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Regulation and Function of Epithelial Secreted Phospholipase A_2 Group X in Asthma

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***Rationale*: Indirect airway hyperresponsiveness (AHR) is a fundamental feature of asthma that is manifest as exercise-induced bronchoconstriction (EIB). Secreted phospholipase A_2 group X ($sPLA_2$-X) plays a key role in regulating eicosanoid formation and the development of inflammation and AHR in murine models.**

***Objectives*: We sought to examine $sPLA_2$-X in the airway epithelium and airway wall of patients with asthma, the relationship to AHR in humans, and the regulation and function of $sPLA_2$-X within the epithelium.**

***Methods*: We precisely phenotyped 34 patients with asthma (19 with and 15 without EIB) and 10 normal control subjects to examine *in vivo* differences in epithelial gene expression, quantitative morphometry of endobronchial biopsies, and levels of secreted protein. The regulation of $sPLA_2$-X gene (*PLA2G10*) expression was examined in primary airway epithelial cell cultures. The function of epithelial $sPLA_2$-X in eicosanoid formation was examined using PLA_2 inhibitors and murine tracheal epithelial cells with *Pla2g10* deletion.**

***Measurements and Main Results*: We found that $sPLA_2$-X protein is increased in the airways of patients with asthma and that epithelial-derived $sPLA_2$-X may be increased in association with indirect AHR. The expression of $sPLA_2$-X increases during *in vitro* epithelial differentiation; is regulated by inflammatory signals including tumor necrosis factor, IL-13, and IL-17; and is both secreted from the epithelium and directly participates in the release of arachidonic acid by epithelial cells.**

***Conclusions*: These data reveal a relationship between epithelial-derived $sPLA_2$-X and indirect AHR in asthma and that $sPLA_2$-X serves as an epithelial regulator of inflammatory eicosanoid formation. Therapies targeting epithelial $sPLA_2$-X may be useful in asthma.**

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Secreted phospholipase A_2 group X ($sPLA_2$-X) is a key regulator of eicosanoid formation in mammalian cells and in murine models of asthma. Prior studies have found that $sPLA_2$-X is the dominant $sPLA_2$ in the airways of patients with asthma and that it is avidly expressed in the airway epithelium. Little is known about the relationship between the epithelial expression of this enzyme and manifestation of airway hyperresponsiveness or the regulation and function of the enzyme in airway epithelial cells.

What This Study Adds to the Field

In precisely phenotyped subjects with and without asthma who underwent bronchoscopic evaluation, we found that $sPLA_2$-X protein is released in increased quantities in asthma and that the protein in the epithelium may be increased in relationship to the severity of indirect airway hyperresponsiveness. Furthermore, epithelial $sPLA_2$-X increases during epithelial differentiation, is regulated by inflammatory cytokines, and serves as a regulator of eicosanoid formation.

Keywords: airway hyperresponsiveness; asthma; eicosanoid; epithelial cell; secretory phospholipase A_2

Indirect airway hyperresponsiveness (AHR) refers to the propensity to develop airway narrowing in response to stimuli such as exercise, osmotic challenge, or adenosine that cause airflow obstruction via inflammatory or neuronal cells that release mediators (1). Exercise-induced bronchoconstriction (EIB) is a prototypical feature of indirect AHR in asthma that occurs in about 30 to 50% of subjects with asthma in cross-sectional studies (2, 3). Prior studies have identified epithelial shedding into the airway lumen and increased production of inflammatory eicosanoids such as leukotrienes in the airways of patients with EIB (4–8). The basis for the dysregulation of eicosanoids in asthma is incompletely understood.

The rate-limiting step in eicosanoid biosynthesis is the release of unesterified arachidonic acid (AA) from the *sn*-2 position of membrane phospholipids by phospholipase A_2 (PLA_2) (9). Recent work has uncovered 10 mammalian secreted PLA_2s ($sPLA_2$s) that may coordinate eicosanoid synthesis with the well-described cytosolic PLA_2-α (i.e., $cPLA_2\alpha$) (10–12). $sPLA_2$s have several other inflammatory functions, including the generation of lysophospholipids and platelet-activating factor (13), and are released into the extracellular fluid by the classical

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secretory pathway (13). The $sPLA_2$ activity in the airways is elevated in asthma (14), and there is an increase in $sPLA_2$ activity after allergen challenge (15, 16). We have found that $sPLA_2$ group X ($sPLA_2$-X) gene (*PLA2G10*) has the highest expression in the airways of patients with asthma (17, 18) and is strongly expressed in the airway epithelium relative to the other $sPLA_2$ genes (18). The $sPLA_2$ with the strongest ability to initiate eicosanoid synthesis in mammalian cells is $sPLA_2$-X (19), and $sPLA_2$-X specifically initiates cysteinyl LT (CysLT) synthesis by eosinophils (20). An important *in vivo* role for $sPLA_2$-X has been demonstrated with protection from ovalbumin-induced airway inflammation, eicosanoid production, and AHR in *Pla2g10*-deficient ($Pla2g10^{-/-}$) mice (21). Insertion of the human $sPLA_2$-X ortholog in $Pla2g10^{-/-}$ mice restores the capacity for the development of airway inflammation and AHR that could be abolished by an active site-directed inhibitor of human $sPLA_2$-X (22). Thus, we hypothesized that patients with EIB would have increased levels of $sPLA_2$-X in the airway epithelium and secreted into the airway lumen and that $sPLA_2$-X plays an important role in eicosanoid regulation by airway epithelial cells.

Some of the results of this study have been previously reported in the form of an abstract (23).

METHODS

Study Subjects and Study Protocol

Three groups of subjects 18 to 59 years of age were recruited for the study. Written informed consent was obtained from all participants. Subjects with asthma used only an inhaled β_2-agonist for asthma treatment during the study. Spirometry, exercise, and methacholine challenges were conducted in accordance with American Thoracic Society standards (24, 25). Patients with asthma were characterized as EIB (+) with greater than or equal to 15% fall in FEV_1 after exercise challenge and EIB (−) with less than or equal to 7% fall in FEV_1 after exercise challenge. Subjects without asthma were enrolled who had normal spirometry and negative methacholine and exercise challenge tests. Study participants had two sputum inductions and a research bronchoscopy, each 2 to 10 days apart.

Immunohistochemistry and Design-based Stereology

Endobronchial biopsy specimens were embedded in isector molds for design-based stereology (26, 27). Using the newCAST Whole Slide Stereology system (Visiopharm, Hoersholm, Denmark), we quantified the volume of epithelial $sPLA_2$-X staining relative to the surface area of the basal lamina and the volume of the epithelium relative to the surface area of the basal lamina.

Quantitative Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction analysis was conducted using TaqMan primer probe sets with quantification relative to a standard curve of the gene copy number (28).

Time-resolved Fluorescence Immunoassay of $sPLA_2$-X

The level of $sPLA_2$-X protein was measured by time-resolved fluorescence immunoassay (18). A linear standard curve was derived from recombinant human $sPLA_2$-X (19).

Eicosanoid Analysis

CysLTs were assayed by ELISA (Cayman, Ann Arbor, MI), whereas the levels of other eicosanoids were determined by charge reversal derivatization followed by liquid chromatography–tandem mass spectrometry (29).

Human Epithelial Cell Culture

Primary bronchial epithelial cells isolated during bronchoscopy were cultured in bronchial epithelial growth media (Lonza, Allendale, NJ). Cells were cryopreserved and/or subcultured at 90 to 95% confluence. Cryopreserved primary epithelial cells at passage one or two were used for differentiated air–liquid interface organotypic cultures (30). In some experiments, cells were cultured in the presence of tumor necrosis factor (TNF), IL-1β, IL-4, IL-13, IL-17, IL-25, IL-33, and thymic stromal lymphopoietin (PeproTech, Rock Hill, NJ).

Murine-derived Tracheal Epithelial Cell Culture

Primary murine tracheal epithelial cells (mTECs) were established from tracheas isolated from $Pla2g10^{-/-}$ mice on a C57BL/6J background (21) and wild-type C57BL/6J control mice (31).

Regulation of Epithelial AA Release and Eicosanoid Formation

Epithelial cells were treated with [^{3}H]arachidonate when the cells reached approximately 80% confluence. The percentage of [^{3}H]arachidonate release to the medium was calculated relative to the total counts in the medium and cell fractions (11).

Statistical Analysis

Differences in the characteristics of each of the groups were assessed with a Chi-square, analysis of variance, or Kruskal-Wallis test. Differences in the $sPLA_2$ levels between the groups were tested with a Kruskal-Wallis test with Dunn *post hoc* tests or the Mann-Whitney test. The association between levels of $sPLA_2$-X in the airway wall with the severity of EIB was assessed by linear regression. Differences across multiple conditions in the cell culture experiments were assessed with an analysis of variance with Bonferroni *post hoc* tests.

RESULTS

Subject Characteristics

The three groups of subjects were well matched with respect to age, sex, ethnicity, race, and baseline lung function as measured by FEV_1 and FVC (Table 1). There were significant differences between the asthma and control groups with respect to baseline airflow obstruction reflected in the FEV_1/FVC ratio and direct AHR measured by methacholine challenge. There were also differences between the two asthma groups regarding the FEV_1/FVC ratio ($P = 0.03$) and provocative concentration of methacholine causing a 20% drop in lung function ($P = 0.03$). Symptoms of dyspnea measured by the Borg scale increased over time during the exercise challenge in all three groups, but the rate of increase in symptoms during exercise was highest in the EIB (+) group (*see* Figure E1A in the online supplement). The severity of EIB was markedly greater in the EIB (+) group than in either of the other groups (Figure E1B).

Although there was no difference in the total number of positive skin tests between the two asthma groups, the percentages of subjects with positive skin prick tests to grass, *Dermatophagoides farinae*, cat dander, alder, and ragweed were higher in the two asthma groups compared with the control group (Table 1). Using a quantitative scale, only cat dander differed between EIB (+) and EIB (−) subjects with asthma ($P < 0.05$, Figure E2). Additional details of the study subject characteristics are in the online supplement.

PLA2G10 Is Strongly Expressed in the Airway Epithelium of Subjects with and without Asthma

In all subjects combined, the median *PLA2G10* expression was about 100-fold higher in airway epithelial brushings than in induced sputum cells (Figure 1A). In airway epithelial brushings from each of the three groups, there was no detectable difference in the expression of *PLA2G10* between subjects with and without asthma and between the two asthma groups (Figure 1B). Similarly,

TABLE 1. STUDY POPULATION

Characteristic	Control ($n = 10$)	Asthma		*P* Value
		EIB (−) ($n = 15$)	EIB (+) ($n = 19$)	
Age, yr	30.4 ± 12.7	24.4 ± 4.5	26.8 ± 8.6	0.25
Sex, male, %	20.0	26.7	31.6	0.80
Ethnicity, %				0.51
Non-Hispanic	100	100	94.7	
Hispanic	0	0	5.3	
Race, %				0.36
White	70.0	93.3	84.2	
Asian	30.0	6.7	10.5	
Multiracial	0	0	5.3	
Lung function				
FEV_1, % predicted	96.5 ± 11.3	91.6 ± 9.0	88.8 ± 10.9	0.18
FVC, % predicted	95.7 ± 13.5	96.7 ± 8.6	103.3 ± 9.6	0.10
FEV_1/FVC	0.87 ± 0.06	0.81 ± 0.09	0.73 ± 0.09	<0.001
Methacholine PC_{20}	>8 ± 0	1.7 ± 1.2	0.6 ± 1.5	<0.001
Exercise challenge				
Maximum fall in FEV_1, %	1.7 ± 2.1	2.6 ± 0.8	27.7 ± 9.5	<0.001
AUC FEV_1	−7.4 ± 58.1	−8.6 ± 71.7	624.7 ± 295.7	<0.001
Atopy				
Number of allergens	0.9 ± 1.5	5.3 ± 4.9	7.4 ± 3.7	0.001
Grass, %	11.1	50.0	76.9	0.01
Dermatophagoides farinae, %	11.1	75.0	92.3	<0.001
Cat dander, %	11.1	50.0	84.6	0.003
Alder, %	0.0	25.0	61.5	0.009
Ragweed, %	0.0	25.0	53.8	0.02

Values represent mean ± standard deviation unless otherwise specified.

Definition of abbreviations: AUC = area under the curve; EIB = exercise-induced bronchoconstriction; PC_{20} = provocative concentration causing a 20% drop in lung function.

there was no clear difference in the *PLA2G10* expression in induced sputum cells between the groups of subjects (Figure 1C).

The $sPLA_2$-X Protein Secreted into the Airway Lumen Is Increased in Asthma

The concentration of $sPLA_2$-X in induced sputum supernatant was increased in both asthma groups relative to the control group without asthma (Figure 2A). Although the difference between the EIB (+) group and the control group was greater than the difference between the EIB (−) group and control group, the difference between the EIB (+) and EIB (−) groups was not statistically significant. In our prior analysis of bronchoalveolar lavage (BAL) fluid, we reported $sPLA_2$-X levels relative to total protein after concentration of BAL fluid (18). Here we demonstrate that the amount of $sPLA_2$-X relative to the total protein is increased in both asthma groups relative to the control group (Figure 2B) and that the levels of $sPLA_2$-X relative to total protein are similar to our prior findings. A Western blot of induced sputum supernatant using representative samples from each of the groups demonstrates that the protein is present as a single band that runs in approximately the same location as the mature $sPLA_2$-X protein (Figure E6).

Epithelial $sPLA_2$-X Protein May Be Increased in Relation to AHR

The amount of $sPLA_2$-X protein relative to the total protein in the epithelial lysate was increased in the EIB (+) asthma group relative to control subjects without asthma and also relative to EIB (−) subjects with asthma (Figure 2C). Similar results were found for the concentration of $sPLA_2$-X in the epithelial lysate and the amount of $sPLA_2$-X protein relative to the number of epithelial cells (data not shown). The levels of $sPLA_2$-X protein in lysates from the induced sputum cell pellet also identify an increase in the amount of $sPLA_2$-X protein in the EIB (+) group of subjects with asthma (Figure 2D).

We further quantified differences in epithelial $sPLA_2$-X protein using design-based stereology. Design-based stereology is a quantitative technique that avoids the usual sources of bias encountered in two-dimensional sections due to differences in cell size and the volume of the reference space (32). The volume of the epithelium relative to the surface area of the basal lamina did not differ between the asthma and control groups (Figure 3A). The volume of $sPLA_2$-X immunostaining relative to the surface area of the basal lamina tended to be higher in the EIB

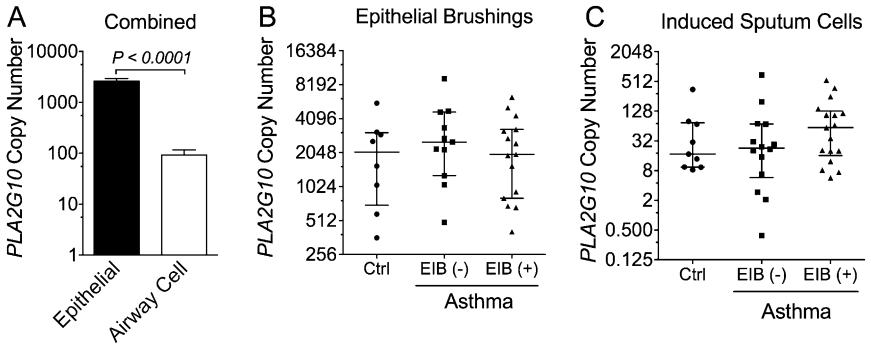

Figure 1. Expression of *PLA2G10* in airway epithelial brushings and induced sputum cells. (*A*) Copy number analysis of *PLA2G10* expression in all groups combined demonstrates higher expression in airway epithelial cells than in airway cells derived from induced sputum. (*B*) There was no difference between the *PLA2G10* expression in airway epithelial brushings between subjects with and without asthma and between subjects with and without exercise-induced bronchoconstriction (EIB). (*C*) There was also no difference in the *PLA2G10* expression in induced sputum cells between each of the groups.

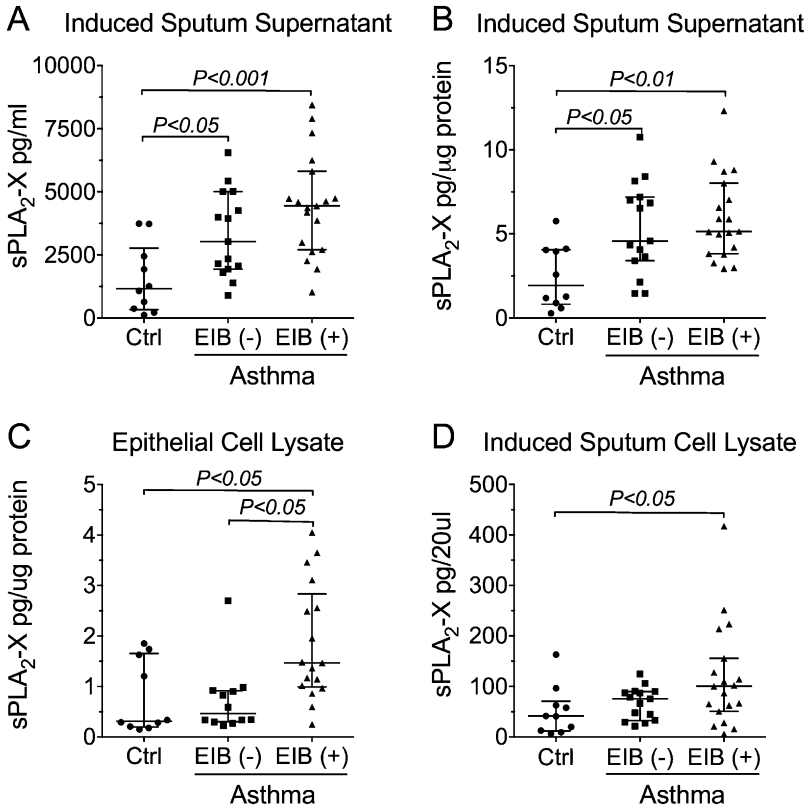

Figure 2. Quantification of secreted phospholipase A_2 group X ($sPLA_2$-X) protein in the airway lining fluid and airway cells. The concentration of $sPLA_2$-X protein in induced sputum supernatant (*A*) as well as the amount of $sPLA_2$-X protein relative to total protein (*B*) was increased in subjects with asthma relative to subjects without asthma. The differences were greater for subjects with exercise-induced bronchoconstriction (EIB) relative to subjects without EIB. (*C*) The amount of $sPLA_2$-X protein relative to total protein in lysates from epithelial cells obtained by bronchoscopic brushings of the airway epithelium was increased in the group of subjects with asthma with EIB. (*D*) The concentration of $sPLA_2$-X in cell lysates from the induced sputum cell pellet was increased in subjects with EIB. Ctrl = control.

(+) group, but these differences did not reach statistical significance (Figure 3B). Further analysis of the relationship between $sPLA_2$-X and the severity of EIB revealed that the volume of epithelial $sPLA_2$-X immunostaining relative to the surface area of the basal lamina tended to be associated with severity of AHR ($P = 0.07$, Figure 3C). The volume of $sPLA_2$-X immunostaining relative to the volume of the epithelium in asthma was increased relative to the control group after exclusion of two outliers (Figure E7, Vv $sPLA_2$-X, epi, 0.64 vs. 0.54 $\mu m^3/\mu m^3$; $P = 0.01$); however, there was no difference between the two asthma groups for this parameter.

Representative images of epithelial $sPLA_2$-X immunostaining reveal $sPLA_2$-X protein throughout the epithelium in many areas (Figure 3D) and prominent basal cell immunostaining in some regions (Figure 3E). Immunostaining at the terminal bar was also identified in fully differentiated ciliated cells (Figure 3F). In contrast to findings in mice (21), there was minimal $sPLA_2$-X staining in goblet cells (Figure 3F). Overall, these results indicate that there is an increase in the secretion of $sPLA_2$-X in the airways of patients with asthma in the absence of differences in epithelial *PLA2G10* gene expression and that there may be an increase in the $sPLA_2$-X protein in epithelial cells in association with the severity of EIB.

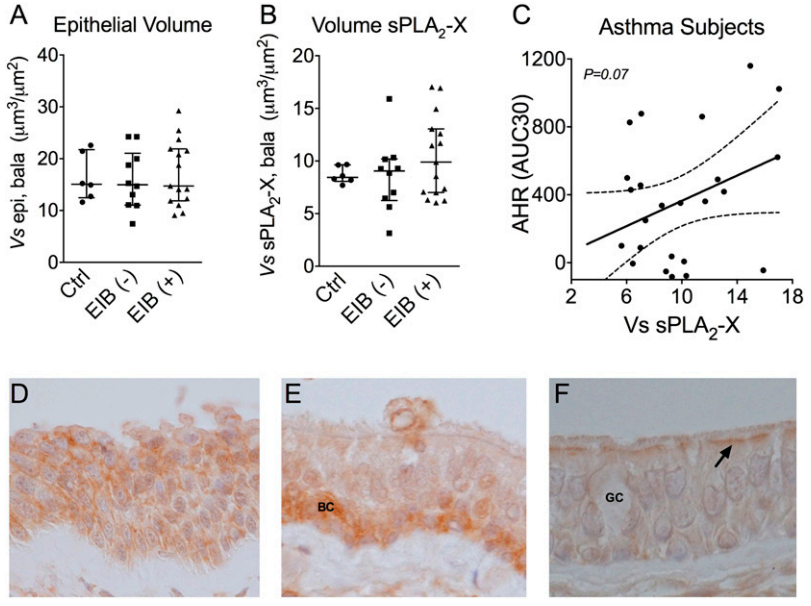

Figure 3. Quantification of epithelial secreted phospholipase A_2 group X ($sPLA_2$-X) immunostaining by design-based stereology. (*A*) The volume (Vs) of the epithelium (epi) relative to the surface area of the basal lamina (bala) was similar between the asthma and control groups. (*B*) The volume of $sPLA_2$-X immunostaining relative to the area of the basal lamina had more variation in the asthma groups and tended to be higher in the exercise-induced bronchoconstriction (EIB [+]) group, but these differences did not reach statistical significance. (*C*) Within the asthma group, the volume of $sPLA_2$-X immunostaining in the epithelium tended to be associated with severity of airway hyperresponsiveness (AHR). Epithelial $sPLA_2$-X immunostaining could be identified through the epithelium in many sections (*D*), but was also prominent in basal cells (BC) in some regions (*E*), as well as staining at the terminal bar (*arrow*) of ciliated cells (*F*). Goblet cells (GC) do not appear to be a significant source of $sPLA_2$-X staining (*F*).

The Expression of PLA2G10 Increases during Epithelial Differentiation and Repair

As epithelial brushings primarily capture fully differentiated ciliated cells (33), we examined the regulation of epithelial *PLA2G10* expression during *in vitro* differentiation in organotypic culture. The *PLA2G10* expression increased over time in epithelial cells in organotypic culture derived from each of the three groups (n = 4–5 per group, Figure 4A). The increase in *PLA2G10* expression during differentiation was lower in the EIB (−) asthma group over time ($P = 0.01$). Because retinoic acid (RA) plays a key role in epithelial differentiation, we treated primary epithelial cells from normal control subjects with RA (50 nM) and found that *PLA2G10* expression increased over time in RA-treated cells relative to untreated cells in basal medium (n = 4 replicates, Figure 4B). We found that $sPLA_2$-X protein accumulates in the apical compartment relative to the amount of intracellular $sPLA_2$-X, demonstrating that the protein is constitutively secreted (n = 3 per group, $P = 0.01$, Figure 4C); however, there were no definite differences between the groups ($P = 0.22$, EIB [−] vs. EIB [+]).

As injury to the airway epithelium has been implicated in asthma, we examined *PLA2G10* expression after an *in vitro* scratch wound in fully differentiated epithelial cells (n = 3 per group). There was a significant increase in *PLA2G10* expression in the time period from 6 to 48 hours after the scratch wound that was similar in both groups (Figure 4D). In the wounding experiment, the epithelial cells from EIB (−) donors did not grow well and were not included; this alteration in growth may be the etiology of the lower expression of $sPLA_2$-X during *in vitro* differentiation (Figures 4A and 4C).

Growth Factors and Inflammatory Cytokines Regulate Epithelial *PLA2G10* Expression

When cells were cultured in growth medium with the full set of serum replacements and growth factors, the expression of *PLA2G10* was suppressed relative to cells cultured in basal medium (n = 4 per condition, Figure 5A). As some of the components of the growth medium have also been implicated in asthma pathogenesis, we added supplements individually to primary epithelial cells cultured in basal medium and found that epinephrine and insulin both markedly reduced *PLA2G10* expression, whereas epidermal growth factor and hydrocortisone decreased the expression to a lesser extent (n = 3 per condition, Figure 5B). We also found that the histone deacetylase inhibitor sodium butyrate markedly increased the expression of *PLA2G10* in primary epithelial cells (n = 5 per condition, Figure 5C).

The combination of TNF and IL-1β increased the expression of *PLA2G10* in primary epithelial cells (n = 4 per condition, Figure 5D). Further analysis revealed that IL-17 and IL-13 increased, whereas IL-4 reduced, the expression of *PLA2G10* (n = 3 per condition, Figure 5E). Epithelial *PLA2G10* expression was slightly decreased by IL-25 and thymic stromal lymphopoietin, whereas IL-33 had no effect on *PLA2G10* expression. We further examined differential effects of sodium butyrate, TNF/IL-1β, RA, IL-13, and IL-17, but found that there were no clear differences between the responses to these factors by epithelial cells derived from each of the groups (n = 3 per group, Figure 5F).

Epithelial $sPLA_2$-X Regulates AA Release but Is Not Essential for Prostaglandin E_2 Synthesis

In initial studies using the BEAS2B human airway epithelial cell line we found that cellular AA is released over time into the extracellular fluid, and the release of AA is increased in response to TNF with IL-1β (Figure 6A). Because studies in human epithelial cells with a lentiviral shRNA knockdown and a small molecule inhibitor of human $sPLA_2$-X (ROC-0929) were not fully informative (*see* online supplement), we examined the effects of $sPLA_2$-X deficiency using primary mTECs from wild-type and *PLa2g10*$^{-/-}$ mice. Compared with wild-type mice, *PLa2g10*$^{-/-}$ mice had reduced AA release over time (Figure 6B). As a comparator, the $cPLA_2$ inhibitor pyrrophenone (Pyr-2) generally decreased AA release to a lesser extent than *PLa2g10* deficiency.

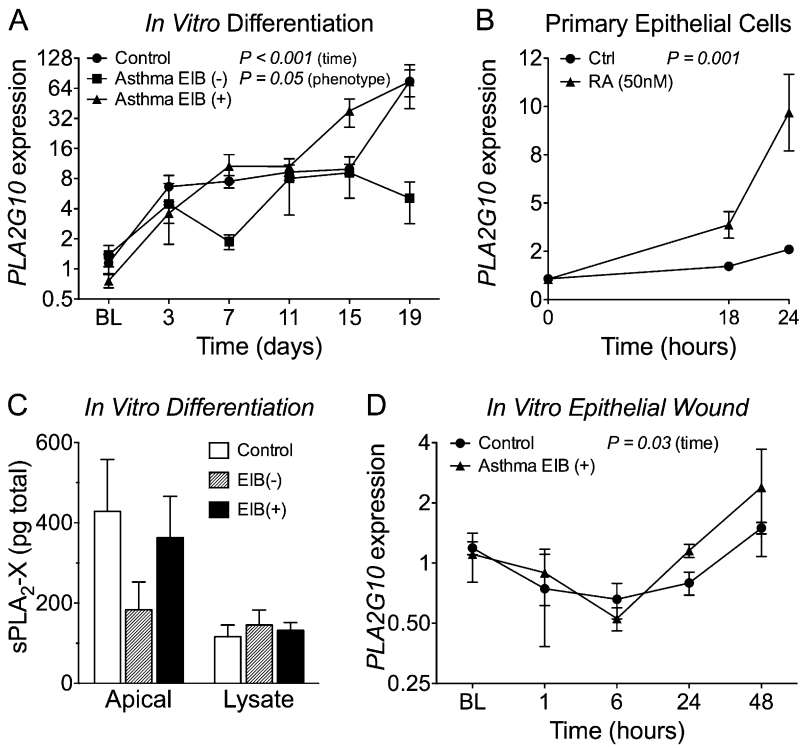

Figure 4. *PLA2G10* expression during differentiation and repair in primary airway epithelial cell organotypic culture. (*A*) The expression of *PLA2G10* increased over multiple time points during *in vitro* epithelial differentiation in organotypic culture in epithelial cells derived from subjects with asthma with exercise-induced bronchoconstriction (EIB [+]), EIB (−) subjects with asthma, and control subjects without asthma. The change in gene expression over time differed by phenotype. (*B*) Treatment with retinoic acid (RA, 50 nM) in primary airway epithelial cells cultured in basal medium increased the *PLA2G10* expression change over time. (*C*) In fully differentiated epithelial cells, the secreted phospholipase A_2 group X ($sPLA_2$-X) protein accumulated in the apical fluid relative to the intracellular protein found in the lysate ($P = 0.01$), but there was no difference by phenotype. (*D*) After an *in vitro* scratch wound in fully differentiated cells the *PLA2G10* expression changed over time but was similar between the two groups studied.

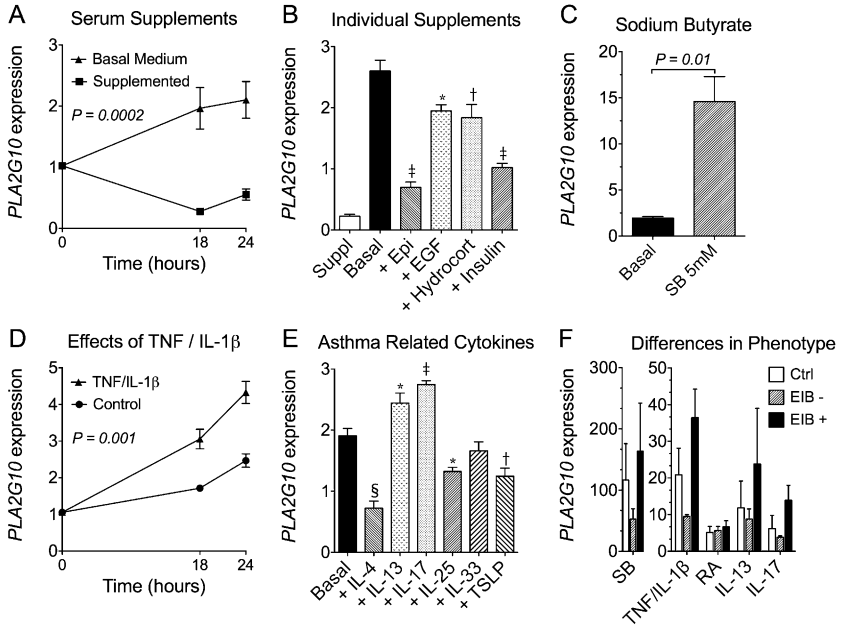

Figure 5. Effects of serum replacements and asthma-related cytokines on *PLA2G10* expression in cultured primary epithelial cells. (*A*) The addition of growth factors and serum supplements in defined bronchial epithelial growth medium had reduced *PLA2G10* expression over time relative to primary airway epithelial cells cultured in basal media. (*B*) The addition of epinephrine (Epi) or insulin, or to a lesser extent epidermal growth factor (EGF) or hydrocortisone (Hydrocort) to basal medium reduced the expression of epithelial *PLA2G10*. (*C*) The histone deacetylase inhibitor sodium butyrate markedly increased the expression of *PLA2G10* in epithelial cells after 18 hours in culture. (*D*) Treatment of primary epithelial cells in basal media with tumor necrosis factor (TNF) and IL-1β increased the expression of *PLA2G10* relative to untreated epithelial cells. (*E*) Treatment of epithelial cells with IL-17 and to a lesser extent IL-13 increased the expression of *PLA2G10*, whereas IL-4 and to a lesser extent IL-25 and thymic stromal lymphopoietin decreased *PLA2G10* expression. (*F*) Treatment of epithelial cells from each of the study groups with SB, TNF/IL-1β, RA, IL-13, and IL-17 did not reveal significant differences in the increase in *PLA2G10* expression between the groups. $^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$, $^{\S}P < 0.0001$ RA = retinoic acid.

In mTECs from *PLa2g10*$^{-/-}$ mice, treatment with TNF plus IL-1β increased AA release in a manner that was partially inhibited by Pyr-2 (Figure 6C). Despite the effect of *PLa2g10* deficiency on AA release, the synthesis of prostaglandin E_2 (PGE_2) was not altered in *PLa2g10*$^{-/-}$ mice; however, TNF plus IL-1β increased PGE_2 synthesis in both mouse strains, and the $cPLA_2$ inhibitor Pyr-2 was effective in blocking PGE_2 synthesis (Figure 6D). These results further support the novel finding that epithelial-derived $sPLA_2$-X serves as a regulator of eicosanoid metabolism through the release of AA before secretion and by acting on target cells such as eosinophil after secretion (20); furthermore, $sPLA_2$-X is selective and does not alter the formation of PGE_2.

DISCUSSION

We found that the levels of the secreted $sPLA_2$-X protein are elevated in the airways of patients with asthma and that there may be an increase in the $sPLA_2$-X protein in epithelial cells, particularly in subjects with EIB. The expression of *PLA2G10* increased markedly during *in vitro* differentiation of epithelial cells in organotypic culture in part via RA that increases the expression of *PLA2G10*. The expression of *PLA2G10* was increased by cytokines implicated in asthma, including TNF/IL-1β, IL-17, and to a lesser extent IL-13, but was suppressed by IL-4. In cultured epithelial cells, $sPLA_2$-X is constitutively secreted, and also contributes to the release of the eicosanoid precursor AA from epithelial cells, but does not regulate the formation of epithelial PGE_2. These findings support a role for epithelial $sPLA_2$-X in indirect AHR and indicate that $sPLA_2$-X functions as an epithelial regulator of eicosanoid metabolism, both within epithelial cells and acting on target cells such as eosinophils in the airways.

Prior studies have identified an increase in $sPLA_2$ activity in BAL fluid from subjects with asthma (14) and an increase in

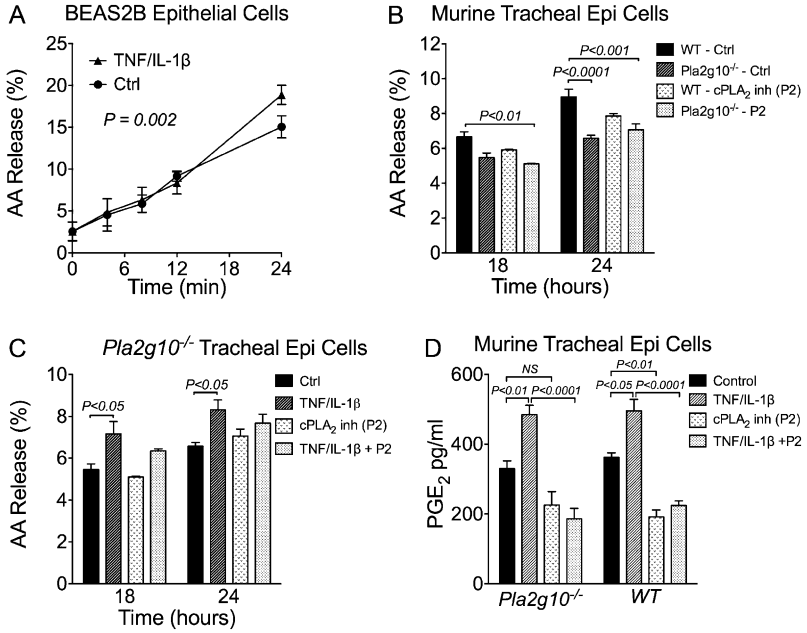

Figure 6. Respective roles of secreted phospholipase A_2 group X ($sPLA_2$-X) and cytosolic phospholipase A_2 alpha ($cPLA_2\alpha$) in epithelial release of arachidonic acid (AA) and prostaglandin E_2 (PGE_2). (*A*) The release of radiolabeled AA into the culture medium increased over time in the BEAS2B human airway epithelial cell line, and treatment with tumor necrosis factor (TNF) in combination with IL-1β increased the release of AA. (*B*) The spontaneous release of AA was reduced at 18 and 24 hours in murine tracheal epithelial cells (mTECs) from *PLa2g10*$^{-/-}$ mice compared with wild-type (WT) mice, whereas the $cPLA_2$ inhibitor pyrrophenone (Pyr-2) inhibited AA release to a lesser extent. (*C*) Treatment with TNF plus IL-1β increased AA release in a manner that was partially inhibited by Pyr-2 in *PLa2g10*$^{-/-}$ mice. (*D*) The generation of PGE_2 by mTECs was increased by the addition of TNF plus IL-1β and decreased by the $cPLA_2$ inhibitor Pyr-2, but the synthesis of PGE_2 was not altered in *PLa2g10*$^{-/-}$ mice.

$sPLA_2$ activity after allergen challenge (15, 16, 34). We previously found that $sPLA_2$-X levels are higher than $sPLA_2$-V and $sPLA_2$-IIA in the BAL fluid of patients with asthma (18). The present study further demonstrates an increase in the secreted $sPLA_2$-X protein in the airways of subjects with asthma and an increase in the protein within epithelial cells in subjects with EIB. The marked increase in the secreted protein levels despite the absence of differences in *PLA2G10* gene expression in epithelial brushings suggests prominent post-translational regulation of $sPLA_2$-X. The $sPLA_2$-X protein is translated with a highly conserved RXn(K/R)R furin-recognition motif at its C-terminus that must be cleaved leading to activation (35). Inhibiting the removal of the propeptide by a cell-permeable furin inhibitor in HEK293 cells transfected with full-length *Pla2 g10* also inhibited some of the secretion of the protein, suggesting that proteolytic conversion regulates $sPLA_2$-X release (36). The regulation of $sPLA_2$-X by furin cleavage could represent a feed-forward loop, as the CysLT LTD_4 induces the expression of furin in HEK293 cells transfected with the $CysLT_1$ receptor (37). We found that $sPLA_2$-X protein is constitutively secreted in epithelial cells in organotypic culture and that the protein in the airways represents the mature form of the protein. Further activation of the mature $sPLA_2$-X protein may also be important in asthma, as we previously found that transglutaminase 2 is overexpressed in the airways of patients with asthma and increases the enzymatic activity of human $sPLA_2$-X (38). A dual inhibitor of transglutaminase 2 and $sPLA_2$ in the lipocortin family inhibited the development of inflammation and AHR in a murine model of asthma (39).

Although the expression of *PLA2G10* was much higher in epithelial cells than other airway cells, it is possible that the elevated level of $sPLA_2$-X is due to secretion of the protein from a nonepithelial source. Both the airway epithelium and airway macrophages demonstrate strong immunostaining for $sPLA_2$-X in both humans and mice (17, 21). Further study using model systems with selective *PLa2g10* deficiency in epithelial cells and macrophages will be needed to resolve the relative importance of each of these cell types in animal models, because the development of airway inflammation and AHR is impaired with global *PLa2g10* deficiency (21, 22). Although transgenic expression of full-length *PLA2G10* in mice is not associated with pathology in the absence of inflammation (40), the transgenic expression of active *PLA2G10* without the propeptide sequence in macrophages causes significant lung dysfunction related to the degradation of surfactant phospholipids (41). Thus, macrophage expression of *PLA2G10* could alternatively be an important source of $sPLA_2$-X in the airways.

The regulation of epithelial *PLA2G10* expression by RA may be important in asthma, as the RA receptors are increased in asthma, and RA has been implicated in the development of airway disease (42, 43). We also found that $sPLA_2$-X was epigenetically regulated by the nonselective histone deacetylase inhibitor sodium butyrate as demonstrated before in a squamous epithelial cell line (44), a finding of relevance as reduced histone deacetylase activity has been identified in asthma (45). Serum replacements, including epinephrine, hydrocortisone, epidermal growth factor, and insulin, suppressed the *PLA2G10* expression in epithelial cells, suggesting that inhaled corticosteroids and β_2-agonists could regulate the expression of epithelial *PLA2G10*. Mediators of inflammation, including TNF plus IL-β, that are known to induce the expression of other $sPLA_2$s (46) increased *PLA2G10* expression. Notably, *PLA2G10* was induced by the key mediator in asthma IL-13 (47) but was induced more strongly by IL-17, which is increasingly recognized as a regulator of asthma (48). It is also notable that IL-4, which can play an antiinflammatory role in some contexts, such as rheumatoid arthritis (49), decreases *PLA2G10* expression in primary epithelial cells. These results also suggest that the IL-13–mediated increase in epithelial *PLA2G10* occurs via the IL-13Rα2 receptor, whereas signaling via IL-13Rα1/IL-4Rα causes a reduction in *PLA2G10* gene expression. These results may be of interest, as epithelial wound repair is mediated in part via IL-13 release and signaling via IL-13Rα2 (50), as we demonstrate that *PLA2G10* gene expression increases after an *in vitro* scratch wound.

The formation of eicosanoids is reduced in *Pla2 g10*-deficient mice during allergic inflammation (21). The epithelium is often overlooked as a regulator of eicosanoids, because the terminal synthetic enzymes for the major eicosanoids implicated in asthma are predominantly expressed in leukocytes; however, structural cells serve as an important source of AA via transcellular metabolism (51). We found that $sPLA_2$-X serves as a key regulator of AA release in mTECs and that the *PLa2g10* knockout had a larger effect on AA release than inhibition of $cPLA_2\alpha$, which is generally believed to be the dominant regulator of AA release in epithelial cells (52). As the release of AA in human primary cells was not inhibited by a cell-impermeant $sPLA_2$-X inhibitor, the results suggest that AA release mediated by $sPLA_2$-X in epithelial cells occurs before secretion, as was recently demonstrated for this enzyme in transfected HEK293 cells (36).

We assessed the levels of PGE_2 in the epithelial cell model because this eicosanoid is directly synthesized via terminal synthetic enzymes that are plentiful in epithelial cells. PGE_2 serves as a protective mediator in the airways in patients with EIB (53), and the levels of PGE_2 actually decline after exercise challenge among patients with asthma (7). We found that the synthesis of PGE_2 in epithelial cells was predominantly under control of $cPLA_2\alpha$, further supporting the discordance between $sPLA_2$-X as a mediator of inflammatory eicosanoids, whereas both inflammatory and protective eicosanoids may be regulated by $cPLA_2\alpha$.

Based on the findings of marked protection from allergen-induced airway immunopathology in murine models, the function of this enzyme as a regulator of inflammatory eicosanoid formation, and the elevated levels in the airways of patients with asthma, the results suggest that therapies targeting $sPLA_2$-X may be useful in the treatment of asthma, particularly indirect AHR.

Author disclosures are available with the text of this article at www.atsjournals.org.

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