goblet cells. MUC5AC or MUC5B messenger RNA (mRNA) expression in cell lysates and MUC5AC or MUC5B protein in supernatants of HBE cells grown with basal exposure to IL-13 (0, 2, 5, or 10 ng/mL) for 0, 5, 10, or 14 d. A, MUC5AC mRNA expression. B, MUC5AC protein. C, MUC5B mRNA expression. D, MUC5B protein. Data are presented as the mean ± SEM from four samples. Significant differences from PBS at each date are indicated by *P < .05, **P < .02, and ***P < .01, and from d 0 are indicated by #P < .05 and ##P < .01. GAPDH = glyceraldehyde-3-phosphate dehydrogenase. See Figure 1 legend for expansion of other abbreviations.*

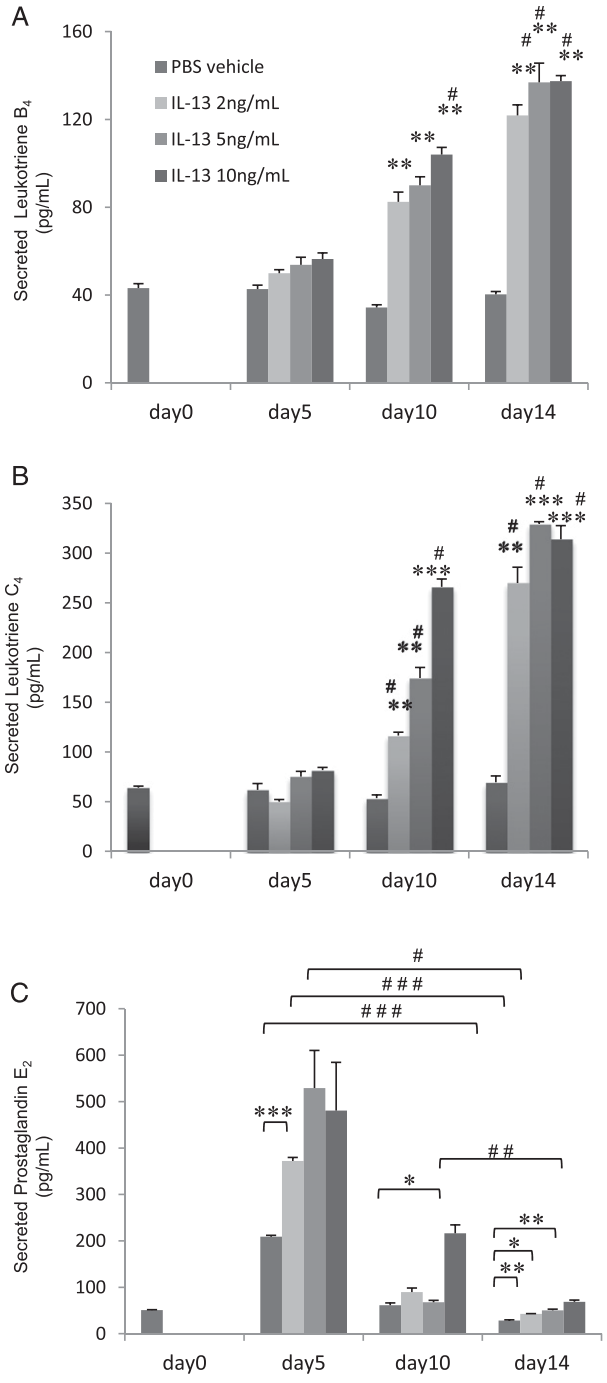

Figure 3 – *Leukotriene B_4 (LTB_4), leukotriene C_4 (LTC_4), and prostaglandin E_2 are secreted primarily from goblet cell-enriched culture. TB_4, LTC_4, or prostaglandin E_2 in supernatants of HBE cells grown for 5, 10, or 14 days with basal exposure to IL-13 (0, 2, 5, or 10 ng/mL). Data are presented as the mean ± SEM from four samples. A, LTB_4. B, LTC_4. Significant differences from control subjects (no IL-13) at each date are indicated by **P < .01, ***P < .001, and from d 0 are indicated by #P < .01. C, Prostaglandin E_2. Significant differences are indicated by #P < .05, ##P < .01, and ###P < .001, and from control subjects at each date are indicated by *P < .05, **P < .01, and ***P < .001. See Figure 1 legend for expansion of abbreviations.*

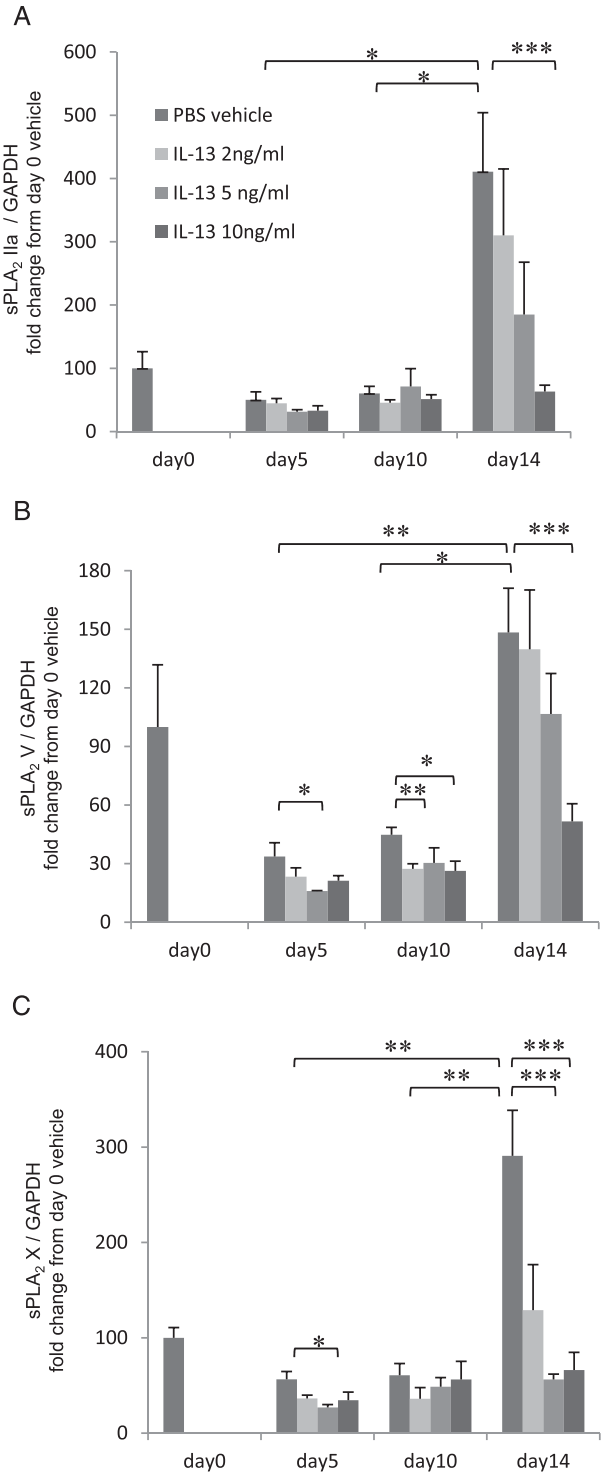

Figure 4 – *$sPLA_2$ are expressed primarily from ciliated cells. $sPLA_2$ group IIa, V, and X mRNA expression in cell lysates from HBE cells with basal exposure to IL-13 (0, 2, 5, or 10 ng/mL). The results are the mean ± SEM from six samples. A, $sPLA_2$ IIa. B, $sPLA_2$ V. C, $sPLA_2$ X. Significant differences are indicated by *P < .05, **P < .01, and ***P < .001. See Figure 1 and 2 legends for expansion of abbreviations.*

increasing the number of goblet cells (from 0.2 to 15.9 cells), PAS-positive cells (from 2.5 to 28.2 cells), and MUC5AC-positive cells (from 0.1 to 25.7 cells/250 μm on slide).[30]

HBE cells cultured at ALI without IL-13 demonstrated a well-differentiated morphology with ciliated cells at the surface of epithelial layers, referred to as ciliated-enriched

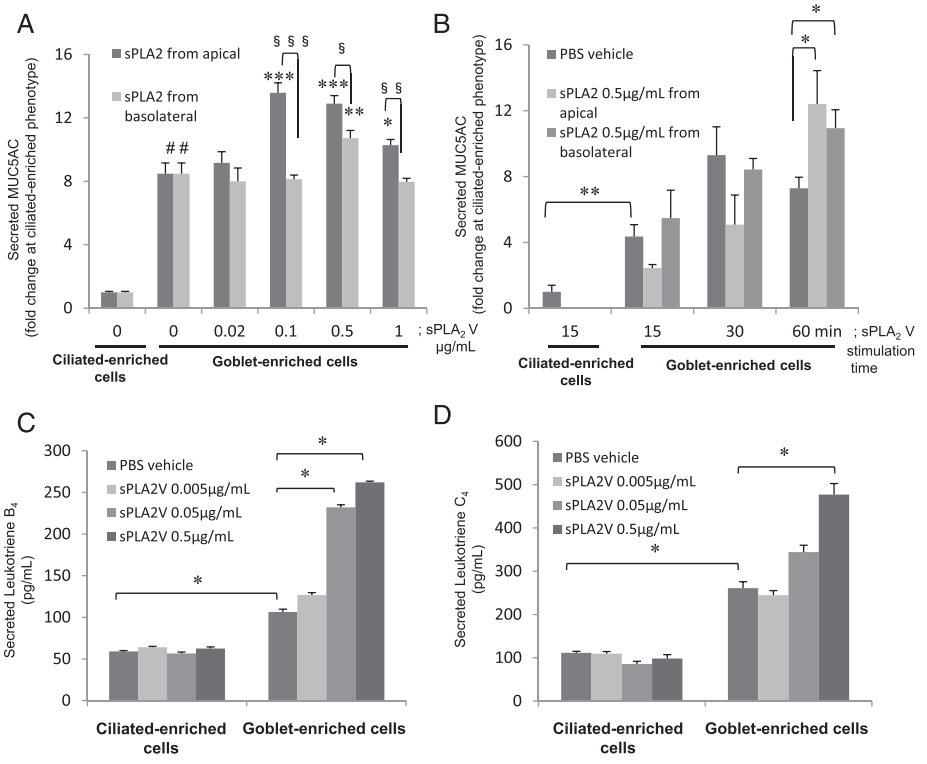

Figure 5 – *sPLA$_2$ V stimulates MUC5AC, LTB$_4$, and LTC$_4$ secretion from goblet cells, but not ciliated cells. A and B, Apical and basolateral sPLA$_2$ V increased MUC5AC secretion from goblet-enriched cell culture. MUC5AC protein was measured in supernatants from ciliated or goblet cells. A, Apical or basal 60-min exposure to sPLA$_2$ V at 0, 0.02, 0.1, 0.5, or 1 μg/mL. Data are presented as mean ± SEM from five samples. #P < .001 for ciliated cells, *P < .05, **P < .02, ***P < .01 for goblet cells compared with PBS, §P < .02, §§P < .01, §§§P < .001. B, Apical or basal 15-, 30-, or 60-min exposure to sPLA$_2$ V (0 or 0.5 μg/mL). Data are presented as mean ± SEM from five samples. *P < .05, **P < .01. C and D, Apical sPLA$_2$ V stimulates LTB$_4$ and LTC$_4$ secretion from goblet and ciliated-enriched cells. Secreted LTB$_4$ or LTC$_4$ in supernatants of ciliated-enriched and goblet-enriched cells stimulated for 24 h with apical sPLA$_2$ (0, 0.005, 0.05, or 0.5 μg/mL). C, LTB$_4$. D, LTC$_4$. Data are presented as mean ± SEM from four samples. *P < .01. See Figure 1 and 3 legends for expansion of abbreviations.*

cells for convenience.[30] These cells weakly stain with MUC5B, PAS, and MUC5AC, and strongly stain with acetylated α-tubulin. In the presence of 5 ng/mL IL-13, HBE cells differentiated into a predominantly goblet cell phenotype with secretory granules (similarly referred to as goblet-enriched cells) strongly stain with PAS, MUC5AC, and MUC5B, but not with acetylated α-tubulin. The apical surface of ciliated cells is shown in red immunofluorescence using acetylated α-tubulin and goblet cells in green using MUC5AC. Images show predominantly goblet cells with few or no ciliated cells after growth of 5 ng/mL IL-13. The ciliated cells in culture do not appear to stain for PAS or MUC5B antibodies. Cells grown in the absence of IL-13 supplementation transform predominantly into a ciliated phenotype, with the occasional goblet cell appearing in the epithelial culture. The ciliated cells themselves stain with antibodies to tubulin, but not with PAS or MUC5B. Images taken at the cell surface, where the antibody was placed, show a small amount of nonspecific staining (Fig 6).

Mucin mRNA Expression and Protein Secretion From Goblet Cells

As we reported previously,[12,23,29,30] mucin (MUC5AC, MUC5B) mRNA expression and protein secretion increased with IL-13 concentration (0, 2, 5, or 10 ng/mL) after 14 days in culture at ALI (Fig 2).

LTB$_4$, LTC$_4$, and PGE$_2$ Secretion From Goblet Cells

LTB$_4$ and LTC$_4$ secretion in apical supernatants was increased with IL-13 (2, 5, or 10 ng/mL) after 14 days in culture compared with ciliated enriched cells grown without IL-13 (Figs 3A, 3B) (LTB$_4$, 137 ± 8.7 pg/mL, and LTC$_4$, 329 ± 2.4 pg/mL, with 5 ng/mL of IL-13; $P < .01$). PGE$_2$ secretion in apical supernatants was decreased with exposure to IL-13 (2, 5, or 10 ng/mL) at 14 days compared with day 5 at each concentration of IL-13 (Fig 3C) ($P < .01$).

sPLA$_2$ mRNA Expression and Secretion in Ciliated Cells

After 14 days of culture at ALI, sPLA$_2$ group IIa (Fig 4A), V (Fig 4B), or X (Fig 4C) mRNA expression was greater in ciliated-enriched cells than in goblet-enriched cells ($P < .001$), and sPLA$_2$ secretion was greater from ciliated-enriched cells than from goblet-enriched cells (Fig 1A) ($P < .001$). The increased sPLA$_2$ secretion from ciliated-enriched cells was greater in apical supernatant than in basolateral culture medium (Fig 1A) ($P < .001$). IL-13 (5 ng/mL) exposure for 24 h at the end of 14 days (after withdrawing IL-13 for 24 h) did not inhibit sPLA$_2$ secretion from ciliated-enriched cells or goblet-enriched cells, but rather, modestly increased sPLA$_2$ secretion in apical supernatant from ciliated-enriched cells ($P < .001$).

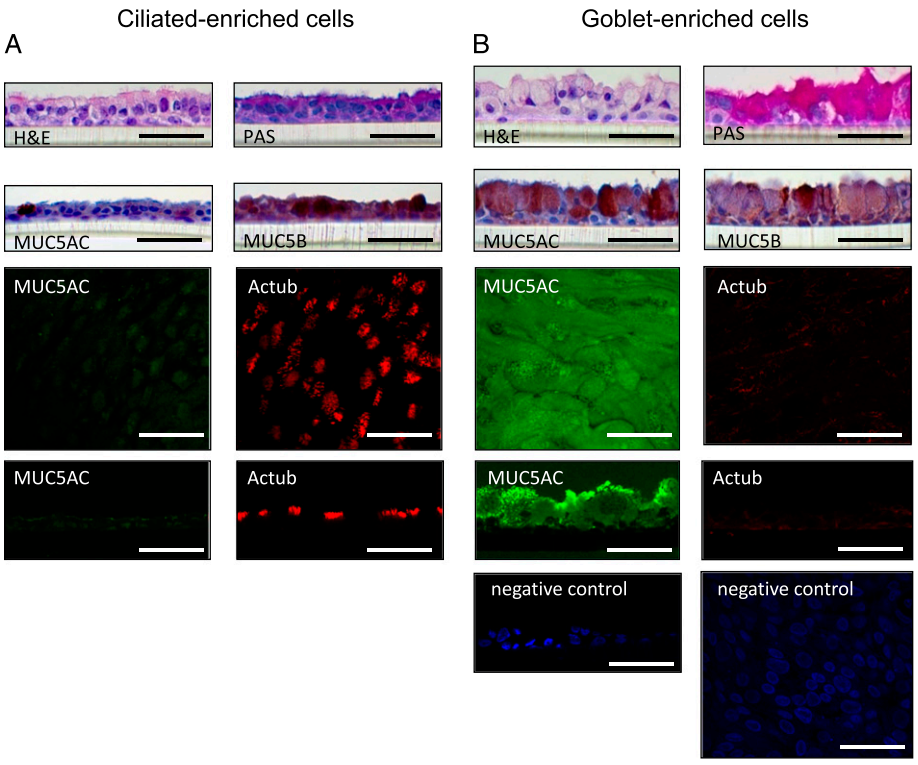

Figure 6 – *A, B, HBE cells were differentiated to ciliated cell-enriched or to goblet cell-enriched phenotypes. H&E staining, PAS staining, MUC5AC immunohistochemical staining, MUC5B immunohistochemical staining, and immunofluorescence with laser confocal microscopy of HBE cells grown for 14 d at an air-liquid interface with 0 or 5 ng/mL IL-13. Representative image of apical surface and z-stack immunofluorescence shows MUC5AC (Alexa Fluor 488, green), acetylated Actub (Alexa Fluor 568, red), and nuclei (DAPI, blue). Bar = 50 μm. Actub = α-tubulin; H&E = hematoxylin and eosin; PAS = periodic acid-Schiff. See Figure 1 legend for expansion of other abbreviations.*

$sPLA_2$ Stimulate MUC5AC, LTB_4, and LTC_4 Secretion in Goblet-Enriched Cells

When goblet-enriched cells were apically exposed to $sPLA_2$ group V or X (0.5 μg/mL), this increased MUC5AC secretion compared with the $sPLA_2$ vehicle (PBS) (Fig 7A) ($P < .05$). LTB_4 (Fig 7B) and LTC_4 (Fig 7C) secretion was increased in goblet-enriched cells compared with ciliated-enriched cells ($P < .01$). Apical exposure to $sPLA_2$ group IIa (0.05 or 0.5 μg/mL), V, or X (0.5 μg/mL) increased LTB_4 in goblet-enriched cells (Fig 7B) ($P < .05$), and apical exposure to 0.5 μg/mL $sPLA_2$ group V increased LTC_4 release compared with PBS (Fig 7C) ($P < .05$). Group V produced a greater increase than did group II or X (Fig 7C) ($P < .05$). $sPLA_2$ group IIa, V, and X, at 0.05 or 0.5 μg/mL had no effect on MUC5AC, LTB_4, or LTC_4 secretion from ciliated-enriched cells (data not shown).

$sPLA_2$ Group V Is Expressed in Ciliated-Enriched Cells and Causes Goblet-Enriched Cells to Secrete MUC5AC, LTB_4, and LTC_4

HBE cells cultured to a ciliated cell morphology (Figs 8A, 8E, 8I) strongly stain with $sPLA_2$ V antibody (Figs 8A, 8C, 8I). However, with differentiation into goblet cells, there was some $sPLA_2$ V antibody staining in the cytoplasm but not in the mucous granules (Figs 8B, 8D, 8J). In these goblet-enriched cells, a 60-min apical exposure to $sPLA_2$ V (0.1, 0.5, or 1 μg/mL) or 0.5 μg/mL exposure at the basolateral side increased MUC5AC secretion (Fig 5A) ($P < .01$ except for $P < .02$ at 1 μg/mL of apical $sPLA_2$ V and $P < .05$ at 0.5 μg/mL of basolateral $sPLA_2$ V). Apical exposure to $sPLA_2$ V increased LTB_4 and LTC_4 secretion in goblet-enriched cells but not in ciliated-enriched cells (Figs 5C, 5D) ($P < .01$ for LTB_4 at 0.05 or 0.5 μg/mL of $sPLA_2$ V and LTC_4 at 0.5 μg/mL of $sPLA_2$ V).

Inhibiting Either Cyclooxygenase or LO Decreases $sPLA_2$ V-Stimulated MUC5AC Secretion

Quercetin 10 μM (an LO inhibitor), indomethacin 1 and 10 μM (a cyclooxygenase inhibitor), and MK-886 10 μM (a specific 5-LO inhibitor) all decreased $sPLA_2$ V-induced MUC5AC secretion from goblet-enriched cells (Fig 9A) ($P < .02$ at 10 μM of quercetin, $P < .05$ at 1 μM, $P < .01$ at 10 μM of indomethacin, $P < .02$ at 10 μM of MK-886). Although quercetin (0.1, 1, or 10 μM) dose dependently decreased $sPLA_2$ V-induced LTB_4 and LTC_4 secretion, indomethacin did not affect LTB_4 or LTC_4 secretion (Figs 9B, 9C).

Discussion

$sPLA_2$ are increased in the plasma and BALF of subjects with asthma,[4] and these enzymes increase cysLTs in the asthmatic airway. LTC_4, leukotriene D_4, and

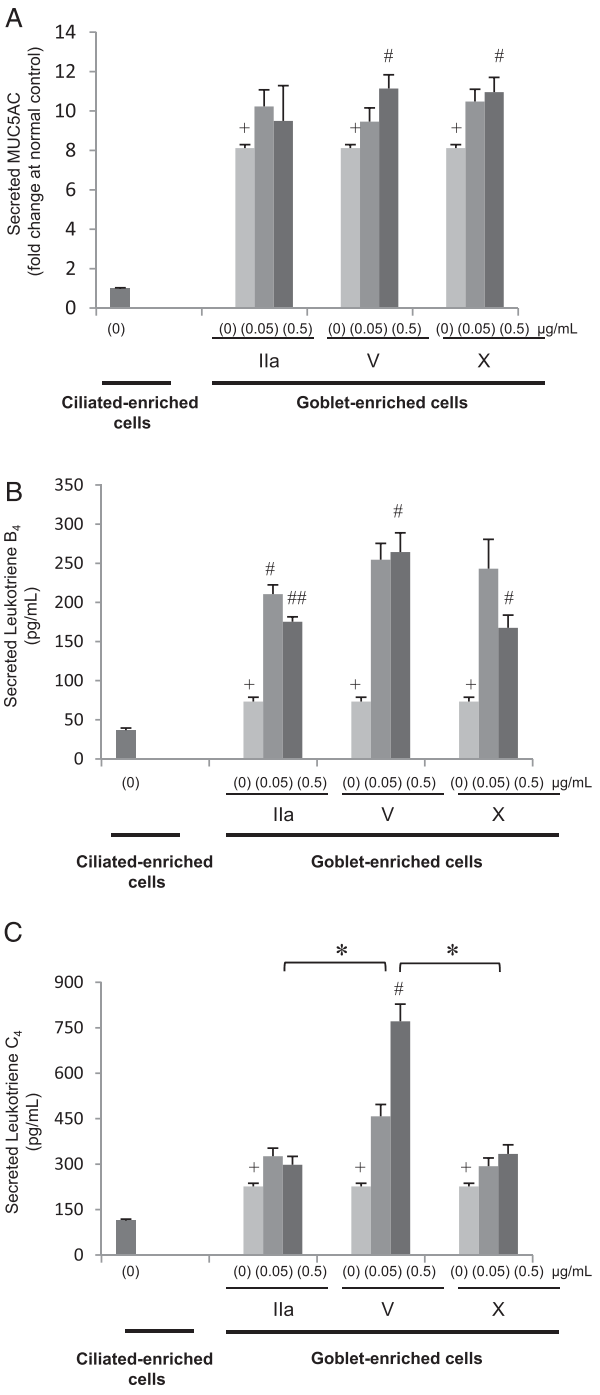

Figure 7 – *sPLA$_2$ stimulates MUC5AC, LTB$_4$, and LTC$_4$ secretion from goblet cells. Apical sPLA$_2$ subgroups IIa, V, or X with 0 (PBS), 0.05, or 0.5 μg/mL stimulate MUC5AC, LTB$_4$, and LTC$_4$ secretion from goblet cell-enriched culture. Secreted MUC5AC, LTB$_4$, and LTC$_4$ in apical supernatants from goblet cells or ciliated cells that were apically exposed for 60 min or 24 h with sPLA$_2$ IIa, V, X, or PBS. A, MUC5AC; 60 min. B, LTB$_4$; 24 h. C, LTC$_4$; 24 h. Data are presented as the mean ± SEM from four samples. +P < .01 to ciliated cells with PBS; #P < .05, ##P < .01 to goblet cells with PBS; *P < .05. See Figure 1 and 3 legends for expansion of abbreviations.*

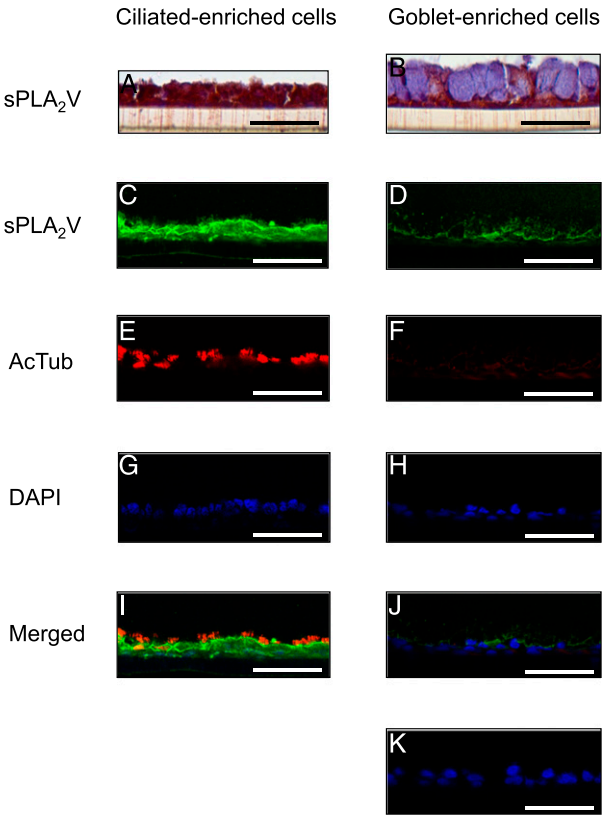

Figure 8 – *sPLA$_2$ V is expressed in ciliated cells. sPLA$_2$ V immunostaining and immunofluorescence with laser confocal microscopy of ciliated or goblet cell-enriched culture containing mucous granules grown for 14 d with PBS or with 5 ng/mL IL-13. A and B, Immunostaining. C-K, Immunofluorescence. A, C, E, G, I, PBS. B, D, F, H, J, K, IL-13. Immunofluorescence shows sPLA$_2$ V (Alexa Fluor 488, green; C, D), acetylated α-tubulin (AcTub, Alexa Fluor 568, red; E, F), nuclei (DAPI, blue; G, H), and merged image of the double staining and DAPI (I, J). K, Negative control is HBE cells without the primary antibodies. Bar = 50 μm. See Figure 1 and 6 legends for expansion of abbreviations.*

leukotriene E$_4$ cause bronchoconstriction, edema, mucus hypersecretion, and eosinophil chemotaxis, and LTB$_4$ is a potent neutrophil chemoattractant.[31] LTB$_4$ and LTC$_4$ are produced in bronchial epithelial cell culture,[32] and other epithelial cell types release LTB$_4$ and LTC$_4$ after inflammatory injury.[33,34] An epithelial cell source of cysLT may contribute to neutrophil migration in the airway.[35]

We have shown previously that sPLA$_2$ dramatically increases mucus secretion from the ferret trachea and we called this "secretory hyperresponsiveness."[11] Mucus accumulation and goblet cell hyperplasia in the airway are closely correlated with morbidity and mortality.[36,37] The results of the current study suggest that sPLA$_2$ from ciliated cells, perhaps acting as airway sensory receptors, can increase mucus secretion and production of inflammatory mediators from IL-13-transformed goblet cells. A 24-h exposure to IL-13 only modestly increased sPLA$_2$ secretion from the ciliated-enriched cell culture and had no further effect on goblet cell culture.

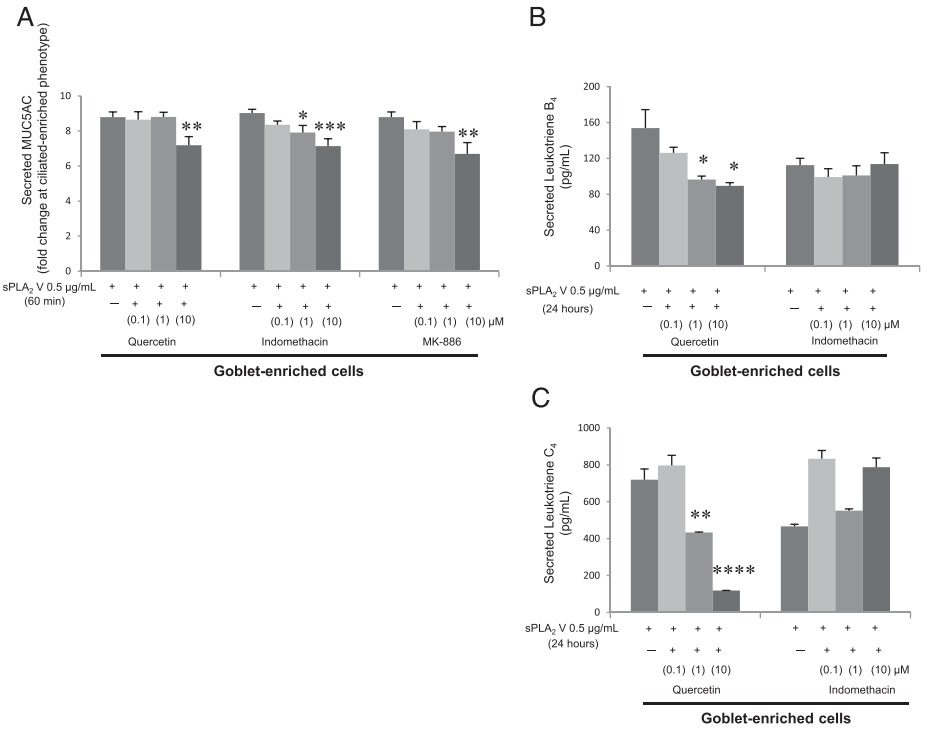

Figure 9 – *Inhibiting either cyclooxygenase or lipoxygenase decreases $sPLA_2$ V-stimulated MUC5AC secretion from goblet cells. Secreted MUC5AC, LTB_4, and LTC_4 in apical supernatants of goblet cells apically stimulated for 60 min or 24 h to $sPLA_2$ V with quercetin, indomethacin, or MK-886 (0.1, 1, or 10 μM). A, MUC5AC; 60 min. B, LTB_4; 24 h. C, LTC_4; 24 h. Data are presented as mean ± SEM from four samples.* *P < .05, ** P < .02, *** P < .01, or **** P < .001 to quercetin, indomethacin, or MK-886 vehicle (dimethyl sulfoxide). See Figure 1 and 3 legends for expansion of abbreviations.*

$sPLA_2$ can act on cellular membranes to release not only arachidonic acid, but also a variety of saturated, monosaturated, and polyunsaturated fatty acids and, in so doing, alter the function of the cell membrane. The physiologic functions of $sPLA_2$ may include a combination of these actions.[38]

In antigen-challenged mice, $sPLA_2$ V is increased in the bronchial epithelial cells, and the intratracheal application of anti-group V $sPLA_2$ antibody can ameliorate airway inflammation.[9] $sPLA_2$ V and X in bronchial cells potentiate arachidonate release and prostaglandin synthesis.[6] Group V and X $sPLA_2$ are widely expressed in the airway epithelium of human lungs.[6] $sPLA_2$ have the ability to liberate arachidonic acid from cell membranes, leading to the production of eicosanoids, with groups V and X reported to be the most potent.[39-43] Although it remains uncertain how $sPLA_2$ in the medium gains access to the inner leaflet of the plasma membrane, the ability of $sPLA_2$ V and X to release cellular arachidonic acid correlates with their high capacity to bind phosphatidylcholine, a major phospholipid in the outer leaflet of the plasma membrane.[41-43]

We showed that in HBE cells, $sPLA_2$ group V stimulated LTC_4 secretion significantly more than either group IIa or X. Immunohistochemical staining was positive for $sPLA_2$ V in ciliated-enriched cells, but spared mucous granules. These results suggest that in the airway, group V $sPLA_2$ production and secretion is predominantly from ciliated cells. Group V $sPLA_2$ may contribute to mucus secretion and to cysLT generation in a paracrine fashion, acting on goblet cells and suggesting a role in the pathogenesis of asthma.

Arachidonic acid is a substrate for LO-generating eicosanoids, including leukotrienes.[44] Quercetin, a LO inhibitor, decreased $sPLA_2$-induced mucus secretion. Although quercetin has other effects as well, including antihistamine and antiinflammatory effects, we also show that MK-886, a specific 5-LO inhibitor, decreased $sPLA_2$-induced mucus secretion, confirming earlier data that $sPLA_2$ probably stimulates mucus secretion by activating the LO pathway. MK-886 binds a membrane-bound 5-LO-activating protein, thereby inhibiting the translocation and activation of 5-LO, LTC_4,[45] and leukotriene D_4.[46,47] These suggest that 5-LO products play a role in $sPLA_2$-induced mucus secretion.

Prostaglandins also stimulate mucus secretion in the airways,[48] stomach,[49] colon,[50] and uterus.[51] We show that indomethacin, a cyclooxygenase inhibitor, decreased $sPLA_2$-induced mucus secretion, suggesting that cyclooxygenase pathway products may play a role in $sPLA_2$-induced mucus secretion.

PGE_2 is an airway smooth muscle relaxant and may have bronchoprotective and antiinflammatory properties.[52-54]

Although it is reported that IL-13 inhibits PGE_2 synthetic pathways and decreases PGE_2 secretion in HBE cells,[55] a clinical study showed that cysLT and PGE_2 concentrations in sputum are higher in those with asthma than in control subjects.[56] PGE_2 also promotes cell growth in bronchial epithelial cells.[57] We showed that, consistent with this, PGE_2 secretion was increased during the cell growth and differentiation phase of HBE cells. We also showed that IL-13 stimulates PGE_2 production and secretion from goblet-enriched cells. Thus, it is possible that PGE_2 or cysLT is released from goblet cells and acts in an autocrine manner to enhance $sPLA_2$-stimulated mucin secretion.

It would have been interesting to evaluate the role of $sPLA_2$ in primary cells from patients with asthma. Although the HBE cells used in this study were from subjects without lung disease, it has been reported that bronchial epithelial cell cultures from subjects with asthma contain many more goblet cells and fewer ciliated cells compared with those from healthy subjects.[28]

Conclusions

The findings in the current study suggest that $sPLA_2$ are released from ciliated airway epithelial cells and act on goblet cells in a paracrine manner to stimulate LTB_4, LTC_4, and MUC5AC secretion. We reported previously that IL-33 acts directly on goblet-enriched cells, but not ciliated-enriched cells, to stimulate IL-8 production, suggesting that beyond secreting mucus, goblet cells are inflammatory effector cells.[30] These data provide additional further support that goblet cells can act as inflammatory effector cells in the airway, and this may contribute to the severity of asthma and other airway diseases.

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