

Airway Lipoxin A₄ Generation and Lipoxin A₄ Receptor Expression Are Decreased in Severe Asthma

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Rationale: Airway inflammation is common in severe asthma despite antiinflammatory therapy with corticosteroids. Lipoxin A₄ (LXA₄) is an arachidonic acid–derived mediator that serves as an agonist for resolution of inflammation.

Objectives: Airway levels of LXA₄, as well as the expression of lipoxin biosynthetic genes and receptors, in severe asthma.

Methods: Samples of bronchoalveolar lavage fluid were obtained from subjects with asthma and levels of LXA₄ and related eicosanoids were measured. Expression of lipoxin biosynthetic genes was determined in whole blood, bronchoalveolar lavage cells, and endobronchial biopsies by quantitative polymerase chain reaction, and leukocyte LXA₄ receptors were monitored by flow cytometry.

Measurements and Main Results: Individuals with severe asthma had significantly less LXA₄ in bronchoalveolar lavage fluids (11.2 ± 2.1 pg/ml) than did subjects with nonsevere asthma (150.1 ± 38.5 pg/ml; *P* < 0.05). In contrast, levels of cysteinyl leukotrienes were increased in both asthma cohorts compared with healthy individuals. In severe asthma, 15-lipoxygenase-1 mean expression was decreased fivefold in bronchoalveolar lavage cells. In contrast, 15-lipoxygenase-1 was increased threefold in endobronchial biopsies, but expression of both 5-lipoxygenase and 15-lipoxygenase-2 in these samples was decreased. Cyclooxygenase-2 expression was decreased in all anatomic compartments sampled in severe asthma. Moreover, LXA₄ receptor gene and protein expression were significantly decreased in severe asthma peripheral blood granulocytes.

Conclusions: Mechanisms underlying pathological airway responses in severe asthma include lipoxin underproduction with decreased expression of lipoxin biosynthetic enzymes and receptors. Together, these results indicate that severe asthma is characterized, in part, by defective lipoxin counterregulatory signaling circuits.

Keywords: severe asthma; lipoxins; eicosanoids

Chronic airway inflammation is common in asthma and linked to disease activity (1). Corticosteroids are the most frequent

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Lipoxins are potent antiinflammatory and proresolving mediators that are generated during inflammatory responses to promote catabasis. Lipoxin A₄ (LXA₄) biosynthetic capacity is decreased in whole blood in severe asthma.

What This Study Adds to the Field

In severe asthma, airway LXA₄ levels and expression of lipoxin biosynthetic enzymes and receptors were markedly decreased. Thus, severe asthma is characterized in part by defective lipoxin counterregulatory signaling circuits.

antiinflammatory agents used to treat asthma, yet some patients are resistant to the actions of corticosteroids, leading to more severe symptoms and adverse disease outcomes (2). Severe asthma is characterized by persistent airway inflammation that is heterogeneous (3), but often differs from mild and moderate asthma by increases in the number of neutrophils (PMNs) (4). The European Network for Understanding Mechanisms of Severe Asthma (ENFUMOSA) has confirmed that severe asthma is characterized by PMN-predominant inflammation (5). The Severe Asthma Research Program (SARP) of the National Heart, Lung, and Blood Institute (Bethesda, MD) proposed a functional definition for severe asthma and reported that a reduced FEV₁, history of pneumonia, and fewer positive skin tests were the strongest independent risk factors for severe asthma (6). Together, these findings suggest that severe asthma has a distinct pathobiology that is not merely an extension of the processes responsible for mild to moderate asthma.

Arachidonic acid (AA) metabolism generates several classes of eicosanoids that serve as bioactive mediators for the regulation of airway tone and inflammation (7). Cysteinyl leukotrienes (CysLTs) are 5-lipoxygenase (5-LO)–derived eicosanoids that carry the most potent bronchoconstrictive activity identified to date (8) and contribute to both early- and late-phase responses to inhaled allergen challenge (9). Leukotriene B₄ (LTB₄) acts as a chemoattractant, proadhesive agent, and secretagogue for PMNs (10), eosinophils (11), and select populations of T lymphocytes (12, 13). Lipoxins (LXs) are eicosanoids that are distinct in structure and function from CysLTs and LTB₄. In many model settings, including experimental asthma (14), LXs serve as

antiinflammatory lipid mediators that orchestrate the resolution of acute inflammation (15), and nebulized LXA₄ protects from LTC₄-mediated bronchoconstriction in asthma (16). A major route for LX biosynthesis at mucosal surfaces (i.e., airways) is via interactions between leukocyte 5-LO and epithelial cell 15-lipoxygenase (15-LO) (15). There are two distinct 15-LO isoforms: 15-LO-1 (GenBank, M23892.1) and 15-LO-2 (GenBank, U78294.1). 15-LO-2 converts AA exclusively to 15(*S*)-hydroxyeicosatetraenoic acid (15-HETE), in contrast to 15-LO-1, which oxygenates AA mainly at carbon-15 but also at carbon-12 (17). 15-LO-1 is increased in asthma in airway epithelial cells and eosinophils (18, 19), and is also present in mast cells in bronchial tissues (20). In addition to 5-LO and 15-LOs, cyclooxygenase-2 (COX-2) is also pivotal to resolution of airway injury and inflammation, in part via prostaglandin-mediated induction of 15-LO-1 and LX formation (21, 22).

LXA₄ acts at antiinflammatory receptors named ALX that are expressed on both leukocytes (23) and airway epithelial cells (21). Subjects with severe asthma have decreased peripheral blood LXA₄ levels and 15-LO-1 expression (24). However, levels of LXs in peripheral whole blood or circulating leukocytes may not reflect the biochemical environment of the respiratory tract, so it was important to determine whether dysregulated LX biosynthesis was also present in the severe asthmatic airway. In view of the protective actions of LXs, the possibility of defective LX counterregulatory signaling in the airways of subjects with severe asthma would have potentially important pathophysiological implications.

Here, we show marked decrements in LX levels in bronchoalveolar lavage fluid (BALF) obtained from subjects with severe asthma recruited and carefully phenotyped by the SARP, and that LX biosynthetic and receptor gene expression differs by both anatomic compartment and disease severity to contribute to decreased LX signaling circuits in severe asthma. Some of the results of these studies have been previously reported in the form of an abstract (25).

METHODS

Participants

Asthma severity was determined on the basis of criteria developed and used by the SARP (*see* Moore and coworkers [6] and Levy and coworkers [24]). The protocol was approved by the Partners Healthcare institutional review board, and written informed consent was obtained from all subjects. All subjects with asthma had chronic persistent asthma and none of the patients undergoing bronchoscopy were experiencing an asthma exacerbation. Healthy individuals were defined as subjects who had no clinical symptoms of asthma and whose lung function did not decrease with methacholine.

Sample Collection

Peripheral venous blood (15 ml) was collected by venipuncture. Samples were drawn into three 5-ml tubes containing acid-citrate-dextrose and processed immediately. During bronchoscopy on willing participants, endobronchial lung biopsies (EBBs) were collected first, followed by BALFs. EBBs were obtained with Olympus biopsy forceps in the lower lobes of the lung (as in Lilly and coworkers [26]) and placed in vials with 1 ml of RNAlater (Ambion, Austin, TX) (4°C) while being protected from ambient light. Samples were then stored at -20°C before analysis.

For BALs, saline (0.9%) was warmed (37°C) and three 50-ml aliquots were introduced into an upper lobe segment. BALF samples were immediately placed on ice and 1 volume of iced methanol was added (1:1, vol/vol). Prostaglandin B₂ (PGB₂) was added to each sample as an internal control. Samples were stored at -80°C before lipid mediator extraction and analysis.

In patients in whom BAL cells gene expression was determined, one 5-ml aliquot of BALF was centrifuged (2,000 rpm, 5 min, 4°C) and the

cell pellet was resuspended in 1 ml of TRIzol (Invitrogen, Carlsbad, CA). After homogenization, samples were stored at -80°C before analysis.

Extraction and Measurement of Lipid Mediators

Eicosanoids from BAL samples were extracted with C₁₈ Sep-Pak cartridges (Waters, Milford, MA) (as in Levy and coworkers [24]). Materials in the methyl formate eluate (i.e., LX and hydroxyeicosatetraenoic acids [HETEs]) and materials in the methanol eluate (i.e., cysteinyl leukotrienes [CysLTs]: LTC₄, LTD₄, and LTE₄) were brought to dryness under a gentle stream of N₂ and each was resuspended in 1 ml of methanol and kept at -80°C until eicosanoids were measured. To estimate losses during lipid extraction, PGB₂ levels were measured by HPLC. Ten percent of the methyl formate fraction was applied to an HPLC (Agilent 1100 series; Agilent Technologies, Palo Alto, CA) equipped with an Ultrasphere C₁₈ column (250 × 4.6 mm, 5 μm; Phenomenex, Torrance, CA) and coupled to a photodiode array detector (ultraviolet and visible range).

The mobile phase was methanol-distilled H₂O-glacial acetic acid (70:30:1, vol/vol/vol) as phase 1 (t₀ to 30 min) and a linear gradient with methanol (100%) as phase 2 (30 to 65 min) at a flow rate of 0.5 ml/minute (t₀ to 30 min), changing to 1 ml/minute (30 to 65 min).

The criteria used for identification of eicosanoids were retention time and ultraviolet spectra. Before ELISA, samples stored in methanol were brought to dryness under a gentle stream of N₂ and then resuspended with ELISA buffer before analysis. LXA₄ and 15-HETE present in the methyl formate fraction and CysLTs present in the methanol fraction were quantitated by ELISAs (Neogen [Lansing, MI] and Cayman Chemical [Ann Arbor, MI]). The measurements were corrected by PGB₂ recovery to account for losses during extraction.

Real-Time Polymerase Chain Reaction

To minimize variation in sample handling and RNA preservation, measurements of gene expression were all performed on materials obtained only at the Boston SARP site. Samples of whole blood from individuals with severe (n = 24) or nonsevere (n = 10) asthma and from healthy subjects (n = 7) were added to buffer EL erythrocyte lysis buffer (Qiagen, Valencia, CA) and an RNeasy mini kit (Qiagen) was used for RNA extraction as per the manufacturer's instructions. Because the principal aim of the Boston SARP investigators was to determine whether differences in eicosanoid metabolism were present in severe versus nonsevere asthma, healthy individuals were not subjected to the risks of bronchoscopy for assessment of eicosanoid-related gene expression. Total RNA in BAL cells from individuals with severe (n = 6) and nonsevere (n = 6) asthma was extracted with TRIzol reagent according to the manufacturer's instructions. EBBs from subjects with severe (n = 14) or nonsevere (n = 17) asthma were homogenized in TRIzol, using 19-, 21-, 23-, and 25-gauge needles sequentially before RNA extraction. DNase treatment was performed with RNase-free DNase (Qiagen) and RNA concentration was determined by ultraviolet absorbance. First-strand cDNA was generated from 1 μg of total RNA with TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). Quantitative real-time PCR (qPCR) was performed to determine gene expression for selected eicosanoid biosynthetic genes. GenBank accession numbers for 5-LO, 15-LO-1, 15-LO-2, COX-2, and ALX are J03600.1, M23892.1, U78294.1, M90100.1, and M84562.1, respectively. qPCR was performed with a Stratagene MX 3000P sequence detection system (Stratagene, La Jolla, CA), using fluorescent TaqMan methodology (Applied Biosystems) as described previously (27). Briefly, cyclophilin A was used as the control gene and quantitation was done by calculating the cycle threshold (C_t). The C_t (mean [SEM]) for cyclophilin A in EBBs, BAL cells, and blood was 25.01 [SEM, 0.52], 25.40 [SEM, 0.97], and 22.40 [SEM, 0.20], respectively. The difference between the C_t value for the gene of interest and the respective C_t value for cyclophilin A was then calculated (ΔC_t). Using the healthy or nonsevere asthma group as calibrator, the fold change for severe asthma was calculated as 2^{-ΔΔC_t}.

Flow Cytometry

Leukocytes were isolated as previously described (24). Briefly, cells were resuspended at about 5 × 10⁶/ml and then incubated with human IgG (Sigma-Aldrich, St. Louis, MO) for 20 minutes at 4°C to block Fc receptors. The cells were then incubated with anti-ALX antibody

(Genovac, Freiburg, Germany) followed by fluorescein isothiocyanate-conjugated anti-mouse secondary antibody (R&D Systems, Minneapolis, MN). To distinguish PMNs from eosinophils, cells were incubated with an α_4 integrin antibody (eBioscience, San Diego, CA) (28). Samples were run on a BD FACSCalibur flow cytometer and data were analyzed with CellQuest software (BD Biosciences, San Jose, CA).

Statistical Analysis

Samples were deidentified before all analyses. Values for lipid mediator levels and gene expression (ΔC_t) were analyzed by one-way analysis of variance for multiple comparisons between three or more groups and by Student *t* test for comparison between two groups. Wilcoxon rank sum tests were used to compare skewed continuous data and patient groups of disparate size. Fisher's exact tests were used to analyze categorical data. Data are presented as the mean and SEM; *P* less than 0.05 was considered significant.

RESULTS

Subject Characteristics

Subjects with severe or mild to moderate ("nonsevere") asthma, as defined by SARP criteria (6), were recruited from all eight SARP participating centers to undergo bronchoscopy. Table 1 reports the clinical profile of the volunteer subjects included in the BAL analysis.

Clinical data indicated that the severe asthma cohort was significantly older and had a higher body mass index compared with those with nonsevere asthma and healthy individuals. Almost half (47%) of subjects with nonsevere asthma and 100% of subjects with severe asthma were taking inhaled corticosteroids and 50% of subjects with severe asthma were also taking oral corticosteroids; 8% of subjects with nonsevere asthma and 14% of subjects with severe asthma were aspirin intolerant by self-report. Asthma Quality of Life Questionnaire, FEV₁, and FVC were all significantly lower in the subjects with severe asthma compared with both nonsevere asthma and healthy individuals (Table 1). Of interest, there were no significant differences in bronchodilator reversibility or provocative concentration of methacholine causing a 20% fall in FEV₁ in patients who underwent these procedures among the asthma cohorts. As

shown in Table 2, there were no significant differences in BAL total cell counts or leukocyte differentials among the cohorts.

Airway Lipoxin Levels Are Lower in Severe Asthma

To determine LO-derived eicosanoid levels in the airways of individuals with severe asthma, lipid mediators were extracted from BALF samples and specific eicosanoids were measured by sensitive ELISAs (see METHODS). LXA₄ levels were significantly increased in nonsevere asthma (150.1 [SEM, 38.1] pg of LXA₄/ml BALF) compared with healthy individuals (11.8 [SEM, 1.4] pg of LXA₄/ml BALF; *P* < 0.05; Figure 1A). Individuals with severe asthma had significantly less LXA₄ (11.2 [SEM, 2.1] pg of LXA₄/ml BALF) than subjects with nonsevere asthma (*P* < 0.05; Figure 1A). Relative to healthy individuals, there was a trend toward increased 15-HETE levels in both nonsevere asthma (804.8 [SEM, 206.7] pg of 15-HETE/ml BALF) and severe asthma (891.9 [SEM, 415.4] pg of 15-HETE/ml BALF), but no significant differences were found (Figure 1B). Levels of the bronchoconstrictive CysLTs were significantly increased in those with both nonsevere asthma (6.9 [SEM, 0.7] pg of CysLTs/ml BALF) and severe asthma (5.7 [SEM, 0.4] pg of CysLTs/ml BALF) compared with subjects without asthma (3.6 [SEM, 0.4] pg of CysLTs/ml BALF); *P* < 0.01; Figure 2A). In addition, a ratio of LXA₄ to CysLTs was determined to reflect each subject's relative metabolism of arachidonic acid to protective (LXA₄) versus provocative (CysLTs) mediators. Similar to LXA₄ alone (Figure 1A), significant differences in the LXA₄/CysLT ratio were also present in severe compared with nonsevere asthma (1.7 [SEM, 0.2] vs. 16.4 [SEM, 4.0], respectively; *P* < 0.05; Figure 2B). The ratio of 15-HETE to CysLTs was calculated, but no significant difference between severe and nonsevere asthma was present (164.1 [SEM, 80] vs. 155.4 [SEM, 43.5]). No significant relationships were detected for LXA₄ or CysLT levels and lung function (data not shown). Because the severe asthma cohort was characterized by a higher prevalence of both inhaled and oral corticosteroid use, the relationship between corticosteroid treatment and arachidonic acid metabolites was next determined. There was a trend toward lower levels of LXA₄ in subjects with nonsevere asthma taking inhaled

TABLE 1. CLINICAL PROFILE OF SUBJECTS UNDERGOING BRONCHOALVEOLAR LAVAGE

	Healthy Subjects	Subjects with Nonsevere Asthma	Subjects with Severe Asthma
No. of subjects	17	38	14
Clinical data			
Age, yr	30 ± 8 (22–52)	30 ± 10 (19–48)	41 ± 13*† (18–58)
Female, %	47	66	71
Race, % white	82	71	57
Ethnicity, % Hispanic	6	5	0
BMI	27 ± 7 (19–48)	27 ± 7 (20–53)	30 ± 5* (20–38)
Inhaled corticosteroids	0	18*	14*
Oral corticosteroids	0	0	7*†
Aspirin intolerant	0	3	2
Lung function			
Asthma duration, yr	N/A	14 ± 10 (1–41)	18 ± 13 (2–50)
AQLQ	6.99 ± 0.03 (6.9–7.0)	5.23 ± 0.99* (2.7–6.9)	3.56 ± 1.56*† (1.5–6.3)
IgE	89 ± 176 (0–688)	260 ± 404* (17–2,290)	294 ± 454* (9–1,650)
FEV ₁ , L	4.12 ± 0.82 (2.7–6.0)	3.37 ± 0.98* (1.7–6.0)	2.42 ± 0.80*† (1.4–4.5)
FEV ₁ , % pred	103 ± 14 (75–123)	92 ± 15* (64–118)	76 ± 17*† (54–98)
FVC, L	4.97 ± 0.95 (3.2–6.7)	4.44 ± 1.20 (2.33–7.99)	3.48 ± 0.88*† (1.9–4.9)
FVC, % pred	103 ± 11 (77–119)	102 ± 14 (74–137)	91 ± 17*† (68–119)
Bronchodilator	2.8 ± 2.2 (0.0–6.3)	8.1 ± 5.6* (1.0–20.8)	12.5 ± 14.2* (0.0–57.1)
Mch PC ₂₀ , mg/ml [‡]	≥50 (50, 50)	1.83* (2.1, 8.6)	2.34* (–0.9, 19.4)

Definition of abbreviations: AQLQ = Asthma Quality of Life Questionnaire; BMI = body mass index; Mch PC₂₀ = provocative concentration of methacholine causing a 20% fall in FEV₁.

Results are expressed as means ± SD (range).

* *P* ≤ 0.05 when compared with healthy individuals.

† *P* ≤ 0.05 when compared with subjects with nonsevere asthma.

‡ Values represent geometric mean (95% confidence interval); *P* calculated for log values.

TABLE 2. BRONCHOALVEOLAR LAVAGE CELL COUNTS AND LEUKOCYTE DIFFERENTIALS

	Healthy Subjects (n = 17)	Subjects with Nonsevere Asthma (n = 38)	Subjects with Severe Asthma (n = 14)
Total cell count, millions/ml	12.3 ± 8.5 (1.8–26.8)	12.0 ± 7.2 (0.3–38.4)	10.9 ± 6.4 (2.8–23.0)
Macrophages, %	91.1 ± 4.8 (81.0–97.7)	86.8 ± 10.1 (56.5–98.0)	91.1 ± 6.0 (80.3–98.2)
Lymphocytes, %	7.3 ± 5.1 (2.0–19.0)	9.2 ± 7.5 (1.0–36.0)	6.3 ± 3.8 (0.7–13.5)
Neutrophils, %	1.3 ± 1.5 (0.0–5.0)	3.1 ± 6.0 (0.0–27.2)	1.9 ± 2.9 (0.0–10.5)
Eosinophils, %	0.4 ± 0.6 (0.0–2.1)	0.9 ± 1.9 (0.0–11.4)	0.7 ± 1.1 (0.0–3.1)

Total cell counts and leukocyte differentials were determined in bronchoalveolar lavage samples from individuals with severe asthma and nonsevere asthma and healthy subjects from all participating Severe Asthma Research Program centers.

corticosteroids ($P = 0.17$) that was statistically significant when normalized with the ratio of LXA₄ to CysLTs ($P = 0.03$). These relationships in subjects with nonsevere asthma taking inhaled corticosteroids were not present for the severe asthma cohort. Moreover, no significant correlation was found for oral corticosteroids and BAL eicosanoid levels. Taken together, these results indicate that levels of LO-derived mediators in BALFs are regulated in asthma and differ by disease severity.

Lipoxin Biosynthetic Gene Expression Differs by Anatomic Compartment and Asthma Severity

To investigate mechanisms for low levels of LXs in severe asthma, we next examined the expression of genes involved in LX biosynthesis. To address the impact of anatomic compartments, gene expression was measured in peripheral blood, BAL cells, and EBBs. To minimize variation in sample handling for RNA preparation, materials for gene expression measurements were obtained only from the Boston site. The clinical profile for these volunteer subjects, including clinical data, lung function, and leukocyte counts (*see* Tables E1–E4 in the online supplement), was without significant difference from the SARP bronchoscopy cohort.

By quantitative PCR, the ΔC_t values for four pivotal LX biosynthetic genes were determined (*see* Table E5 in the online supplement; and *see* METHODS). 5-LO was the most abundant RNA (lowest ΔC_t) in all three compartments sampled, particularly in blood and BAL cells (Figure 3A). In contrast, the least abundant gene (highest ΔC_t) was 15-LO-1 in blood and BAL cells (Figure 3B) and 15-LO-2 in EBBs (Figure 3C). By asthma severity, the ΔC_t values for 5-LO, 15-LO-1, 15-LO-2, and COX-2 were all significantly increased in blood from subjects with severe asthma compared with subjects with nonsevere asthma (*see* Table E5), indicating decreased expression of all these regulatory genes in severe asthma whole blood (Figure 4, *top*). In all compartments sampled, 15-LO-2 and COX-2 expression was lower in subjects with severe asthma compared with sub-

jects with nonsevere asthma, with marked changes in particular in blood and EBBs (Figures 3C and 3D, and *see* Table E5). No significant differences in expression of either 5-LO or 15-LO-1 in EBBs were present between asthma cohorts. Relative to healthy individuals, 15-LO-2 expression in severe asthma blood was decreased and 5-LO expression in nonsevere asthma blood was increased (*see* Table E5). In severe asthma blood and BAL cells, the fold change in 15-LO-1 RNA was decreased by 14- and 5-fold, respectively (Figure 4, *top* and *middle*). In contrast, in EBBs, 15-LO-1 was increased approximately threefold in severe asthma, but expression levels of the other important LX biosynthetic enzyme (5-LO) and a second isoform of 15-LO (15-LO-2) were both decreased in these tissues (Figure 4, *bottom*). Together, these findings indicate that LX biosynthetic gene expression is uncoupled in severe asthma, with significant differences that vary by anatomic compartment and disease severity.

Because of the potential for corticosteroid treatment to regulate gene expression, the impact of corticosteroid dose on LX biosynthetic gene ΔC_t was next determined. In blood, a weak correlation was present for inhaled corticosteroid dose and decreased expression of 15-LO-1 ($r = 0.47$, $P = 0.01$) and 15-LO-2 ($r = 0.44$, $P = 0.01$), but not for 5-LO or COX-2 ($r = 0.2$ and $r = 0.35$, respectively; $P > 0.05$). No significant correlation was found between inhaled corticosteroid dose and any of the four genes in BAL cells or EBBs. In addition, no significant correlation was present for these genes and oral corticosteroid use. Total and differential leukocyte counts were also not significantly different in blood and BAL cells between the asthma cohorts (*see* Table E4). Of note, there was no significant correlation between subjects with self-reported aspirin-intolerant asthma and either BALF eicosanoid levels or gene expression.

Leukocyte ALX Receptors Are Decreased in Severe Asthma

Because LXs act at specific receptors to transduce their pro-resolving effects (14), we next examined gene expression of the antiinflammatory LXA₄ receptor (ALX) in peripheral blood

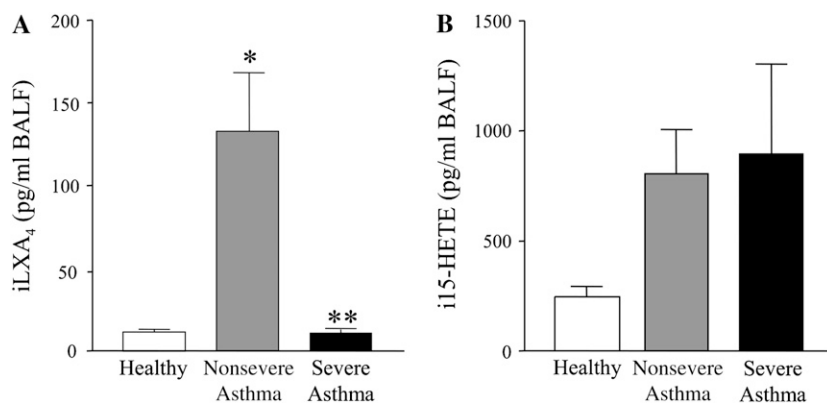


Figure 1. Airway lipoxin A₄ (LXA₄) and 15(S)-hydroxy-eicosatetraenoic acid (15-HETE) levels in severe asthma. Samples of bronchoalveolar lavage fluid (BALF) were obtained from healthy individuals and from subjects with asthma, the latter separated into severe asthma and mild to moderate (“nonsevere”) cohorts on the basis of criteria stated by the Severe Asthma Research Program of the National Heart, Lung and Blood Institute. After extraction, materials were analyzed by HPLC and ELISA (*see* METHODS) for (A) LXA₄ and (B) 15-HETE. Results are expressed as means ± SEM (healthy, n = 17; nonsevere, n = 38; severe, n = 14) for immunoreactive materials. * $P < 0.05$ in comparison with healthy subjects; ** $P < 0.05$ in comparison between severe and nonsevere asthma cohorts.

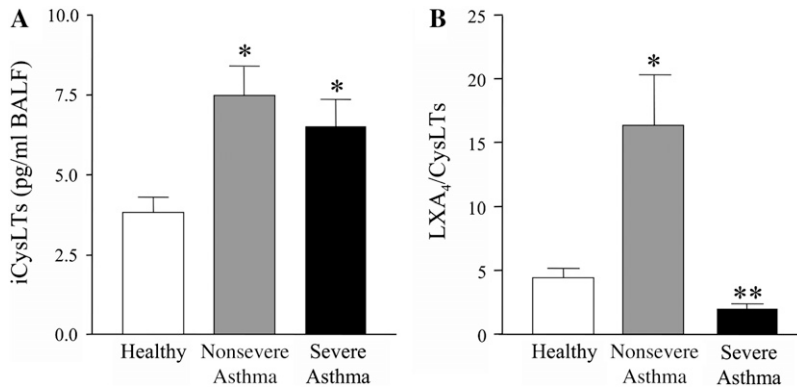


Figure 2. Airway generation of cysteinyl leukotrienes (CysLTs) relative to LXA₄ in severe asthma. (A) Levels of immunoreactive CysLTs in bronchoalveolar lavage fluid (BALF) from healthy individuals and subjects with asthma were determined (see METHODS). (B) To normalize comparisons of eicosanoid levels between cohorts, individual ratios of LXA₄ to CysLT levels in BALF were measured. Results are expressed as means \pm SEM (healthy, n = 17; nonsevere, n = 38; severe, n = 14). **P* < 0.05 for subjects with nonsevere and severe asthma compared with healthy individuals; ***P* < 0.05 for subjects with severe asthma compared with nonsevere asthma.

from subjects with severe and nonsevere asthma and healthy individuals. ALX gene expression was decreased by 56 and 78% in subjects with nonsevere and severe asthma, respectively, compared with healthy individuals (Figure 5A). ΔC_t values for subjects with severe asthma (4.6 [SEM, 0.3]) and subjects with nonsevere asthma (3.6 [SEM, 0.4]) were both significantly increased compared with healthy individuals (2.4 [SEM, 0.2]; *P* < 0.001). In addition, significant differences were also present for ALX ΔC_t values of severe compared with nonsevere asthma (*P* < 0.001).

To determine whether these changes in gene expression impacted ALX protein expression, we measured ALX cell surface expression by flow cytometric analysis of peripheral blood leukocytes (Figure 5B). PMN ALX expression was significantly decreased in both severe asthma (1.36 [SEM, 0.51], *P* < 0.01) and nonsevere asthma (1.01 [SEM, 0.35]; *P* < 0.01) compared with healthy individuals (3.8 [SEM, 0.77]) (Figure 5C). ALX expression was also decreased in eosinophils from subjects with both severe asthma (*P* < 0.01) and nonsevere asthma (*P* < 0.01) compared with healthy individuals. No significant differences were identified between asthma cohorts or in other leukocyte classes, and no correlation was observed for corticosteroids and ALX expression. Together, these findings indicate that ALX

receptor expression was downregulated in granulocytes in the peripheral blood of subjects with severe asthma.

DISCUSSION

The present findings are the first to demonstrate that individuals with severe asthma have diminished BALF LXs and to determine LX biosynthetic gene and receptor expression in asthma by quantitative PCR. In contrast to decrements in LXs, the 5-LO-derived CysLTs and 15-LO-derived 15-HETE levels in BALF were increased in individuals with asthma independent of severity. Decreased LXs without concomitant decreases in CysLTs in severe asthma would lead to an imbalance in bioactive lipid mediators that promoted the airway inflammation and airflow obstruction typical of severe asthma. Similar to whole blood (24), a decreased ratio of BALF LXA₄ to CysLTs sharply distinguished severe from nonsevere asthma. Decreased LX levels in severe asthma were related to dysregulated expression of LX biosynthetic genes. In addition to LXs, LXA₄ receptor expression was also decreased in granulocytes from asthmatic whole blood.

Lipoxins are eicosanoid mediators that promote resolution of cytokine-driven acute inflammation (22) and reduce airway

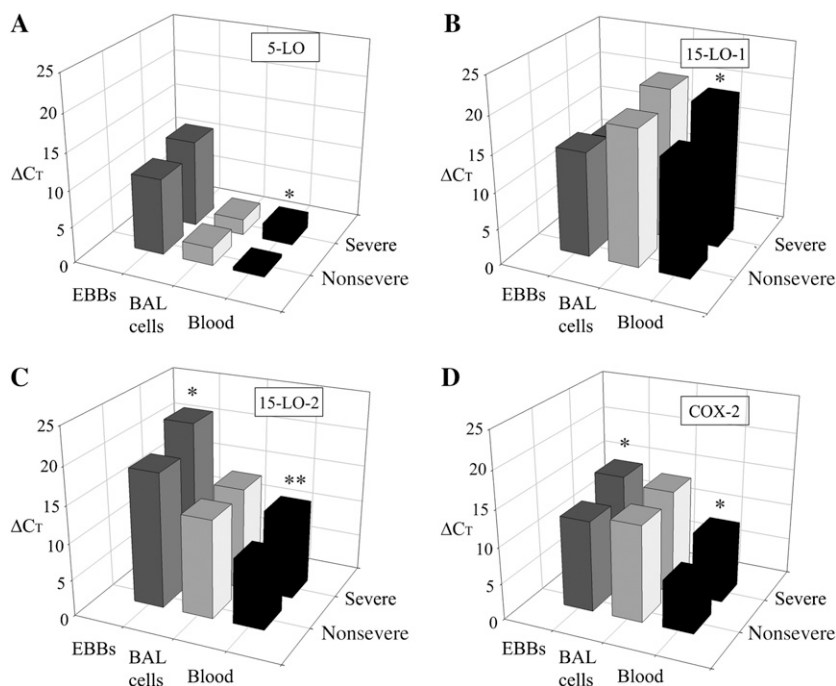


Figure 3. Lipoxin biosynthetic gene expression in blood, bronchoalveolar lavage (BAL) cells, and endobronchial lung biopsies (EBBs). Changes in cycle threshold (ΔC_t) values for (A) 5-lipoxygenase (5-LO), (B) 15-lipoxygenase (15-LO)-1, (C) 15-LO-2, and (D) cyclooxygenase (COX)-2 in subjects with severe and nonsevere asthma were obtained from quantitative polymerase chain reaction results, using cyclophilin A as the control gene (see METHODS). Results are expressed as means of ΔC_t values (nonsevere [n = 10], severe [n = 24] for blood; nonsevere [n = 6], severe [n = 6] for BAL cells; and nonsevere [n = 17], severe [n = 14] for EBBs). **P* < 0.05 for subjects with severe versus nonsevere asthma; ***P* < 0.01 for subjects with severe versus nonsevere asthma.

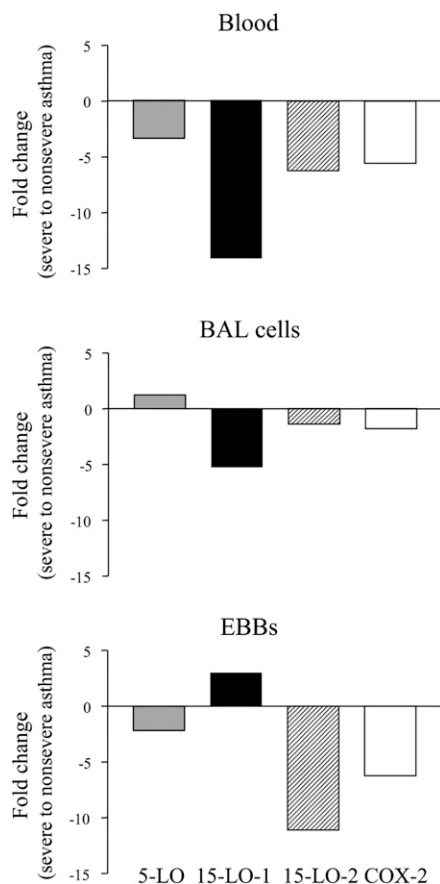


Figure 4. Relative lipoxin biosynthetic gene expression in peripheral blood, bronchoalveolar lavage (BAL) cells, and endobronchial lung biopsies (EBBs). 5-LO (shaded columns), 15-LO-1 (solid columns), 15-LO-2 (hatched columns), and COX-2 (open columns) gene expression in samples from individuals with severe asthma was analyzed by quantitative polymerase chain reaction, using fluorescence TaqMan technology (see METHODS), and compared with samples from individuals with nonsevere asthma; samples represented peripheral whole blood (top), BAL cells (middle), and EBBs (bottom). Results are expressed as means of fold change for severe asthma relative to nonsevere asthma (blood: healthy [n = 7], nonsevere [n = 10], severe [n = 24]; BAL cells: nonsevere [n = 6], severe [n = 6]; EBBs: nonsevere [n = 17], severe [n = 14]).

inflammation in experimental asthma (14). In allergic airway inflammation, LXA₄ blocks both airway hyperresponsiveness and pulmonary inflammation via ALX, leading to decreases in the numbers of eosinophils and T lymphocytes and lower levels of IL-5, IL-13, eotaxin, IgE, prostanoids, and CysLTs (14). Lipoxins are also formed *in vivo* and are associated with inflammatory events, as LXA₄ is present in BALF from patients with respiratory inflammation (29). Individuals with asthma possess the capacity to generate LXs, but activated whole blood from aspirin-intolerant subjects with asthma and subjects with severe asthma displays relative decrements in LXA₄ biosynthesis (24, 30, 31). In contrast to LXs, leukotriene levels (LTB₄ and CysLTs) are increased in activated whole blood from subjects with severe asthma compared with those with nonsevere asthma (24). In addition, the normalized values of LO-derived eicosanoids (i.e., the ratio of LXA₄ to CysLTs) in nonstimulated whole blood correlates with airflow obstruction. The significant decrements in LXA₄ in severe asthma BALF samples described here now extend to the respiratory tract the earlier findings of

dysregulated LX biosynthesis in severe asthma whole blood (24). Of interest, induced sputum samples from individuals with severe asthma also have lower concentrations of LXA₄ than do those from subjects with mild or moderate asthma (32), indicating similar changes in LX production in both upper and lower airways. Protectin D1 is a 15-LO-derived product of docosahexaenoic acid with counterregulatory properties that is also generated in asthma and serves as a potent inhibitor of both airway inflammation and hyperresponsiveness (33). Of interest, protectin D1 levels are lower in exhaled breath condensates from subjects with asthma during acute exacerbations (33), suggesting, together with the LX decrements in severe asthma, an overall decrease in the capacity to generate 15-LO-derived counterregulatory lipid mediators during uncontrolled asthma. Levels of 15-HETE and CysLTs were higher in subjects with asthma. Unlike these eicosanoids, LX biosynthesis requires the actions of more than one LO. 15-LO is a key enzyme capable both of initiating LX biosynthesis and converting 5-LO-derived LTA₄ to LXs (34). In animal models, increased 15-LO expression protects from atherosclerosis (35), renal injury (36), and periodontitis (37) and promotes wound healing (38) via increased *in vivo* formation of LXs. Of interest, a polymorphism in human 15-LO-1 has been identified that leads to increased 15-LO-1 mRNA levels and activity that are associated with a lower risk of atherosclerosis (39). 15-LO-2 expression is also increased in the airway mucosa of smokers with chronic bronchitis (40). As in peripheral blood (24), expression of 15-LO-1 mRNA was decreased in BAL cells in severe compared with nonsevere asthma.

The decrements in LX BALF levels in subjects with severe asthma were 15-fold less than in subjects with nonsevere asthma, but quantitative gene expression analysis of 15-LO-1 and 15-LO-2 combined in BAL cells revealed only a sixfold decrease, indicating that post-transcriptional factors in addition to changes in gene expression were also likely impacting LX formation in severe asthma. In this regard, changes in RNA expression may not fully reflect changes in 15-LO-1 activity (41), as its regulated expression can be controlled by a range of pretranslational, translational, and post-translational mechanisms (reviewed in Kuhn and coworkers [42]). The relatively poor correlations between 15-LO RNA or protein and its product are likely to be the result of a complex regulatory process involving multiple different cell types, levels of enzyme activation, and cellular uptake and metabolism of the product of 15-HETE (43). 15-HETE can be generated by either 15-LO-1 or 15-LO-2, but LX biosynthesis requires both 15-LO and 5-LO activity. Transcellular LX biosynthesis occurs most effectively during cell-cell interactions and decreases when direct cell contact is prevented (44). In contrast to 15-LO, levels of the 5-LO-derived CysLTs in BALF were without significant change in subjects with severe compared with nonsevere asthma, correlating with the 5-LO gene expression data. Because LTA₄ can serve as a biosynthetic intermediate for either CysLTs or LXs, there is a reciprocal relationship between CysLT and LX formation that can be influenced by several factors, such as redox state (45). Thus, the severe asthmatic lung microenvironment may display alterations in eicosanoid biosynthetic gene expression, cell-cell interactions, and redox state; any one of which could disrupt LX biosynthesis without also decreasing CysLT or HETE production.

Corticosteroid resistance defines severe asthma (6), and here subjects with severe asthma differed from the nonsevere cohort by exposure to larger amounts of systemic and inhaled corticosteroids. Relationships between corticosteroids and either 15-LO expression or LXA₄ were not identified in severe asthma. Of potential interest for nonsevere asthma, a significant corre-

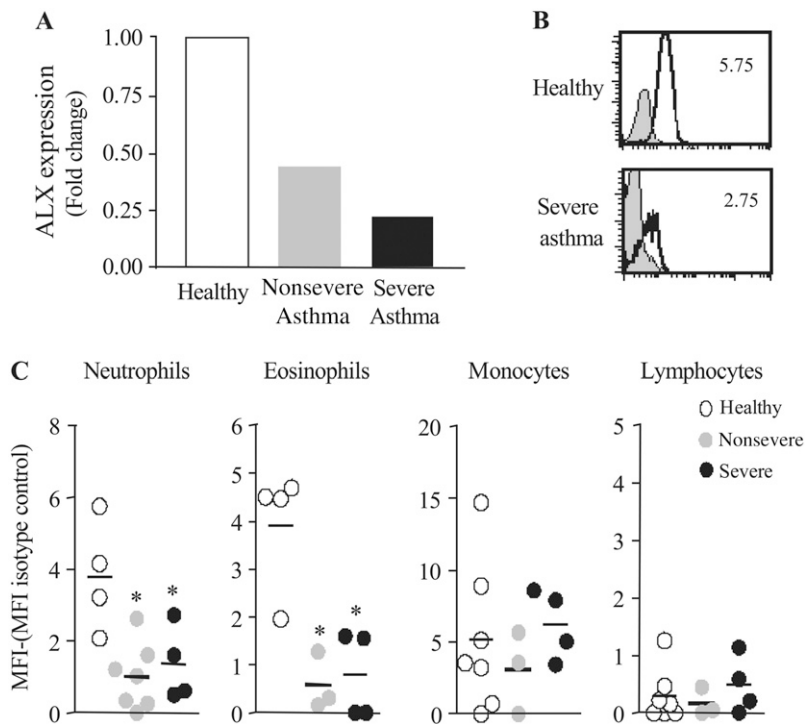


Figure 5. Expression of LXA₄ receptor (ALX) RNA and protein in peripheral blood leukocytes. (A) ALX RNA in whole blood from subjects with severe asthma, subjects with nonsevere asthma, and healthy individuals was analyzed by quantitative polymerase chain reaction (see METHODS). Results are expressed as the fold change in ALX expression ($2^{-\Delta\Delta C_t}$), using the healthy group as the calibrator arbitrarily set at 1 (healthy, n = 7; nonsevere, n = 10; severe, n = 24). (B) ALX surface expression on peripheral blood leukocytes was determined by flow cytometry (ALX antibody, 1:50 dilution) (see METHODS). Histograms show the isotype control (shaded) overlaid in boldface by ALX expression in neutrophils. Inset: Numbers indicate the median fluorescence intensity (MFI) minus the MFI of the isotype control. (C) Quantitation of ALX surface expression in leukocyte subsets (neutrophils, eosinophils, lymphocytes, and macrophages) in subjects with severe and nonsevere asthma and healthy individuals. Results are expressed as individual values. **P* < 0.05 for asthma subjects compared with healthy individuals.

lation was observed between inhaled corticosteroids and decreased 15-LO-1 and 15-LO-2 expression as well as decreased LXA₄ relative to CysLTs. Although not seen in the respiratory compartments sampled or with oral corticosteroids, the relationship in blood may be explained in part by corticosteroid-induced decreases in viable circulating leukocytes, in particular eosinophils and cytokine-primed monocytes that can express 15-LO (19, 46). Although significant differences in cell number were not evident in stained peripheral blood smears, this would not have been sensitive enough to detect functional changes in the leukocytes. Although little information is available about the direct regulation of 15-LO by corticosteroids, the induction of 15-LO-1 expression *in vitro* by IL-4 and IL-13 can be attenuated by steroids in orbital fibroblasts (47). COX-2 expression was downregulated in all samples from subjects with severe asthma. Because COX-2 expression is sensitive to glucocorticoids (48) and linked to 15-LO expression and LX formation (21, 22), an indirect effect of steroid treatment on LX pathways via repression of COX-2 cannot be excluded. Thus, it is possible that decreased LX levels in asthma relate in part to corticosteroid actions either on leukocyte viability or cellular 15-LO expression, but the increased BALF levels of the 15-LO-derived product 15-HETE in both asthma cohorts argue against an important effect for corticosteroids on airway 15-LO or LX generation.

COX-2-derived eicosanoids also regulate expression of LXA₄ receptors in airway epithelial cells (49). ALX receptors are G protein-coupled proteins that bind LXA₄ with high affinity (K_D , 1.7 nM) (50). ALX is expressed in leukocytes and structural cells of the lung, and IL-13 and IFN- γ can dramatically induce ALX expression *in vitro* in epithelial cells (51). Transgenic expression of human ALX coupled to a component of the CD11b promoter blocks both allergic airway inflammation and acute airway injury from acid aspiration (14, 21). Of note, the coordinate changes in LXA₄ levels and ALX expression in severe asthma were an example of positive feedback between ligand and receptor; a phenomenon previously observed with other eicosanoids, including LTB₄ and its recep-

tor BLT1 (52). The present findings are the first to uncover a defect in ALX expression in severe asthma.

In summary, our results indicate that severe asthma is characterized by decreased airway LXA₄ levels and leukocyte ALX receptor availability. These findings suggest that more severe variants of asthma may result from a defect in LX-mediated counterregulatory signaling. In experimental model systems, disruption of LX biosynthesis is resolution "toxic" for acute inflammation (21, 53). Thus, this LX defect in severe asthma would serve to perpetuate the chronic inflammation and airway hyperresponsiveness typical of this disease. Corticosteroids do not rescue this LX defect and may further decrease LX generation. LXA₄ analogs have been prepared that are potent regulators of airway inflammation and hyperresponsiveness (14, 54), providing potentially new therapeutic strategies for asthma control.

Conflict of Interest Statement: A.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. G.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. O.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.J.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.R.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.C.-E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.C.E. is a principal investigator of an industry-sponsored grant of bronchial thermoplasty for asthma from Alair/Asthmatx, but received no personal compensation for any part of the study. W.J.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. K.F.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. N.N.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. W.W.B. serves on advisory boards for Wyeth, Isis, CV Therapeutics, Pfizer, Amgen, Genentech, and Abbot. Speaker's fees were received from Novartis, Merck, AstraZeneca, and GlaxoSmithKline. Grants-in-aid were received from Novartis, Centocor, MedImmune, and GlaxoSmithKline. S.E.W. has consulted and served on advisory boards for Merck and received \$8,000 from Merck in 2007, \$10,000 from Merck in 2006, and \$10,000 in 2005. S.E.W. has also consulted and spoken for Critical

Therapeutics, receiving \$4,000 in 2007, \$12,000 in 2006, and \$10,000 in 2005. B.D.L. is a coinventor on a patent owned by Brigham and Women's Hospital that has been licensed to Bayer Healthcare for the use of lipoxin analogs in the treatment of airway diseases and asthma and has received \$10,000 in licensing fees.

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