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


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 LXA4, 15-epi-LXA4, and LTC4 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 LXA4 and 15-epi-LXA4 were generated in whole blood activated by the divalent cation ionophore, A23187. Higher levels of LXA4 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 LXA4. Generation of a bronchoconstrictor, LTC4, was similar in both AIA and ATA. Consequently, the ratio of LXA4:LTC4 quantitatively favoured the bronchoconstrictor for AIA and differed from both ATA and healthy subjects. In addition, the capacity for 15-epi-LXA4 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-tolerant asthmatics display a lower biosynthetic capacity than aspirin-intolerant 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.


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 LTC4 and LTD4 [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 LTC4 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 lipooxygenase (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]. LXA4 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 LXA4 by human asthmatics blocks LTC4 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-activated" 15-epi-LX share LX bioactions, are potent counter-regulatory lipid mediators that block LT-mediated inflammatory responses in vivo and can compete for LTD4 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 LXA4, 15-epi-LXA4 and LTC4 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 LXA4 and 15-epi-LXA4 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.

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 (Krako Âw, Poland). Venepuncture from volunteer subjects who had given written informed consent to a protocol approved by the Jagellonian University Ethical Committee (Krako Âw, Poland). 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 LXA4, 15-epi-LXA4 and LTC4 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 LXA4 and 15-epi-LXA4 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.

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 (Krako Âw, Poland). 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).

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 LXA4 in amounts that were not statistically different from that generated by healthy volunteers ((mean±SEM) 5.7±2.0 vs. 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 LXA4 (12.9±5.4 ng mL\(^{-1}\) than from AIA (5.7±2.0 ng mL\(^{-1}\)). The addition of exogenous substrate was not required for either LXA4 or 15-epi-LXA4 formation in activated whole blood, indicating that both ATA and AIA utilized endogenous sources of arachidonate to generate these counterregulatory eicosanoids.

Table 1. – Characteristics of subjects

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

Values are the mean±sd. NA: not applicable; NM: not measured; FEV1: forced expiratory volume in one second; IgE: immunoglobulin E. *: geometric mean±sd.
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 LXA4 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 LXA4 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) of 1.4 ± 0.2 versus 1.4 ± 0.2 ng mL\(^{-1}\)). Moreover, the relationship between LX and LT production, and together, their sum (4.5 ng mL\(^{-1}\)) were increased in aspirin-treated samples from AIA, less than in ATA (mean 11.1 ng mL\(^{-1}\)).

Response in ATA, neither 15-epi-LXA4 nor LXA4 formation was present (fig. 1a).

In addition to LXA4, activated whole blood also generated 15-epi-LXA4 (fig. 2). When activated in the presence of aspirin, values for 15-epi-LXA4 increased ~80% in ATA whole blood (1.4 to 2.6 ng mL\(^{-1}\)). In contrast to the response in ATA, neither 15-epi-LXA4 nor LXA4 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 LXA4 and 15-epi-LXA4 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-LXA4, 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 LXA4 and LXB4 were present in each study group. Native LXA4 and its all-trans isomer (11-trans-LXA4) eluted at 15.0 and 12.7 min (consistent with authentic synthetic materials), respectively, and were further identified on the basis of their molecular ions ([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 LXA4 were identified at m/z 333 ([M-H]-H\(_2\)O), m/z 315 ([M-H]-2H\(_2\)O), m/z 307 ([M-H]-CO\(_2\)), m/z 289 ([M-H]-H\(_2\)O, -CO\(_2\)), m/z 251 ([M-H]-CHO (CH\(_2\)_2CH\(_3\)), m/z 233 ([M-H]-H\(_2\)O, -CHO (CH\(_2\)_2CH\(_3\)), m/z 135 (CH\(_3\)CH(CH=CH\(_3\))CO\(_2\))). Native LXB4 and its all-trans isomer (8-trans-LXB4) 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\(_2\)O), m/z 315 ([M-H]=2H\(_2\)O), m/z 307 ([M-H]-CO\(_2\)), m/z 289 ([M-H]=H\(_2\)O, -CO\(_2\)), m/z 251 ([M-H]-CHO (CH\(_2\)_2CH\(_3\))), m/z 233 ([M-H]=H\(_2\)O, -CHO (CH\(_2\)_2CH\(_3\))), and m/z 221 ([M-H]=CHO (CH\(_2\)_2CH\(_3\))) LC/MS/MS analysis revealed a ratio for LXA4: LXB4 generation of ~1.2:1. (Note: LXB4 and its aspirin-triggered epimeric form, 15-epi-LXB4 levels were not monitored in individual samples because the authors have not yet prepared immunosassays for these eicosanoids.)

In addition to both LXA4 and LXB4, 15S-HETE (stereochimistry at the carbon 15 alcohol was determined by chiral HPLC analysis) was also present in samples from patients with AIA. Patient-derived 15S-HETE was eluted at 37.9 min (consistent with authentic 15S-HETE in this LC/
MS/MS system) and was identified by its molecular ion ([M-H]=m/z 319) and MS/MS ions present at m/z 301 ([M-H]-H2O), m/z 275 ([M-H]-CO2), m/z 257 ([M-H]-H2O, -CO2), and m/z 219 ([M-H]-CHO(CH2)3CH3). 15S-HETE is predominantly a 15-LO derived eicosanoid that can serve as a biosynthetic intermediate for LX. Formation of its epimer, 15R-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 15S-HETE present in materials pooled from AIA, levels of 15R-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-lypoxigenase product, 15S-HETE, but not the aspirin-acetylated COX-2 product, 15R-HETE. This difference is of interest because 15-epi-LX was generated while its precursor 15R-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 LXA4:LTC4 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. LXA4) 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 AIA and ATA 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 LX [14]. Eosinophilic asthma is characterized by mucosal eosinophilia, airway hyperreactivity, and increased numbers of eosinophils in bronchoalveolar lavage fluid [14]. The presence of eosinophils in asthmatic airways is associated with increased levels of eosinophilic mediators, such as COX-2 and LTB4, which can induce smooth muscle contraction and increase airway hyperreactivity [14]. The results shown in figure 2 demonstrate that AIA peripheral blood has diminished biosynthetic capacity for 15-epi-LX, a 5-LO
product of 15R-HETE. Low levels of 15-epi-LXA4 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-LXA4 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 acetaminophen by aspirin (for 15R-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.

LXA4 and 15-epi-LXA4 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, LXA4 also interacts in the airway with functionally characterized Cys-LT1 receptors (LTD4 receptor) to block airway smooth muscle contraction [29]. LXA4 also competes with LTD4 specific binding to endothelial cells in culture [21] and inhibits LTC4-mediated airway hyperresponsiveness in asthmatics [13]. LXA4 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 responses in asthmatics, as documented here, may serve as a convenient means to distinguish between aspirin-tolerant asthmatics and aspirin-intolerant asthma (ATA) generated increased levels of 15-epi-LXA4 from ATA generated increased levels of 15-epi-LXA4 in response to bronchial provocation with allergen, aspirin, leukotriene D4, and histamine in asthmatics. Am Rev Respir Dis 1992; 146: 96–103.


