HHS Public Access

Author manuscript

J Allergy Clin Immunol. Author manuscript; available in PMC 2017 January 01.

Published in final edited form as:

J Allergy Clin Immunol. 2016 January ; 137(1): 268–277.e8. doi:10.1016/j.jaci.2015.05.026.

Endogenous secreted phospholipase A_2 group X regulates cysteinyl leukotrienes sy nthesis by human eosinophils

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Abstract

Background—Phospholipase A_2s mediate the rate-limiting step in the formation of eicosanoids such as cysteinyl leukotrienes (CysLT). Group IVA cytosolic PLA_2 (cPLA$_2\alpha$) is thought to be the dominant PLA_2 in eosinophils; however, eosinophils also have secreted PLA_2 (sPLA$_2$) activity that has not been fully defined.

Objectives—To examine the expression of sPLA$_2$ group X (sPLA$_2$-X) in eosinophils, the participation of sPLA$_2$-X in CysLT formation, and the mechanism by which sPLA$_2$-X initiates CysLT synthesis in eosinophils.

Methods—Peripheral blood eosinophils were obtained from volunteers with asthma and/or allergy. A rabbit polyclonal anti-sPLA$_2$-X antibody identified sPLA$_2$-X by western blot. We used confocal microscopy to co-localize the sPLA$_2$-X to intracellular structures. An inhibitor of sPLA$_2$-X (ROC-0929) that does not inhibit other mammalian sPLA$_2$s, as well as inhibitors of the mitogen activated kinase cascade (MAPK) and cPLA$_2\alpha$ were used to examine the mechanism of N-formyl-methionyl-leucyl-phenylalanine (fMLP)-mediated CysLT formation.

Results—Eosinophils express the sPLA$_2$-X gene (*PLA2G10*). The sPLA$_2$-X protein is located in the endoplasmic reticulum (ER), golgi, and granules of eosinophils and moves to the granules and lipid bodies during fMLP-mediated activation. Selective sPLA$_2$-X inhibition attenuated the fMLP-mediated release of arachidonic acid and CysLT formation by eosinophils. Inhibitors of p38, ERK1/2, JNK and cPLA$_2\alpha$ also attenuated the fMLP-mediated CysLT formation. The sPLA$_2$-X inhibitor reduced the phosphorylation of p38 and ERK1/2 as well as cPLA$_2\alpha$ during cellular activation, indicating that sPLA$_2$-X is involved in activating the MAPK cascade leading to CysLT

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formation via cPLA$_2$α. We further demonstrate that sPLA$_2$-X is activated prior to secretion from the cell during activation. Short-term priming with IL-13 and TNF/IL-1β increased the expression of *PLA2G10* by eosinophils.

Conclusions—These results demonstrate that sPLA$_2$-X plays a significant role in CysLTs formation by human eosinophils. The predominant role of the enzyme is the regulation of MAPK activation that leads to phosphorylation of cPLA$_2$α. The sPLA$_2$-X protein is regulated by proteolytic cleavage suggesting that an inflammatory environment may promote the formation of CysLTs through this mechanism. These results have important implications for the treatment of eosinophilic disorders such as asthma.

Keywords

eosinophil; leukotriene; phospholipase A$_2$; mitogen-activated kinase; asthma; allergy

INTRODUCTION

Cysteinyl leukotrienes (CysLTs, C$_4$, D$_4$ and E$_4$) are generated in increased quantities in the airways of patients with asthma (1–3). Eosinophils accumulate in the airways of many patients with asthma and serve as a key source of CysLTs (4). The rate-limiting step in CysLT formation is phospholipase A$_2$ (PLA$_2$)-mediated release of arachidonate from the *sn*-2 position of membrane phospholipids. It has been assumed that group IVA cytosolic PLA$_2$ (i.e. cPLA$_2$α) serves as the key regulator of endogenous CysLT synthesis in myeloid cells such as eosinophils (5,6); however, 10 mammalian secreted PLA$_2$s (sPLA$_2$s) have been identified, and in transfected cells, several sPLA$_2$s have been found to coordinate eicosanoid synthesis with cPLA$_2$α (7–9). Among the sPLA$_2$s, groups V and X (i.e. sPLA$_2$-V and sPLA$_2$-X) have unique functional capacities to cleave phospholipids and initiate cellular eicosanoid synthesis (10,11). We have demonstrated that sPLA$_2$-X is elevated in the airways of patients with asthma (12,13), and serves to activate CysLT synthesis in eosinophils when the recombinant enzyme is added exogenously to eosinophils (14).

Eosinophils also have endogenous sPLA$_2$ activity that contributes to surfactant dysfunction through the cleavage of surfactant phospholipids (15,16). The gene expression of sPLA$_2$ group IIA (sPLA$_2$-IIA, *PLA2G2A*) and sPLA$_2$-X (*PLA2G10*) were previously identified in human eosinophils (16). The sPLA$_2$-IIA protein was identified in eosinophils (17), but does not contribute to leukotriene formation in these cells (18,19). The other high activity sPLA$_2$, sPLA$_2$-V, is not present in eosinophils (20). In the present study, we investigated the contribution of the sPLA$_2$-X enzyme to sPLA$_2$ activity in human primary eosinophils, and the role of this enzyme as a key control point in CysLT formation. The results support an important role of sPLA$_2$-X in endogenous CysLT synthesis in eosinophils, and that sPLA$_2$-X is involved in the activation of the MAPK cascade leading to the activation of cPLA$_2$α.

METHODS

Materials

Ficoll-Paque PLUS (d 1.077) was from GE Healthcare (Piscataway, NJ). Antibodies for immunomagnetic selection were from Miltenyi Biotec (Auburn, CA). [^{3}H]Arachidonate was

from American Radiolabeled Chemicals (St. Louis, MO). N-formyl-methionyl-leucyl-phenylalanine (fMLP) was from Sigma-Aldrich (St. Louis, MO). Antibodies against phospho-cPLA$_2\alpha$ [Ser505], cPLA$_2\alpha$, phospho-p44/42 MAPK (Erk 1/2)[Thr202/Tyr204], p44/42 MAPK (Erk 1/2), phospho-p38 MAPK [Thr180/Tyr182], p38 MAPK, phospho-stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK)[thr183/tyr185], and SAPK/JNK were from Cell Signaling Technologies (Beverly, MA). Inhibitors of MEK 1/2 (U0126), p38 (SB203580), JNK (SP600125), Furin I (344930, D-RVKR-CMK) and Furin II (344931, 6-D-R) were purchased from EMD Biosciences (San Diego, CA). Rabbit polyclonal anti-sPLA$_2$-X antibody was developed and characterized in our laboratory (21). Dr. James J. Lee (Mayo Clinic, Scottsdale, AZ) provided the murine monoclonal anti-EPX antibody. Murine monoclonal anti-GM130 (35/GM130) was from BD Bioscience (San Jose, CA). Murine monoclonal anti-PDI (RL90) was from Abcam (Cambridge, MA). Murine monoclonal anti-ADRP (AP125) was from Fitzgerald (Acton, MA). Rabbit anti-5-LO antiserum was purchased from Cayman Chemical (Ann Arbor, MI) and rabbit anti-cPLA$_2\alpha$ anti-serum was purchased from Santa Cruz Biotechnology (Dallas, TX). Cy3-conjugated goat anti-rabbit IgG was from Jackson ImmunoReasearch (West Grove, PA). Alexa Fluor 647 goat anti-mouse IgG was purchased from Invitrogen.

Isolation of human blood eosinophils and differentiation of HL15 cells

The University of Washington Institutional Review Board approved the study, and written informed consent was obtained from all participants. Peripheral blood eosinophils were obtained from volunteers with a physician diagnosis of asthma and/or allergy and $\geq 1.2 \times 10^5$ eosinophil/ml of peripheral blood. Granulocytes were isolated from peripheral blood by density gradient centrifugation followed by hypotonic lysis of red blood cells. Eosinophils were removed from the granulocyte fraction by negative immunomagnetic selection. The purity of eosinophils was determined by differential counts of Romanowski-stained (Diff-Quick) cytospin preparations. Eosinophil viability was assessed by trypan blue exclusion.

HL-60 clone-15 (HL-60-C15) and AML14.3D10-CCR3 cells were purchased from ATCC. Cells were cultured in RPMI with 10% FCS. HL-60-C15 cells were treated with and without 0.5 mM sodium butyrate for 7 days.

Selective PLA$_2$ inhibitors

Because human eosinophils contain sPLA$_2$ group IIA (sPLA$_2$-IIA) (17) we used a sPLA$_2$ inhibitor, known as ROC-0929, that is selective for sPLA$_2$-X and does not inhibit other mammalian sPLA$_2$s at nanomolar concentrations (22). The compound ROC-0929 is an analog of the well-known sPLA$_2$ inhibitor LY315920 (22). Docking studies revealed that the isobutyl group of ROC-0929 sterically excludes this compound from the active site of sPLA$_2$-IIA, but not the active site of sPLA$_2$-X, resulting in > 80-fold difference in inhibitory potency of ROC-0929 between the sPLA$_2$-X and sPLA$_2$-IIA enzymes (22). To test for off target effects, we used a control compound, known as ROC-0428, that differs from ROC-0929 by one methyl group and is essentially devoid of sPLA$_2$ inhibition (22). We also used a pan-sPLA$_2$ inhibitor (0509A) and an inhibitor that is selective for the group IIA/IIE enzymes (0320). The inhibitory a ctivities of the sPLA$_2$ inhibitors and the control compound

are shown in Supplemental Table 1. Inhibition of cPLA$_2\alpha$ was achieved with Pyr-2 (pyrrophenone) and Wyeth-2 (gifipladib) (23,24).

Secreted PLA$_2$ activity assay

^{3}H-labelled *E. coli* membranes were resuspended with reaction buffer at a final concentration of 2.0×10^6 dpm/mL (25). The eosinophil lysate was treated with inhibitors or DMSO control for 10 minutes at room temperature. The radio-labeled *E. coli* membrane substrate was added to each reaction tube and incubated at 37°C for 1 hour. The reaction was stopped by the addition of 0.1 M EDTA and the sample centrifuged at 14, 000 × *g*. The supernatant was submitted to scintillation counting.

Assessment of arachidonate release by eosinophils

The release of arachidonate and CysLT from eosinophils was determined in 24-well plates coated with 0.01% BSA. Eosinophils (1.8×10^5 per well) were resuspended in RPMI with 0.01% BSA, and the cells were incubated for 24 h with [^{3}H]arachidonate (0.1 μCi/well). After the unincorporated arachidonate was washed three times, the cells were preincubated with inhibitors for 20 min at 37°C, and then stimulated with fMLP for 20 min. Supernatants were submitted to scintillation counting after being centrifuged to remove detached cells. The remaining eosinophils were detached with 0.25% trypsin with EDTA for 30 min at 37°C, pelleted, and submitted to scintillation counting. Arachidonate release was expressed as a percentage of counts/min in the supernatant to the total counts/min in the cells and supernatant.

Assessment of CysLT formation by eosinophils

Eosinophils were resuspended in HBSS with Ca^{2+} and Mg^{2+} at a concentration of 1.5×10^5 per well in a 5% CO_2 incubator at 37°C. The cells were preincubated with either inhibitor or DMSO control for 20 min at 37°C and then stimulated with fMLP for 20 min. The synthesis of eicosanoids was stopped by the addition of 4 volumes of ice-cold methanol with 0.2% formic acid. An ELISA assay measured LTC_4 or total CysLT levels after removal of the methanol by evaporation (Cayman Chemical, Ann Arbor, MI).

Selective Kinase Inhibitors

To examine the role of the MAPKs in LTC_4 synthesis, we used a series of inhibitors that are specific with little cross inhibition of the other kinases (26–28). Eosinophils (1.5×10^5 per well) resuspended in HBSS with Ca^{2+} and Mg^{2+} were preincubated with either DMSO control or inhibitors of MEK1/2 (U0126, 10 μM), p38 (SB203580, 30 μM), or JNK (SP600125, 20 μM) for 20 min at 37°C in a 5% CO_2 incubator and then stimulated with fMLP (100 nM). The synthesis of eicosanoids was stopped by the addition of 4 volumes of ice-cold methanol with 0.2% formic acid after 20 min.

Measurement of MAPK and cPLA$_2\alpha$ phosphorylation

Phosphorylation of ERK1/2, p38, JNK and cPLA$_2\alpha$ in eosinophils (2.0×10^6 cells) pretreated with DMSO control or ROC-0929 and then stimulated with 100nM fMLP for 20 min was determined by western blots of the phosphorylated protein relative to the total

protein. The cells were pelleted and treated with lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, with protease and phosphatase inhibitors) on ice for 30 min. Equal amounts of cell lysate were heated for 10 min in LDS loading buffer with DTT and β-mercaptoethanol, and separated on 4–12% Bis-Tris gel under reducing conditions. The resolved proteins were transferred onto a polyvinylidene fluoride membrane using a semidry apparatus, blocked with 5% non-fat milk, and incubated with the primary antibody overnight at 4°C. ECL visualized the proteins of interest.

Pro-peptide convertase inhibitors

Eosinophils (1.5×10^5 per well) resuspended in HBSS with Ca^{2+} and Mg^{2+} were preincubated with either DMSO control or Furin I (D-RVKR-CMK, 10 μM) and Furin II (344931, 6-D-R, 10 μM) for 20 min at 37°C in a 5% CO_2 incubator and then stimulated with fMLP (100 nM). The synthesis of eicosanoids was stopped by the addition of 4 volumes of iced methanol with 0.2% formic acid after 20 min.

Confocal Microscopy

Eosinophils (1.0×10^6 per well) were allowed to adhere to cover glasses coated with 0.01% BSA and treated with DMSO vehicle control or ROC-0929 for 20 min, and then treated with either vehicle control or fMLP (100 nM) for 20 min at 37°C in a 5% CO_2 incubator. The cells were fixed in 3.7% formaldehyde in PBS at room temperature for 30 min. Eosinophils were permeabilized with 0.1% Triton X-100 and blocked with 10% goat serum. Immunostained cells were visualized with a Zeiss LSM510 Confocal Microscope.

Quantitative PCR

Real-time PCR analysis was conducted using TaqMan primer probe sets with quantification relative to an endogenous control gene using the delta Ct method. Primer-probe sets were obtained from the Applied Biosystems using a FAM probe for *PLA2G10* (Hs00358567_m1) and a primer-limited VIC probe for *HPRT1* (4326321E) as an endogenous control (Applied Biosystems, Foster City, CA). Real-time PCR was performed using a Mastercycler ep realplex system (Eppendorf, Hauppauge, NY). In some experiments, eosinophils were placed in RPMI at 37°C in a 5% CO_2 incubator for 4 hours during treatment with buffer control, or TNFα, IL-β, IL-4, IL-5, IL-13, GMCSF, IL-33, and/or thymic stromal lymphopoietin (TSLP) (Peprotech, Rock Hill, NJ). Following cytokine treatment, Trizol isolated RNA was used to assay *PLA2G10* gene expression.

Statistical Analysis

A one-way ANOVA assessed differences in eicosanoid formation with fMLP stimulation in the presence of specific inhibitors. Post-hoc comparisons were made between fMLP treated cells and other conditions with Dunnett's multiple comparison test. A paired t-test assessed differences between *PLA2G10* gene expression when two conditions were present.

Results

Subject characteristics

We isolated peripheral blood eosinophils 54 times from a total of 45 distinct donors for these studies. The mean viability was 99.5% (SD 1.58) and purity was 99.4% (SD 0.64) eosinophils. The mean age of the donors was 30.2 years old and 55.6% were female. The study population was 8.9% Hispanic, and 62.2% Caucasian, 8.9% African American, and 6.7% Asian. Both asthma and allergic diseases were present in 53.3% of the population, while 11.1% had asthma alone, and 35.6% had allergic diseases. The mean concentration of eosinophils in peripheral blood was 3.0×10^5 eosinophils/ml, and the mean percentage of eosinophils was 4.5%.

sPLA$_2$-X is expressed by human eosinophils and localizes to structures involved in leukotriene formation

We assessed the expression of the sPLA$_2$-X gene *PLA2G1*0 in human eosinophils from 3 donors and found that human eosinophils strongly express *PLA2G10* (Fig 1A). The positive control for *PLA2G10* expression was RNA obtained from a human epithelial cell line as epithelial cells strongly express *PLA2G10* (13). In cell lysates of eosinophils, the sPLA$_2$-X protein can be identified on western blot using a rabbit polyclonal antibody that is specific for sPLA$_2$-X (Fig 1B) (21,29). The native sPLA$_2$-X protein runs on the gel at about 13.5 kDa, slightly larger than the recombinant mature sPLA$_2$-X protein, indicating that some post-translational modification of the protein has occurred. Treatment with N-Glycosidase A to cleave N-glycans did not significantly change the gel shift of the protein (data not shown). A second larger band identified in the blot appears to be a dimer of the sPLA$_2$-X enzyme that is also seen with the recombinant protein when run at a higher concentration (**see** Fig E3E). Either a sPLA$_2$-X-specific inhibitor (ROC-0929) or a pan-sPLA$_2$ inhibitor (0509A) significantly decreased the total sPLA$_2$ activity in lysates from human eosinophils; however, the inhibitory activity of the sPLA$_2$-IIA/IIE inhibitor (0320) did not reach statistical significance (Fig 1C; Table S1). It should be noted that the high-sensitivity PLA$_2$ assay used in the present study has a specific activity for sPLA$_2$-X that is relatively low due to the anionic phospholipid composition in the *E. coli* radiometric assay (30). In contrast, the release of free arachidonate from mammalian HEK293 cells is ~1,000 times higher for sPLA$_2$-X over the sPLA$_2$-IIA enzyme (11). These results indicate that sPLA$_2$-X is expressed in human eosinophils and comprises the majority of sPLA$_2$ activity in these cells.

We used confocal microscopy to characterize the location of sPLA$_2$-X in resting and fMLP-stimulated eosinophils. In resting cells the sPLA$_2$-X protein co-localized predominantly to the endoplasmic reticulum (ER) and golgi (Fig 2 and Fig E1). There may also be some colocalization to eosinophil granules identified by immunostaining for EPX. The antibody used to identify lipid bodies (i.e. anti-ADRP) demonstrated only background immunostaining on the plasma membrane in resting cells.

Following stimulation of the cells with fMLP, there was persistent colocalization to the ER, and less co-localization to the golgi (Fig 2 and Fig E2). In stimulated cells, the granule staining by anti-EPX became more distinct, and there was some colocalization with sPLA$_2$-

X to the granules (arrow in middle panel of Fig 2). The immunostaining for $sPLA_2$-X in granules is further delineated in Figure E2 demonstrating red immunostaining for $sPLA_2$-X in structures labeled with the anti-EPX antibody for granules in green (arrows, third panel). With the anti-ADRP immunostaining of lipid bodies, fMLP stimulation caused the formation of lipid bodies that could be observed both within the cytoplasm of eosinophils as well as some lipid bodies that appear extracellular. Although there was limited colocalization of $sPLA_2$-X to lipid bodies (arrow in lower panel of Fig 2), it was apparent that the $sPLA_2$-X protein resides in these structures, but in a slightly different location from the target for the anti-ADRP (arrows in lower panel of Fig E2). These results indicate that $sPLA_2$-X is located predominantly in the ER of resting eosinophils, but enters the secretory system and lipid bodies upon stimulation and may be externalized during activation either through release or eosinophil necrosis.

An active site-directed inhibitor of $sPLA_2$-X inhibits fMLP-mediated eicosanoid synthesis

We activated human eosinophils with fMLP based in part on the observation that well-established allergens, house dust mite proteins, activate eosinophils via the formyl peptide receptors (31). After eosinophils were incubated with radiolabeled arachidonate, the percent of [^{3}H]arachidonate released in response to fMLP was attenuated by pre-treatment of the cells with the $sPLA_2$-X-specific inhibitor ROC-0929 (Fig 3A, $P=0.004$). In this assay, there was no significant difference between the effects of the inhibitor at concentrations ranging from 10 to 1000 nM. The release of the CysLT LTC_4 from fMLP activated eosinophils was reduced in a dose dependent manner by increasing concentrations of ROC-0929 ranging from 1–1000 nM, although only the 100 and 1000 nM concentrations of the inhibitor caused a statistically significant reduction in LTC_4 formation based on *post hoc* tests (Fig 3B, $P<0.0001$). The ROC-0929 inhibitor did not have any effect on the basal release of LTC_4 from human eosinophils. We further quantified the total CysLT release from eosinophils following fMLP stimulation and found that there was dose dependent inhibition of CysLT formation starting at a concentration of just 1 nM (Fig 3C). As a further control for this experiment, we found that there was no alteration in CysLT formation mediated by the control inhibitor (ROC-0428) that is structurally similar to ROC-0929, but is unable to bind to the active site of $sPLA_2$-X due to the addition of a methyl group (14,22).

We assessed the potential use of HL-60-C15 and AML14.3D10-CCR3 cells as potential cell lines that could be used to further understand the biology of $sPLA_2$-X in eosinophils, but found that *PLA2G10* expression in these cells was much lower than in primary human eosinophils (see supplemental results, Fig E3D). However, the HL-60-C15 cell line provides a further control for our results in primary human eosinophils (Fig 3) studies since this cell line has minimal expression of *PLA2G10* and the ROC-0929 inhibitor had a negligible impact on CysLT formation in these cells (Fig E3B). We also assessed the prolonged maintenance of primary eosinophils in culture for potential RNA interference (RNAi) studies, and found that although the cells appeared viable, they failed to strongly activate in response to fMLP after 24 hours in culture, either with or without the addition of fetal calf serum (FCS) to the medium (Fig E4). We further found that treatment of eosinophils with IL-5 and GMCSF to prolong survival would not serve as an adequate model because of the impact on *PLA2G10* expression (see below and Fig 5).

sPLA$_2$-X is involved in the activation of the MAPK cascade and cPLA$_2\alpha$

As we have demonstrated that exogenously added sPLA$_2$-X acts in part via the activation of the MAPK cascade leading to phosphorylation at Ser^{505} of cPLA$_2\alpha$ (14), we investigated the possibility that endogenous sPLA$_2$-X serves as an initiator of the MAPK cascade and cPLA$_2\alpha$ phosphorylation. The role of cPLA$_2\alpha$ was examined using two different cPLA$_2\alpha$ inhibitors Pyr-2 (pyrrophenone) and Wyeth-2 (girfiladib) during fMLP-mediated activation of eosinophils. Treatment of eosinophils with either cPLA$_2\alpha$ inhibitor at a concentration of 5 μM decreased the release of radiolabeled arachidonate from eosinophils (Fig 4A, *P=0.08*). The generation of CysLTs in response to fMLP was also significantly inhibited in a dose-dependent manner by Pyr-2 in concentrations ranging from 1–10 μM, and by 5 μM Wyeth-2 (Fig. 4B, *P=0.03*). Selective inhibitors of MEK 1/2 (U0126 10 μM), p38 (SB203580 30 μM), and JNK (SP600125 20 μM) each suppressed LTC$_4$ formation initiated by fMLP treated eosinophils (Fig 4C, *P<0.0001*).

To connect the activation of CysLT formation by cPLA$_2\alpha$ and the MAPK pathway to the endogenous role of sPLA$_2$-X in this process, we examined western blots of eosinophil lysates following stimulation with fMLP, with or without the selective sPLA$_2$-X inhibitor ROC-0929. We found that fMLP strongly initiated ERK1/2 and p-38 phosphorylation, and further that pre-treatment of the cells with ROC-0929 inhibited the phosphorylation of ERK1/2 and p-38 (Fig 4D). We were unable to identify any effects of fMLP or the ROC-0929 inhibitor on JNK phosphorylation, but did find that JNK was phosphorylated in these cells prior to fMLP treatment. We also found that the sPLA$_2$-X inhibitor decreased phosphorylation of cPLA$_2\alpha$ at Ser^{505} by approximately half as compared to the fMLP-stimulated cells without this inhibitor (Fig 4D). These results indicate that sPLA$_2$-X initiates the phosphorylation of ERK1/2 and p-38, that is known to initiate phosphorylation of cPLA$_2\alpha$, leading to eicosanoid synthesis (32).

To further examine the sPLA$_2$-X mediated activation of CysLT synthesis, we examined the effects of sPLA$_2$-X inhibition on the localization of 5-LO and cPLA$_2\alpha$ that form a complex in close proximity to nuclear membranes and lipid bodies during eosinophil leukotriene formation (33–35). Following fMLP-mediated activation, there was an increase in the signal for 5-LO and cPLA$_2\alpha$ in the cytoplasmic space surrounding the nucleus and lipid bodies (Fig E5). Pre treatment of the cells with the sPLA$_2$-X inhibitor ROC-0929 resulted in partial attenuation of the changes in 5-LO and cPLA$_2\alpha$ immunostaining in these cells. These results further indicate that sPLA$_2$-X is involved in the cascade of events leading to leukotriene formation. As we found that sPLA$_2$-X resides in the secretory apparatus, and the ROC-0929 inhibitor is not readily cell permeable (8), we assessed western blots of the sPLA$_2$-X protein in eosinophil supernatant and found that while the protein could be detected in extracellular fluid, there was no apparent difference in the amount of sPLA$_2$-X in the eosinophil supernatant with activation (Fig E6A). One checkpoint in the activation of sPLA$_2$-X is the cleavage of a pro-peptide that occurs at a furin consensus sequence (36,37). We found that a competitive inhibitor of furin-like propeptide convertases (6-D-R) (38) inhibited fMLP-mediated LTC$_4$ formation while an irreversible pro-peptide convertase inactivator (D-RVKR-CMK) (39) did not alter LTC$_4$ formation (Fig E6B).

PLA2G10 expression is increased by inflammatory signals and reduced by a HDAC inhibitor

Because $sPLA_2$-X is upregulated by inflammatory stimuli such as TNF (40) and it is well known that eosinophils are ”primed” in the airways under the influence of IL-5 and GMCSF (41), we tested the effects of cytokines implicated in asthma on the expression of *PLA2G10* in primary eosinophils. We found that the expression of *PLA2G10* was increased by treatment of the cells with TNF and IL-1β together, but not by either cytokine alone (Fig 5A). Although the expression of *PLA2G10* tended to be increased by both IL-4 and IL-13, the increase only reached statistical significance for IL-13 (Fig 5B). In contrast to our expectations, the expression of *PLA2G10* was decreased by treatment with IL-5 with or without GMCSF (Fig 5C), and was also decreased by treatment with thymic stromal lymphopoietin (TSLP) and IL-33 (Fig 5D). In accord with our expectation, treatment with the HDAC inhibitor SB decreased the expression of *PLA2G10* in primary eosinophils (Fig 5E). We also examined the effects of selected cytokines on the fMLP-mediated formation of CysLTs, and found that priming the eosinophils with TNF/IL- β was the only factor that led to a statistically significant increase in CysLTs (*P=0.01*, Fig 5F), while IL-5 tended to increase CysLT formation but this difference was not statistically significant (*P=0.17*). These data indicate that TNF/IL-1β as well as IL-13 increases the gene expression of *PLA2G10* in eosinophils, and that TNF/IL-1β treatment increased fMLP-mediated CysLT formation.

DISCUSSION

Eosinophils are known to have endogenous $sPLA_2$ activity (15,16), but the only previously characterized $sPLA_2$ enzyme in eosinophils is $sPLA_2$-IIA (17), an enzyme that does not contribute to leukotriene formation in these cells (18,19). A prior study found that the $sPLA_2$-X gene *PLA2G10* is expressed in eosinophils (16). In this study, we confirmed that *PLA2G10* is expressed in human eosinophils, and demonstrate for the first time that $sPLA_2$-X significantly contributes to $sPLA_2$ activity, and is involved in the endogenous release of arachidonic acid and the formation of CysLTs. Based on confocal microscopy, we found that $sPLA_2$-X resides within the secretory compartment, and appears to be externalized during activation. As it is known that fMLP-mediated CysLT formation is mediated by $cPLA_2\alpha$, we found that $sPLA_2$-X was involved in the activation of the MAPK cascade, specifically ERK1/2 and p-38, which activate $cPLA_2\alpha$ by phosphorylation at a Ser^{505} (32,42,43). These results show that $sPLA_2$-X serves as a key regulator of CysLT formation by eosinophils, in part through the regulation of the MAPK cascade and $cPLA_2\alpha$. We further demonstrate that the gene expression of *PLA2G10* is increased in response to TNFα/IL-β, and IL-13, and that TNFα/IL-β increased CysLT formation by eosinophils. These findings have important implications for asthma and allergic disease through the identification of a novel PLA_2 enzyme that we show for the first time to regulate LT formation by eosinophils.

Human eosinophils undergo piecemeal degranulation in response to fMLP stimulation (44), suggesting that the $sPLA_2$-X that we identified in the secretory compartment should be externalized during activation. Externalization may be very important since $sPLA_2$-X has

very high activity for phosphatidylcholine (PC) that is enriched on the extracellular facing plasma membrane (11) and a large portion of eicosanoid synthesis in transfected HEK293 cells occurred after secretion on the extracellular face of the cell (45). We did not observe immunostaining on the outer cell membrane by confocal studies probably because sPLA$_2$-X does not bind to the plasma membrane or accumulate on the cell surface (8). The relatively large volume of cell culture medium that was used *in vitro* for the experiment also reduces cell surface binding. Because the ROC-0929 sPLA$_2$-X inhibitor is cell impermeant and blocked CysLT formation, these studies support the finding that endogenous sPLA$_2$-X is externalized where it can act on the outer cell membrane to generate free fatty acids including arachidonic acid, generate lysophospholipids and contribute to surfactant dysfunction. These results indicate the endogenous sPLA$_2$-X acts in a manner that we have identified previously for exogenous sPLA$_2$-X, causing CysLT formation via the MAPK cascade and cPLA$_2\alpha$, and also further amplifying CysLT formation by providing an additional source of arachidonic acid (14,46). The release of lysophospholipids is critical in the initiation of the MAPK cascade as we have previously demonstrated in eosinophils (14), and lysophospholipids were recently implicated as a major regulator of innate lymphoid cell responses in the periphery (47).

A key control point in sPLA$_2$-X-mediated activation of eicosanoid synthesis is the proteolytic cleavage of a propeptide that must be removed to generate the active enzyme (48,49). Transgenic expression of full-length *Pla2g10* in a mouse created no alteration in phenotype in the absence of an inflammatory environment (37). In contrast, transgenic expression of *Pla2g10* without the propeptide (i.e. active sPLA$_2$-X) in macrophages causes unrestrained inflammation (50). The propeptide contains a highly conserved RXn(K/R)R furin-recognition motif at its C-terminus (36). We found that a competitive furin propeptide convertase inhibitor (i.e. 6-D-R) that can act either within or following secretion from cells (38) inhibits LTC$_4$ formation in these cells. In the cell lysate or the extracellular fluid, only a single band for the mature protein is seen (Figs 1B and **E4E**). These results suggest that sPLA$_2$-X is activated within the cell prior to secretion. Intracellular inhibition of sPLA$_2$-X could have even greater effects on eicosanoid formation in these cells; however, this hypothesis could not be further tested since a cell permeate sPLA$_2$-X inhibitor is not available. As seen in supplemental Fig E3 the eosinophil-like cell lines HL-60-C15 and AML14.3D10-CCR3 have much lower expression of *PLA2G10* compared to primary human eosinophils; thus, shRNA gene silencing studies were not attempted. We also found that the reduced activation of eosinophils after 24 hours in cell culture limited our ability to perform short-term siRNA studies (Fig E4). In a prior study, an irreversible furin propeptide convertase inhibitor (i.e. D-RVKR-CMK) prevented the secretion of the protein in HEK293 cells transfected with full-length human *PLA2G10* or murine *Pla2g10*, suggesting that proteolytic conversion is regulating sPLA$_2$-X release (36). This same irreversible furin propeptide convertase inhibitor did not alter LTC$_4$ formation in eosinophils, suggesting that different pro-peptide convertases regulate LT formation in HEK293 cells and primary human eosinophils (38,51–53). As furins serve as checkpoints of inflammation, these results suggest that proteolytic control of sPLA$_2$-X may be important in eosinophil-mediated inflammation (54).

It is well established that airway eosinophils relative to circulating eosinophils have increased *ex vivo* survival and markers of activation (41,55–57). Although IL-5 and GM-CSF have the most established effects on the production, differentiation and survival of eosinophils (58–60), eosinophil migration is also regulated by IL-4 and IL-13 (61,62). We found that IL-13 and IL-1β in combination with TNF were the major cytokines that increased *PLA2G10* expression. We also found that TNF/IL-1β priming of eosinophils increased CysLT formation suggesting that such an increase in *PLA2G10* may be involved in increasing CysLT formation by eosinophils. A number of cytokines including IL-5 and GMCSF actually decreased *PLA2G10* expression. Further, the expression of *PLA2G10* in eosinophils was decreased by a HDAC inhibitor and by IL-33 and TSLP. These findings were unexpected since IL-33 activates eosinophils via the transmembrane ST2 receptor (63–65), and TSLP activates both CD34+ progenitor cells and mature eosinophils (66,67).

A major question raised by these results is whether the increased levels of $sPLA_2$-X identified in asthma (12,13) could be the result of secretion of this enzyme from eosinophils as suggested by the present study. Because we have shown that the epithelium (13) and airway macrophages (29) immunostain in human airway samples for $sPLA_2$-X, and that primary epithelial cells in organotypic culture secrete the mature $sPLA_2$-X protein (13), the precise cellular source of the enzyme in humans is unclear. The deletion of the murine $sPLA_2$-X gene (*pla2g10*) (68) or an active site directed inhibitor of human $sPLA_2$-X in a transgenic mouse model (69) inhibits the development of airway inflammation, hyperresponsiveness, and structural remodeling. Thus it will be important to identify the relative importance of the epithelium, macrophages and eosinophils in future murine studies examining the *in vivo* effects of $sPLA_2$-X on allergic inflammation.

These studies have important potential therapeutic implications for asthma, as it is clear that lipid mediators including CysLTs play an important role in asthma (70,71), but therapies targeting CysLTs have only been modestly effective by virtue of blocking only one receptor (i.e. $CysLT_1$ receptor that predominantly binds to LTD_4), while these lipid mediators bind to multiple receptors (i.e. $CysLT_2$ that preferentially binds LTC_4, and the LTE_4 receptors, $CysLT_E$, $P2Y_{12}$ and GPR99)(72,73). In this paper, we show for the first time in a primary human cell that an inhibitor of $sPLA_2$-X can effectively block the formation of CysLTs. Further, the products of $sPLA_2$s including lysophospholipids may also serve as important regulators of Th2-mediated inflammation (47). These findings further support $sPLA_2$-X as a potential therapeutic target in asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by National Institutes of Health grants R21HL111845 (TSH), and R37HL036235 (MHG).

Abbreviations used in this paper

5-LO	5-Lipoxygenase
cPLA$_2$α	Cytosolic phospholipase A$_2$α
CysLT	Cysteinyl leukotriene
ER	endoplasmic reticulum
ERK 1/2	extracellular-signal-regulated kinases 1/2 (p44/42 MAPK)
HL-60-C15	HL-60 clone-15 cells
LT	Leukotriene
MAPK	mitogen activated protein kinase
PC	Phosphatidylcholine
PLA$_2$	Phospholipase A$_2$
PLA2G10	mammalian sPLA$_2$-X gene
pla2g10	murine sPLA$_2$-X gene
RNAi	RNA interference
SAPK/JNK	stress-activated protein kinase/Jun-amino-terminal kinase
SB	sodium butyrate
sPLA$_2$	Secreted phospholipase A$_2$
sPLA$_2$-IIA	sPLA$_2$ group IIA
sPLA$_2$-V	sPLA$_2$ group V
sPLA$_2$-X	sPLA$_2$ group X
TSLP	thymic stromal lymphopoietin

References

1. Hallstrand TS, Moody MW, Aitken ML, Henderson WR Jr. Airway immunopathology of asthma with exercise-induced bronchoconstriction. J Allergy Clin Immunol. 2005; 116:586–593. [PubMed: 16159628]
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. [PubMed: 10588604]
3. Romagnoli M, Vachier I, Tarodo de la Fuente P, Meziane H, Chavis C, Bousquet J, Godard P, Chanez P. Eosinophilic inflammation in sputum of poorly controlled asthmatics. Eur Respir J. 2002; 20:1370–1377. [PubMed: 12503691]
4. Hallstrand TS, Henderson WR Jr. An update on the role of leukotrienes in asthma. Curr Opin Allergy Clin Immunol. 2010; 10:60–66. [PubMed: 19915456]
5. Evans JH, Spencer DM, Zweifach A, Leslie CC. Intracellular calcium signals regulating cytosolic phospholipase A$_2$ translocation to internal membranes. J Biol Chem. 2001; 276:30150–30160. [PubMed: 11375391]
6. Glover S, de Carvalho MS, Bayburt T, Jonas M, Chi E, Leslie CC, Gelb MH. Translocation of the 85-kDa phospholipase A$_2$ from cytosol to the nuclear envelope in rat basophilic leukemia cells

stimulated with calcium ionophore or IgE/antigen. J Biol Chem. 1995; 270:15359–15367. [PubMed: 7797525]

7. 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 P388D1 macrophage-like cells. J Biol Chem. 2003; 278:48059–48065. [PubMed: 12963740]
8. Mounier CM, Ghomashchi F, Lindsay MR, James S, Singer AG, Parton RG, Gelb MH. Arachidonic acid release from mammalian cells transfected with human groups IIA and X secreted phospholipase A_2 occurs predominantly during the secretory process and with the involvement of cytosolic phospholipase A_2-α. J Biol Chem. 2004; 279:25024–25038. [PubMed: 15007070]
9. Satake Y, Diaz BL, Balestrieri B, Lam BK, Kanaoka Y, Grusby MJ, Arm JP. Role of group V phospholipase A_2 in zymosan-induced eicosanoid generation and vascular permeability revealed by targeted gene disruption. J Biol Chem. 2004; 279:16488–16494. [PubMed: 14761945]
10. Calabrese C, Triggiani M, Marone G, Mazzarella G. Arachidonic acid metabolism in inflammatory cells of patients with bronchial asthma. Allergy. 2000; 55(Suppl 61):27–30. [PubMed: 10919502]
11. Singer AG, Ghomashchi F, Le Calvez C, Bollinger J, Bezzine S, Rouault M, Sadilek M, Nguyen E, Lazdunski M, Lambeau G, Gelb MH. Interfacial kinetic and binding properties of the complete set of human and mouse groups I, II, V, X, and XII secreted phospholipases A_2. J Biol Chem. 2002; 277:48535–48549. [PubMed: 12359733]
12. Hallstrand TS, Lai Y, Ni Z, Oslund RC, Henderson WR Jr, Gelb MH, Wenzel SE. Relationship between levels of secreted phospholipase A_2 groups IIA and X in the airways and asthma severity. Clin Exp Allergy. 2011
13. Hallstrand TS, Lai Y, Altemeier WA, Appel CL, Johnson B, Frevert CW, Hudkins KL, Bollinger JG, Woodruff PG, Hyde DM, Henderson WR Jr, Gelb MH. Regulation and function of epithelial secreted phospholipase A2 group X in asthma. Am J Respir Crit Care Med. 2013; 188:42–50. [PubMed: 23614662]
14. Lai Y, Oslund RC, Bollinger JG, Henderson WR Jr, Santana LF, Altemeier WA, Gelb MH, Hallstrand TS. Eosinophil cysteinyl leukotriene synthesis mediated by exogenous secreted phospholipase A_2 group x. J Biol Chem. 2011; 285:41491–41500. [PubMed: 20974857]
15. Ackerman SJ, Kwatia MA, Doyle CB, Enhorning G. Hydrolysis of surfactant phospholipids catalyzed by phospholipase A_2 and eosinophil lysophospholipases causes surfactant dysfunction: a mechanism for small airway closure in asthma. Chest. 2003; 123:355S. [PubMed: 12628972]
16. Kwatia MA, Doyle CB, Cho W, Enhorning G, Ackerman SJ. Combined activities of secretory phospholipases and eosinophil lysophospholipases induce pulmonary surfactant dysfunction by phospholipid hydrolysis. J Allergy Clin Immunol. 2007; 119:838–847. [PubMed: 17321580]
17. Blom M, Tool AT, Wever PC, Wolbink GJ, Brouwer MC, Calafat J, Egesten A, Knol EF, Hack CE, Roos D, Verhoeven AJ. Human eosinophils express, relative to other circulating leukocytes, large amounts of secretory 14-kD phospholipase A_2. Blood. 1998; 91:3037–3043. [PubMed: 9531617]
18. Triggiani M, Granata F, Balestrieri B, Petraroli A, Scalia G, Del Vecchio L, Marone G. Secretory phospholipases A_2 activate selective functions in human eosinophils. J Immunol. 2003; 170:3279–3288. [PubMed: 12626587]
19. 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. [PubMed: 16785555]
20. Munoz NM, Kim YJ, Meliton AY, Kim KP, Han SK, Boetticher E, O'Leary E, Myou S, Zhu X, Bonventre JV, Leff AR, Cho W. 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. [PubMed: 12796497]
21. Degousee N, Ghomashchi F, Stefanski E, Singer A, Smart BP, Borregaard N, Reithmeier R, Lindsay TF, Lichtenberger C, Reinisch W, Lambeau G, Arm J, Tischfield J, Gelb MH, Rubin BB. Groups IV, V, and X phospholipases A2s in human neutrophils: role in eicosanoid production and gram-negative bacterial phospholipid hydrolysis. J Biol Chem. 2002; 277:5061–5073. [PubMed: 11741884]

22. Oslund RC, Cermak N, Gelb MH. Highly specific and broadly potent inhibitors of mammalian secreted phospholipases A_2. J Med Chem. 2008; 51:4708–4714. [PubMed: 18605714]

23. Seno K, Okuno T, Nishi K, Murakami Y, Yamada K, Nakamoto S, Ono T. Pyrrolidine inhibitors of human cytosolic phospholipase A_2. Part 2: synthesis of potent and crystallized 4-triphenylmethylthio derivative 'pyrrophenone'. Bioorg Med Chem Lett. 2001; 11:587–590. [PubMed: 11229777]

24. Lamothe J, Lee K, Schelling S, Stedman N, Leach M, McKew J, Clark J, Nickerson-Nutter C, Hegen M. Sa.29. Efficacy of Giripladib, a Novel Inhibitor of Cytosolic Phospholipase $A_2\alpha$, in Two Different Mouse Models of Rheumatoid Arthritis. Clinical Immunology. 2008; 127:S89–S90.

25. Weiss J, Inada M, Elsbach P, Crowl RM. Structural determinants of the action against Escherichia coli of a human inflammatory fluid phospholipase A2 in concert with polymorphonuclear leukocytes. J Biol Chem. 1994; 269:26331–26337. [PubMed: 7929350]

26. Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A. 2001; 98:13681–13686. [PubMed: 11717429]

27. Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC. SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett. 1995; 364:229–233. [PubMed: 7750577]

28. DeSilva DR, Jones EA, Favata MF, Jaffee BD, Magolda RL, Trzaskos JM, Scherle PA. Inhibition of mitogen-activated protein kinase kinase blocks T cell proliferation but does not induce or prevent anergy. J Immunol. 1998; 160:4175–4181. [PubMed: 9574517]

29. Hallstrand TS, Chi EY, Singer AG, Gelb MH, Henderson WR Jr. Secreted phospholipase A_2 group X overexpression in asthma and bronchial hyperresponsiveness. Am J Respir Crit Care Med. 2007; 176:1072–1078. [PubMed: 17901411]

30. Touaibia M, Djimde A, Cao F, Boilard E, Bezzine S, Lambeau G, Redeuilh C, Lamouri A, Massicot F, Chau F, Dong CZ, Heymans F. Inhibition of secreted phospholipase A2. 4-glycerol derivatives of 4,5-dihydro-3-(4- tetradecyloxybenzyl)-1,2,4-4H-oxadiazol-5-one with broad activities. J Med Chem. 2007; 50:1618–1626. [PubMed: 17335183]

31. Svensson L, Redvall E, Bjorn C, Karlsson J, Bergin AM, Rabiet MJ, Dahlgren C, Wenneras C. House dust mite allergen activates human eosinophils via formyl peptide receptor and formyl peptide receptor-like 1. Eur J Immunol. 2007; 37:1966–1977. [PubMed: 17559171]

32. Zhu X, Sano H, Kim KP, Sano A, Boetticher E, Munoz NM, Cho W, Leff AR. Role of mitogen-activated protein kinase-mediated cytosolic phospholipase A_2 activation in arachidonic acid metabolism in human eosinophils. J Immunol. 2001; 167:461–468. [PubMed: 11418683]

33. Ago H, Kanaoka Y, Irikura D, Lam BK, Shimamura T, Austen KF, Miyano M. Crystal structure of a human membrane protein involved in cysteinyl leukotriene biosynthesis. Nature. 2007; 448:609–612. [PubMed: 17632548]

34. Martinez Molina D, Wetterholm A, Kohl A, McCarthy AA, Niegowski D, Ohlson E, Hammarberg T, Eshaghi S, Haeggstrom JZ, Nordlund P. Structural basis for synthesis of inflammatory mediators by human leukotriene C_4 synthase. Nature. 2007; 448:613–616. [PubMed: 17632546]

35. Mandal AK, Jones PB, Bair AM, Christmas P, Miller D, Yamin TT, Wisniewski D, Menke J, Evans JF, Hyman BT, Bacskai B, Chen M, Lee DM, Nikolic B, Soberman RJ. The nuclear membrane organization of leukotriene synthesis. Proc Natl Acad Sci U S A. 2008; 105:20434–20439. [PubMed: 19075240]

36. Jemel I, Ii H, Oslund RC, Payre C, Dabert-Gay AS, Douguet D, Chargui K, Scarzello S, Gelb MH, Lambeau G. Group X secreted phospholipase A_2 proenzyme is matured by a furin-like proprotein convertase and releases arachidonic acid inside of human HEK293 cells. The Journal of biological chemistry. 2011; 286:36509–36521. [PubMed: 21878635]

37. Ohtsuki M, Taketomi Y, Arata S, Masuda S, Ishikawa Y, Ishii T, Takanezawa Y, Aoki J, Arai H, Yamamoto K, Kudo I, Murakami M. 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. [PubMed: 17008322]

38. Peinado JR, Kacprzak MM, Leppla SH, Lindberg I. Cross-inhibition between furin and lethal factor inhibitors. Biochem Biophys Res Commun. 2004; 321:601–605. [PubMed: 15358148]
39. Garten W, Hallenberger S, Ortmann D, Schafer W, Vey M, Angliker H, Shaw E, Klenk HD. Processing of viral glycoproteins by the subtilisin-like endoprotease furin and its inhibition by specific peptidylchloroalkylketones. Biochimie. 1994; 76:217–225. [PubMed: 7819326]
40. 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. [PubMed: 16603549]
41. Sedgwick JB, Calhoun WJ, Vrtis RF, Bates ME, McAllister PK, Busse WW. Comparison of airway and blood eosinophil function after in vivo antigen challenge. J Immunol. 1992; 149:3710–3718. [PubMed: 1358975]
42. Geijsen N, Dijkers PF, Lammers JJ, Koenderman L, Coffer PJ. Cytokine-mediated cPLA(2) phosphorylation is regulated by multiple MAPK family members. FEBS Lett. 2000; 471:83–88. [PubMed: 10760518]
43. Myou S, Leff AR, Myo S, Boetticher E, Meliton AY, Lambertino AT, Liu J, Xu C, Munoz NM, Zhu X. Activation of group IV cytosolic phospholipase A_2 in human eosinophils by phosphoinositide 3-kinase through a mitogen-activated protein kinase-independent pathway. J Immunol. 2003; 171:4399–4405. [PubMed: 14530366]
44. Malm-Erjefalt M, Persson CG, Erjefalt JS. Degranulation status of airway tissue eosinophils in mouse models of allergic airway inflammation. Am J Respir Cell Mol Biol. 2001; 24:352–359. [PubMed: 11245636]
45. Murakami M, Kambe T, Shimbara S, Higashino K, Hanasaki K, Arita H, Horiguchi M, Arita M, Arai H, Inoue K, Kudo I. Different functional aspects of the group II subfamily (Types IIA and V) and type X secretory phospholipase A_2s in regulating arachidonic acid release and prostaglandin generation. Implications of cyclooxygenase-2 induction and phospholipid scramblase-mediated cellular membrane perturbation. J Biol Chem. 1999; 274:31435–31444. [PubMed: 10531345]
46. Feltenmark S, Gautam N, Brunnstrom A, Griffiths W, Backman L, Edenius C, Lindbom L, Bjorkholm M, Claesson HE. Eoxins are proinflammatory arachidonic acid metabolites produced via the 15-lipoxygenase-1 pathway in human eosinophils and mast cells. Proc Natl Acad Sci U S A. 2008; 105:680–685. [PubMed: 18184802]
47. Palm NW, Rosenstein RK, Yu S, Schenten DD, Florsheim E, Medzhitov R. Bee venom phospholipase A2 induces a primary type 2 response that is dependent on the receptor ST2 and confers protective immunity. Immunity. 2013; 39:976–985. [PubMed: 24210353]
48. Hanasaki K, Ono T, Saiga A, Morioka Y, Ikeda M, Kawamoto K, Higashino K, Nakano K, Yamada K, Ishizaki J, Arita H. Purified group X secretory phospholipase A_2 induced prominent release of arachidonic acid from human myeloid leukemia cells. J Biol Chem. 1999; 274:34203–34211. [PubMed: 10567392]
49. Cupillard L, Koumanov K, Mattei MG, Lazdunski M, Lambeau G. Cloning, chromosomal mapping, and expression of a novel human secretory phospholipase A_2. J Biol Chem. 1997; 272:15745–15752. [PubMed: 9188469]
50. Curfs DM, Ghesquiere SA, Vergouwe MN, van der Made I, Gijbels MJ, Greaves DR, Verbeek JS, Hofker MH, de Winther MP. Macrophage secretory phospholipase A_2 group X enhances anti-inflammatory responses, promotes lipid accumulation, and contributes to aberrant lung pathology. J Biol Chem. 2008; 283:21640–21648. [PubMed: 18511424]
51. Becker GL, Sielaff F, Than ME, Lindberg I, Routhier S, Day R, Lu Y, Garten W, Steinmetzer T. Potent inhibitors of furin and furin-like proprotein convertases containing decarboxylated P1 arginine mimetics. J Med Chem. 2010; 53:1067–1075. [PubMed: 20038105]
52. Sarac MS, Peinado JR, Leppla SH, Lindberg I. Protection against anthrax toxemia by hexa-D-arginine in vitro and in vivo. Infection and immunity. 2004; 72:602–605. [PubMed: 14688144]
53. Komiyama T, Coppola JM, Larsen MJ, van Dort ME, Ross BD, Day R, Rehemtulla A, Fuller RS. Inhibition of furin/proprotein convertase-catalyzed surface and intracellular processing by small molecules. J Biol Chem. 2009; 284:15729–15738. [PubMed: 19332539]

54. Seidah NG. What lies ahead for the proprotein convertases? Annals of the New York Academy of Sciences. 2011; 1220:149–161. [PubMed: 21388412]
55. Johansson MW, Kelly EA, Busse WW, Jarjour NN, Mosher DF. Up-regulation and activation of eosinophil integrins in blood and airway after segmental lung antigen challenge. J Immunol. 2008; 180:7622–7635. [PubMed: 18490765]
56. Liu LY, Jarjour NN, Busse WW, Kelly EA. Chemokine receptor expression on human eosinophils from peripheral blood and bronchoalveolar lavage fluid after segmental antigen challenge. J Allergy Clin Immunol. 2003; 112:556–562. [PubMed: 13679815]
57. Liu LY, Mathur SK, Sedgwick JB, Jarjour NN, Busse WW, Kelly EA. Human airway and peripheral blood eosinophils enhance Th1 and Th2 cytokine secretion. Allergy. 2006; 61:589–597. [PubMed: 16629789]
58. Rothenberg ME. Eotaxin. An essential mediator of eosinophil trafficking into mucosal tissues. Am J Respir Cell Mol Biol. 1999; 21:291–295. [PubMed: 10460744]
59. Sedgwick JB, Quan SF, Calhoun WJ, Busse WW. Effect of interleukin-5 and granulocyte-macrophage colony stimulating factor on in vitro eosinophil function: comparison with airway eosinophils. J Allergy Clin Immunol. 1995; 96:375–385. [PubMed: 7560640]
60. Vliagoftis H, Befus AD, Hollenberg MD, Moqbel R. Airway epithelial cells release eosinophil survival-promoting factors (GM-CSF) after stimulation of proteinase-activated receptor 2. J Allergy Clin Immunol. 2001; 107:679–685. [PubMed: 11295658]
61. Horie S, Okubo Y, Hossain M, Sato E, Nomura H, Koyama S, Suzuki J, Isobe M, Sekiguchi M. Interleukin-13 but not interleukin-4 prolongs eosinophil survival and induces eosinophil chemotaxis. Intern Med. 1997; 36:179–185. [PubMed: 9144009]
62. Luttmann W, Matthiesen T, Matthys H, Virchow JC Jr. Synergistic effects of interleukin-4 or interleukin-13 and tumor necrosis factor-alpha on eosinophil activation in vitro. Am J Respir Cell Mol Biol. 1999; 20:474–480. [PubMed: 10030846]
63. Cherry WB, Yoon J, Bartemes KR, Iijima K, Kita H. A novel IL-1 family cytokine, IL-33, potently activates human eosinophils. J Allergy Clin Immunol. 2008; 121:1484–1490. [PubMed: 18539196]
64. Pecaric-Petkovic T, Didichenko SA, Kaempfer S, Spiegl N, Dahinden CA. Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. Blood. 2009; 113:1526–1534. [PubMed: 18955562]
65. Stolarski B, Kurowska-Stolarska M, Kewin P, Xu D, Liew FY. IL-33 exacerbates eosinophil-mediated airway inflammation. J Immunol. 2010; 185:3472–3480. [PubMed: 20693421]
66. Allakhverdi Z, Comeau MR, Smith DE, Toy D, Endam LM, Desrosiers M, Liu YJ, Howie KJ, Denburg JA, Gauvreau GM, Delespesse G. CD34+ hemopoietic progenitor cells are potent effectors of allergic inflammation. J Allergy Clin Immunol. 2009; 123:472–478. [PubMed: 19064280]
67. Wong CK, Hu S, Cheung PF, Lam CW. Thymic stromal lymphopoietin induces chemotactic and prosurvival effects in eosinophils: implications in allergic inflammation. Am J Respir Cell Mol Biol. 2010; 43:305–315. [PubMed: 19843704]
68. Henderson WR Jr, Chi EY, Bollinger JG, Tien YT, Ye X, Castelli L, Rubtsov YP, Singer AG, Chiang GK, Nevalainen T, Rudensky AY, Gelb MH. Importance of group X-secreted phospholipase A_2 in allergen-induced airway inflammation and remodeling in a mouse asthma model. J Exp Med. 2007; 204:865–877. [PubMed: 17403936]
69. Henderson WR Jr, Oslund RC, Bollinger JG, Ye X, Tien YT, Xue J, Gelb MH. Blockade of human group X secreted phospholipase A2 (GX-sPLA2)-induced airway inflammation and hyperresponsiveness in a mouse asthma model by a selective GX-sPLA2 inhibitor. J Biol Chem. 2011; 286:28049–28055. [PubMed: 21652694]
70. 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. [PubMed: 6823253]
71. Gauvreau GM, Parameswaran KN, Watson RM, O'Byrne PM. Inhaled leukotriene E_4, but not leukotriene D_4, increased airway inflammatory cells in subjects with atopic asthma. Am J Respir Crit Care Med. 2001; 164:1495–1500. [PubMed: 11704602]

72. Kanaoka Y, Maekawa A, Austen KF. Identification of GPR99 protein as a potential third cysteinyl leukotriene receptor with a preference for leukotriene E4 ligand. J Biol Chem. 2013; 288:10967–10972. [PubMed: 23504326]

73. Austen KF, Maekawa A, Kanaoka Y, Boyce JA. The leukotriene E4 puzzle: finding the missing pieces and revealing the pathobiologic implications. J Allergy Clin Immunol. 2009; 124:406–414. quiz 415–406. [PubMed: 19647860]

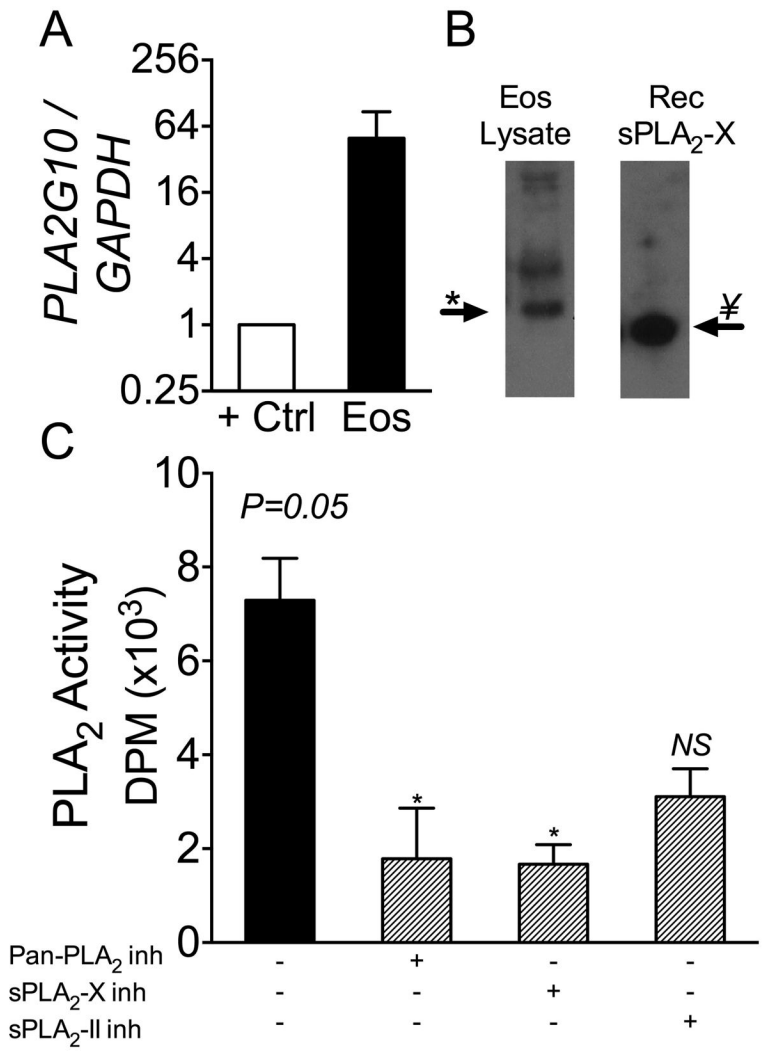

Figure 1.

Identification of $sPLA_2$-X in human eosinophils. **A**, Human eosinophils strongly express the $sPLA_2$-X gene *PLA2G10* relative to the positive control consisting of RNA isolated from an epithelial cell line. **B**, Western blot of eosinophil lysate demonstrates the $sPLA_2$-X protein running as a single band at 13.5 kDa just above the mature recombinant protein. C, Total $sPLA_2$ activity in eosinophils lysates was inhibited by a $sPLA_2$-X-specific inhibitor (ROC-0929) or a pan-$sPLA_2$ in hibitor (0509A), but the inhibition by a $sPLA_2$ group IIA/IIE inhibitor did not reach statistical significance (*P=0.05* 0verall ANOVA, * $P < 0.05$ by post-hoc tests).

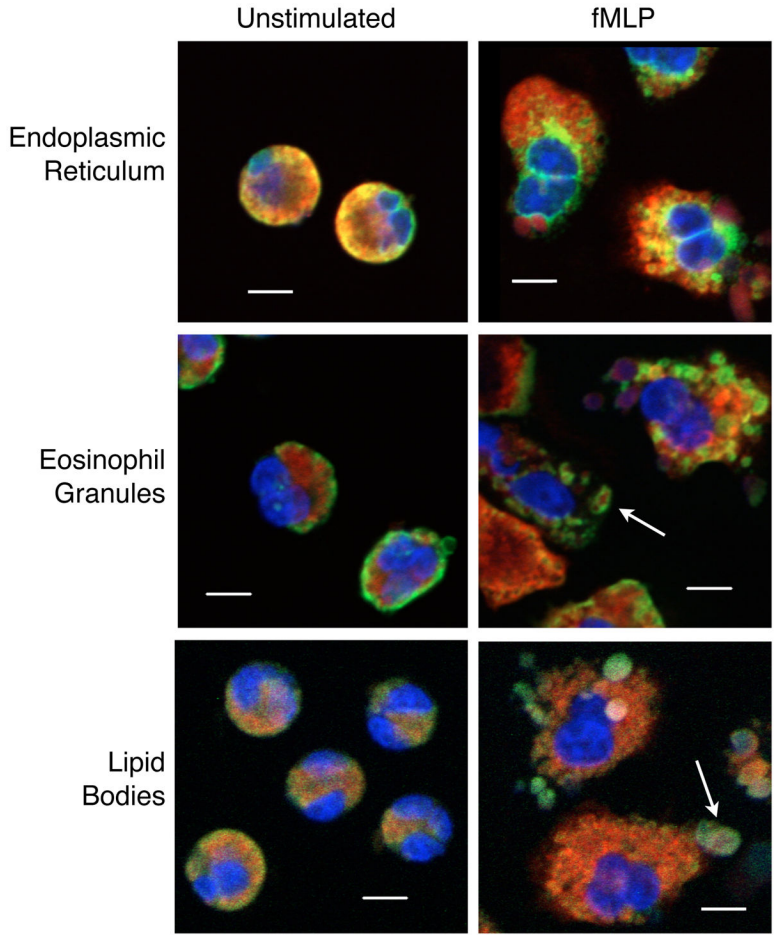

Figure 2.

Localization of $sPLA_2$-X in human eosinophils by confocal microscopy. Immunostaining demonstrates co-localization in yellow using antibodies directed against $sPLA_2$-X (red) and endoplasmic reticulum (ER, anti-PDI), granules (anti-EPX) and lipid bodies (anti-ADRP), all in green. $sPLA_2$-X co-localized predominantly to the endoplasmic reticulum (ER) in unstimulated eosinophils. Following fMLP stimulation, there is persistent colocalization to the ER and some colocalization to granules (arrow middle panel), and lipid bodies (arrow lower panel) that form after stimulation with fMLP. Some of the lipid bodies containing $sPLA_2$-X appear extracellular. Further details of the immunostaining for $sPLA_2$-X and eosinophil structures are presented in Figures E1 and E2. Scale bar is 5 μm.

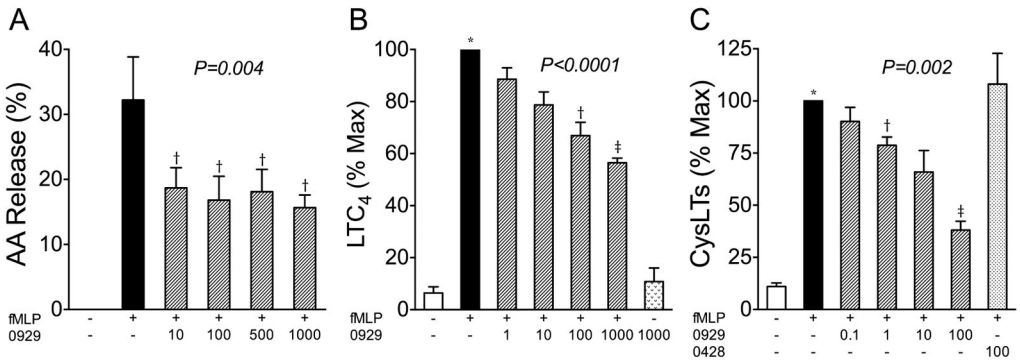

Figure 3.

Eicosanoid formation mediated by endogenous $sPLA_2$-X. A, An active-site directed $sPLA_2$-X inhibitor (0929), reduced the amount of arachidonic acid released in response to fMLP across all concentrations of the inhibitor ranging from 10–1000 nM. B, The fMLP-mediated formation of LTC_4 was reduced in a dose-related manner by the $sPLA_2$-X inhibitor, but had no affect on basal LTC_4 synthesis. C, The formation of total CysLTs was inhibited by the $sPLA_2$-X inhibitor, but a structural analog that is not active as an inhibitor (0428), did not affect CysLT formation. $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$.

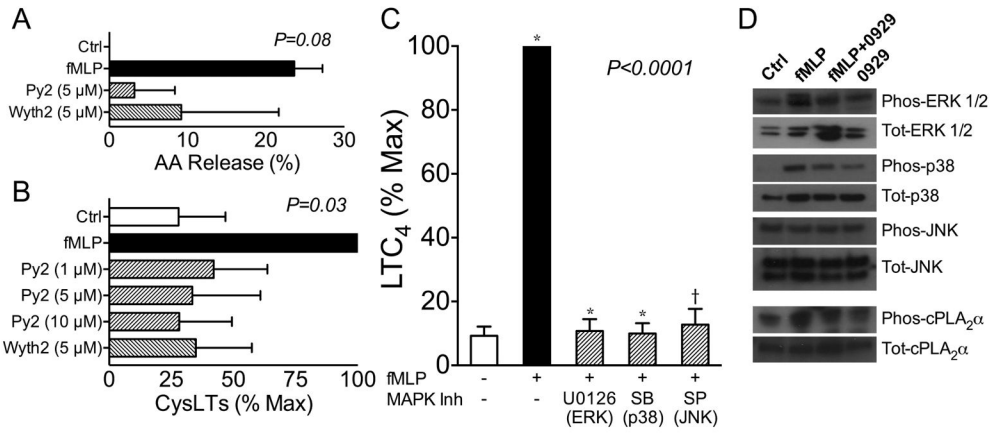

Figure 4.

Effects of $sPLA_2$-X mediated through the MAPK cascade. A, The fMLP-mediated release of [^{3}H]arachidonate was attenuated by the $cPLA_2\alpha$ inhibitors Pyr-2 (5 μM) and Wyeth-2 (5 μM). B, The formation of CysLTs in response to fMLP was also decreased by Pyr-2 (1–10 μM) and Wyeth-2 (5 μM). C, The fMLP-mediated LTC4 formation was inhibited by a MEK 1/2 inhibitor (U0126 10 μM), a p38 inhibitor (SB203580 30 μM), and by a JNK inhibitor (SP600125 20 μM). D, Western blots of cell lysates from eosinophils treated with buffer control (Ctrl), fMLP, or fMLP plus the $sPLA_2$-X inhibitor (0929) demonstrates that the 0929 inhibitor attenuated the phosphorylation of ERK1/2 and p38 in response to fMLP. There were no changes in the phosphorylation of JNK, while the phosphorylation of $cPLA_2\alpha$ at Ser^{505} relative to total $cPLA_2\alpha$ was modestly reduced.

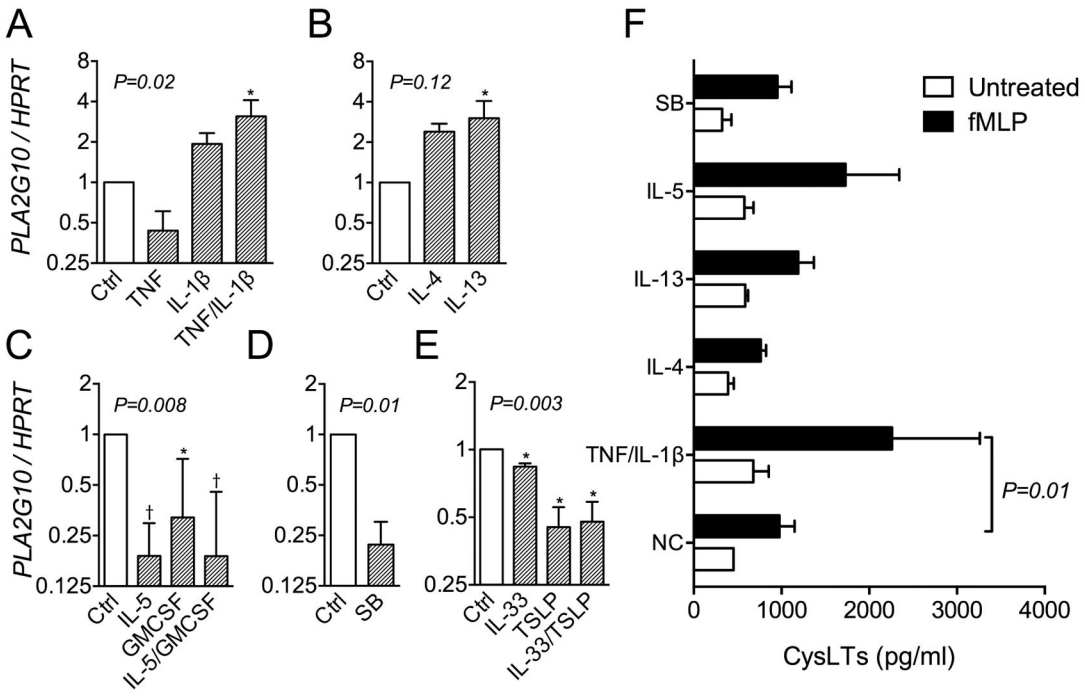

Figure 5.

Consequences of short-term cytokine treatment on *PLA2G10* expression in eosinophils. A-E, Expression of *PLA2G10* by eosinophils was increased by TNF and IL-1β and by IL-13 alone; while the expression was decreased by treatment with IL-5 with or without GMCSF, sodium butyrate, and TSLP, IL-33 or a combination of IL-33 and TSLP. F, The formation of CysLTs by fMLP-stimulated eosinophils was increased after priming with TNF/IL- β (P=0.01), but did not reach statistical significance for other selected cytokines. $^{*}P<0.05$, $^{\dagger}P<0.01$