

Relationship between levels of secreted phospholipase A₂ groups IIA and X in the airways and asthma severity

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Summary

Background Secreted phospholipase A₂s (sPLA₂) may be important mediators of asthma, but the specific sPLA₂s involved in asthma are not known.

Objective To evaluate sPLA₂ group IIA, V, and X proteins (sPLA₂-IIA, sPLA₂-V, and sPLA₂-X) in bronchoalveolar lavage (BAL) fluid, BAL cells, and airway epithelial cells of subjects with and without asthma, and examine the relationship between the levels of specific sPLA₂ enzymes and airway inflammation, asthma severity, and lung function.

Methods The expression of sPLA₂-IIA, sPLA₂-V, and sPLA₂-X in BAL cells and epithelial brushings was assessed by qPCR. The levels of these sPLA₂ proteins and sPLA₂ activity with and without group II and group X-specific inhibitors were measured in BAL fluid from 18 controls and 39 asthmatics.

Results The airway epithelium expressed sPLA₂-X at higher levels than either sPLA₂-IIA or sPLA₂-V, whereas BAL cells expressed sPLA₂-IIA and sPLA₂-X at similar levels. The majority of sPLA₂ activity in BAL fluid was attributed to either sPLA₂-IIA or sPLA₂-X. After 10-fold concentration of BAL fluid, the levels of sPLA₂-X normalized to total protein were increased in asthma and were associated with lung function, the concentration of induced sputum neutrophils, and prostaglandin E₂. The levels of sPLA₂-IIA were elevated in asthma when normalized to total protein, but were not related to lung function, markers of airway inflammation or eicosanoid formation.

Conclusions and Clinical Relevance These data indicate that sPLA₂-IIA and sPLA₂-X are the major sPLA₂s in human airways, and suggest a link between the levels of sPLA₂-X in the airways and several features of asthma.

Keywords asthma, eicosanoid, epithelial cell, leukotriene, phospholipase

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Introduction

Inflammatory eicosanoids such as leukotrienes (LTs) and certain prostaglandins (PGs) such as PGD₂ have important pathophysiological roles in airway inflammation, hyper-responsiveness (AHR), and structural remodelling in asthma [1]. The first rate-limiting step in eicosanoid biosynthesis is the release of unesterified arachidonate from the *sn*-2 position of membrane phospholipids by phospholipase A₂ (PLA₂) [2]. Recently, 10 mammalian secreted PLA₂s (sPLA₂s) have been identified, and at least some of these enzymes may coordinate eicosanoid synthesis along with the well-described cytosolic PLA₂ (i.e.

cPLA₂α) [3–5]. Early studies demonstrated an increased sPLA₂ enzymatic activity in bronchoalveolar lavage (BAL) fluid and nasal lavage fluid following allergen challenge in patients with asthma and allergic rhinitis, and more recently total sPLA₂ activity was found to be increased in BAL fluid from asthmatic subjects, but the specific sPLA₂s were not identified in these studies [6–9]. We demonstrated previously that the gene expression of sPLA₂ group X (sPLA₂-X) is increased in induced sputum cells of asthmatics and that the level of sPLA₂-X in induced sputum supernatant increased following exercise challenge among asthmatics with exercise-induced bronchoconstriction (EIB) [10]. Deletion of sPLA₂-X inhibits the development

of airway inflammation, AHR, and structural remodelling, and deletion of sPLA₂ group V (sPLA₂-V) reduces cellular airway inflammation and AHR in murine asthma models [11, 12].

Severe asthma is characterized by poorly reversible structural alterations of the lung, persistent symptoms and exacerbation, and a poor response to controller therapies, resulting in high health care utilization [13]. The pathophysiological mechanisms of severe asthma remain incompletely understood; however, alterations in eicosanoid metabolism may play a significant role in the disease. An increase in the production of inflammatory eicosanoids such as the cysteinyl leukotrienes (CysLT)s, C₄, D₄, and E₄ has been demonstrated in BAL fluid of poorly controlled asthmatics [14, 15]. Stable severe asthma is characterized by an increase in LTB₄ and an increase in airway neutrophils [16]. Thus, sPLA₂s may play significant roles in dysregulated eicosanoid synthesis in asthma, but the specific sPLA₂s involved in asthma are not known.

We assessed the sPLA₂ enzymatic activity and the levels of selected sPLA₂s in BAL fluid of asthmatics with severe asthma as compared with asthmatics with mild-to-moderate asthma and non-asthmatic controls. We focused on the levels of sPLA₂ groups IIA, V, and X as these groups have been most strongly implicated in asthma. The levels of the sPLA₂s in BAL fluid were quantitatively measured by a time-resolved fluoroimmunoassay [17]. We used selective enzyme inhibitors to determine the contribution of the different sPLA₂ enzymes to BAL fluid sPLA₂ enzymatic activity. Our goals were to (1) characterize the expression of sPLA₂-X relative to sPLA₂-IIA and sPLA₂-V in the airway epithelium and immune cells present in BAL fluid, (2) determine the relative amounts of these sPLA₂s in BAL fluid supernatant from asthmatic and normal subjects, and (3) determine if specific sPLA₂s are increased in asthma in relation to asthma severity, lung function, and airway inflammation.

Some of the results of this study have been reported previously in the form of an abstract [18].

Methods

Study subjects

Subjects for this study were recruited at National Jewish Medical and Research Center, and University of Pittsburgh Medical Center. The Institutional Review Boards at each centre approved the study procedures, and subjects provided written informed consent. All subjects were non-smokers (<5 pack-years of cigarette use). Severe asthmatics met the ATS workshop definition of severe asthma based on the presence of one of two major and two of seven minor criteria notable for poor asthma control despite treatment with high doses of corticosteroids [19]. Mild-to-moderate asthmatics were identified based on

lung function, medication use, and symptom frequency in accordance with published guidelines [20]. Non-asthmatic controls were enrolled who had no prior history of asthma, a forced expiratory volume in 1 s (FEV₁) ≥ 80% predicted, and a negative methacholine challenge.

Bronchoscopy and induced sputum

Bronchoscopy with BAL and endobronchial epithelial brushing was performed as described previously [21]. BAL was performed in subsegments of the lingula or right middle lobe by using warm sterile saline. Airway brushings were obtained from subsegmental airways and processed for epithelial cell RNA. Induced sputum was conducted with 3% saline for 20 min [22]. Total cell count was determined with a haemocytometer, and slides for differential cell counts were prepared with a cytocentrifuge. The levels of PGE₂ in induced sputum supernatant were assayed following solid phase extraction by ELISA (Cayman Chemical, Ann Arbor, MI, USA).

Quantitative real-time PCR

Real-time PCR was performed on the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA). Primer-probe sets for selected genes were obtained from the Applied Biosystems Assays-on-Demand repository (Applied Biosystems). The reference numbers are as follows: sPLA₂-IIA, Hs00179898_m1; sPLA₂-V, Hs01099100_m1; and sPLA₂-X, Hs00358567_m1. The primer for sPLA₂-IIA was validated against K562 cell cDNA with a C_t value of 38 for 100 ng per reaction. The primer for sPLA₂-V was validated against recombinant plasmid DNA with a C_t value of 17.5 for 0.1 ng per reaction. The primer for sPLA₂-X was validated using 16HBE140-cell cDNA with a C_t value of 28 for 41 ng per reaction. The values obtained for each transcript of interest were normalized to the level of GAPDH mRNA detected in each sample. An identical threshold cycle (C_t) was applied for each gene of interest. Relative mRNA expression levels were calculated using the ΔC_t method.

Quantification of selected sPLA₂s in BAL fluid

The sPLA₂ proteins in BAL fluid were measured after 10-fold concentration using a centrifugal concentrator with a 3 kDa molecular weight cut-off (Millipore, Danvers, MA, USA). Triton-X 0.5% was added before concentration to improve recovery. The recoveries of recombinant proteins measured by time-resolved fluoroimmunoassay (TRFIA) before and after concentration were 90.3% for sPLA₂-X, 22.4% for sPLA₂-IIA, and 84.8% for sPLA₂-V. The assay is based on sPLA₂-specific polyclonal rabbit anti-sPLA₂ capture antibodies and anti-sPLA₂ detection antibodies that are Eu³⁺-labelled to measure time-resolved

immunofluorescence [23]. Ninety-six-well microplates were coated with anti-sPLA₂ antibody and blocked with Tris-buffer containing 1.0% bovine serum albumin (BSA), 6% D-sorbitol, 0.9% NaCl and 1 mM CaCl₂. For the assay, 50 µL of 10-fold concentrated BAL fluid or standard solution and 50 µL of assay buffer were added to the anti-sPLA₂ antibody-coated microplate wells and incubated at room temperature for 30 min with shaking at 240 cycles/min. After washing, 100 µL of detecting antibody (Eu³⁺-labelled anti-sPLA₂ antibody containing >5 × 10⁵ counts per second (cps)/µL) was added to the wells and incubated for 30 min with shaking. After washing, 100 µL of Delfia enhancement solution (Perkin-Elmer, Waltham, MA, USA) was incubated in the wells for 15 min with shaking, and fluorescence was measured at excitation and emission wavelengths of 340 and 615 nm. The lower limit of detection is 8 pg/mL for sPLA₂-X, 2 pg/mL for sPLA₂-IIA, and 96 pg/mL for sPLA₂-V. Linear standard curves were derived from recombinant human sPLA₂s [24].

Measurement and selective inhibition of sPLA₂ activity in BAL fluid

Preliminary studies using the fluorometric assay with the pyrene-labelled phospholipid analogue showed that the assay was not sensitive enough to measure sPLA₂ activity in all samples without further concentration, so the sPLA₂ enzymatic activity in BAL fluid was assessed by monitoring the release of free fatty acid from [³H]oleate-labelled *Escherichia coli* membranes [25]. Unconcentrated BAL fluid or recombinant sPLA₂ proteins were incubated at 37 °C for 1 h with ~2 000 000 dpm of [³H]oleate-labelled *E. coli* membranes in 100 µL of reaction buffer (0.1 M Tris-HCl, pH 8.0, 10 mM CaCl₂, 0.1% fatty acid-free BSA). The reaction was stopped with the addition of 300 µL of 0.1 M EDTA, pH 8.0, and 1% fatty acid-free BSA. The samples were centrifuged at 13 000 g for 3 min, and 400 µL of the supernatant was used for scintillation counting. Control incubations in the absence of sPLA₂ were carried out in parallel to calculate specific hydrolysis. Recombinant human sPLA₂ group IIA, V, and X proteins that were generated in *E. coli*, purified, and refolded to the active enzymes were used to determine the specific activity of the assay for each enzyme (Table 1) [23]. The total sPLA₂ activity of 100 µL of BAL fluid was assessed, and the samples that had activity above the linear range were identified and diluted to 1 : 2 or 1 : 10 for the final assay. All samples were assayed simultaneously during the final measurement of sPLA₂ activity. Separate aliquots of BAL fluid were measured simultaneously in the presence of inhibitors that are selective for sPLA₂ groups IIA/III designated ROC-0320 and sPLA₂-X designated ROC-0929 (Table 2). The inhibitory activities of these inhibitors for the recombinant proteins at different concentrations of each inhibitor are shown in Table 1. To assess the effects of

Table 1. Specific activity of sPLA₂ proteins in the sPLA₂ activity assay and the effects of the inhibitors on the recombinant proteins

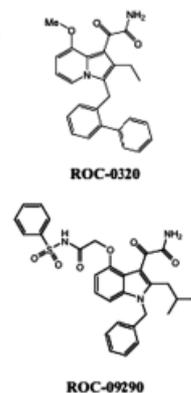
Recombinant human protein	sPLA ₂ -IIA (10 pg)	sPLA ₂ -X (25 pg)
Specific activity (dpm ± SD)		
No Inhibitor	39844.9 ± 498.3	11644.6 ± 2036.8
Inhibition (%)		
sPLA ₂ -II Inhibitor (ROC-0320) (µM)		
0.5	90.2	16.4
1	98.5	11.2
2	99.5	28.9
5	98.7	23.5
10	102.8	15.0
sPLA ₂ -X Inhibitor (ROC-0929) (µM)		
0.5	7.1	103.8
1	-2.6	105.9
2	24.0	109.0
5	3.5	95.8
10	17.5	112.7

Table 2. Selectivity of inhibitors [IC₅₀ (nm)*]

sPLA ₂	ROC-0929	ROC-0320
hGIB	> 1600	> 1600
hGIIA	> 1600	35 ± 2
hGIID [†]	700 ± 200	> 1300
hGIII	> 1600	50 ± 10
hGIIF	> 1600	> 1600
hGIII	> 1600	> 1600
hGV	> 1600	> 1600
hGX	20 ± 10	> 1600
hGXIIA	> 1600	> 1600

*IC₅₀ values obtained using fluorometric assay with pyrene-labelled phosphatidylglycerol as substrate.

[†]IC₅₀ value obtained using radiolabelled *E. coli* membrane assay.



the inhibitors in BAL fluid, each inhibitor was used at a 2 µM final concentration and pre-incubated for 10 min at room temperature before the addition of the reaction mixture containing [³H]oleate-labelled *E. coli* membranes.

Statistical analysis

We compared differences in the characteristics of each of the groups with either a chi-square test for categorical variables or an ANOVA or Kruskal-Wallis test for continuous variables. To test for differences in the sPLA₂ levels and sPLA₂ activity between the groups, we used a Kruskal-Wallis test with Dunn's post-hoc tests. The difference between the subjects based on asthma status or atopy status were assessed with the Mann-Whitney test. The changes in sPLA₂ activity due to inhibitors were assessed by the Wilcoxon signed-rank test. The sPLA₂ protein

levels were associated with other parameters such as lung function by linear regression after log transformation. The primary hypothesis related to differences in sPLA₂-X was assessed without adjustment of the *P*-value for multiple comparisons. The additional studies evaluating the levels of sPLA₂-IIA were also unadjusted, and can be viewed as hypothesis generating.

Results

Subject characteristics

The studies were conducted on BAL fluid samples from 18 non-asthmatic controls, 19 mild to moderate asthmatics, and 20 severe asthmatics (Table 3). The subjects with asthma were older, especially those with severe asthma. There were marked differences between the groups with regard to lung function. Differences in airway inflammation between the groups were notable for lower percentages of macrophages and higher percentages of eosinophils and neutrophils in BAL fluid. Induced sputum data were available from 35 subjects, demonstrating that there were higher concentrations of lymphocytes and columnar epithelial cells in induced sputum in the asthma groups (Table 4). The levels of PGE₂ in induced sputum supernatant were increased in the asthma groups, particularly severe asthma.

Epithelial and BAL cell sPLA₂ gene expression

The expression of sPLA₂ groups IIA, V, and X in epithelial brushings and BAL cells were assessed in five representative samples from each group. In airway epithelial brushings, the expression of sPLA₂-X overall from all groups combined was significantly higher than the expression of

sPLA₂-V and sPLA₂-IIA (Fig. 1a). In the epithelium, sPLA₂-V and sPLA₂-IIA were near the threshold of detection and there was no difference between these two sPLA₂s. In BAL cells, there was no difference in the expression of sPLA₂-X and sPLA₂-IIA, and both of these sPLA₂s had higher expression than sPLA₂-V (Fig. 1b). In BAL cells, the expression of sPLA₂-V was near the threshold of detection. In this modest number of samples, differences in the expression of sPLA₂ groups IIA, V, and

Table 3. Comparison of the characteristics of study groups*

	Asthma			<i>P</i> Value
	Control (<i>n</i> = 18)	Mild-Mod (<i>n</i> = 19)	Severe (<i>n</i> = 20)	
Age (years)(range)	31.7 (20–58)	33.7(19–55)	43.6 (23–62)	0.01
Gender [†]				1.00
Male (%)	52.9%	53.3%	52.9%	
Female (%)	47.1%	46.7%	47.1%	
Race [†]				0.19
Caucasian	64.7%	83.3%	68.8%	
African American	11.8%		31.3%	
Native American	5.9%			
Mixed/Other	17.6%	16.7%		
Lung Function				
FEV ₁ (% pred)	100.9±8.1	87.1±15.0	60.4±19.5	<0.001
FVC (% pred)	103.6±11.5	97.2±12.7	72.8±15.4	<0.001
FEV ₁ /FVC	0.8±0.1	0.7±0.1	0.6±0.1	<0.001
Atopic (%)	43.8%	83.3%	47.1%	0.03
Blood Eosinophils (%) [‡]	3.0 (1.2–4.2)	4.5 (2.8–7.3)	3.8 (1.9–7.5)	0.17

*Comparisons made by ANOVA except where otherwise specified.

[†]Chi-square test.

[‡]Comparisons made by Kruskal–Wallis Test. Data are expressed as medians (interquartile ranges).

Table 4. Comparison of airway inflammation between the study groups*

	Asthma			<i>P</i> Value
	Control (<i>n</i> = 18)	Mild-Mod (<i>n</i> = 19)	Severe (<i>n</i> = 20)	
Bronchoalveolar lavage				
Macrophages (%)	87.3 (81.2–92.5)	87.7 (83.7–92.4)	80.9 (74.7–84.8)	0.03
Lymphocytes (%)	7.8 (4.7–14.3)	11.0 (6.8–14.3)	8.9 (6.6–18.2)	0.40
Neutrophils (%)	1.7 (1.0–3.6)	0.9 (0.5–1.5)	1.7 (0.9–5.8)	0.06
Eosinophils (%)	0.4 (0.0–0.7)	0.2 (0.0–0.8)	1.7 (0.1–4.8)	0.03
Protein (µg/mL)	13.5 (10.1–21.5)	9.8 (7.5–14.9)	8.8 (7.4–10.2)	0.01
Induced sputum [†]				
Macrophages/mL	196.6 (58.4–223.5)	231.1 (154.7–1013.0)	421.6 (75.8–779.4)	0.23
Lymphocytes/mL	14.2 (3.0–21.8)	21.8 (6.7–69.2)	81.8 (8.7–159.8)	0.06
Neutrophils/mL	72.1 (20.7–131.6)	79.8 (35.1–223.3)	384.3 (60.9–1185.1)	0.19
Eosinophils/mL	0.0 (0.0–0.7)	3.8 (0.3–23.2)	9.4 (4.4–54.5)	0.01
Epithelial cells/mL	14.4 (0.0–29.9)	54.4 (18.9–81.0)	60.3 (20.9–182.3)	0.01
PGE ₂ (pg/mL)	251.0 (199.0–463.5)	373.0 (235.5–840.8)	710.0 (356.0–1582.5)	0.03

*Comparisons made by Kruskal–Wallis test. Data are expressed as medians (interquartile ranges).

[†]Induced sputum cells × 10³.

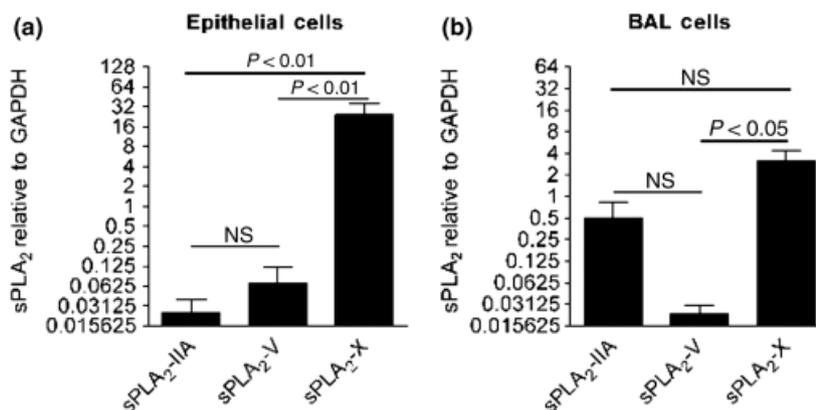


Fig. 1. Expression of the sPLA₂ enzymes in the airway epithelium and BAL cells. The gene expression of sPLA₂-IIA, sPLA₂-V, and sPLA₂-X was assessed in 15 samples, representing five subjects from each group. The expression of sPLA₂-X was significantly higher than the expression of either sPLA₂-V or sPLA₂-IIA in the epithelium (a). There was no difference in the expression of sPLA₂-IIA and sPLA₂-V in the epithelium. The expression of sPLA₂-X in BAL cells was significantly higher than sPLA₂-V, but no different than sPLA₂-IIA (b). There was no statistically significant difference between the expression of sPLA₂ groups IIA and V in BAL cells.

X between the groups of subjects were not apparent either in the airway epithelium or in the BAL cell pellet.

Differences in sPLA₂ protein in the airways

Following 10-fold concentration of BAL fluid, the sPLA₂-X protein was detectable by TRFIA in all but four BAL fluid samples. The undetectable samples were set at the low level of detection of 8 pg/mL for the assay. The median concentration of sPLA₂-X in BAL fluid following concentration was no different between the groups (median 58.4 pg/mL severe asthma, 46.1 pg/mL mild-to-moderate asthma, 45.7 pg/mL normal control, $P = 0.42$). However, the protein concentration measured after 10-fold concentration of BAL fluid varied widely from 3.9 to 89.0 µg/mL across the study population and varied between the three groups (Table 4). Because the recovery of total protein following concentration was variable, the sPLA₂-X levels were normalized to total protein levels in BAL fluid and expressed as pg of sPLA₂-X per µg of BAL protein. The concentration of sPLA₂-X relative to total protein was elevated in asthma relative to control (median 4.6 pg/µg protein vs. 3.1 pg/µg protein, $P = 0.02$), and varied between the three groups of subjects ($P = 0.03$ Kruskal-Wallis test, Fig. 2a), with the greatest difference in the severe asthma group relative to control ($P < 0.05$ Dunn's post-hoc test, Fig. 2a).

We assessed associations between the levels of sPLA₂-X in BAL fluid with lung function and inflammatory parameters among the asthma group. For these regression analyses, we excluded 2 BAL samples that were below the detection limit of the TRFIA assay but had readily detectable sPLA₂-X-specific activity in the activity assay described below before concentration, suggesting that the sPLA₂-X was lost during the concentration step. These samples were not excluded from the primary comparison

of differences between the groups to avoid any possibility of bias, but were removed from the regression analysis because they served as outliers with an influential effect on the analysis. The level of sPLA₂-X tended to be inversely correlated with the % predicted FEV₁ ($P = 0.06$, Fig. 2b). The level of sPLA₂-X was correlated with the number of neutrophils/mL in induced sputum (Fig. 2c), but not the neutrophils/mL in BAL fluid or other cellular parameters in induced sputum or BAL fluid. The level of sPLA₂-X was correlated with the concentration of PGE₂ in induced sputum ($P = 0.03$, Fig. 2d). There was no difference in the levels of sPLA₂-X between atopic and non-atopic asthmatics (median 4.1 pg/µg protein atopic vs. 4.9 pg/µg protein non-atopic, $P = 0.46$). The levels of sPLA₂-V in 10-fold concentrated BAL fluid from five representative asthmatics were all below the detection limit of 96 pg/mL, so comparisons between the groups for sPLA₂-V protein were not performed.

Sufficient BAL fluid was available to measure the level of sPLA₂-IIA by TRFIA in 43 of the subjects (13 controls, 15 mild-to-moderate asthma, and 15 severe asthma). The sPLA₂-IIA protein was detectable in all BAL samples, but values were below the linear range of the assay of 2 pg/mL in four controls and eight asthmatics following 10-fold concentration of the samples. These low values were set to 2 pg/mL. The levels of sPLA₂-IIA protein normalized to total protein in BAL fluid tended to be increased in asthma relative to the control group (median 0.9 pg/µg protein vs. 0.5 pg/µg protein, $P = 0.06$), and there were differences between the three groups ($P = 0.03$ Kruskal-Wallis test), with the main difference between severe asthma and other groups ($P < 0.05$, Fig. 3). The levels of sPLA₂-IIA were not associated with the % predicted FEV₁ ($n = 30$, $r^2 = 0.01$, $P = 0.60$), neutrophils/mL in induced sputum ($n = 19$, $r^2 = 0.00$, $P = 0.92$) or BAL fluid, or PGE₂ in induced sputum ($n = 19$, $r^2 = 0.00$, $P = 0.99$). There was no

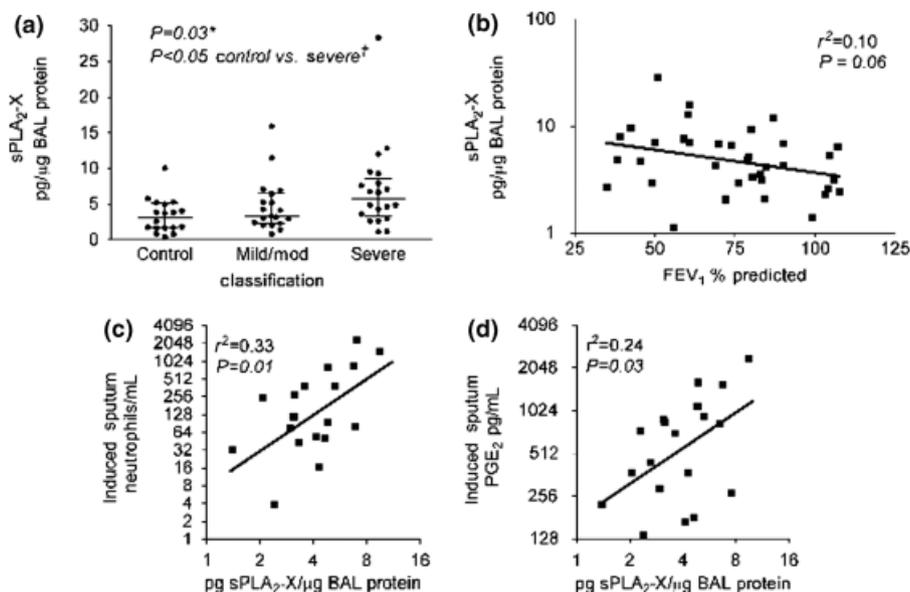


Fig. 2. Levels of sPLA₂-X protein in BAL fluid in relation to measures of lung function and inflammation. The levels of sPLA₂-X in BAL fluid normalized to total protein were higher in asthmatics relative to controls, with the largest difference in the severe asthma group relative to controls (a). The comparison between the three groups was made with the Kruskal–Wallis test (*), and comparison between the subgroups using the Dunn's post-hoc test (†). The figure shows the medians and interquartile ranges. The levels of sPLA₂-X were associated with lung function among asthmatics by regression analysis (b). Among asthmatics, the sPLA₂-X level in BAL fluid was associated with neutrophils in induced sputum (c), and levels of PGE₂ in induced sputum supernatant (d).

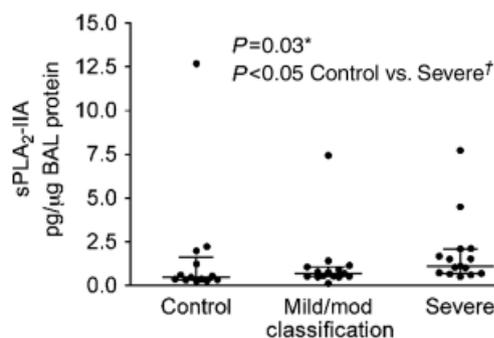


Fig. 3. Levels of sPLA₂-IIA protein in BAL fluid in asthma subjects and normal controls. The levels of sPLA₂-IIA in BAL fluid normalized to total protein were higher in the severe asthma group relative to the control group. The comparison between the three groups was made with the Kruskal–Wallis test (*), and comparison between the subgroups using the Dunn's post-hoc test (†). The figure shows the medians and interquartile ranges.

difference in the levels of sPLA₂-IIA between atopic and non-atopic asthmatics (median 0.8 pg/μg protein atopic vs. 1.0 pg/μg protein non-atopic, $P = 0.45$).

Total and group-specific sPLA₂ activity in BAL fluid

The total sPLA₂ activity per 100 μL of BAL fluid was no different between the groups of subjects (Fig. 4a). A number of samples had very high sPLA₂ activity, including six samples that required 10-fold dilution, and 13

samples that required two-fold dilution to keep the activity levels within the linear range of the assay. The majority of this high activity was due to sPLA₂-IIA because of the high specific activity for sPLA₂-IIA being ~8- to 9-fold higher than sPLA₂-X in this assay. To measure the contribution of the group II and X sPLA₂ enzymes to the total sPLA₂ activity, in separate aliquots we measured the activity after treatment with 2.0 μM ROC-0320 a sPLA₂-II inhibitor, and with 2.0 μM ROC-0929 a specific sPLA₂-X inhibitor. At these molar concentrations, the ROC-0320 compound completely inhibits the recombinant human sPLA₂-IIA enzyme, while inhibiting recombinant human sPLA₂-X by about 28%, and the ROC-0929 compound completely inhibits sPLA₂-X, while inhibiting the sPLA₂-IIA enzyme by 24% (Table 1). In BAL fluid, there was a 76.9% ($P < 0.0001$) decrease in sPLA₂ activity with the sPLA₂-II inhibitor; the percent change in sPLA₂ activity with this inhibitor was different between the three groups, reflecting a difference between the control and mild-to-moderate groups in a post hoc analysis ($P = 0.04$ with Dunn's post-hoc test, Fig. 4b). With the sPLA₂-X inhibitor, there was an 11.9% ($P < 0.0001$) decrease in sPLA₂ activity overall; however, there was no difference in the percent change in sPLA₂ activity with this inhibitor between the groups of subjects (Fig. 4b). Because of the higher specific activity for sPLA₂-IIA over sPLA₂-X in this assay, these results are consistent with the protein analysis showing an increased amount of sPLA₂-X protein over sPLA₂-IIA protein; however, this high sPLA₂-

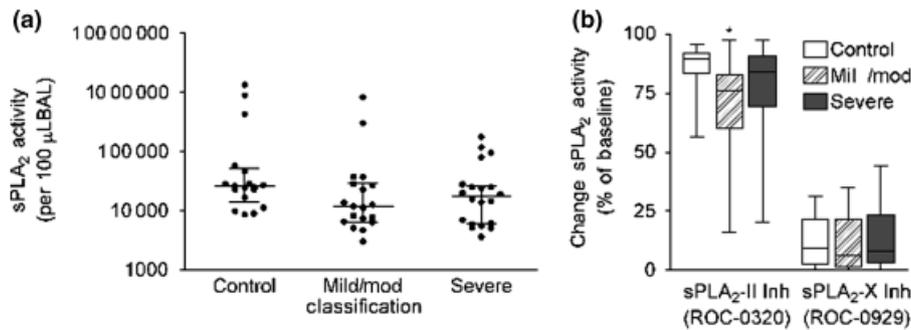


Fig. 4. Assessment of enzyme-specific sPLA₂ activity in BAL fluid. No differences in total sPLA₂ enzymatic activity in BAL fluid were identified between asthmatic and non-asthmatic groups (a). The sPLA₂ enzymatic activity was inhibited 76.9% ($P < 0.0001$) by sPLA₂-II inhibitor (ROC-0320) and 11.9% ($P < 0.0001$) by sPLA₂-X inhibitor (ROC-0929) (b). The percent change in sPLA₂ activity with the ROC-0320 inhibitor differed among the three groups, primarily between the control and mild-to-moderate asthmatics based on a post hoc analysis ($*P = 0.04$).

IIA activity and the overlap in the specificity of the inhibitors make it difficult to detect differences between the groups based on enzyme-specific activity.

Regression analyses were conducted to demonstrate that the sPLA₂ activity assay primarily reflects sPLA₂-IIA enzyme activity in BAL fluid and to determine if the inhibitor data were concordant with the protein analysis. We conducted these secondary analyses of the sPLA₂ inhibitor activity after exclusion of samples with very high sPLA₂ activity that required a 1 : 10 dilution because we felt that the inhibitory assays were less specific in the high-activity samples, especially given the large differences in specific activity for each enzyme in the assay. The total sPLA₂ activity in BAL fluid was associated with the concentration of sPLA₂-IIA enzyme in BAL fluid measured by the TRFIA assay (Fig. 5a). The change in sPLA₂ activity with the sPLA₂-II inhibitor ROC-0320 was also associated with the concentration of the sPLA₂-IIA enzyme (Fig. 5b), while the change in activity due to the sPLA₂-X inhibitor ROC-0929 was not associated with the levels of the sPLA₂-IIA protein ($r^2 = 0.02$, $P = 0.45$). The total sPLA₂ activity in BAL fluid was not associated with the concentration of sPLA₂-X enzyme in BAL fluid (Fig. 5c). Similarly, there was no association between the change in sPLA₂ activity with the sPLA₂-II inhibitor ROC-0320 and the levels of sPLA₂-X ($r^2 = 0.01$, $P = 0.48$); however, the change in sPLA₂ activity with the sPLA₂-X inhibitor ROC-0929 tended to be associated with the levels of sPLA₂-X protein in BAL fluid (Fig. 5d). These findings indicate that the sPLA₂ activity before concentration is consistent with the sPLA₂ protein concentrations that were measured after concentration; however, the marked differences in specific activity make the sPLA₂-IIA activity dominant and the contribution of sPLA₂-X difficult to observe in the activity analysis.

Discussion

Although prior studies have identified an increase in sPLA₂ activity in asthma [9] and following allergen

inhalation in the upper and lower respiratory tract [6–8], the specific sPLA₂s involved in asthma have not been delineated previously. In this study, we found that sPLA₂-X and sPLA₂-IIA predominate in the airways of patients with asthma, as well as non-asthmatic controls. In particular, sPLA₂-X is avidly expressed in the airway epithelium, while BAL cells expressed both sPLA₂-IIA and sPLA₂-X. The sPLA₂-V protein was below the level of detection in concentrated BAL fluid, although the lower limit of detection for sPLA₂-V is higher than sPLA₂-IIA and sPLA₂-X in the TRIFA assay; however, the gene expression of sPLA₂-V was also at the threshold of detection in both the airway epithelium and BAL cells suggesting that if sPLA₂-V is present, it is present at very low levels. The results also suggest that sPLA₂-X and sPLA₂-IIA may both be increased in asthma, but the magnitude of this increase in the airways is modest and only clearly apparent after normalization to the total protein concentration in BAL fluid. The sPLA₂ levels in this study were normalized to protein levels because it is likely that variable loss of protein during the 10-fold concentration step needed to measure these enzymes in BAL fluid led to heterogeneity in the data. Although it is not possible to determine from these data if sPLA₂s play a causal role in asthma, there is strong preclinical evidence of an *in vivo* role of sPLA₂-X, and we found that the levels of sPLA₂-X were correlated with lung function, neutrophilic inflammation, and the levels of the eicosanoid PGE₂ in the present study suggesting that sPLA₂-X may play a role in asthma pathogenesis. The sPLA₂-IIA protein was also elevated, especially in the severe asthma group; however, correlations between the levels of sPLA₂-IIA and features of asthma were not apparent in the present study.

Our major hypothesis was that sPLA₂-X would be elevated in asthma based on the high functional capacity of this enzyme [24] and *in vivo* evidence that sPLA₂-X plays a key role in airway inflammation, eicosanoid formation, AHR, and structural remodelling of the lung [11]. In humans, we demonstrated previously that the gene expression of sPLA₂-X is increased in induced sputum

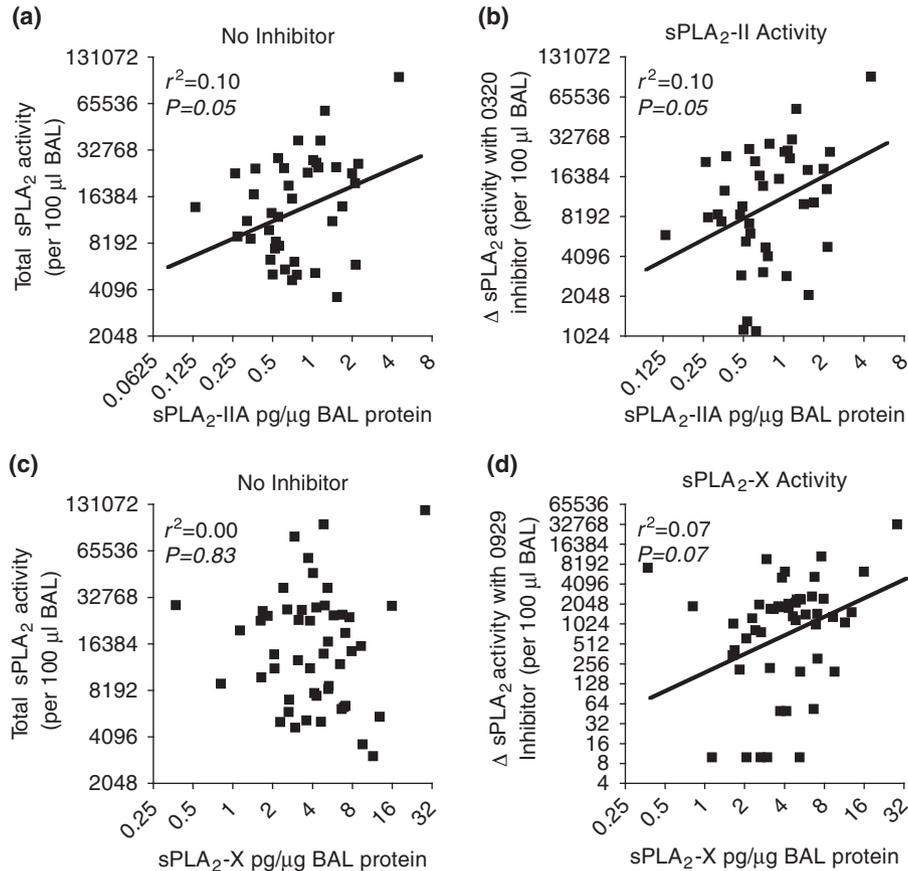


Fig. 5. Comparison of enzyme-specific sPLA₂ activity to sPLA₂ protein levels in BAL fluid. The sPLA₂-IIA protein levels were associated with the total sPLA₂ enzymatic activity (a) and the sPLA₂-II activity (b) based on inhibition by the ROC-0320 inhibitor. The sPLA₂-X protein level was not associated with total sPLA₂ enzymatic activity (c), but the sPLA₂-X protein level tended to be associated with the activity attributed to sPLA₂-X based on inhibition of sPLA₂ activity by ROC-0929 (d).

cells of asthmatics with EIB relative to non-asthmatic controls, and observed an increase in sPLA₂-X protein in the airway lining fluid and immunostaining in epithelial cells and macrophages in the airways following exercise challenge [10]. Asthmatics with EIB have elevated levels of inflammatory eicosanoids in induced sputum and exhaled breath condensate [26, 27], and the release of eicosanoids following exercise challenge plays a major role in disease pathogenesis [28, 29]. The overproduction of eicosanoids has also been described in severe asthma [14, 15]. The results presented here further support an increase in sPLA₂-X in the airways in association with markers of lung function and eicosanoid formation in asthma.

In contrast to some other studies [9], we were unable to identify differences in total sPLA₂ activity between the asthmatic and non-asthmatic subjects. A major advance in this study is the demonstration that both the sPLA₂-X (ROC-0929) and sPLA₂-IIA (ROC-0320) inhibitors result in significant decreases in sPLA₂ activity in BAL fluid, and that there is little residual sPLA₂ activity once these two enzymes are inhibited. One reason we were not able to

demonstrate differences in sPLA₂s activity between the groups is that the assay primarily reflects the sPLA₂-IIA activity in the sample due to the tight binding of sPLA₂-IIA to anionic phospholipid membranes such as those of the *E. coli* radiometric assay used in the present study resulting in specific activity of sPLA₂-IIA that is ~8- to 9-fold higher than for sPLA₂-X in this assay. This higher specific activity for sPLA₂-IIA in the assay is also reflected in the association between total sPLA₂ activity and the levels of sPLA₂-IIA protein in BAL fluid. The difference in enzyme activity in this assay does not signify a larger amount of sPLA₂-IIA enzyme in the airways relative to sPLA₂-X. Taking the difference in specific activity into account, the activity assay is consistent with a slightly larger amount of sPLA₂-X protein than sPLA₂-IIA protein in BAL fluid normalized to total protein. Because of the modest overlap in specificity of the inhibitors (Table 1) and the marked differences in specific activity, it is not possible to determine if there is greater sPLA₂-X activity in the airways of asthmatics using this assay at present. It is notable that in contrast to the specific activity on *E. coli* membranes, sPLA₂-X is ~30 times more active on the

mammalian major outer cell wall phospholipid phosphatidylcholine than sPLA₂-IIA, and the release of free arachidonate from mammalian HEK293 cells is ~1000 times higher for sPLA₂-X over the sPLA₂-IIA enzyme [24]. Thus, the modest increase in sPLA₂-X protein identified in this study may have a major biological effect on eicosanoid metabolism *in vivo* in human airways because of the high activity and also because target cells with the critical enzymes involved in eicosanoid formation are increased in the airways of patients with asthma. High functional activity is also present *in vitro* and *in vivo* for sPLA₂-V, but the apparent levels of sPLA₂-V in the present study were significantly lower than the sPLA₂-X protein, a finding that was further confirmed at the level of gene expression in the epithelium and in BAL cells.

The significance of the increased sPLA₂-IIA in severe asthma is uncertain because this enzyme is ubiquitous and elevated in a wide range of inflammatory conditions including the peripheral blood of patients with pancreatitis and joint fluid from patients with arthritis. Recently, Misso et al. [30] demonstrated that sPLA₂ activity in the blood may be increased in severe asthmatics and is increased during acute asthma. Although the Misso study did not specifically measure sPLA₂-IIA activity, the lipid vesicles used in the Misso study have higher specific activity for IIA as compared with groups V or X [24], and the heparin affinity column used in that study would not have captured sPLA₂-X. Further uncertainty exists regarding the relation of sPLA₂-IIA to asthma because an sPLA₂ inhibitor (LY333013) that is active predominantly against sPLA₂-II (i.e. IIA, D, E, and F) failed to inhibit either the early or late response to allergen challenge [31].

There are several limitations that should be considered in interpreting these data. There was a significant overlap between the levels of sPLA₂ between the different groups of subjects. Although we show that the sPLA₂-X level was related to a number of markers of inflammation, there may be other aspects of asthma such as the severity of indirect AHR that could be more strongly associated with

the levels of sPLA₂-X in the airways. Although we measured the sPLA₂s down to levels as low as 2 pg per sample, measurement of sPLA₂s required a 10-fold concentration step that resulted in the loss of protein and introduced variability into the measurement.

In summary, we found that sPLA₂ groups IIA and X were responsible for the majority of sPLA₂ enzymatic activity in BAL fluid, based on specific activity and protein levels. In a heterogeneous population of asthmatics, the sPLA₂-X normalized to total BAL protein is modestly increased in asthma and associated with asthma severity, lung function, and multiple measures of airway inflammation. These results indicate that sPLA₂-X may be an important mediator of airway inflammation in asthma, particularly in patients with severe asthma who have poor asthma control despite treatment with currently available treatments. Therapies that target sPLA₂-X might provide therapeutic benefit in asthma through the inhibition of eicosanoid-mediated airway inflammation.

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