Leukotriene E₄ in urine in patients with asthma and COPD—The effect of smoking habit

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Summary
Leukotriene E₄ (LTE₄) is implicated in asthma pathophysiology and possibly in chronic obstructive pulmonary disease (COPD) as one of the causes of persistent bronchoconstriction and mucus hypersecretion. Cigarette smoking stimulates cysteinyl leukotrienes (CysLTs) production. We investigated whether LTE₄ is equally increased in asthma and COPD and whether smoking significantly affects LTE₄ levels. Secondary outcomes involved correlations with inflammatory and functional parameters.

We studied 40 patients with COPD (20 smokers), 40 asthmatics (20 smokers) and 30 healthy subjects (15 smokers). Spirometry (FEV₁% pred., FEV₁/FVC) was performed, urine was collected for measurement of LTE₄ and creatinine, induced sputum was collected for differential cell counts and serum for ECP.

LTE₄/creatinine levels (pg/mg) were increased in asthmatic patients compared to COPD and controls, [125.6(54.5) vs. 54.5(19) vs. 55.9(18.9) pg/mg, respectively, P<0.0001 for asthma]. Smoking significantly affects LTE₄ levels only in asthmatic patients [164 (48) vs. 87 (26.3), P<0.0001 for smokers]. The only significant correlation was between eosinophils in induced sputum and LTE₄/creatinine levels in asthmatics.

In conclusion, patients with asthma presented higher LTE₄ values compared to normals and patients with COPD. Smoking significantly affects LTE₄ values only in asthmatics indicating a different underlying CysLTs inflammatory process in this condition.

Introduction
Cysteinyl leukotrienes (CysLTs) are a group of inflammatory mediators, which has received interest in the recent years. CysLTs (leukotrienes C₄, D₄ and E₄) are central mediators in...
asthma pathophysiology. Several inflammatory cells have the biosynthetic capacity to produce CysLTs and Leukotriene E4 (LTE4) is a stable end product of CysLTs metabolism in the human lung. Urine LTE4 is a reliable marker of endogenous CysLTs formation and measurement of LTE4 offers a possibility to monitor changes in the rate of CysLTs production. Cigarette smoking stimulates CysLTs production in animal models but also in humans. The role of CysLTs in chronic obstructive pulmonary disease (COPD) remains uncertain despite previous but limited evidence which indicates increased production in different fluids in this disorder. However, there is evidence that CysLTs increase microvascular permeability, evoke mucus hypersecretion and are potent smooth muscle constriction agents, features that are present in the airways of COPD patients, and this suggests that they might play a role in this disorder.

We tested the hypothesis that LTE4 values are equally increased in both asthma and COPD patients, and smoking habit significantly affects the above measurements. Therefore, we assessed the levels of LTE4 in the urine of patients with COPD and asthma, as well as in healthy controls, in an attempt to clarify the in vivo role of this mediator. Smoking as a possible factor that might affect LTE4 values was also tested after subdividing the study groups according to their smoking habit. Additionally, we investigated whether airway or systemic eosinophilic inflammation is implicated in the pathophysiology of this mediator in the two diseases, by assessing sputum differential cell counts and serum eosinophil cationic protein (ECP) levels. Finally, we examined whether the functional status, as assessed by forced expiratory volume in 1 s (FEV1%) predicted and the FEV1/FVC (FVC—forced vital capacity) ratio, is implicated in the measurements of LTE4.

Materials and methods

Subjects

Subjects’ characteristics are summarized in Table 1. The diagnosis of COPD was established according to the GOLD Guidelines, whereas the GINA Guidelines were used for the diagnosis of asthma. All subjects in the three study groups were subdivided according to their smoking habit (for COPD patients this subdivision referred to current and ex-smokers). A total of 145 subjects were initially recruited in order to achieve the final number of patients.

Forty COPD patients (20 current smokers, all non-atopic and steroid-naïve) were recruited. All COPD patients were characterized by the absence of eosinophils in induced sputum. All patients were receiving occasionally short acting β2-agonists as relief medication. Ex-smokers with COPD stopped smoking at least 2 years before entering the study.

Forty atopic asthmatics, matched for age, BMI and lung function with COPD patients (20 current smokers, 20 never smokers, matched for age, BMI, lung function and smoking habit with the COPD patients who were current smokers; all steroid-naïve), were also studied. All asthmatic patients had positive bronchodilation tests (increase of pre-bronchodilator FEV1 > 12% after administration of salbutamol at dose 400 mcg by meter dose inhaler). They were occasionally receiving short-acting β2-agonists as relief medication.

Thirty normal subjects (15 current smokers, 15 never smokers, matched for the smoking habit with the respective asthmatic and COPD smokers), with no history of any disease, served as controls. All were non-atopic, had normal lung function and negative bronchial challenge testing, and none of them was receiving any kind of medication at the time of the study. Normal subjects were younger compared to asthma and COPD patients since it is difficult to find healthy subjects without any comorbidities in the age of 65 years old.

All patients were clinically stable, i.e. they had no evidence of acute exacerbation for at least 4 weeks prior to the study. None of our patients was receiving any anti-inflammatory treatment (including corticosteroids, long-acting β2 agonists, or leukotriene receptor antagonists), theophylline, inhaled or oral mucolytics, or long-term oxygen therapy. Atopy was defined by the high values of total IgE and on the basis of positive skin tests to six common aeroallergens.

Study protocol details

Lung function measurements were performed in all subjects one day before the urine and blood collection and sputum induction. In all subjects, the collection of urine and blood
preceded the sputum induction procedure, in order to avoid any effect of sputum induction procedure on the urine and blood measurements. All subjects were asked not to smoke 2 h before each procedure. In order to ensure the subjects’ compliance to this recommendation, they were asked to stay in our laboratory for 2 h under the supervision of one of the investigators. Sputum induction was not performed in normal subjects. Metacholine test was performed only in normal subjects on a separate day of the protocol. All subjects were asked not to receive short-acting bronchodilators at least 12 h before the collection of samples and the lung function tests.

**LTE4/creatinine values were compared among patients with COPD, patients with asthma and normal subjects.** Similar comparisons were performed in the study subgroups. Correlations between LTE4/creatinine values and study parameters were also performed in all study groups.

All subjects were recruited from the outpatient clinics of the Athens Veterans Hospital. This is a general hospital that serves veterans and civilians. The Scientific Committee of the hospital approved the study protocol, and all participants gave written informed consent.

**Metacholine challenge**

Bronchial hyperresponsiveness to metacholine was measured and performed by a rapid metacholine inhalation test for the determination of PD20, as previously described for histamine. PD20 was determined by linear interpolation on a semi-logarithmic scale. The test was performed according to the instructions established by the American Thoracic Society guidelines.

**Lung function**

Pulmonary function tests were measured with a dry spirometer (Vica-test, Model VEP2; Mijnhardt; Rotterdam, Holland). FEV1 and FVC were measured according to the American Thoracic Society guidelines.

**Sputum induction and processing**

Sputum was induced as previously described. Subjects were asked to blow their noses, rinse their mouths, and swallow the water in the aerosol to minimize contamination with postnasal drip and saliva. An induction procedure using inhalation of an aerosol of hypertonic saline solution (3.5%) generated by a DeVilbiss ultrasonic nebulizer (2696 Somerset PA, USA) was chosen. Induction was performed for periods of 30 s, and 1, 2, 4 and 8 min. The patients were asked to expectorate sputum after the 4 and 8 min period. At least 2 mL of sputum were collected in a sterile container. The person who did the differential cell counts in sputum (G.P.) was not aware of the clinical and functional status of the patients or the results of bronchial challenge testing.

**LTE4 measurements**

LTE4 concentrations were measured in a non-purified urine morning sample with a specific competitive enzyme immunoassay (EIA) (Cayman Chemical; Ann Arbor, MI, USA, No. 520411). The detection limit of the assay was 25 pg/mL. The results were normalized to the creatinine concentration determined in the same sample and the LTE4/creatinine ratios were used for subsequent studies. All urine samples were tested for protein, bilirubin, ketone, urobilinogen, glucose and hemoglobin and if any of these substances were presented the sample was excluded from the study. According to manufacturer’s guidelines and in order to check the need of purification, all samples diluted 1:3 with distilled water. LTE4 was measured in diluted and undiluted samples. The two different dilutions of the samples showed differences less than 18% in the final calculated LTE4 concentration, so the samples were measured without purification.

**Creatinine measurement**

Creatinine concentration in urine was measured with a kinetic method. The complex formed by creatinine and picric acid (8.8 mmol/L) in an alkaline medium (NaOH 0.4M) was measured for 1 min at 492 nm.

**ECP measurement**

The concentrations of ECP in the serum were determined by a specific enzyme immuno-assay (Pharmacia Diagnostics AB; Uppsala, Sweden) in an auto analyzer (Uni CAP 100) with a lower limit of detection of 2.0 μg/L.

**LTE4/creatinine repeatability measurements**

Repeatability of LTE4/creatinine measurements was evaluated in 20 subjects [4 controls (2 smokers), 8 COPD patients (4 from each subgroup) and 8 asthmatics (4 smokers)]. Repeatability of LTE4/creatinine measurements was tested in samples from the same subjects collected on two consecutive days.

**Statistical analysis**

Data are expressed as mean (SD), unless otherwise mentioned. Values among three or more groups were evaluated using one-way analysis of variance (ANOVA) with an appropriate post hoc test for multiple comparisons (Bonferroni). For comparisons between two groups, Mann–Whitney U tests were used. Spearman’s correlation coefficient was used to investigate correlations between parameters. Repeatability of LTE4/creatinine measurements was assessed with the Bland and Altman test. A P-value <0.05 was considered significant.

**Results**

**Subjects characteristics in induced sputum**

Asthmatic patients were mainly characterized by a predominant eosinophilia in induced sputum whereas COPD patients were characterized by a predominance of neutrophilic inflammation (Table 1). Asthmatic smokers presented
with significantly higher values of % eosinophils in induced sputum compared to non-smokers (Table 2, P < 0.003). Asthmatic smokers presented a trend for higher values of % neutrophils in induced sputum compared to non-smokers, that was marginally statistically significant (Table 2, P = 0.048). No significant differences were observed between current and ex-smokers patients with COPD in regard to % neutrophils in induced sputum (Table 2, P = 0.79).

**LTE₄/creatinine levels in major study groups**

The levels of LTE₄/creatinine (pg/mg) were increased in asthmatic subjects compared to COPD and controls [125.6(54.5) vs. 54.5(19) vs. 55.9(18.9) pg/mg, respectively, P < 0.0001 for asthma, Fig. 1]. No statistically significant difference was found between COPD patients and normal subjects [54.5(19) vs. 55.9(18.9) pg/mg, P = 0.3, Fig. 1]. However, a trend for higher values in COPD smokers compared to ex-smokers was evident.

**Effect of smoking**

The levels of LTE₄/creatinine (pg/mg) were increased in asthmatic smokers compared to non-smokers and controls [164 (48) vs. 87 (26.3) pg/mg, P < 0.0001, Fig. 2]. No significant difference was observed between smokers and non-smokers in the COPD and control groups [58.3 (23) vs. 50.5 (13) pg/mg, 0.0001, Fig. 1]. No statistically significant difference was found between current and ex-smokers patients with COPD in regard to % neutrophils in induced sputum (Table 2, P = 0.79).

**Correlations**

Major correlation data are summarized in Table 3. Briefly, the values of LTE₄/creatinine presented a significant correlation with eosinophils in induced sputum in asthmatics (r = 0.83, P < 0.0001). This significant correlation was also observed in smoking asthmatics, but was not expressed in asthmatic non-smokers (Table 3, Figs. 3A and B). No correlation with ECP levels was found. No significant correlation was observed between the levels of LTE₄/creatinine and any of the parameters tested in COPD patients. No significant correlation was observed between LTE₄ and the degree of lung function impairment or the smoking habit in pack-years in all study groups. Neutrophils in COPD patients did not present any significant correlations with LTE₄/creatinine (r = 0.02, P = 0.77).

**Repeatability of LTE₄/creatinine measurements**

The measurements of LTE₄/creatinine on 2 consecutive days presented good repeatability. LTE₄/creatinine on days 1 and 2 were 80.96(52) pg/mg and 82.04(52) pg/mg, respectively. The correlation between LTE₄/creatinine measurements on two consecutive days was significant (r = 0.94, P < 0.0001). The mean difference with limits of agreement was -1.08 ± 4.9 (mean ± 2 SD) in the Bland and Altman plot.

**Discussion**

In this prospective cross-sectional study we have found significant elevation of LTE₄ levels in the urine of patients with asthma and COPD.
with asthma compared to matched COPD patients and healthy controls. Smoking habit significantly affects LTE4 levels only in asthmatic subjects. The values of LTE4 in urine present significant correlations with eosinophils in induced sputum only in asthmatics; interestingly, the absence of smoking habit seems to affect negatively the above significance. The measurements of LTE4/creatinine on 2 consecutive days are repeatable.

CysLTs are synthesized from arachidonic acid through a 5-lipoxygenase pathway and are mainly generated by many inflammatory cells, particularly eosinophils, mast cells and macrophages. The implication of LTE4 in asthmatic inflammation is well established, and the increased levels of LTE4 in the urine of asthmatic subjects reported in this study are comparable to those published by previous investigators. However, this is the first study to our knowledge that evaluates the levels of LTE4 in urine of patients with COPD. Two previous studies have reported increased CysLTs levels in patients with COPD; however, in the first study the mediator that was used (LTC4) is not considered as a stable end product in CysLT production, and in the second the biological fluids studied did not represent sources which would definitely express airway inflammation. Interestingly, the levels measured were not significantly higher compared to normal subjects, indicating that the 5-lipoxygenase pathway of arachidonic acid metabolism may not be activated in COPD patients. The reason for this observation might be attributed to the capability and/or the activation state of the inflammatory cells which participate in the CysLTs production. This is partially supported by the findings of our study that show a positive correlation between sputum eosinophils and LTE4 values in asthma and the absence of any correlation between induced sputum cells and LTE4 values in COPD. However, this positive correlation might also express the CysLTs-induced prolonged survival of eosinophils in the airways that contributes to the maintenance of the inflammatory process in asthma. The absence of similar correlation with ECP might be related to the low sensitivity of this mediator for the assessment of eosinophilic inflammation. A previous observation, where the use of a CysLT1 receptor antagonist led to improvement of some COPD patients with less fixed airflow obstruction was not tested in this study, since the methodology used did not include a proper approach for this purpose.

Table 3  Correlations of LTE4 in COPD and asthmatic patients as well as in asthmatic smokers and non-smokers.

<table>
<thead>
<tr>
<th></th>
<th>Asthma</th>
<th>COPD</th>
<th>Asthma smokers</th>
<th>Asthma non-smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>P-value</td>
<td>r</td>
<td>P-value</td>
</tr>
<tr>
<td>FEV1 (% predicted)</td>
<td>−0.02</td>
<td>0.80</td>
<td>−0.04</td>
<td>0.74</td>
</tr>
<tr>
<td>FEV1/FVC ratio</td>
<td>−0.014</td>
<td>0.35</td>
<td>−0.21</td>
<td>0.10</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>0.83</td>
<td>&lt;0.0001</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Smoking (py)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ECP (µg/L)</td>
<td>0.10</td>
<td>0.50</td>
<td>0.002</td>
<td>0.83</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.03</td>
<td>0.86</td>
<td>0.02</td>
<td>0.77</td>
</tr>
</tbody>
</table>

Bold letters indicate significant correlations. ND = not done.
placebo-controlled design; thus, any conclusions arising were not strong enough to confirm a potential role of CysLT\textsubscript{1} receptor antagonists in COPD.\textsuperscript{17}

Smoking affects significantly LTE\textsubscript{4} levels in asthma but not in COPD and normal subjects. The positive effect of smoking in CysLTs production in asthma, irrespective of the degree of smoking habit, might be related to the number and/or the activation state of cells responsible for producing CysLTs. Cells like alveolar macrophages and mast cells, which are activated in asthma and also increased by smoking, might stimulate the production of CysLTs via the preexisting activated 5-lipoxygenase pathway. This theory is supported by previous observations where in vivo data showed the protective effect of a leukotriene receptor antagonist on cigarette smoking-induced lung injury.\textsuperscript{18} Similar findings regarding the concentration of the eicosanoid PGE\textsubscript{2} in smoking asthmatics\textsuperscript{19} indicated that eicosanoid metabolism in asthmatics is significantly influenced by smoking. Data from the present study showing absence of significant correlation between eosinophils in induced sputum and the levels of LTE\textsubscript{4} in the urine of non-smoking asthmatics may represent further evidence in this direction. However a possible limitation of our study by combining the above observations might be that they just represent the prolonged survival of eosinophils that is induced by the increased CysLTs or the eosinophil-induced CysLTs production. The above observation about the effect of smoking on asthmatics might have practical implications, since it might partially explain the difference in the response to inhaled steroid treatment in smoking asthmatics, once the CysLTs-related pathophysiology is not sensitive to the treatment with steroids.\textsuperscript{20,21} Interestingly, the above observations were not present in normal subjects and COPD patients. This finding does not seem reasonable, but it could be attributed to the previously inactivated 5-lipoxygenase pathway. In normal subjects it might also be explained by previous observations where the increase in LTE\textsubscript{4} was attributed to acute effects rather than to indirect effects resulting from chronic smoking exposure, since 5-lipoxygenase is not in a proper state to be activated and to produce CysLTs.\textsuperscript{2} However, some previous data supports that smoking in a dose-dependent manner significantly affects LTE\textsubscript{4} values in normal subjects.\textsuperscript{22}

The repeatability data presented in this study are similar to data reported for COPD using another biological fluid,\textsuperscript{23} indicating that, in stable patients with chronic inflammatory airway diseases and persistent inflammation, the measurement of mediators involved in the inflammatory process may be repeatable. This study presents one possible limitation, regarding the measurement of LTE\textsubscript{4} in urine, which was performed by an EIA without purification. However, LTE\textsubscript{4} has been shown to be stable in urine samples stored at –20°C for months without the addition of preservatives. Additionally, LTE\textsubscript{4} levels in crude urine samples assessed by EIA have been shown to present significant correlation with LTE\textsubscript{4} measurements performed after purification on solid-phase extraction followed by separation on reversed-phase high-performance liquid chromatography\textsuperscript{24} and finally a specific procedure assessing interference in diluted and undiluted samples as suggested by the manufacturer was performed.

In summary, we report that patients with asthma present higher urine LTE\textsubscript{4} values compared to patients with COPD and to healthy controls. Smoking in asthmatics seems to be a critical parameter that might influence the above values. Eosinophils as a cellular source or as a result of prolonged survival are probably the key inflammatory cells in CysLTs-related inflammatory process in smoking asthmatics.

![Figure 3](image_url)

**Figure 3** Correlations between the concentration of LTE\textsubscript{4}/creatinine and eosinophils (A) in asthmatic smokers ($r = 0.77$, $P < 0.0001$) and (B) in asthmatic non-smokers ($r = 0.35$, $P = 0.1$).

References


