Relation between bronchial responsiveness to inhaled leukotriene D₄ and markers of leukotriene biosynthesis

P Gyllfors, M Kumlin, S-E Dahlén, F Gaber, P-O Ehrs, B Dahlén

Background: While clinical trials with antileukotrienes have shown overall beneficial effects in asthma, the factors that determine leukotriene dependent asthma are still unclear. A study was undertaken to determine whether or not leukotriene responsiveness in the airways correlates with endogenous leukotriene biosynthesis.

Methods: Bronchial responsiveness to leukotriene (LT) D₄ was assessed as PD⁰₂₀FEV₁ in 20 subjects with mild asthma and 10 healthy controls, and compared with bronchial responsiveness to methacholine and two global measures of leukotriene production—urinary LTE₄ and ex vivo production of LTB₄ in whole blood.

Results: In patients with asthma the bronchoconstrictor activity of LTD₄ was about 1300 times greater than methacholine (geometric mean PD⁰₂₀ 0.69 nmol v 887 nmol). Those who were most responsive to LTD₄ were relatively less responsive to methacholine (p<0.01). There was, however, no correlation between bronchial responsiveness to LTD₄ and urinary LTE₄ or blood ex vivo LTB₄ levels in asthmatic subjects or healthy controls. Subjects with asthma treated with inhaled corticosteroids produced higher levels of LTB₄ (p<0.05).

Conclusions: General measures of leukotriene production cannot predict bronchial responsiveness to LTD₄. The unique bronchoconstrictive potency of LTD₄ on human airways may relate to the locally regulated expression of the cysteinyll LT₁ receptor. However, in patients with AIA there was no correlation between U-LTE₄ and the treatment response to the leukotriene antagonist montelukast.

While it is uncertain whether the production of leukotrienes correlates with the beneficial effect of antileukotrienes, the responsiveness to leukotrienes in the airways has not been taken into account. It is not known if increased bronchial responsiveness to leukotrienes is an indicator of a predominant leukotriene component in asthmatic inflammation. We have therefore embarked on a series of studies of factors that determine leukotriene responsiveness in the airways. The aim of the first investigation in this project was to assess whether or not the responsiveness of an individual to inhaled LTD₄ is related to the general propensity to synthesise leukotrienes. As CysLTs cause bronchoconstriction and many other biological effects by activation of the G protein coupled CysLT₁ receptor, it was hypothesised that there might be a relation between the endogenous level of agonist (CysLTs) and the airway responsiveness to inhaled LTD₄. It is common for agonist levels to influence the degree of receptor expression. For this study, two different global measures of leukotriene production—U-LTE₄ and blood ex vivo LTB₄ generation—were assessed. LTE₄ is the end product of the CysLTs excreted into the urine and its level is a reflection of whole body biosynthesis of CysLTs. As circulating levels of leukotrienes in the blood are very low and far below the detection limit of reliable assays, standardised ex vivo stimulation of whole blood with secretagogues such as a calcium ionophore has been...
introduced as an indirect method of assessing the biosynthetic capacity of blood cells for leukotriene production. In this setting LTB₄ is the main leukotriene formed, and its synthesis is thought to reflect the degree of 5-lipoxygenase activity.

This cross sectional study of 20 subjects with asthma and 10 healthy controls therefore tested whether bronchial responsiveness to LTD₄, expressed as the PD₂₀ value, was correlated with leukotriene production measured as U-LTE₄ and blood ex vivo production of LTB₄. Bronchial responsiveness to LTD₄ was also compared with responsiveness to methacholine (MCh), selected as a general marker of airway responsiveness, and with the fraction of expired nitric oxide (FeNO) as a surrogate marker of airway inflammation, both being common asthma outcome variables in treatment studies. Although descriptive data on LTD₄ responsiveness are available in the literature, there is almost no mechanistic information about factors that determine leukotriene responsiveness in humans.¹⁷

**METHODS**

**Subjects**

Twenty subjects with intermittent to mild asthma according to GINA criteria and documented airway hyperresponsiveness to MCh and 10 healthy individuals were included in the study. Subjects with asthma were recruited from a general practitioner’s clinic and the healthy volunteers through advertisements. All subjects were never smokers or non-smokers for the last 2 years with a smoking history of less than 5 pack years who had not had a respiratory tract infection during the 4 weeks before screening. Atopic subjects were not studied during seasonal allergen exposure and subjects with allergy to animal dander were required to avoid animal contact for 2 weeks before and during the study.

The 20 subjects with asthma were in a stable condition with forced expiratory volume in 1 second (FEV₁) ≥70% of predicted. Ten of the asthmatic subjects used a stable dose of inhaled corticosteroids (ICS) and short acting β₂ agonists as rescue, and 10 used short acting β₂ agonists as their sole medication (table 1).

The healthy individuals were skin prick test negative to 10 common allergen extracts and did not display significant bronchoconstriction with a fall of ≥20% in FEV₁ when challenged with a cumulative dose of 45 282 nmol MCh.

The study was approved by the ethical review board at the Karolinska University Hospital (EtDnr 00-267) and the study was registered at ClinicalTrials.gov (NCT00296026). All patients gave their written informed consent.

### Table 1 Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>Healthy (n=10)</th>
<th>Asthma (no ICS) (n=10)</th>
<th>Asthma (ICS) (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F/M</td>
<td>5/5</td>
<td>5/5</td>
<td>6/4</td>
</tr>
<tr>
<td>Mean (SD) age (years)</td>
<td>35 (10.1)</td>
<td>32 (8.6)</td>
<td>31 (6.9)</td>
</tr>
<tr>
<td>Mean (SD) FEV₁ (l)</td>
<td>4.1 (0.7)</td>
<td>3.2 (0.6)</td>
<td>3.3 (1.0)</td>
</tr>
<tr>
<td>Mean (SD) FEV₁ % predicted</td>
<td>110 (11.6)</td>
<td>90 (9.4)</td>
<td>91 (14.7)</td>
</tr>
<tr>
<td>Medication</td>
<td>–</td>
<td>SABA</td>
<td>Budesonide + SABA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(n=9; daily dose 400 µg n=7, 800 µg n=1, 1200 µg n=1)</td>
</tr>
<tr>
<td>Mean (SD) SPT (out of 10)</td>
<td>–</td>
<td>3 (2.1)</td>
<td>3 (2.3)</td>
</tr>
<tr>
<td>Median (range) FeNO</td>
<td>11.0 (6.3–19.7)</td>
<td>25.2 (6.8–198.6)</td>
<td>17.2 (6.1–86.5)</td>
</tr>
<tr>
<td></td>
<td>n=9</td>
<td>n=8</td>
<td>n=9</td>
</tr>
<tr>
<td>Mean (SD) asthma specific QoL</td>
<td>–</td>
<td>5.9 (0.88)</td>
<td>6.0 (0.73)</td>
</tr>
</tbody>
</table>

FEV₁, forced expiratory volume in 1 second; ICS, inhaled corticosteroid; SABA, short acting β₂ agonist; SPT, skin prick test; FeNO, fraction of expired nitric oxide; QoL, quality of life.

Lung function data at baseline (visit 1).

**Study design**

The screening visit included subject characterisation with documentation of history, a MCh challenge, and a skin prick test. Three further visits were scheduled 2 weeks apart, and the subjects always reported to the clinic at the same time in the morning. At visit 1 Feso was measured according to the ATS/ERS standard, dynamic spirometric tests were performed, blood samples were collected for analysis of whole blood ex vivo LTD₄ production, and urinary samples were collected for analysis of baseline U-LTE₄ concentrations. In the subjects with asthma, quality of life was measured using the Asthma Quality of Life Questionnaire (AQLQ) devised by Juniper et al. The patients were asked to indicate the extent to which their quality of life was limited on a 7-point scale where 1 indicates maximal impairment and 7 no impairment at all. At visit 2, blood and urine samples were collected and spirometric tests performed as the subjects underwent a bronchoscopic examination as part of a separate ongoing mechanistic investigation of the leukotriene pathway in the lung. At visit 3, inhalation challenge with LTD₄ was performed in addition to blood and urine sampling. Short acting β₂ agonists were withheld for at least 6 hours before visits except for visit 2.

**Inhalation challenge**

Pulmonary function was measured as FEV₁ on a spirometer (Vitalograph MDI Compact; Förbandsmaterial, Stockholm, Sweden) and the baseline defined as the best of three recordings. All bronchoprovocation tests were performed using a dosimeter controlled jet nebuliser (Spira Elektro 2; Intramedic, Bålstå, Sweden). Challenges always began with inhalation of the respective diluent. Provided FEV₁ did not change by more than 10%, incremental doses of the provocative agent were administered until FEV₁ had fallen by at least 20% from post-diluent baseline. For LTD₄ bronchoprovocation tests, approximately half-log increments in the cumulative dose (3, 10, 30 pmol) were inhaled every 10 minutes (dose range 3–335 780 pmol). This was achieved by using six solutions of good manufacturing practice (GMP) grade LTD₄ (sealed colour coded vials each containing 1 ml of solution; concentrations increasing by tenfold from 4.2×10⁻⁹ M to 4.2×10⁻³ M; 4:1 solvent water:ethanol; Cascade Biochemicals; Reading, UK) and a varying number of breaths (2–7) from each solution. Spirometric tests were performed 5 and 10 minutes after each dose and the peak fall used for calculation of PD₂₀. Airway responsiveness to MCh was assessed with a similar protocol but with dose increments every 3 minutes and single FEV₁ measurements.
Three concentrations (6.24, 50, 400 mM prepared at Norrlands University Hospital Pharmacy) were used to create increasing doses (range 89–45 282 nmol).

**Collection of urine samples and analysis of urinary LTE4**

Urine was collected on arrival on each of the three study days, two samples at visit 1 with 1 hour in between and one sample at visits 2 and 3. The total volumes of the urine samples were measured, aliquoted, immediately frozen without preservatives, and stored at $-20^\circ$C until analysis. The concentration of LTE4 was determined using a previously validated enzyme immunoassay method with data expressed in relation to creatinine excretion determined colorometrically. The rabbit polyclonal CysLT antiserum used in the present study cross reacted with LTE4 (67%), LTC4 (100%), and LTD4 (100%). Acetylcholine esterase linked LTE4 was used as tracer and unlabelled LTE4 as standard. Baseline U-LTE4 refers to the mean value of the four baseline samples.

**Ex vivo ionophore induced formation of LTB4 in whole blood**

Blood samples were obtained by venepuncture into heparinised vaccutainer tubes upon arrival at visits 1, 2 and 3. Ex vivo stimulation of freshly drawn peripheral whole blood was performed with a modified version of previously described protocols. The blood was kept at room temperature for 1 hour before incubation to minimise fluctuations in values due to decreased capacity for leukotriene formation within the first hour of blood collection. The calcium ionophore ionomycin was dissolved in 95% ethanol to a stock concentration of 10 mM. The stock solution and vehicle (95% ethanol) were diluted 10 times with autologous plasma. Aliquots of blood (1 ml) were preincubated at 37°C for 2 minutes, followed by addition of vehicle or ionomycin in 50 μl autologous plasma. The final concentration of ionomycin was 50 μM. Incubations were continued for 15 minutes at 37°C and interrupted on ice. Plasma was obtained by centrifugation at 714g for 5 minutes at 4°C and stored at $-70^\circ$C until assayed for LTB4 by enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA). Data were expressed in relation to the white blood cell (WBC) count on each blood collection day. The mean values of the three visit days were used for comparisons between groups, whereas correlation with PD20LTD4 was tested for values obtained on the day of the bronchoprovocation (visit 3).

**Statistical analysis**

The provocative doses causing falls of 10%, 15% and 20% in FEV1 (PD10, PD15, PD20) were derived by linear interpolation from the respective log cumulated dose-response curves. Calculations of geometric mean values were performed on log transformed raw data. Urinary LTE4 and ex vivo LTB4 concentrations are expressed as median values with ranges. Correlations between bronchial challenges were performed with Pearson product moment correlation and all others with Spearman rank order correlation. The Mann-Whitney rank sum test was used for comparison between groups and Kruskal-Wallis one way analysis of variance on ranks was used to assess variability in values of ex vivo LTB4 and U-LTE4 at baseline. Differences were considered significant if p<0.05.

**RESULTS**

**Bronchial responsiveness to LTD4 and MCh**

The dose-response relations for inhaled LTD4 and MCh in all individuals are shown in fig 1A–C, and group mean data for different measures of responsiveness are given in table 2. All subjects with asthma produced PD20 values for LTD4 and MCh with no significant difference in responsiveness between the group taking ICS and the group that did not.
The geometric mean (range) PD$_{20}$LTD$_4$ and PD$_{20}$MCh for all subjects with asthma (n = 20) was 0.69 (0.062–37.05) nmol and 887 (89–37 188) nmol, respectively. Thus, on a molar basis, LTD$_4$ was over a 1000 times more potent than MCh (PD$_{20}$ ratio 877/0.69 = 1285; fig 1B and C).

There was a linear relation between airway responsiveness to methacholine and the dose ratio of MCh to LTD$_4$ (table 2). As in the asthmatic subjects, LTD$_4$ was more than 1000 times more potent than MCh when PD$_{10}$ values for LTD$_4$ and MCh were compared in the seven healthy individuals who responded to MCh (fig 1A and table 2).

There was no correlation between the responsiveness to inhaled LTD$_4$ and FeNO in subjects with asthma or in healthy individuals at the PD$_{15}$ level (data not shown).

**Blood ex vivo LTB$_4$ production and urinary excretion of LTE$_4$**

Urinary LTE$_4$ excretion and ex vivo LTB$_4$ production were consistent within each study group with no significant differences between the three visit days (table 3), nor were there differences in baseline U-LTE$_4$ excretion between the three study groups (p>0.05, table 3). However, asthmatic subjects taking ICS had higher ex vivo LTB$_4$ production than those with asthma not taking ICS (median (range) 10.7 (4.3–21.5) v 7.0 (2.5–18.1) ng/10$^6$ WBC, p<0.05). There was no correlation between ex vivo LTB$_4$ production and U-LTE$_4$ in either the asthmatic subjects (p>0.05) or in the healthy individuals (p>0.05, data not shown).

Furthermore, there was no correlation between airway responsiveness to LTD$_4$ and ex vivo LTB$_4$ generation on the day of provocation in subjects with asthma (n = 20, r = 0.36, p = 0.12) or in the healthy individuals (n = 7, r = −0.12, p = 0.80; fig 3A). Likewise, there was no correlation between responsiveness to inhaled LTD$_4$ and baseline U-LTE$_4$ concentrations in subjects with asthma (n = 20, r = −0.22, p = 0.36) or in healthy individuals (n = 7, r = −0.17, p = 0.71; fig 3B).

**DISCUSSION**

No relationship was found between two global measures of leukotriene biosynthesis and bronchial responsiveness to inhaled LTD$_4$. Although baseline data (including asthma specific quality of life) indicated that the subjects with asthma had relatively similar disease severity, their responsiveness to LTD$_4$ varied by almost 1000 times (PD$_{20}$ from 60 pmol to 40 nmol (30 ng to 20 μg). There was also no relation between LTD$_4$,PD$_{20}$ and exhaled NO, although the latter displayed the expected difference between healthy subjects and asthmatics (table 1).
In the search for factors that determine leukotriene responsiveness in the airways, the effects of inhaled LTD₄ were examined in one of the largest bronchoprovocation studies (n = 30) completed with this agent. Subjects with mild asthma, half of whom were receiving treatment with ICS, were chosen because they represent the vast majority (approximately 70%) of all asthma patients. Thus, in subjects with asthma, LTD₄ was (on a molar basis) more potent than MCh (geometric mean PD₂₀ values obtained after inhalation: 650 pmol LTD₄ (325 ng) v >900 nmol MCh (270 μg)). This potency ratio compares well with other bronchoprovocation studies and with investigations in isolated human airways.²⁷ ²⁸ In the asthmatic subjects there was, however, no difference in absolute or relative responsiveness to LTD₄ or MCh between subjects on ICS and those not on ICS.

On the other hand, the asthmatic subjects who were the most responsive to MCh had the lowest relative airway responsiveness to LTD₄ compared with MCh. The relation between responsiveness to LTD₄ and standard direct bronchodilators has been debated.²⁴–²⁶ The relationship we found between responsiveness to MCh and LTD₄ supports previous findings.²⁷ ²⁸ Adelroth et al hypothesised that asthmatic subjects with more severe airway inflammation and greater MCh responsiveness somehow had developed a specific tachyphylaxis to LTD₄, possibly as a result of increased local biosynthesis of CysLTs, as well as of bronchoprotective factors such as nitric oxide or prostaglandin E₂. The conclusion that LTD₄ has a very specific mode of action on the airways is also supported by the current observation that there was no relation between LTD₄ PD₂₀ and exhaled NO, whereas MCh responsiveness has been reported to be correlated with exhaled NO levels.³⁰

It was confirmed that LTD₄ is also a potent bronchoconstrictor in subjects without asthma.²⁴–²⁶ Interestingly, a PD₂₀ value for LTD₄ could be obtained in seven out of 10 healthy individuals whereas only two subjects had a fall in FEV₁ of more than 12% after inhalation of the highest dose of MCh used in this protocol. The latter observation is in line with the established plateau to MCh in healthy subjects.³¹ The less apparent plateau to LTD₄ might relate to its longer duration of action in airway smooth muscle compared with agonists such as histamine or MCh.³² Bel et al found that the maximal airway narrowing to LTD₄ was much greater than that produced by MCh.³³ In agreement with these fundamental differences in action on the airways, the potency ratio between LTD₄ and MCh (1827) could only be determined on a PD₁₀ level in the healthy controls. However, when LTD₄ responsiveness was compared for the groups, the healthy controls were about 40 times less responsive than the subjects with asthma.

Baseline urinary excretion of LTE₄ was the same in all three groups studied, and there was no relation between baseline U-LTE₄ and PD₂₀ for inhaled LTD₄ in subjects with asthma or in healthy individuals (Spearman rank order correlation): (A) blood ex vivo LTB₄ production; (B) baseline U-LTE₄ concentration.

![Figure 3](http://thorax.bmj.com/)
asthma and healthy controls. Measurements of ex vivo LTB₄ formation in whole blood also failed to establish a relation between this measure and airway responsiveness to LTD₄ in asthmatics or healthy controls. Taken together, the study therefore refuted the hypothesis that there is a relation between airway responsiveness to LTD₄ and the global propensity of individuals to generate leukotrienes measured either as U-LTE₄ or blood ex vivo LTB₄ production. It is likely that the expression of CySLT₁ receptors in the airways is the primary determinant of responsiveness to inhaled LTD₄. CySLT₁ receptor expression and hence responsiveness to the inhaled agonist may, however, still be regulated by the local biosynthesis of leukotrienes in the airways measured, for example, in sputum, bronchoalveolar lavage (BAL) fluid or cells recovered from BAL fluid. Mechanistic studies to address these possibilities are ongoing.

Interestingly, we found that asthmatic patients who were receiving ICS produced somewhat higher levels of LTB₄ whereas their urinary excretion of LTE₄ was the same as in asthmatic patients not treated with ICS. The finding with LTE₄ is new and, together with the confirmatory data on urinary LTE₄, adds to the knowledge that ICS have complex effects on the leukotriene pathway with no or minimal effects on leukotriene biosynthesis generally observed in vivo. The increased levels of steroid treatment might relate to the previous finding that 5-lipoxygenase inhibitor and LTD₄ receptor expression in cells may be upregulated by ICS. As there was no correlation between ex vivo generation of LTD₄ and U-LTE₄ for the individual subjects, the study also confirms the hypothesis that these two measures represent two different ways to assess the leukotriene pathway.

In conclusion, the lack of relationship between global markers of biosynthesis of leukotrienes and responsiveness to LTD₄ is noteworthy in view of the limited usefulness of these markers to predict the response to antileukotriene drugs. Since subjects with asthma in the current study displayed a wide three log order of magnitude variability in responsiveness to inhaled LTD₄ variability in the sensitivity of the target tissue to the effects of CySLT may perhaps be a more decisive determinant of the treatment response to antileukotriene drugs. We therefore propose that future trials aimed at the establishment of responders to antileukotriene treatment should include PD₂₀FEV₁ for LTD₄. We hypothesise that such a direct measure of airway responsiveness may be more predictive than the markers of biosynthesis currently established of responders to antileukotriene treatment.

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REFERENCES

LUNG ALERT ......................................................................................................

Roflumilast as a treatment for COPD

慢性炎症是慢性阻塞性肺病发展的关键病理生理机制，并且越来越多地引起了潜在治疗药物的注意。这项由多个中心参与的双盲随机对照试验关注了多氟尼索，一种口服磷酸二酯酶4抑制剂。

1411名稳定为中等至严重的慢性阻塞性肺病患者被随机分配接受安慰剂，多氟尼索250μg/次，每日，或多氟尼索500μg/次，每日24周。主要结局指标是支气管扩张剂后第一秒用力呼气量（FEV1）和圣乔治呼吸问卷（SGRQ）总分。次要结局指标包括COPD恶化事件的数量。结果显示，与安慰剂相比，多氟尼索500μg组在24周后的FEV1平均提高了74（SD 18）ml，而500μg组则为97（SD 18）ml。与安慰剂组相比，500μg组的COPD恶化事件数平均减少了34%（p<0.0001）。没有显著的差异出现在SGRQ分数中。在多氟尼索和安慰剂组之间的比较中，平均恶化事件数的减少由34%在500μg组与安慰剂组比较中引起的。由于在轻度恶化事件中减少（42%，p = 0.004），主要的不良反应是胃肠道的性质和自我限制性。

这项研究提供了早期的数据来支持使用多氟尼索治疗COPD的使用。需要进一步的研究来确认这些结果是否在24周后保持不变，并评估它对健康相关生活质量的影响。

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