Secreted Phospholipase A2 Group X Overexpression in Asthma and Bronchial Hyperresponsiveness

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Rationale: Secreted phospholipase A2 enzymes (sPLA2s) play key regulatory roles in the biosynthesis of eicosanoids, such as the cysteinyl leukotrienes, 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 patients with asthma, and to determine if these enzymes may play a role in the generation of eicosanoids in exercise-induced bronchoconstriction.

Methods: Induced sputum samples were obtained from subjects with asthma with exercise-induced bronchoconstriction and nonasthmatic control subjects at baseline, and on a separate day 30 minutes after exercise challenge. The expression of the PLA2 in induced sputum cells and supernatant was determined by quantitative polymerase chain reaction, immunocytochemistry, and Western blot.

Measurements and Main Results: The sPLA2s expressed at the highest levels in airway cells of subjects with asthma 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, whereas the levels were lower and unchanged after challenge in nonasthmatic control subjects.

Conclusions: Increased expression of sPLA2-X may play a key role in the dysregulated eicosanoid synthesis in asthma.

Keywords: asthma; eicosanoid; epithelial cell; leukotriene; macrophage

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 (LTs), hydroxyeicosatetraenoic acids (HETEs), and prostaglandins (PGs). LTs, such as the cysteinyl LTs (CysLTs), are produced at increased levels in the airways of patients with asthma (2), and inhalation of CysLTs reproduces many of the features of asthma (3). Members of the PG family include PGE2, which serves as a bronchoconstrictor (4), and PGE2, which has a bronchodilatory and bronchoprotective role in the airways (5). A major manifestation of asthma is exercise-induced bronchoconstriction (EIB), which 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 patients with asthma with EIB than in patients with asthma without EIB. In particular, the ratio of CysLTs to PGE2 is increased in induced sputum of patients with asthma with EIB (11). On the basis of our prior work (8) and the results of Mickleborough and colleagues (9) demonstrating a sustained increase in CysLTs and PGD2 in the airways, and concurrent decrease in the level of PGE2 (8) after exercise challenge, we undertook this investigation to evaluate the upstream regulators of eicosanoid production in the airways of patients with asthma and EIB.

A key regulatory mechanism for eicosanoid biosynthesis is through phospholipase A2 (PLA2), which hydrolyzes the sn-2 position of membrane phospholipids, liberating unesterified AA, the precursor to the eicosanoids (12). Although the well-described cytosolic PLA2-α (cPLA2-α) is necessary for efficient eicosanoid biosynthesis (13), a group of secreted PLA2 enzymes (sPLA2s) has been identified that coordinate with cPLA2-α to augment synthesis of eicosanoids (14–16). The sPLA2-mediated release of eicosanoids is up-regulated by treatment with the proinflammatory cytokines, transforming growth factor-α, and IL-1β (17). In cultured cells, sPLA2s are strongly linked to the generation of the proinflammatory eicosanoids, such as CysLTs, whereas cPLA2-α is more closely associated with the generation of PGD2 (18–20). The sPLA2s are small (14–16kD), Ca2+-dependent (KCa<sub>Ca<sup>2</sup></sub>-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, which have the highest AA-releasing activities when added to mammalian cells (21, 22). An increase in PLA2 enzyme activity in bronchoalveolar lavage fluid and nasal lavage fluid was identified after 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 subjects with asthma and EIB. On the basis of the results, we determined if selected sPLA2s were differentially expressed in subjects with asthma compared with...
nonasthmatic control subjects 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 as follows: (1) to determine the identities of the sPLA₂s expressed in airway cells of subjects with asthma, and whether these enzymes are overexpressed in asthma; (2) to localize the cellular sources of sPLA₂s in airway inflammatory and epithelial cells; and (3) to 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 aged 12 to 59 years who had a physician diagnosis of asthma for 1 year or more before the study, used only an inhaled b₂-agonist for asthma, and had a 15% or greater fall in FEV₁ after exercise challenge (8). The control group consisted of persons aged 18 to 59 years with no history of asthma or atopy, a baseline FEV₁ of 80% predicted or greater, and a less than 7% fall in FEV₁ after exercise challenge. Induced sputum was obtained using the induced sputum protocol (25). The sample was dispersed with an equal volume of dithiothreitol 0.1% in a shaking water bath at 37°C for 10 minutes. Slides were prepared of the dispersed induced sputum with a cytocentrifuge and fixed in methanol, and then in methyl Carnoy’s solution for immunocytochemistry. The remaining dispersed induced sputum sample was centrifuged at 250 × g for 10 minutes, and portions of the supernatant were treated with protease inhibitors and iced methanol with 0.2% formic acid for protein and eicosanoid analysis, respectively. The cell pellet was resuspended in cell lysis buffer (RLT buffer; Qiagen, Valencia, CA) with protein and eicosanoid analysis, respectively. The cell pellet was re-suspended in cell lysis buffer (RLT buffer; Qiagen, Valencia, CA) with protease inhibitors and iced methanol with 0.2% formic acid for protein and eicosanoid analysis, respectively. The cell pellet was resuspended in cell lysis buffer (RLT buffer; Qiagen, Valencia, CA) with β-mercaptoethanol, and underwent mechanical lysis. Total RNA was extracted from the lysed cell pellet using the RNaseasy protocol (Qiagen).

First-strand cDNA was transcribed from total RNA using oligo(dT)₁₂₋₁₈ primers. Initially, semiquantitative polymerase chain reaction (PCR) was conducted using primers for the secreted and cytosolic PLA₂s, 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 sPLA₂ groups V, X, and XIIA using SYBR green method. Human K-562 cell line cDNA was used for the standard curve.

Immunostaining for sPLA₂ groups V, X, and XIIA was performed on cytocrfluorid preparations of induced sputum cells using polyclonal rabbit anti-human sPLA₂ antibodies that were raised against the recombinant proteins (27). The immunostaining was colocalized to specific inflammatory and epithelial cells based on morphologic criteria (28).

Western blots were conducted on induced sputum supernatant using polyclonal rabbit anti-human sPLA₂ groups V, X, and XIIA antibodies. The detection of sPLA₂-V was confirmed with a murine monoclonal antibody (Cayman, Ann Arbor, MI).

Statistical Analysis

The PLA₂ expression data are reported as the mean ± SD. Differences between the baseline and postexercise 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 sPLA₂s at baseline and postexercise were analyzed with a paired t test. Comparisons between the groups were made with unpaired t tests.

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

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<th>TABLE 1. CHARACTERISTICS OF STUDY PARTICIPANTS</th>
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Definition of abbreviations: EIB = exercise-induced bronchoconstriction; F = female; M = male.

Values reported are mean ± SD unless otherwise specified.

* P < 0.001.

Statistical signifigance was determined by a paired t test. Comparisons between the groups were made with unpaired t tests.

Figure 1. Expression of secreted phospholipase A₂ enzymes (sPLA₂s) in induced sputum cells from subjects with asthma and exercise-induced bronchoconstriction. Semiquantitative polymerase chain reaction was used to assess the expression of the secreted (A) and cytosolic PLA₂s (cPLA₂) (B) relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The bars represent the mean ± SD.
a group of 10 nonasthmatic control subjects. 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 to 63.1%. Induced sputum was collected at baseline and then on a separate day 30 minutes after dry-air exercise challenge. The average time between baseline and post–exercise-induced sputum visits was 9.3 days (range, 4–18 d) in the asthma group, and 10.0 days (range, 5–19 d) 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 with levels of eicosanoids in the control group in Figures E1 and E2 of the online supplement.

Expression and Localization of Secreted PLA2 in Induced Sputum Cells

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

On the basis of the results of the semiquantitative PCR analysis in subjects with asthma, we determined differences in selected sPLA2s between subjects with asthma and control subjects by quantitative real-time PCR. The expression of sPLA2-X was increased in subjects with asthma relative to control subjects (Figure 2A), whereas there was no difference in the expression of sPLA2-XIIA (B). The percentage of cells immunostaining for 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 subjects with asthma relative to control subjects.

Effects of Exercise Challenge on sPLA2 in the Airways

To assess the potential role of the identified sPLA2s in the marked release of proinflammatory eicosanoids that occurs after exercise in subjects with asthma and EIB, we evaluated paired induced sputum samples obtained at baseline and 30 minutes post–exercise challenge in subjects with asthma and normal control subjects. The gene expression of sPLA2-X in induced sputum cells increased after 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 subjects with asthma after challenge, but not in control subjects (Figure 4B). The increase in immunostaining for sPLA2-X in subjects with asthma was in bronchial macrophages and columnar epithelial cells but not eosinophils (Figure 4C). No changes occurred in these cells after exercise challenge in the control group (Figure 4D). The secreted sPLA2-X protein measured in induced sputum increased in the asthma group after exercise challenge (Figure 4E), but not in the control group (Figure 4F). An additional Western blot comparing the levels of sPLA2-X after exercise challenge in subjects with asthma compared with control subjects found that the level of sPLA2-X was higher in the subjects with asthma (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 the control group (Figure 5A). There was a marked increase in the percentage of induced sputum cells immunostaining for sPLA2-V after exercise challenge that occurred in the asthma group, whereas no changes were observed in the control group (Figure 5B). The increase in immunostaining in the asthma group was predominantly in columnar epithelial cells and bronchial macrophages, but also occurred in eosinophils, whereas no similar changes were observed in induced sputum cell subtypes in the control group (Figures E5A and E5B). 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 subjects with asthma after challenge, whereas no changes were observed in columnar epithelial cells or eosinophils in subjects with asthma, and no changes were identified in any induced sputum cell type in of the control subjects (Figures E5C and E5D). The sPLA2-V protein was difficult to detect in induced sputum supernatant of subjects with asthma by Western blot, and could not be detected in normal control subjects. Exercise challenge did not alter the levels of sPLA2-V by Western blot in subjects with asthma (Figure 6B).

**Figure 3.** Immunostaining for secreted phospholipase A2 (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 subjects with asthma after exercise challenge.

**DISCUSSION**

In this study, we evaluated the expression of the complete set of functional PLA2 enzymes in the lower airways of subjects with asthma and BHR, and determined if sPLA2 groups V, X, and XIIA were differentially expressed in subjects with asthma compared with nonasthmatic control subjects. Marked differences in the levels of proinflammatory eicosanoids (e.g., CysLTs and 15S-HETEs) in the airways were present between these two groups, particularly after exercise challenge. Of the two sPLA2 enzymes with high AA-releasing capacity (i.e., groups V and X), only sPLA2-X was overexpressed in subjects with asthma relative to control subjects. 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, whereas the levels remained constant after challenge in nonasthmatic control subjects. These results indicate that sPLA2-X may play a role in regulating the levels of proinflammatory eicosanoids in asthma, particularly in manifestations of indirect BHR, in which increased production of proinflammatory eicosanoids is critical (7–9).

The sPLA2s are attractive therapeutic targets because these enzymes may be preferentially involved in the production of proinflammatory AA metabolites (e.g., LTs, HETEs, and PGD2), which are key for asthma immunopathogenesis (12). Two prior studies identified increased sPLA2 enzyme activity in bronchoalveolar lavage fluid after whole lung allergen challenge in allergic asthma (23, 24), and in 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 nine distinct functional sPLA2s that have been identified in the human genome (12). LY335031, an 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, whereas 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, together 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), indicate 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 the three cPLA2s in subjects with asthma, including the well-known cPLA2α, which 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 up-regulated 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 that sustain 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 after exercise challenge, in contrast to our prior findings that the ratio of CysLTs to PGE2 increases after exercise challenge in subjects with asthma and EIB (8). This altered balance of eicosanoids is important because PGE2 serves protective and bronchodilator functions (5), and inhaled PGE2 before 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), whereas sPLA2s have been strongly implicated in the release of proinflammatory eicosanoids, such as CysLTs (18, 19), which 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 up-regulation 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 products, such as CysLTs (41), the epithelium can augment CysLT production in leukocytes through mechanisms involving sPLA2s. The synthesis of LTs 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, because 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 subjects with asthma is important because bronchial macrophages are the most prevalent cell in the airways of patients with asthma 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). Up-regulation of sPLA2-X in alveolar macrophages was also identified after 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 sPLA2-X in macrophages alters the production of eicosanoids in asthma.

An unexpected finding of the present study was that the sPLA2 with the highest expression by quantitative PCR and immunocytochemistry was sPLA2-XIIA. Although it was unclear if sPLA2-XIIA is differentially overexpressed in asthma, there was a marked increase in immunostaining for epithelial cell and bronchial macrophage sPLA2-XIIA in subjects with asthma that was not seen in the control group after exercise challenge. sPLA2-XIIA displays homology to other sPLA2s 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 sPLA2-XIIA either through the M-type or N-type receptor are also possible. Cells transfected with sPLA2-XIIA exhibit morphologic alterations in HEK293 cells, which are unique among the sPLA2 transfected cells (52).
Among the sPLA₂s, sPLA₂-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 sPLA₂-X is differentially overexpressed in subjects with asthma and EIB, a component of asthma that is a manifestation of indirect BHR. We found strong evidence of an increase in sPLA₂-X in columnar epithelial cells and bronchial macrophages and an increase in extracellular sPLA₂-X protein in response to exercise challenge in subjects with asthma, but not in control subjects. Collectively, these results indicate that sPLA₂-X may play a role in the generation of proinflammatory eicosanoids in the airways and in the development of BHR. Inhibition of sPLA₂-X may represent an important novel therapeutic target in human asthma.

Conflict of Interest Statement: T.S.H. served as a consultant for GlaxoSmithKline, gave lectures sponsored by Merck and Schering Plough, and was the recipient of a medical school grant from Merck. E.Y.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.H.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.G.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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