Secreted phospholipase A₂ group X overexpression in asthma and bronchial hyperresponsiveness


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What is the current scientific knowledge on this subject?
Secreted phospholipase A₂s (sPLA₂)s have recently emerged as critical regulators of eicosanoid synthesis, but the specific sPLA₂ enzymes expressed in human airways and the role of these enzymes in asthma pathogenesis are not known.

What does this study add to the field?
Group X sPLA₂ (sPLA₂-X) is overexpressed in airway epithelial cells and bronchial macrophages of asthmatic subjects, and released in response to exercise challenge, a stimulus that causes dysregulated eicosanoid synthesis.
Abstract

Rationale: Secreted PLA2s (sPLA2)s play key regulatory roles in the biosynthesis of eicosanoids such as the cysteinyl leukotrienes (CysLT)s, but the role of these enzymes in the pathogenesis of asthma is not known. Objectives: To establish if sPLA2s are overexpressed in the airways of asthmatics, and determine if these enzymes may play a role in the generation of eicosanoids in exercise-induced bronchoconstriction (EIB). Methods: Induced sputum samples were obtained from asthmatic subjects with EIB, and non-asthmatic controls at baseline, and on a separate day 30 min after exercise challenge. The expression of the PLA2s in induced sputum cells and supernatant was determined by quantitative PCR, immunocytochemistry, and Western blot. Main Results: The sPLA2s expressed at the highest levels in airway cells of asthmatics were groups X and XIIA. Group X sPLA2 (sPLA2-X) was differentially overexpressed in asthma and localized to airway epithelial cells and bronchial macrophages. The gene expression, immunostaining in airway epithelial cells and bronchial macrophages, and the level of the extracellular sPLA2-X protein in the airways increased in response to exercise challenge in the asthma group, while the levels were lower and unchanged following challenge in non-asthmatic controls. Conclusions: Increased expression of sPLA2-X may play a key role in the dysregulated eicosanoid synthesis in asthma.

Abstract Word Count: 207

Key Words: Asthma, Eicosanoid, Epithelial Cell, Leukotriene, and Macrophage.
INTRODUCTION

The biosynthesis of eicosanoids in the airways is a key component of asthma pathogenesis (1). Eicosanoids are the products of arachidonic acid (AA), including the leukotrienes (LT)s, hydroxyeicosatetraenoic acids (HETE)s, and prostaglandins (PG)s. LTs such as the cysteinyl leukotrienes (CysLT)s are produced at increased levels in the airways of asthmatics (2), and inhalation of CysLTs reproduces many of the features of asthma (3). Members of the PG family include PGD_2 that serves as a bronchoconstrictor (4), and PGE_2 that has a bronchodilatory and bronchoprotective role in the airways (5). A major manifestation of asthma is exercise-induced bronchoconstriction (EIB) that reflects the degree of indirect bronchial hyperresponsiveness (BHR) (6). Dysregulated eicosanoid synthesis is prominent in indirect BHR (7-9). The levels of CysLTs in the airways, measured in exhaled breath condensate (10) or in induced sputum (11), are higher in asthmatics with EIB than asthmatics without EIB. In particular, the ratio of CysLTs to PGE_2 is increased in induced sputum of asthmatics with EIB (11). Based on our prior work (8) and the results of Mickleborough and colleagues (9) demonstrating a sustained increase in CysLTs and PGD_2 in the airways, and concurrent decrease in the level of PGE_2 (8) following exercise challenge, we undertook this investigation to evaluate the upstream regulators of eicosanoid production in the airways of asthmatics with EIB.

A key regulatory mechanism for eicosanoid biosynthesis is through phospholipase A_2 (PLA_2) that hydrolyzes the sn-2 position of membrane phospholipids, liberating unesterified AA the precursor to the eicosanoids (12). Although the well described cytosolic PLA_2α (cPLA_2α) is necessary for efficient eicosanoid biosynthesis (13), a group of secreted PLA_2s (sPLA_2)s has been identified that coordinate with cPLA_2α to augment synthesis of eicosanoids (14-16). The sPLA_2-mediated release of eicosanoids is upregulated by treatment with the pro-inflammatory
cytokines, transforming growth factor-α (TGF-α) and IL-1β (17). In cultured cells, sPLA₂s are strongly linked to the generation of the pro-inflammatory eicosanoids such as CysLTs, while cPLA₂ α is more closely associated with the generation of PGE₂ (18-20). The sPLA₂s are small (14-16 kDa), Ca²⁺-dependent (Kₐ₉ ≈ μM to mM) enzymes released into the extracellular fluid by the classical secretory pathway or by degranulation (12). Nine functional human sPLA₂s have been characterized, including human sPLA₂ groups V and X that have the highest AA releasing activities when added to mammalian cells (21, 22). An increase in sPLA₂ enzyme activity in bronchoalveolar lavage (BAL) fluid and nasal lavage fluid was identified following allergen challenge in patients with asthma and allergic rhinitis respectively, but the identities of the sPLA₂s involved in asthma remain unknown (23-25).

We assessed the expression of the full set of cytosolic and secreted PLA₂s in lower airway cells obtained by induced sputum in a group of asthmatics with EIB. Based on the results, we determined if selected sPLA₂s were differentially expressed in asthmatic subjects compared to non-asthmatic controls based on gene expression, immunostaining in induced sputum cells, and Western blots of the secreted protein. We examined levels of these selected enzymes at baseline and after exercise challenge in both groups. Our goals were to 1) determine the identities of the sPLA₂s expressed in airway cells of asthmatics, and if these enzymes are overexpressed in asthma, 2) localize the cellular sources of sPLA₂s in airway inflammatory and epithelial cells, and 3) determine if selected sPLA₂s may play a role in the dysregulated synthesis of eicosanoids in response to exercise challenge in asthma.

Some of the results of this study have been previously reported in the form of an abstract (26).
METHODS

Study Subjects and Protocol

A detailed description of these methods can be found in the online supplement. The University of Washington Institutional Review Board approved the study protocol, and written informed consent was obtained from all participants. The asthma group consisted of persons 12-59 years of age who had a physician diagnosis of asthma for ≥ 1 year, used only an inhaled β₂-agonist for asthma, and had ≥ 15% fall in forced expiratory volume in one second (FEV₁) following exercise challenge (8). The control group consisted of persons 18-59 years of age with no history of asthma or atopy, baseline FEV₁ ≥ 80% predicted, and < 7% fall in FEV₁ after exercise challenge. Induced sputum was conducted with 3% saline for 12 min at baseline and on a separate day 4-20 days later 30 min after exercise challenge. Albuterol (180 µg via MDI) was administered 15 min prior to the induced sputum.

Induced Sputum Analysis

Induced sputum was placed on ice and processed within 30 min. The sample was dispersed with an equal volume of DTT 0.1% in a shaking water bath at 37°C for 15 min. Slides were prepared of the dispersed induced sputum with a cytocentrifuge, and fixed in methanol and then methyl Carnoy’s solution for immunocytochemistry. The remaining dispersed induced sputum sample was centrifuged at 250 g for 10 min, and portions of the supernatant treated with protease inhibitors, and with iced methanol with 0.2% formic acid for protein and eicosanoid analysis respectively. The cell pellet was resuspended in RLT buffer (Qiagen, Valencia, CA) with β-mercaptoethanol, and underwent mechanical lysis. Total RNA was extracted from the lysed cell pellet using the RNeasy protocol (Qiagen, Valencia, CA).
First strand cDNA was transcribed from total RNA using oligo(dT)$_{12-18}$ primers. Initially, semi-quantitative PCR was conducted using primers for the secreted and cytosolic PLA$_2$s, based on the PCR product after 23, 27, 31, and 35 cycles of amplification relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantitative real-time PCR was conducted for sPLA$_2$ groups V, X, and XIIA using SYBR green method. Human K-562 cell line cDNA was used for the standard curve.

Immunostaining for sPLA$_2$ groups V, X, and XIIA was performed on cytocentrifuge preparations of induced sputum cells using polyclonal rabbit anti-human sPLA$_2$ antibodies that were raised against the recombinant proteins (27). The immunostaining was co-localized to specific inflammatory and epithelial cells based on morphological criteria (28).

Western blots were conducted on induced sputum supernatant using polyclonal rabbit anti-human sPLA$_2$ groups V, X, and XIIA antibodies. The detection of sPLA$_2$-V was confirmed with a murine monoclonal antibody (Cayman, Ann Arbor, MI).

**Statistical Analysis**

The PLA$_2$ expression data are reported as the mean +/- SD. Differences between the baseline and post-exercise gene expression, protein, and eicosanoid products were analyzed with a paired $t$ test after log transformation. The differences in the percentage of cells immunostaining for the different sPLA$_2$s at baseline and post-exercise were analyzed with a paired $t$ test. Comparisons between the groups were made with unpaired $t$ tests.

**Methods Word Count:** 500
RESULTS

Subject Characteristics and Eicosanoid Levels

The studies were conducted on induced sputum samples obtained from a cohort of 25 subjects with asthma and EIB, and a group of 10 non-asthmatic controls. The baseline lung function, response to short-acting bronchodilator, and severity of EIB were markedly different between the two groups (Table 1). The severity of EIB in the asthmatic group ranged from a maximum fall in FEV₁ after exercise of 15.1-63.1%. Induced sputum was collected at baseline and then on a separate day 30 min after dry-air exercise challenge. The average time between baseline and post-exercise induced sputum visits was 9.3 days (range 4-18) in the asthma group, and 10.0 days (range 5-19) in the control group. The levels of the eicosanoids CysLTs, 15S-HETE and PGE₂ were previously reported in the asthma group (8, 29), and are compared to levels of eicosanoids in the control group in Figures E1 and E2 of the online repository.

Expression and Localization of Secreted PLA₂s in Induced Sputum Cells

Semi-quantitative PCR analysis of induced sputum cells from asthmatic subjects was used to assess the expression of all the known cytosolic and secreted PLA₂s (Figure 1). Induced sputum cells from asthmatics expressed sPLA₂ groups IB, IIA, IID, IIF, X, and XIIA. Of note, sPLA₂ groups X and XIIA were expressed at high levels, while groups IIE, III, and V were below the level of detection. Isoenzymes of cPLA₂ were also identified including groups IVα, IVβ, and IVγ.

Based on the results of the semi-quantitative PCR analysis in asthmatics, we determined differences in selected sPLA₂s between asthmatics and controls by quantitative real-time PCR. The expression of sPLA₂-X was increased in asthmatics relative to controls (Figure 2A), while
there was no difference in the expression of sPLA2-XIIA between asthmatics and controls (Figure 2B). The expression of sPLA2-V was above the threshold cycle (Ct) for detection. Although we did not detect sPLA2-V by PCR, we conducted ICC for sPLA2-V because of its high AA releasing capacity. The concentrations of leukocytes and epithelial cells in induced sputum from both of the groups are presented in the online repository (Figure E3). Immunostaining for sPLA2-V in induced sputum cells at baseline was no different between asthmatics and controls (Figure 2C). In contrast, the percentages of cells immunostaining for sPLA2 groups X and XIIA were greater in induced sputum cells of asthmatics compared to controls (Figures 2D and 2E). Immunostaining for sPLA2 groups V, X, and XIIA localized predominantly in columnar epithelial cells and bronchial macrophages, and to a lesser extent in eosinophils (Figure 3 and Figure E4, online repository).

Effects of Exercise Challenge on sPLA2s in the Airways

To assess the potential role of the identified sPLA2s in the marked release of pro-inflammatory eicosanoids that occurs following exercise in asthmatics with EIB, we evaluated paired induced sputum samples obtained at baseline and 30 min post-exercise challenge in asthmatics and normal controls. The gene expression of sPLA2-X in induced sputum cells increased following exercise challenge in the asthma group, but not in the control group (Figure 4A). The percentage of cells in induced sputum immunostaining for sPLA2-X increased in asthmatic subjects following challenge, but not in controls (Figure 4B). The increase in immunostaining for sPLA2-X in asthmatic subjects was in bronchial macrophages and columnar epithelial cells but not eosinophils (Figure 4C). No changes occurred in these cells following exercise challenge in the control group (Figure 4D). The secreted sPLA2-X protein measured in
induced sputum increased in the asthma group following challenge (Figure 4E), but not in the control group (Figure 4F). An additional Western blot comparing the levels of sPLA2-X following exercise challenge in asthmatic subjects to controls found that the level of sPLA2-X was higher in the asthmatic subjects (Figure 4G). Reprobing the blot with an antibody to the intracellular protein β-actin did not reveal any β-actin signal indicating that the source of the sPLA2 was protein secreted from the cells.

Although the sPLA2-XIIA gene expression was no different in the asthma and control groups at baseline, the gene expression increased after exercise challenge in the asthma group, but not in controls (Figure 5A). There was a marked increase in the percentage of induced sputum cells immunostaining for sPLA2-XIIA following exercise challenge that occurred in the asthma group that was not seen in the control group (Figure 5B). The increase in immunostaining in the asthma group was predominantly in bronchial macrophages and columnar epithelial cells, but also occurred in eosinophils, while no similar changes were observed in induced sputum cell subtypes in the control group (Figure E5A and B, online repository). The level of sPLA2-XIIA could not be measured accurately by Western blot as the protein was difficult to detect in either group by this method.

The percentage of cells immunostaining for sPLA2-V did not increase in either of the groups in response to exercise challenge (Figure 6A). Immunostaining for sPLA2-V in bronchial macrophages tended to increase in asthmatic subjects following challenge; while no changes were observed in columnar epithelial cells or eosinophils in asthmatics, and no changes were identified in any induced sputum cell type in controls (Figure 5C and D, online repository). The sPLA2-V protein was difficult to detect in induced sputum supernatant of asthmatics by Western blot, and could not be detected in normal controls. Exercise challenge did not alter the levels of
sPLA$_2$-V by Western blot in asthmatic subjects (Figure 6B).
DISCUSSION

In this study, we evaluated the expression of the complete set of functional PLA₂ enzymes in the lower airways of asthmatic subjects with BHR, and determined if sPLA₂ groups V, X, and XIIA were differentially expressed in asthmatic subjects compared to non-asthmatic controls. Marked differences in the levels of pro-inflammatory eicosanoids (e.g. CysLTs, and 15S-HETE) in the airways were present between these two groups, particularly following exercise challenge. Of the two sPLA₂ enzymes with high AA releasing capacity (i.e. groups V and X), only sPLA₂-X was overexpressed in asthmatics relative to controls. The gene expression, immunostaining in airway epithelial cells and bronchial macrophages, and the level of the sPLA₂-X protein in the airways all increased in response to exercise challenge in the asthma group, while the levels remained constant following challenge in non-asthmatic controls. These results indicate that sPLA₂-X may play a role in regulating the levels of pro-inflammatory eicosanoids in asthma, particularly in manifestations of indirect bronchial hyperresponsiveness where increased production of pro-inflammatory eicosanoids is critical (7-9).

The sPLA₂s are attractive therapeutic targets because these enzymes may be preferentially involved in the production of pro-inflammatory AA metabolites (e.g. LTs, HETEs, and PGD₂) that are key for asthma immunopathogenesis (12). Two prior studies identified increased sPLA₂ enzyme activity in bronchoalveolar lavage (BAL) fluid following whole lung allergen challenge in allergic asthma (23, 24), and nasal lavage fluid after nasal allergen challenge in subjects with allergic rhinitis (25). The specific sPLA₂s involved in asthma have not been determined. There are 9 distinct functional sPLA₂s that have been identified in the human genome (12). LY333013, a sPLA₂ inhibitor 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.
suggesting that sPLA2 group II enzymes may not be the key sPLA2 enzymes in human asthma (30). The group II enzymes were expressed at relatively low levels in the present study. Among the two sPLA2s with high AA releasing capacity (22), we found that only sPLA2-X was differentially overexpressed in asthma, while sPLA2-V was present at low levels and was not differentially expressed in asthma. The difference in sPLA2-X function may be greater than the difference in sPLA2-X protein levels identified in this study because sPLA2-X becomes activated in inflammatory tissues (31). These results, along with the recent demonstration by our research group that sPLA2-X deficiency significantly inhibits the development of BHR, airway inflammation and remodeling in acute and chronic murine models of allergen-induced asthma (32) indicates that sPLA2-X is a potential therapeutic target in asthma.

Although we focused on the sPLA2 enzymes in this study, we also identified the expression of 3 cPLA2s in asthmatics, including the well-known cPLA2α that has a pivotal role in eicosanoid production (13), and two other enzymes cPLA2β and cPLA2γ. In cultured cells sPLA2s coordinate with cPLA2α to augment the production of eicosanoids (15). Relatively little is known about the two other cPLA2s, but both enzymes function in eicosanoid synthesis (33, 34). cPLA2β is implicated in lipid mediator release in response to cardiac ischemia (35), and cPLA2γ is involved in peroxide-induced release of eicosanoids (36). The expression of cPLA2γ is upregulated in macrophages by the adipocyte-derived cytokine leptin, enhancing cellular LT synthesis (37).

Dysregulated eicosanoid synthesis is prominent in asthma with EIB as indicated by elevated basal levels of CysLTs in the lower airways (10, 11), and release of CysLTs and PGD2 into the airways during EIB sustaining bronchoconstriction (8, 9). Selective functions of the different PLA2 enzymes may be involved in this dysregulated eicosanoid synthesis. We found in
the present study that the ratio of CysLTs to PGE₂ decreased in controls following exercise challenge, in contrast to our prior findings that the ratio of CysLTs to PGE₂ increases following exercise challenge in asthmatics with EIB (8). This altered balance of eicosanoids is important because PGE₂ serves protective and bronchodilator functions (5), and inhaled PGE₂ prior to exercise and allergen challenge significantly attenuates bronchoconstriction (38-40). In cultured cells, activation of cPLA₂α alone is more closely associated with the generation of PGE₂ (18, 19), while sPLA₂s have been strongly implicated in the release of pro-inflammatory eicosanoids such as CysLTs (18, 19) that can occur in the absence of cPLA₂α activation (20).

Localization of sPLA₂-X to the epithelium was prominent in the present study, consistent with the upregulation of sPLA₂-X in the epithelium identified in a murine model of asthma (32). Although the epithelium has limited synthetic capacity for the generation of 5-lipoxygenase (5-LO) products such as CysLTs (41), the epithelium can augment CysLT production in leukocytes through mechanisms involving sPLA₂s. The synthesis of leukotrienes (LT)s in alveolar macrophages co-cultured with alveolar epithelial cells is augmented by the transfer of free AA from the epithelium to the macrophages, shunting AA away from PGE₂ synthesis (42). Similarly, release of sPLA₂-V from epithelial cells augments CysLT synthesis in eosinophils without activation of cPLA₂α (43, 44). These findings have strong implications for the pathogenesis of EIB, since the stimulus for EIB may be the transfer of water and resultant osmotic stress in the epithelium during exercise (45).

The increase in sPLA₂-X in bronchial macrophages in asthmatics is important since bronchial macrophages are the most prevalent cell the airways of asthmatics under most circumstances, and there are phenotypic alterations in bronchial macrophages in asthma (46, 47). In rodent models, bronchial macrophages are important in the development of BHR in response
to allergen sensitization (48). Upregulation of sPLA$_2$-X in alveolar macrophages was also identified following allergen sensitization in the murine model of asthma (32). After migration to the lungs, bronchial macrophages have increased capacity for LT synthesis (49), although alterations in eicosanoid synthesis between asthmatic and normal macrophages have not been identified (50). Further study is necessary to determine if increased expression of sPLA$_2$-X in macrophages alters the production of eicosanoids in asthma.

An unexpected finding of the present study was that the sPLA$_2$ with the highest expression by quantitative PCR and ICC was sPLA$_2$-XIIA. Although it was unclear if sPLA$_2$-XIIA is differentially overexpressed in asthma, there was a marked increase in immunostaining for epithelial cell and bronchial macrophage sPLA$_2$-XIIA in asthmatics that was not seen in the control group after exercise challenge. sPLA$_2$-XIIA displays homology to other sPLA$_2$s only over a short stretch in the active site region (51), has low AA releasing capacity (22), and does not contribute to AA release in cultured cells that express this enzyme (17). Receptor-mediated effects of human sPLA$_2$-XIIA either through the M-type or N-type receptor are also possible. Cells transfected with sPLA$_2$-XIIA exhibit morphological alterations in HEK293 cells that are unique among the sPLA$_2$ transfected cells (52). Among the sPLA$_2$s, sPLA$_2$-XIIA is also unique as a bactericidal protein that has activity against both gram-positive and gram-negative bacteria (53).

In summary, we found that sPLA$_2$-X is differentially overexpressed in asthmatics with EIB, a component of asthma that is a manifestation of indirect BHR. We found strong evidence of an increase in sPLA$_2$-X in columnar epithelial cells and bronchial macrophages and an increase in extracellular sPLA$_2$-X protein in response to exercise challenge in asthmatics, but not in controls. Collectively, these results indicate that sPLA$_2$-X may play a role in the generation of
pro-inflammatory eicosanoids in the airways and the development of BHR. Inhibition of sPLA$_2$-X may represent an important novel therapeutic target in human asthma.
REFERENCES


Figure Legends

**Figure 1.** Expression of PLA2s in induced sputum cells from asthmatics with EIB. Semi-quantitative PCR was used to assess the expression of the secreted (A) and cytosolic PLA2s (B) relative to the expression of GAPDH. The bars represent the mean +/- SD.

**Figure 2.** Baseline differences in sPLA2 groups V, X, and XIIA between asthmatics with EIB and non-asthmatic controls. The gene expression of sPLA2-X was increased relative to control (A), while there was no difference in the expression of sPLA2-XIIA (B). The expression of sPLA2-V was below the level of detection. No difference was detected for immunostaining in induced sputum cells for sPLA2-V (C). The percentage of cells immunostaining for sPLA2-X (D) and sPLA2-XIIA (E) was increased in asthmatic subjects relative to controls.

**Figure 3.** Immunostaining for sPLA2 groups V, X, and XIIA in induced sputum cells. Representative photomicrographs of immunocytochemistry for sPLA2-V (A, B), sPLA2-X (C, D), and sPLA2-XIIA (E, F) demonstrate immunostaining predominantly in columnar epithelial cells (arrows) and macrophages (arrowheads). The representative slides were from asthmatic subjects following exercise challenge.

**Figure 4.** Effects of exercise challenge on sPLA2-X in asthmatics with EIB and non-asthmatic controls. The gene expression (A) and percentage of cells in induced sputum immunostaining (B) for sPLA2-X increased in asthmatic subjects following challenge, but not in controls. Immunostaining for sPLA2-X in asthmatic subjects increased in bronchial macrophages (Mac) and columnar epithelial cells (Epi), but not eosinophils (EOS) (C); while no changes were
observed in the control group (D). Western blots of induced sputum supernatant revealed an increase in sPLA2-X protein in asthmatic subjects (E), but not in controls (F), resulting in a higher level of sPLA2-X in asthmatic subjects than controls post-exercise (G).

**Figure 5.** Effects of exercise challenge on sPLA2-XIIA in asthmatics with EIB and nonasthmatic controls. The gene expression (A) and percentage of cells in induced sputum immunostaining (B) for sPLA2-XIIA increased in asthmatic subjects following challenge, but not in controls.

**Figure 6.** Effects of exercise challenge on sPLA2-V in asthmatics with EIB and non-asthmatic controls. The percentage of cells in induced sputum immunostaining for sPLA2-V was no different post-exercise compared to baseline in either asthmatics or controls (A). Western blot of induced sputum supernatant had no increase in sPLA2-V protein in asthmatic subjects (B), and could not be detected in controls.
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<th>Normal Control (n=10)</th>
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<td>6/4</td>
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<td><strong>Post Bronchodilator</strong></td>
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<td>Max Δ FEV&lt;sub&gt;1&lt;/sub&gt; (%)&lt;sup&gt;‡&lt;/sup&gt;</td>
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* Values reported are mean +/- standard deviation unless otherwise specified

† p < 0.001
‡ p < 0.01
Figure 1.
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Secreted phospholipase A$_2$ group X overexpression in asthma and bronchial hyperresponsiveness


ONLINE DATA SUPPLEMENT
METHODS

Subjects

The University of Washington Institutional Review Board approved the study protocol, and written informed consent was obtained from all participants. Induced sputum samples from asthmatic for this study were obtained from a cohort of 25 patients enrolled in a study on the pathogenesis of exercise-induced bronchoconstriction (EIB) (E1). Study subjects consisted of persons 12-59 years of age who had a physician diagnosis of asthma for ≥ 1 year, used only an inhaled β₂-agonist for asthma treatment, and had ≥ 15% fall in forced expiratory volume in one second (FEV₁) following exercise challenge. Potential participants with asthma were excluded if baseline FEV₁ was ≤ 65% predicted, they had received treatment for acute asthma within the prior month, or hospitalized for asthma within the prior 3 months, or had a history of life-threatening asthma. In addition, induced sputum samples were obtained from 10 non-asthmatic controls, consisting of persons 18-59 years of age with no history of asthma or atopy, baseline FEV₁ ≥ 80% predicted, and < 7% fall in FEV₁ after exercise challenge. Potential participants were excluded if they had a history of smoking cigarettes within the prior year or ≥ 7 pack-year smoking history. Participants were excluded if they had used an inhaled corticosteroid, leukotriene modifier, long-acting antihistamine, cromone, or long-acting β₂-agonist in the 30 days prior to the start of the study.

Study Protocol

The first visit consisted of a physical examination, spirometry, and in the asthma group an exercise challenge to determine eligibility for the study. Each subject had 2 induced sputums. One visit consisted of spirometry before and 15 min after administration of 180 μg of albuterol
via a metered dose inhaler, followed by induced sputum. On the other visit, conducted at the same time of day, 4-10 days apart, participants had an exercise challenge followed by induced sputum 30 min after the end of exercise. Albuterol (180 μg via MDI) was administered 15 min prior to the induced sputum. Subjects were asked not to exercise, use short-acting antihistamines for 48 hours, and β2-agonist and caffeinated beverages for 6 hours prior to each study visit.

**Spirometry, Exercise Challenge, and Sputum Induction**

Spirometry and exercise challenge tests were conducted in accordance with American Thoracic Society (ATS) standards (E2, E3). Exercise challenge was performed on a motorized treadmill such that each subject sustained ≥85% of their maximum heart rate for the final 6 min of exercise (E2). Subjects wore nose clips and breathed dry air (0% relative humidity, 22°C) delivered from a weather balloon reservoir through a one-way valve (Hans Rudolph, Kansas City, MO) during exercise. Spirometry was conducted 20 and 5 min before each exercise challenge, and repeated at 0, 3, 6, 10, and 15 min after the end of exercise. The better of at least 2 FEV₁ maneuvers within 5% of each other was recorded at each time point. Induced sputum was conducted with 3% hypertonic saline via an ultrasonic nebulizer (DeVilbiss, Somerset, PA) (E4). At 2-min intervals, subjects were asked to clear saliva from their mouth and then expectorate sputum. Sputum was collected over 12 min and was pooled into a single sample container. The induced sputum was placed on ice immediately and processed within 30 min of collection. Samples were coded with a subject number, visit number, and date.
**Induced Sputum Processing**

Following the addition of an equal volume of DTT 0.1% (Calbiochem, La Jolla, CA), the induced sputum sample was mixed gently with a vortex mixer and placed in a shaking water bath at 37°C for 15 min. A plastic transfer pipette was used periodically to further mix and disperse the sample. A 1 ml portion of the sample was removed for cell counts and cytocentrifuge preparations. The remaining dispersed induced sputum sample was centrifuged at 250 x g for 10 min and the supernatant removed from the cell pellet. The supernatant was centrifuged a second time at 4,000 g for 20 min and the supernatant stored for protein analysis with the addition of PMSF and EDTA, and for eicosanoid analysis by the addition of 4 volumes of methanol with 0.2% formic acid (E5). The cell pellet was resuspended in RLT buffer (Qiagen, Valencia, CA) with β-mercaptoethanol, and passed through an 18g needle 5-6 times. After lysis, the cell pellet was stored at –80°C. Total RNA was extracted from the lysed cell pellet using the RNeasy protocol (Qiagen).

**Eicosanoid Measurements**

The levels of cysteinyl leukotriene (CysLT)s, prostaglandin (PG)D2, 15S-hydroxyeicosatetraenoic acid (15S-HETE) and PGE2 were measured in the control group for this study and compared to values previously measured in the asthma group (E1). The samples were brought to ~13% methanol by the addition of 5 volumes of 0.03% formic acid in high purity water, and loaded onto a Oasis HLB column (Waters, Milford, MA). The samples were washed with 0.03% formic acid in water, 0.03% formic acid in 10% ethanol, and hexanes, and then eluted with 0.2% formic acid in methanol (E6). The samples were evaporated to dryness in a
centrifugal concentrator, and resuspended in assay buffer at their original volume. The levels of CysLTs, PGD2, 15S-HETE, and PGE2 were measured by enzyme immunoassay (Cayman, Ann Arbor, MI). PGD2 was measured as the stable PGD2-MOX derivative after treatment of the sample with methoxylamine hydrochloride. Recovery was 78% for 15S-HETE, and 100% for CysLTs and PGD2 after solid phase extraction.

**Semi-quantitative RT-PCR**

Semi-quantitative PCR was conducted on 5 representative asthmatic subjects from RNA isolated from the baseline induced sputum sample. Ribogreen reagent was used to measure the RNA concentration. The RNA was precipitated with ethanol, and redissolved in nuclease-free Tris-EDTA buffer to give an RNA concentration of 100 ng/μl. First strand cDNA was transcribed from 10 μl of this RNA with SuperScript II RT and oligo(dT)12-18 primers (Invitrogen, Carlsbad, CA). PCR was conducted using primers for the secretory and cytosolic PLA2s and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specified in Table EI. The amount of cDNA solution from each induced sputum sample was determined empirically by the amount of cDNA solution that was sufficient to give a just perceptible band from the GAPDH primers after 23 cycles of PCR. PCR was performed with 0.6 U of Qiagen Hotstar Taq polymerase and 200 μM of each primer under the following conditions: 95°C, 15 min; (94°C, 45 sec; 60°C, 1 min; 68°C, 45 sec) for 23 cycles; 68°C, 5 min. At the end of this program 5 μl of reaction product was removed for agarose gel analysis and PCR was continued under the following conditions: (95°C, 45 sec; 60°C, 1 min; 68°C, 45 sec) for 4 cycles; 68°C, 5 min. A 5 μl analytical sample was taken and this modified PCR program was repeated twice more. The PCR products after 23, 27, 31, and 35 total cycles were run on separate 2% agarose gels with
detection by ethidium bromide. The relative amounts of the mRNAs were calculated based on
the observation that four cycles of PCR results in a ten fold increase in PCR product under these
conditions after the band has become perceptible on an agarose gel through at least eight more
cycles of PCR, corresponding to a linear range of 100-fold increase in product.

**Quantitative Real-time PCR**

Quantitative PCR was conducted on paired samples from 8 representative asthma
subjects and 10 control subjects collected at baseline and 30 min post-exercise. The primers for
human sPLA₂ groups V, X, XIIA, and GAPDH were designed using Primer Express Version 1.0
(Applied Biosystems, Foster City, CA) and are specified in Table EII. All primers crossed large
intronic sequences. Human K-562 cell line cDNA was used to make standard curve. All cDNA
samples were diluted to 50 ng/μl. Quantitative real-time PCR was conducted using SYBR Green
master mix, and monitored on an ABI Model 7900 real-time PCR system (Applied Biosystems,
Foster City, CA). Thermal cycler conditions were as follows: Stage 1: 50°C, 2 min; Stage 2: 95°C, 10 min; Stage 3: step 1: 95°C 0.15 min, step 2: 60°C 1 min, for 40 cycles.

**Immunocytochemistry**

The total and differential cell counts were conducted for this study in the control group
and compared to values previously reported in the asthma group (E1). The total cell count was
determined with a hemocytometer on a 1 ml portion of the induced sputum supernatant, and
slides for differential cell counts and immunocytochemistry were prepared with a cytocentrifuge.
Slides were stained (Hema 3, Fisher Diagnostics, Middletown, VA) and at least 400 non-
squamous cells counted per slide. The differential cell count of inflammatory and epithelial cells was based on morphological criteria (E4).

**Immunocytochemistry** was conducted on 8 representative asthma subjects and 5 representative control subjects from cytospin preparations of induced sputum obtained at baseline and 30 min post-exercise. The cytospin slides were initially fixed in methanol, and then methyl Carnoy’s solution. All immunolabeling was conducted at 24°C. Endogenous peroxidase activity was inactivated by 0.75% hydrogen peroxide for 30 min. Slides were washed and incubated with polyclonal rabbit antibodies raised against human sPLA₂ groups V, X, and XIIA at a dilution of 1:100 for 30 min (E7). The slides were washed and incubated with a horseradish peroxidase (HRP) conjugated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) at a dilution of 1:40 for 30 min. Control preparations were prepared with pre-immune rabbit sera. Detection of peroxidase was with 0.5% 3’,3’-diaminobenzidine tetrachloride and 0.15% hydrogen peroxide, which stains the cells brown/black. The nuclei were counterstained with Vector Hematoxylin QS. For each slide, 280-500 cells were counted and the results were expressed as the percentages of the total cells.

**Western Blots**

Western blots were conducted on paired induced sputum samples from 5 representative subjects at baseline and post-exercise from each of the groups. The protein concentration of the induced sputum supernatant was measured with the Coomassie Plus Braddford assay (Pierce, Rockford, IL). Between 3 and 5 μg of total protein was loaded into each well. The same amount of total protein was loaded for each pair of samples. The optimal loading condition for each of the proteins was determined empirically using the recombinant protein (data not shown). sPLA₂-
V was prepared under reducing conditions without heating. sPLA2-X was prepared under reducing conditions and was heated to 70°C for 10 min. sPLA2-XIIA was not reduced or heated. The proteins were separated on a 12% NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA) at 200V for 28 min. Proteins were transferred by semi-dry transfer to polyvinylidifluoride (PVDF) membranes at 110 mAmp for 40 min. For sPLA2-X, non-specific binding was blocked with 5% nonfat milk in Tris-buffered saline with 0.1% tween-20 (TBST) for 2 hours at RT. The membrane was incubated with rabbit polyclonal anti-sPLA2-X antibody overnight at 4°C, and then subsequently incubated with goat anti-rabbit HRP-linked antibody (Cell Signaling, Danvers, MA) for 1 hour at RT (Figures E6A and B). For sPLA2 groups V and XIIA, non-specific binding was blocked with NETG-buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5, 0.05% triton-X, 0.25% gelatin) for 30 min at RT. The membrane was incubated with rabbit polyclonal anti-sPLA2 antibody for 2 hours at RT, and then subsequently incubated with goat anti-rabbit HRP-linked antibody for 1 hour at RT (Figure E6C). For sPLA2-V, this procedure was repeated on samples from one subject with a murine monoclonal anti-sPLA2 hG V antibody and a horse anti-mouse HRP-linked antibody (Figure E6D). The peroxidase activity of the blots was detected using LumiGLO ECL reagents (Cell Signaling, Danvers, MA). One membrane was reprobed with a murine monoclonal anti-β-actin antibody and horse anti-mouse HRP-linked antibody to detect evidence of cell lysis releasing this intracellular protein during induced sputum processing.

**Statistical Analysis**

The PLA2 expression data are reported as the mean +/- SD. Differences between the baseline and post-exercise gene expression, protein, and eicosanoid products were analyzed with
a paired $t$ test after log transformation. The differences in the percentage of cells immunostaining for the different sPLA$_2$s at baseline and post-exercise were analyzed with a paired $t$ test. Comparisons between the groups were made with unpaired $t$ tests. Comparisons of the concentrations of cells in induced sputum between the groups were made with a Mann-Whitney U test. Changes in the concentrations of cells in induced sputum between baseline and post-exercise were made with the Wilcoxon signed-rank test.
RESULTS

Comparison of Eicosanoid Levels

At baseline the ratio of CysLTs to PGE\textsubscript{2} was elevated in the asthma group compared to control (p=0.03), and this ratio increased markedly in the asthmatics, while this ratio decreased following exercise challenge in controls (Figure E1). The geometric mean levels of CysLTs (167.6 vs. 132.8 pg/ml, p=0.17), PGD\textsubscript{2} (486.5 vs. 483.6 pg/ml, p=0.97), 15S-HETE (26.2 vs. 24.4 ng/ml, p=0.50) in the control group did not increase following exercise challenge as had been observed in the cohort of asthmatics in this study and in other studies (E1, E8, E9). The geometric mean level of PGE\textsubscript{2} did not decline following exercise challenge in the control group (125.5 vs. 177.7 pg/ml, p=0.26) as had been observed in the asthma group (E1). A comparison of the mean baseline level of eicosanoids between the asthma and control groups demonstrated a trend towards higher levels of CysLTs in the asthma group (641.5 vs. 284.0 pg/ml, p=0.06), but no detectible difference in the basal levels of 15S-HETE (64.9 vs. 33.0 ng/ml, p=0.32) or PGE\textsubscript{2} (321.9 vs. 241.0 pg/ml, p=0.54). Following exercise challenge the levels of CysLTs and 15S-HETE were markedly elevated in the asthma group compared to control (Figures E2A and B), while the level of PGE\textsubscript{2} was no different between the groups (Figure E2C). The level of PGD\textsubscript{2} had not been measured in the asthma group and could not be compared between the groups.

Comparison of Cellular Constituents of Induced Sputum

There were no differences in the volume (4.7 vs. 3.8 ml, p=0.18), concentration of lower airway cells excluding squamous epithelial cells (1.3 x 10\textsuperscript{6} vs. 1.0 x 10\textsuperscript{6} cells, p=0.67), or percentage of squamous epithelial cells (23.4 vs. 24.6 %, p=0.57) in the baseline induced sputum between asthma and control groups respectively. The median concentration of eosinophils was
increased in the asthma group (2.51 x 10⁴ vs. 0.9 x 10³ cells/ml, p<0.01); however, there were no
differences in the median concentrations of lymphocytes (1.7 x 10⁴ vs. 1.9 x 10⁴ cells/ml,
p=0.33), macrophages (4.8 x 10⁵ vs. 5.6 x 10⁵ cells/ml, p=0.65), neutrophils (4.6 x 10⁵ vs. 3.2 x
10⁵ cells/ml, p=0.32), or columnar epithelial cells (6.2 x 10⁴ vs. 1.7 x 10⁴ cells/ml, p=0.62)
between the groups. The concentration of epithelial cells in induced sputum increased in both the
asthma and control groups following exercise challenge (Figure E3). In the asthma group, there
were no changes in the concentration of leukocytes following exercise challenge (Figure E3A);
however, in the control group there was a decrease in the concentration of lymphocytes and an
increase in the concentration of neutrophils following exercise challenge (Figure E3B).
Comparison of the median concentrations of induced sputum cells between the groups following
exercise challenge showed that only eosinophils were higher in the asthma group relative to
controls (2.4 x 10⁴ vs. 0.0, p=0.01), but no differences were identified in the concentrations of
lymphocytes, macrophages, neutrophils, or epithelial cells.

**Secreted PLA₂ Immunocytochemistry**

Immunostaining for sPLA₂ groups V, X, and XIIA in the baseline induced sputum
samples localized predominantly to columnar epithelial cells and bronchial macrophages, and to
a lesser degree eosinophils (Figure E4). Control slides immunolabeled with pre-immune rabbit
serum did not have any immunostaining (data not shown). The percentage of cells
immunostaining for sPLA₂-V did not increase in either of the groups in response to exercise
challenge (Figure 6A). Immunostaining for sPLA₂-V in bronchial macrophages tended to
increase in asthmatic subjects following challenge; while no changes were observed in columnar
epithelial cells or eosinophils in asthmatics (Figure E5A), and no changes were identified in any
induced sputum cell type in controls (Figure E5B). The percentage of cells in induced sputum immunostaining for sPLA$_2$-X increased in asthmatic subjects following challenge, but not in controls (Figure 4B). The increase in immunostaining for sPLA$_2$-X in asthmatic subjects was in bronchial macrophages and columnar epithelial cells but not eosinophils (Figure 4C). No changes occurred in these cells following exercise challenge in the control group (Figure 4D). There was a marked increase in the percentage of induced sputum cells immunostaining for sPLA$_2$-XIIA following exercise challenge that occurred in the asthma group that was not seen in the control group (Figure 5B). The increase in immunostaining in the asthma group was predominantly in bronchial macrophages and columnar epithelial cells, but also occurred in eosinophils (Figure E5C). No similar changes were observed in induced sputum cell subtypes in the control group (Figure E5D).
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<th>Group</th>
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<th>Antisense strand primer</th>
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**Table EII.** Primers sequences for quantitative real-time PCR

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<th>Group</th>
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<th>Antisense strand primer</th>
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Figure E1. Opposing effects of exercise challenge on the CysLT to PGE₂ ratio in induced sputum. Asthmatics with EIB have an increase in the CysLT to PGE₂ ratio in response to exercise (A), while normal controls have a decrease in the CysLT to PGE₂ ratio following exercise challenge (B). The levels of CysLTs and PGE₂ in the asthma group were previously reported (E1).
Figure E2. Comparison of the levels of eicosanoids in induced sputum between asthmatics and normal controls. The level of CysLTs tended to be higher at baseline, while there were no baseline differences in the levels of 15S-HETE and PGE₂ between the groups (see text). Following exercise challenge, the levels of CysLTs (A) and 15S-HETE (B) were elevated in the asthma group compared to the control group, while no difference was detected for PGE₂ (C). The levels of CysLTs, 15S-HETE and PGE₂ in the asthma group were previously reported (E1, E8).
Figure E3. Concentration of leukocytes and epithelial cells in induced sputum at baseline and on a separate day 30 min after exercise challenge in asthmatics with EIB (A) and non-asthmatic controls (B). The concentrations of leukocytes and epithelial cells in the asthma group were previously reported (E1).
Figure E4. Comparison of the baseline immunostaining of sPLA₂ groups V, X, and XIIA in induced sputum cells in asthmatics and controls. The figure shows the percentage of induced sputum cells immunostaining positive for the sPLA₂ out of all induced sputum cells.
Figure E5. Effects of exercise challenge on the percentage of induced sputum cells immunostaining for sPLA₂ groups V and XIIA. Immunostaining for sPLA₂-V in bronchial macrophages (Mac) tended to increase in asthmatic subjects following challenge (A), while no changes were observed in columnar epithelial cells (Epi) or eosinophils (EOS) in asthmatics, and no changes were identified in any cell type in controls (B). Immunostaining for sPLA₂-XIIA in asthmatic subjects increased in bronchial macrophages (Mac) and columnar epithelial cells (Epi), and tended to increase in eosinophils (EOS) (C); while no changes were observed in the control group (D).
Figure E6. Western blots of sPLA₂ groups V and X. Western blots from asthmatic induced sputum samples were conducted in 5 representative subjects (labeled 1-5) at baseline (left) and post-exercise (right) for sPLA₂-X (A) and sPLA₂-V (C). A Western blot was also conducted on 5 representative asthmatic (left) and non-asthmatic (right) induced sputum samples post-exercise (B). Western blots for sPLA₂-X were conducted with a rabbit polyclonal antibody (A, B). Western blots for sPLA₂-V were conducted with the rabbit polyclonal antibody (C) and a murine monoclonal antibody (D). The recombinant protein (rhG V or rhG X) is to the right of the ladder.
REFERENCES


