

Inflammatory Basis of Exercise-induced Bronchoconstriction

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Rationale: Exercise-induced bronchoconstriction (EIB) is a highly prevalent condition with unclear pathogenesis. Two competing theories of the pathogenesis of EIB differ regarding the inflammatory basis of this condition. **Objectives:** Our goals were to establish whether epithelial cell and mast cell activation with release of inflammatory mediators occurs during EIB and how histamine and cysteinyl leukotriene antagonists alter the airway events occurring during EIB. **Methods:** Induced sputum was used to measure mast cell mediators and eicosanoids at baseline and 30 minutes after exercise challenge in 25 individuals with asthma with EIB. In a randomized, double-blind crossover study, the cysteinyl leukotriene antagonist montelukast and antihistamine loratadine or two matched placebos were administered for two doses before exercise challenge. **Main Results:** The percentage of columnar epithelial cells in induced sputum at baseline was associated with the severity of EIB. After exercise challenge, histamine, tryptase, and cysteinyl leukotrienes significantly increased and prostaglandin E₂ and thromboxane B₂ significantly decreased in the airways, and there was an increase in columnar epithelial cells in the airways. The concentration of columnar epithelial cells was associated with the levels of histamine and cysteinyl leukotrienes in the airways. Treatment with montelukast and loratadine inhibited the release of cysteinyl leukotrienes and histamine into the airways, but did not inhibit the release of columnar epithelial cells into the airways. **Conclusions:** These data indicate that epithelial cells, mast cell mediators, and eicosanoids are released into the airways during EIB, supporting an inflammatory basis for EIB.

Keywords: asthma; eicosanoid; epithelial cell; exercise-induced bronchoconstriction; mast cell

Exercise-induced bronchoconstriction (EIB) is a highly prevalent condition present in approximately half of patients with asthma (1) and in other individuals who have EIB in the absence of additional features of asthma (2). The pathogenesis of EIB is poorly understood. Although conditioning of the inspired air, leading to drying and cooling of the intrathoracic airways, may serve as the initial trigger for EIB (3, 4), the subsequent events in the airways are unclear. Two competing hypotheses of the

mechanism of EIB are: (1) loss of heat leads to vascular engorgement as the airways rewarm after exercise initiating bronchoconstriction; and (2) loss of water leads to a change in airway osmolarity that initiates epithelial cell and mast cell activation, leading to the release of inflammatory mediators in the airways that cause bronchoconstriction (3, 4). Although many studies suggest a noninflammatory basis of EIB related to thermal fluxes in the airways (5, 6), animal models of EIB indicate that dry air hyperpnea initiates an increase in airway osmolarity (7), resulting in release of epithelial cells and eicosanoids into the airways (8, 9). Isocapnic hyperpnea is a model of EIB that leads to an increase in epithelial cells and eicosanoids in bronchoalveolar lavage (BAL) fluid in human subjects with asthma (10). However, studies designed to determine if EIB has an inflammatory basis in persons with asthma have been inconclusive (11–13) due to a limited number of subjects studied and the use of BAL that samples the distal airways, rather than the segmental airways involved in EIB (14). Our objective was to determine the inflammatory basis of EIB using induced sputum, a technique that collects airway lining fluid from the conducting airways (15). Our primary goal was to determine if epithelial cell and mast cell activation with release of inflammatory mediators such as histamine, cysteinyl leukotrienes (CysLTs), and tryptase occurs during EIB in humans. We also determined how pharmacologic antagonists of CysLTs and histamine alter the airway events in the development of EIB.

Some of the results of this study have been previously reported in the form of an abstract (16).

METHODS

Subjects and Study Protocol

The study population consisted of persons aged 12–59 with asthma treated only with a short-acting β_2 -agonist as needed, an FEV₁ greater than 65% predicted, and a fall in FEV₁ of 15% or more after dry air exercise challenge (0% relative humidity, 22°C). Spirometry (17) and exercise challenges (18) were conducted in accordance with American Thoracic Society standards. Potential participants were excluded if they smoked cigarettes within 1 year, had 7 pack-years or more of smoking history, or had used an inhaled corticosteroid, leukotriene modifier, long-acting antihistamine, cromone, or long-acting β_2 -agonist in the past 30 days.

Participants had a total of four visits at the same time of day separated by 4 to 10 days. Airway events were characterized by analysis of induced sputum at baseline and 30 minutes after exercise challenge on separate days. In a randomized, double-blind crossover study, the CysLT₁ antagonist montelukast and H₁-antihistamine loratadine or two matched placebos were administered for two doses 36 and 12 hours before exercise challenge. The study protocol was approved by the University of Washington institutional review board, and written informed consent and, when applicable, assent was obtained from all participants (additional details in the online supplement).

Induced Sputum Analysis

Induced sputum was conducted using hypertonic saline (3%) via an ultrasonic nebulizer for 12 minutes (19). At 2-minute intervals, subjects

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were asked to clear saliva from their mouth and then expectorate sputum. The induced sputum was placed on ice and processed within 30 minutes. The sample was dispersed with an equal volume of DTT 0.1% in a shaking water bath at 37°C for 15 minutes. Total cell count was determined with a hemocytometer, and slides for differential cell counts were prepared with a cytocentrifuge. Slides were stained and at least 400 nonsquamous cells counted per slide. A portion of the supernatant was treated for eicosanoid analysis by the addition of 4 vols of methanol, precipitated, centrifuged at $300 \times g$ for 20 minutes, evaporated to near dryness at 0.7 mm Hg, and resuspended in methanol and distilled water (20). The concentrations of mediators were determined by ELISA for histamine, tryptase, CysLTs, LTB₄, PGE₂, and thromboxane B₂. The concentrations of interleukin (IL)-4, IL-5, IL-6, IL-8, tumor necrosis factor- α , RANTES (regulated upon activation, normal T-cell expressed and secreted), and vascular endothelial growth factor were determined by protein multiplex assay, and the concentration of IL-13 was determined by ELISA (additional details in the online supplement).

Statistical Analysis

Characteristics of the study participants were expressed as the mean and standard deviation. The area under the FEV₁/time curve (AUC) (21) quantified the severity of EIB over 0 to 30 minutes after exercise (AUC₃₀) during the screening visit and over 0 to 15 minutes after exercise (AUC₁₅) during the subsequent study visits. An ANOVA model was used to assess the effects of treatment and determine if there were carry over effects of treatment according to the sequence of randomization. The medians of differential cell counts were compared between different conditions with the Wilcoxon signed-rank test. The levels of inflammatory mediators in induced sputum were compared between different conditions with a paired *t* test of the log-transformed values. The relationship of cellular constituents and inflammatory mediators to each other and to severity of EIB was assessed after log transformation using Pearson's correlation coefficient.

RESULTS

Subject Characteristics

One hundred eleven potential participants with physician-diagnosed asthma were screened for this study. Four subjects were excluded because baseline FEV₁ was less than or equal to 65% predicted. One hundred seven subjects had an exercise challenge test; this identified 28 subjects with fall in FEV₁ of 15% or more after exercise challenge. One subject was not entered into the study because symptoms after exercise challenge required β_2 -agonist treatment. Two participants were excluded from the study because of time constraints or noncompliance. A total of 25 patients with mild to moderate asthma who had fall in FEV₁ of 15% or more after exercise challenge completed this study and were included in the analysis (Table 1). Twenty-one of the 25 participants had mild asthma, with a baseline FEV₁ greater than or equal to 80% of predicted and symptoms primarily during exercise. The severity of EIB ranged from a maximum decline in FEV₁ of 15% to 63% after exercise challenge during the screening visit. The average time between the screening exercise challenge and the baseline induced sputum was 14.7 days (range, 4–61 days). The intraclass correlation coefficient for the severity of EIB measured by the AUC₁₅ on the screening day compared with the AUC₁₅ on the placebo visit was 0.843 (*p* = 0.0002). The screening and placebo visits were conducted on average 24.2 days apart (range, 11–69 days).

Characteristics of Airway Inflammation in EIB

The median percentage of lower airway cells at baseline were 2.4% eosinophils, 1.6% lymphocytes, 41.3% macrophages, 42.3% neutrophils, and 5.3% columnar epithelial cells. Airway eosinophilia greater than or equal to 2% was present in 14 of 25 and greater than or equal to 4% in 4 of 25 subjects. There was no relationship between the percentage of airway eosinophils,

TABLE 1. CHARACTERISTICS OF STUDY PARTICIPANTS

Characteristic	Asthma with EIB (<i>n</i> = 25)
Age, yr	
Mean	28.1
Range	14–55
Sex, % Male	44%
Race	
White	92%
African American	4%
Asian	4%
Baseline	
FEV ₁ , %	84.8 ± 8.4
FVC, %	100.1 ± 10.3
FEV ₁ /FVC	0.78 ± 0.06
FEF _{25–75} , %	78.5 ± 19.2
Postbronchodilator	
Δ FEV ₁ , %	11.0 ± 6.2
Δ FVC, %	2.5 ± 6.1
Δ FEV ₁ /FVC, %	9.05 ± 5.5
Δ FEF _{25–75} , %	33.3 ± 16.8
Postexercise	
Maximum decrease in FEV ₁	29.2 ± 11.9
Area under FEV ₁ curve*	681.4 ± 321.5

Definition of abbreviation: EIB = exercise-induced bronchoconstriction.

Values reported are mean ± SD unless otherwise specified.

* Area under the FEV₁ curve over the first 30 min after exercise (% change · min).

neutrophils, lymphocytes, or macrophages in the baseline induced sputum and the severity of EIB as measured by the maximum decline in FEV₁ after exercise challenge during the placebo visit. Similarly, there was no relationship between the concentration of eosinophils, neutrophils, lymphocytes, or macrophages in the baseline induced sputum and the maximum fall in FEV₁ after exercise challenge. The percentage of columnar epithelial cells at baseline was associated with the severity of EIB measured by the maximum fall in FEV₁ after exercise challenge (r^2 = 0.174, *p* = 0.043; Figure 1). A trend was noted between the concentration of columnar epithelial cells in induced sputum and the maximum fall in FEV₁ after exercise challenge (r^2 = 0.135, *p* = 0.077). The concentration of lower airway cells in induced sputum (i.e., excluding squamous epithelial cells) was not associated with the severity of EIB measured by the maxi-

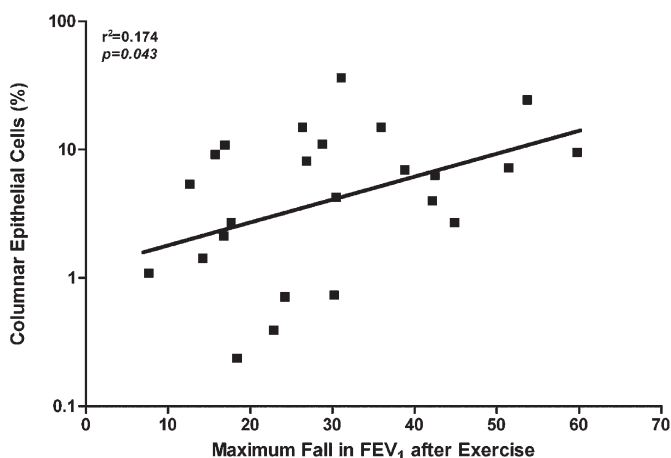


Figure 1. Relationship between the percentage of columnar epithelial cells in induced sputum at baseline and the severity of exercise-induced bronchoconstriction (EIB) as measured by the maximum fall in FEV₁ over the first 15 minutes after exercise challenge.

num fall in FEV₁ after exercise challenge ($r^2 = 0.008$, $p = 0.662$). However, the induced sputum volume was associated with the severity of EIB ($r^2 = 0.156$, $p = 0.051$) and a trend was observed between the total number of lower airway cells in induced sputum and the severity of EIB ($r^2 = 0.105$, $p = 0.115$). No relationship was seen between baseline lung function ($r^2 = 0.056$, $p = 0.25$) or bronchodilator response ($r^2 = 0.094$, $p = 0.13$) and the severity of EIB.

Effects of Exercise Challenge on Airway Inflammatory Mediators

Effects of exercise challenge on airway cells and mediators were assessed by comparing induced sputum samples obtained at baseline and 30 minutes after exercise challenge during the administration of placebo. The induced sputa at baseline and during placebo administration were conducted an average of 9.9 days apart (range, 4–18 days). There were no differences in induced sputum volume (median 4.69 ml vs. 4.45 ml, $p = 0.17$) and concentration of lower airway cells in induced sputum (median 1.24×10^6 cells/ml vs. 1.53×10^6 cells/ml, $p = 0.82$) between the baseline and post-exercise samples. There was a significant increase in the concentration of columnar epithelial cells and trends toward decreased concentrations of lymphocytes and macrophage in induced sputum after exercise challenge (Figure 2). No changes were observed in the concentration of neutrophils (median 4.6×10^5 vs. 4.5×10^5 , $p = 0.64$) or eosinophils (median 2.5×10^4 vs. 2.2×10^4 , $p = 0.56$) after exercise challenge. There was a significant increase in the percentage of columnar epithelial cells (median 5.3% vs. 13.7%, $p = 0.001$) and significant decreases in the percentage of macrophages (median 41.3% vs. 28.0%, $p = 0.01$) and lymphocytes (median 1.6% vs. 1.2%, $p = 0.05$). No changes were observed in the percentage of neutrophils (median 42.3% vs. 41.3%, $p = 0.78$) or eosinophils (median 2.4% vs. 1.6%, $p = 0.72$) after exercise.

The levels of histamine, tryptase, and CysLTs increased in induced sputum after exercise challenge, whereas PGE₂ and TXB₂ decreased after exercise challenge (Figure 3). The ratio of CysLTs to PGE₂ increased from a median of 3.87 at baseline to 7.54 after exercise challenge ($p = 0.002$). The concentrations of CysLTs and histamine in induced sputum were associated with the concentration of epithelial cells in induced sputum after exercise challenge (Figure 4). There was a trend toward an association between the increase in CysLTs in induced sputum and the severity of EIB measured by the AUC₁₅ ($r^2 = 0.133$, $p = 0.15$). The levels of LTB₄, IL-6 (19.3 vs. 26.1 pg/ml, $p = 0.14$),

IL-8 (1,007 vs. 1,528 pg/ml, $p = 0.55$), and vascular endothelial growth factor (380.9 vs. 594.9 pg/ml, $p = 0.43$) were no different compared with baseline after exercise challenge. The levels of IL-4, IL-5, IL-13, RANTES, and tumor necrosis factor- α were below the level of detection in the majority of the subjects.

Treatment Effects on Lung Function and Severity of EIB

Before exercise challenge, there was modest improvement in FEV₁ (3.50 L versus 3.33 L, $p = 0.008$) during treatment with montelukast and loratadine as compared with matched placebo. The severity of EIB was markedly attenuated by treatment with montelukast and loratadine as seen by a 62% reduction in AUC₁₅ during the first 15 min after exercise challenge (138.3 vs. 363.8, $p < 0.001$) and a 53.7% reduction in the maximum fall in FEV₁ after exercise (−13.6% versus −29.3%, $p < 0.001$) (Figure 5). Symptoms of dyspnea after exercise were reduced at all time points after exercise challenge during treatment ($p < 0.01$) (Figure 6).

Treatment Effects on Airway Events During EIB

Effects of treatment with montelukast and loratadine on airway cells and mediators were assessed by comparing induced sputum samples obtained 30 minutes after exercise challenge during the administration of placebo and during treatment with montelukast and loratadine. The placebo- and treatment-induced sputa were conducted an average of 6.8 days apart (range, 4–14 days). There were no differences in induced sputum volume (median 4.45 ml vs. 4.33 ml, $p = 0.49$) and concentration of lower airway cells in induced sputum (median 1.53×10^6 cells/ml vs. 1.11×10^6 cells/ml, $p = 0.17$) between the postexercise samples during placebo and treatment. The concentration of lymphocytes was decreased in induced sputum after exercise challenge during treatment with montelukast and loratadine as compared with placebo (median 1.57×10^4 cells/ml vs. 0.79×10^4 cells/ml, $p = 0.02$). The concentrations of eosinophils (median 2.16×10^4 cells/ml vs. 2.01×10^4 cells/ml, $p = 0.37$), macrophages (median 3.69×10^5 cells/ml vs. 3.02×10^5 cells/ml, $p = 0.53$), neutrophils (median 4.46×10^5 cells/ml vs. 4.36×10^5 cells/ml, $p = 0.55$), and columnar epithelial cells (median 1.47×10^5 cells/ml vs. 1.59×10^5 cells/ml, $p = 0.56$) in induced sputum after exercise challenge were no different during placebo as compared with treatment with montelukast and loratadine. There were no differences in the percentages of eosinophils (median 1.63% vs. 1.78%, $p = 0.28$), lymphocytes (median 1.18% vs. 0.91%, $p = 0.10$), macrophages (median 28.00% vs. 26.42%, $p = 0.23$), neutrophils (median 41.27% vs. 40.58%, $p = 0.68$), and columnar epithelial cells

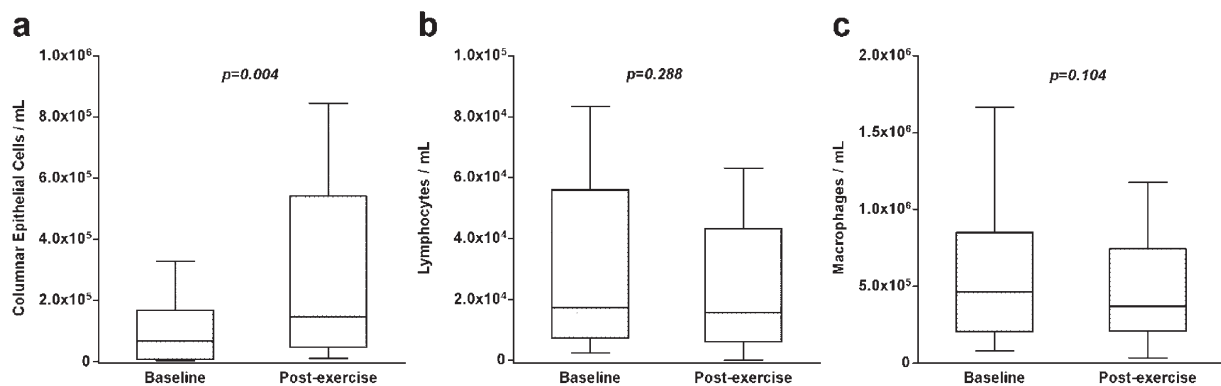


Figure 2. Effects of exercise challenge on the concentrations of columnar epithelial cells (a), lymphocytes (b), and macrophages (c) in persons with asthma and EIB. Comparisons were made between baseline and 30 minutes after exercise separated by an average of 9.9 days (range, 4–18 days). The box plots show the median (line), interquartile range (box), and the 10th and 90th percentiles (whiskers).

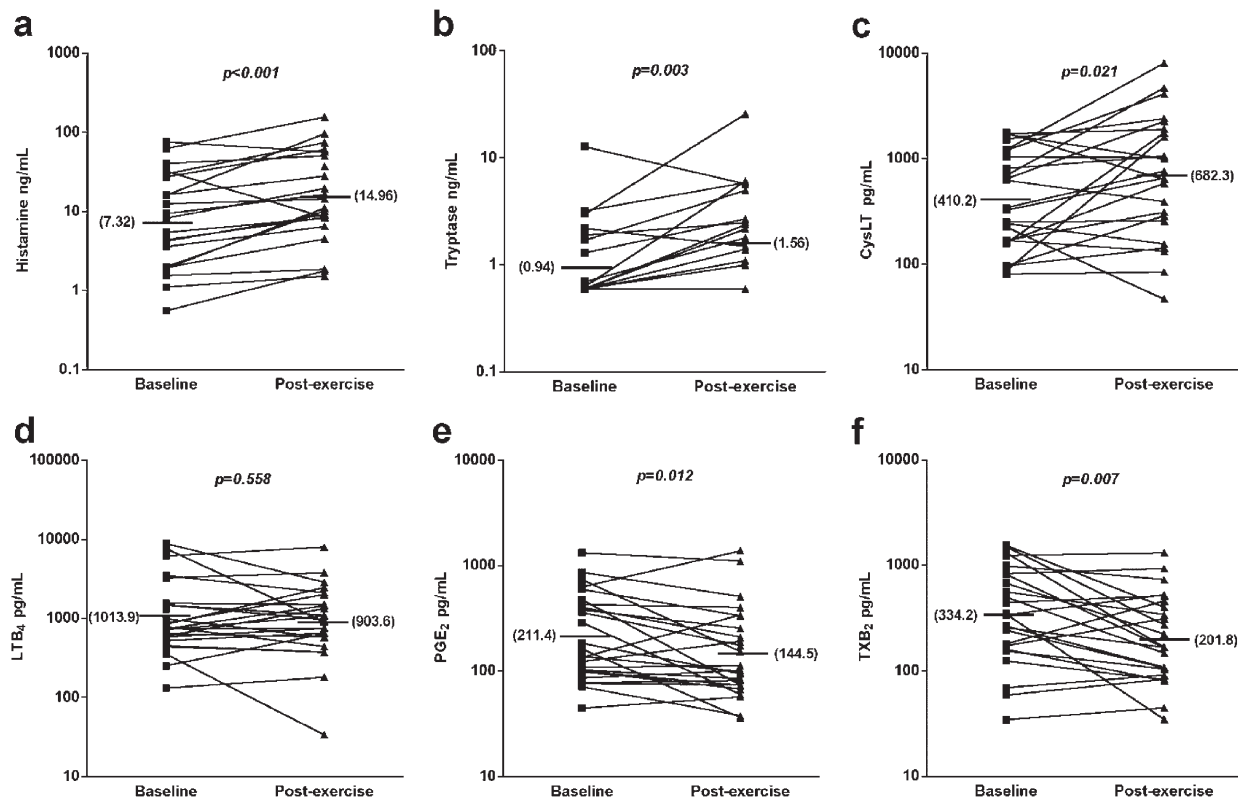


Figure 3. Effects of exercise challenge on the levels of mast cell mediators (*a* and *b*) and eicosanoids (*c–f*) in induced sputum of individuals with asthma with EIB. Comparisons were made between baseline and 30 minutes after exercise challenge on separate days an average of 9.9 days apart (range, 4–18 days). The figures show the individual data points and the geometric mean value. The levels of histamine, tryptase, and CysLTs significantly increased, and PGE₂ and TXB₂ significantly decreased.

(median 13.74% vs. 16.72%, $p = 0.24$) between placebo and treatment.

The levels of histamine and CysLTs were lower and there was a trend toward a decrease in the level of tryptase after exercise challenge during treatment with montelukast and loratadine as compared with placebo (Figure 7). The ratio of CysLTs to PGE₂ was lower during treatment with montelukast and loratadine compared with placebo (median 4.95 vs. 7.54, $p = 0.003$). No differences were observed in the levels of LTB₄, PGE₂, and TXB₂ between treatment and placebo. No differences were observed in the levels of IL-6 (16.9 vs. 26.1 pg/ml, $p = 0.15$), IL-8 (1,240 vs. 1,528 pg/ml, $p = 0.84$), and vascular endothelial growth factor (414.2 vs. 594.9 pg/ml, $p = 0.13$) between treatment and placebo.

The levels of histamine (geometric mean 9.33 ng/ml vs. 7.56 ng/ml, $p = 0.16$), CysLTs (geometric mean 373.25 pg/ml vs. 423.64 pg/ml, $p = 0.59$), LTB₄ (geometric mean 1,061.70 pg/ml vs. 1,002.30 pg/ml, $p = 0.65$), and PGE₂ (geometric mean 177.42 pg/ml vs. 218.27 pg/ml, $p = 0.23$) in induced sputum during treatment with montelukast and loratadine were no different than the baseline induced sputum. The concentration of tryptase was increased (geometric mean 1.21 ng/ml vs. 0.94 pg/ml, $p = 0.04$) and the concentration of TXB₂ was decreased (geometric mean 225.42 pg/ml vs. 356.45 pg/ml, $p = 0.05$) during treatment with montelukast and loratadine compared with the baseline induced sputum.

DISCUSSION

Exercise-induced bronchoconstriction occurs after a brief period of vigorous exercise as a result of conditioning the inspired air,

which leads to both drying and cooling of the conducting airways in temperate climates (3, 4). Because the subsequent events in the airways remain poorly defined, there is ongoing debate about the mechanism of EIB. To characterize the airway inflammatory responses during EIB, we measured the concentrations of airway cells, mast cell mediators, and eicosanoids in induced sputum at baseline and 30 minutes after exercise challenge in subjects with asthma with EIB. A randomized, double-blind crossover study was conducted to determine the effect of a combination of CysLT₁ and histamine antagonists on the airway events occurring during EIB. This study demonstrates in human subjects with asthma and EIB that exercise challenge initiates the release of mediators from mast cells and other airway cells, which occurs simultaneously with release of columnar epithelial cells into the airways. The levels of CysLTs and histamine in the airways are associated with the concentration of columnar epithelial cells released into the airways. The combination of CysLT₁ and histamine antagonists inhibits the release of histamine and CysLTs into the airways, but does not prevent the release of columnar epithelial cells into the airways.

Our hypothesis was that exercise challenge in susceptible individuals leads to the release of mediators such as histamine and tryptase from mast cells, eicosanoids from a variety of airway cells including mast cells, eosinophils, and the airway epithelium (4). Release of eicosanoids into the airways occurs in animal models of EIB (8, 9) and after isocapnic hyperventilation in human subjects with asthma (10). Whether these findings are applicable to EIB has remained controversial because studies of BAL fluid after the development of EIB in humans have failed to demonstrate the release of mast cell mediators and

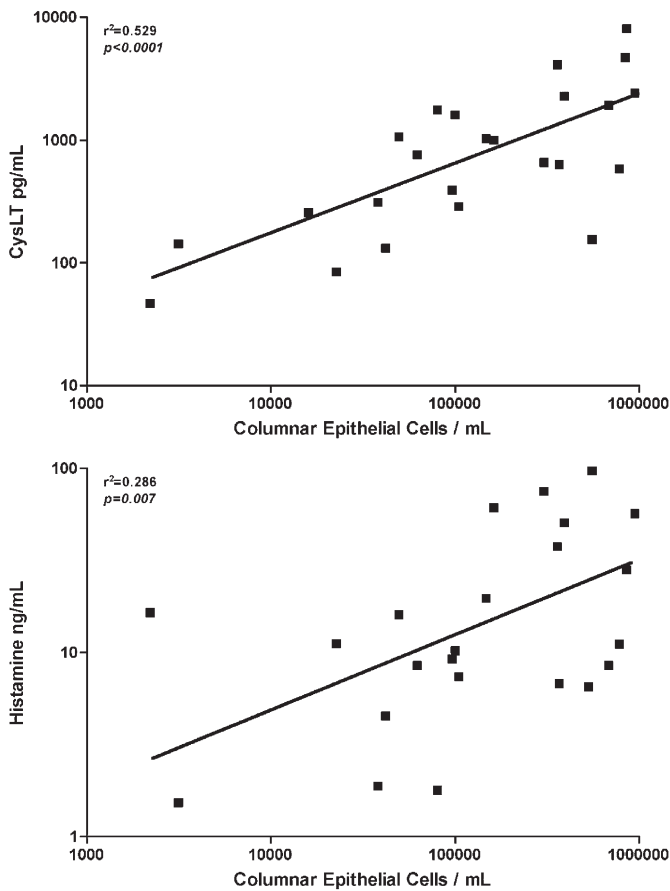


Figure 4. Relationships between the concentrations of CysLTs (*upper panel*) and histamine (*lower panel*) and the concentration of epithelial cells in induced sputum after exercise challenge in individuals with asthma with EIB.

eicosanoids into the airways after exercise challenge (11, 22). Because EIB is initiated by drying and cooling of the airways, which causes bronchoconstriction in the segmental airways (14), these studies may have been unable to identify the relevant airway events because BAL fluid is collected in the distal airways. Our study was conducted using induced sputum, which provides a sample from the conducting airways (15), and shows that mast cell mediators, histamine, and tryptase are released in the airways during EIB; CysLTs, products of mast cells and other airway cells, are also generated during EIB. These findings, together with histologic evidence of mast cell degranulation after exercise challenge (12), indicate that mast cell activation occurs during EIB. Alternatively, basophils could contribute to release of both histamine and tryptase, although basophils only contain about 1% of the tryptase content of mast cells (23). Evidence of a mast cell mechanism is also supported by measurements of mediators in the peripheral blood and urine. Some studies have shown an increase in plasma histamine after EIB, although a number of studies conflict with this result (24). Similarly, some studies have identified an increase in CysLTs (25, 26) and the mast cell prostaglandin PGD_2 (27) in the urine after the development of EIB, although not all studies have concurred (28). The decrease in PGE_2 identified may be critical because an alteration in the balance between CysLTs and PGE_2 favors bronchoconstriction in the period after exercise. Inhaled PGE_2 has been shown to reduce the severity of EIB (29). The airway epithelium

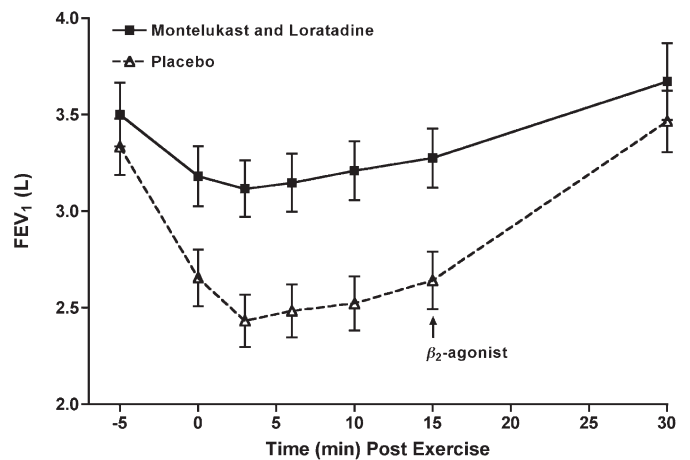


Figure 5. FEV₁ (mean $\pm$ SEM) before and after exercise challenge after two doses of montelukast and loratadine or two matched placebos 36 and 12 hours before exercise challenge in a randomized, double-blind crossover study. Treatment with montelukast and loratadine significantly reduced the severity of EIB ($p < 0.001$).

is a major source of PGE_2 which inhibits mast cell activation and relaxes airway smooth muscle (30). Injury to the airway epithelium reduces the synthesis of PGE_2 , favoring production of CysLTs through transcellular cooperation leading to an increased ratio of CysLTs to PGE_2 and bronchoconstriction (31).

We did not identify an early increase in LTB_4 in the airways; however, these data do not exclude the possibility that LTB_4 may be released at a latter time point, contributing to neutrophil recruitment to the airways (13). An increase in LTB_4 in the peripheral blood was recently identified 60 minutes after exercise (26). Studies in the canine model show an increase in LTB_4 in the airways after repeated challenges of the airways with dry air (32). It is unlikely that TXB_2 is an important mediator of EIB, because TXB_2 levels decreased in the airways after exercise challenge. Although one study suggested that a thromboxane inhibitor reduced the severity of EIB (33), a number of other studies have shown no benefit (34). An important distinction between the findings in the present study and the findings in the canine model of EIB is that CysLTs, LTB_4 , PGE_2 , and TXB_2 all increase in the airways in response to dry air challenge in the canine model (35), whereas in human subjects with asthma an increase in CysLTs and a reduction in PGE_2 and TXB_2 occurs.

An increase in columnar epithelial cells released into the airways occurs in response to dry air challenge in unsensitized guinea pigs and canines (8, 9), in response to isocapnic hyperpnea in human subjects with asthma (10) and in response to exercise challenge in individuals with asthma with EIB in the present study. In the canine model, dry air challenge induces histologic evidence of injury and increased permeability of the airway epithelium (36). These data suggest that injury of the airway epithelium occurs in response to exercise challenge in individuals with asthma with EIB. The concentration of epithelial cells released into the airways was correlated with the magnitude of eicosanoid release in response to dry air challenge in canines (35) and associated with the levels of CysLTs and histamine in the present study, suggesting that release of epithelial cells is involved in the pathogenesis of EIB. However, the increase in epithelial cells is only weakly correlated with the increase in airway resistance in the canine model (35), and not associated with the severity of bronchoconstriction induced by isocapnic

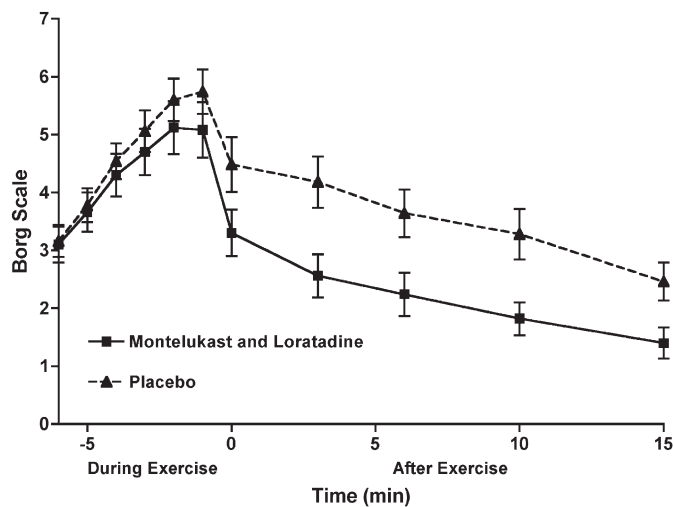


Figure 6. Symptoms of dyspnea (mean $\pm$ SEM) according to the Borg scale (0–10) during the final 6 minutes of exercise at greater than 85% of maximum heart rate and over the 15 minutes after exercise challenge in persons with asthma and EIB. After exercise challenge there was a significant reduction in symptoms of dyspnea at all time points during treatment with montelukast and loratadine ($p < 0.01$).

hyperventilation (10) or with severity of EIB in the present study. Leukotriene inhibition markedly reduced the severity of bronchoconstriction in the canine model (9) and in the present study without reducing the number of epithelial cells released into the airways.

Chronic epithelial injury may be an important factor mediating the susceptibility to EIB. Measures of airway permeability have previously been associated with the severity of EIB (37). We identified a relationship between the percentage of epithelial cells in the baseline induced sputum and the severity of EIB, but the magnitude of this association was small. The magnitude of this association may be small because the number of columnar epithelial cells in induced sputum is an imprecise measure of epithelial disruption, or simply indicate that factors other than epithelial disruption are more strongly associated with EIB. Epithelial remodeling was previously described in cold weather athletes who developed asthma-like symptoms and bronchial hyperresponsiveness after repeated bouts of exercise in cold dry air (38), and occurs after repeated challenges with dry air in canine airways (32).

Although prior reports have associated eosinophilic airway inflammation with the severity of EIB (39), airway eosinophilia may not be essential for the development of EIB. Airway neutrophilia is prominent in these patients with EIB, a finding noted previously in athletes with and without respiratory symptoms (40). The generation of IL-8 from epithelial cells is increased after cooling and hyperosmolar stimuli *in vitro* (41); however, an increase in IL-8 production after exercise was not identified in the present study, possibly because 30 minutes was too early

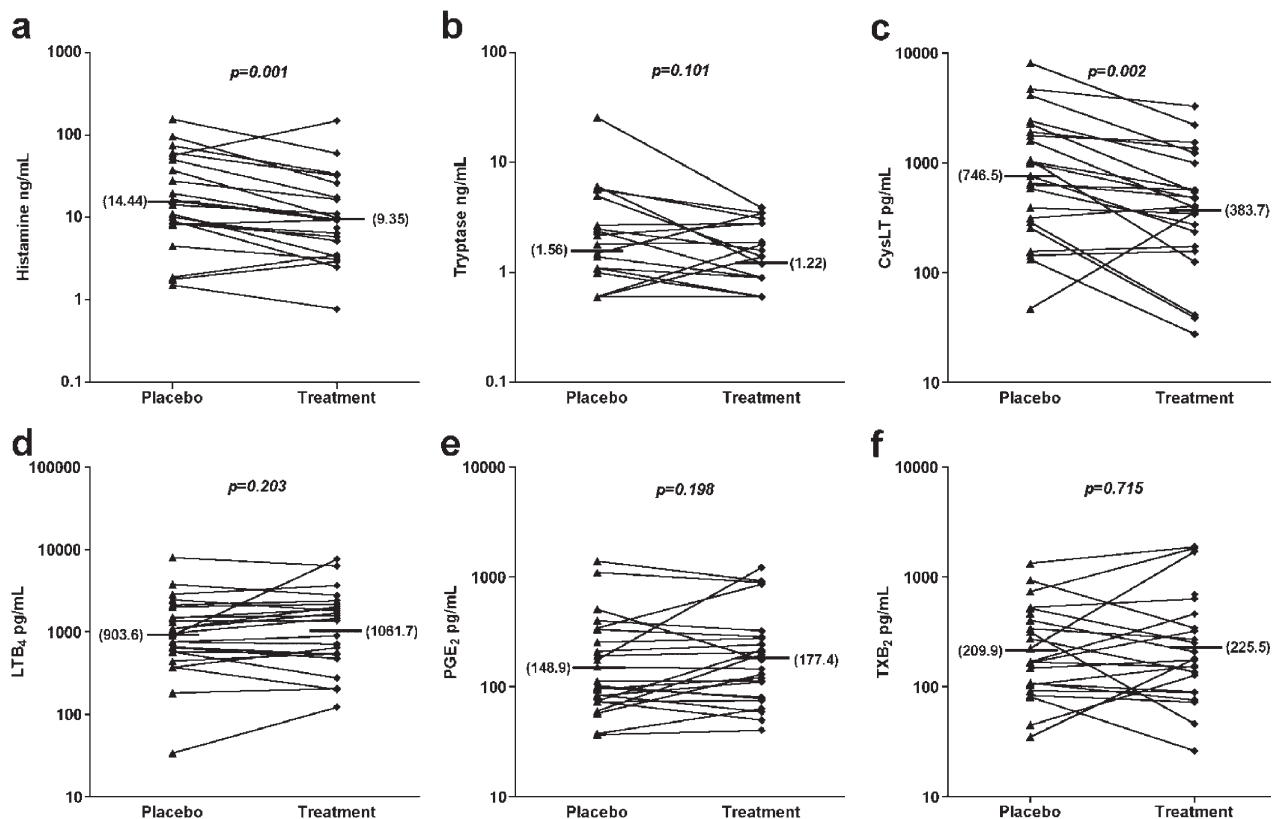


Figure 7. Effects of treatment with montelukast and loratadine on the levels of mast cell mediators (a and b) and eicosanoids (c–f) in induced sputum 30 minutes after exercise challenge in individuals with asthma with EIB. Comparisons were made between treatment with montelukast and loratadine as compared with administration of two matched placebos an average of 6.8 days apart (range, 4–14 days). The figures show the individual data points and the geometric mean value. The levels of histamine and CysLTs significantly decreased.

for such protein production to be measurable. The decrease in the percentage of airway lymphocytes and macrophages after exercise challenge may indicate that airway lymphocytes are activated during exercise challenge. A similar decrease in the concentration of lymphocytes and macrophages in BAL fluid also occurs in response to dry air hyperpnea in the canine model of EIB (9). We previously identified an increase in activated T-helper cells in the peripheral blood after exercise challenge in individuals with asthma (42).

These data indicate that short-term treatment with a combination of CysLT₁ and histamine antagonists is highly effective in reducing the severity of EIB. Two recent studies evaluating short-term administration of a CysLT₁ antagonist with or without the concurrent administration of loratadine showed that there was little additional effect of the combination treatment over the CysLT₁ antagonist alone (43, 44). The modest additional benefit of adding a histamine antagonist for EIB is in contrast to *in vitro* and clinical studies of allergen-induced bronchoconstriction, which show an additive benefit of CysLT₁ and histamine antagonists (45). These results do not exclude a beneficial role of histamine antagonists in the management of EIB. Histamine was elevated in the airways during EIB in this study and histamine antagonists alone reduce the severity of EIB, especially EIB of modest severity (46). Histamine is released as a preformed mediator, whereas the CysLTs are newly generated in response to a stimulus (47). This suggests that histamine is important in the initiation of bronchoconstriction and that CysLTs are important in sustaining bronchoconstriction, a concept that is supported by the findings in a model of EIB using a hyperosmolar mannitol solution (48).

Pretreatment with a combination of CysLT₁ and histamine antagonists reduced the levels of histamine and CysLTs, indicating that these drugs may inhibit mast cell activation, and demonstrating *in vivo* findings identified *in vitro* for both CysLT₁ and histamine antagonists. The CysLT₁ receptor is present on mast cells, and inhibition of the CysLT₁ receptor prevents activation of mast cells (49, 50). Recent evidence indicates that montelukast also inhibits the enzyme 5-lipoxygenase, although the IC₅₀ was one order of magnitude higher than the estimated tissue concentrations of the drug (50). Several *in vitro* studies also demonstrate that antihistamines inhibit histamine and CysLT release from mast cells (51). Alternatively, inhibition of airway inflammation could be responsible for these findings (52, 53).

This study was designed to clarify the pathogenesis of EIB. The results show that mast cell activation occurs during EIB and the balance of bronchoconstricting CysLTs and histamine to bronchodilating PGE₂ is altered, favoring bronchoconstriction in the period after exercise challenge in subjects with asthma with EIB. Injury to the airway epithelium may be a predisposing factor for the development of EIB. Release of columnar epithelial cells into the airways occurs during EIB and is associated with the release of CysLTs and histamine into the airways. Treatment with CysLT₁ and histamine antagonists reduces the severity of EIB and decreased the release of CysLTs and histamine into the airways.

Conflict of Interest Statement: T.S.H. has given lectures (2001–2004) in sessions at regional and national meetings, which were funded in part by Merck & Co., Inc. and was given the placebo tablets for montelukast for this study by Merck & Co. through a medical school grant awarded to the University of Washington; M.W.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.M.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; L.B.S. receives royalties from Pharmacia Diagnostics for the UniCAP Tryptase Assay; W.R.H. has given lectures (2001–2004) in sessions at regional, national, and international meetings, which were funded in part by Merck & Co., Inc. and received research support from a Merck & Co. medical school grant awarded to the University of Washington in 2002; M.L.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Online Data Supplement

Inflammatory Basis of Exercise-induced Bronchoconstriction

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Subjects

Subjects 12-59 years of age were recruited for this study who had a physician diagnosis of asthma for ≥ 1 year, symptoms of EIB, used only an inhaled short-acting β_2 -agonist for asthma treatment and had a $\geq 15\%$ decrease in forced expiratory volume in 1 second (FEV_1) following a standardized exercise challenge. Excluded were subjects who had a $FEV_1 \leq 65\%$, smoked cigarettes within the prior year or ≥ 7 pack-year smoking history, been treated for acute asthma within the prior month, been hospitalized for asthma within the prior 3 months, a history of life threatening asthma, or used an inhaled corticosteroid, leukotriene modifier, long-acting antihistamine, cromone, or long-acting β_2 -agonist in the 30 days prior to the study. The study protocol was approved by the University of Washington institutional review board, and written informed consent and, when applicable, assent was obtained from all participants.

Study Protocol

Each subject had a total of 4 study visits separated by 4-10 days. The visits were conducted at the same time of day for each subject. Subjects were asked to avoid physical activity and caffeinated beverages for 6 hours prior to each study visit. Short-acting antihistamines and β_2 -agonist were permitted during the trial except for 48 hours and 6 hours prior to each visit respectively. The first visit consisted of a physical examination, spirometry, and exercise challenge to determine study eligibility. The second visit consisted of an induced sputum and spirometry. The third and fourth visits each consisted of an exercise challenge followed by an induced sputum 30 min after the end of exercise. Subjects received 2 doses of montelukast 10 mg and loratadine 10 mg

orally or 2 matched placebos 36 and 12 hours prior to visits 3 and 4 in a randomized, double-blind, crossover design.

Spirometry and Exercise Challenge

Spirometry and exercise challenge were conducted according to American Thoracic Society (ATS) standards (E1, E2). Exercise challenge was performed on a motorized treadmill such that each subject sustained $\geq 85\%$ of their maximum heart rate for the final 6 min of exercise (E1). Subjects wore nose clips and breathed dry air (0% RH, 22°) delivered from a weather balloon reservoir through a one-way valve (Hans Rudolph, Kansas City, MO, USA) during exercise. The incline and speed of the treadmill required to sustain $\geq 85\%$ of maximum heart rate on the first visit was subsequently repeated during visits 3 and 4. Spirometry was conducted 20 and 5 min before each *exercise challenge, and repeated at 0, 3, 6, 10, 15, and 30 min after the end of exercise. The better of at least 2 FEV₁ maneuvers within 5% of each other was recorded at each time point. The pre-exercise FEV₁ was taken as the average of the 2 spirometry tests recorded before exercise. Symptoms of dyspnea were recorded on a visual analog scale (Borg scale) every minute during the exercise challenge and 0, 3, 6, 10, and 15 min after exercise challenge.

Sputum Induction

Induced sputum was conducted using hypertonic saline (3%) via an ultrasonic nebulizer (DeVilbiss, Somerset, PA, USA) for 12 min as previously described (E3). Subjects were premedicated with 180 µg of albuterol via a metered dose inhaler 15 min

before the procedure. At 2-minute intervals, subjects were asked to clear saliva from their mouth and then expectorate sputum. The induced sputum was placed on ice immediately and processed within 30 min of collection.

Induced Sputum Processing and Analysis

Following the addition of an equal volume of DTT 0.1% (Calbiochem, La Jolla, CA, USA), the induced sputum sample was mixed gently with a vortex mixer and placed in a shaking water bath at 37°C for 15 min. A plastic transfer pipette was used periodically to further mix and disperse the sample. Total cell count was determined with a hemocytometer on a 1 mL portion of the sample and slides for differential cell counts were prepared with a cytocentrifuge. Slides were stained (Hema 3, Fisher Diagnostics, Middletown, VA, USA) and at least 400 non-squamous cells counted per slide. The remaining dispersed induced sputum sample was centrifuged at 250 *g* for 10 min and the supernatant removed from the cell pellet. The supernatant was centrifuged a second time at 4,000 *g* for 20 min and the supernatant removed. A portion of the supernatant was treated for eicosanoid analysis by the addition of 4 volumes of methanol, precipitated for 5-min on ice and centrifuged at 300 *g* for 20 min (E4). The supernatant was then evaporated to near dryness in a speed-vac (ThermoSavant, Holbrook, NY, USA) at 0.7 torr, resuspended in a 1:1 mixture of methanol and distilled water, and stored at –80°C for later analysis. The supernatant not used for the eicosanoid analysis was separated into 2 portions stored immediately at –80°C for later analysis, one containing protease inhibitors PMSF (100 µM final) and EDTA (5 mM final) (Sigma-Aldrich, St. Louis, MO, USA). A portion of the supernatant for tryptase analysis was

brought to 1 M NaCl by addition of 5 M NaCl, vortexed and centrifuged at 10,000 *g* for 30 min. Total tryptase concentration was measured by ELISA as described (E5). The concentrations of mediators were determined by ELISA for histamine (Beckman Coulter, Fullerton, CA, USA), and CysLTs, leukotriene (LT)₄, Prostaglandin (PG)_{E2} and thromboxane (TX)_{B2} (Cayman Chemical, Ann Arbor, MI, USA). The concentrations of IL-4, IL-5, IL-6, IL-8, TNF- α , VEGF, and RANTES were determined by protein multiplex assay, and the concentration of IL-13 was determined by ELISA (R&D Systems, Minneapolis, MN, USA).

Statistical Analysis

Baseline characteristics of the study participants were expressed as the mean and SD. The area under the FEV₁/time curve (AUC) (E6) quantified the severity of EIB over 0-30 min after exercise (AUC₃₀) during the screening visit and over 0-15 min after exercise (AUC₁₅) during the following study visits. An ANOVA model was used to assess the effects of treatment and determine if there were carry-over effects of treatment according to the sequence of randomization (E7). The medians of differential cell counts were compared between different conditions with the Wilcoxon signed-rank test. The levels of inflammatory mediators in induced sputum were compared between different conditions with a paired t-test of the log-transformed values. Only complete pairs of samples were considered in the analysis, therefore, the number of samples varied from 23 to 25 pairs. The relationship of cellular constituents and inflammatory mediators to each other and to severity of EIB was assessed after log transformation using Pearson's correlation coefficient.

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