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Relationship between levels of secreted phospholipase A_2 groups IIA and X in the airways and asthma severity

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Abstract

Background—Secreted phospholipase A_2s ($sPLA_2$) may be important mediators of asthma, but the specific $sPLA_2$s involved in asthma are not known.

Objectives—To evaluate $sPLA_2$ group IIA, V, and X proteins ($sPLA_2$-IIA, $sPLA_2$-V and $sPLA_2$-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 $sPLA_2$ enzymes and airway inflammation, asthma severity, and lung function.

Methods—The expression of $sPLA_2$-IIA, $sPLA_2$-V and $sPLA_2$-X in BAL cells and epithelial brushings was assessed by qPCR. The levels of these $sPLA_2$ proteins and $sPLA_2$ 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 $sPLA_2$-X at higher levels than either $sPLA_2$-IIA or $sPLA_2$-V, whereas BAL cells expressed $sPLA_2$-IIA and $sPLA_2$-X at similar levels. The majority of $sPLA_2$ activity in BAL fluid was attributed to either $sPLA_2$-IIA or $sPLA_2$-X. After 10-fold concentration of BAL fluid, the levels of $sPLA_2$-X normalized to total protein were increased in asthma and were associated with lung function, the concentration of induced sputum neutrophils, and prostaglandin E_2. The levels of $sPLA_2$-IIA were elevated in asthma when normalized to total protein, but where not related to lung function, markers of airway inflammation or eicosanoid formation.

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Conclusions and Clinical Relevance—These data indicate that sPLA$_2$-IIA and sPLA$_2$-X are the major sPLA$_2$s in human airways, and suggest a link between the levels of sPLA$_2$-X in the airways and several features of asthma.

Keywords

Asthma; Eicosanoid; Epithelial Cell; Leukotriene; Phospholipase

INTRODUCTION

Inflammatory eicosanoids such as leukotrienes (LT)s and certain prostaglandins (PG)s such as PGD_2 have important pathophysiological roles in airway inflammation, hyperresponsiveness (AHR) and structural remodeling 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 A_2 (PLA$_2$) [2]. Recently, 10 mammalian secreted PLA$_2$s (sPLA$_2$s) have been identified, and at least some of these enzymes may coordinate eicosanoid synthesis along with the well-described cytosolic PLA$_2$ (i.e. cPLA$_2$α) [3–5]. Early studies demonstrated increased sPLA$_2$ 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 sPLA$_2$ activity was found to be increased in BAL fluid from asthmatic subjects, but the specific sPLA$_2$s were not identified in these studies [6–9]. We previously demonstrated that the gene expression of sPLA$_2$ group X (sPLA$_2$-X) is increased in induced sputum cells of asthmatics and that the level of sPLA$_2$-X in induced sputum supernatant increased following exercise challenge among asthmatics with exercise-induced bronchoconstriction [10]. Deletion of sPLA$_2$-X inhibits the development of airway inflammation, AHR, and structural remodeling, and deletion of sPLA$_2$ group V (sPLA$_2$-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, C_4, D_4 and E_4 has been demonstrated in BAL fluid of poorly controlled asthmatics [14,15]. Stable severe asthma is characterized by an increase in LTB_4 and a increase in airway neutrophils [16]. Thus, sPLA$_2$s may play significant roles in dysregulated eicosanoid synthesis in asthma, but the specific sPLA$_2$s involved in asthma are not known.

We assessed the sPLA$_2$ enzymatic activity and the levels of selected sPLA$_2$s in BAL fluid of asthmatics with severe asthma as compared to asthmatics with mild-to-moderate asthma and non-asthmatic controls. We focused on the levels of sPLA$_2$ groups IIA, V, and X as these groups have been most strongly implicated in asthma. The levels of the sPLA$_2$s in BAL fluid were quantitatively measured by time-resolved fluoroimmunoassay [17]. We used selective enzyme inhibitors to determine the contribution of the different sPLA$_2$ enzymes to BAL fluid sPLA$_2$ enzymatic activity. Our goals were to 1) characterize the expression of sPLA$_2$-X relative to sPLA$_2$-IIA and sPLA$_2$-V in the airway epithelium and immune cells present in BAL fluid, 2) determine the relative amounts of these sPLA$_2$s in BAL fluid supernatant from asthmatic and normal subjects, and 3) determine if specific sPLA$_2$s are increased in asthma in relation to asthma severity, lung function, AHR, and airway inflammation.

Some of the results of this study have been previously reported 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 center 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 1 of 2 major and 2 of 7 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 one second (FEV_1) ≥ 80% predicted, and a negative methacholine challenge.

Bronchoscopy and Induced Sputum

Bronchoscopy with bronchoalveolar lavage (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 hemocytometer, and slides for differential cell counts were prepared with a cytocentrifuge. The levels of PGE_2 in induced sputum supernatant were assayed following solid phase extraction by ELISA (Cayman Chemical, Ann Arbor, MI).

Quantitative Real-time PCR

Real-time PCR was performed on the ABI Prism 7700 sequence detection system (Applied Biosystems). Primer-probe sets for selected genes were obtained from the Applied Biosystems Assays-on-Demand repository (Applied Biosystems, Foster City, CA). The reference numbers are as follows: $sPLA_2$-IIA, Hs00179898_m1; $sPLA_2$-V, Hs01099100_m1; and $sPLA_2$-X, Hs00358567_m1. The primer for $sPLA_2$-IIA was validated against K562 cell cDNA with a Ct value of 38 for 100 ng per reaction. The primer for $sPLA_2$-V was validated against recombinant plasmid DNA with a Ct value of 17.5 for 0.1 ng per reaction. The primer for $sPLA_2$-X was validated using 16HBE14o- cell cDNA with a Ct 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 (Ct) was applied for each gene of interest. Relative mRNA expression levels were calculated using the delta Ct method.

Quantification of Selected $sPLA_2$s in BAL Fluid

The $sPLA_2$ proteins in BAL fluid were measured after 10-fold concentration using a centrifugal concentrator with a 3 kDa molecular weight cutoff (Millipore, Danvers, MA). Triton-X 0.5% was added prior to concentration to improve recovery. The recoveries of recombinant proteins measured by time-resolved fluoroimmunoassay (TRFIA) before and after concentration were 90.3% for $sPLA_2$-X, 22.4% for $sPLA_2$-IIA, and 84.8% for $sPLA_2$-V. The assay is based on $sPLA_2$-specific polyclonal rabbit anti-$sPLA_2$ capture antibodies and anti-$sPLA_2$ detection antibodies that are Eu^{3+}-labeled to measure time resolve immunofluorescence [23]. Ninety-six well microplates were coated with anti-$sPLA_2$ antibody and blocked with Tris-buffer containing 1.0% bovine serum albumin (BSA), 6%

D-sorbitol, 0.9% NaCl and 1 mM $CaCl_2$. 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-$sPLA_2$ 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 (Eu^{3+}-labeled anti-$sPLA_2$ antibody containing > 5×10^5 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) was incubated in the wells for 15 min with shaking, and fluorescence was measured at excitation and emission wavelengths of 340 nm and 615 nm. The lower limit of detection is 8 pg/ml for $sPLA_2$-X, 2 pg/ml for $sPLA_2$-IIA, and 96 pg/ml for $sPLA_2$-V. Linear standard curves were derived from recombinant human $sPLA_2$s [24].

Measurement and Selective Inhibition of $sPLA_2$ Activity in BAL Fluid

Preliminary studies using the fluorometric assay with the pyrene-labeled phospholipid analog showed that the assay was not sensitive enough to measure $sPLA_2$ activity in all samples without further concentration, so the $sPLA_2$ enzymatic activity in BAL fluid was assessed by monitoring the release of free fatty acid from [^{3}H]oleate-labeled *E. coli* membranes [25]. Unconcentrated BAL fluid or recombinant $sPLA_2$ proteins were incubated at 37°C for 1 hour with ~200,000 dpm of [^{3}H]oleate-labeled *E. coli* membranes in 100 μl of reaction buffer (0.1 M Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 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 13,000g for 3 min, and 400 μL of the supernatant was used for scintillation counting. Control incubations in the absence of $sPLA_2$ were carried out in parallel to calculate specific hydrolysis. Recombinant human $sPLA_2$ 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 $sPLA_2$ 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 $sPLA_2$ activity. Separate aliquots of BAL fluid were measured simultaneously in the presence of inhibitors that are selective for $sPLA_2$ groups IIA/IIE designated ROC-0320 and $sPLA_2$-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 the inhibitors in BAL fluid, each inhibitor was used at a 2 μM final concentration and preincubated for 10 min at room temperature prior to the addition of the reaction mixture containing [^{3}H]oleate-labeled *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 $sPLA_2$ levels and $sPLA_2$ 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 $sPLA_2$ activity due to inhibitors were assessed by the Wilcoxon signed-rank test. The $sPLA_2$ protein levels were associated with other parameters such as lung function by linear regression after log transformation. The primary hypothesis related to differences in $sPLA_2$-X was assessed without adjustment of the *P*-value for multiple comparisons. The additional studies evaluating the levels of $sPLA_2$-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 regards 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 PGE_2 in induced sputum supernatant were increased in the asthma groups, particularly severe asthma.

Epithelial and BAL Cell $sPLA_2$ Gene Expression

The expression of $sPLA_2$ groups IIA, V and X in epithelial brushings and BAL cells were assessed in 5 representative samples from each group. In airway epithelial brushings the expression of $sPLA_2$-X overall from all groups combined was significantly higher than the expression of $sPLA_2$-V and $sPLA_2$-IIA (Fig 1A). In the epithelium $sPLA_2$-V and $sPLA_2$-IIA were near the threshold of detection and there was no difference between these two $sPLA_2$s. In BAL cells, there was no difference in the expression of $sPLA_2$-X and $sPLA_2$-IIA, and both of these $sPLA_2$s had higher expression than $sPLA_2$-V (Fig 1B). In BAL cells, the expression of $sPLA_2$-V was near the threshold of detection. In this modest number of samples, differences in the expression of $sPLA_2$ groups IIA, V, and X between the groups of subjects were not apparent either in the airway epithelium or in the BAL cell pellet.

Differences in $sPLA_2$ Protein in the Airways

Following 10-fold concentration of BAL fluid, the $sPLA_2$-X protein was detectable by TRFIA in all but 4 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 $sPLA_2$-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 μg/mL to 89.0 μg/mL across the study population and varied between the three groups (Table 4). Since the recovery of total protein following concentration was variable, the $sPLA_2$-X levels were normalized to total protein levels in BAL fluid and expressed as pg of $sPLA_2$-X per μg of BAL protein. The concentration of $sPLA_2$-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 $sPLA_2$-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 detectible $sPLA_2$-X-specific activity in the activity assay described below prior to concentration, suggesting that the $sPLA_2$-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 influential effect on the analysis. The level of $sPLA_2$-X tended to be inversely correlated with the % predicted FEV_1 ($P=0.06$, Fig 2B). The level of $sPLA_2$-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 $sPLA_2$-X was correlated with the concentration of PGE_2 in induced sputum (*P=0.03*, Fig 2D). There was no difference in the levels of $sPLA_2$-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 $sPLA_2$-V in 10-fold concentrated BAL fluid from 5 representative asthmatics were all below the detection limit of 96 pg/mL, so comparisons between the groups for $sPLA_2$-V protein were not performed.

Sufficient BAL fluid was available to measure the level of $sPLA_2$-IIA by TRFIA in 43 of the subjects (13 controls, 15 mild-to-moderate asthma, and 15 severe asthma). The $sPLA_2$-IIA protein was detectable in all BAL samples, but values were below the linear range of the assay of 2 pg/mL in 4 controls and 8 asthmatics following 10-fold concentration of the samples. These low values were set to 2 pg/mL. The levels of $sPLA_2$-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 the severe asthma and other groups (*P<0.05*, Fig 3). The levels of $sPLA_2$-IIA were not associated with the % predicted FEV_1 (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 PGE_2 in induced sputum (n=19, r^2=0.00, *P=0.99*). There was no difference in the levels of $sPLA_2$-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 $sPLA_2$ Activity in BAL Fluid

The total $sPLA_2$ activity per 100 μl of BAL fluid was no different between the groups of subjects (Fig 4A). A number of samples had very high $sPLA_2$ activity, including 6 samples that required 10-fold dilution, and 13 samples that required 2-fold dilution to keep the activity levels within the linear range of the assay. The majority of this high activity was due to $sPLA_2$-IIA because of the high specific activity for $sPLA_2$-IIA is ~8- to 9-fold higher than $sPLA_2$-X in this assay. To measure the contribution of the group II and X $sPLA_2$ enzymes to the total $sPLA_2$ activity, in separate aliquots we measured the activity after treatment with 2.0 μM ROC-0320 a $sPLA_2$-II inhibitor, and with 2.0 μM ROC-0929 a specific $sPLA_2$-X inhibitor. At these molar concentrations the ROC-0320 compound completely inhibits the recombinant human $sPLA_2$-IIA enzyme, while inhibiting recombinant human $sPLA_2$-X by about 28%, and the ROC-0929 compound completely inhibits $sPLA_2$-X, while inhibiting the $sPLA_2$-IIA enzyme by 24% (Table 1). In BAL fluid, there was 76.9% (*P<0.0001*) decrease in $sPLA_2$ activity with the $sPLA_2$-II inhibitor; the percent change in $sPLA_2$ 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 $sPLA_2$-X inhibitor, there was an 11.9% (*P<0.0001*) decrease in $sPLA_2$ activity overall; however there was no difference in the percent change in $sPLA_2$ activity with this inhibitor between the groups of subjects (Fig 4B). Because of the higher specific activity for $sPLA_2$-IIA over $sPLA_2$-X in this assay, these results are consistent with the protein analysis showing an increased amount of $sPLA_2$-X protein over $sPLA_2$-IIA protein; however, this high $sPLA_2$-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 $sPLA_2$ activity assay primarily reflects $sPLA_2$-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 $sPLA_2$ inhibitor activity after exclusion of samples with very high $sPLA_2$ 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 $sPLA_2$ activity in BAL fluid was associated with the concentration of

$sPLA_2$-IIA enzyme in BAL fluid measured by the TRFIA assay (Fig 5A). The change in $sPLA_2$ activity with the $sPLA_2$-II inhibitor ROC-0320 was also associated with the concentration of the $sPLA_2$-IIA enzyme (Fig 5B), while the change in activity due to the $sPLA_2$-X inhibitor ROC-0929 was not associated with the levels of the $sPLA_2$-IIA protein (r^2=0.02, *P=0.45*). The total $sPLA_2$ activity in BAL fluid was not associated with the concentration of $sPLA_2$-X enzyme in BAL fluid (Fig 5C). Similarly, there was no association between the change in $sPLA_2$ activity with the $sPLA_2$-II inhibitor ROC-0320 and the levels of $sPLA_2$-X (r^2=0.01, *P=0.48*); however, the change in $sPLA_2$ activity with the $sPLA_2$-X inhibitor ROC-0929 tended to be associated with the levels of $sPLA_2$-X protein in BAL fluid (Fig 5D). These findings indicate that the $sPLA_2$ activity prior to concentration is consistent with the $sPLA_2$ protein concentrations that were measured after concentration; however, the marked differences in specific activity makes the $sPLA_2$-IIA activity dominant and the contribution of $sPLA_2$-X difficult to observe in the activity analysis.

DISCUSSION

Although prior studies have identified an increase in $sPLA_2$ activity in asthma [9] and following allergen inhalation in the upper and lower respiratory tract [6–8], the specific $sPLA_2$s involved in asthma have not been previously delineated. In this study, we found that $sPLA_2$-X and $sPLA_2$-IIA predominate in the airways of patients with asthma, as well as non-asthmatic controls. In particular, $sPLA_2$-X is avidly expressed in the airway epithelium, while BAL cells expressed both $sPLA_2$IIA and $sPLA_2$-X. The $sPLA_2$-V protein was below the level of detection in concentrated BAL fluid, although the lower limit of detection for $sPLA_2$-V is higher than $sPLA_2$ IIA and $sPLA_2$-X in the TRIFA assay; however, the gene expression of $sPLA_2$-V was also at the threshold of detection in both the airway epithelium and BAL cells suggesting that if $sPLA_2$-V is present, it is present at very low levels. The results also suggest that $sPLA_2$-X and $sPLA_2$-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 $sPLA_2$ 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 $sPLA_2$s play a causal role in asthma, there is strong preclinical evidence of an *in vivo* role of $sPLA_2$-X, and we found that the levels of $sPLA_2$-X were correlated with lung function, neutrophilic inflammation, and the levels of the eicosanoid PGE_2 in the present study suggesting that $sPLA_2$-X may play a role asthma pathogenesis. The $sPLA_2$-IIA protein was also elevated, especially in the severe asthma group; however, correlations between the levels of $sPLA_2$-IIA and features of asthma were not apparent in the present study.

Our major hypothesis was that $sPLA_2$-X would be elevated in asthma based on the high functional capacity of this enzyme [24] and *in vivo* evidence that $sPLA_2$-X plays a key role in airway inflammation, eicosanoid formation, AHR, and structural remodeling of the lung [11]. In humans we previously demonstrated that the gene expression of $sPLA_2$-X is increased in induced sputum cells of asthmatics with exercise-induced bronchoconstriction (EIB) relative to non-asthmatic controls, and observed an increase in $sPLA_2$-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 $sPLA_2$-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 $sPLA_2$ activity between the asthmatic and non-asthmatic subjects. A major advance in this study is the demonstration that both the $sPLA_2$-X (ROC-0929) and $sPLA_2$-IIA (ROC-0320) inhibitors result in significant decreases in $sPLA_2$ activity in BAL fluid, and that there is little residual $sPLA_2$ activity once these two enzymes are inhibited. One reason we were not able to demonstrate differences in $sPLA_2$s activity between the groups is that the assay primarily reflects the $sPLA_2$-IIA activity in the sample due to the tight binding of $sPLA_2$-IIA to anionic phospholipid membranes such as those of the *E. coli* radiometric assay used in the present study resulting in specific activity of $sPLA_2$-IIA that is ~8- to 9-fold higher than for $sPLA_2$-X in this assay. This higher specific activity for $sPLA_2$-IIA in the assay is also reflected in the association between total $sPLA_2$ activity and the levels of $sPLA_2$-IIA protein in BAL fluid. The difference in enzyme activity in this assay does not signify a larger amount of $sPLA_2$-IIA enzyme in the airways relative to $sPLA_2$-X. Taking the difference in specific activity into account, the activity assay is consistent with a slightly larger amount of $sPLA_2$-X protein than $sPLA_2$-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 $sPLA_2$-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, $sPLA_2$-X is ~30 times more active on the mammalian major outer cell wall phospholipid phosphatidylcholine than $sPLA_2$-IIA, and the release of free arachidonate from mammalian HEK293 cells is ~1,000 times higher for $sPLA_2$-X over the $sPLA_2$-IIA enzyme [24]. Thus, the modest increase in $sPLA_2$-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 eicosanoids formation are increased in the airways of patients with asthma. High functional activity is also present *in vitro* and *in vivo* for $sPLA_2$-V, but the apparent levels of $sPLA_2$-V in the present study were significantly lower than the $sPLA_2$-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 $sPLA_2$-IIA in severe asthma is uncertain since 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. demonstrated that $sPLA_2$ activity in the blood may be increased in severe asthmatics and is increased during acute asthma [30]. Although the Misso study did not specifically measure $sPLA_2$-IIA activity, the lipid vesicles used in the Misso study have higher specific activity for IIA as compared to groups V or X [24], and the heparin affinity column used in that study would not have captured $sPLA_2$-X. Further uncertainty exists regarding the relation of $sPLA_2$-IIA to asthma since a $sPLA_2$ inhibitor (LY333013) that is active predominantly against $sPLA_2$-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 significant overlap between the levels of $sPLA_2$ between the different groups of subjects. Although we show that the $sPLA_2$-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 $sPLA_2$-X in the airways. Although we measured the $sPLA_2$s down to levels as low as 2 pg per sample, measurement of $sPLA_2$s required a 10-fold concentration step that resulted in the loss of protein and introduced variability into the measurement.

In summary, we found that $sPLA_2$ groups IIA and X were responsible for the majority of $sPLA_2$ enzymatic activity in BAL fluid, based on specific activity and protein levels. In a

heterogeneous population of asthmatics, the $sPLA_2$-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 $sPLA_2$-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 $sPLA_2$-X might provide therapeutic benefit in asthma through inhibition of eicosanoid-mediated airway inflammation.

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Abbreviations used

AHR	Airway hyperresponsiveness
BAL	Bronchoalveolar lavage
BMI	Body mass index
EIB	Exercise-induced bronchoconstriction
FEV_1	Forced expiratory volume in one second
$cPLA_2$	Cytosolic phospholipase A_2
CysLT	Cysteinyl leukotriene
$CysLTR_1$	Cysteinyl leukotriene 1 receptor
LT	Leukotriene
PG	Prostaglandin
$sPLA_2$	Secreted phospholipase A_2
TRFIA	Time-resolved fluoroimmunoassay

References

1. Hallstrand TS, Henderson WR Jr. An update on the role of leukotrienes in asthma. Curr Opin Allergy Clin Immunol. 2010; 10:60–66. [PubMed: 19915456]
2. Triggiani M, Granata F, Giannattasio G, Marone G. Secretory phospholipases A_2 in inflammatory and allergic diseases: not just enzymes. J Allergy Clin Immunol. 2005; 116:1000–1006. [PubMed: 16275367]
3. Balboa MA, Shirai Y, Gaietta G, Ellisman MH, Balsinde J, Dennis EA. Localization of group V phospholipase A_2 in caveolin-enriched granules in activated $P388D_1$ macrophage-like cells. J Biol Chem. 2003; 278:48059–48065. [PubMed: 12963740]
4. Mounier CM, Ghomashchi F, Lindsay MR, James S, Singer AG, Parton RG, Gelb MH. Arachidonic acid release from mammalian cells transfected with human groups IIA and X secreted phospholipase A_2 occurs predominantly during the secretory process and with the involvement of cytosolic phospholipase A_2-α. J Biol Chem. 2004; 279:25024–25038. [PubMed: 15007070]
5. Satake Y, Diaz BL, Balestrieri B, Lam BK, Kanaoka Y, Grusby MJ, Arm JP. Role of group V phospholipase A_2 in zymosan-induced eicosanoid generation and vascular permeability revealed by targeted gene disruption. J Biol Chem. 2004; 279:16488–16494. [PubMed: 14761945]
6. Bowton DL, Seeds MC, Fasano MB, Goldsmith B, Bass DA. Phospholipase A_2 and arachidonate increase in bronchoalveolar lavage fluid after inhaled antigen challenge in asthmatics. Am J Respir Crit Care Med. 1997; 155:421–425. [PubMed: 9032172]

7. Chilton FH, Averill FJ, Hubbard WC, Fonteh AN, Triggiani M, Liu MC. Antigen-induced generation of lyso-phospholipids in human airways. J Exp Med. 1996; 183:2235–2245. [PubMed: 8642333]
8. Stadel JM, Hoyle K, Naclerio RM, Roshak A, Chilton FH. Characterization of phospholipase A_2 from human nasal lavage. Am J Respir Cell Mol Biol. 1994; 11:108–113. [PubMed: 8018333]
9. Triggiani M, Giannattasio G, Calabrese C, Loffredo S, Granata F, Fiorello A, Santini M, Gelb MH, Marone G. Lung mast cells are a source of secreted phospholipases A_2. J Allergy Clin Immunol. 2009; 124:558–565. 65 e1–3. [PubMed: 19541351]
10. Hallstrand TS, Chi EY, Singer AG, Gelb MH, Henderson WR Jr. Secreted phospholipase A_2 group X overexpression in asthma and bronchial hyperresponsiveness. Am J Respir Crit Care Med. 2007; 176:1072–1078. [PubMed: 17901411]
11. Henderson WR Jr, Chi EY, Bollinger JG, Tien YT, Ye X, Castelli L, Rubtsov YP, Singer AG, Chiang GK, Nevalainen T, Rudensky AY, Gelb MH. Importance of group X-secreted phospholipase A_2 in allergen-induced airway inflammation and remodeling in a mouse asthma model. J Exp Med. 2007; 204:865–877. [PubMed: 17403936]
12. Munoz NM, Meliton AY, Arm JP, Bonventre JV, Cho W, Leff AR. Deletion of secretory group V phospholipase A_2 attenuates cell migration and airway hyperresponsiveness in immunosensitized mice. J Immunol. 2007; 179:4800–4807. [PubMed: 17878379]
13. Moore WC, Bleecker ER, Curran-Everett D, Erzurum SC, Ameredes BT, Bacharier L, Calhoun WJ, Castro M, Chung KF, Clark MP, Dweik RA, Fitzpatrick AM, Gaston B, Hew M, Hussain I, Jarjour NN, Israel E, Levy BD, Murphy JR, Peters SP, Teague WG, Meyers DA, Busse WW, Wenzel SE. Characterization of the severe asthma phenotype by the National Heart, Lung, and Blood Institute's Severe Asthma Research Program. J Allergy Clin Immunol. 2007; 119:405–413. [PubMed: 17291857]
14. Pavord ID, Ward R, Woltmann G, Wardlaw AJ, Sheller JR, Dworski R. Induced sputum eicosanoid concentrations in asthma. Am J Respir Crit Care Med. 1999; 160:1905–1909. [PubMed: 10588604]
15. Romagnoli M, Vachier I, Tarodo de la Fuente P, Meziane H, Chavis C, Bousquet J, Godard P, Chanez P. Eosinophilic inflammation in sputum of poorly controlled asthmatics. Eur Respir J. 2002; 20:1370–1377. [PubMed: 12503691]
16. Wenzel SE, Szefler SJ, Leung DY, Sloan SI, Rex MD, Martin RJ. Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids. Am J Respir Crit Care Med. 1997; 156:737–743. [PubMed: 9309987]
17. Nevalainen TJ, Eerola LI, Rintala E, Laine VJ, Lambeau G, Gelb MH. Time-resolved fluoroimmunoassays of the complete set of secreted phospholipases A_2 in human serum. Biochim Biophys Acta. 2005; 1733:210–223. [PubMed: 15863368]
18. Hallstrand TS, Lai YL, Ni Z, Oslund RC, Henderson WR Jr, Gelb MH, Wenzel SE. Relationship between airway levels of secreted phospholipase A_2 group X ($sPLA_2$-X) and asthma severity. Proc Am Thorac Soc. 2009 (abstract).
19. Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. American Thoracic Society. Am J Respir Crit Care Med. 2000; 162:2341–2351. [PubMed: 11112161]
20. National Institutes of Health NH, Lung, and Blood Institute, National Asthma Education and Prevention Program. Bethesda (MD): National Institutes of Health; 2007. Expert Panel report 3: guidelines for the diagnosis and management of asthma.
21. Trudeau J, Hu H, Chibana K, Chu HW, Westcott JY, Wenzel SE. Selective downregulation of prostaglandin E_2-related pathways by the Th2 cytokine IL-13. J Allergy Clin Immunol. 2006; 117:1446–1454. [PubMed: 16751012]
22. Fahy JV, Boushey HA, Lazarus SC, Mauger EA, Cherniack RM, Chinchilli VM, Craig TJ, Drazen JM, Ford JG, Fish JE, Israel E, Kraft M, Lemanske RF, Martin RJ, McLean D, Peters SP, Sorkness C, Szefler SJ. Safety and reproducibility of sputum induction in asthmatic subjects in a multicenter study. Am J Respir Crit Care Med. 2001; 163:1470–1475. [PubMed: 11371420]
23. Degousee N, Ghomashchi F, Stefanski E, Singer A, Smart BP, Borregaard N, Reithmeier R, Lindsay TF, Lichtenberger C, Reinisch W, Lambeau G, Arm J, Tischfield J, Gelb MH, Rubin BB.

Groups IV, V, and X phospholipases A_2s in human neutrophils: role in eicosanoid production and gram-negative bacterial phospholipid hydrolysis. J Biol Chem. 2002; 277:5061–5073. [PubMed: 11741884]

24. Singer AG, Ghomashchi F, Le Calvez C, Bollinger J, Bezzine S, Rouault M, Sadilek M, Nguyen E, Lazdunski M, Lambeau G, Gelb MH. Interfacial kinetic and binding properties of the complete set of human and mouse groups I, II, V, X, and XII secreted phospholipases A_2. J Biol Chem. 2002; 277:48535–48549. [PubMed: 12359733]
25. Touaibia M, Djimde A, Cao F, Boilard E, Bezzine S, Lambeau G, Redeuilh C, Lamouri A, Massicot F, Chau F, Dong CZ, Heymans F. Inhibition of secreted phospholipase A_2. 4-glycerol derivatives of 4,5-dihydro-3-(4-tetradecyloxybenzyl)-1,2,4-4H-oxadiazol-5-one with broad activities. J Med Chem. 2007; 50:1618–1626. [PubMed: 17335183]
26. Carraro S, Corradi M, Zanconato S, Alinovi R, Pasquale MF, Zacchello F, Baraldi E. Exhaled breath condensate cysteinyl leukotrienes are increased in children with exercise-induced bronchoconstriction. J Allergy Clin Immunol. 2005; 115:764–770. [PubMed: 15805996]
27. Hallstrand TS, Moody MW, Aitken ML, Henderson WR Jr. Airway immunopathology of asthma with exercise-induced bronchoconstriction. J Allergy Clin Immunol. 2005; 116:586–593. [PubMed: 16159628]
28. Hallstrand TS, Moody MW, Wurfel MM, Schwartz LB, Henderson WR Jr, Aitken ML. Inflammatory basis of exercise-induced bronchoconstriction. Am J Respir Crit Care Med. 2005; 172:679–686. [PubMed: 15947280]
29. Mickleborough TD, Lindley MR, Ray S. Dietary salt, airway inflammation, and diffusion capacity in exercise-induced asthma. Med Sci Sports Exerc. 2005; 37:904–914. [PubMed: 15947713]
30. Misso NL, Petrovic N, Grove C, Celenza A, Brooks-Wildhaber J, Thompson PJ. Plasma phospholipase A_2 activity in patients with asthma: association with body mass index and cholesterol concentration. Thorax. 2008; 63:21–26. [PubMed: 17573441]
31. Bowton DL, Dmitrienko AA, Israel E, Zeiher BG, Sides GD. Impact of a soluble phospholipase A_2 inhibitor on inhaled allergen challenge in subjects with asthma. J Asthma. 2005; 42:65–71. [PubMed: 15801331]

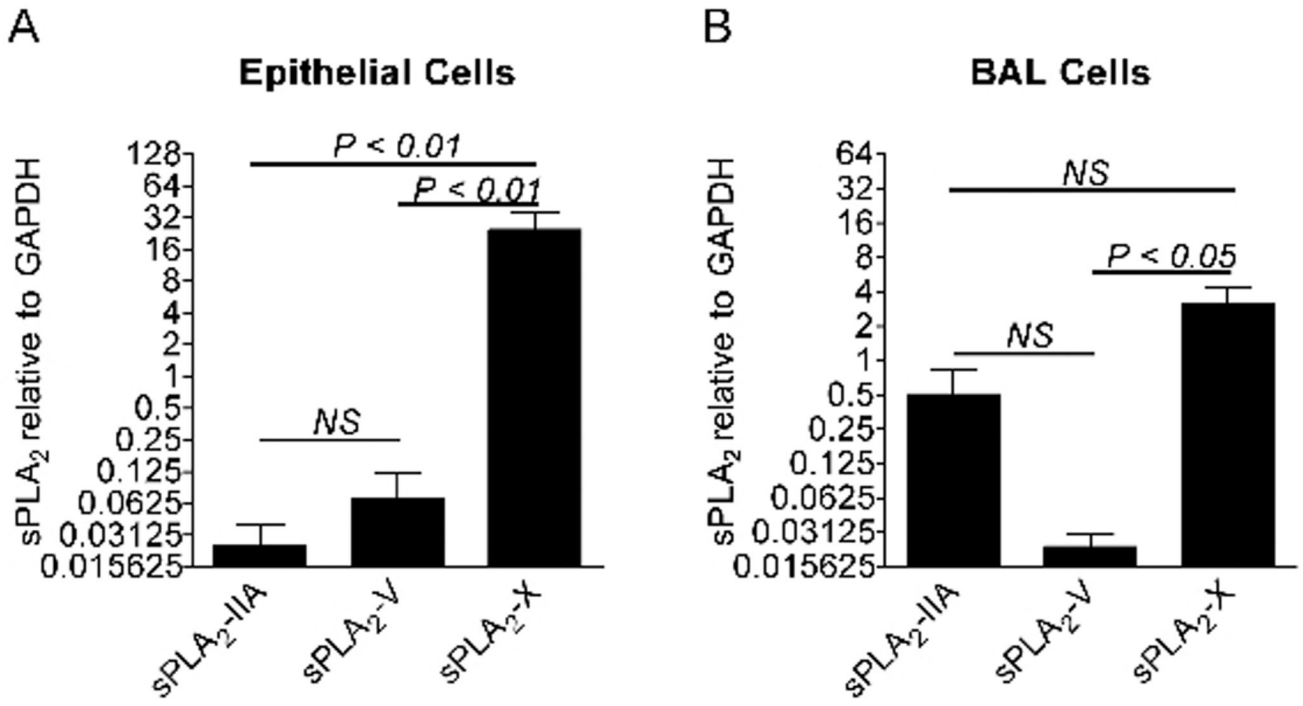


Figure 1.

Expression of the $sPLA_2$ enzymes in the airway epithelium and BAL cells. The gene expression of $sPLA_2$-IIA, $sPLA_2$-V and $sPLA_2$-X was assessed in 15 samples, representing five subjects from each group. The expression of $sPLA_2$-X was significantly higher than the expression of either $sPLA_2$-V or $sPLA_2$-IIA in the epithelium (A). There was no difference in the expression of $sPLA_2$-IIA and sPLA2-V in the epithelium. The expression of $sPLA_2$-X in BAL cells was significantly higher than $sPLA_2$-V, but no different than $sPLA_2$-IIA (B). There was no statistically significant difference between the expression of $sPLA_2$ groups IIA and V in BAL cells.

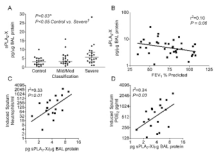


Figure 2.

Levels of $sPLA_2$-X protein in BAL fluid in relation to measures of lung function and inflammation. The levels of $sPLA_2$-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 $sPLA_2$-X were associated with lung function among asthmatics by regression analysis (B). Among asthmatics, the $sPLA_2$-X level in BAL fluid was associated with neutrophils in induced sputum (C), and levels of PGE_2 in induced sputum supernatant (D).

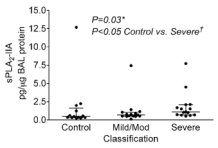


Figure 3.

Levels of $sPLA_2$-IIA protein in BAL fluid in asthma subjects and normal controls. The levels of $sPLA_2$-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.

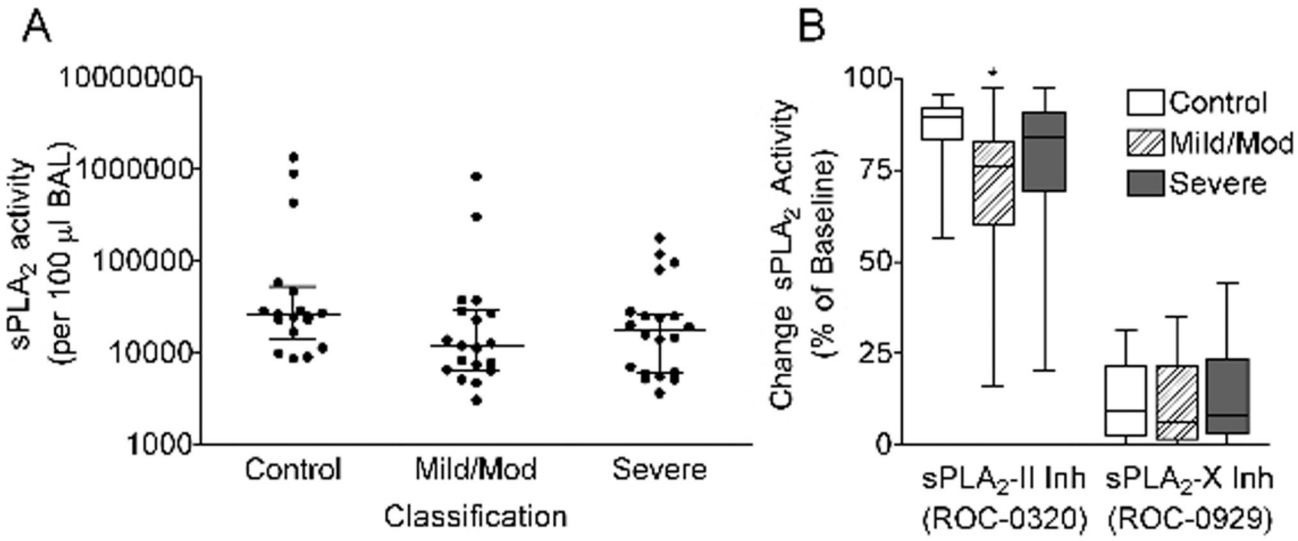


Figure 4.

Assessment of enzyme-specific $sPLA_2$ activity in BAL fluid. No differences in total $sPLA_2$ enzymatic activity in BAL fluid were identified between asthmatic and non-asthmatic groups (A). The $sPLA_2$ enzymatic activity was inhibited 76.9% (*P<0.0001*) by $sPLA_2$-II inhibitor (ROC-0320) and 11.9% (*P<0.0001*) by $sPLA_2$-X inhibitor (ROC-0929)(B). The percent change in $sPLA_2$ 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*).

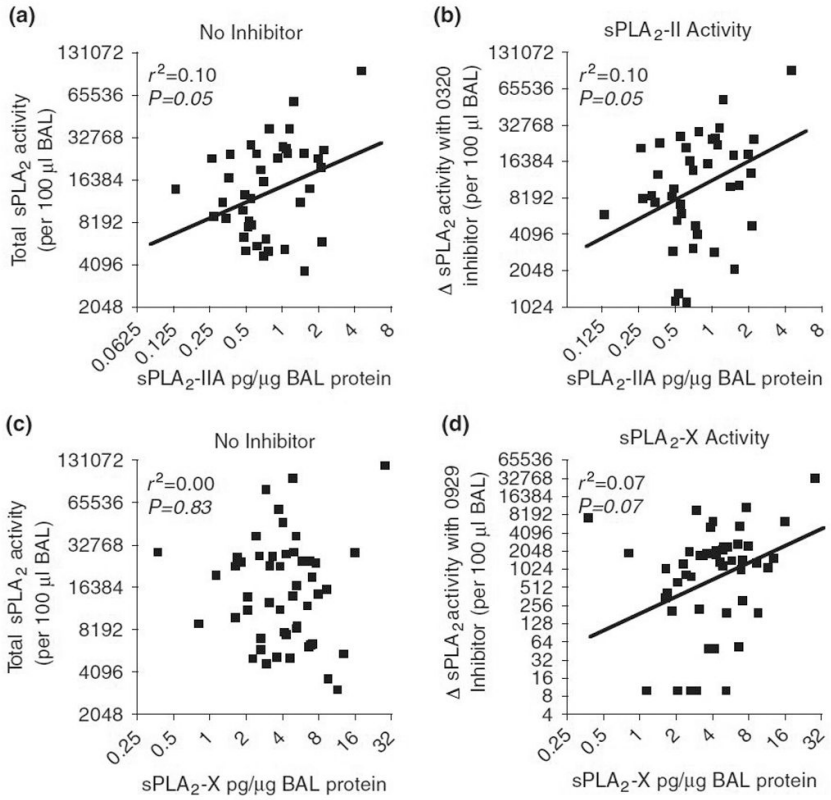


Figure 5.

Comparison of enzyme-specific $sPLA_2$ activity to $sPLA_2$ protein levels in BAL fluid. The $sPLA_2$-IIA protein levels were associated with the total $sPLA_2$ enzymatic activity (A) and the $sPLA_2$-II activity (B) based on inhibition by the ROC-0320 inhibitor. The $sPLA_2$-X protein level was not associated with total $sPLA_2$ enzymatic activity (C), but the $sPLA_2$-X protein level tended to be associated with the activity attributed to $sPLA_2$-X based on inhibition of $sPLA_2$ activity by ROC-0929 (D).

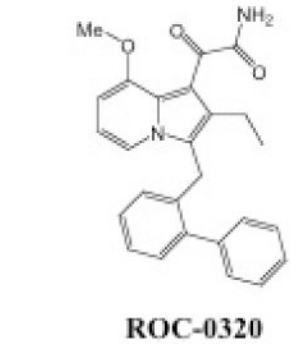
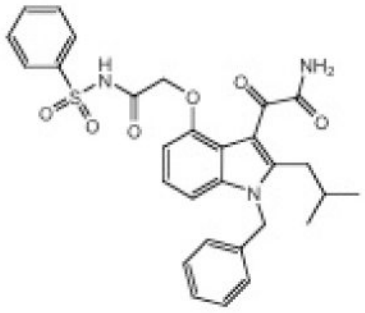
Table 1

Specific activity of $sPLA_2$ proteins in the $sPLA_2$ 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)	0.5μM	90.2	16.4
	1μM	98.5	11.2
	2μM	99.5	28.9
	5μM	98.7	23.5
	10μM	102.8	15.0
$sPLA_2$-X Inhibitor (ROC-0929)	0.5μM	7.1	103.8
	1μM	−2.6	105.9
	2μM	24.0	109.0
	5μM	3.5	95.8
	10μM	17.5	112.7

Table 2

Selectivity of inhibitors [IC50 (nM)*]

sPLA$_2$	**ROC-0929**	**ROC-0320**	
hGIB	>1600	>1600	 ROC-0320  ROC-09290
hGIIA	>1600	35±2	
hGIID**	700±200	>1300	
hGIIE	>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-labeled phosphatidylglycerol as substrate.

** IC50 value obtained using radiolabeled *E. coli* membrane assay.

Table 3

Comparison of the characteristics of study groups.*

		Asthma		
	Control (n=18)	**Mild-Mod (n=19)**	**Severe (n=20)**	***P Value***
Age (yrs)(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		
	Control (n=18)	**Mild-Mod (n=19)**	**Severe (n=20)**	***P Value***
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 × 10^3