

The Influence of Aspirin on Release of Eoxin C₄, Leukotriene C₄ and 15-HETE, in Eosinophilic Granulocytes Isolated from Patients with Asthma

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Key Words

Asthma · Arachidonic acid · Lipoxygenase · Eoxin · Leukotriene · Aspirin hypersensitivity

Abstract

Background: The effect of aspirin on the release of key arachidonic acid metabolites in activated eosinophils from subjects with aspirin-intolerant asthma (AIA) has not been investigated previously, despite the characteristic eosinophilia in AIA. **Methods:** Peripheral blood eosinophils were isolated from four groups of subjects: healthy volunteers (HV; n = 8), mild asthma (MA; n = 8), severe asthma (SA; n = 9) and AIA (n = 7). In the absence or presence of lysine-aspirin, eosinophils were stimulated with arachidonic acid or calcium ionophore to trigger the 15-lipoxygenase-1 (15-LO) and 5-lipoxygenase (5-LO) pathways, respectively. 15(S)-hydroxy-eicosatetraenoic acid (15-HETE) and eoxin C₄ (EXC₄) were measured as 15-LO products and leukotriene (LT)C₄ as a product of the 5-LO pathway. **Results:** Activated eosinophils from patients with SA and AIA produced approximately five times more 15-HETE than eosinophils from HV or MA patients. In the presence of lysine-aspirin, eosinophils from AIA, MA and SA patients generated higher levels of 15-HETE than in the absence of lysine-aspirin. Furthermore, in the presence of ly-

sine-aspirin, formation of EXC₄ was also significantly increased in eosinophils from AIA patients, and LTC₄ synthesis was increased both in AIA and SA patients. **Conclusions:** Taken together, this study shows an increased release of the recently discovered lipid mediator EXC₄, as well as the main indicator of 15-LO activity, 15-HETE, in activated eosinophils from severe and aspirin-intolerant asthmatics, and also elevated EXC₄ and LTC₄ formation in eosinophils from AIA patients after cellular activation in the presence of lysine-aspirin. The findings support a pathophysiological role of the 15-LO pathway in SA and AIA.

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Introduction

Aspirin-intolerant asthma (AIA) is arguably the most well-defined phenotype of asthma. With an adult onset and preponderance among women, subjects characteristically suffer from chronic rhinosinusitis and non-allergic asthma [1]. Ingestion of aspirin and other non-steroidal anti-inflammatory drugs will trigger mast cell activation

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and release of cysteinyl leukotrienes (CysLTs) and other mediators that induce bronchoconstriction [2]. The adverse reaction is due to inhibition of the cyclooxygenase (COX) enzyme responsible for the biosynthesis of prostaglandins (PG) [3, 4], although the precise cellular and molecular events that cause the adverse reaction remain undetermined. It is, however, well established that baseline biosynthesis of CysLTs is increased in AIA as reflected by elevated levels in urine, sputum, or incubations of mixed leukocytes [2, 5–8].

A clinically salient and often early recognised feature of AIA is an increase in blood eosinophils [1, 9]. This particular cell is known to have an efficient pathway for the biosynthesis of CysLTs [10], and we recently observed that activated sputum cells derived from subjects with AIA produced increased levels of CysLTs *ex vivo* that correlated with sputum eosinophil counts [11]. However, to date there are no published studies examining the release of CysLTs or other arachidonic acid metabolites in purified eosinophils from patients with AIA.

Previous studies of AIA have also suggested deviations in the metabolism of arachidonic acid along pathways initiated by the 15-lipoxygenase (15-LO) enzyme, which incidentally is also very active in eosinophils [12]. It was thus reported that leukocytes from patients with AIA responded to an *ex vivo* aspirin challenge with increased formation of the main 15-LO product, 15S-hydroxy-eicosatetraenoic acid (15-HETE) [13], and this challenge has been suggested as an *in vitro* test of AIA [14].

More recently, it was discovered that eoxins (EX) represent a new group of metabolites biosynthesised from arachidonic acid in reactions initiated by the 15-LO enzyme [15]. The main metabolites, EXC₄, EXD₄ and EXE₄, are biosynthesised in a pathway analogous to that responsible for the formation of CysLTs, hence, they contain corresponding cysteinyl conjugates at C-14, as compared with C-6 in LTC₄, LTD₄ and LTE₄. The formation of EXC₄ and LTC₄ are both catalysed by LTC₄ synthase (LTC₄-S), an enzyme which is highly expressed in eosinophils [16]. Although the biological role of the EX remains unclear, they are detectable in bronchoalveolar lavage fluid from patients with respiratory disease, and furthermore, increased levels of EX have been reported in exhaled breath condensates from children with asthma [17, 18].

As there have been no previous studies of the release of key arachidonic acid metabolites in eosinophils isolated from subjects with properly diagnosed AIA, we initiated the present study to determine the activity of the two major lipoxygenase pathways in eosinophils from these patients. Whereas the activation of the 5-lipoxygen-

ase (5-LO) that initiates CysLT biosynthesis *in vitro* is stimulated by the calcium ionophore A23187 [10, 15], 15-LO is specifically activated by the addition of exogenous arachidonic acid [15]. In this initial investigation of the activities of the 5-LO and 15-LO pathways in eosinophils from subjects with AIA, we challenged cells both in the absence and presence of acetylsalicylic acid in order to test the hypothesis that eosinophils from subjects with AIA responded differently after COX inhibition.

The *ex vivo* responsiveness of the eosinophils from the AIA patients was compared to that of eosinophils derived from healthy volunteers, as well as two other similar-sized groups of subjects with asthma. One group had mild-to-moderate asthma controlled by low-to-medium doses of inhaled glucocorticosteroids only, whereas the other group had severe disease, which was poorly controlled despite treatment with high doses of steroids and other asthma medications. The inclusion of these four groups permitted assessments of the effects of AIA as well as asthma severity on the release of the measured arachidonic acid metabolites in eosinophils, with an additional control being whether or not any observed differences were related to the level of steroid treatment.

Materials and Methods

Subjects

Twenty-four subjects with diagnosed asthma and eight healthy non-atopic volunteers participated in the study (table 1). All asthmatics were recruited from the Lung and Allergy outpatient clinic at Karolinska University Hospital, Huddinge, and included 8 subjects with mild-persistent asthma, 9 subjects with severe-persistent asthma and another 7 subjects with AIA. Subjects in the AIA group either had an unequivocal history documented in hospital files, or were assigned to the AIA group after a positive aspirin inhalation test according to EAACI/GA²LEN guidelines [1]. The 8 healthy volunteers were recruited by means of advertisement. All subjects were never smokers or non-smokers for the last 2 years with a smoking history of less than 5 pack years, and had not had a respiratory tract infection in the 6 weeks prior to the study. The asthmatic subjects had stable asthma and had not suffered any exacerbations or required any change in maintenance therapy, nor any additional therapy, in the 6 weeks prior to the study. Diagnosis of severe asthma was made according to ENFUMOSA criteria [19]. The study was approved by the local ethics committee at Karolinska University Hospital in Stockholm (Dnr 2006/728-31/2) and the participants provided written informed consent.

Study Design

The first visit involved collection of baseline data, including documentation of medical history, treatments, physical examination, dynamic spirometry, collection of blood samples for differential cell counts and measurement of exhaled nitric oxide, which was analysed using an NIOX analyser (Aerocrine AB, Solna,

Table 1. Subject characteristics

Group	Gender	Age	Eosinophils × 10 ⁹ /l	FEV ₁ , L	FEV ₁ , % predicted	FEV ₁ /FVC ratio, %	ICS dose, µg budesonid equivalent	SPT (positive)	F _e NO, ppb
HV (n = 8)	5F/3M	36 (23–48)	0.2 (0.1–0.3)	4.1 (3.0–5.3)	117 (107–133)	82.4 (79–89)	No use	No pos	11.1 (5.1–20.4)
MA (n = 8)	7F/1M	38.5 (24–58)	0.2 (0.1–0.4)	2.95 (2.0–4.0)	98.6 (82–122)	80.3 (69–93)	495 (320–800)	4.1 (0–5)	11.3 (4.1–23.2)
SA (n = 9)	5F/4M	46 (30–60)	0.8 (0.1–3.6)	2.59 (1.6–4.6)	75.6 (40–99)	69.8 (42–85)	2018 (1,280–3,200)	3 (0–5)	40.7 (7.3–121)
AIA (n = 7)	4F/3M	39.6 (23–49)	0.5 (0.3–0.7)	3.33 (2.2–3.3)	95 (73–123)	69.1 (58–77)	560 (160–1,200)	0.3 (0–2)	27.9 (9.9–56)

Data are shown as mean value and range. FEV₁ = Forced expiratory volume in 1 s; ICS = inhaled corticosteroid; SPT = skin prick test; F_eNO = fraction of exhaled nitric oxide; M = male; F = female.

Sweden) at a flow rate of 50 ml/s as described in ATS/ERS guidelines [20]. All patients and the healthy volunteers underwent skin prick testing (if not previously performed) against common aero-allergens including *Dermatophagoides pteronyssinus*, *Aspergillus fumigatus*, grass pollen, cat fur, horse and dog hair (ALK, Hørsholm, Denmark). At the second visit, blood was taken for isolation of eosinophilic granulocytes.

Isolation of Eosinophils

Following collection of 100 ml of venous blood, an initial centrifugation at 400 g for 15 min was performed. The upper phase was discarded, and the lower phase was collected and subjected to dextran sedimentation for 30 min at 20°C. After sedimentation, the upper phase containing white blood cells was centrifuged once at 400 g for 10 min. The pellet was suspended in lysis buffer (100 mM NH₄Cl, 10 mM Tris-HCl, pH 7.4) and incubated for 30 min at room temperature to remove erythrocytes. After incubation, density gradient centrifugation using Lymphoprep was performed. The polymorphonuclear fraction, containing neutrophils and eosinophils was collected and subjected to magnetic cell sorting. Eosinophils were isolated by negative selection using CD16 antibodies conjugated to magnetic microbeads. The eosinophils constituted more than 99% of all leukocytes in this preparation.

Incubation of Eosinophils

Isolated eosinophils were resuspended in PBS at a concentration of 1×10^6 cells per ml and incubated for 5 min in the presence or absence of lysine-aspirin (200 µM) before further incubation with arachidonic acid (10 µM) or A23187 (1 µM) for 10 min. The reactions were terminated by rapid cool off on ice and centrifugation at 1,200 g for 5 min. Supernatants were stored at –80°C prior to analysis. Due to the low differential count of eosinophils in healthy subjects and the order in which the incubations were carried out, insufficient amounts of eosinophils were recovered for comparable analysis of LTC₄ in the HV group.

Quantitative Analysis by Enzyme Immunoassay

Levels of EXC₄, LTC₄ and 15-HETE were measured by enzyme immunoassay. Supernatants were diluted in enzyme immunoassay buffer and analyses were performed according to the protocol

of the manufacturer (EXC₄ and 15-HETE from Cayman Chemicals, Ann Arbor, Mich., USA; LTC₄ from GE Healthcare, Little Chalfont, UK). Standard curves ranging from 7.8 to 1,000, from 16 to 960 and from 39 to 5,000 pg/ml were generated and used to determine concentrations of EXC₄, LTC₄ and 15-HETE, respectively.

Identification of Eicosanoids by Liquid Chromatography-Mass Spectrometry

For this analysis on a subset of samples, certain incubations were terminated by the addition of acetonitrile containing internal standards and the samples were processed as described in the online supplementary material (for all online suppl. material, see www.karger.com/doi/10.1159/000351422) for measurements of a broader range of metabolites by liquid chromatography-mass spectrometry.

Statistical Analyses

All statistical tests were performed using GraphPad Prism software (GraphPad Software Inc., La Jolla, Calif., USA). The majority of datasets showing 15-HETE, EXC₄ and LTC₄ production were normally distributed according to the Kolmogorov-Smirnov test and were therefore compared using either Student's paired or unpaired t test (for within-group comparisons or between-group comparisons, respectively). Certain clinical characteristics were not normally distributed and all correlations were therefore examined using the Spearman rank test. A p value of <0.05 was accepted as statistically significant. The sizes of the subject groups included were small yet similar to previous studies performed by our group where significant differences regarding eicosanoid production were observed between patients with AIA and aspirin-tolerant asthmatics [8, 11].

Results

Subjects

The characteristics of the four study groups are displayed in table 1. The patients with severe asthma (SA) were older than those in the three other groups, used

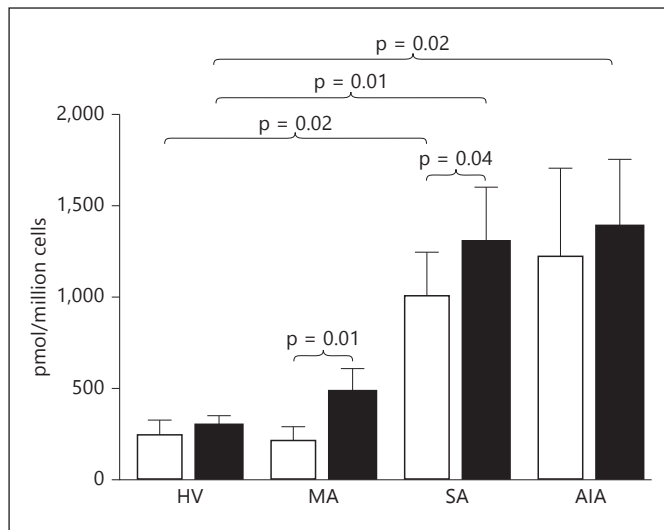


Fig. 1. Formation of 15-HETE in eosinophils isolated from HV, MA, SA and AIA subjects. Isolated eosinophils were resuspended in PBS at a concentration of 1×10^6 cells per ml and incubated for 5 min in the presence (■) or absence (□) of lysine-aspirin (200 μ M), prior to incubation with arachidonic acid (10 μ M) for 10 min. The capacity of eosinophils to produce 15-HETE was calculated as pmol 15-HETE per million eosinophils. Results are shown as mean + SEM. The level of significance for comparisons is indicated above each bar.

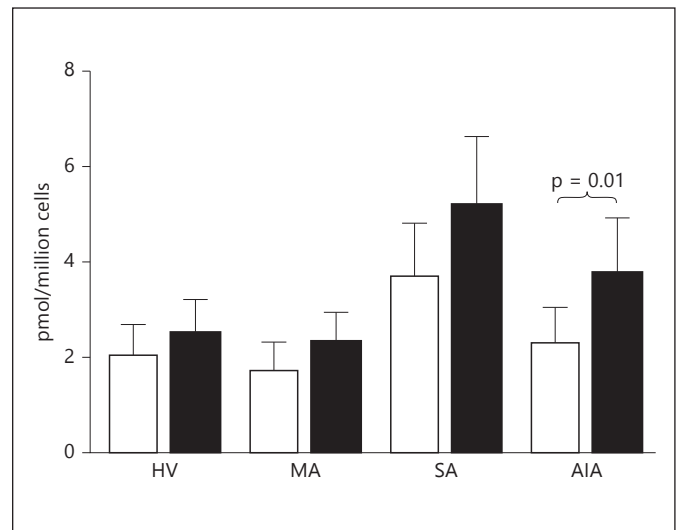


Fig. 2. Biosynthesis of EXC₄ in eosinophils isolated from HV, MA, SA and AIA subjects. Isolated eosinophils were resuspended in PBS at a concentration of 1×10^6 cells per ml and incubated for 5 min in the presence (■) or absence (□) of lysine-aspirin (200 μ M), prior to incubation with arachidonic acid (10 μ M) for 10 min. The capacity of eosinophils to produce EXC₄ was calculated as pmol EXC₄ per million eosinophils. Results are shown as mean + SEM. The level of significance for comparisons is indicated above each bar.

higher doses of inhaled steroids and had lower lung function compared with the groups with mild asthma (MA) and AIA. The patients in the groups with SA and AIA had higher exhaled nitric oxide than MA and HV. The number of eosinophils in peripheral blood was higher in all asthma groups compared with healthy volunteers (HV), and higher in SA and AIA compared with MA, without significant differences between SA and AIA (table 1).

Release of 15-HETE by the Eosinophils

Suspensions of purified eosinophils were pre-incubated with lysine-aspirin or PBS for 5 min before challenge for 10 min with arachidonic acid. In the PBS incubations, eosinophils from SA and AIA patients produced approximately five times higher levels of 15-HETE than the amounts produced by cells isolated from HV or MA patients (fig. 1). In the presence of lysine-aspirin, eosinophils from all asthma groups produced higher levels of 15-HETE than after pre-incubation with PBS (fig. 1), although the difference compared to PBS did not reach statistical significance for the AIA patients ($p = 0.33$). However, after pre-incubation with lysine-aspirin, the highest amounts of 15-HETE were produced by SA and AIA eosinophils (fig. 1).

Release of EXC₄ by the Eosinophils

Using the same protocol as above in which cells were stimulated with arachidonic acid, a similar basal release of EXC₄ was found in PBS incubations from all groups (fig. 2). After pre-incubation with lysine-aspirin, eosinophils from AIA patients produced significantly more EXC₄ than after PBS (fig. 2). This effect was not observed in eosinophils from HV and MA subjects. Eosinophils from SA patients incubated with lysine-aspirin also showed a tendency to produce more EXC₄ than in the absence of aspirin ($p = 0.06$).

Release of LTC₄ by the Eosinophils

Suspensions of purified eosinophils were pre-incubated with lysine-aspirin or PBS for 5 min before challenge for 10 min with the ionophore A23187. The baseline release of LTC₄ was lowest in the MA and highest in AIA groups, but the differences between the groups did not reach statistical significance in the presence of PBS (fig. 3). There was, however, a significantly greater release of LTC₄ when incubations were performed in the presence of lysine-aspirin in the SA and AIA groups. This effect of lysine aspirin was not seen in the MA group. As circulat-

Table 2. Correlations between 15-HETE, EXC₄ and LTC₄ release and clinical characteristics in all asthmatic patients

	15-HETE (AA)		15-HETE (AA + ASA)		EXC ₄ (AA)		EXC ₄ (AA + ASA)		LTC ₄ (A23187)	LTC ₄ (A23187 + ASA)
	p	r _s	p	r _s	p	r _s	p	r _s		
Blood eosinophils, 10 ⁹ /l	<0.01	0.58	<0.01	0.56	0.04	0.43	0.02	0.48	NS	NS
FEV ₁ , l	0.06	-0.39	0.04	-0.42	NS		0.03	-0.46	NS	NS
FEV ₁ , % predicted	<0.01	-0.53	<0.01	-0.56	0.06	-0.40	0.01	-0.55	NS	NS
F _e NO	0.09	0.35	0.04	0.42	NS		NS		NS	NS

NS = Not significant; r_s = Spearman's rank correlation coefficient; AA = arachidonic acid; ASA = acetylsalicylic acid; A23187 = calcium ionophore; FEV₁ = forced expiratory volume in 1 s; F_eNO = fraction of exhaled nitric oxide.

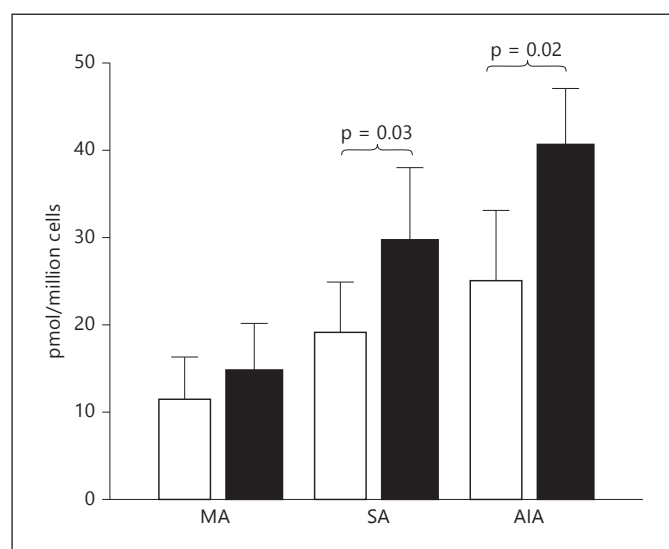


Fig. 3. Biosynthesis of LTC₄ in eosinophils isolated from MA, SA and AIA subjects. Isolated eosinophils were resuspended in PBS at a concentration of 1×10^6 cells per ml and incubated for 5 min in the presence (■) or absence (□) of lysine-aspirin (200 μ M), prior to incubation with calcium ionophore A23187 (1 μ M) for 10 min. The capacity of eosinophils to produce LTC₄ was calculated as pmol LTC₄ per million eosinophils. Results are shown as mean + SEM. Insufficient amounts of eosinophils were recovered from the HV group for a meaningful analysis of LTC₄ in that group. The level of significance for comparisons is indicated above each bar.

ing eosinophil numbers in the healthy subjects were low, sufficient cell numbers were not available for complete analyses of LTC₄ release in this group.

Relations between Subject Characteristics and the Release of 15-HETE, EXC₄ and LTC₄ by Eosinophils

When all asthmatic subjects were analysed together there were several significant correlations between the

release of the lipid mediators and clinical characteristics (table 2). First, the release of 15-HETE and EXC₄ both in the presence and absence of lysine-aspirin showed significant correlations with the total number of blood eosinophils. Second, 15-HETE levels in particular showed significant correlations with lung function and exhaled nitric oxide. Finally, EXC₄ levels in incubations in the presence of lysine-aspirin also correlated with lung function.

Identification of Metabolites by Liquid Chromatography-Mass Spectrometry

Material was available from 2 patients with AIA to perform an extended analysis of metabolites in incubates by mass spectrometry. Challenge of the eosinophil suspensions with arachidonic acid led to significant levels of TXB₂ (84 pmol/million cell, mean of two experiments), lower but detectable amounts of PGE₂, PGD₂ and PGF_{2 α} , as well as EXC₄ and LTC₄ (all ranging between 1.5 and 10 pmol/million cells). The level of EXC₄ was, however, about five times higher than that of LTC₄ after challenge with arachidonic acid. In contrast, when cells from the same subjects were stimulated with calcium ionophore, the levels of most prostanoids and EXC₄ were close to or below the lower limit of detection, whereas there was substantial release of LTC₄, and also significant amounts of TXB₂ in ionophore-stimulated incubates (13 pmol/million cells).

Discussion

In this first assessment of the biosynthesis of key 5- and 15-LO products in highly purified eosinophils from subjects with AIA we have shown that eosinophils from patients with AIA are able to synthesise the novel 15-LO

product EXC₄ and also that its level was higher when cells were stimulated in the presence of aspirin. Under these conditions, eosinophils from patients with AIA and severe asthma behaved similarly, also showing an increased release of both LTC₄ and 15-HETE. The similar effects of aspirin on arachidonic acid-induced EXC₄ and 15-HETE release on the one hand, and ionophore-induced LTC₄ release on the other, are unlikely to be explained by the effects of aspirin on 15-LO activity. As we essentially observed the same effect of aspirin on the release of EXC₄ and LTC₄ in subjects with AIA as in subjects with severe asthma, it is also unlikely that these results relate to the specific intolerance reaction to aspirin that occurs in the AIA group. Thus, the most likely explanation for the effects of the aspirin-treatment, is that the COX inhibition removed PGE₂ which is known to exert an important negative regulatory role on mediator release in inflammatory cells in general [21–23].

Furthermore, there were significant correlations between the levels of EXC₄ and 15-HETE, and blood eosinophil numbers (table 2). The magnitude of the differences in capacity for mediator production between the groups is in fact greater than perceived when levels are expressed per million cells (fig. 1–3). Considering the hypereosinophilia of the subjects with severe asthma and AIA (table 1), the released amounts correspond to approximately 50 nM of 15-HETE per litre of blood from mild asthmatics or healthy subjects, compared to 1 µM per litre of blood from severe or aspirin-intolerant asthmatics. In addition to increased numbers of eosinophils in these two patient groups, another factor contributing to this remarkable degree of 15-LO activity would be increased numbers of hyperactive hypodense eosinophils in the AIA and SA groups. Subjects with blood eosinophilia are known to have a greater proportion of hypodense eosinophils [24], and the number of hypodense eosinophils is increased in patients with asthma in relation to clinical severity [25].

In addition, the extended analysis of eicosanoid metabolism by mass spectrometry of samples from 2 donors with AIA raised another speculative explanation for the increased release of LTC₄ and EXC₄ observed in severe asthma and AIA. The highest level of any primary COX product found was TXB₂, a metabolite indicating biosynthesis of the platelet-specific arachidonic acid product TXA₂ [26, 27]. This finding may seem unexpected as we worked with a highly purified eosinophil preparation, and there are no other cells in the investigated cell suspension that primarily express TXA₂ synthase, but it is well known that leukocytes carry adherent

platelets on their surface [28]. Their presence creates favourable conditions for transcellular metabolism as platelets also express LTC₄-S and have been shown to produce LTC₄ from LTA₄ provided by other cells [29, 30]. A similar mechanism could catalyse the formation of EXC₄ from its eoxin intermediate. Interestingly, it was recently shown that eosinophils from aspirin-intolerant asthmatics have a far greater degree of adherent platelets than aspirin-tolerant asthmatics [31], and these platelets account for a significant proportion of total 5-LO products, as proven by experiments where platelets were removed. It is possible that the EXC₄ and LTC₄ formed in our suspensions could be derived both from eosinophils and adherent platelets that have remained during the eosinophil isolation procedure. We therefore suggest that one factor explaining the greater production of LTC₄ and EXC₄ in AIA and severe asthma is enhanced transcellular metabolism of cysteine-containing products (LTC₄ and EXC₄) due to platelet-eosinophil interactions.

Our findings may seem contradictory to previous studies where aspirin has been shown to specifically increase the release of 15-HETE but not of LTC₄ in blood leukocytes and nasal polyps from AIA patients [13, 32]. However, these studies are not directly comparable due to the different cell types examined, how the cells were activated and, unlike our study, they did not include groups of severe asthmatic patients without aspirin intolerance. A limitation of the current study was that we did not have sufficient eosinophils to study the effects of aspirin in the absence of challenge with arachidonic acid or ionophore, which would have enabled examination of whether aspirin itself could activate mediator release from the eosinophils.

Taken together, the study provides new evidence that metabolites formed via the 15-LO pathway may contribute to asthma pathogenesis. We found significant correlations between levels of 15-HETE production and lung function (FEV₁) as well as exhaled nitric oxide. Levels of 15-HETE were high both in SA and AIA patients, but as the dose of steroids used by the severe asthmatics was significantly higher than that of the patients with AIA, who actually received similar doses to the mild asthmatics (table 1), we conclude that the level of steroid treatment was a marker of asthma severity [19] rather than contributing to the increased 15-LO activity.

Previous publications support a role for 15-LO products in asthma, for example, 15-HETE is the major metabolite of arachidonic acid in asthmatic lung tissue [33, 34]. The amounts and activity of 15-LO in the bronchial

mucosa of adult patients with asthma are increased compared to control subjects, and to a greater extent in patients with severe asthma compared to milder disease [34, 35]. These results indicate that the capacity of eosinophils to produce 15-HETE could be used as a biomarker for asthma severity. An increased activity of the 15-LO pathway has also been described in childhood asthma, with increased levels of EX in breath condensate [18]. 15-HETE has also been found to conjugate to phosphatidylethanolamine and to stimulate ERK phosphorylation in epithelial cells from asthmatics, leading to activation of inflammatory pathways in asthmatic lung tissue [36]. Overexpression of 15-LO in airway epithelial cells in vitro leads to increased release of pro-inflammatory cytokines [37] and mice deficient in 12/15-LO, the ortholog to human 15-LO, displayed attenuated allergic airway inflammation and mucus secretion compared to wild-type controls [38]. Consistent with this latter finding, 15-HETE has also been reported to be involved in mucus secretion in humans [39, 40].

The possible involvement of 15-HETE in AIA suggests that therapies targeting the 15-LO pathway might be considered for the treatment not only of AIA, but also severe aspirin-tolerant asthmatics, in which an increased 15-HETE level was also found. Such therapies may hypothetically reduce the physiological effects of 15-HETE of relevance to asthma as mentioned above, including mucus secretion and inflammatory cell influx.

In summary, our findings in eosinophils are in line with previous observations of a high capacity for 15-HETE generation in mixed leukocytes from subjects with AIA [13] and extend this information by showing that the eosinophil is likely to be the main source of 15-LO products. This higher biosynthetic activity is in part due to increased numbers of eosinophils, but the data also support enhanced eosinophil function, possibly involving transcellular interactions with platelets. Moreover, the increased 15-LO activity was present both in AIA and severe asthma, and incubation with aspirin was able to enhance this activity in both patient groups, suggesting that this effect of aspirin not was restricted to the specific intolerance reaction in AIA, nor was it related to the level of steroid treatment. Finally, this first documentation of EXC₄ formation in activated eosinophils from patients with asthma encourages further explorations of the biological role of this novel lipid mediator.

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Material and Methods

Reagents

Lymphoprep was bought from Medinor (Stockholm, Sweden). Microbeads CD16 and MACS column was bought from Göteborgs Termometerfabrik (Göteborg, Sweden). Dextran (T500) was bought from GE Healthcare (Uppsala, Sweden). NH_4Cl was bought from Merck Solna (Sweden). Tris-HCl was bought from Scharlau (Spain) Arachidonic acid was bought from Nu-Check-prep (Elysian, MN, USA). Lysin-aspirin was bought from Apotektet (Huddinge, Sweden). A23187 was bought from Sigma (Stockholm, Sweden). Enzyme Immunoassays from Cayman, 15-HETE and EXC_4 , was bought from AH Diagnostics (Stockholm, Sweden) and Cayman Chemicals (Ann Arbor, Michigan, USA) respectively. Enzyme immunoassay LTC_4 was bought from GE Healthcare (Uppsala, Sweden). Synthetic Prostaglandin E_2 (PGE_2), D_2 (PGD_2), $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$), 15-Ketoprostaglandin E_2 (15-Keto- PGE_2), 6-Keto prostaglandin $\text{F}_{1\alpha}$ (6-Keto- $\text{PGF}_{1\alpha}$), Thromboxane B_2 (TxB_2), Eoxin C_4 (EXC_4), Leukotriene C_4 (LTC_4) and Lipoxin A_4 (LXA_4) were bought from the SMS-gruppen (Denmark).

Identification of eicosanoids by LC-MS/MS

The comprehensive identification by LC-MS/MS was performed on a Surveyor MS coupled to a TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Electron Corporation, Sweden). Reverse phase LC was performed using a Gemini-NX $3\mu\text{m}$ C18 column 2.0×50 mm (Phenomenex) with flow rate constantly held at $400\ \mu\text{l}/\text{min}$. Mobile phase A consisted of 2% acetonitril in water and 0.1% acetic acid, and mobile phase B consisted of 80% acetonitrile in water and 0.1% acetic acid. All water used was of MilliQ grade and organic solvents of analysis grade. Three different analyses methods were performed on prostaglandins, $\text{EXC}_4/\text{LTC}_4$ and LXA_4 . Incubations were stopped by adding acetonitrile containing internal standard for quantitative analyses of prostaglandins and LXA_4 by LC-MS/MS.

For prostaglandins the chromatography started isocratically for 1 min at 90 % A was followed by a 7 min linear gradient reaching 70% of B. After direct switching to 100 % B the system

was washed for 2 min and subsequently equilibrated at 90% A for 2 min. The mass spectrometer was operated using electrospray atmospheric pressure ionization source in negative mode. The spray voltage was set to 3300 V, capillary temperature was 350°C and sheath and auxiliary gas was optimal at 50 and 6 respectively (arbitrary units). Skimmer offset was at 10-12 V and tube lens 128 V. The transition utilized in the SRM scan was for PGE₂ and PGD₂ 351.2 → 271.0 m/z, PGF_{2α} 353.2 → 309.0 m/z, 15-Keto-PGE₂ 349.1 → 287.1 m/z, 6-Keto-PGF_{1α} 369.2 → 245.2 m/z, TxB₂ 369.2 → 169.0 m/z at collision energies between of 20-26 eV.

For EXC₄/LTC₄ the chromatography started isocratically for 1.5 min at 90 % A was followed by a 4.5 min linear gradient reaching 100% of B. The system was washed at 100 % B for 2 min and subsequently equilibrated at 90% A for 2 min. The mass spectrometer was operated using electrospray atmospheric pressure ionization source in positive mode. The spray voltage was set to 4500 V, capillary temperature was 350°C and sheath, ion sweep gas and auxiliary gas pressure was optimal at 60, 5 and 5 respectively (arbitrary units). Skimmer offset was at 10 V and tube lens 122 V. The transition utilized in the SRM scan for LTC₄ was 626.2 → 189.0 m/z and for EXC₄ 626.2 → 205.1 m/z at a collision energy of 19 and 21 eV, respectively. The EXC₄ and LTC₄ were also separated chromatographically by approximately 0.4 min.

For LXA₄ an Sunfire 3.5 μm C18 column 2.1 x 30 mm (Waters, Sweden) was used and the chromatography started isocratically for 1 min at 90 % A was followed by a 5 min linear gradient reaching 70% of B. After direct switching to 100 % B the system was washed for 2 min and subsequently equilibrated at 90% A for 2 min. The mass spectrometer was operated using electrospray atmospheric pressure ionization source in negative mode. The spray voltage was set to 3000 V, capillary temperature was 350°C and sheath ion, sweep gas and auxiliary gas was optimal at 60, 5 and 5 respectively (arbitrary units). Skimmer offset was at 10 V and tube lens 96 V. The transition utilized in the MRM scans was for LXA₄ 351.2 → 289.2 and 217.1 m/z at collision energy of 15 and 21 eV, respectively. Standard calibration curve at ten concentration levels was prepared in PBS (0.5 ml) in the following interval; 1.2-600 pmol/ml for all prostaglandins, 0.6-320 pmol/ml for EXC₄ and LTC₄ and 0.9-473 pmol/ml. Three qualitative controls at 3 different concentration levels were used for each analyte. Warfarin was used as internal standard at 16 nM added to the acetonitril solution used to stop the incubations. The transition utilized for warfarin in the SRM scan was in negative mode 307.0 → 161.0 m/z and in positive mode 309.0 → 163.1 m/z. The standard samples were

treated in the same manner as the eosinophil incubation samples. One standard series was analysed in the beginning and one at the end of the analysis list. Limit of quantification (S/N 5) was for PGE₂ 1.2 pmol/ml, PGD₂ 1.2 pmol/ml, PGF_{2α} 4.7 pmol/ml, 15-Keto-PGE₂ 4.7 pmol/ml, 6-Keto-PGF_{1α} 18.8 pmol/ml, TxB₂ 1.2 pmol/ml, EXC₄ 1.3 pmol/ml, LTC₄ 1.3 pmol/ml and LXA₄ 7.4 pmol/ml.