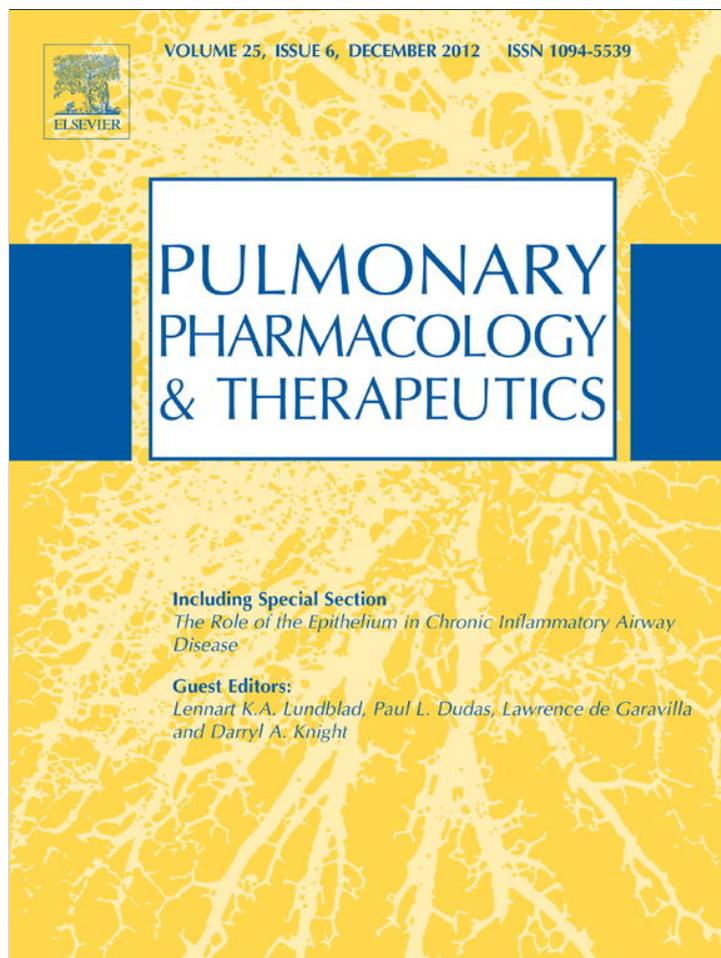


Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/copyright>



Contents lists available at SciVerse ScienceDirect

Pulmonary Pharmacology & Therapeutics

journal homepage: www.elsevier.com/locate/ypupt

Epithelial regulation of eicosanoid production in asthma

Teal S. Hallstrand^{a,*}, Ying Lai^a, William R. Henderson Jr.^b, William A. Altemeier^a, Michael H. Gelb^{c,d}^a University of Washington, Division of Pulmonary and Critical Care, Box 356522, 1959 NE Pacific Street, Seattle, WA 98195, USA^b Department of Medicine, Division of Allergy and Infectious Diseases, University of Washington, Seattle, WA, USA^c Department of Chemistry, University of Washington, Seattle, WA, USA^d Department of Biochemistry, University of Washington, Seattle, WA, USA

ARTICLE INFO

Article history:

Received 15 September 2011

Received in revised form

10 January 2012

Accepted 13 February 2012

Keywords:

Asthma

Exercise-induced bronchoconstriction

Eicosanoid

Leukotriene

Prostaglandin

Epithelium

Mast cell

Eosinophil

Phospholipase A2

Transglutaminase

ABSTRACT

Alterations in the airway epithelium have been associated with the development of asthma in elite athletes and in subjects that are susceptible to exercise-induced bronchoconstriction (EIB). The syndrome of EIB refers to acute airflow obstruction that is triggered by a period of physical exertion. Asthmatics who are susceptible to EIB have increased levels of cysteinyl leukotrienes (CysLTs, i.e., LTs C₄, D₄, and E₄) in induced sputum and exhaled breath condensate, and greater shedding of epithelial cells into the airway lumen. Exercise challenge in individuals susceptible to this disorder initiates a sustained increase in CysLTs in the airways, and secreted mucin release and smooth muscle constriction, which may be mediated in part through activation of sensory nerves. We have identified a secreted phospholipase A₂ (sPLA₂) with increased levels in the airways of patients with EIB called sPLA₂ group X (sPLA₂-X). We have found that sPLA₂-X is strongly expressed in the airway epithelium in asthma. Further, we discovered that transglutaminase 2 (TGM2) is expressed at increased levels in asthma and serves as a regulator of sPLA₂-X. Finally, we demonstrated that sPLA₂-X acts on target cells such as eosinophils to initiate cellular eicosanoid synthesis. Collectively, these studies identify a novel mechanism linking the airway epithelium to the production of inflammatory eicosanoids by leukocytes.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

Asthma can be viewed as a group of related phenotypes with significant heterogeneity in both the underlying genetic and environmental determinants as well as in the clinical manifestations of the disease [1]. A prominent manifestation of asthma is exercise-induced bronchoconstriction (EIB), a syndrome where a brief period of exercise or increase in ventilation triggers airflow obstruction that lasts 30–90 min in the absence of treatment [2]. We have conducted a number of studies that examine the

immunological basis of EIB, and mediator release in the airways following exercise challenge. Here we review the underlying immunopathology that leads to EIB, and the nature of mediator release in the airways following exercise challenge. These studies serve as the foundation of our work examining the regulation of mediator formation in the airways, particularly the identification of sPLA₂-X as an important regulator of eicosanoid formation [3–6], the discovery that transglutaminase 2 (TGM2) is expressed at increased levels in asthma and serves as a regulator of secreted phospholipase A₂ group X (sPLA₂-X) [7], and that sPLA₂-X acts on target cells such as eosinophils to initiate cellular eicosanoid synthesis [8]. Collectively, these studies identify a novel mechanism linking the airway epithelium to the production of inflammatory eicosanoids by leukocytes in the airways.

2. Clinical implications

The importance of EIB within the spectrum of asthma is due to the impact of the exercise-related symptoms and airflow obstruction. Exercise-related asthma symptoms are associated with reduced health-related quality of life in children [9], and exercise challenge can serve as a stimulus for severe bronchoconstriction

Abbreviations: 5-LO, 5-Lipoxygenase; 15S-HETE, 15S-Hydroxyeicosatetraenoic Acid; AA, Arachidonic acid; AERD, Aspirin exacerbated respiratory disease; AHR, Airway Hyperresponsiveness; BAL, Bronchoalveolar Lavage; cPLA₂, Cytosolic Phospholipase A₂; CysLT, Cysteinyl Leukotrienes; CST1, Cystatin 1; ECP, Eosinophilic Cationic Protein; EIB, Exercise-induced Bronchoconstriction; F_{ENO}, Fraction of exhaled nitric oxide; FEV₁, Forced Expiratory Volume in One Second; HIB, Hyperpnea-induced Bronchoconstriction; ICS, Inhaled Corticosteroid; IL-13, Interleukin-13; LT, Leukotriene; MUC5AC, Mucin 5AC; PG, Prostaglandin; PUFA, Polyunsaturated Fatty Acid; PLA₂, Phospholipase A₂; SLP, Secretory leukocyte peptidase inhibitor; sPLA₂, Secreted Phospholipase A₂; TGM2, Transglutaminase 2.

* Corresponding author. Tel.: +1 206 543 3166; fax: +1 206 685 8673.

E-mail address: tealh@u.washington.edu (T.S. Hallstrand).

[10]. Hyperpolarized helium images of regional ventilation following exercise challenge demonstrate closure or near closure of segmental airways of the lungs during EIB [11]. Bronchoconstriction induced by exercise can be life-threatening as demonstrated by a population-based study that found 61 of 263 sports-related fatalities in young adults were caused by asthma exacerbation during exercise [12]. Two recent large cohort studies with long-term longitudinal follow-up demonstrated that exercise-induced wheeze prior to the age of 5 [13] and airway hyperresponsiveness (AHR) to cold dry air hyperpnea in childhood [14] are among the strongest predictors of persistent asthma in adulthood.

3. Immunopathology of asthma with Exercise-induced Bronchoconstriction (EIB)

Cross sectional studies in adults suggest that EIB is a discrete phenotype with distinct pathophysiology that is most strongly related to other aspects of indirect AHR [15]. Whether or not this phenotype is a durable clinical phenotype awaits further longitudinal epidemiological studies. A modest size cross-sectional study established a prevalence of EIB of 46% out of 164 asthmatic children who were not using any daily controller therapy, and found that the prevalence of EIB was increased in the children whose asthma was more severe [16]. The severity of EIB is generally not associated with the baseline forced expiratory volume in 1 s (FEV_1) [2,16,17], and is only weakly associated with the degree of direct AHR [18,19].

An inflammatory basis of EIB is suggested by an increase in the fraction of exhaled nitric oxide (F_{ENO}) among asthmatics who are susceptible to EIB [20], especially in subjects with atopy [21]. In a comparison that we conducted between two groups of asthmatics, one with EIB and the other without EIB, we found that the concentration and number of columnar epithelial cells in induced sputum was much higher in asthmatics with EIB, suggesting that the epithelium is disrupted, and epithelial cells are shed into the airway lumen in this disorder [2]. We found that the concentration of cysteinyl leukotrienes (CysLTs, LTs C_4 , D_4 and E_4) are increased in induced sputum of adults with EIB [2], and another group found that the levels of CysLTs are increase in exhaled breath condensate of children with EIB [17]. Eicosanoids such as LTs and prostaglandins (PG)s are formed from arachidonic acid (AA) that is released by the hydrolysis of the sn-2 position of membrane phospholipids by a family of phospholipase A_2 (PLA_2) enzymes. Although AA and many of the products of AA readily move across the cell membrane, the formation of inflammatory eicosanoids such as CysLTs and PGD_2 is largely restricted to myeloid cells, especially mast cells and eosinophils that contain LTC_4 synthase and mast cells that contain PGD_2 synthase [22]. The connection between epithelial shedding and increased production of inflammatory lipid mediators has led us to consider mechanisms involving the epithelial regulation of leukocyte mediator production and function. One aspect of this relationship between the epithelium and leukocytes is that PGE_2 , an eicosanoid produced in large quantities by the epithelium that inhibits EIB [23] is consistently decreased in relation to the generation of CysLTs in subjects with EIB [2].

We found that the number of eosinophils was increased overall in subjects with EIB, but sputum eosinophilia *per se* does not appear to be required for EIB [2]. In line with these observations the magnitude and onset of the suppression of EIB in response to high dose but not low dose inhaled corticosteroid (ICS) therapy was associated with the degree of sputum eosinophilia [24]. Subjects with EIB who did not have sputum eosinophilia were less likely to have an improvement in EIB during ICS therapy compared to those with evidence of sputum eosinophilia [24]. We also found in a genome-wide expression study of airway cells of patients with

EIB relative to patients with asthma that did not have EIB, that the expression of the mast cell genes tryptase and carboxypeptidase A3 (CPA3) were significantly increased in the EIB positive group [7]. These data are consistent with the recent findings of a unique intraepithelial mast cell phenotype in asthma notable for the high expression of tryptase and CPA3, but low expression of chymase that was described particularly in the Th2 high molecular phenotype [25,26]. Collectively these studies indicate that patients with EIB represent a group of subjects with prominent cellular inflammation and epithelial shedding into the airway lumen in association with increased production of inflammatory eicosanoids.

4. Inflammatory mediator release in the airways during EIB

Studies conducted in our lab and others indicate that exercise challenge initiates the release of inflammatory mediators into the airways in asthmatics with EIB, but the precise mechanism that initiates these events remains an area of controversy [27]. Under most conditions, heat and water are transferred out of the airways during exercise as the inspired air is equilibrated to the temperature and humidity of the lower airways. The amount of water transferred out of the airways during exercise is strongly associated with the severity of bronchoconstriction following exercise challenge. It is likely that water transfer from the airways during exercise serves as a stimulus to the epithelium and leukocytes residing within the epithelium to initiate the release of mediators.

Studies from our laboratory and others indicate that there is a sustained increase in CysLTs and other bronchoconstrictive eicosanoids such as PGD_2 in the airways following exercise challenge to induce EIB [28,29]. The levels of CysLTs are elevated at 30 min, 1 h, and 6 h after exercise challenge in asthmatics with EIB [28,29]. It is clear from pharmacological inhibitor studies blocking either 5-lipoxygenase (5-LO) or the CysLT1 receptor that CysLTs, especially LTD_4 plays a pathological role in this disorder [28,30–33]. However, the inhibition of EIB by CysLT inhibitors alone is incomplete, implicating other bronchoconstrictive eicosanoids and/or the reduction in bronchoprotective mediators such as PGE_2 in the pathogenesis of EIB. Fish oil supplementation, high in n-3 polyunsaturated fatty acids (PUFA) that inhibit synthesis of 2-series PGs and the 4-series LTs, inhibits EIB and the increase in both CysLTs and PGD_2 in the airways [34], and the mast cell product $9\alpha,11\beta$ - PGF_2 in the urine following exercise challenge [35]. The epithelium itself may play a key role in the regulation and production of inflammatory mediators. Following exercise challenge the level of PGE_2 declines in the airways of asthmatics with EIB [28], altering the balance of bronchoconstrictive to bronchoprotective mediators favoring bronchoconstriction in the period following exercise challenge [3]. A unifying explanation for these findings is that the epithelium triggers the production of inflammatory eicosanoids by leukocytes that are in close contact, and that there is shunting of epithelial-derived AA away from the epithelium and toward the production of inflammatory eicosanoids by adjacent leukocytes. Inflammatory cells co-cultured with epithelial cells *in vitro* have increased synthesis of leukocyte-derived eicosanoids [36]. Under the influence of interleukin-13 (IL-13) *in vitro*, the epithelium has reduced capacity for PGE_2 synthesis through a reduction in the synthetic enzymes cyclooxygenase-2 (COX-2) and PGE synthase 1 [37]. The epithelium itself may also serve directly as an important source of inflammatory mediators such as the eicosanoid 15S-Hydroxyeicosatetraenoic Acid (15S HETE) that is increased in the airways of patients with EIB after exercise challenge [3]. Studies in asthma have found that the key enzyme in the 15S HETE synthetic pathway, 15-Lipoxygenase-1, has increased expression in the airway epithelium of patients with asthma [38,39]. These findings suggest

that alterations in the airway epithelium may serve to regulate the production of inflammatory eicosanoids.

Mast cells and eosinophils are strongly implicated as the cellular sources of CysLTs and other eicosanoids in EIB. The eosinophil product eosinophilic cationic protein (ECP) is released into the airways following challenge, and the amount of ECP release varies with the severity of the EIB under different experimental conditions [29]. Following exercise challenge, histamine and the mast cell protease tryptase are released into the airways, and inhibition of EIB with a CysLT₁ receptor inhibitor along with an antihistamine reduced the amount of histamine released after exercise challenge [28]. In an analogous situation using manitol challenge, pharmacological inhibitors indicate that histamine is responsible for bronchoconstriction early after challenge, while the release of CysLTs is responsible for sustained bronchoconstriction [40].

5. Sensory nerve involvement in EIB

The production of eicosanoids such as CysLTs in the airway may initiate bronchoconstriction in part through the activation of sensory airway nerves. Sensory nerves release neurokinins when activated through a process called retrograde axonal transmission leading to bronchoconstriction and mucus release. Sensory nerves may be activated directly by osmotic stimuli, but several eicosanoids can significantly alter the activation threshold of these nerves. In a guinea pig model of hyperpnea-induced bronchoconstriction (HIB), either a 5-LO inhibitor or a CysLT₁ antagonist inhibited HIB and the release of neurokinins, while a neurokinin 2 receptor antagonist inhibited HIB, but not the release of leukotrienes, suggesting that leukotrienes are involved in the release of neurokinins that cause bronchoconstriction [41]. Similarly in a dog model, a combination neurokinin 1 and 2 receptor antagonist inhibited HIB and the generation of LTs that are known in this model to cause HIB [42]. We demonstrated that mucin 5AC (MUC5AC), the predominant gel-forming mucin of goblet cells is released into the airways during EIB and is associated with the levels of CysLTs in the airways [43]. Further, the levels of neurokinin A and CysLTs in these individuals post-exercise challenge are correlated, suggesting that CysLTs mediate the activation of sensory nerves and mucus release during EIB in humans [43].

6. Identification of secreted PLA₂ group X (sPLA₂-X) in the airway epithelium as a potential regulator of eicosanoid production

The first rate-limiting step in the formation of the CysLTs and other eicosanoids is the release of arachidonic acid (AA) from membrane phospholipids that is regulated by the PLA₂ enzymes. It is clear from many studies that cytosolic PLA₂ α (cPLA₂ α) has a major function in efficient eicosanoid synthesis, evidenced by the marked reduction in eicosanoid production when the gene is knocked out in a murine model of asthma [44]. However, in the presence of cPLA₂ α , several secreted PLA₂s (sPLA₂)s have been shown to significantly increase AA release over cPLA₂ α alone, and may preferentially direct eicosanoid production toward LT synthesis [45]. Although the identities of specific sPLA₂s were not characterized, increased sPLA₂ activity was identified in nasal lavage fluid and in bronchoalveolar lavage (BAL) fluid following allergen challenge [46–48]. To determine the identities of the sPLA₂s in human airways, we examined induced sputum samples from asthmatics with EIB as well as a non-asthmatic control group and found that sPLA₂ groups X and XIIA predominate at the level of gene expression [3]. Immunocytochemistry indicated that groups X and XIIA are primarily present in columnar epithelial cells and bronchial macrophages. Of the mammalian sPLA₂s, groups V and X have generated the

most interest because of their capacity to initiate cellular eicosanoid synthesis [49], particularly sPLA₂ group X (sPLA₂-X) since it is the most potent of the sPLA₂s at releasing AA from membrane phospholipids. Because sPLA₂-X is able to hydrolyze phosphatidylcholine-rich vesicles at a rate comparable with its action on anionic phospholipids, sPLA₂-X releases AA when added exogenously to the phosphatidylcholine-rich extracellular plasma membrane of mammalian cells. In murine models of asthma, genetic deficiency of either sPLA₂-V or sPLA₂-X attenuates the development of allergen-induced inflammation, mucus release, and AHR [6,50], as does inhibition of human sPLA₂-X expressed in a transgenic mouse model [5]. In our initial study we found that, following exercise challenge in asthmatics with EIB, there were increases in sPLA₂-X protein in induced sputum supernatant and the percentage of epithelial cells immunostaining for sPLA₂-X, suggesting that activation or release of sPLA₂-X may be involved in the generation of eicosanoids following exercise challenge [3]. In subsequent work to better understand the identities of the sPLA₂s in human airways, we have found that sPLA₂-X and sPLA₂-IIA are the predominant sPLA₂s in human BAL fluid both in subjects with and without asthma [4]. In the airway epithelium, the expression of sPLA₂-X predominated (Fig. 1), while both sPLA₂-X and sPLA₂-IIA were expressed in BAL cells [4]. The levels of sPLA₂-X in BAL fluid were increased in asthma, particularly in severe asthma and correlated with lung function and eicosanoid formation in the airways. In contrast, although sPLA₂-IIA was elevated in asthma, it was not associated with lung function, cellular inflammation or eicosanoid levels [4]. Taken together, these results suggest a prominent role of sPLA₂-X in asthma as a regulator of cellular inflammation and eicosanoid formation. Studies are currently underway to better understand the distribution of sPLA₂-X expression within the epithelium of patients with asthma and non-asthmatic subjects. It is notable that in the murine model, the expression of sPLA₂-X colocalizes to cells expressing MUC5AC suggesting prominent expression in secretory cells such as goblet cells [6].

7. Transglutaminase 2 (TGM2) is increased in the airways of patients with asthma and regulates the activity of sPLA₂-X

In our comparison of genes expressed in airway cells, we found that the expression of TGM2 is increased in asthmatics with EIB relative to asthmatics without EIB, and that TGM2 is markedly increased in either asthma group relative to non-asthmatic controls [7]. Immunostaining for TGM2 in endobronchial biopsies from patients with asthma demonstrated TGM2 throughout the airway epithelium. In addition, primary epithelial cells proliferating in culture contain high amounts of TGM2. Although TGM2 has been implicated in a number of inflammatory diseases, our study was the first to clearly implicate TGM2 in asthma. Of interest is that TGM2 is upregulated by retinoic acid in transformed airway epithelial cells [51]. The TGM2 gene is located on chromosome 20q11.2–12 near a cluster of genes related to epithelial barrier function in close proximity to a region linked to both atopic dermatitis and asthma [52]. Two of the other differentially expressed genes in our study, secretory leukocyte peptidase inhibitor (SLPI) and cystatin 1 (CST1), are also located in this region of chromosome 20. TGM2 is a calcium-dependent enzyme that modifies protein structure through the transfer of an acyl group from glutamine to lysine or free amines resulting in a new inter- or intra-molecular amidic cross-link [53]. TGM2 is also known to activate the transcription factor NF κ B, which induces expression of pro-inflammatory cytokines [54]. Using an *in vitro* assay of PLA₂ activity, we found that recombinant human TGM2 enzymatically modifies sPLA₂-X leading to a substantial increase in the PLA₂ activity of the enzyme, suggesting that one of the mechanisms of TGM2 action in asthma

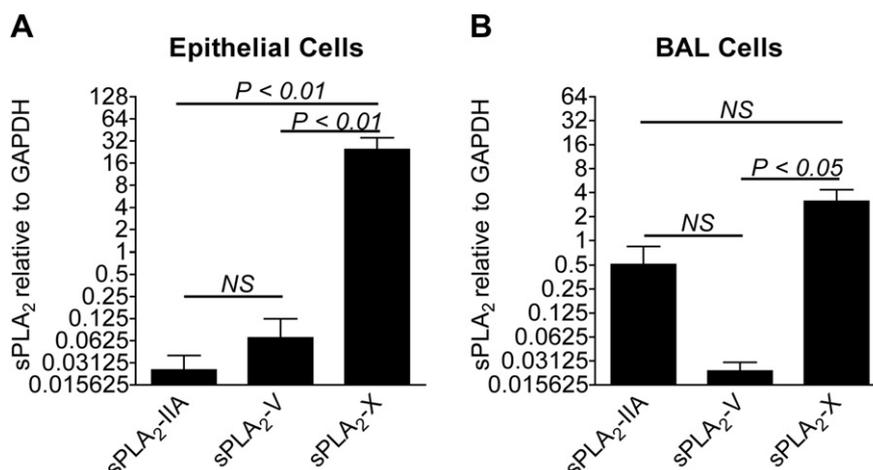


Fig. 1. Expression of the sPLA₂ enzymes in the airway epithelium and BAL cells. The gene expression of sPLA₂-IIA, sPLA₂-V, and sPLA₂-X was assessed in epithelial brushings and BAL fluid from asthmatic and non-asthmatic subjects. The expression of sPLA₂-X was significantly higher than the expression of either sPLA₂-V or sPLA₂-IIA in the epithelium (A). There was no difference in the expression of sPLA₂-IIA and sPLA₂-V in the epithelium. The expression of sPLA₂-X in BAL cells was significantly higher than sPLA₂-V, but no different than sPLA₂-IIA (B). There was no statistically significant difference between the expression of sPLA₂ groups IIA and V in BAL cells (Adapted from [4]).

is regulation of eicosanoid and lysophospholipid synthesis (Fig. 2). In a prior investigation, dual inhibitors of TGM2 and sPLA₂ reduced ocular inflammation in a rabbit model of allergen-induced conjunctivitis [53]. It is now clear from more recent animal models that TGM2 is induced in the airways of mice sensitized and challenged with ovalbumin in the presence of adjuvant [55], as well as in mouse models of PMA-induced atopic dermatitis and IgE-dependent passive cutaneous anaphylaxis [56]. In one study, a peptide that inhibits both TGM2 and PLA₂ reduced allergen-induced airway inflammation and eicosanoid formation, but the specific role of TGM2 remains to be fully elucidated [55]. In the studies of TGM2 in cutaneous anaphylaxis and atopic dermatitis, a chemical inhibitor of TGM2 partially inhibited PMA-induced dermatitis and IgE-dependent cutaneous anaphylaxis [55].

8. Secreted PLA₂ group X (sPLA₂-X) initiates eicosanoid production by human eosinophils

Eosinophils have been implicated as a significant source of CysLTs involved in the pathogenesis of EIB based on increased levels

of eosinophils in association with both high levels of CysLTs [2] and the severity of EIB [24] as well as evidence of activation of eosinophils in the airways following exercise challenge [29]. The critical enzyme in CysLT formation, LTC₄ synthase, is predominantly present in mast cells and eosinophils in the airways in subjects with asthma [22], and eosinophils are the predominant source of LTC₄ synthase in aspirin exacerbated respiratory disease (AERD) [57]. Following allergen challenge, the amount of CysLT formation in the airways is associated with the eosinophil count, further implicating eosinophils as a major source of CysLT production [58].

Based on the identification of sPLA₂-X in the airways of patients with EIB and the evidence that eosinophils are a major source of CysLTs, we examined the ability of sPLA₂-X to efficiently activate CysLT formation by human eosinophils [8]. It is well known that group IVA cytosolic PLA₂ (i.e. cPLA₂α) plays a major role in endogenous CysLT synthesis in myeloid cells such as eosinophils [59,60]; however, it is reported that sPLA₂ group V (sPLA₂-V) initiates CysLT synthesis by human eosinophils in the absence of cPLA₂α activation [61,62]. Although sPLA₂-V and sPLA₂-X both have high capacity to initiate cellular eicosanoid synthesis

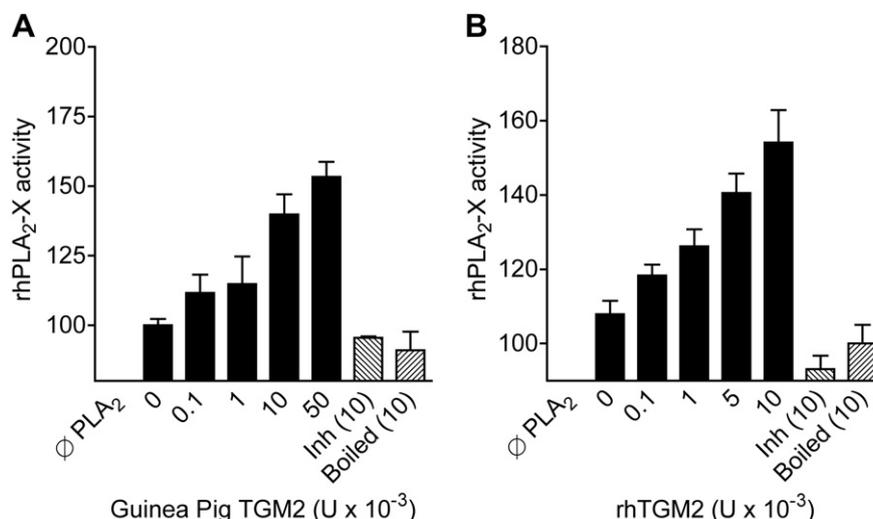


Fig. 2. *In vitro* increase in secreted PLA₂ group X activity by TGM2. Pre-incubation of recombinant human sPLA₂-X with purified TGM2 from guinea pig liver (A) or with recombinant human TGM2 (B) causes an increase in the PLA₂ activity of the sPLA₂-X enzyme. Denaturing the TGM2 with heat (boiled) or inhibiting the activity of the enzyme by saturating the enzyme with N-carbobenzoxy-Gln-Gly (Inh) demonstrate that the *in vitro* findings are due to the enzymatic activity of TGM2 (Adapted from [7]).

[49], sPLA₂-V has been difficult to identify in the airways of patients with asthma [4] or EIB [3]. To examine the role of sPLA₂-X in eosinophil CysLT synthesis, we used recombinant human sPLA₂-X to activate human eosinophils isolated from donors with a physician diagnosis of asthma and/or allergy. Exogenous sPLA₂-X rapidly caused release of a large portion of labeled AA and CysLT synthesis that was related to the amount of sPLA₂-X added exogenously to eosinophils. A specific, active site-directed inhibitor of sPLA₂-X inhibited both CysLT synthesis and AA release indicating that sPLA₂-X was responsible for the AA release and CysLT synthesis. In addition to AA release, sPLA₂-X caused marked lysophospholipid release from eosinophils including LysoPC species known to induce a Ca²⁺ flux in eosinophils. Other lysophospholipid species enriched in AA in human eosinophils, including phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE) [63], and plasmenyl PC and PE species [64], were released by the addition of sPLA₂-X. Although it is clear that sPLA₂-X serves as a major source of AA and lysophospholipids, the mechanism of CysLT formation is more complex. We found that selective inhibitors of cPLA₂α suppressed CysLT formation mediated by sPLA₂-X suggesting that sPLA₂-X or a product of sPLA₂-X activates cPLA₂α. Activation of cPLA₂α involves an intracellular calcium flux and phosphorylation of a serine residue by MAP kinases. Treatment with sPLA₂-X initiated Ser⁵⁰⁵-phosphorylation of cPLA₂α and an intracellular Ca²⁺ flux in eosinophils, as well as translocation of cPLA₂ and 5-LO to focal locations in the cytoplasm and in the perinuclear space. CysLT formation in response to sPLA₂-X was attenuated by pharmacological inhibition of p38 and JNK MAP kinases; further LysoPC initiated CysLT formation that was similarly dependent upon p38 and JNK MAP kinases. Despite the finding of cPLA₂α involvement, it was also apparent that AA release by sPLA₂-X may still contribute to additional CysLT synthesis since the addition of sPLA₂-X to eosinophils during fMLP-mediated CysLT synthesis further increased CysLT synthesis during these conditions of strong cPLA₂α activation. Thus, we have demonstrated that sPLA₂-X initiates CysLT synthesis in eosinophils through AA and lysophospholipid release through a mechanism involving cPLA₂α and

resulting in the amplification of CysLT synthesis in cells that are actively synthesizing CysLTs induced by another stimulus (Fig. 3). These results imply that sPLA₂-X mediated activation of eosinophils in the airways may be an important source of CysLTs in asthma.

9. Conclusions

We have found that EIB is a distinct syndrome in asthma that is related to indirect AHR, and is notable for increased production of CysLTs and shedding of epithelial cells into the airway lumen. Exercise challenge serves as a stimulus to the airway epithelium and adjacent leukocytes resulting in sustained CysLT and PGD₂ release in association with smooth muscle contraction and the release of MUC5AC that may be the consequence of sensory nerve activation. Based on several lines of evidence, mast cells and eosinophils serve as the principal sources of inflammatory eicosanoids in this disorder. Our work in this area led to the identification of sPLA₂-X that is strongly expressed in the airway epithelium and is released into the airway lumen in asthma. In a genome-wide expression study, we identified increased TGM2 and demonstrated that TGM2 serves as a regulator of sPLA₂-X. Finally, we demonstrated that sPLA₂-X acts on target cells such as eosinophils to initiate cellular eicosanoid synthesis. Collectively, these studies identify a novel mechanism linking the airway epithelium to the production of inflammatory eicosanoids by leukocytes.

Acknowledgment

Supported by National Institutes of Health grant HL089215.

References

- [1] Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, et al. Identification of asthma phenotypes using cluster analysis in the severe asthma research program. *Am J Respir Crit Care Med* 2010;181:315–23.
- [2] Hallstrand TS, Moody MW, Aitken ML, Henderson Jr WR. Airway immunopathology of asthma with exercise-induced bronchoconstriction. *J Allergy Clin Immunol* 2005;116:586–93.
- [3] Hallstrand TS, Chi EY, Singer AG, Gelb MH, Henderson Jr WR. Secreted phospholipase A2 group X overexpression in asthma and bronchial hyperresponsiveness. *Am J Respir Crit Care Med* 2007;176:1072–8.
- [4] Hallstrand TS, Lai Y, Ni Z, Oslund RC, Henderson Jr WR, Gelb MH, et al. Relationship between levels of secreted phospholipase A groups IIA and X in the airways and asthma severity. *Clin Exp Allergy* 2011;41:801–10.
- [5] Henderson Jr WR, Oslund RC, Bollinger JG, Ye X, Tien YT, Xue J, et al. Blockade of human group X secreted phospholipase A2-induced airway inflammation and hyperresponsiveness in a mouse asthma model by a selective group X secreted phospholipase A2 inhibitor. *J Biol Chem*; 2011.
- [6] Henderson Jr WR, Chi EY, Bollinger JG, Tien YT, Ye X, Castelli L, et al. Importance of group X-secreted phospholipase A₂ in allergen-induced airway inflammation and remodeling in a mouse asthma model. *J Exp Med* 2007;204:865–77.
- [7] Hallstrand TS, Wurfel MM, Lai Y, Ni Z, Gelb MH, Altemeier WA, et al. Transglutaminase 2, a novel regulator of eicosanoid production in asthma revealed by genome-wide expression profiling of distinct asthma phenotypes. *PLoS One* 2010;5:e8583.
- [8] Lai Y, Oslund RC, Bollinger JG, Henderson Jr WR, Santana LF, Altemeier WA, et al. Eosinophil cysteinyl leukotriene synthesis mediated by exogenous secreted phospholipase A2 group X. *J Biol Chem* 2010;285:41491–500.
- [9] Hallstrand TS, Curtis JR, Aitken ML, Sullivan SD. Quality of life in adolescents with mild asthma. *Pediatr Pulmonol* 2003;36:536–43.
- [10] Marotel C, Natali F, Heyraud JD, Vaylet F, L'Her P, Bonnet D, et al. Severe forms of effort-induced asthma. *Allerg Immunol (Paris)* 1989;21:61–4.
- [11] Samee S, Altes T, Powers P, de Lange EE, Knight-Scott J, Rakes G, et al. Imaging the lungs in asthmatic patients by using hyperpolarized helium-3 magnetic resonance: assessment of response to methacholine and exercise challenge. *J Allergy Clin Immunol* 2003;111:1205–11.
- [12] Becker JM, Rogers J, Rossini G, Mirchandani H, D'Alonzo Jr GE. Asthma deaths during sports: report of a 7-year experience. *J Allergy Clin Immunol* 2004;113:264–7.
- [13] Frank PI, Morris JA, Hazell ML, Linehan MF, Frank TL. Long term prognosis in preschool children with wheeze: longitudinal postal questionnaire study 1993–2004. *BMJ* 2008;336:1423–6.
- [14] Stern DA, Morgan WJ, Halonen M, Wright AL, Martinez FD. Wheezing and bronchial hyper-responsiveness in early childhood as predictors of newly

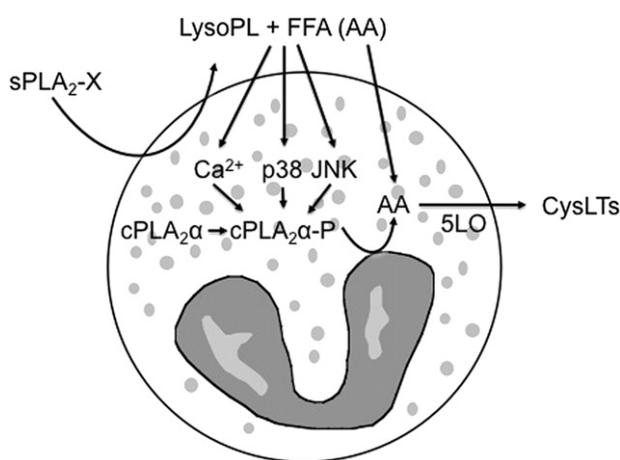


Fig. 3. Schematic representation of sPLA₂-X-mediated CysLT synthesis by eosinophils. sPLA₂-X causes the release of lysophospholipids (LysoPL) and free fatty acids (FFA), including arachidonic acid (AA) from membrane phospholipids species. sPLA₂-X causes CysLT synthesis that is dependent upon cPLA₂α and initiates a Ca²⁺ flux and cPLA₂α phosphorylation in eosinophils. We found that the sPLA₂-X causes a Ca²⁺ flux and that sPLA₂-X- and LysoPC-induced CysLT synthesis could be inhibited by p38 and JNK inhibitors but not by a MEK 1/2 inhibitor. Free AA released by sPLA₂-X may contribute to additional CysLT synthesis based on the observation that the addition of sPLA₂-X to eosinophils treated with fMLP leads to additional CysLT synthesis (Adapted from [8]).

- diagnosed asthma in early adulthood: a longitudinal birth-cohort study. *Lancet* 2008;372:1058–64.
- [15] Joos GF, O'Connor B, Anderson SD, Chung F, Cockcroft DW, Dahlen B, et al. Indirect airway challenges. *Eur Respir J* 2003;21:1050–68.
- [16] Cabral AL, Conceicao GM, Fonseca-Guedes CH, Martins MA. Exercise-induced bronchospasm in children: effects of asthma severity. *Am J Respir Crit Care Med* 1999;159:1819–23.
- [17] Carraro S, Corradi M, Zanconato S, Alinovi R, Pasquale MF, Zacchello F, et al. Exhaled breath condensate cysteinyl leukotrienes are increased in children with exercise-induced bronchoconstriction. *J Allergy Clin Immunol* 2005;115:764–70.
- [18] Freezer NJ, Croasdell H, Doull IJ, Holgate ST. Effect of regular inhaled beclomethasone on exercise and methacholine airway responses in school children with recurrent wheeze. *Eur Respir J* 1995;8:1488–93.
- [19] Holzer K, Anderson SD, Douglass J. Exercise in elite summer athletes: challenges for diagnosis. *J Allergy Clin Immunol* 2002;110:374–80.
- [20] Scollo M, Zanconato S, Ongaro R, Zaramella C, Zacchello F, Baraldi E. Exhaled nitric oxide and exercise-induced bronchoconstriction in asthmatic children. *Am J Respir Crit Care Med* 2000;161:1047–50.
- [21] Malmberg LP, Pelkonen AS, Mattila PS, Hammaren-Malmi S, Makela MJ. Exhaled nitric oxide and exercise-induced bronchoconstriction in young wheezy children - interactions with atopy. *Pediatr Allergy Immunol* 2009;20:673–8.
- [22] Cai Y, Bjermer L, Halstensen TS. Bronchial mast cells are the dominating LTC₄S-expressing cells in aspirin-tolerant asthma. *Am J Respir Cell Mol Biol* 2003;29:683–93.
- [23] Melillo E, Woolley KL, Manning PJ, Watson RM, O'Byrne PM. Effect of inhaled PGE₂ on exercise-induced bronchoconstriction in asthmatic subjects. *Am J Respir Crit Care Med* 1994;149:1138–41.
- [24] Duong M, Subbarao P, Adelroth E, Obminski G, Strinich T, Inman M, et al. Sputum eosinophils and the response of exercise-induced bronchoconstriction to corticosteroid in asthma. *Chest* 2008;133:404–11.
- [25] Dougherty RH, Sidhu SS, Raman K, Solon M, Solberg OD, Caughey GH, et al. Accumulation of intraepithelial mast cells with a unique protease phenotype in T(H)2-high asthma. *J Allergy Clin Immunol* 2010;125:1046–53. e1048.
- [26] Woodruff PG, Boushey HA, Dolganov GM, Barker CS, Yang YH, Donnelly S, et al. Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. *Proc Natl Acad Sci U S A* 2007;104:15858–63.
- [27] Anderson SD, Kippelen P. Airway injury as a mechanism for exercise-induced bronchoconstriction in elite athletes. *J Allergy Clin Immunol* 2008;122:225–35. quiz 236–227.
- [28] Hallstrand TS, Moody MW, Wurfel MM, Schwartz LB, Henderson Jr WR, Aitken ML. Inflammatory basis of exercise-induced bronchoconstriction. *Am J Respir Crit Care Med* 2005;172:679–86.
- [29] Mickleborough TD, Lindley MR, Ray S. Dietary salt, airway inflammation, and diffusion capacity in exercise-induced asthma. *Med Sci Sports Exerc* 2005;37:904–14.
- [30] Meltzer SS, Hasday JD, Cohn J, Bleecker ER. Inhibition of exercise-induced bronchospasm by zileuton: a 5-lipoxygenase inhibitor. *Am J Respir Crit Care Med* 1996;153:931–5.
- [31] Melo RE, Sole D, Naspitz CK. Exercise-induced bronchoconstriction in children: montelukast attenuates the immediate-phase and late-phase responses. *J Allergy Clin Immunol* 2003;111:301–7.
- [32] Pearlman DS, van Adelsberg J, Philip G, Tilles SA, Busse W, Hendeles L, et al. Onset and duration of protection against exercise-induced bronchoconstriction by a single oral dose of montelukast. *Ann Allergy Asthma Immunol* 2006;97:98–104.
- [33] Philip G, Villaran C, Pearlman DS, Loeyes T, Dass SB, Reiss TF. Protection against exercise-induced bronchoconstriction two hours after a single oral dose of montelukast. *J Asthma* 2007;44:213–7.
- [34] Mickleborough TD, Lindley MR, Ionescu AA, Fly AD. Protective effect of fish oil supplementation on exercise-induced bronchoconstriction in asthma. *Chest* 2006;129:39–49.
- [35] Mickleborough TD, Murray RL, Ionescu AA, Lindley MR. Fish oil supplementation reduces severity of exercise-induced bronchoconstriction in elite athletes. *Am J Respir Crit Care Med* 2003;168:1181–9.
- [36] Wijewickrama GT, Kim JH, Kim YJ, Abraham A, Oh Y, Ananthanarayanan B, et al. Systematic evaluation of transcellular activities of secretory phospholipases A₂. High activity of group V phospholipases A₂ to induce eicosanoid biosynthesis in neighboring inflammatory cells. *J Biol Chem* 2006;281:10935–44.
- [37] Trudeau J, Hu H, Chibana K, Chu HW, Westcott JY, Wenzel SE. Selective downregulation of prostaglandin E₂-related pathways by the Th2 cytokine IL-13. *J Allergy Clin Immunol* 2006;117:1446–54.
- [38] Bradding P, Redington AE, Djukanovic R, Conrad DJ, Holgate ST. 15-lipoxygenase immunoreactivity in normal and in asthmatic airways. *Am J Respir Crit Care Med* 1995;151:1201–4.
- [39] Chu HW, Balzar S, Westcott JY, Trudeau JB, Sun Y, Conrad DJ, et al. Expression and activation of 15-lipoxygenase pathway in severe asthma: relationship to eosinophilic phenotype and collagen deposition. *Clin Exp Allergy* 2002;32:1558–65.
- [40] Currie GP, Haggart K, Lee DK, Fowler SJ, Wilson AM, Brannan JD, et al. Effects of mediator antagonism on mannitol and adenosine monophosphate challenges. *Clin Exp Allergy* 2003;33:783–8.
- [41] Lai YL, Lee SP. Mediators in hyperpnea-induced bronchoconstriction of guinea pigs. *Naunyn Schmiedebergs Arch Pharmacol* 1999;360:597–602.
- [42] Freed AN, McCulloch S, Meyers T, Suzuki R. Neurokinins modulate hyperventilation-induced bronchoconstriction in canine peripheral airways. *Am J Respir Crit Care Med* 2003;167:1102–8.
- [43] Hallstrand TS, Debley JS, Farin FM, Henderson Jr WR. Role of MUC5AC in the pathogenesis of exercise-induced bronchoconstriction. *J Allergy Clin Immunol* 2007;119:1092–8.
- [44] Uozumi N, Kume K, Nagase T, Nakatani N, Ishii S, Tashiro F, et al. Role of cytosolic phospholipase A₂ in allergic response and parturition. *Nature* 1997;390:618–22.
- [45] Hallstrand TS, Henderson Jr WR. An update on the role of leukotrienes in asthma. *Curr Opin Allergy Clin Immunol* 2010;10:60–6.
- [46] Bowton DL, Seeds MC, Fasano MB, Goldsmith B, Bass DA. Phospholipase A₂ and arachidonate increase in bronchoalveolar lavage fluid after inhaled antigen challenge in asthmatics. *Am J Respir Crit Care Med* 1997;155:421–5.
- [47] 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;183:2235–45.
- [48] Stadel JM, Hoyle K, Naclerio RM, Roshak A, Chilton FH. Characterization of phospholipase A₂ from human nasal lavage. *Am J Respir Cell Mol Biol* 1994;11:108–13.
- [49] Singer AG, Ghomashchi F, Le Calvez C, Bollinger J, Bezzine S, Rouault M, et al. Interfacial kinetic and binding properties of the complete set of human and mouse groups I, II, V, X, and XII secreted phospholipases A₂. *J Biol Chem* 2002;277:48535–49.
- [50] Munoz NM, Meliton AY, Arm JP, Bonventre JV, Cho W, Leff AR. Deletion of secretory group V phospholipase A₂ attenuates cell migration and airway hyperresponsiveness in immunosensitized mice. *J Immunol* 2007;179:4800–7.
- [51] Ma Y, Koza-Taylor PH, DiMattia DA, Hames L, Fu H, Dragnev KH, et al. Microarray analysis uncovers retinoid targets in human bronchial epithelial cells. *Oncogene* 2003;22:4924–32.
- [52] Cookson W. The immunogenetics of asthma and eczema: a new focus on the epithelium. *Nat Rev Immunol* 2004;4:978–88.
- [53] Sohn J, Kim TI, Yoon YH, Kim JY, Kim SY. Novel transglutaminase inhibitors reverse the inflammation of allergic conjunctivitis. *J Clin Invest* 2003;111:121–8.
- [54] Lee J, Kim YS, Choi DH, Bang MS, Han TR, Joh TH, et al. Transglutaminase 2 induces nuclear factor- κ B activation via a novel pathway in BV-2 microglia. *J Biol Chem* 2004;279:53725–35.
- [55] Kim DY, Park BS, Hong GU, Lee BJ, Park JW, Kim SY, et al. Anti-inflammatory effects of the R2 peptide, an inhibitor of transglutaminase 2, in a mouse model of allergic asthma, induced by ovalbumin. *Br J Pharmacol* 2011;162:210–25.
- [56] Kim Y, Eom S, Kim K, Lee YS, Choe J, Hahn JH, et al. Transglutaminase II interacts with rac1, regulates production of reactive oxygen species, expression of snail, secretion of Th2 cytokines and mediates in vitro and in vivo allergic inflammation. *Mol Immunol* 2010;47:1010–22.
- [57] Cowburn AS, Sladek K, Soja J, Adamek L, Nizankowska E, Szczeklik A, et al. Overexpression of leukotriene C₄ synthase in bronchial biopsies from patients with aspirin-intolerant asthma. *J Clin Invest* 1998;101:834–46.
- [58] Macfarlane AJ, Dworski R, Sheller JR, Pavord ID, Kay AB, Barnes NC. Sputum cysteinyl leukotrienes increase 24 hours after allergen inhalation in atopic asthmatics. *Am J Respir Crit Care Med* 2000;161:1553–8.
- [59] Evans JH, Spencer DM, Zweifach A, Leslie CC. Intracellular calcium signals regulating cytosolic phospholipase A₂ translocation to internal membranes. *J Biol Chem* 2001;276:30150–60.
- [60] Glover S, de Carvalho MS, Bayburt T, Jonas M, Chi E, Leslie CC, et al. Translocation of the 85-kDa phospholipase A₂ from cytosol to the nuclear envelope in rat basophilic leukemia cells stimulated with calcium ionophore or IgE/antigen. *J Biol Chem* 1995;270:15359–67.
- [61] Munoz NM, Kim YJ, Meliton AY, Kim KP, Han SK, Boetticher E, et al. Human group V phospholipase A₂ induces group IVA phospholipase A₂-independent cysteinyl leukotriene synthesis in human eosinophils. *J Biol Chem* 2003;278:38813–20.
- [62] Munoz NM, Meliton AY, Lambertino A, Boetticher E, Learoyd J, Sultan F, et al. Transcellular secretion of group V phospholipase A₂ from epithelium induces β_2 -integrin-mediated adhesion and synthesis of leukotriene C₄ in eosinophils. *J Immunol* 2006;177:574–82.
- [63] Chilton FH, Westcott JY, Zapp LM, Henson JE, Voelkel NF. Incorporation of arachidonic acid into lipids of the isolated perfused rat lung. *J Appl Physiol* 1989;66:2763–71.
- [64] Albert CJ, Thukkani AK, Heuertz RM, Slungaard A, Hazen SL, Ford DA. Eosinophil peroxidase-derived reactive brominating species target the vinyl ether bond of plasmalogens generating a novel chemoattractant, alpha-bromo fatty aldehyde. *J Biol Chem* 2003;278:8942–50.