Leukotriene B₄ in Exhaled Breath Condensate and Sputum Supernatant in Patients With COPD and Asthma*

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Study objectives: Some patients with COPD present with significant reversibility of airflow limitation after receiving bronchodilation therapy. Leukotriene B₄ (LTB₄) has been implicated in the pathophysiology of both COPD and asthma. We tested the hypothesis that COPD patients with airflow reversibility and asthmatic patients who smoke might have similar levels of LTB₄ in exhaled breath condensate (EBC) and sputum supernatant. The repeatability and stability of LTB₄ measurements were additionally studied.

Design: Prospective, cross-sectional study.

Patients or participants: We studied 30 patients with COPD (15 smokers [FEV₁, 56% predicted; SD, 6% predicted]; 15 patients with significant reversibility in airway obstruction after bronchodilation [FEV₁, 14% predicted; SD, 2% predicted]). Fifteen asthmatic patients who smoked, with similar FEV₁ and reversibility were also studied. Ten healthy smokers served as control subjects.

Setting: A hospital research laboratory.

Interventions: Spirometry and reversibility testing were performed on the first visit. On the following day, EBC was collected for the measurement of LTB₄, and induced sputum was collected for differential cell counts and LTB₄ measurement in the sputum supernatant.

Measurements and results: LTB₄ levels in EBC (mean [SD]) were increased in COPD patients (mean, 86.7 pg/mL; SD, 19 pg/mL) and asthmatic patients (mean, 97.5 pg/mL; SD, 15 pg/mL) compared to control subjects (mean, 32.3 pg/mL; SD, 10 pg/mL; p < 0.0001 for both groups). COPD patients with airflow reversibility (mean, 99.8 pg/mL; SD, 12 pg/mL) had values similar to those of asthmatic patients (mean, 97.5 pg/mL; SD, 15 pg/mL; p = 0.2) and higher than those of COPD patients without airflow reversibility (mean, 73.7 pg/mL; SD, 17 pg/mL; p = 0.002). Similar results were observed in the sputum supernatant. Measurements of LTB₄ in EBC and sputum were repeatable on two consecutive days, but measurements in the frozen samples of EBC and sputum were not stable after 3 weeks.

Conclusions: Patients with asthma and reversible COPD presented with higher LTB₄ values compared to patients with nonreversible COPD and healthy smokers. This difference may be mainly attributed to the presence of reversibility in airway obstruction, probably as part of a common underlying inflammatory process.

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Key words: asthma; COPD; exhaled breath condensate; induced sputum; leukotriene B₄; reversibility

Abbreviations: EBC = exhaled breath condensate; LTB₄ = leukotriene B₄

COPD is currently defined by the presence of airflow limitation that shows little or no improvement after the administration of inhaled bronchodilators.¹ However, some patients with COPD present with significant reversibility of airway obstruction after the inhalation of β₂-agonist agents.¹ The distinction of those patients from smokers with chronic asthma who had persistent airflow limitation remains difficult, and only data from the underlying inflammatory pattern can safely distinguish the two situations.²

Leukotrienes are potent bronchoconstrictors that

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may be implicated in bronchial obstruction in both asthma and COPD patients, and their role has been evaluated in both diseases in the past. Cysteinyll

leukotrienes have been connected with the asthmatic response, whereas the role of leukotriene B₄ (LTB₄) has been connected to neutrophilic inflammation. LTB₄ is produced by constitutive cells (eg, mast cells and macrophages) and infiltrating cells (eg, neutrophils and eosinophils). LTB₄ has no direct action on airway smooth muscle, but it may contribute to bronchoconstriction by increasing vascular permeability and mucus secretion.

Sputum induction and exhaled breath condensate (EBC) collection are safe techniques that have been used for the assessment of airway inflammation in patients with obstructive airway diseases. Increased levels of LTB₄ have been found in the sputum and EBC of stable patients and patients experiencing acute exacerbations of COPD. Furthermore, LTB₄ levels are increased in the EBC of asthmatic patients, and this has been associated with more severe forms of the disease.

The aim of this study was to assess the levels of LTB₄ in the EBC and sputum supernatant of COPD patients with reversible and nonreversible airway obstruction after bronchodilation and to compare them with the levels of matched asthmatic patients who smoke and healthy smokers in an attempt to clarify the in vivo role of this mediator. We tested the hypothesis that COPD patients with reversible airway obstruction might have similar levels of LTB₄ as asthmatic smokers, possibly through some common pathophysiologic mechanism. Furthermore, we investigated whether the cellular source of this mediator was similar in the two diseases, using sputum differential cell counts. Additionally, we checked for correlations with either lung function impairment, as expressed by FEV₁, or the degree of airflow reversibility.

Finally, we validated the measurements of LTB₄ in EBC and induced sputum supernatant, and examined whether there was a correlation between the levels of this mediator in these two specimens in order to test whether these two techniques provide comparable measurements in the assessment of airways inflammation.

**Materials and Methods**

**Subjects**

Subject characteristics are summarized in Table 1. The diagnosis of COPD was established according to the Global Initiative for Chronic Obstructive Lung Disease guidelines, whereas the Global Initiative for Asthma guidelines were used for the diagnosis of asthma.

Thirty male COPD patients, who were current smokers, nonatopic, and steroid-naïve, were divided in two subgroups of 15 patients each, according to the presence of reversibility in airway obstruction after bronchodilation. All COPD patients were characterized by the predominance of neutrophils and the absence of eosinophils in induced sputum. All patients were occasionally receiving short-acting β₂-agonists as relief medication.

Fifteen atopic male asthmatic patients, with similar smoking histories, were also studied. All asthmatic patients were characterized by sputum eosinophilia and had positive bronchodilation test results. They were occasionally receiving short-acting β₂-agonists as relief medication.

The differentiation of asthmatic and COPD patients with reversibility of airway obstruction was based on the following criteria: (1) asthmatic patients had a long-standing history of asthma from their childhood or adolescence, preceding the onset of smoking, whereas COPD patients had later onset of their symptoms, which was closely related to smoking; (2) asthmatic patients had a history of atopy, which was confirmed with the measurement of elevated total IgE levels and positive skin prick test results for six common aeroallergens; and (3) COPD patients did not present with sputum eosinophilia, a finding that was present in the asthmatic group. The classification of the patients in the two groups was performed by two experienced clinicians (K.K. and T.K.) who were not aware of the LTB₄ measurements at the time of study enrollment.

All patients were clinically stable (ie, they had no evidence of acute exacerbation for at least 4 weeks prior to entering the study). None of our patients was receiving any antiinflammatory

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control Subjects (n = 10)</th>
<th>COPD Patients (n = 30)</th>
<th>COPD Patients (n = 15)</th>
<th>Asthma Patients (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>58 (8) [43–77]</td>
<td>56 (7) [45–69]</td>
<td>57 (7) [48–69]</td>
<td>57 (8) [45–68]</td>
</tr>
<tr>
<td>FEV₁, % predicted</td>
<td>86 (6) [52–96]</td>
<td>56 (6) [46–65]</td>
<td>55 (6) [47–63]</td>
<td>57 (5) [46–65]</td>
</tr>
<tr>
<td>FEV₁/FVC ratio, %</td>
<td>88 (7) [50–94]</td>
<td>64 (9) [51–73]</td>
<td>65 (6) [51–72]</td>
<td>63 (7) [54–73]</td>
</tr>
<tr>
<td>Smoking habit, pack-yr</td>
<td>46 (3) [34–62]</td>
<td>44 (6) [37–71]</td>
<td>43 (5) [40–70]</td>
<td>45 (8) [37–71]</td>
</tr>
<tr>
<td>Reversibility, %</td>
<td>0</td>
<td>9 (6) [0–19]</td>
<td>14 (2) [12–19]</td>
<td>4 (2) [0–7]</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td>41 (4) [33–57]</td>
<td>63 (5) [53–72]</td>
<td>62 (6) [53–71]</td>
<td>64 (5) [55–72]</td>
</tr>
<tr>
<td>Eosinophils, %</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>12 (3) [7–18]</td>
</tr>
<tr>
<td>Atopy</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td>(+)</td>
</tr>
</tbody>
</table>

*Values given as mean (SD) [range]. R = reversible; NR = nonreversible; −, negative; +, positive.*
treatment (including corticosteroids, long-acting β-agonists, or leukotriene antagonists), theophylline, inhaled or oral mucolitics, or long-term oxygen therapy.

Ten male subjects, who were current smokers with no history of any disease, served as control subjects. All were nonatopic, which was determined on the basis of negative responses to skin-prick tests for six common aeroallergens and a negative result for bronchial challenge testing, and none of them was receiving any kind of medication at the time of the study.

All subjects were recruited from the outpatient clinics of the Army General Hospital of Athens over a period of 6 months. The Army General Hospital serves army personnel, veterans, and civilians. The Scientific Committee of the hospital approved the study protocol, and all participants gave written informed consent.

Lung Function

Pulmonary function and reversibility tests were measured with a dry spirometer (Vica-test, model VE52; Mijnhardt; Rotterdam, the Netherlands). FEV₁ and FVC were measured according to American Thoracic Society criteria. Reversibility testing was performed according to Global Initiative for Chronic Obstructive Lung Disease guidelines. Briefly, 400 µg of short-acting β₂-agonist was administered. Increases in FEV₁ 30 min after bronchodilator administration of > 12% above the prebronchodilator FEV₁ were considered to indicate reversible airway obstruction. The alternative increase of 200 mL in FEV₁ was not used as a reversible response in this study.

Collection and Measurements of EBC

The collection of EBC was performed as previously described. Approximately 2 mL of condensate was stored at −70°C. All condensate samples were tested for salivary contamination by the determination of amylase activity. LTB₄ was measured by a commercial kit (Cayman Chemical; Ann Arbor, MI), as previously described.

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 the inhalation of an aerosol of an isotonic saline solution (0.9%) generated by an ultrasonic nebulizer (model 2696; DeVilbiss; Somerset, PA) was chosen, since some of our subjects had significant airflow limitation by the determination of amylase activity. LTB₄ was measured by a commercial kit (Cayman Chemical; Ann Arbor, MI), as previously described.

Statistical Analysis

Data are expressed as the mean (SD), unless otherwise mentioned. For comparisons of values between two groups, the Mann-Whitney U tests were used. The Spearman correlation coefficient was used to investigate correlations between parameters. The repeatability of LTB₄ measurements was assessed using the Bland-Altman test. The stability of LTB₄ was evaluated using one-way analysis of variance with an appropriate post hoc test for multiple comparisons (Bonferroni correction). A p value of < 0.05 was considered to be significant.

RESULTS

LTB₄ Values in EBC and Sputum

The levels of LTB₄ in EBC were increased in both COPD patients (mean, 86.7 pg/mL; SD, 19 pg/mL) and asthmatic patients (mean, 97.5 pg/mL; SD, 15 pg/mL) compared to LTB₄ levels in control subjects (mean, 32.3 pg/mL; SD, 10 pg/mL; p < 0.0001 for both groups). COPD patients with reversible airway obstruction had values similar to those of asthmatic patients (mean, 99.8 pg/mL [SD, 12 pg/mL] vs 97.5 pg/mL [SD, 15 pg/mL], respectively; p = 0.39) and significantly higher values compared to COPD patients without airflow reversibility (mean 73.7 pg/mL; SD, 17 pg/mL; p = 0.002) [Fig 1].

Similar results were observed for the LTB₄ values in sputum supernatant. LTB₄ sputum levels were increased in both COPD patients (mean, 2.64 pg/mL; SD, 0.6 pg/mL) and asthmatic patients (mean, 3.05 pg/mL; SD, 0.2 pg/mL) compared to those in healthy subjects who smoked (mean, 1.45 pg/mL; SD, 0.4 pg/mL; p < 0.0001 for both groups). COPD patients with reversible airway obstruction had values similar to those of asthmatic patients (mean, 3.07 pg/mL [SD, 0.3 pg/mL] vs 3.05 pg/mL [SD, 0.2 pg/mL]; p = 0.95) and significantly higher...
values compared to COPD patients without airflow reversibility (mean, 2.24 pg/mL; SD, 0.5 pg/mL; \( p < 0.002 \)) \[Fig 2\].

**Correlations**

Major correlation data are summarized in Tables 2 to 4. Briefly, the values of LTB4 in both the sputum supernatant and the EBC of patients with reversible and nonreversible COPD presented a significant positive correlation with sputum neutrophil levels. The levels of LTB4 in the sputum and EBC of COPD patients with both reversible and nonreversible COPD, were not significantly correlated with the degree of reversibility. No significant correlation was observed between LTB4 levels and the degree of lung function impairment. A significant positive correlation was observed between LTB4 values in the EBC and sputum of COPD patients (\( r = 0.83; p < 0.0001 \)) \[Fig 3, top\].

LTB4 values, in both the sputum supernatant and EBC of patients with asthma, did not correlate either with the degree of lung function impairment or reversibility, or with the number of sputum inflammatory cells. Furthermore, a significant positive correlation was observed between LTB4 values in the EBC and sputum of asthmatic patients (\( r = 0.87; p < 0.0001 \)) \[Fig 3, bottom\].

**Repeatability and Stability Measurements**

The measurements of LTB4 in EBC on 2 consecutive days showed good repeatability. The mean LTB4 levels in EBC on days 1 and 2 were 64.2 pg/mL (SD, 38 pg/mL) and 65.4 pg/mL (SD, 37 pg/mL), respectively. The correlation between LTB4 measurements on 2 consecutive days was significant (\( r = 0.96; p < 0.0001 \)). The mean (± 2 SDs) difference with limits of agreement was \(-0.56 ± 9.3\) in the Bland-Altman plot (Fig 4, top).

The evaluation of the stability of LTB4 in the frozen EBC samples showed significant differences among the four measurements performed (first day, 61.4 pg/mL [SD, 39 pg/mL]; after 1 week, 65.2 pg/mL [SD, 38 pg/mL]; after 2 weeks, 66.3 pg/mL [SD, 35 pg/mL]; after 3 weeks, 56.3 pg/mL [SD, 14 pg/mL]; \( p = 0.05 \) [significantly lower after 3 weeks]).

**Table 2—Correlations of LTB4 in EBC of COPD Patients**

<table>
<thead>
<tr>
<th>Variables</th>
<th>R</th>
<th>NR</th>
<th>R</th>
<th>NR</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV1, % predicted</td>
<td>-0.07</td>
<td>0.79</td>
<td>-0.17</td>
<td>0.09</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.85</td>
<td>0.0001†</td>
<td>0.85</td>
<td>&lt; 0.0001†</td>
</tr>
<tr>
<td>Macrophages</td>
<td>-0.76</td>
<td>0.0009†</td>
<td>-0.85</td>
<td>&lt; 0.0001†</td>
</tr>
<tr>
<td>Reversibility, %</td>
<td>0.19</td>
<td>0.51</td>
<td>0.27</td>
<td>0.32</td>
</tr>
<tr>
<td>LTB4 sputum</td>
<td>0.96</td>
<td>&lt; 0.0001†</td>
<td>0.61</td>
<td>0.01</td>
</tr>
</tbody>
</table>

*See Table 1 for abbreviations not used in the text.
†Significant correlations.
The measurements of LTB4 in sputum on 2 consecutive days showed good repeatability. The mean LTB4 levels in sputum on days 1 and 2 were 2.48 pg/mL (SD, 0.8 pg/mL) and 2.43 pg/mL (SD, 0.8 pg/mL), respectively. The correlation between LTB4 measurements on the 2 consecutive days was significant ($r = 0.94; p < 0.0001$). The mean (± 2 SDs) difference with limits of agreement was 0.06 ± 0.3 in the Bland-Altman plot (Fig 4, bottom).

The evaluation of the stability of LTB4 in the frozen sputum samples showed significant differences among the four measurements performed (first day, 2.41 pg/mL [SD, 0.8 pg/mL]; after 1 week, 2.45 pg/mL [SD, 0.8 pg/mL]; after 2 weeks, 2.47 pg/mL [SD, 0.85 pg/mL]; after 3 weeks, 2.27 pg/mL [SD, 0.9 pg/mL]; $p = 0.03$ [significantly lower after 3 weeks]).

**Discussion**

In this prospective, cross-sectional study, we have found significant elevations in LTB4 levels in the EBC and sputum supernatant of patients with COPD and asthma who smoked compared to matched healthy control subjects who smoked. Furthermore, COPD patients with reversible airway obstruction showed levels of LTB4 similar to those of asthmatic patients who smoked, and both groups had higher levels of LTB4 than COPD patients without reversibility of airway obstruction. The levels of LTB4 were not related to the degree of reversibility or lung function impairment. The values of LTB4 in sputum and EBC showed significant correlations with the number of neutrophils in COPD patients, but did not provide evidence for a possible cellular source of LTB4 in both diseases. The measurements of LTB4 in EBC and sputum seem to be repeatable, but the stability of this mediator is not preserved in frozen samples after a period of 3 weeks.

The levels of LTB4 both in EBC and sputum supernatant that were reported in this study are comparable to those published by previous investigators.$^7$,$^11$,$^18$ However, this is the first study, to our knowledge, that compares the levels of LTB4 in both samples in patients with asthma and COPD. Interestingly, the levels measured in EBC were much higher than the levels in sputum. This may be attributed either to the physical properties of LTB4, which make it more detectable in EBC, or to the
dilution required for the processing of induced sputum. The strong correlations between the LTB4 values in EBC and sputum in all study groups suggest that these two techniques may provide comparable results for the evaluation of airways inflammation, despite the different origins of the collected samples.

Previous studies\textsuperscript{11,12,18} have reported that LTB4 levels are increased in patients with both asthma and COPD, but the increase was more remarkable in the latter. The implication of LTB4 in asthmatic inflammation is not well-established, although there is evidence that supports an important role for LTB4 in more severe forms of asthma, which is probably associated with the presence of neutrophilia.\textsuperscript{12,21} Our results, along with the previously published data, suggest that LTB4 represents a significant mediator in the inflammatory process of both asthma and COPD. The similar levels of LTB4 in asthmatic patients and patients with reversible COPD confirm our initial hypothesis that these two diseases may share some common pathophysiologic mechanism related to this mediator. Despite the absence of significant correlation between LTB4 values and the degree of reversibility in patients with reversible and nonreversible COPD, we speculate that the critical point for the evaluation of LTB4 in COPD patients is the reversibility of airway obstruction, probably not as a functional parameter but as the consequence of a common underlying inflammatory process. An important factor that one would have to take into account for the interpretation of these results is the reported low reproducibility of bronchodilation testing through time in COPD patients.\textsuperscript{22} However, the measurements of LTB4 in our patients were performed on the day following the reversibility test, thus representing an inflammatory profile that practically corresponds to the reversibility data.

The underlying inflammatory process that is responsible for the comparable levels of LTB4 in patients with asthma and reversible COPD might be either a common cellular source of LTB4 or a similar effect on the activity of 5-lipoxygenase in airway cells. The data from the present study do not identify a cellular source that could be responsible for the production of LTB4 in those two distinct groups. Despite the significant correlations of LTB4 with neutrophils in COPD, the absence of correlations with either eosinophils or neutrophils in asthmatic patients does not allow us to detect this source in the infiltrating cells of the airways. If we consider neutrophils as one probable cellular source of LTB4 in COPD patients, we would have to seek another cellular source that might be implicated in the production of LTB4 both in patients with reversible COPD and in asthmatic patients. A plausible answer to this question, and a possible additional source of LTB4, might be the structural cells that are implicated in the pathophysiology of both asthma and COPD, especially mast cells or macrophages.\textsuperscript{23,24}

The repeatability data presented in this study are similar to data that have been reported for COPD,\textsuperscript{11} 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. Although it is hard to specify the exact reason for the instability of the frozen LTB4 samples observed in this study, one plausible explanation might be the physical loss of this mediator, probably due to its binding to nonspecific sites of the container.\textsuperscript{25} Our data support this hypothesis, as they indicate that this physical \textit{in vitro} loss of LTB4 in the frozen samples is time-related.

A possible limitation of this study may be the classification of patients with reversible COPD and asthmatic patients who smoked. However, we think that the fact that the characterization of patients was performed by two experienced clinicians who were

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\textbf{Figure 4.} Repeated measures of LTB4 on 2 consecutive days in 13 subjects, presented in Bland-Altman plots (differences against mean values) in EBC (top) and in sputum supernatant (bottom). Dotted lines represent the mean difference value and the limits of agreement (± 2 SDs).
not aware of the LTB₄ measurements at the time of enrollment, as well as the use of specific criteria, to minimize the chance for misclassification.

In summary, we have reported that patients with asthma and reversible COPD show higher levels of LTB₄ compared to patients with nonreversible COPD and to healthy control subjects who smoke. This difference may be attributed mainly to the presence of reversibility in airway obstruction, probably not as a functional factor but as a component that reflects a common underlying inflammatory process. On that perspective, we suggest that reversible airway obstruction in COPD might share some common mechanisms with asthma.

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