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ORIGINAL ARTICLE

Lipoxin Generation Is Related to Soluble Epoxide Hydrolase Activity in Severe Asthma

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Abstract

Rationale: Severe asthma is characterized by airway inflammatory responses associated with aberrant metabolism of arachidonic acid. Lipoxins (LX) are arachidonate-derived pro-resolving mediators that are decreased in severe asthma, yet mechanisms for defective LX biosynthesis and a means to increase LXs in severe asthma remain to be established.

Objectives: To determine if oxidative stress and soluble epoxide hydrolase (sEH) activity are linked to decreased LX biosynthesis in severe asthma.

Methods: Aliquots of blood, sputum, and bronchoalveolar lavage fluid were obtained from asthma subjects for mediator determination. Select samples were exposed to *t*-butyl-hydroperoxide or sEH inhibitor (sEHI) before activation. Peripheral blood leukocyte–platelet aggregates were monitored by flow cytometry, and bronchial contraction was determined with cytokine-treated human lung sections.

Measurements and Main Results: 8-Isoprostane levels in sputum supernatants were inversely related to LXA_4 in severe asthma ($r = -0.55$; $P = 0.03$) and *t*-butyl-hydroperoxide decreased LXA_4 and 15-epi-LXA_4 biosynthesis by peripheral blood leukocytes. LXA_4 and 15-epi-LXA_4 levels were inversely related to sEH activity in sputum supernatants and sEHIs significantly increased 14,15-epoxy-eicosatrienoic acid and 15-epi-LXA_4 generation by severe asthma whole blood and bronchoalveolar lavage fluid cells. The abundance of peripheral blood leukocyte–platelet aggregates was related to asthma severity. In a concentration-dependent manner, LXs significantly inhibited platelet-activating factor–induced increases in leukocyte–platelet aggregates (70.8% inhibition [LXA_4 100 nM], 78.3% inhibition [15-epi-LXA_4 100 nM]) and 15-epi-LXA_4 markedly inhibited tumor necrosis factor-α–induced increases in bronchial contraction.

Conclusions: LX levels were decreased by oxidative stress and sEH activity. Inhibitors of sEH increased LXs that mediated antiphlogistic actions, suggesting a new therapeutic approach for severe asthma.
Clinical trial registered with www.clinicaltrials.gov (NCT 00595114).

Keywords: resolution; asthma; pro-resolving mediators; inflammation

Severe asthma (SA) is distinguished from milder variants by reduced corticosteroid responsiveness (1, 2) that derives in part from increased airway oxidative stress (3). No matter the cause of this heterogeneous condition (4), most patients with SA experience excess, chronic airway inflammation with defective resolution (5, 6).

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*A complete list of members may be found before the beginning of the References.

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At a Glance Commentary

Science Knowledge on the Subject: In severe asthma, the levels of lipoxins (LXs) are decreased by post-transcriptional mechanisms that have yet to be defined. Because LXs are pro-resolving mediators, defective LX generation may underlie the chronic inflammatory airway changes in severe asthma. Because LX formation occurs via transcellular biosynthesis during cell–cell interactions, these biosynthetic pathways are potentially vulnerable to oxidative stress.

What This Study Adds to the Field: In severe asthma, LX generation in the airway was inversely related to oxidative stress and soluble epoxide hydrolase activity. Highly selective inhibitors of soluble epoxide hydrolase increased 14,15-epoxy-eicosatrienoic acid and 15-epi-LXA_4 generation by severe asthma whole blood and bronchoalveolar lavage cells. 15-epi-LXA_4 decreased leukocyte–platelet aggregates and both 15-epi-LXA_4 and 14,15-epoxy-eicosatrienoic acid decreased bronchial contraction.

The resolution of inflammation is an active process governed by specialized pro-resolving mediators and cellular events (7). In model systems, pro-resolving mediators can potently decrease further recruitment of leukocytes to inflamed tissues and promote a return of the tissue to homeostasis (8). The genus of endogenous pro-resolving mediators includes lipoxins (LXs), which are enzymatically derived from arachidonic acid during cell-cell interactions in inflammation (9). The multistep enzymatic process of LX biosynthesis with transcellular exchange of biosynthetic intermediates may increase its vulnerability to corruption by nonenzymatic, oxidative attack on the arachidonate backbone in the presence of increased oxidative stress.

LXs are generated in asthmatic airways (10); however, LX generation in SA is defective (11–13). LXs can regulate allergic airways responses, including airway inflammation, mucus metaplasia, and hyperresponsiveness to methacholine (MCh) (14). At a cellular level, LXs inhibit eosinophil trafficking (14) and neutrophil chemotaxis, transendothelial and transepithelial migration, generation of superoxide anions, and degranulation of neutrophil azurophilic granules (7). In addition, inhaled LXA_4 blocks leukotriene (LT) C_4 (LTC_4)-mediated bronchoprovocation of subjects with asthma (15) and a LXA_4 stable analog markedly decreases infantile eczema (16). SA is a uniquely human disease and important species-specific differences are present between LX biosynthetic enzymes, emphasizing the importance of human translational research in this condition.

Here, we determine the relationship between LX levels and oxidative stress, identify a post-transcriptional mechanism to increase LX biosynthesis in SA, and uncover protective actions for LXs on two important prophlogistic features of SA, namely platelet-leukocyte interactions for lung leukocyte recruitment and proinflammatory cytokine induced *ex vivo* bronchial reactivity.

Methods

See the online supplement for detailed methods.

Participants

Aliquots of materials were collected from a random subset of individuals enrolled in the Macrolides In Asthma (MIA; NCT 00318708) trial or the cKit Inhibition in Severe Asthma (KIA; NCT 01097694) trial for this ancillary mechanistic study. Asthma severity was graded nonsevere asthma (NSA) or SA based on criteria developed by the NHLBI Severe Asthma Research Program (4). The Brigham and Women's Hospital human subjects institutional review board approved the study, and all subjects provided written consent in accordance with the Declaration of Helsinki.

Sample Collection

Peripheral venous blood was collected in heparinized tubes and used immediately. Induced sputum was prepared as in (13) with supernatants stored at −80°C for later analysis. Bronchoalveolar lavage fluids (BALF) were collected as in (17) with cell-free supernatants stored at −80°C as methanolic extracts (1:1, vol/vol BALF/methanol). All samples were collected from volunteer subjects before the initiation of study medication or placebo. Prostaglandin B_2 was added as an internal control.

Effects of *t*-Butyl-Hydroperoxide and Soluble Epoxide Hydrolase Inhibitors

Partially purified peripheral blood leukocytes were warmed (5 min, 37°C) and exposed (15 min, 37°C) to *t*-butyl-hydroperoxide (*t*-BuOOH; 2 mM) or vehicle before incubation with A23187 (50 μM) or vehicle (0.1% ethanol) for 15 minutes (37°C). Incubations were stopped with 5 vols of iced methanol and stored at −80°C. In separate experiments, peripheral venous blood (700 μl per incubation) was warmed (5 min, 37°C) and exposed (10 min, 37°C) to either a soluble epoxide hydrolase inhibitor (sEHI) (500 nM; 12-[3-adamantan-1yl-ureido] dodecanoic acid [AUDA], 1-[1-acetypiperidin-4-yl]-3-adamantanylurea, 1-trifluoromethoxyphenyl-3-[1-propionylpiperidin-4-yl] urea, and *trans*-4-{4-[3-(4-trifluoromethoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid [*t*-TUCB]; structures given in online supplement) or vehicle (0.1% dimethyl sulfoxide) before A23187 (50 μM, 15 min, 37°C). Incubations were stopped with 5 vols of iced methanol. BALF cells (1×10^5 cells per incubation) in phosphate-buffered saline were warmed (5 min, 37°C) and exposed (10 min, 37°C) to either a sEHI (500 nM) or vehicle before A23187 (5 μM, 15 min, 37°C). Incubations were stopped with 2 vols of iced methanol.

Extraction and Measurement of Lipid Mediators

Whole blood and BALF cell incubations were extracted as in (12, 17). Quantitation of LXA_4, 15-epi-LXA_4, LTB_4, 8-isoprostane (8-IP), 14,15-epoxy-eicosatrienoic acid (14,15-EET), and 14,15-dihydroxy-eicosatrienoic acid (14,15-DHET) was determined in parallel by ELISA (Neogen, Lansing, MI; Cayman Chemical, Ann Arbor, MI; Detroit R&D, Detroit, MI). LXA_4 levels were confirmed by liquid chromatography–mass spectrometry in select samples as in (18).

Flow Cytometry

Freshly obtained peripheral venous blood was obtained from subjects enrolling at the Boston site for the MIA or KIA trials or from healthy control (HC) subjects. Leukocyte-platelet aggregates (LPAs) were analyzed by flow cytometry as in (19). LPAs were identified as $CD16^+CD41^+$. To some aliquots of whole blood, samples were exposed to either LXA_4, 15-epi-LXA_4, 14,15-EET, 8-IP (Cayman Chemical), or

vehicle (0.1% ethanol) (5 min, 37°C) before activation with platelet-activating factor (PAF; 5 μM) or vehicle (0.1% ethanol) (15 min, 37°C). To stimulate endogenous LX generation, samples of whole blood were first exposed to granulocyte-macrophage colony–stimulating factor (200 pM, 90 min, 37°C) before sEHI or vehicle (10 min, 37°C) and followed by activation with PAF (15 min, 37°C). At least 20,000 $CD41^+$ cells were recorded for each sample on a FACS Canto II (BD Biosciences, San Jose, CA) and data were analyzed using FloJo software (Tree Star, Ashland, OR).

Human Lung Tissue Samples

The study was approved by the Université de Sherbrooke's Ethics Committee (protocol number 05-088-S1-R2). Human lung tissues were obtained from 10 patients undergoing resection and bronchi were dissected and cultured for 48 hours with 10 ng/ml tumor necrosis factor (TNF)-α (R&D Systems, Minneapolis, MN) in the presence of 100 nM 15-epi-LXA_4, 14,15-EET, or vehicle.

Isometric Tension Measurements

Mechanical tension measurements were performed using an isolated organ bath system (Radnoti Glass Tech., Monrovia, CA) as previously described (20). Briefly, bronchial rings were mounted in baths filled with 6 ml of Krebs solution, at pH 7.4 (37°C, 5% CO_2). A basal tension of 0.8 g was applied to each bronchi. Passive and active tensions were assessed using FT03 Grass transducer systems coupled to Polyview software (Grass-Astro-Med Inc., West Warwick, RI) to allow data acquisition and analysis.

Statistical Analysis

Samples were deidentified before analysis. Analysis was based on the Mann-Whitney test, the Kruskal-Wallis analysis of variance followed by the Mann-Whitney test with Bonferroni correction to compare the ranks of continuous variables across the levels of a categorical variable with more than two groups, the Spearman rank correlation test to compare the ranks of two continuous variables, and chi-square tests and Fisher exact test for comparisons of categorical variables. Data are presented as the mean ± SD or SEM where indicated. P less than 0.05 was regarded as statistically significant. Prism (GraphPad Software, La Jolla, CA) or Sigma Plot 12.0 (SPSS-Science, Chicago, IL) were used to manage and analyze the data.

Results

Subject Characteristics

As an ancillary study, samples were obtained from a random subset of subjects with asthma participating in either the MIA or KIA trials. Using criteria developed by the Severe Asthma Research Program (4), participants could be separated into cohorts of SA and NSA. The clinical profiles of the enrolled subjects with NSA and SA whose materials were studied here are provided in Table 1. Relative to NSA, subjects with SA used significantly more inhaled corticosteroids and long-acting bronchodilators with higher Asthma Control Questionnaire scores. Healthy subjects were also recruited as control subjects (HC) (*see* online supplement for detailed methods).

Lipid Mediators and 8-IP in NSA Plasma and Sputum

To determine if the abundance of lipoxygenase-derived (LXs and LTs) and nonenzymatically derived (IPs) eicosanoids differ by anatomic compartment, levels of representative members of these families (i.e., LXA_4, LTB_4, and 8-IP) were measured in samples of plasma and sputum from subjects with NSA (Figure 1). LXA_4, LTB_4, and 8-IP were detected in all samples (Figures 1A and 1B). Plasma levels of LXA_4 (mean ± SD, 463.3 ± 152.6 pg/ml) were significantly higher than LTB_4 (mean ± SD, 16.8 ± 18.8 pg/ml; $P < 0.05$) (Figure 1A), but not in sputum supernatants (Figure 1B). Substantial amounts of 8-IP were present in both plasma (mean ± SD, 254.0 ± 125.6 pg/ml) and sputum (mean ± SD, 292.4 ± 51.0 pg/ml) (Figures 1A and 1B). There was no significant relationship between levels of LXA_4, LTB_4, and 8-IP in plasma and sputum (Figure 1C), suggesting important anatomic differences in arachidonic acid availability and metabolism. The levels of these mediators were not significantly related to the clinical parameters listed in Table 1.

LXA_4 Generation Is Inversely Related to Oxidative Stress in the SA Airway

To determine if there was a relationship between LX generation and oxidative stress, levels of LXA_4 and 8-IP, a sensitive biomarker for oxidative stress, were measured in samples of plasma and sputum. Of interest, there was a significant positive correlation between LXA_4 and 8-IP in NSA plasma ($r = 0.68$; $P = 0.002$) (Figure 2A), suggesting that plasma levels of these compounds were related to arachidonic acid availability. Unlike plasma, there was a negative relationship and no significant correlation between LXA_4 and 8-IP levels in NSA sputum supernatants ($r = -0.40$; $P = 0.28$) (Figure 2B).

In sharp contrast with NSA, a significant inverse relationship between LXA_4 and 8-IP levels in SA sputum supernatants was present ($r = -0.55$; $P = 0.03$) (Figure 2C), indicative of an inverse relationship between LX generation and oxidative stress in the airway, in particular in SA. To this end, the mean levels of 8-IP were higher in SA sputum supernatants

Table 1. Subject Characteristics

	Mild Asthma	Severe Asthma
Number of subjects	24	19
Clinical data		
Age, yr	37 ± 10 (22–58)	40 ± 11 (20–52)
Sex, M/F	7/17	10/9
Race, % white	37.5	57.9
Ethnicity, % Hispanic	20.8	15.8
Inhaled steroids dose, μg	166 ± 72 (0–320)	922 ± 320* (427–1807)
LABA/Tio, %	0	100*
Lung function		
ACQ score	1.5 ± 0.65 (0.43–3.4)	2.6 ± 0.76* (2.0–4.7)
FEV_1, L	2.4 ± 0.68 (1.1–3.9)	2.4 ± 0.74 (1.3–4.1)
FEV_1, % predicted	77 ± 18 (38–106)	72 ± 19 (40–101)

Definition of abbreviations: ACQ = Asthma Control Questionnaire; LABA = long-acting β agonist. Results are expressed as mean ± SD (range).
*$P < 0.05$ when compared with subjects with mild asthma.

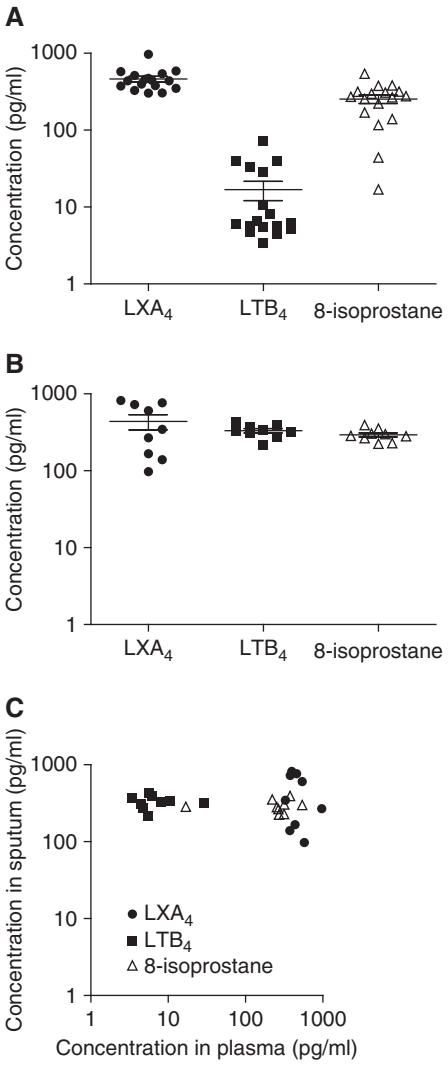

Figure 1. The relationship between lipoxin (LX) A_4, leukotriene (LT) B_4, and 8-isoprostane levels differs by anatomic compartment in asthma. Samples were obtained from a subset of subjects with nonsevere asthma enrolled in the Macrolides In Asthma trial. (*A*) Plasma (n = 17) and (*B*) sputum supernatants (n = 9) were extracted and levels of LXA_4, LTB_4, and 8-isoprostane were determined by ELISA (*see* METHODS). Results are expressed as individual values. The mean ± SEM are indicated by overlay in (*A*) plasma and (*B*) sputum. (*C*) The concentrations of LXA_4 (*circles*), LTB_4 (*squares*), and 8-isoprostane (*triangles*).

(mean ± SD, 421.8 ± 195.4 pg/ml) than NSA sputum supernatants (mean ± SD, 292.4 ± 51.0 pg/ml), but these differences did not reach statistical significance at this sample size (Figure 2D). To determine if LX biosynthesis was susceptible to oxidative stress, leukocytes from healthy subjects were partially purified from peripheral blood and activated with A23187 in the presence or absence of *t*-BuOOH. Both LXA_4 and 15-epi-LXA_4 biosynthesis increased with A23187, but LX production was significantly inhibited by *t*-BuOOH (Figures 2E and 2F). Together, these findings are consistent with disruption of LX biosynthesis by oxidative stress.

Relationship between LXA_4 Levels and sEH Activity

sEH expression is increased by oxidative stress (21), abundant in asthmatic airways (22), and its activity can influence LX generation by human cells *in vitro* and in rodent lung *in vivo* (18, 23), so we next determined if there was a relationship between LXA_4 levels and sEH activity in the sputum supernatants from subjects with asthma (Figure 3). Because 14,15-EET is a prominent cytochrome P-450–derived eicosanoid in the lung that is converted by sEH to 14,15-DHET (24), the product/precursor ratio of 14,15-DHET to 14,15-EET was used as an index for sEH activity. Significant inverse relationships were identified between the ratio of 14,15-DHET to 14,15-EET and LXA_4 levels in both NSA ($r = -0.81$; $P = 0.01$) (Figure 3A) and SA sputum supernatants ($r = -0.72$; $P = 0.002$) (Figure 3B), indicative of a relationship between increased sEH activity and decreased LX levels. No significant correlations between ratio of 14,15-DHET to 14,15-EET and 8-IP were identified in these samples (data not shown).

sEHI Decreases the Ratio of 14,15-DHET to 14,15-EET

In view of this inverse relationship between sEH activity and LXA_4 levels, we first tested the capacity of four potent and selective sEHIs (Table 2) to block sEH activity in samples from subjects with SA (Figure 4). In activated whole blood and BAL cells, all four sEHI significantly decreased sEH activity relative to control incubations ($P < 0.05$) (Figures 4A and 4B). In addition to immunologic detection by ELISA, the identification of 14,15-EET and 14,15-DHET, and the impact of the sEHI on their formation was confirmed by physical methods with liquid chromatography–mass spectrometry (Figures 4C–4F) (*see* METHODS).

sEHI Increases 15-Epi-LXA_4 Biosynthesis

To determine if the sEHIs could increase LX generation, materials from the same incubations in Figure 4 were also analyzed by ELISA for 15-epi-LXA_4. In the presence of A23187, 15-epi-LXA_4 was detectable in the incubations with whole blood (mean ± SD, 78.9 ± 30.9 pg/ml) and BAL cells (mean ± SD, 110.7 ± 65.2 pg/ml). There was a significant increase in 15-epi-LXA_4 levels with AUDA and *t*-TUCB in SA whole blood (Figure 5A) and with AUDA, 1-(1-acetypiperidin-4-yl)-3-adamantanylurea, and *t*-TUCB with BALF cells (Figure 5B). The most active sEHIs in these samples (Figures 4A and 4B) were generally also the most potent inducers of 15-epi-LXA_4 generation (Figures 5A and 5B). Similar to the relationship in SA and NSA sputum supernatants between endogenous LXA_4 and the 14,15-DHET/14,15-EET ratio (Figures 3A and 3B), there was also a significant inverse relationship between 15-epi-LXA_4 and the 14,15-DHET/14,15-DHET ratio with sEHIs (Figure 5C).

Leukocyte–Platelet Aggregates Are Increased in SA and Regulated by LXs

Because leukocyte–platelet interactions are present in asthmatic blood (25) and involved in lung leukocyte recruitment (26, 27), we next determined if these heterotypic aggregates were related to asthma severity and regulated by LXs and sEH activity (Figure 6). LPAs were quantitated by determining the percent of granulocytes (identified by forward and side scatter, confirmed by CD16) that also expressed the platelet-specific marker CD41a (Figure 6A). The percentage was increased in SA relative to HC and could be increased even further by whole blood incubation with PAF (5 μM), a proinflammatory agonist for both leukocytes and platelets (Figures 6A and 6B). The percentage of LPAs was linked to asthma severity in the presence and absence of PAF (Figure 6B). LXA_4 (1–100 nM) inhibited PAF-induced LPAs in a concentration-dependent manner, but there was still segregation between the cohorts by asthma severity (Figure 6C). LXA_4 and 15-epi-LXA_4 (100 nM) were equipotent at inhibiting the PAF-induced formation of LPAs (mean percent inhibition; 70.8% for LXA_4, 78.3% for 15-epi-LXA_4), whereas 14,15-EET and 8-IP were inactive in this bioassay (Figure 6D). Incubation of whole blood from HC in the presence of *t*-TUCB and TPPU (chosen based on their ability to inhibit sEH and induce 15-epi-LXA_4) led to

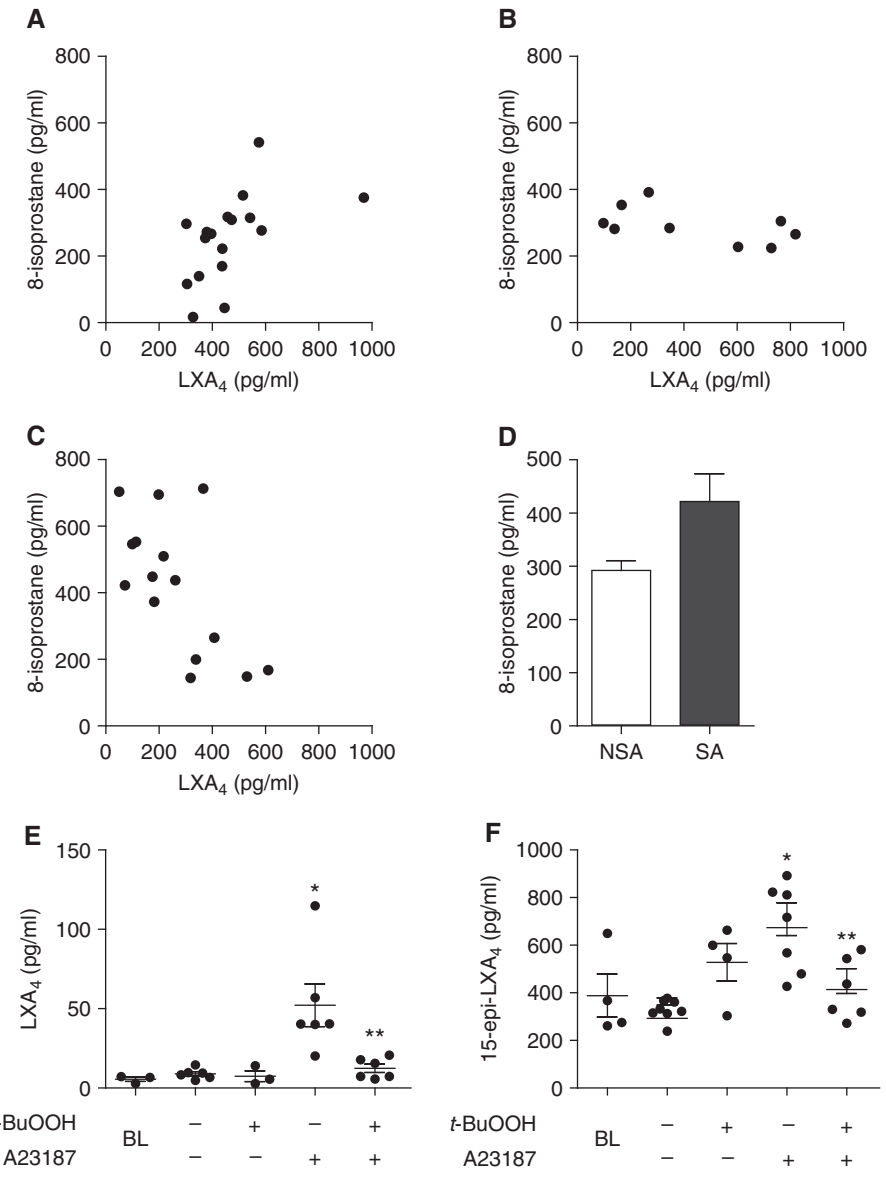

Figure 2. Oxidative stress is inversely related to lipoxin (LX) levels. Samples were obtained from a subset of subjects with nonsevere asthma enrolled in the Macrolides In Asthma trial (same samples as in Figure 1) and subjects with severe asthma enrolled in the cKit Inhibition in Severe Asthma trial. Materials in (*A*) plasma and (*B–D*) sputum supernatants were extracted and levels of LXA_4 and 8-isoprostane, a sensitive indicator of oxidative stress, were determined by ELISA (*see* METHODS). Values for each individual sample are shown. Analysis was based on the Spearman rank correlation test (*A*, $r = 0.68$, $P = 0.002$; *B*, $r = -0.40$, $P = 0.28$; *C*, $r = -0.55$, $P = 0.03$). *Black bar* shows the 8-IP levels in sputum from SA subjects and *white bar* shows those from NSA subjects (*D*, $P = 0.17$). (*E* and *F*) To determine if LX production is disrupted by oxidative stress, peripheral blood leukocytes from healthy subjects were exposed to *t*-butyl-hydroperoxide (*t*-BuOOH) to purposely increase oxidative stress before activation of LX biosynthesis with A23187 (*see* METHODS). Results are expressed as the mean ± SEM for n ⩾ 3. *$P < 0.05$ compared with vehicle/vehicle control, **$P < 0.05$ compared with vehicle/A23187.

different levels of inhibition of PAF-induced cell-cell interactions (Figure 6E) that reflected their capacity to increase endogenous 15-epi-LXA_4 levels (Figure 6F).

Effect of 15-Epi-LXA_4 on TNFα–induced Bronchial Reactivity

In addition to LXs' potent regulation of LPAs for tissue inflammation, their direct actions on lung structural cells was next determined with an *ex vivo* model of bronchial reactivity using human lung sections (Figure 7). Surgical specimens were obtained and exposed (48 h) to 15-epi-LXA_4 (100 nM), 14,15-EET (100 nM), or vehicle with TNF-α (10 ng/ml) to increase bronchial reactivity as in (28). Bronchi were challenged *in vitro* with a range of relevant pharmacologic agonists. Histamine (1 μM) (Figure 7A) and U-46619 (30 nM, thromboxane receptor agonist) (Figure 7B) induced rapid increases in tension within seconds that reached a plateau by 5 minutes. With media alone, the mean tension for MCh (1 μM), histamine (1 μM), or U-46619 (30 nM) was 0.19 ± 0.03 g, 0.28 ± 0.04 g, and 0.34 ± 0.04 g, respectively (Figure 7C). TNF-α increased bronchial contractile responses to the same agents to 0.31 ± 0.04 g, 0.45 ± 0.07 g, and 0.55 ± 0.07 g, which represent significant increases in the active tension of 63%, 61%, and 62%, respectively, compared with media alone (Figure 7C). Exposure to 15-epi-LXA_4 (100 nM) markedly reversed the TNF-α–induced increases in mean tension on challenge with MCh to 0.17 ± 0.02 g, histamine to 0.27 ± 0.03 g, and U-46619 to 0.36 ± 0.04 g (Figure 7C). These findings with 15-epi-LXA_4 represent a significant reduction of 44.5, 38.6, and 34.4%, respectively, in TNF-α–mediated increases in bronchial contraction to a diverse range of stimuli. In addition to 15-epi-LXA_4, 14,15-EET also carried bioactivity in these assays. Direct exposure to 15-epi-LXA_4 reduced TNF-α–triggered responses by 113.5, 106.3, and 90.1% for MCh, histamine, and U-46619, respectively. For purposes of comparison with data included in our prior publication (28), exposure to 14,15-EET reduced TNF-α–induced bronchial contraction to these same agonists by 78.4, 73.4, and 85.2%, respectively (Figure 7D).

15-epi-LXA_4 can interact with multiple receptors in the airway, including as an agonist at ALX/FPR2 (29), so WRW4, a receptor antagonist for ALX/FPR2, was used to determine if the LX actions were ALX/FPR2-dependent. Paired recordings (Figures 7E and 7F) and quantitative analyses (Figure 7C) of bronchial responses to MCh revealed concentration-dependent bronchial relaxation by 15-epi-LXA_4 with partial inhibition by WRW4. Similar experiments were performed to assess the putative effects of WRW4 on the relaxing effects induced by 14,15-EET on MCh

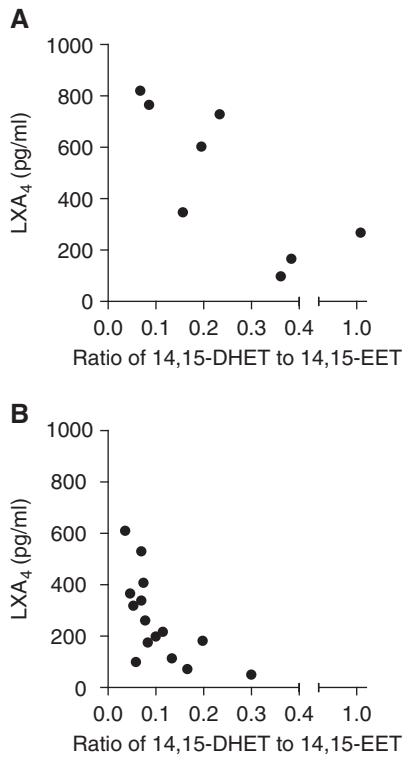

Figure 3. Relationship between lipoxin (LX) A_4 levels and soluble epoxide hydrolase activity in asthma. Samples were obtained from (*A*) subjects with nonsevere asthma enrolled in the Macrolides In Asthma trial (same samples as in Figures 1 and 2) and (*B*) subjects with severe asthma enrolled in the cKit Inhibition in Severe Asthma trial (same samples as in Figure 2). Materials in sputum supernatants were extracted and levels of LXA_4, 14,15-epoxy-eicosatrienoic acid (14,15-EET), and 14,15-dihydroxy-eicosatrienoic acid (14,15-DHET) were determined by ELISA. The ratio of 14,15-DHET to 14,15-EET was calculated as an index of soluble epoxide hydrolase activity. Values for each individual sample are shown. Analysis was based on the Spearman rank correlation test (*A*, $r = -0.81$, $P = 0.02$; *B*, $r = -0.72$, $P = 0.002$).

precontracted human bronchi (*see* Figure E1 in the online supplement). 14,15-EET induced concentration-dependent relaxation of mean MCh responses that were also partially inhibited by WRW4 (Figure 7F; *see* Figure E1). Together, these findings add direct tissue protective actions to 15-epi-LXA_4's and 14,15-EET's antiasthmatic activities that are partially ALX/FPR2-dependent.

Discussion

The present findings provide evidence that oxidative stress and sEH activity are linked to decreased LX generation in SA. 8-IP levels were used as an arachidonic acid–based indicator of oxidative stress. This F2-IP is nonenzymatically synthesized by free radical–catalyzed lipid peroxidation (30). 8-IP is a sensitive biomarker of oxidative stress that is increased in asthma (30, 31) and related to disease activity (32). In contrast, LXs are generated enzymatically from arachidonic acid in a highly orchestrated, multistep biosynthesis that includes exchange of arachidonic acid and biosynthetic intermediates between cell types (33). These cell-cell interactions occur between leukocytes and platelets in the vasculature (33) and between leukocytes and mucosal epithelial cells in the airway (23). We hypothesized that the shuttling of polyunsaturated fatty acid intermediates between cells would increase their vulnerability in oxidative environments to nonenzymatic attack and subsequent disruption of LX biosynthesis. This susceptibility for LXs differs from LT biosynthesis, which often proceeds in single cells (9).

To address our hypothesis, we compared closely related eicosanoids (i.e., 8-IP, LXA_4, and LTB_4) in samples from patients with asthma. Levels of 8-IP were increased in SA sputum supernatants relative to NSA, and there was a significant inverse relationship between 8-IP and LXA_4. Increased oxidative stress may lead to corticosteroid resistance, which is common in SA (3). LX levels are decreased in SA, yet corticosteroid dosing is not related to LX production in SA (17). Because the changes in LX levels here were linked to oxidative stress, indirectly reflected in the 8-IP levels, we purposefully induced oxidative stress in healthy leukocytes using t-BuOOH, which significantly disrupted LXA_4 and 15-epi-LXA_4 biosynthesis. In NSA sputum, there was also a trend toward an inverse relationship between LXA_4 and 8-IP, but it did not reach statistical significance and the slope was less marked. In sharp contrast, there was a positive correlation between 8-IP and LXA_4 in NSA plasma, which was more consistent with arachidonate availability as the limiting factor for plasma 8-IP, LTB_4, and LXA_4 and indicative of an important anatomic-specific influence of oxidative stress on LX biosynthesis.

LX levels in asthmatic sputum were also inversely related to sEH activity and were increased *in vitro* by sEHIs. sEH is expressed in asthmatic lung (22) and plays important roles in arachidonic acid metabolism (24). Expression of sEH is increased by oxidative stress (21). Cytochrome P-450 enzymes convert arachidonic acid to EETs, resulting in the production of four regioisomeric EETs: 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET. Among these regioisomers, 14,15-EET is the most abundant in lung tissues (34). Based on protective actions of EETs and their sEH-mediated transformation to biologically inactive diols (DHETs), sEHIs have been developed (21, 35, 36). In rodents, inhibition of sEH decreases cigarette smoke–induced lung inflammation and increases LXA_4 levels (18). In addition, proinflammatory cytokine-exposed human airway epithelial cells can donate

Table 2. Four Specific Soluble Epoxide Hydrolase Inhibitors*

Compound	Abbreviation	Target Enzymes	IC_{50}	Compound Name
EHI 700	AUDA	sEH and EH3	3 nM	12-(3-adamantan-1yl-ureido) dodecanoic acid
EHI 1153	APAU	sEH	15 nM	1-(1-acetypiperidin-4-yl)-3-adamantanylurea
EHI 1770	TPPU	sEH	1 nM	1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea
EHI 1728	*t*-TUCB	sEH	2 nM	*trans*-4-{4-[3-(4-trifluoromethoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid

Definition of abbreviations: EHI = epoxide hydrolase inhibitor; IC_{50} = half maximal 50% inhibitory concentration; sEH = soluble epoxide hydrolase.
*Structures of the sEHIs are shown in the online supplement.

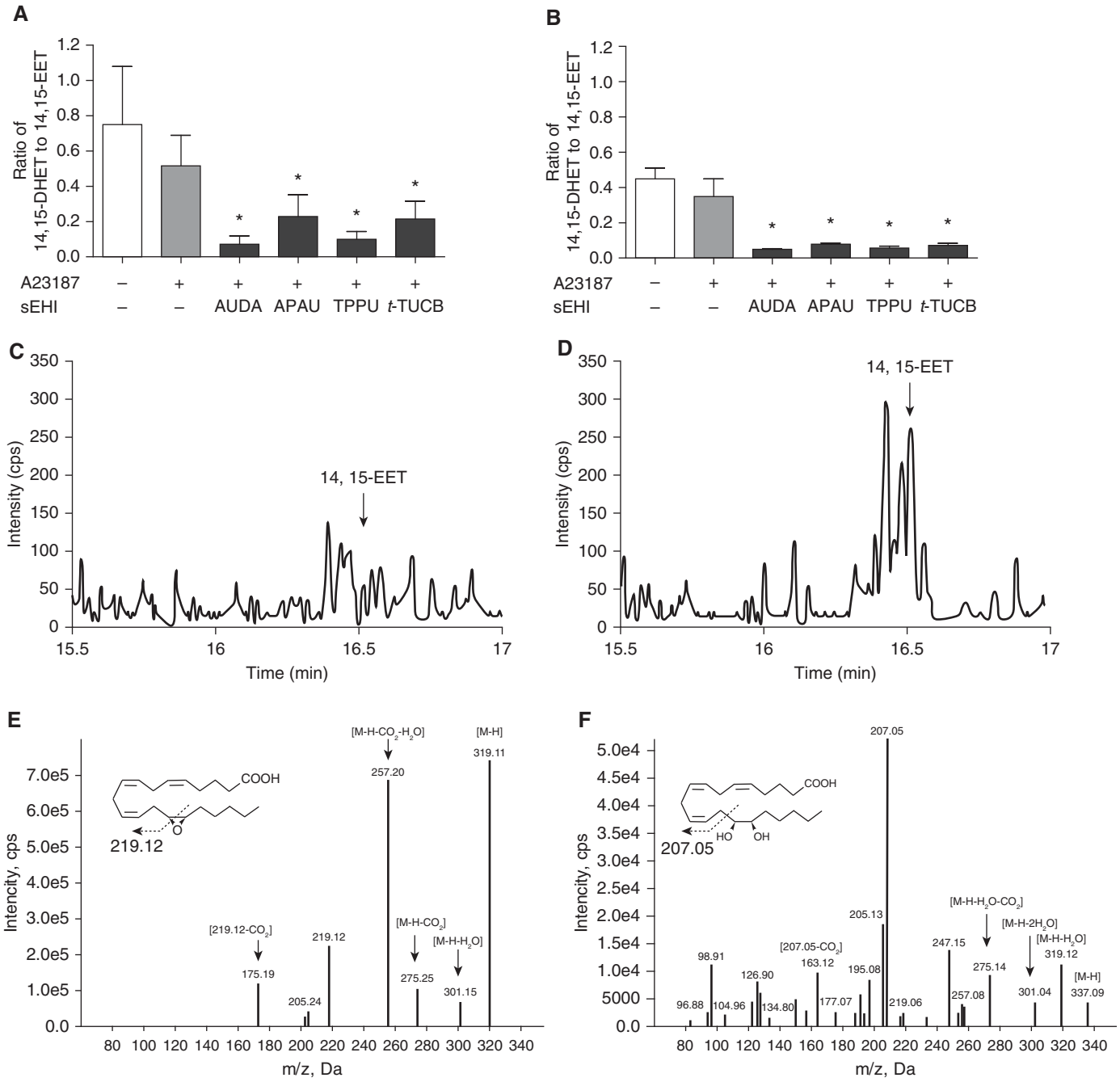

Figure 4. Soluble epoxide hydrolase inhibitors decreased the ratio of 14,15-dihydroxy-eicosatrienoic acid (14,15-DHET) to 14,15-epoxy-eicosatrienoic acid (14,15-EET) in severe asthma. Samples of (*A*) whole blood and (*B*) bronchoalveolar lavage fluids were obtained from subjects with severe asthma and exposed to a soluble epoxide hydrolase inhibitor (sEHI) or vehicle before cell activation with A23187 (*see* METHODS). Materials from these incubations were extracted and a ratio of 14,15-DHET to 14,15-EET was determined as an indicator of sEH activity. Results are expressed as mean ± SEM ($n \geq 6$). *$P < 0.05$ in comparison with samples activated by A23187 in the absence of sEHI by one-way analysis of variance. (*C–F*) Liquid chromatography–mass spectrometry (representative of n = 3). (*C*) Vehicle, (*D*) sEHI 700, (*E*) 14,15-EET, and (*F*) 14,15-DHET. APAU =1-(1-acetypiperidin-4-yl)-3-adamantanylurea; AUDA =12-(3-adamantan-1yl-ureido) dodecanoic acid; TPPU =1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea; *t*-TUCB = *trans*-4-{4-[3-(4-trifluoromethoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid.

14,15-EET to human neutrophils during coincubation for transcellular 15-epi-LXA_4 biosynthesis (23).

Because LX generation is deficient in SA, we determined the relationship between LXA_4 and sEH activity in cells from subjects with asthma. In both NSA and SA sputum, there was a significant inverse relationship between LXA_4 and endogenous

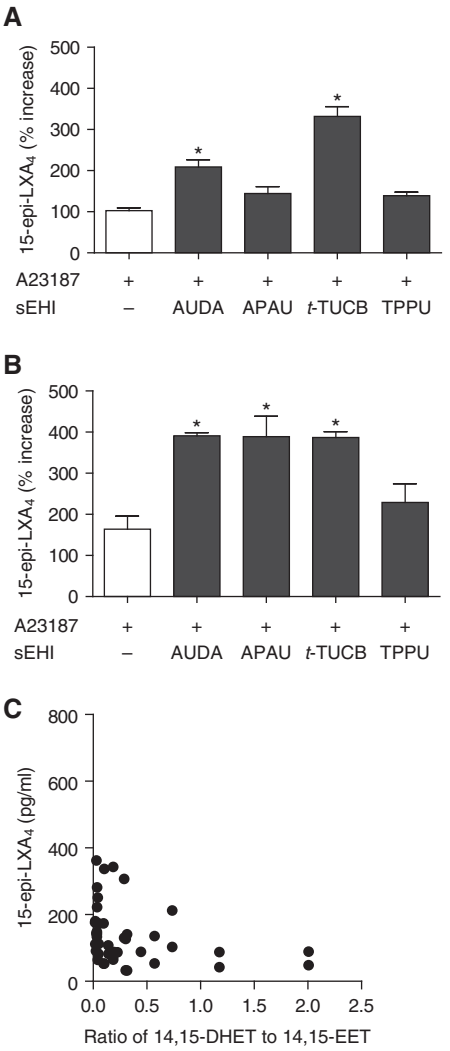

Figure 5. Soluble epoxide hydrolase inhibition increases 15-epi-LXA_4 generation in severe asthma. Samples of (*A*) whole blood and (*B*) bronchoalveolar lavage fluids (BALF) were obtained from subjects with severe asthma and exposed to a soluble epoxide hydrolase inhibitor (sEHI) or vehicle before cell activation with A23187 (*see* METHODS). Materials from these incubations were extracted and 15-epi-LXA_4 was determined by ELISA. The change in 15-epi-LXA_4 is expressed as an increase relative to control samples exposed only to vehicle (whole blood, 74.0 ± 40.6 pg 15-epi-LXA_4/ml; BALF, 77.8 ± 55.4 pg 15-epi-LXA_4/ml). Results are expressed as mean ± SEM ($n \geq 6$). *$P < 0.05$ in comparison with samples activated with A23187 in the absence of sEHI by one-way analysis of variance. (*C*) 15-epi-LXA4, 14,15-EET, and 14,15-DHET were determined by ELISA. The ratio of 14,15-DHET to 14,15-EET was calculated as an index of SEH activity. Values for each individual sample are shown for whole blood. Analysis was based on the Kruskal-Wallis analysis of variance followed by the Mann-Whitney test with Bonferroni correction (*A* and *B*). Spearman rank correlation test was used to compare the ranks of two continuous variables (*C*, $r = -0.39$, $P = 0.01$). APAU =1-(1-acetypiperidin-4-yl)-3-adamantanylurea; AUDA =12-(3-adamantan-1yl-ureido) dodecanoic acid; 14,15-DHET =14,15-dihydroxy-eicosatrienoic acid; 14,15-EET = 14,15-epoxy-eicosatrienoic acid; LX = lipoxin; TPPU =1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea; *t*-TUCB = *trans*-4-{4-[3-(4-trifluoromethoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid.

sEH activity (14,15-DHET/14,15-EET ratio). Moreover, sEHIs blocked conversion of 14,15-EET to 14,15-DHET and significantly increased 15-epi-LXA_4 levels by SA whole blood and BALF cells. For these experiments, four structurally different sEHIs were used. These sEHIs are tight binding inhibitors that approach stoichiometric interaction with sEH. They are competitive and reversible inhibitors that have high target occupancy and a slow off rate (37). The consistent findings with all four compounds indicate a class effect for sEH inhibition, rather than an off-target action of a single compound. Detailed information on the synthesis, physical properties, and pharmacokinetics of each of these sEHIs has been published (22, 35–37). Together, these findings support sEH inhibition as a new pharmacologic mechanism for enhancing endogenous LX generation in SA.

To assess the potential therapeutic impact of sEHIs and LXs in asthma, two disease-related functional responses were selected for preclinical studies of human cells and lung tissues: leukocyte recruitment for airway inflammation and cytokine-mediated bronchial reactivity. For leukocyte recruitment, LPAs were quantitated in whole blood. LPAs play important roles in secondary capture for leukocyte entry into inflamed tissues, including lung (26, 27, 38). Asthmatic responses are associated with platelet activation (39, 40) and increased circulating LPAs have been detected in asthma attacks and after allergen challenge (39, 41). These LPAs can also serve as important sources for bioactive lipid mediators, including LTs and LXs (25, 33).

Here, we found that LPAs were increased in asthma and related to disease severity. PAF can activate both leukocytes and platelets (42) and *in vitro* initiated a marked increase in LPAs. LXA_4 gave concentration-dependent inhibition of PAF-initiated LPAs. LXA_4 and 15-epi-LXA_4 inhibitory responses were similar; however, neither 14,15-EET nor 8-IP led to significant inhibition. During incubations designed for LX generation from endogenous sources (as in [33]), sEHIs partially inhibited PAF-induced LPAs, the amplitude of which was related to induction of 15-epi-LXA_4 generation. These findings indicated that LPAs are useful biomarkers of asthma activity and severity and that LXs can regulate platelet activation and interaction with peripheral blood leukocytes. If sEHIs increase vascular LX generation, then subsequent lung inflammation could be decreased by disrupting LPAs. Thus, LPAs might also serve as a useful indicator of sEHI efficacy. In addition, these results also suggest that some of the antiinflammatory actions attributed to EETs may relate to LX generation and action. The potential value of LPAs as cellular biomarkers of asthma activity and severity requires further

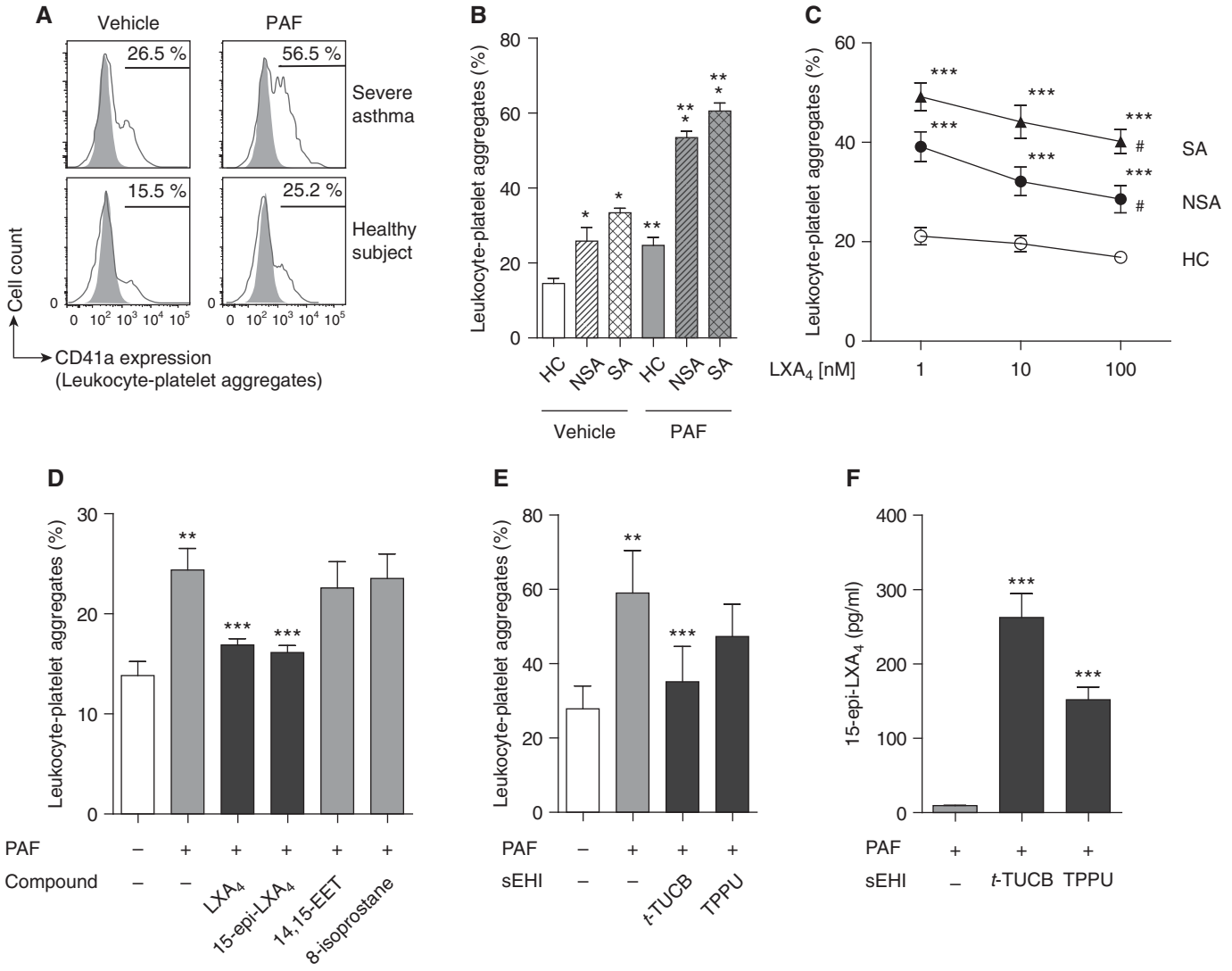

Figure 6. Leukocyte-platelet aggregates are associated with asthma severity and inhibited by lipoxins (LXs) and soluble epoxide hydrolase inhibition. Leukocyte–platelet aggregates in peripheral venous blood were detected and enumerated by flow cytometry before and after incubation with either platelet activating factor (PAF, 5 μM) or vehicle control (0.1% ethanol). (*A*) Representative histograms of leukocyte–platelet aggregates (as determined by cells with forward and side scatter properties of granulocytes staining with the platelet-specific molecule CD41) in blood from a subject with severe asthma (SA; *top*) and a healthy subject (*bottom*). *Inset*: Percentages of leukocyte–platelet aggregates are shown. (*B*) Leukocyte–platelet aggregates (%) in blood from healthy subjects (HC, *open*) or subjects with nonsevere asthma (NSA, *striped*) or SA (*cross-striped*) that were exposed to vehicle (*white*) or PAF (*gray*) ($n \geq 3$). (*C*) Samples of whole blood were exposed to LXA_4 (1–100 nM, 5 min) before activation with PAF (5 μM) in samples from HC, subjects with NSA, or SA ($n > 3$). (*D*) Samples of whole blood from HC were exposed (5 min) to either vehicle (0.1% ethanol) or 100 nM of LXA_4, 15-epi-LXA_4, 14,15-epoxy-eicosatrienoic acid (14,15-EET), or 8-isoprostane before activation with PAF (5 μM), and leukocyte–platelet aggregates (%) were determined. (*E* and *F*) Samples of whole blood from HC were exposed to granulocyte-macrophage colony–stimulating factor (200 pM, 50 min) followed by select soluble epoxide hydrolase inhibitors (sEHI) and then activated (15 min) with fMLP (10^{-7} M) and thrombin (0.1 U/ml). Leukocyte–platelet aggregates (%) and levels of 15-epi-LXA_4 were determined ($n \geq 3$) (*see* METHODS). (*B–F*) Data are expressed as mean ± SEM. $^{*}P < 0.05$ in comparison with HC, $^{**}P < 0.05$ in comparison with vehicle, $^{***}P < 0.05$ in comparison with PAF, $^{\#}P < 0.05$ in comparison with 1 nM. TPPU =1-trifluoromethoxyphenyl-3-(1-propionylpiperidin-4-yl) urea; *t*-TUCB = *trans*-4-{4-[3-(4-trifluoromethoxy-phenyl)-ureido]-cyclohexyloxy}-benzoic acid.

investigation in a larger, independent clinical study.

To investigate a direct role for LXs on lung tissue, we chose to investigate its effects on increased bronchial contraction, which is a major feature of asthma and is a principal cause of symptomatic dyspnea (1). When given to subjects with asthma by inhalation, LXA_4 can block LTC_4-induced bronchoprovocation (15), and LXA_4 and its stable analogs can dampen MCh-initiated bronchial hyperreactivity in mice (14). Because sEHI-mediated LX generation requires cell-cell interactions between leukocytes and airway cells (23) and leukocytes were not present in the lung tissue sections, the direct impact of 15-epi-LXA_4 on bronchial responses in human lung was determined via isometric tension measurements on human bronchi in an isolated organ bath system. Exposure

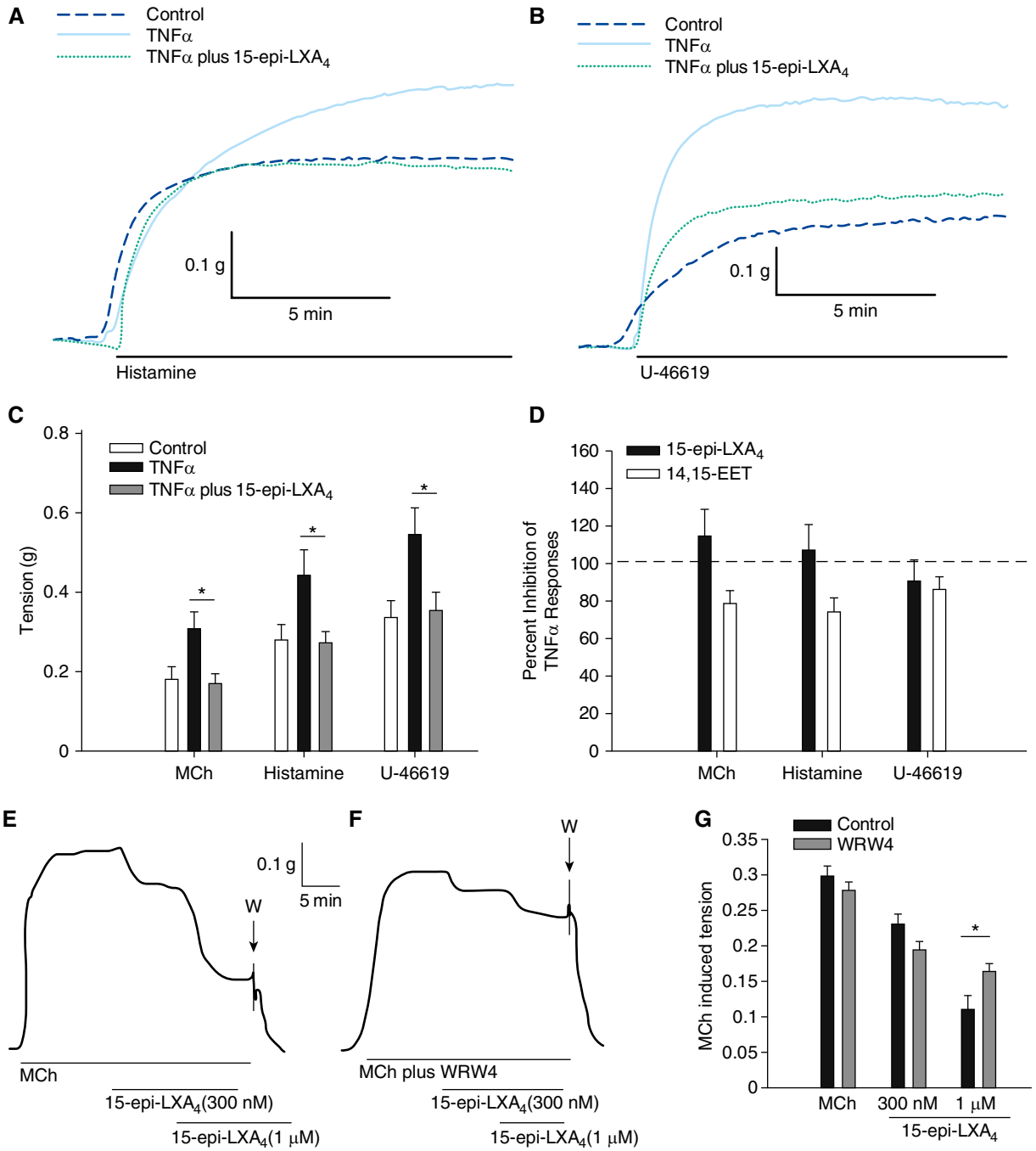

Figure 7. Effect of 15-epi-LXA4 on tumor necrosis factor (TNF)-α–induced increases in bronchial contraction. (*A*) Representative traces of histamine (1 μM) induced tension on human bronchi cultured for 48 hours in either control media (*blue dashed line*), media containing TNF-α (10 ng/ml, *blue solid line*), or media with TNF-α and 15-epi-LXA_4 (100 nM, *green dotted line*) (*see* METHODS). (*B*) Representative traces of U-46619 induced tension on human bronchi under the same experimental conditions as described in *A*. (*C*) The impact of 15-epi-LXA_4 (100 nM) on tension induced by methacholine (MCh) (1 μM), histamine (1 μM), or U-46619 (30 nM) was measured with TNF-α–exposed human bronchi from multiple donors. (*D*) Percent inhibition of the TNF-α–triggered bronchial responses was determined for 15-epi-LXA_4 and 14,15-epoxy-eicosatrienoic acid (14,15-EET) (100 nM). Values represent the mean ± SD for n = 13. *$P < 0.05$ by analysis of variance. (*E*) Representative recording of the MCh response and the concentration-dependent relaxing effects induced by LXA_4 in control condition and (*F*) in a paired recording in the presence of the ALX/FPR2 receptor antagonist WRW4 (1 μM). (*G*) Results are expressed as the mean and SD for the effect of cumulative addition of 15-epi-LXA_4 in the absence (control) or presence of WRW4 on the tension induced by MCh (1 μM) on human bronchi. n = 12; * $P < 0.05$. LX = lipoxin.

to 15-epi-LXA_4 significantly reduced TNF-α–induced increases in bronchial contraction to a wide range of agonists, including MCh, histamine, and a thromboxane receptor agonist (U-46619). LXs principally mediate their actions as agonists at ALX/FPR2 and/or antagonists at CysLT1 receptors (29). Here, an ALX/FPR2-selective antagonist partially blocked the actions of 15-epi-LXA_4 and 14,15-EET, suggesting roles for both LX receptors in regulating bronchial responses. Together, these findings suggest that a sEHI that increased 15-epi-LXA_4 in SA would have beneficial actions on bronchial relaxation in inflamed airways.

In summary, LX generation in the asthmatic airway is adversely impacted by oxidative stress and sEH activity, in particular in SA. Specific inhibitors of sEH increased 14,15-EET and 15-epi-LXA_4 generation by SA whole blood and BALF cells, and 15-epi-LXA_4 decreased PAF-initiated LPAs and TNF-α–mediated increases in bronchial contraction, suggesting that sEHIs or LX stable analogs could serve as a novel pro-resolving therapeutic strategy in SA. ■

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