Leukotriene E_4 induces airflow obstruction and mast cell activation through the cysteinyl leukotriene type 1 receptor

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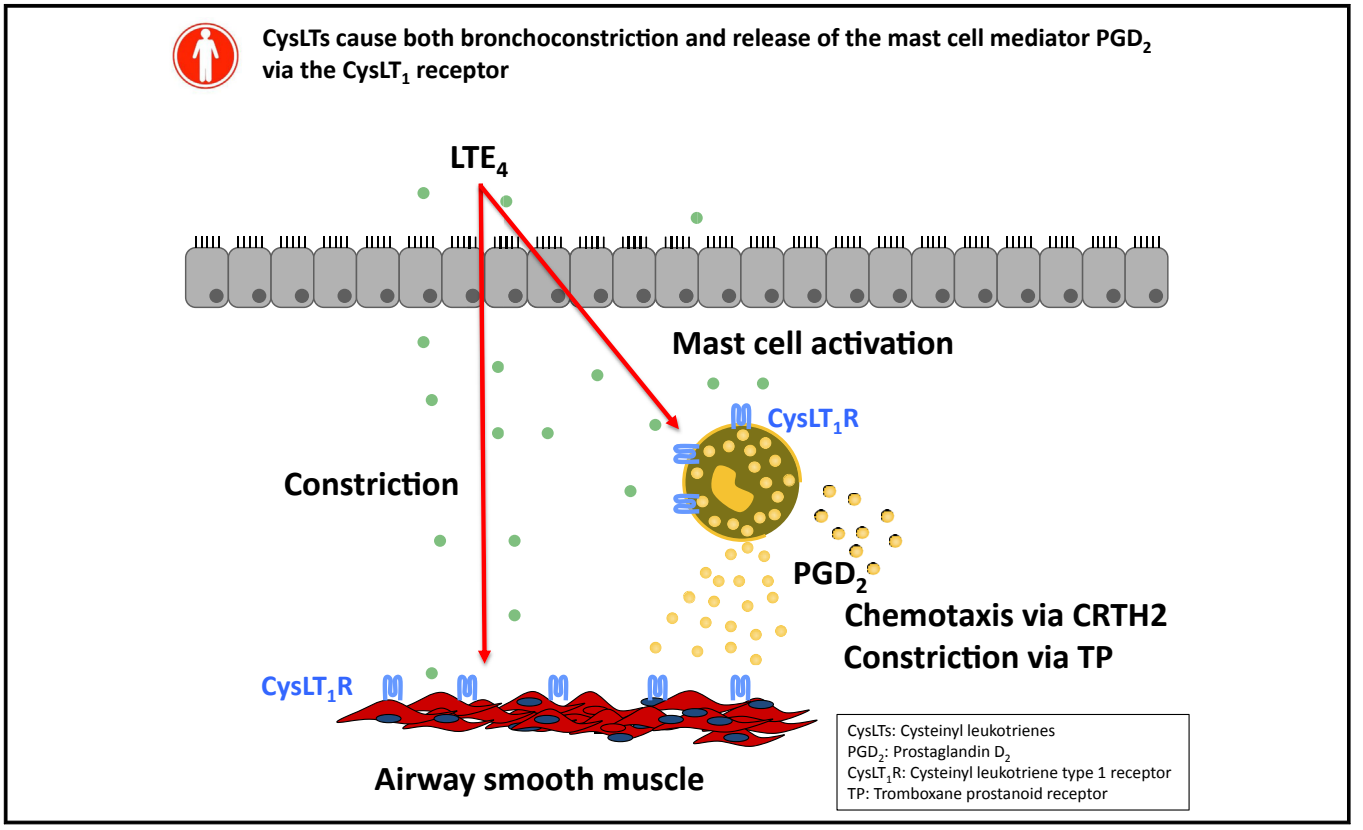
GRAPHICAL ABSTRACT



Background: Leukotriene (LT) E_4 is the final active metabolite among the cysteinyl leukotrienes (CysLTs). Animal studies have identified a distinct LTE_4 receptor, suggesting that current cysteinyl leukotriene type 1 ($CysLT_1$) receptor antagonists can provide incomplete inhibition of CysLT responses.

Objective: We tested this hypothesis by assessing the influence of the $CysLT_1$ antagonist montelukast on responses induced by means of inhalation of LTE_4 in asthmatic patients.

Methods: Fourteen patients with mild intermittent asthma and 2 patients with aspirin-exacerbated respiratory disease received

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20 mg of montelukast twice daily and placebo for 5 to 7 days in a randomized, double-blind, crossover study (NCT01841164). The PD_{20} value was determined at the end of each treatment period based on an increasing dose challenge. Measurements included lipid mediators in urine and sputum cells 4 hours after LTE_4 challenge.

Results: Montelukast completely blocked LTE_4-induced bronchoconstriction. Despite tolerating an at least 10 times higher dose of LTE_4 after montelukast, there was no difference in the percentage of eosinophils in sputum. Urinary excretion of all major lipid mediators increased after LTE_4 inhalation. Montelukast blocked release of the mast cell product prostaglandin (PG) D_2, as well as release of $PGF_{2\alpha}$ and thromboxane (Tx) A_2, but not increased excretion of PGE_2 and its metabolites or isoprostanes.

Conclusion: LTE_4 induces airflow obstruction and mast cell activation through the $CysLT_1$ receptor. (J Allergy Clin Immunol 2018;142:1080-9.)

Key words: *Asthma, cysteinyl leukotrienes, receptors, bronchoconstriction, mast cells, sputum cells, leukotrienes, mass spectrometry, lipid mediators in urine*

The cysteinyl leukotrienes (CysLTs; leukotriene [LT] C_4, LTD_4, and LTE_4) are biosynthesized in mast cells and other inflammatory cells and contribute to the pathobiology of asthma by producing bronchoconstriction, increased vascular permeability, inflammatory cell infiltration, mucus production, and smooth muscle proliferation.[1] Although LTC_4 and LTD_4 cause bronchoconstriction and many of the proinflammatory effects in human subjects through activation of the cysteinyl leukotriene type 1 ($CysLT_1$) receptor that is blocked by clinically used antagonists, such as montelukast, the mode of action of LTE_4 in asthmatic patients remains unclear. For example, the relative bronchoconstrictive potency of LTE_4 compared with histamine, methacholine, and the other CysLTs has been reported to be greater in asthmatic patients than in healthy subjects,[2,3] although its absolute potency was lower than those of LTC_4 and LTD_4.[4,5] Furthermore, studies in genetically modified mice have demonstrated a receptor that is distinctly sensitive to LTE_4[6] and mediates mucin secretion in the trachea[7] and vascular permeability in the skin.[6] Taken together, the possibility of a distinct E-type receptor in human airways has gained recognition as a potential new target for the treatment of asthma. However, there are no studies in human subjects confirming the presence of such a receptor.

Therefore in this randomized, placebo-controlled, crossover study we set out to characterize the effects of inhaled LTE_4 in asthmatic patients with a particular focus on whether the $CysLT_1$ receptor was involved. Accordingly, the primary aim of the investigation was to establish the effect of the intervention with the prototype $CysLT_1$ receptor antagonist montelukast on bronchial responsiveness to increasing dose challenges with inhaled LTE_4. The secondary aim of the study was to determine the influence of montelukast intervention on airway inflammation after LTE_4 inhalation. This was assessed based on sputum cell counts made after provocations once treatment with placebo or active drug had occurred. We hypothesized that if LTE_4 was inducing airway inflammation through a different receptor than $CysLT_1$, its effects on sputum cells would be dose dependent for LTE_4 but independent of whether treated with placebo or montelukast.

Abbreviations used
AERD: Aspirin-exacerbated respiratory disease
CysLT: Cysteinyl leukotriene
$CysLT_1$: Cysteinyl leukotriene receptor type 1
FENO: Fraction of exhaled nitric oxide
IOS: Impulse oscillometry
LT: Leukotriene
PG: Prostaglandin
TX: Thromboxane

Finally, as an exploratory end point, we collected urine during provocations for analysis of lipid mediator excretion. This led to the serendipitous discovery of increased urinary excretion of metabolites of prostaglandin (PG) D_2, as well as several other potent lipid mediators, after inhalation of LTE_4. The findings add a new dimension, namely that LTE_4, in addition to having a direct bronchoconstrictive action, also activates mast cells and other cells to produce secondary responses that can amplify or modify its primary effect.

METHODS

Fourteen patients with mild intermittent asthma according to Global Initiative for Asthma guidelines[8] and 2 patients with aspirin-exacerbated respiratory disease (AERD) were recruited from our clinic and through advertisement. Both patients with AERD had a history of unequivocal severe bronchoconstriction after intake of aspirin-like drugs, and one of them had a positive lysine-aspirin inhalation challenge test result.[9] Inclusion and exclusion criteria and medications are reported in the Methods section in this article's Online Repository at www.jacionline.org.

The Ethical Review Board in Stockholm (Dnr 2011/1016-31/1) and the Swedish Medical Products Agency (Dnr 151:2011/49661) approved the study. The participants provided written informed consent. The study is registered at ClinicalTrials.gov (NCT01841164).

Study design

The study was a double-blind, randomized, placebo-controlled study with a crossover design involving two 5- to 7-day treatment periods during which the subjects received 20 mg of montelukast twice daily and matching placebo, respectively (Fig 1). The dosing and duration of treatment was selected from published data on montelukast in LTD_4 challenges.[10]

At screening, patients' baseline characteristics and airway sensitivity to methacholine and inhaled LTE_4 were obtained (Table I). A washout period of 7 to 14 days preceded the start of the crossover phase during which inhalation challenge with LTE_4 was repeated on the last day of each period. Urine samples were collected before, during, and after the end of the challenge at hourly intervals for up to 4 hours. For analysis of circulating white blood cells, venous blood samples were drawn before LTE_4 inhalation and at 5 minutes after the last dose. Sputum induction was performed 4 hours after the end of the LTE_4 challenge.

Lung function tests, fraction of exhaled nitric oxide measurement, skin prick tests, and sputum induction

Lung function, including spirometry and impulse oscillometry (IOS), measurement of fraction of exhaled nitric oxide (FENO), skin prick tests, and sputum induction were performed as outlined in the Methods section in this article's Online Repository.

Drugs and materials

The $CysLT_1$ receptor antagonist montelukast (Singulair; Merck Sharp & Dohme, Stockholm, Sweden) was purchased from the Karolinska University

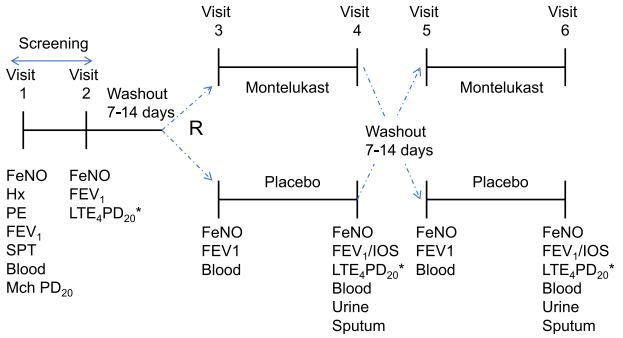

FIG 1. Study design. *Inhalation challenge with LTE_4. *Mch,* Methacholine; *SPT,* skin prick test.

Hospital Pharmacy, and matching placebo was obtained from Recipharm Pharmaceutical Development AB (Stockholm, Sweden). High-purity LTE_4 for human use was purchased from Cayman Chemicals (Ann Arbor, Mich). For safety reasons, LTE_4 solutions were dispersed in color-coded vials in 10-fold increasing concentrations from 0.042 to 4200 μmol/L (see the Methods section in this article's Online Repository).

Bronchoprovocation

LTE_4 was administered for inhalation after nebulization by using a dosimeter-controlled jet nebulizer (Spira Elektro 2; Respiratory Care Center, Hameenlinna, Finland), with pulmonary function measured as FEV_1. IOS measurements were done before and 5 minutes after the end of the challenge. Challenges were performed in the morning and started by inhalation of the diluent. Provided FEV_1 was stable (ie, not deviating >10%), the postdiluent FEV_1 value was used as a baseline value. Half-log dose increments of LTE_4 solution were inhaled every 10 minutes, and FEV_1 was obtained after each dose increment. Provocation was terminated when FEV_1 had decreased by 20% from the baseline value or the maximum dose of LTE_4 was reached. The PD_{20} value was derived by means of linear interpolation from the log-cumulated dose-response curve. Bronchial hyperresponsiveness to methacholine was assessed with a similar protocol but with dose increments every third minute in doubling doses (88.6-45,274 nmol). For the 2 patients with AERD, asthma medications were withheld 24 hours before provocation. The protocols for the provocations are in Tables E3 and E4 in this article's Online Repository at www.jacionline.org.

Measurement of urinary metabolites

Urine was stored at −70°C until assay. COX metabolites of arachidonic acid, LTE_4, and isoprostanes were measured by using in-house ultraperformance liquid chromatography/tandem mass spectrometry method.[11] Mass spectrometry measurements of PGD_2 metabolites were compared with the enzyme immunoassay for 11β-$PGF_{2\alpha}$ (Cayman Chemical), as described previously.[12]

Urinary samples from the validation cohort

Urinary samples collected during a previous bronchoprovocation study with LTD_4 in healthy subjects and patients with asthma were analyzed.[13] Samples had been biobanked at −20°C for 12 years.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 6.0c software (GraphPad Software, La Jolla, Calif). PD_{20} FEV_1 values for LTE_4 and FENO values were transformed logarithmically, data were presented as geometric means and ranges, and repeated-measures ANOVA was performed. Correlations were performed with Pearson correlation on log-transformed data. Because of nonnormal distribution, data from IOS measurements and total sputum cell counts were presented as medians with ranges, and the Wilcoxon matched-pairs signed rank test was used for analysis. Paired *t* tests were used to determine changes in percentages of blood differential cell counts, as well as changes in levels of urinary metabolites.

RESULTS

Effects of montelukast on baseline lung function and FENO values

There was no difference in prechallenge baseline lung function and FENO values between the 2 treatment periods (see Table E5 in this article's Online Repository at www.jacionline.org).

Effects of montelukast on airway response to inhaled LTE_4

Montelukast protected effectively against LTE_4-induced bronchoconstriction: mean maximal FEV_1 decrease was 0.45% (95% CI, −3% to 2.1%) with montelukast compared with 26.3% (95% CI, −23% to −29.6%) after placebo ($P < .001$) and 28.7% (95% CI, −25.3% to −32%) at screening (Fig 2, *A*). After treatment with montelukast, all subjects tolerated the highest cumulated dose of inhaled LTE_4 (geometric mean, 380,277 pmol), which was, on average, 10-fold higher compared with placebo (geometric mean, 37,006 pmol; $P < .001$; Fig 2, *B*). There was no significant difference in LTE_4 PD_{20} between screening and placebo treatment (geometric mean, 23,610 vs 20,923 pmol; Fig 2, *B*); likewise, Bland-Altman analysis indicated excellent repeatability for the challenge (see Fig E8 in this article's Online Repository at www.jacionline.org). Compared with methacholine, LTE_4 was about 77 times more potent on a molar basis (Table I). Furthermore, LTE_4 was a relatively more potent bronchoconstrictor in the subjects with the lowest airway hyperresponsiveness to methacholine (Fig 2, *C*).

IOS measurements were performed in 11 subjects, including both patients with AERD, to extend the information about the influence of LTE_4 on the airway tree. There was a mean 58.9% change (95% CI, 41.1% to 76.8%) in total airway resistance at 5 Hz after inhalation of LTE_4 with placebo compared with a mean 7.1% change (95% CI, −3.2% to 17.2%) with montelukast ($P < .001$). With respect to the sensitive markers of small-airway obstruction,[14] frequency-dependent resistance and reactance area, there were 7- and 10-fold increases, respectively, after inhalation of LTE_4 in the presence of placebo. This conspicuous small-airway constriction caused by LTE_4 was abolished by montelukast (Fig 3, *A* and *B*). Changes in all measured IOS parameters during the 2 treatment periods are presented in Table E6 in this article's Online Repository at www.jacionline.org.

Effects of montelukast on white blood cell counts

Five minutes after inhalation of the last dose of LTE_4, there was an increase in numbers of circulating white blood cells, which was driven by increased numbers of total lymphocytes and associated with a distinct decrease in numbers of eosinophils, whereas neutrophil and monocyte numbers remained the same (see Fig E1 in this article's Online Repository at www.jacionline.org). However, there was no significant difference between placebo and montelukast with respect to these changes in white blood cell counts (see Fig E1).

Effects of montelukast on sputum cells

Paired data on viable sputum cells were obtained in 9 subjects (including 1 patient with AERD) 4 hours after LTE_4 challenge on the last day of each treatment period. There was no significant difference between placebo and montelukast

TABLE I. Patient characteristics at screening

Patient ID	Age (y)	M/F	FENO (ppb)	FEV_1 (L)	FEV_1 (% predicted)	MCh PD_{20} (nmol)	Screening LTE_4 PD_{20} (nmol)	MCh PD_{20}/LTE_4 PD_{20} ratio
1	45	F	8	2.1	79	4,986	3.8	1,326
2	24	M	68	4.2	86	1,654	54.9	30.2
3	37	F	50	2.9	114	499	39.4	12.7
4	28	F	13	2.8	81	2,565	30.8	83.4
5	38	M	116	4.3	106	916	70.2	13
6	29	M	24	4.3	90	2,378	19.8	120.6
7	46	F	10	2.7	99	3,532	49.4	71.5
8	20	F	11	3.5	95	2,153	31.9	67.4
9	52	F	14	2.6	101	12,188	43.2	282
10	45	F	36	2.4	96	924	8.6	107.3
11	25	F	30	2.9	92	899	11.3	79.5
12	44	F	52	4.1	129	4,281	22.7	188.4
13	38	F	11	2.5	103	2,197	12.6	173.8
14	28	F	48	2.6	93	649	2.9	224
AERD 1	48	M	14	3.8	97	17,206	87.4	197
AERD 2	35	M	21	3.8	79	2,528	55.7	45.4
Mean	36.4		24.2*	3.2	96.3	1,815*	23.6*	76.9
Range	20-52		8-116	2.1-4.3	79-129	499-17,206	2.9-54.9	12.7-1,326

F, Female; *M*, male; *MCh*, methacholine.
*Geometric mean.

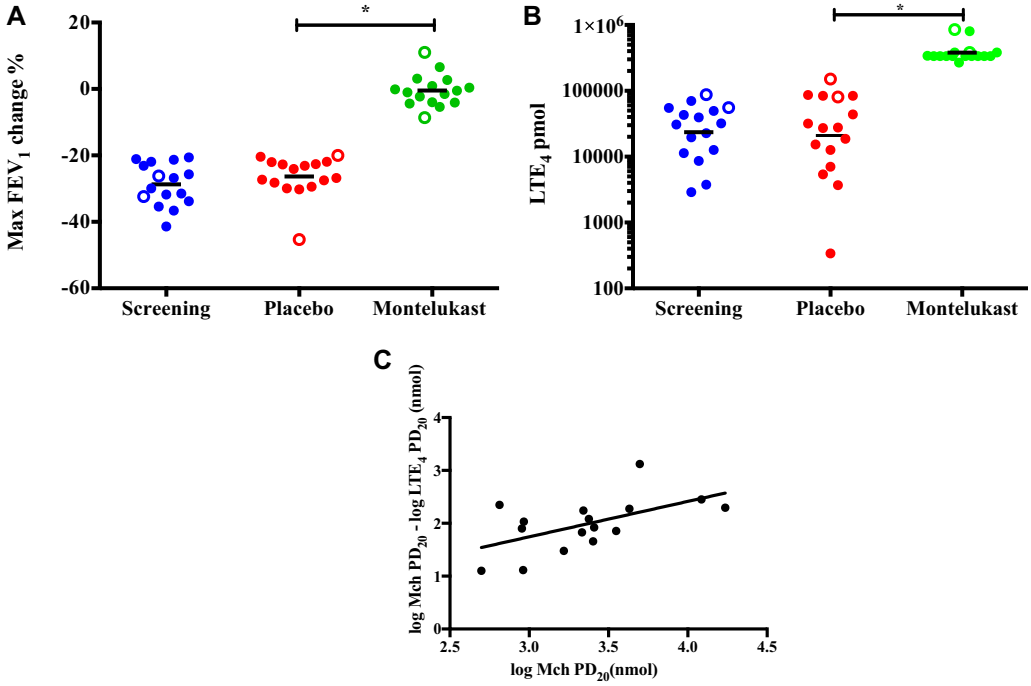

FIG 2. A, Maximal FEV_1 change percentage at screening *(blue plot)* and after treatment with placebo *(red plot)* and montelukast *(green plot). Open circles* represent values for patients with AERD. *Horizontal bars* indicate mean values. **P* < .001 between treatments. **B,** LTE_4 PD_{20} values at screening *(blue plot)* and after treatment with placebo *(red plot)* and total dose of inhaled LTE_4 after treatment with montelukast *(green plot). Open circles* represent values for patients with AERD. *Horizontal bars* indicate geometric mean values. **P* < .001 between treatments. **C,** Relation between airway responsiveness to methacholine (PD_{20}) and relative potency of LTE_4 compared with methacholine (*Mch*; difference in logPD_{20} methacholine and logLTE_4 PD_{20} values). $r = 0.57$, $P = .002$ (Pearson).

with respect to the percentage of eosinophils (mean, 4.5% [95% CI, 1.8% to 7.1%] for placebo vs 3.2% [95% CI, 1.4% to 4.9%] for montelukast; $P = .11$; Fig 3, *C*) or neutrophils (mean, 28.8% [95% CI, 18.5% to 39%] for placebo vs 30.4% [95% CI, 12.9% to 47.9%] for montelukast; $P = .82$), although there was a small increase in the total number of cells (median, 4.27 [interquartile range, 2.4-8.8 $\times$ 10^6/g sputum] vs 5.3 [interquartile range, 3.6-9.9 $\times$ 10^6/g sputum] for placebo and montelukast, respectively; $P = .039$; Fig 3, *D*). Other differential cell counts were also similar between the 2 treatment periods (see Fig E2 in this article's Online Repository at www.jacionline.org).

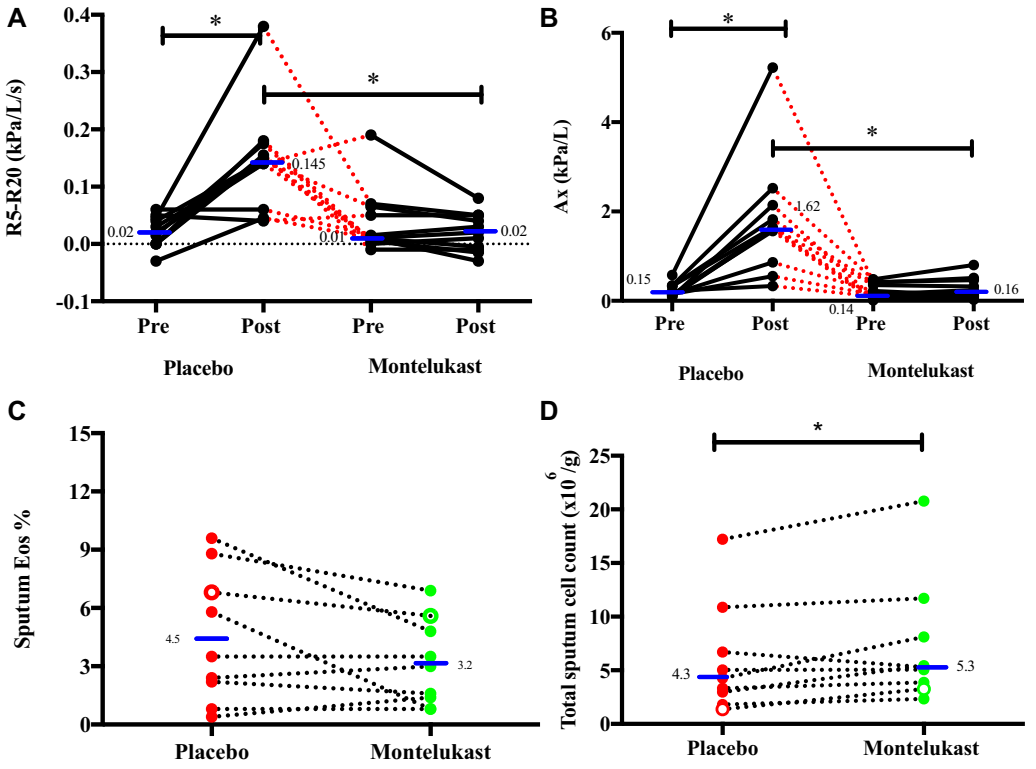

FIG 3. **A** and **B,** Changes in frequency-dependent resistance (R_5–R_{20}; Fig 3, *A*) and reactance area (Ax; Fig 3, *B*) before and 5 minutes after LTE_4 inhalation challenge for both treatment periods. *Horizontal bars* indicate median values. *$P < .01$, Wilcoxon signed rank test. **C** and **D,** Effects of montelukast on sputum eosinophil (*Eos*; Fig 3, *C*) and total sputum cell (Fig 3, *D*) counts from 9 subjects with paired data. The *open circle* represents values in patient number 2 with AERD. *Horizontal bars* indicate median (Fig 3, *C*) and mean (Fig 3, *D*) values. *$P = .039$.

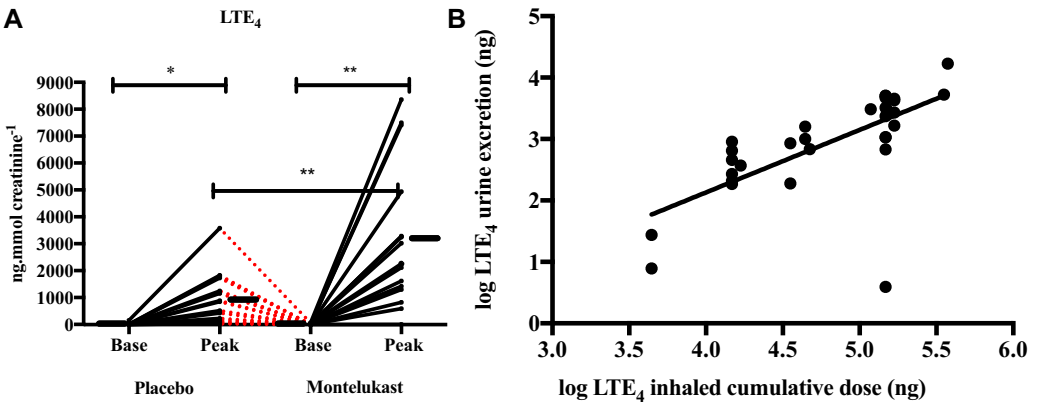

FIG 4. **A,** Urinary excretion of LTE_4 before and after challenge with inhaled LTE_4 during the 2 treatment periods. *Horizontal bars* indicate mean values, with *upper bars* for before versus after values during each period and *lower bars* for comparison between periods. Measurements were performed with ultraperformance liquid chromatography/tandem mass spectrometry, and values are expressed as nanograms per millimole of creatinine *$P < .01$ and **$P < .001$. **B,** Relation between inhaled and excreted LTE_4 (both expressed as nanograms) in the urine for both treatment periods ($r = 0.68$, $P < .0001$ [Pearson]).

Mediator excretion in urine (all data are presented as nanograms per nanomole of creatinine)

LTE_4. As expected, urinary excretion of LTE_4 increased compared with baseline values after inhalation of LTE_4 during both treatment periods (981 ± 240 after placebo vs 15 ± 10 at baseline, $P = .0015$; 3201 ± 645 after montelukast vs 11 ± 5 at baseline, $P = .0002$). Peak excretion during montelukast treatment was significantly greater ($P = .002$; Fig 4, *A*). There was a consistent 2% recovery of inhaled LTE_4 (1.7% on placebo and 2.4% on montelukast) across the range of inhaled concentrations (Fig 4, *B*).

PGD_2 metabolites. Levels of the most abundant metabolite, tetranor-PGDM, increased after LTE_4 inhalation on placebo

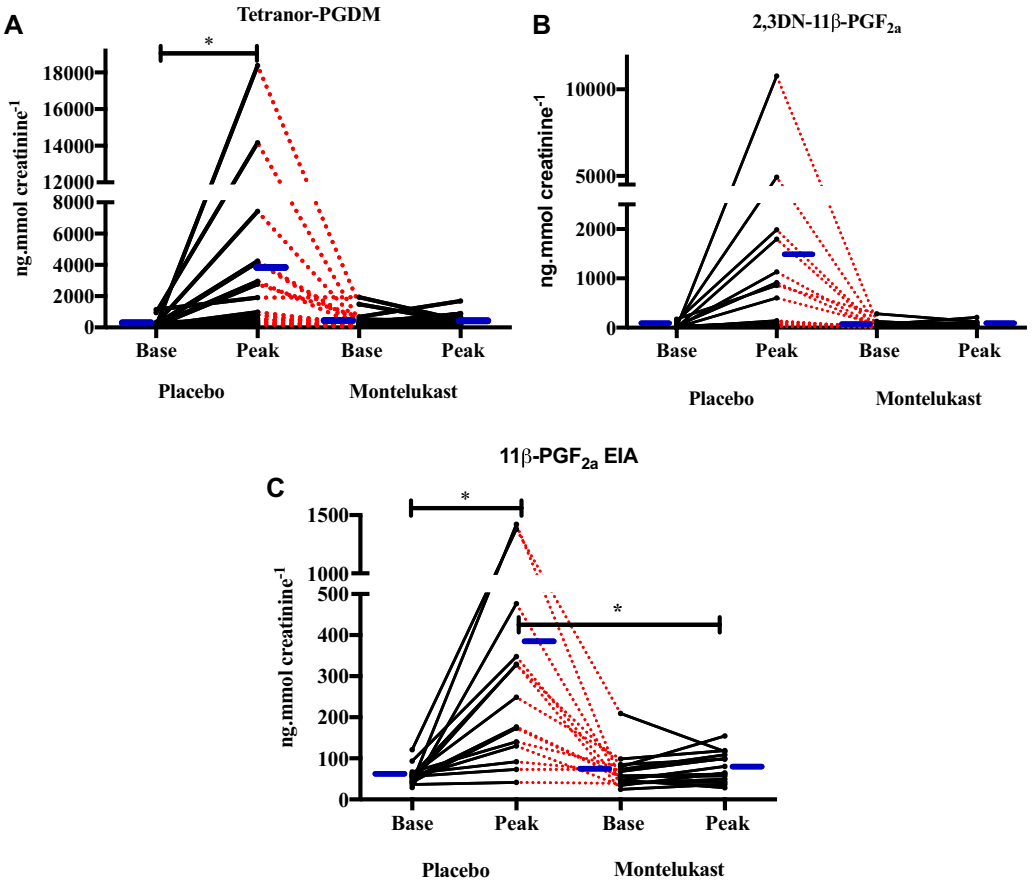

FIG 5. Urinary excretion of the major PGD_2 metabolites, as measured by using ultraperformance liquid chromatography/tandem mass spectrometry (*$P < .05$; **A** and **B**) and an EIA **(C)**. *Horizontal bars* indicate mean values. *$P < .05$. All values are expressed as nanograms per millimole of creatinine.

(3743 ± 1335 vs 272 ± 77 at baseline, $P = .0189$). This increase was completely blocked during montelukast treatment (509 ± 101 vs 418 ± 135 at baseline, not significant; Fig 5, *A*). The early metabolite of the mast cell mediator PGD_2, 2,3-dinor-11β-$PGF_{2\alpha}$, showed an identical pattern (Fig 5, *B*), although the numeric mean increase during placebo did not reach statistical significance ($P = .072$).

We replicated the data for urinary excretion of PGD_2 metabolites observed in the ultraperformance liquid chromatography/tandem mass spectrometry analysis by using a commercially available EIA for the very early PGD_2 metabolite 11β-$PGF_{2\alpha}$ (Fig 5, *C*). However, this metabolite was not detected by using mass spectrometry, extending earlier data indicating that the 10% cross-reactivity of the antibody against 11β-$PGF_{2\alpha}$ with 2,3-dinor-11β-$PGF_{2\alpha}$ explains the results of using that particular EIA on urine.[12,15] We confirmed good agreement between the 2 measurements by using Bland-Altman analysis (see Fig E3 in this article's Online Repository at www.jacionline.org).

Thromboxane B_2 and its metabolites. Levels of the most abundant metabolite, 2,3DN-TXB_2, increased after LTE_4 inhalation challenge during both treatments (1853 ± 460 for placebo vs 201 ± 74 at baseline, $P = .0019$; 561 ± 116 for montelukast vs 159 ± 41 at baseline, $P = .0021$). Peak excretion was significantly greater with placebo ($P = .0092$; Fig 6, *A*). Measurements of primary thromboxane (TX) B_2 and 11DH-TXB_2 levels are presented in the Results section and Fig E4 in this article's Online Repository at www.jacionline.org.

$PGF_{2\alpha}$. $PGF_{2\alpha}$ levels increased significantly after challenge during placebo treatment (711 ± 158 vs 209 ± 34 at baseline, $P = .0029$) but did not reach a significant increase during montelukast treatment (411 ± 67 vs 251 ± 74 at baseline, $P = .06$). Peak excretion was greater during placebo treatment compared with that during the montelukast period ($P = .0350$; Fig 6, *B*).

Prostacyclin metabolite 2,3-dinor-6-keto-$PGF_{1\alpha}$. This metabolite did not increase significantly after LTE_4 inhalation with either treatment. However, there was a trend ($P = .055$) toward an increased level during placebo treatment (Fig 6, *C*).

PGE_2 and its metabolites. Urinary excretion of primary PGE_2 increased to the same extent after challenge during both treatment periods (143 ± 31 for placebo vs 57 ± 21 at baseline, $P = .0219$; 147 ± 34 for montelukast vs 69 ± 39 at baseline, $P = .0188$; Fig 6, *D*). The same was true for the later and more abundant metabolite tetranor-PGEM (1555 ± 365 for placebo vs 363 ± 111 at baseline, $P = .0056$; 1823 ± 407 for montelukast vs 388 ± 90 at baseline, $P = .0037$; Fig 6, *E*).

Isoprostanes. Levels of the most abundant metabolite, 8,12-iso-$PF_{2\alpha}$-VI, increased after LTE_4 inhalation challenge during both treatments (2139 ± 347 for placebo vs 858 ± 166 at baseline, $P = .0035$; 1756 ± 273 for montelukast vs 1081 ± 220 at baseline, $P = .0274$; Fig 6, *F*). Results of 8iso-$PGF_{2\alpha}$ and

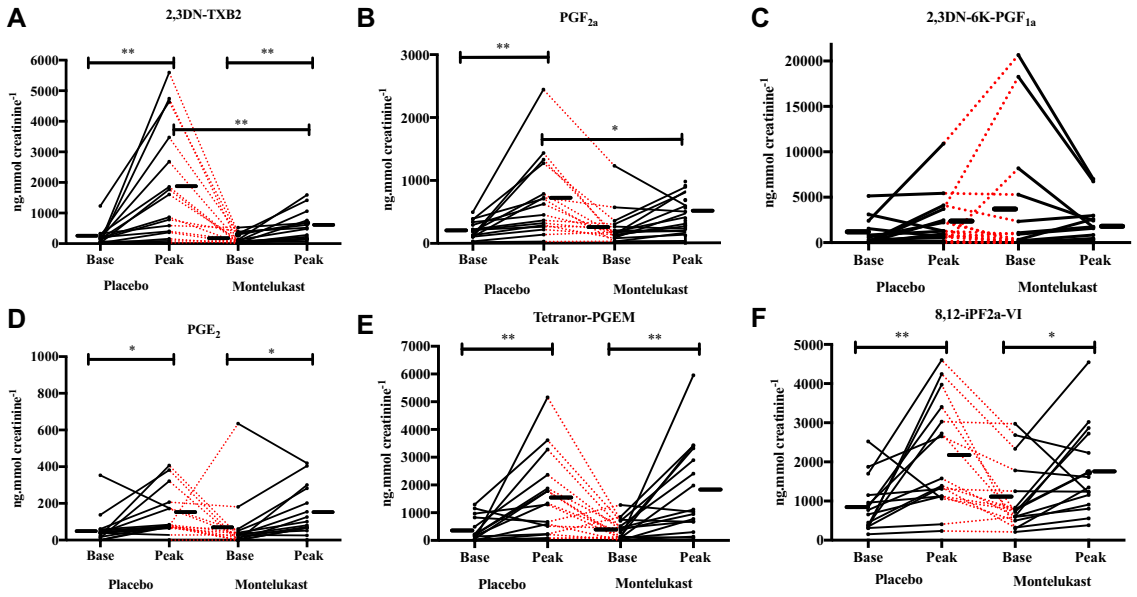

FIG 6. Urinary excretion of other important eicosanoids and their major metabolites measured with ultraperformance liquid chromatography/tandem mass spectrometry during the 2 treatment periods. **(A)** 2,3DN-TXB2, **(B)** PGF_{2a}, **(C)** 2,3DN-6K-PGF_{1a}, **(D)** PGE_2, **(E)** Tetranor-PGEM, **(F)** 8,12-iPF_{2a}-VI. All values are expressed as nanograms per millimole of creatinine. *Horizontal bars* indicate mean values, with *upper bars* for before versus after differences during each period and *lower bars* displaying significant differences between periods. $*P < .05$ and $**P < .01$.

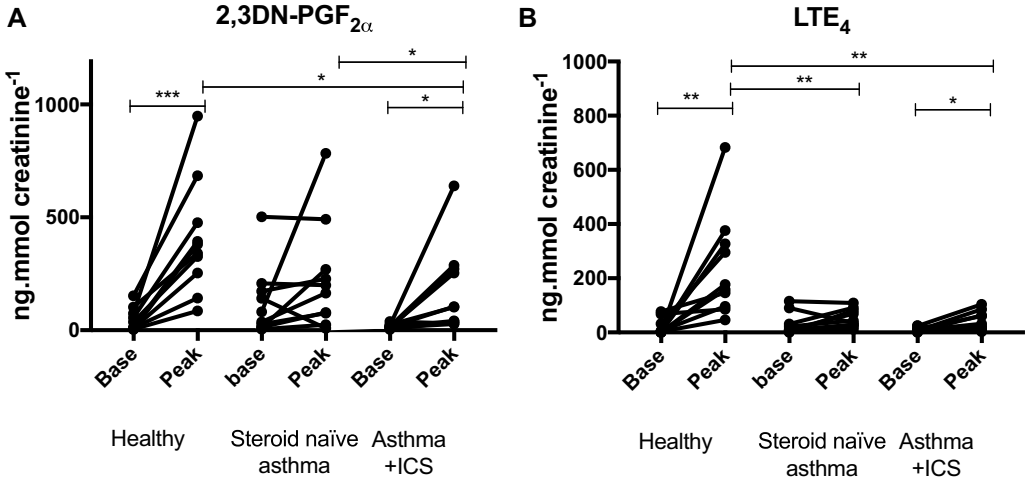

FIG 7. Urinary excretion of the PGD_2 metabolite 2,3-dinor-$PGF_{2\alpha}$ **(A)** and LTE_4 **(B)** after LTD_4 inhalation challenge, as measured with ultraperformance liquid chromatography/tandem mass spectrometry. All values are expressed as nanograms per millimole of creatinine. $*P < .05$, $**P < .01$, and $***P < .001$. *ICS*, Inhaled corticosteroid.

2,3DN-8iso-$PGF_{2\alpha}$ measurement are presented in the Results section and Fig E5 in this article's Online Repository.

Validation of urinary mediator excretion in biobanked samples from bronchoprovocation with LTD_4

Analysis of urine collected in a previous LTD_4 bronchoprovocation study[13] found increased urinary excretion of the following compounds after LTD_4: the PGD_2 metabolite 2,3DN-$PGF_{2\alpha}$; the TXB_2 metabolite 2,3-DN-TXB_2; the isoprostanes 8iso-$PGF_{2\alpha}$, 2,3DN-b8iso-$PGF_{2\alpha}$, and 8,12-$iPF_{2\alpha}$; LTE_4. Data for 2,3DN-$PGF_{2\alpha}$ and LTE_4 are shown in Fig 7, and data for the other metabolites are reported in Fig E6 in this article's Online Repository at www.jacionline.org. No significant increases were seen for the following detected mediators: primary TXB_2 and 11DH-TXB_2, $PGF_{2\alpha}$, and the prostacyclin metabolite 2,3DN-6K-$PGF_{1\alpha}$ (see Fig E7 in this article's Online Repository at www.jacionline.org). We were unable to detect measurable amounts of primary PGE_2, tetranor-PGEM, or tetranor-PGDM, which is in line with previous studies reporting on their degradation during storage.[16]

DISCUSSION

In this controlled study we demonstrate that treatment for 5 to 7 days with the selective $CysLT_1$ receptor antagonist montelukast effectively inhibited a broad range of responses induced by LTE_4 inhalation. This is the first study in which montelukast, the clinically most widely used $CysLT_1$ antagonist, has been assessed for its efficacy against airway obstruction induced by LTE_4. In addition, we discovered that inhaled LTE_4 led to increased urinary levels of several major metabolites of eicosanoid-derived lipid mediators; in particular, increased excretion of metabolites of PGD_2 suggests that the $CysLT_1$ receptor *in vivo* can activate mast cells. Furthermore, LTE_4-induced release of PGD_2 metabolites was blocked by montelukast and replicated by inhalation of LTD_4, which together lend strong support to the interpretation that this presumed mast cell activation was mediated by the $CysLT_1$ receptor. In fact, this new finding heralds a new awareness that CysLTs should be reclassified as both directly and indirectly acting bronchoconstrictors.

As expected, this study confirmed that LTE_4 is a more potent bronchoconstrictor than methacholine. Although the bronchoconstrictive properties of LTD_4 have been studied extensively, there are only a handful studies of how the terminal CysLT LTE_4 affects human airways *in vivo*. Compared with methacholine, LTE_4 was about 70 times more potent on a molar basis. Using an identical challenge protocol, we found that inhaled LTD_4 is approximately 1000 times more potent than methacholine.[13] This confirms the potency differences determined in the few studies in which inhaled LTE_4 has been compared with methacholine or histamine on the one hand and LTC_4 or LTD_4 on the other hand.[3] We also found that the asthmatic patients with the highest hyperresponsiveness for methacholine had the lowest relative airway responsiveness to LTE_4, which is in perfect agreement with relations established between methacholine and inhaled LTC_4 or LTD_4 levels in previous studies.[13,17]

Furthermore, pretreatment with montelukast abolished bronchoconstriction after inhalation of LTE_4 in all study subjects (14 patients with mild asthma and 2 patients with AERD). During treatment with montelukast, on average, asthmatic patients tolerated a 10-fold higher dose of inhaled LTE_4 compared with placebo without any decrease in lung function. In contrast, during the placebo-treated period, the subjects showed an excellent repeatability of LTE_4 PD_{20} measurements determined during screening. Our findings are in agreement with those of previous studies using 2 other LT receptor antagonists against inhaled LTE_4.[5,18] The results support that the *in vivo* effect of LTE_4 on airway smooth muscle in human subjects is mediated exclusively by the $CysLT_1$ receptor. This agrees with *in vitro* data in isolated human bronchi and other models in which LTE_4 acts through the $CysLT_1$ receptor.[1,19-21]

Because FEV_1 reflects mainly changes to the larger airways, for the first time, this study applied IOS measurements in subjects challenged with LTE_4. This approach revealed pronounced peripheral airway obstruction after LTE_4 inhalation in the presence of placebo similar to that seen in patients with severe asthma and small-airways disease.[22] In contrast, during treatment with montelukast, there were no signs of small-airways impairment and essentially no significant changes in IOS parameters after LTE_4 inhalation. Interestingly, previous treatment studies with montelukast have indicated that small-airway function in asthmatic patients was improved by montelukast,[23,24] implicating that endogenous CysLTs contribute to small-airway disease. The small-airway obstruction induced by LTE_4 might be due to both smooth muscle constriction and mucosal edema because CysLTs are potent inducers of plasma exudation,[25] which is also shown in the airways.[26,27]

We deliberately used a higher dose of montelukast (40 mg) than the ordinary clinical 10 mg once-daily dosing. This was because we wished to ensure effective $CysLT_1$ antagonism to avoid ambiguous data caused by incomplete receptor inhibition by this competitive antagonist. In fact, the only published dose-ranging study of montelukast in the LTD_4 provocation setting has shown progressively increasing shifts in the dose-response relation for LTD_4 up to 250 mg of montelukast. It should be appreciated that montelukast is a very selective $CysLT_1$ antagonist and that the initial clinical treatment studies used much higher doses (100-200 mg).[28,29] Interestingly, lung function improvement in these high-dose studies was greater than in subsequent studies using 10 mg,[30] although performed on comparable patient populations. Perhaps it is time to revisit the dosing of montelukast?

Furthermore, despite exposure to one order of magnitude higher dose of LTE_4 in the presence of montelukast, the sputum eosinophil count was the same as during the placebo period. If another receptor had been sensitive to LTE_4, the substantially higher agonist concentration would have been expected to result in an amplified response. Therefore the finding argues against additional receptors being involved in the proinflammatory effects of LTE_4 on airway cells. Inhaled LTD_4 and LTE_4 levels have been reported to differ with respect to the ability to cause airway eosinophilia.[31] Our study suggests that the outstanding explanation for this observation is unlikely to be related to a different receptor for LTE_4.

Original work on LT mechanisms in animal airways showed that CysLTs can cause profound release of PGs and other COX products that contribute to the overall biological responses[32,33]; however, this mechanism has never been confirmed in human subjects *in vivo*. For the first time, our study demonstrated that inhaled LTE_4 induces increased urinary excretion of metabolites indicative of pulmonary release of PGE_2, PGD_2, $PGF_{2\alpha}$, and TXA_2, as well as several isoprostanes being markers of oxidative stress.[34]

Increased excretion of PGD_2 is considered a sign of mast cell activation because this cell is the major source of PGD_2 in human subjects.[12,35] This increased excretion was abolished by montelukast treatment, confirming it to be $CysLT_1$ dependent. There are previous experimental data supporting that CysLTs can activate mast cells and cause secondary release of prostanoids.[36,37] Moreover, we were able to replicate the effect of LTE_4 on PGD_2 release by measurements in urine that had been biobanked after a previous LTD_4 inhalation study.[13] That study also supported dose-dependent mast cell activation because it included healthy subjects who tolerated a higher dose of LTD_4 and displayed a more pronounced mediator excretion in urine. Because mast cells also produce CysLTs, the study results raise the hypothesis that $CysLT_1$-dependent mast cell activation might represent a positive feedback loop sustaining the response to the initial stimulus. Another implication of this new finding is that eosinophils, which are proficient producers of CysLTs, could prime mast cells by this particular mechanism. The allegation of a prostanoid component in LT-induced airway hyperresponsiveness was previously indicated by the finding of Christie et al[38] that indomethacin blocked the increase in histamine responsiveness that followed LTE_4 inhalation.

TXA_2 release and, to some extent, also $PGF_{2\alpha}$ showed similar sensitivity to inhibition by montelukast. This could be also be due to $CysLT_1$-mediated release from mast cells, but secondary release from other sources is also possible. It remains poorly characterized which lipid mediators are formed in human mast cells *in vivo*. In contrast, increased urinary excretion of PGE_2 and its major metabolite, tetranor-PGEM, were not blocked by montelukast. It is possible that another receptor might be involved in that release, perhaps from the airway epithelium, which is a major source of PGE_2.[39,40] However, because the patients received a 10-fold greater dose of LTE_4 in the presence of montelukast without an increase in urinary excretion of PGE_2, it is also possible that the effect partly involved the $CysLT_1$ receptor. Irrespective of the receptor involved, it is of considerable interest that inhalation of LTE_4 triggered secondary release of PGE_2, which has many anti-inflammatory effects on key targets in asthmatic patients, such as mast cells[41,42] and type 2 innate lymphoid cells.[43,44] Therefore the finding of increased PGE_2 excretion might be a protective negative feedback response intended to aid resolution.

LTE_4 inhalation was also followed by an immediate increase in numbers of circulating lymphocytes and a decrease in eosinophil counts, both effects that were unaffected by montelukast, suggesting that they might be mediated by another receptor. Future studies are required to characterize this new observation in greater detail.

Two patients with AERD were included in the study. Their responses to LTE_4 in no respect differed from the rest of the asthmatic patients, which fits with the remarkably good therapeutic response observed when montelukast was added to conventional therapy with inhaled steroids in this particular asthma phenotype.[45]

In conclusion, montelukast inhibited both the bronchoconstriction and mast cell activation that was induced by inhalation of LTE_4. However, PGE_2 release was not blocked by montelukast, suggesting involvement of another LT receptor for this particular effect, at least in part. Nevertheless, the study unequivocally demonstrates that airway obstruction in response to inhalation of LTE_4 in asthmatic patients is mediated solely by the $CysLT_1$ receptor.

Clinical implications: Clinically available LT antagonists protect against the airway obstruction and proinflammatory effects of the terminal CysLT LTE_4.

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