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Aspirin-tolerant asthmatics generate more lipoxins than aspirin-intolerant asthmatics

M. Sanak*, B.D. Levy#, C.B. Clish#, N. Chiang#, K. Gronert#, L. Mastalerz*, C.N. Serhan#, A. Szczeklik*

Aspirin-tolerant asthmatics generate more lipoxins than aspirin-intolerant asthmatics. M. Sanak, B.D. Levy, C.B. Clish, N. Chiang, K. Gronert, L. Mastalerz, C.N. Serhan, A. Szczeklik. ©ERS Journals Ltd 2000.

ABSTRACT: Asthma is characterized by chronic airway inflammation resulting from overproduction of pro-inflammatory mediators, such as leukotrienes (LT). The authors questioned the biosynthetic capacity of asthmatic patients for lipoxins (LX) and 15-epimer lipoxins (15-epi-LX), endogenous regulators of inflammatory responses that inhibit pro-inflammatory events.

Levels of LXA_4, 15-epi-LXA_4 and LTC_4 were determined in 14 clinically characterized aspirin-intolerant asthmatics (AIA), 11 aspirin-tolerant asthmatics (ATA) and eight healthy volunteers using a stimulated whole blood protocol.

Both LXA_4 and 15-epi-LXA_4 were generated in whole blood activated by the divalent cation ionophore, A23187. Higher levels of LXA_4 were produced in ATA than either AIA or healthy volunteers. Exposure of AIA whole blood to interleukin-3 prior to A23187 did not elevate their reduced capacity to generate LXA_4. Generation of a bronchoconstrictor, LTC_4, was similar in both AIA and ATA. Consequently, the ratio of LXA_4:LTC_4 quantitatively favoured the bronchoconstrictor for AIA and differed from both ATA and healthy subjects. In addition, the capacity for 15-epi-LXA_4 generation was also diminished in AIA, since whole blood stimulated in the presence of aspirin gave increased levels only in samples from ATA.

The present results indicate that asthmatics possess the capacity to generate both lipoxins and 15-epimer-lipoxins, but aspirin-intolerant asthmatics display a lower biosynthetic capacity than aspirin-tolerant asthmatics for these potentially protective lipid mediators. This previously unappreciated, diminished capacity for lipoxin formation by aspirin-intolerant asthmatic patients may contribute to their more severe clinical phenotype, and represents a novel paradigm for the development of chronic inflammatory disorders.

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*Dept of Medicine, Jagellonian University School of Medicine, Kraków, Poland. #Center for Experimental Therapeutics and Reperfusion Injury, Dept of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA. *,#M. Sanak and B.D. Levy contributed equally.

Correspondence: C.N. Serhan, Center for Experimental Therapeutics and Reperfusion Injury, Thorn Building for Medical Research, Room 724, Brigham and Women's Hospital, 75 Francis Street, Boston MA 02115 USA. Fax: 1 6172786957

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Aspirin (acetylsalicylic acid)-intolerance is a distinct clinical syndrome observed in 5–10% of adult patients with asthma [1, 2]. In these patients, aspirin and several other nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit cyclo-oxygenase (COX) precipitate asthmatic attacks. Despite avoidance of NSAIDs, aspirin-intolerant asthmatics (AIA) experience protracted symptoms with many requiring systemic corticosteroids for control. This syndrome's pathophysiology is characterized in part by excess production of cysteinyl (Cys)-leukotrienes (LT), such as LTC_4 and LTD_4 [3–5], which primarily serve as bronchoconstrictors [6] as well as pro-inflammatory mediators [7]. Bronchial biopsies of asthmatic airways reveal marked accumulation of eosinophils [8] and overexpression of LTC_4 synthase [9], the terminal enzyme for Cys-LT production. Drugs that prevent the formation or action of Cys-LT effectively prevent aspirin-precipitated attacks of asthma [7, 10] and, on prolonged administration, improve control of the disease [11].

Lipoxins (LX) and 15-epimer-lipoxins (15-epi-LX) are also lipoxygenase (LO)-derived eicosanoids, yet their biological actions differ dramatically from LT [12]. In sharp contrast to LT, LX inhibit bronchoconstriction [13] and carry local anti-inflammatory signals [14]. LX possess a distinctive trihydroxytetraene structure, and are produced by transcellular biosynthesis during cell-cell interactions (*e.g.* between infiltrating leukocytes and resident cells) that occur in pathological states [14], such as in human airways [15, 16] and nasal polyps [17]. LXA_4 inhibits polymorphonuclear neutrophil (PMN) and eosinophil chemotaxis [18, 19] and PMN transmigration into inflammatory tissues [14], responses of interest in airway disease. Of particular interest, inhalation of LXA_4 by human asthmatics blocks LTC_4 stimulated bronchoconstriction [13]. Thus, LX formation in the human lung may, in view of LX actions, play a role in airway disease.

Recently, a novel mechanism of action was uncovered for aspirin revealing that, when inhibiting prostaglandin formation, aspirin-acetylated COX-2 can participate in the generation of new endogenous lipid mediators that are the carbon 15-epimers of LX [20]. These "aspirin-triggered" 15-epi-LX share LX bioactions, are potent counter-regulatory lipid mediators that block LT-mediated inflammatory responses *in vivo* and can compete for LTD_4 specific binding [21]. Thus, formation of 15-epi-LX within the local inflammatory milieu may represent a novel molecular

mechanism underlying some of aspirin′s salutary effects. In the present study, the authors questioned whether aspirin-tolerant and aspirin-intolerant asthmatic patients, who develop chronic airway inflammation, can generate both LX and the aspirin-triggered 15-epi-LX.

Methods

Study subjects

Between December 1997 and June 1998, 14 AIA and 11 aspirin-tolerant asthmatics (ATA) who presented consecutively to one of the authors (A. Szczeklik) at the Dept of Medicine at Jagellonian University in Kraków, Poland were prospectively enrolled for the study. The diagnosis of aspirin intolerance was confirmed by aspirin provocation tests [22] performed during the 12 months directly preceding the study. In addition, ATA patients reported use of NSAIDs without adverse effects. All patients were non-smokers, in stable clinical condition and not using systemic anti-inflammatory drugs during the study (table 1).

Study design

After stimulation of each subject′s whole blood, duplicate determinations of plasma levels of LXA_4, 15-epi-LXA_4 and LTC_4 were performed using specific enzyme-linked immunosorbent assays (ELISA) in tandem (Neogen Corp., Lexington, KY, USA and Cayman Chemical, Co., Ann Arbor, MI, USA). The LXA_4 and 15-epi-LXA_4 ELISAs were validated for these experiments by physical methods, including high-performance liquid chromatography (HPLC) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) [16, 23]. Here, the identities of the immunoreactive eicosanoids were confirmed using LC/MS/MS.

Methods

Peripheral venous blood (~15 mL) was obtained by venepuncture from volunteer subjects who had given written informed consent to a protocol approved by the Jagellonian University Ethical Committee (Kraków, Poland). Blood samples were drawn into three standard 5 mL tubes containing sodium heparin (Vacutainer; Becton Dickinson, Franklin Lakes, NJ, USA) and processed immediately. Whole blood samples were exposed (37°C, 30 min with gentle shaking (40 rpm) in a vertical position) to recombinant human interleukin (IL)-3 (20 ng·mL; Genzyme, Cambridge, MA, USA) in the presence (tube 1) or absence (tube 2) of lysyl-aspirin (100 μM) followed by activation (45 min, 37°C) with the divalent cation ionophore, A23187 (50 μM; Sigma Chemical Co., St. Louis, MO, USA). Note that this concentration of A23187 is required to activate eicosanoid generation in whole blood [24]. The third blood sample was not exposed to IL-3 and warmed (37°C, 5 min) directly prior to activation. After stimulation, plasma was separated from blood by centrifugation ($3{,}000 \times g$, 10 min, 25°C) and stored at -80°C until eicosanoid levels were measured by ELISA.

Samples of plasma from several donors were pooled to obtain quantities that permitted physical identification and 10 ng of deuterium-labelled LTB_4 (d_4-LTB_4; Cayman Chemical Co.) was added as an internal standard. Eicosanoids were extracted as in LEVY *et al.* [25] and methyl formate fractions obtained from these samples were taken to LC/MS/MS for analysis of LX and other eicosanoids [25]. The stereochemistry of 15-hydroxyeicosatetraenoic acid (15-HETE) (% *R versus S* epimer) was determined by chiral HPLC [25].

Table 1. – Characteristics of subjects

	Healthy volunteers	Aspirin -tolerant asthmatics	Aspirin -intolerant asthmatics
Subjects n	10	11	14
Age yrs	43.8±6.5	45.7±9.5	47.6±9.6
Male/female	4/6	3/8	8/6
Duration of asthma yrs	NA	10.4±8.9	8.0±4.8
Leukocyte cells·mm^{-3}	NM	6440±160	6490±1200
Eosinophils cells·mm^{-3}	NM	215±150	279±138
Platelets cells·mm^{-3}	NM	234500 ±69800	247200 ±53030
FEV1 L	NM	2.70±0.73	2.72±0.61
FEV1 %	NM	92.4±13.4	86.4±11.7
Total IgE IU·mL^{-1}	55.2±72.2	91.3±215.9	58.6±82.5
Inhaled steroids %	0	78	65

Values are the mean±SD. NA: not applicable; NM: not measured; FEV1: forced expiratory volume in one second; IgE: immunoglobulinE. *: geometric mean±SD.

Analysis

Samples were coded so that patient identities were not revealed. Collected data were analysed for significance using an unpaired t-test that was performed with statistical software (Microsoft Excel, version 97 SR-1; Microsoft Corp., Redmond, WA, USA). Probability values were two-tailed, with $p<0.05$.

Results

Patient characteristics

Characteristics of the subjects on enrolment are presented in table 1. Although peripheral blood eosinophilia was more pronounced in AIA ((mean±SD) 279±138·mm^{-3}) than ATA (215±150·mm^{-3}), there was no statistical difference. In addition, the asthmatics had no statistical differences with respect to age, duration of asthma, cell counts, baseline spirometry, serum total immunoglobulin E or treatment. At the time of sample acquisition, asthma symptoms were well-controlled in all subjects and none were taking oral glucocorticoids.

Lipoxin and 15-epimer-lipoxin biosynthetic capacity

Activated whole blood from AIA patients produced LXA_4 in amounts that were not statistically different from that generated by healthy volunteers ((mean±SEM) 5.7±2.0 *versus* 7.2±2.8 ng·mL^{-1}). This observation contrasts with the LX biosynthetic capacity of ATA whole blood that generated approximately two-fold more LXA_4 (12.9±5.4 ng·mL^{-1}) than that from AIA (5.7±2.0 ng·mL^{-1}). The addition of exogenous substrate was not required for either LXA_4 or 15-epi-LXA_4 formation in activated whole blood, indicating that both ATA and AIA utilized endogenous sources of arachidonate to generate these counterregulatory eicosanoids.

IL-3 is elaborated by helper type 2 lymphocytes and held to be a pro-inflammatory cytokine that increases the generation of eicosanoids by stimulated granulocytes [26]. Here, the addition of IL-3 increased LXA_4 from 7.2 to 12.1 ng·mL^{-1} in activated whole blood from healthy volunteers. In contrast, neither ATA nor AIA whole blood gave increased LX biosynthetic capacity when exposed to IL-3 and thus these two asthma cohorts still showed a difference in LXA_4 generation when a stimulatory cytokine was present (fig. 1a).

LTC_4 was present in all samples following activation of whole blood (range: 0.26–3.3; median 1.2; mean 1.4 ng·mL^{-1}). The levels of LTC_4 in AIA and ATA samples did not differ ((mean±SEM)1.4±0.2 *versus* 1.4±0.2 ng·mL^{-1}). Moreover, the relationship between LX and LT production, expressed as the ratio of LXA_4 to LTC_4, was significantly reduced in AIA below that observed with either ATA or healthy subjects (fig. 1b).

In addition to LXA_4, activated whole blood also generated 15-epi-LXA_4 (fig. 2). When activated in the presence of aspirin, values for 15-epi-LXA_4 increased ~80% in ATA whole blood (1.4 to 2.6 ng·mL^{-1}). In contrast to the response in ATA, neither 15-epi-LXA_4 nor LXA_4 formation were increased in aspirin-treated samples from AIA, and together, their sum (4.5 ng·mL^{-1}) was significantly less than in ATA ((mean) 11.1 ng·mL^{-1}, $p<0.05$). The levels of both LXA_4 and 15-epi-LXA_4 generated in whole blood were decreased in AIA compared to ATA (fig. 2). These findings indicate that whole blood from both ATA and AIA can generate 15-epi-LXA_4, but the biosynthetic capacity for 15-epi-LX is lower in whole blood from AIA patients.

To further validate the identification of these potential anti-inflammatory eicosanoids, samples from each patient group were pooled and analysed by LC/MS/MS for identification of retention times, signature ions and diagnostic product ion mass spectrometry (MS/MS) spectra. Both LXA_4 and LXB_4 were present in each study group. Native LXA_4 and its all-*trans* isomer (11-*trans*-LXA_4) eluted at 15.0 and 12.7 min (consistent with authentic synthetic materials), respectively, and were further identified on the basis of their molecular anions ($[M-H]^-$ mass-to-change ratio (*m/z*) 351) and diagnostic product ions present in their corresponding MS/MS spectra (fig. 3). Prominent diagnostic ions for LXA_4 were identified at *m/z* 333 ($[M-H]^-$-H_2O), *m/z* 315 ($[M-H]^-$-2H_2O), *m/z* 307 ($[M-H]^-$-CO_2), *m/z* 289 ($[M-H]^-$-H_2O, -CO_2), *m/z* 251 ($[M-H]^-$-CHO $(CH_2)_4CH_3$), *m/z* 233 ($[M-H]^-$-H_2O, -CHO$(CH_2)_4CH_3$), *m/z* 135 ($[CH_2CH(CH{=}CH)_3CHOH]^-$), and *m/z* 115 (CHO $(CH_2)_3COO^-$). Native LXB_4 and its all-*trans* isomer (8-*trans*-LXB_4) were eluted at 10.9 and 9.8 min, respectively, and were also identified on the basis of their molecular anions ($[M-H]^-$=*m/z* 351) and diagnostic product ions in their MS/MS spectra (fig. 3) occurring at *m/z* 333 ($[M-H]^-$-H_2O), *m/z* 315 ($[M-H]^-$-2H_2O), *m/z* 307 ($[M-H]^-$-CO_2), *m/z* 289 ($[M-H]^-$-H_2O, -CO_2), *m/z* 271 ($[M-H]^-$-2H_2O, -CO_2), *m/z* 251 ($[M-H]^-$-CHO$(CH_2)_4CH_3$), *m/z* 233 ($[M-H]^-$-H_2O, -CHO $(CH_2)_4$ CH_3), and *m/z* 221 ($[M-H]^-$-CHOCHOH $(CH_2)_4$ CH_3). LC/MS/MS analysis revealed a ratio for LXA_4:LXB_4 generation of ~1.2:1. (Note: LXB_4 and, its aspirin-triggered epimeric form, 15-epi-LXB_4 levels were not monitored in individual samples because the authors have not yet prepared immunoassays for these eicosanoids.)

In addition to both LXA_4 and LXB_4, 15*S*-HETE (stereochemistry at the carbon 15 alcohol was determined by chiral HPLC analysis) was also present in samples from patients with AIA. Patient-derived 15-HETE was eluted at 37.9 min (consistent with authentic 15*S*-HETE in this LC/

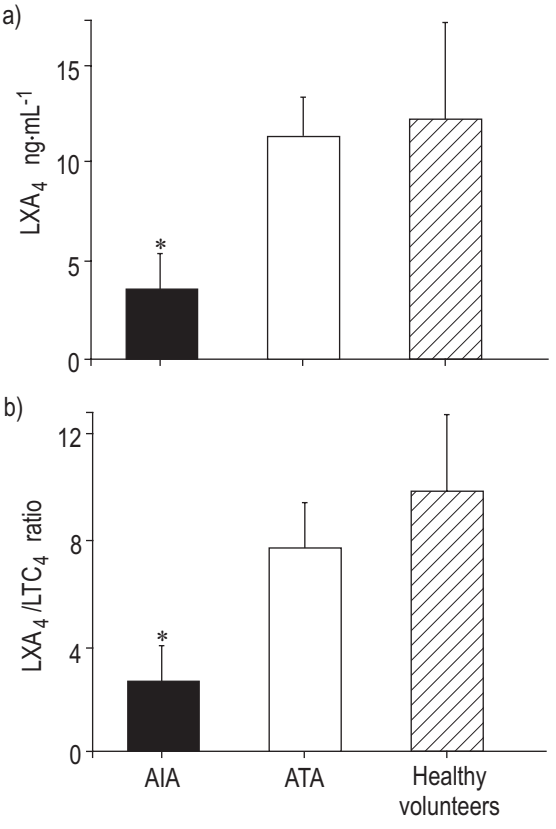

Fig. 1. – Diminished lipoxin (LX)A_4 biosynthetic capacity in aspirin-intolerant asthmatics (AIA). Whole blood samples from AIA or aspirin-tolerant asthmatics (ATA) were exposed to interleukin-3 (20 ng·mL^{-1}, 30 min, 37°C) and activated (see *Methods* section) (45 min, 37°C) as in [24]. LXA_4 and leukotriene (LT)C_4 were measured by specific enzyme linked immunosorbent assays in tandem. Values for LXA_4 (a) and the ratio of LXA_4: LTC_4 (b) represent the mean±SEM for n=11 (ATA), n=14 (AIA) and n=8 (healthy volunteers). *: $p<0.05$ by unpaired *t*-test.

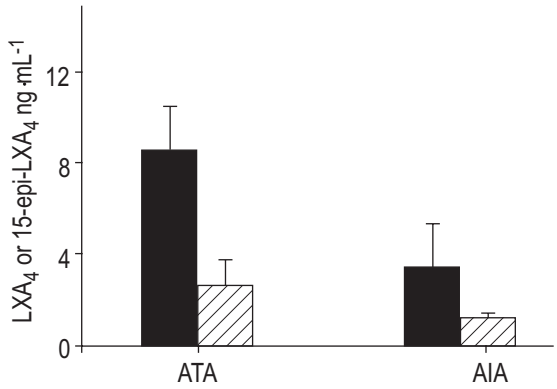

Fig. 2. – Aspirin-intolerant asthmatics (AIA) have a decreased capacity to generate lipoxin (LX) A_4 and 15-epimer (epi)-LXA_4: impact of aspirin. Whole blood from patients with aspirin-tolerant asthmatics (ATA) or AIA was activated (see *Methods* section) in the presence of lysyl-aspirin (100 µM). Values for LXA_4 and 15-epi-LXA_4 were determined by specific enzyme linked immunosorbent assays in tandem (mean±SEM). ■: LXA_4; ▨: 15-epi-LXA_4.

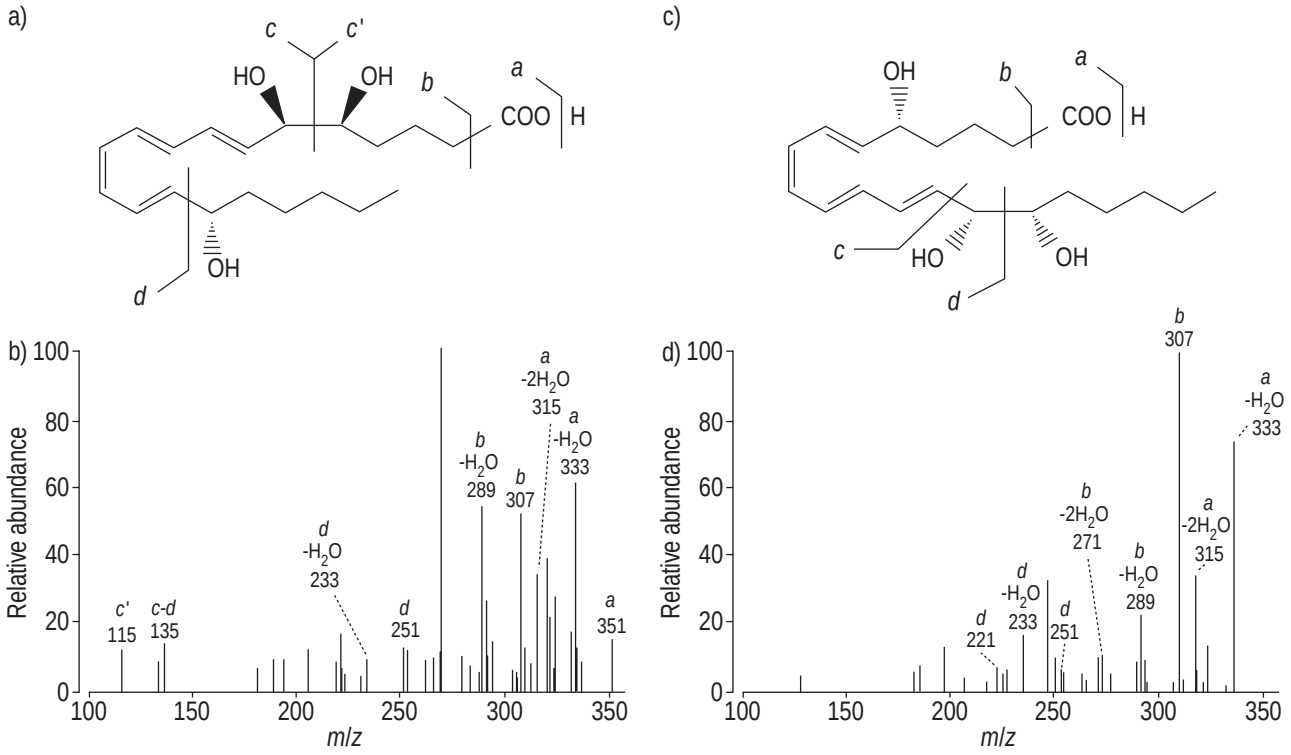

Fig. 3. – Mass spectra and structures of lipoxin (LX)A_4 and LXB_4. Product ion mass spectrometry (MS/MS) spectra for LXA_4 (a and b) and LXB_4 (c and d), which were identified by their ultraviolet spectra and retention times during high-performance liquid chromatography and diagnostic fragmentation in MS/MS, which are highlighted in a and c. Product ion cleavage sites are denoted *a*, *b*, *c*, *c'*and *d*. Diagnostic product ions in the MS/MS spectra of LXA_4 (b) were mass-to-charge ratio (*m/z*) 351 (*a*: molecular anion $[M\text{-}H]^-$); *m/z* 333 (*a* -H_2O); *m/z* 315 (*a* -$2H_2O$); *m/z* 307 (*b*: $[M\text{-}H]^-$-CO_2); *m/z* 289 (*b*-H_2O); *m/z* 251 (*d*: $[M\text{-}H]^-$-$CHO(CH_2)_4CH_3$); *m/z* 233 (*d*-H_2O); *m/z* 135 (*c*-*d*: $[CH_2CH(CH{=}CH)_3CHOH]^-$), and *m/z* 115 (*c'*: $CHO(CH_2)_3COO^-$). Diagnostic product ions in the MS/MS spectra of LXB_4 (d) were *m/z* 333 (*a*: $[M\text{-}H]^-$-H_2O); *m/z* 315 (*a*-$2H_2O$); *m/z* 307 (*b*: $[M\text{-}H]^-$-CO_2); *m/z* 289 (*b*-H_2O); *m/z* 271 (*b*-$2H_2O$); *m/z* 251 (*d*: $[M\text{-}H]^-$-$CHO(CH_2)_4CH_3$); *m/z* 233 (*d*-H_2O); and *m/z* 221 (*c*: $[M\text{-}H]^-$-$CHOCHOH(CH_2)_4CH_3$).

MS/MS system) and was identified by its molecular ion ($[M\text{-}H]^-$=*m/z* 319) and MS/MS ions present at *m/z* 301 ($[M\text{-}H]^-$-H_2O), *m/z* 275 ($[M\text{-}H]^-$-CO_2), *m/z* 257 ($[M\text{-}H]^-$-H_2O, -CO_2), and *m/z* 219 ($[M\text{-}H]^-$-$CHO(CH_2)_4CH_3$). 15*S*-HETE is predominantly a 15-LO derived eicosanoid that can serve as a biosynthetic intermediate for LX. Formation of its epimer, 15*R*-HETE, is catalysed by aspirin-acetylated COX-2 and serves as an intermediate in 15-epi-LX biosynthesis [14, 20]. In contrast to the prominent amounts of 15*S*-HETE present in materials pooled from AIA, levels of 15*R*-HETE in these samples were below the limits of detection for these physical methods and not observed. Not only do these results confirm and extend the antibody-based identification, together they suggest that individuals with AIA accumulate the 15-lipoxygenase product, 15*S*-HETE, but not the aspirin-acetylated COX-2 product, 15*R*-HETE. This difference is of interest because 15-epi-LX was generated while its precursor 15*R*-HETE, a product of aspirin treatment, apparently was not accumulated in these individuals.

Discussion

The present results indicate that asthmatic patients can generate both LX and 15-epi-LX (figs. 1 and 2). Of interest, AIA whole blood showed reduced LX and 15-epi-LX biosynthetic capacity and lower ratios for LXA_4:LTC_4 generation (figs. 1 and 2) when compared to those values obtained for ATA. Taken together, these results indicate that the ability of asthmatics with AIA to mount "stop signals" (*i.e.* LXA_4) is diminished and strikingly different from individuals with ATA.

The first biosynthetic pathway elucidated for LX identified interactions between 15- and 5-LO leading to the sequential insertion of molecular oxygen into the carbon 15 and carbon 5 positions in arachidonic acid [14]. Eosinophils carry prominent 15-LO activity and can generate LX during cell-cell interactions that occur with leukocytes possessing 5-LO [6]. Eosinophils are enriched in asthmatics [7] and here were present in approximately equal numbers in both ATA and AIA whole blood (table 1). Since LX are potent in the picomole to nanomole range in cellular and animal models [14], physiologically relevant amounts of LX can be generated during transcellular eicosanoid biosynthesis.

While aspirin or NSAIDs precipitate symptoms in AIA, individuals with ATA do not experience increased symptoms when challenged with these same agents. In addition to inhibiting COX-catalysed prostanoid formation, the acetylation of COX-2 by aspirin triggers the generation of 15*R*-HETE, a biosynthetic precursor for 15-epi-LX [20]. COX-2 messenger ribonucleic acid was down-expressed in AIA nasal polyps in a study by Piacado *et al.* [27], and here 15*R*-HETE was not detected in aspirin-treated samples from AIA (see *Results* section). The results shown in figure 2 demonstrate that AIA peripheral blood has diminished biosynthetic capacity for 15-epi-LX, a 5-LO

product of 15*R*-HETE. Low levels of 15-epi-LXA_4 were detectable by immunoassay in both AIA and ATA whole blood activated in the absence of aspirin. Only samples from ATA generated increased levels of 15-epi-LXA_4 when stimulated in the presence of aspirin. This reduced 15-epi-LX biosynthetic capacity in AIA may reflect lower COX-2 expression [27], altered COX-2 sensitivity to acetylation by aspirin (for 15*R*-HETE formation) or COX-2 inhibition by asthma medications (*e.g.*, corticosteroids). Since allergic lung responses are heightened in COX deficient mice [28] and 15-epi-LX are more potent than native LX as inhibitors of PMN trafficking and PMN-mediated inflammation *in vivo* [21], generation of 15-epi-LX and LX may distinguish the ability of individuals with asthma to tolerate aspirin.

LXA_4 and 15-epi-LXA_4 act *via* the same specific receptor termed ALXR to mediate their counter regulatory actions important in anti-inflammation and resolution of inflammatory sequelae [14]. In addition to ALXR on human leukocytes, LXA_4 also interacts in the airway with functionally characterized Cys-LT_1 receptors (LTD_4 receptor) to block airway smooth muscle contraction [29]. LXA_4 also competes with LTD_4 specific binding to endothelial cells in culture [21] and inhibits LTC_4-mediated airway hyperresponsiveness in asthmatics [13]. LXA_4 is rapidly metabolized by isolated leukocytes and several organs, including lung and liver [14]. This observation led to the design of novel LX and 15-epi-LX analogues that are highly bioavailable, metabolically stable and even more potent inhibitors of inflammatory responses *in vivo* [21]. In view of the present findings, LX and 15-epi-LX analogues may represent a novel strategy for limiting airway hyperresponsiveness and inflammation in AIA and possibly other lung diseases associated with inflammation.

In summary, the present findings indicate that both aspirin-tolerant asthmatics and aspirin-intolerant asthmatics can produce lipoxin and 15-epimer-lipoxin, but when compared to aspirin-tolerant asthmatics and healthy subjects, individuals with aspirin-intolerant asthma show a diminished ability to generate these protective local mediators. It follows that the decreased capacity to form endogenous mediators of anti-inflammation or resolution could facilitate an influx of leukocytes to perpetuate the local inflammatory response and expose bronchial smooth muscle to relatively unopposed actions of bronchoconstricting substances such as cysteinyl leukotrienes [6]. The mechanisms that underlie this diminished lipoxin production were not examined in this initial study and remain the subject for further investigation. Nevertheless, the present results suggest that testing for 15-epimer-lipoxin A_4 generation by asthmatics, as documented here, may serve as a convenient means to distinguish between aspirin-tolerant asthmatics and aspirin-intolerant asthmatics and warrants further consideration.

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