Secreted Phospholipase A_2 Group X Overexpression in Asthma and Bronchial Hyperresponsiveness

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Rationale: **Secreted phospholipase A_2 enzymes ($sPLA_2$s) 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 $sPLA_2$s 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 PLA_2s in induced sputum cells and supernatant was determined by quantitative polymerase chain reaction, immunocytochemistry, and Western blot.**

Measurements and Main Results: **The $sPLA_2$s expressed at the highest levels in airway cells of subjects with asthma were groups X and XIIA. Group X $sPLA_2$ ($sPLA_2$-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 $sPLA_2$-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 $sPLA_2$-X may play a key role in the dysregulated eicosanoid synthesis in asthma.**

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

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Secreted phospholipase A_2 ($sPLA_2$) enzymes have recently emerged as critical regulators of eicosanoid synthesis, but the specific $sPLA_2$ enzymes expressed in human airways and the role of these enzymes in asthma pathogenesis are not known.

What This Study Adds to the Field

Group X $sPLA_2$ is overexpressed in airway epithelial cells and bronchial macrophages of subjects with asthma, and released in response to exercise challenge, a stimulus that causes dysregulated eicosanoid synthesis.

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 PGD_2, which serves as a bronchoconstrictor (4), and PGE_2, 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 PGE_2 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 PGD_2 in the airways, and concurrent decrease in the level of PGE_2 (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 A_2 (PLA_2), which hydrolyzes the sn-2 position of membrane phospholipids, liberating unesterified AA, the precursor to the eicosanoids (12). Although the well-described cytosolic $PLA_2\alpha$ ($cPLA_2\alpha$) is necessary for efficient eicosanoid biosynthesis (13), a group of secreted PLA_2 enzymes ($sPLA_2$s) has been identified that coordinate with $cPLA_2\alpha$ to augment synthesis of eicosanoids (14–16). The $sPLA_2$-mediated release of eicosanoids is up-regulated by treatment with the proinflammatory cytokines, transforming growth factor-α, and IL-1β (17). In cultured cells, $sPLA_2$s are strongly linked to the generation of the proinflammatory eicosanoids, such as CysLTs, whereas $cPLA_2\alpha$ is more closely associated with the generation of PGE_2 (18–20). The $sPLA_2$s are small (14–16 kD), Ca^{2+}-dependent ($K_{Ca} \sim \mu M$ to mM) enzymes released into the extracellular fluid by the classical secretory pathway or by degranulation (12). Nine functional human $sPLA_2$s have been characterized, including human $sPLA_2$ groups V and X, which have the highest AA-releasing activities when added to mammalian cells (21, 22). An increase in $sPLA_2$ 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 $sPLA_2$s involved in asthma remain unknown (23–25).

We assessed the expression of the full set of cytosolic and secreted PLA_2s 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 $sPLA_2$s were differentially expressed in subjects with asthma compared with

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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 $sPLA_2$s expressed in airway cells of subjects with asthma, and whether these enzymes are overexpressed in asthma; (*2*) to localize the cellular sources of $sPLA_2$s in airway inflammatory and epithelial cells; and (*3*) to determine if selected $sPLA_2$s 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 β_2-agonist for asthma, and had a 15% or greater fall in FEV_1 after exercise challenge (8). The control group consisted of persons aged 18 to 59 years with no history of asthma or atopy, a baseline FEV_1 of 80% predicted or greater, and a less than 7% fall in FEV_1 after exercise challenge. Induced sputum was conducted with 3% saline for 12 minutes at baseline, and on a separate day 4 to 20 days later 30 minutes after exercise challenge. Albuterol (180 μg via metered dose inhaler) was administered 15 minutes before the induced sputum sample was obtained.

Induced Sputum Analysis

Induced sputum was placed on ice and processed within 30 minutes The sample was dispersed with an equal volume of dithiothreitol 0.1% in a shaking water bath at 37°C for 15 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 β-mercaptoethanol, and underwent mechanical lysis. Total RNA was extracted from the lysed cell pellet using the RNeasy 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 PLA_2s, 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 cytocentrifuged preparations of induced sputum cells using polyclonal rabbit anti-human $sPLA_2$ 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 $sPLA_2$ 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 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 $sPLA_2$s 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

TABLE 1. CHARACTERISTICS OF STUDY PARTICIPANTS

	Asthma with EIB (*n* = *25*)	Normal Control (*n* = *10*)
Age, yr (range)	28.1 (14–55)	31.3 (18–57)
Sex, M/F	11/14	6/4
Baseline		
FEV_1, % pred*	84.8 ± 8.4	102.7 ± 10.7
FVC, % pred	100.1 ± 10.3	105.2 ± 13.9
$FEV_1/FVC^{\dagger}$	0.78 ± 0.06	0.81 ± 0.04
Post-bronchodilator		
Δ FEV_1, %†	11.0 ± 6.2	3.4 ± 2.0
Postexercise		
Max Δ FEV_1, %*	−29.2 ± 11.9	−0.4 ± 3.1

Definition of abbreviations: EIB = exercise-induced bronchoconstriction; F = female; M = male.

Values reported are mean ± SD unless otherwise specified.

* $P < 0.001$.

† $P < 0.01$.

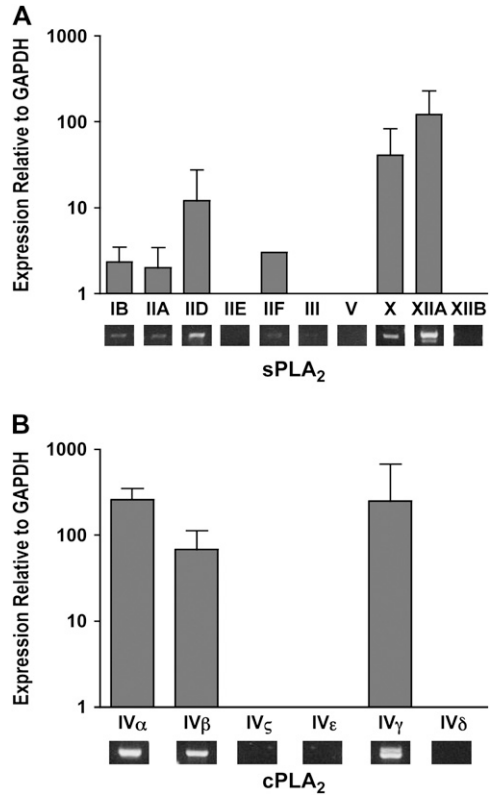

Figure 1. Expression of secreted phospholipase A_2 enzymes ($sPLA_2$s) in induced sputum cells from subjects with asthma and exercise-induced bronchoconstriction. Semiquantitative polymerase chain reaction was used to assess the expression of the secreted (*A*) and cytosolic PLA_2s ($cPLA_2$) (*B*) relative to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The *bars* represent the mean ± SD.

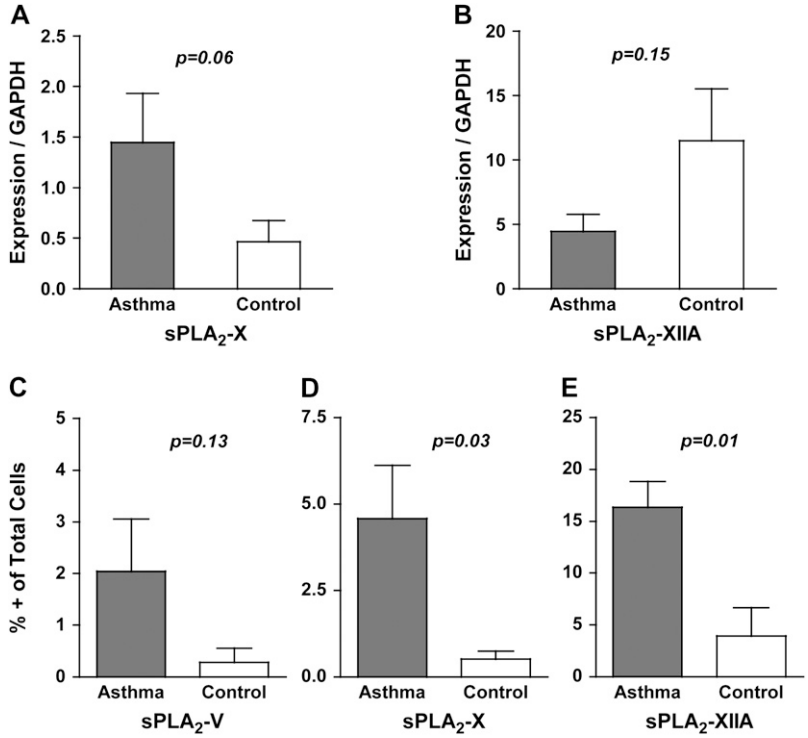

Figure 2. Baseline differences in secreted phospholipase A_2 ($sPLA_2$) groups V, X, and XIIA between subjects with asthma and exercise-induced bronchoconstriction and nonasthmatic control subjects. The gene expression of $sPLA_2$-X was increased relative to control (*A*), whereas there was no difference in the expression of $sPLA_2$-XIIA (*B*). The expression of $sPLA_2$-V was below the level of detection. No difference was detected for immunostaining in induced sputum cells for $sPLA_2$-V (*C*). The percentage of cells immunostaining for $sPLA_2$-X (*D*) and $sPLA_2$-XIIA (*E*) was increased in subjects with asthma relative to control subjects.

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 FEV_1 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, 15*S*-HETE, and PGE_2) 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 PLA_2s 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 PLA_2s (Figure 1). Induced sputum cells from subjects with asthma expressed $sPLA_2$ groups IB, IIA, IID, IIF, X, and XIIA. Of note, $sPLA_2$ groups X and XIIA were expressed at high levels, whereas groups IIE, III, and V were below the level of detection. Isoenzymes of $cPLA_2$ 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 $sPLA_2$s between subjects with asthma and control subjects by quantitative real-time PCR. The expression of $sPLA_2$-X was increased in subjects with asthma relative to control subjects (Figure 2A), whereas there was no difference in the expression of $sPLA_2$-XIIA between subjects with asthma and control subjects (Figure 2B). The expression of $sPLA_2$-V was above the threshold cycle for detection. Although we did not detect $sPLA_2$-V by PCR, we conducted immunocytochemistry for $sPLA_2$-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 supplement (Figure E3). Immunostaining for $sPLA_2$-V in induced sputum cells at baseline was no different between subjects with asthma and control subjects (Figure 2C). In contrast, the percentages of cells immunostaining for $sPLA_2$ groups X and XIIA were greater in induced sputum cells of subjects with asthma compared with control subjects (Figures 2D and 2E). Immunostaining for $sPLA_2$ 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).

Effects of Exercise Challenge on $sPLA_2$s in the Airways

To assess the potential role of the identified $sPLA_2$s 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 $sPLA_2$-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 $sPLA_2$-X increased in subjects with asthma after challenge, but not in control subjects (Figure 4B). The increase in immunostaining for $sPLA_2$-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 $sPLA_2$-X protein measured in induced sputum increased in the asthma group after challenge (Figure 4E), but not in the control group (Figure 4F). An additional Western blot comparing the levels of $sPLA_2$-X after exercise challenge in subjects with asthma compared with control subjects found that the level of $sPLA_2$-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 $sPLA_2$ was protein secreted from the cells.

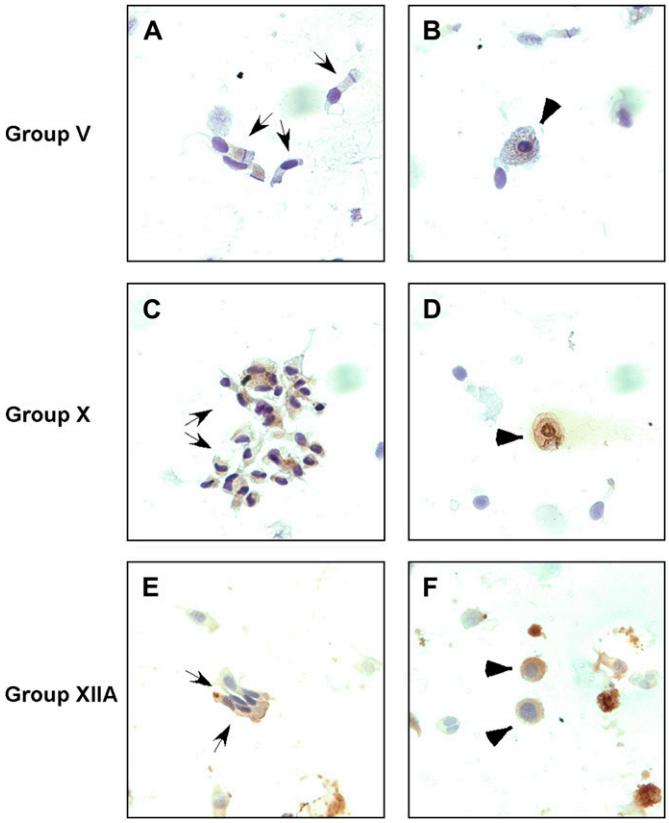

Figure 3. Immunostaining for secreted phospholipase A_2 ($sPLA_2$) groups V, X, and XIIA in induced sputum cells. Representative photomicrographs of immunocytochemistry for $sPLA_2$-V (*A*, *B*), $sPLA_2$-X (*C*, *D*), and $sPLA_2$-XIIA (*E*, *F*) demonstrate immunostaining predominantly in columnar epithelial cells (*arrows*) and macrophages (*arrowheads*). The representative slides were from subjects with asthma after exercise challenge.

Although the $sPLA_2$-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 $sPLA_2$-XIIA after exercise challenge that occurred in the asthma group, which 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, whereas no similar changes were observed in induced sputum cell subtypes in the control group (Figures E5A and E5B). The level of $sPLA_2$-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 $sPLA_2$-V did not increase in either of the groups in response to exercise challenge (Figure 6A). Immunostaining for $sPLA_2$-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 $sPLA_2$-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 $sPLA_2$-V by Western blot in subjects with asthma (Figure 6B).

DISCUSSION

In this study, we evaluated the expression of the complete set of functional PLA_2 enzymes in the lower airways of subjects with asthma and BHR, and determined if $sPLA_2$ 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 15*S*-HETE) in the airways were present between these two groups, particularly after exercise challenge. Of the two $sPLA_2$ enzymes with high AA-releasing capacity (i.e., groups V and X), only $sPLA_2$-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 $sPLA_2$-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 $sPLA_2$-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 $sPLA_2$s are attractive therapeutic targets because these enzymes may be preferentially involved in the production of proinflammatory AA metabolites (e.g., LTs, HETEs, and PGD_2), which are key for asthma immunopathogenesis (12). Two prior studies identified increased $sPLA_2$ 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 $sPLA_2$s involved in asthma have not been determined. There are nine distinct functional $sPLA_2$s that have been identified in the human genome (12). LY333013, an $sPLA_2$ inhibitor 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, suggesting that $sPLA_2$ group II enzymes may not be the key $sPLA_2$ enzymes in human asthma (30). The group II enzymes were expressed at relatively low levels in the present study. Among the two $sPLA_2$s with high AA-releasing capacity (22), we found that only $sPLA_2$-X was differentially overexpressed in asthma, whereas $sPLA_2$-V was present at low levels and was not differentially expressed in asthma. The difference in $sPLA_2$-X function may be greater than the difference in $sPLA_2$-X protein levels identified in this study because $sPLA_2$-X becomes activated in inflammatory tissues (31). These results, together with the recent demonstration by our research group that $sPLA_2$-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 $sPLA_2$-X is a potential therapeutic target in asthma.

Although we focused on the $sPLA_2$ enzymes in this study, we also identified the expression of three $cPLA_2$s in subjects with asthma, including the well-known $cPLA_2\alpha$, which has a pivotal role in eicosanoid production (13), and two other enzymes, $cPLA_2\beta$ and $cPLA_2\gamma$. In cultured cells, $sPLA_2$s coordinate with $cPLA_2\alpha$ to augment the production of eicosanoids (15). Relatively little is known about the two other $cPLA_2$s, but both enzymes function in eicosanoid synthesis (33, 34). $cPLA_2\beta$ is implicated in lipid mediator release in response to cardiac ischemia (35), and $cPLA_2\gamma$ is involved in peroxide-induced release of eicosanoids (36). The expression of $cPLA_2\gamma$ 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 PGD_2 into the

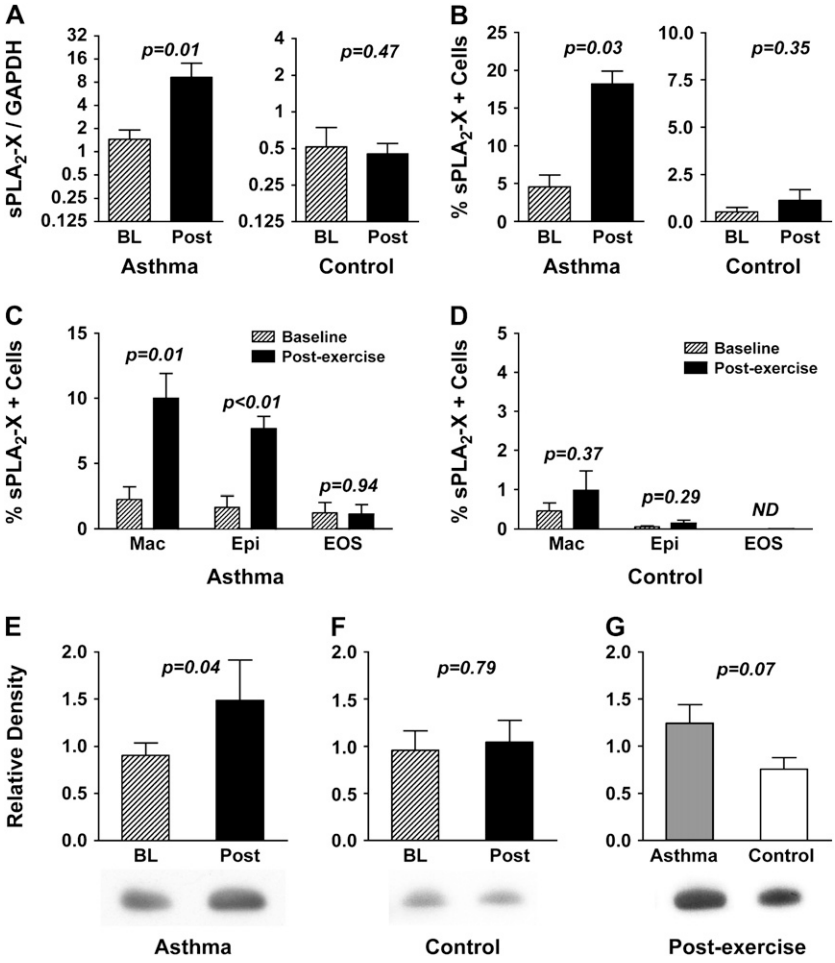

Figure 4. Effects of exercise challenge on group X secreted phospholipase A_2 ($sPLA_2$-X) in subjects with asthma with exercise-induced bronchoconstriction and nonasthmatic control subjects. The gene expression (*A*) and percentage of cells in induced sputum immunostaining (*B*) for $sPLA_2$-X increased in subjects with asthma after challenge, but not in control subjects. Immunostaining for $sPLA_2$-X in subjects with asthma increased in bronchial macrophages (Mac) and columnar epithelial cells (Epi), but not in eosinophils (EOS) (*C*), whereas no changes were observed in the control group (*D*). Western blots of induced sputum supernatant revealed an increase in $sPLA_2$-X protein in subjects with asthma (*E*), but not in control subjects (*F*), resulting in a higher level of $sPLA_2$-X in subjects with asthma than in control subjects after exercise (*G*). BL = baseline; post = postexercise.

airways during EIB that sustain bronchoconstriction (8, 9). Selective functions of the different PLA_2 enzymes may be involved in this dysregulated eicosanoid synthesis. We found in the present study that the ratio of CysLTs to PGE_2 decreased in controls after exercise challenge, in contrast to our prior findings that the ratio of CysLTs to PGE_2 increases after exercise challenge in subjects with asthma and EIB (8). This altered balance of eicosanoids is important because PGE_2 serves protective and bronchodilator functions (5), and inhaled PGE_2 before exercise and allergen challenge significantly attenuates bronchoconstriction (38–40). In cultured cells, activation of $cPLA_2\alpha$ alone is more closely associated with the generation of PGE_2 (18, 19), whereas $sPLA_2$s have been strongly implicated in the release of proinflammatory eicosanoids, such as CysLTs (18, 19), which can occur in the absence of $cPLA_2\alpha$ activation (20).

Localization of $sPLA_2$-X to the epithelium was prominent in the present study, consistent with the up-regulation of $sPLA_2$-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 $sPLA_2$s. 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 PGE_2 synthesis (42). Similarly, release of $sPLA_2$-V from epithelial cells augments CysLT synthesis in eosinophils without activation of $cPLA_2\alpha$ (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 $sPLA_2$-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 $sPLA_2$-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 $sPLA_2$-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 immunocytochemistry 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 subjects with asthma 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 morphologic alterations in HEK293 cells, which are unique among the $sPLA_2$ transfected cells (52).

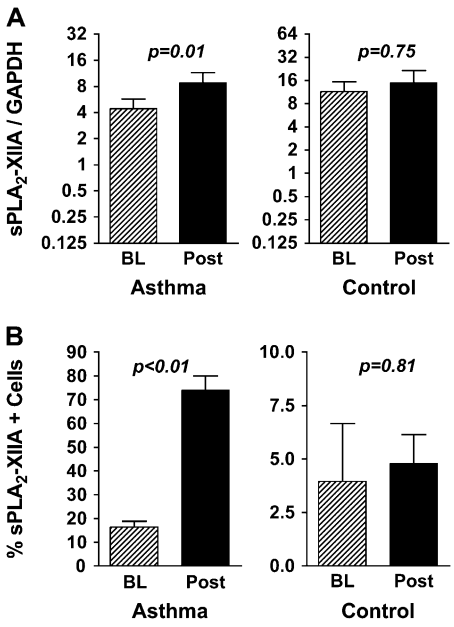

Figure 5. Effects of exercise challenge on group XIIA secreted phospholipase A_2 ($sPLA_2$-XIIA) in subjects with asthma and exercise-induced bronchoconstriction and nonasthmatic control subjects. The gene expression (*A*) and percentage of cells in induced sputum immunostaining (*B*) for $sPLA_2$-XIIA increased in subjects with asthma after challenge, but not in control subjects. BL = baseline; post = postexercise.

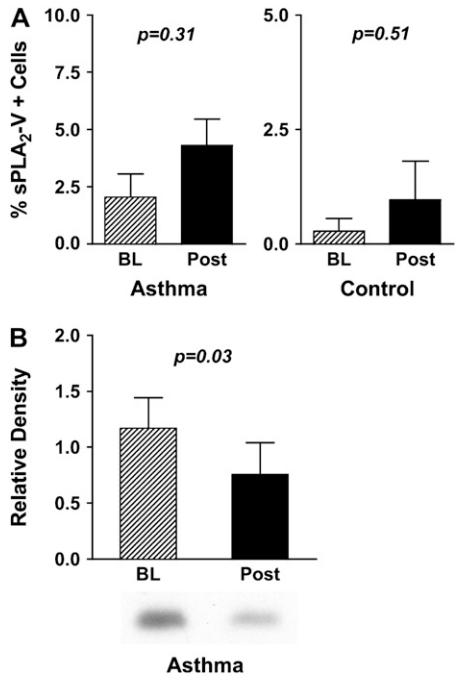

Figure 6. Effects of exercise challenge on group V secreted phospholipase A_2 ($sPLA_2$-V) in subjects with asthma and exercise-induced bronchoconstriction and nonasthmatic control subjects. The percentage of cells in induced sputum immunostaining for $sPLA_2$-V was no different postexercise compared with baseline in either the subjects with asthma or control subjects (*A*). Western blot of induced sputum supernatant had no increase in $sPLA_2$-V protein in subjects with asthma (*B*), and could not be detected in control subjects. BL = baseline; post = postexercise.

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 subjects with asthma and 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 subjects with asthma, but not in control subjects. Collectively, these results indicate that $sPLA_2$-X may play a role in the generation of proinflammatory eicosanoids in the airways and in the development of BHR. Inhibition of $sPLA_2$-X may represent an important novel therapeutic target in human asthma.

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References

1. Hallstrand TS, Henderson WR Jr. Leukotriene modifiers. *Med Clin North Am* 2002;86:1009–1033.
2. 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.
3. Griffin M, Weiss JW, Leitch AG, McFadden ER Jr, Corey EJ, Austen KF, Drazen JM. Effects of leukotriene D on the airways in asthma. *N Engl J Med* 1983;308:436–439.
4. Hardy CC, Robinson C, Tattersfield AE, Holgate ST. The bronchoconstrictor effect of inhaled prostaglandin D_2 in normal and asthmatic men. *N Engl J Med* 1984;311:209–213.
5. Mathe AA, Hedqvist P. Effect of prostaglandins F_2 alpha and E_2 on airway conductance in healthy subjects and asthmatic patients. *Am Rev Respir Dis* 1975;111:313–320.
6. Joos GF, O'Connor B, Anderson SD, Chung F, Cockcroft DW, Dahlen B, DiMaria G, Foresi A, Hargreave FE, Holgate ST, *et al.* Indirect airway challenges. *Eur Respir J* 2003;21:1050–1068.
7. Currie GP, Haggart K, Lee DK, Fowler SJ, Wilson AM, Brannan JD, Anderson SD, Lipworth BJ. Effects of mediator antagonism on mannitol and adenosine monophosphate challenges. *Clin Exp Allergy* 2003;33:783–788.
8. 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.
9. 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.
10. 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.
11. 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.
12. 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.
13. Uozumi N, Kume K, Nagase T, Nakatani N, Ishii S, Tashiro F, Komagata Y, Maki K, Ikuta K, Ouchi Y, *et al.* Role of cytosolic phospholipase A_2 in allergic response and parturition. *Nature* 1997;390:618–622.
14. 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.
15. 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.
16. 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.
17. Ni Z, Okeley NM, Smart BP, Gelb MH. Intracellular actions of group IIA secreted phospholipase A_2 and group IVA cytosolic phospholipase A_2 contribute to arachidonic acid release and prostaglandin production in rat gastric mucosal cells and transfected human embryonic kidney cells. *J Biol Chem* 2006;281:16245–16255.
18. Kessen UA, Schaloske RH, Stephens DL, Killermann Lucas K, Dennis EA. PGE_2 release is independent of upregulation of group V phospholipase A_2 during long-term stimulation of P388D1 cells with LPS. *J Lipid Res* 2005;46:2488–2496.
19. Marshall LA, Bolognese B, Winkler JD, Roshak A. Depletion of human monocyte 85-kDa phospholipase A_2 does not alter leukotriene formation. *J Biol Chem* 1997;272:759–765.
20. Munoz NM, Kim YJ, Meliton AY, Kim KP, Han SK, Boetticher E, O'Leary E, Myou S, Zhu X, Bonventre JV, *et al.* Human group V phospholipase A_2 induces group IVA phospholipase A_2-independent cysteinyl leukotriene synthesis in human eosinophils. *J Biol Chem* 2003;278:38813–38820.
21. Calabrese C, Triggiani M, Marone G, Mazzarella G. Arachidonic acid metabolism in inflammatory cells of patients with bronchial asthma. *Allergy* 2000;55:27–30.
22. Singer AG, Ghomashchi F, Le Calvez C, Bollinger J, Bezzine S, Rouault M, Sadilek M, Nguyen E, Lazdunski M, Lambeau G, *et al.* 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.
23. 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.
24. 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.
25. 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.
26. Hallstrand TS, Chi EY, Gelb MH, Henderson WR Jr. Phospholipase A_2 isoenzymes in airway inflammatory cells of patients with mild asthma and exercise-induced bronchoconstriction. *Am J Respir Crit Care Med* 2004;169:A569.
27. Degousee N, Ghomashchi F, Stefanski E, Singer A, Smart BP, Borregaard N, Reithmeier R, Lindsay TF, Lichtenberger C, Reinisch W, *et al.* 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.
28. Fahy JV, Boushey HA, Lazarus SC, Mauger EA, Cherniack RM, Chinchilli VM, Craig TJ, Drazen JM, Ford JG, Fish JE, *et al.* Safety and reproducibility of sputum induction in asthmatic subjects in a multicenter study. *Am J Respir Crit Care Med* 2001;163:1470–1475.
29. Hallstrand TS, Debley JS, Farin FM, Henderson WR Jr. Role of MUC5AC in the pathogenesis of exercise-induced bronchoconstriction. *J Allergy Clin Immunol* 2007;119:1092–1098.
30. 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.
31. Ohtsuki M, Taketomi Y, Arata S, Masuda S, Ishikawa Y, Ishii T, Takanezawa Y, Aoki J, Arai H, Yamamoto K, *et al.* Transgenic expression of group V, but not group X, secreted phospholipase A_2 in mice leads to neonatal lethality because of lung dysfunction. *J Biol Chem* 2006;281:36420–36433.
32. Henderson WR Jr, Chi EY, Bollinger JG, Tien YT, Ye X, Castelli L, Rubtsov YP, Singer AG, Chiang GK, Nevalainen T, *et al.* 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.
33. Song C, Chang XJ, Bean KM, Proia MS, Knopf JL, Kriz RW. Molecular characterization of cytosolic phospholipase A_2-β. *J Biol Chem* 1999; 274:17063–17067.
34. Underwood KW, Song C, Kriz RW, Chang XJ, Knopf JL, Lin LL. A novel calcium-independent phospholipase A_2, $cPLA_2$-γ, that is prenylated and contains homology to $cPLA_2$. *J Biol Chem* 1998;273: 21926–21932.
35. Mancuso DJ, Abendschein DR, Jenkins CM, Han X, Saffitz JE, Schuessler RB, Gross RW. Cardiac ischemia activates calcium-independent phospholipase A_2β, precipitating ventricular tachyarrhythmias in transgenic mice: rescue of the lethal electrophysiologic phenotype by mechanism-based inhibition. *J Biol Chem* 2003;278:22231–22236.
36. Asai K, Hirabayashi T, Houjou T, Uozumi N, Taguchi R, Shimizu T. Human group IVC phospholipase A_2 ($cPLA_2\gamma$). Roles in the membrane remodeling and activation induced by oxidative stress. *J Biol Chem* 2003;278:8809–8814.
37. Mancuso P, Canetti C, Gottschalk A, Tithof PK, Peters-Golden M. Leptin augments alveolar macrophage leukotriene synthesis by increasing phospholipase activity and enhancing group IVC $iPLA_2$ ($cPLA_2\gamma$) protein expression. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L497–L502.
38. Melillo E, Woolley KL, Manning PJ, Watson RM, O'Byrne PM. Effect of inhaled PGE_2 on exercise-induced bronchoconstriction in asthmatic subjects. *Am J Respir Crit Care Med* 1994;149:1138–1141.
39. Gauvreau GM, Watson RM, O'Byrne PM. Protective effects of inhaled PGE_2 on allergen-induced airway responses and airway inflammation. *Am J Respir Crit Care Med* 1999;159:31–36.
40. Hartert TV, Dworski RT, Mellen BG, Oates JA, Murray JJ, Sheller JR. Prostaglandin E_2 decreases allergen-stimulated release of prostaglandin D_2 in airways of subjects with asthma. *Am J Respir Crit Care Med* 2000;162:637–640.
41. Jame AJ, Lackie PM, Cazaly AM, Sayers I, Penrose JF, Holgate ST, Sampson AP. Human bronchial epithelial cells express an active and inducible biosynthetic pathway for leukotrienes B_4 and C_4. *Clin Exp Allergy* 2007;37:880–892.
42. Peters-Golden M, Feyssa A. Transcellular eicosanoid synthesis in cocultures of alveolar epithelial cells and macrophages. *Am J Physiol* 1993;264:L438–L447.
43. Munoz NM, Meliton AY, Lambertino A, Boetticher E, Learoyd J, Sultan F, Zhu X, Cho W, Leff AR. Transcellular secretion of group V phospholipase A_2 from epithelium induces β_2-integrin-mediated adhesion and synthesis of leukotriene C_4 in eosinophils. *J Immunol* 2006; 177:574–582.
44. Wijewickrama GT, Kim JH, Kim YJ, Abraham A, Oh Y, Ananthanarayanan B, Kwatia M, Ackerman SJ, Cho W. Systematic evaluation of transcellular activities of secretory phospholipases A_2: high activity of group V PLA_2 to induce eicosanoid biosynthesis in neighboring inflammatory cells. *J Biol Chem* 2006;281:10935–10944.
45. Anderson SD. How does exercise cause asthma attacks? *Curr Opin Allergy Clin Immunol* 2006;6:37–42.
46. Plummeridge MJ, Armstrong L, Birchall MA, Millar AB. Reduced production of interleukin 12 by interferon γ primed alveolar macrophages from atopic asthmatic subjects. *Thorax* 2000;55:842–847.
47. Alexis NE, Soukup J, Nierkens S, Becker S. Association between airway hyperreactivity and bronchial macrophage dysfunction in individuals with mild asthma. *Am J Physiol Lung Cell Mol Physiol* 2001;280: L369–L375.
48. Careau E, Proulx LI, Pouliot P, Spahr A, Turmel V, Bissonnette EY. Antigen sensitization modulates alveolar macrophage functions in an asthma model. *Am J Physiol Lung Cell Mol Physiol* 2006;290:L871–L879.
49. Balter MS, Toews GB, Peters-Golden M. Different patterns of arachidonate metabolism in autologous human blood monocytes and alveolar macrophages. *J Immunol* 1989;142:602–608.
50. Balter MS, Eschenbacher WL, Peters-Golden M. Arachidonic acid metabolism in cultured alveolar macrophages from normal, atopic, and asthmatic subjects. *Am Rev Respir Dis* 1988;138:1134–1142.
51. Gelb MH, Valentin E, Ghomashchi F, Lazdunski M, Lambeau G. Cloning and recombinant expression of a structurally novel human secreted phospholipase A_2. *J Biol Chem* 2000;275:39823–39826.
52. Murakami M, Masuda S, Shimbara S, Bezzine S, Lazdunski M, Lambeau G, Gelb MH, Matsukura S, Kokubu F, Adachi M, *et al.* Cellular arachidonate-releasing function of novel classes of secretory phospholipase A_2s (groups III and XII). *J Biol Chem* 2003;278:10657–10667.
53. Koduri RS, Gronroos JO, Laine VJ, Le Calvez C, Lambeau G, Nevalainen TJ, Gelb MH. Bactericidal properties of human and murine groups I, II, V, X, and XII secreted phospholipases A_2. *J Biol Chem* 2002;277:5849–5857.