

EXERCISE INDUCED

Exercise Increases Exhaled Breath Condensate Cysteinyl Leukotriene Concentration in Asthmatic Patients

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Background. Although the importance of cysteinyl leukotrienes (Cys-LTs) in exercise-induced bronchoconstriction (EIB) is supported by various sources of evidence, how the concentration of these mediators change during the development of EIB has not been investigated. **Objectives.** Our goal was to determine the effect of exercise on the concentration of airway Cys-LT in asthmatic patients by measuring Cys-LT in exhaled breath condensate (EBC). **Methods.** Seventeen atopic asthmatic patients with a previous history of EIB and six healthy volunteers were studied. Before and two times within 10 minutes after exercise challenge, FEV₁ was measured and EBC was collected for Cys-LT measurement. Exhaled nitric oxide level, a marker of airway inflammation, was also determined at baseline. **Results.** Baseline Cys-LT level was higher in the asthmatic group versus healthy subjects (168 pg/mL /112–223/ vs. 77 pg/mL /36–119/, $p = .03$). EBC Cys-LT concentration increased in all asthmatic patients post-exercise ($n = 17$, $p = .03$), with the increase significantly greater in patients developing exercise-induced bronchospasm ($n = 7$, $p = .03$), whereas no change was observed in healthy controls ($p = .59$). The exercise-induced fall in FEV₁ in asthmatics was related to the increase in EBC Cys-LT concentration ($r = -0.40$, $p = .03$). **Conclusions.** Our study shows that Cys-LT concentration of EBC is elevated minutes after physical exercise in asthmatic patients and strongly supports the concept that the release of this mediator is involved in the development of EIB.

Keywords asthma, cysteinyl leukotrienes, exercise-induced bronchoconstriction, exhaled breath condensate, exhaled nitric oxide

INTRODUCTION

Asthma is a chronic inflammatory disorder of the airways characterized by variable bronchoconstriction. Various inflammatory cells and mediators play a role in disease development with bronchial hyperresponsiveness (BHR) being a key feature. Although several methodologies are utilized to diagnose BHR, there is only a slight overlap among patient groups with positive test outcomes confirmed by these methods. According to cross-sectional studies, provocation with exercise causes airway narrowing in 30–50% of asthmatic patients. This clinically diagnosed phenomenon is called exercise-induced bronchoconstriction (EIB). The pathomechanism of how exercise leads to bronchospasm is complex, but it is widely accepted that exercise-induced hyperpnoea plays a prominent role as an initiating stimulus by cooling (heat-loss theory) and drying (water-loss theory) the airways. In line with the water-loss theory, dehydration stimulates inflammatory cells in the airways to release bronchoconstrictor mediators.

The fact that provocation tests such as those with mannitol or methacholine have a limited utility in diagnosing EIB (1) suggests that this phenomenon is not general

in asthma and occurs only in a subgroup of asthmatic patients. Exercise provocation test is more commonly reported to be positive in patients with higher severity of airway inflammation (2). Supporting this observation, a high basal exhaled nitric oxide level—an accepted marker of asthmatic airway inflammation—was shown to be related with EIB in some studies (3–5). Other groups, however, found contradictory results (6, 7). Furthermore, the relationship between exhaled nitric oxide and EIB was confirmed only in atopic subjects (5, 8).

Although several inflammatory mediators have been shown to be present at elevated concentration a few hours (or a day) after exercise challenge, only one study has investigated these changes during the development of bronchospasm (9). Invasive (or semi-invasive) sampling techniques such as bronchoalveolar lavage or sputum induction cannot be repeated within a short period of time as these procedures *per se* might influence mediator levels measured in airway samples. Furthermore, both techniques have been described to trigger low-grade inflammation in the airways (10).

Collection of exhaled breath condensate (EBC) is a simple, noninvasive mode of obtaining samples from the airways. This method can be repeated within short intervals without side or carryover effects of sampling, thus providing a unique method to follow changes in airway mediator levels (11). Many biomarkers have previously been successfully measured in EBC, including leukotrienes (12), histamine (13), cytokines (14), enzymes (15), and ATP (16).

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The major role of cysteinyl leukotrienes (Cys-LT) in the development of exercise-related airway narrowing is supported by several lines of evidence (17). Pharmacological studies with leukotriene antagonists show a significant improvement of exercise-induced bronchospasm (18–20). In patients with EIB, the level of LTE₄ in urine (21) and the level of Cys-LTs in induced sputum (2) were increased hours after exercise, but Cys-LT concentration has not been investigated during the development of EIB. EBC collection has been successfully applied minutes after exercise challenge to test adenosine concentration (9), but only pre-exercise EBC Cys-LT concentrations have been determined in subjects with EIB patients, previously (12).

In this study, we investigated the changes in Cys-LT concentration in EBC caused by exercise in asthmatic patients. We also compared the change in Cys LT concentration with airway inflammation as expressed by exhaled NO concentration and with the degree of bronchospasm. We focused on the time period (the first 10 minutes after exercise) when the development of EIB is usually observed.

MATERIALS AND METHODS

Subjects

We studied 17 nonsmoking asthmatic patients (28 ± 10 years, mean $\pm$ SD) with a history of exercise-related dyspnea who were attending the outpatient clinic of National Koranyi Institute for TB and Pulmonology, Budapest, Hungary. All patients met the Global Initiative for Asthma diagnostic criteria for bronchial asthma. Subjects had $\geq 12\%$ increase in FEV₁ to 400 μ g inhaled salbutamol in their history. Asthma was stable and considered clinically well-controlled in all patients. Subjects were atopic, as demonstrated by at least two positive results to skin prick test with the following common aeroallergens: house dust mite, cat dander, dog dander, grass pollen, and *Aspergillus fumigatus*. None of them were receiving inhaled corticosteroids therapy within 2 weeks prior to the study; β_2 -agonists were withdrawn ≥ 12 h before the exercise. We included six healthy, nonsmoking volunteers as a control group (25 ± 6 years).

Subjects were instructed not to consume caffeinated drinks and foods, and refrain from exercise > 12 h before the study. Subjects presenting an upper respiratory tract infection within 6 weeks before the study or patients with a history of chronic upper airway/sinus disease were excluded from the study (22).

The research was carried out according to the Declaration of Helsinki. The protocol was approved by the local ethics committee, and written informed consent was obtained from each subject before the study.

Study Design

The study protocol was used as previously described (9). Briefly, first EBC was collected for 5 minutes. This was followed by the measurement of exhaled NO and FEV₁

in each study participant. Participants were then subjected to a standardized, 8-minute treadmill exercise test. Afterwards, two cycles of EBC collection for 5 minutes immediately followed by FEV₁ measurement was performed. All measurements were performed indoor with fixed temperature and humidity (25°C, 75%).

Collection of EBC

EBC was collected using the EcoScreen condenser (Jaeger, Hoechberg, Germany), as reported previously (9). At rest, subjects breathed at normal frequency and tidal volume. Subjects were instructed not to alter their breathing pattern volume and route of inhalation during EBC collection and to swallow their saliva periodically. There was no significant difference in minute volume between the groups during EBC collection. Collected samples were stored frozen (-70°C) until analysis.

Lung Function Test and Fractional Exhaled Nitric Oxide (FE_{NO50}) Measurement

FEV₁ was measured by means of an electronic spirometer (PDD-301/s, Piston, Budapest, Hungary) according to the latest guidelines (23). Three technically acceptable maneuvers were performed and the highest value was used.

FE_{NO} levels were recorded with a chemiluminescence analyzer (Model LR2000, Logan Research, Rochester, UK) without wearing a nose-clip by slow exhalation (250 mL/s) from total lung capacity for 20–30 seconds against a resistance (5 cm H₂O) to prevent nasal contamination. Three successive recordings were made and the mean of these values was used in all calculations. To make our results comparable with other studies measuring exhaled nitric oxide level at 50 mL/s, we calculated FE_{NO50} using a previously described and validated formula (24) and presented FE_{NO} also as FE_{NO50}.

Exercise Challenge

Subjects exercised on an electrically driven treadmill at room temperature wearing a nose-clip. The speed, gradient, time of exercise and heart rate were displayed continuously during running. Exercise intensity was ramped over 2 minutes to 80–90% of predicted heart rate (220-age) and sustained for 6 minutes (25). A greater than 15% drop in FEV₁ because of exercise was used as a criterion for a positive BHR test.

Determination of Cys-LT

Cys-LT concentrations were measured by using a specific enzyme immunoassay (Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions. The detection limit was 13 pg/mL. The assay was validated for measurements in EBC and calibration curves were created in the buffer solution provided by the manufacturer.

Statistical Analysis

Results are expressed as a mean $\pm$ SD and for FE_{NO}, FE_{NO50}, and Cys-LT concentration median/range/.

Normality was tested with Shapiro–Wilk normality test. Because Cys-LT concentrations and FE_{NO} levels were not normally distributed, these values were logarithmically transformed and log transformed data were used for further analysis. Repeated measures ANOVA and two-way ANOVA together with Bonferroni post hoc test were used to compare baseline versus post-exercise and asthmatic versus healthy and EIB versus non-EIB subgroup data, respectively. Pearson test was performed to analyze the correlation between the different variables. *t*-Tests were used to compare asthmatic versus control groups and EIB versus non-EIB subgroups. The maximal increase/decrease in Cys-LT concentration and FEV₁ was calculated by using the highest/lowest post-exercise value and plotted as percentage of the baseline value. *P*-values <.05 were considered significant.

RESULTS

All subjects performed the exercise provocation and the simultaneous collection of EBC without any adverse effects.

Baseline Comparisons between Asthmatic and Healthy Groups

There was no difference in the baseline FEV₁ percentage predicted between the asthmatic and healthy groups (3.49 ± 0.77 L, $99 \pm 11\%$ pred. vs. 3.53 ± 0.61 L, $95 \pm 3\%$ pred., $p = .33$). However, baseline Cys-LT concentration (157 pg/mL / 43 – 400 / vs. 69 pg/mL / 28 – 141 /, $p = .03$) was significantly higher in asthmatic subjects compared with healthy individuals but no significant difference was seen in FE_{NO} between the two groups (8.8 ppb / 3.6 – 30.1 / vs. 5.8 ppb / 3.0 – 12.7 /, $p = .10$). The calculated FE_{NO}50 values were 21.1 ppb / 9.3 – 65.45 / versus 14.4 ppb / 7.8 – 29.6 / for asthmatics and healthy subjects, respectively; without significant difference between groups ($p = .12$).

Effect of Exercise on FEV₁ and Cys-LT Concentration

As a result of the exercise challenge, FEV₁ dropped from that of the baseline value ($-9\% \pm 13\%$), yet there was no significant change in the time course of FEV₁ in the asthmatic group ($n = 17$, $p = .20$). In the healthy group a slight, but not significant, increase in FEV₁ was observed ($n = 6$, $p = .60$, $4 \pm 5\%$).

However, in asthmatic patients exercise caused an increase in EBC Cys-LT levels ($n = 17$, $p = .03$, repeated measures ANOVA) with no difference between time points evaluated by a post hoc test (130 pg/mL / 42 – 410 / and 180 pg/mL / 58 – 1000 /, 0 and 10 minutes after exercise, respectively). On the contrary, no change was found in healthy subjects ($n = 6$, $p = .34$; 68 pg/mL / 52 – 147 / and 93 pg/mL / 25 – 140 /, 0 and 10 minutes after exercise, respectively). There was a significant difference in the time course of changes in Cys-LT concentrations between asthmatic and healthy control groups ($p = .02$, two-way ANOVA; Figure 1).

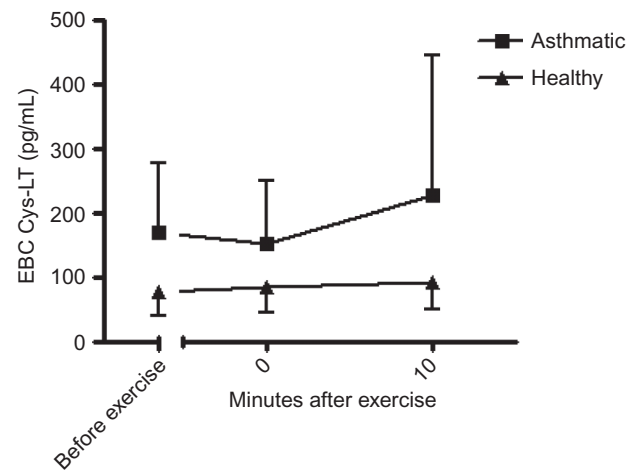


FIGURE 1.—Time course of Cys-LT concentration changes in asthmatic and healthy groups. Exercise caused significant change in the asthmatic group, whereas no change was found in the healthy subjects ($p = .03$ and $p = .34$, repeated measures ANOVA). There was a significant difference in the time course of changes between the two groups ($p = .02$, two-way ANOVA).

The exercise-induced change in EBC Cys-LT concentration at each time point was matched with the corresponding change in FEV₁ values. In this way two pairs of data were obtained from each subject. There was a significant negative correlation between these variables in the asthmatic group ($r = -0.40$, $p = .03$), whereas no relationship was found in the control group (18 matched pairs, $r = -0.10$, $p = .60$).

Relation between FE_{NO} Levels and Exercise-Induced Changes in FEV₁ and Cys-LTs

Although there was a strong negative correlation between baseline FE_{NO} values and the maximal decrease in FEV₁ in asthmatic group ($n = 17$, $r = -0.77$, $p < .001$; Figure 2), no correlation was found in healthy volunteers ($n = 6$, $r = -0.76$, $p = .07$). Furthermore, a positive correlation was found between the baseline FE_{NO} levels and

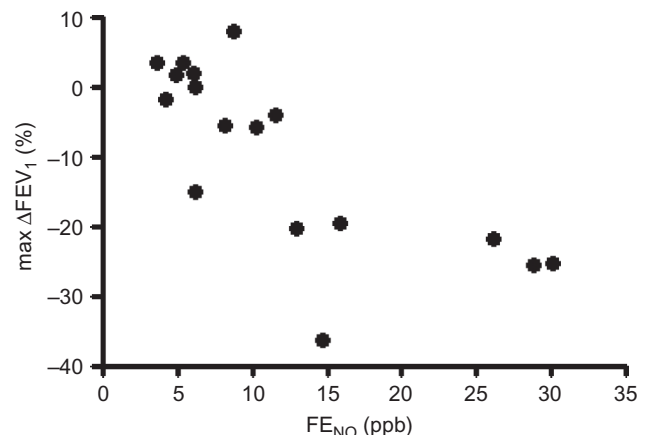


FIGURE 2.—Relationship between basal FE_{NO} level and maximal decrease in FEV₁ in asthmatic patients. Pre-exercise levels of fractional exhaled nitric oxide (FE_{NO}) are plotted against the maximal exercise-induced decrease in forced expiratory volume in 1 second (FEV₁) ($n = 17$, $r = -0.77$, $p < .001$).

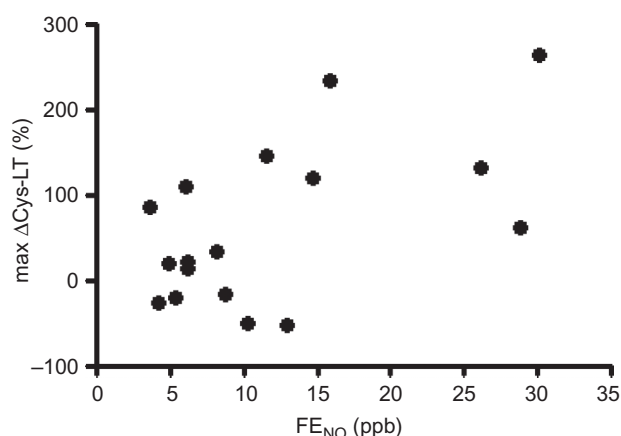


FIGURE 3.—Relationship between basal FE_{NO} level and maximal increase in EBC Cys-LT concentration in asthmatic patients. Pre-exercise levels of fractional exhaled nitric oxide (FE_{NO}) are plotted against the maximal exercise-induced increase in exhaled breath condensate (EBC) cysteinyl leukotriene concentrations ($n = 17$, $r = 0.57$, $p = .01$).

the maximal increase in Cys-LT concentration in the asthmatic group ($n = 17$, $r = 0.57$, $p = .01$; Figure 3) with no relation observed in the healthy subjects ($n = 6$, $r = 0.27$, $p = .60$).

Comparisons between the EIB and the Non-EIB Subgroups

EIB developed in 41% of patients ($n = 7$, EIB group), with no significant bronchoconstriction occurring in the rest of the patients ($n = 10$, non-EIB group).

There was no significant difference between the EIB and the non-EIB groups regarding baseline FEV₁% predicted (3.30 ± 0.98 L, $98 \pm 14\%$ pred. and 3.58 ± 0.67 L, $99 \pm 10\%$ pred., $p = .47$ and $p = .87$) and baseline EBC Cys-LT concentrations (90 pg/mL /43–375/ vs. 158 pg/mL /86–400/, $p = .46$). However, FE_{NO} was significantly higher in the EIB group compared with the non-EIB group (15.9 ppb /6.2–30.1/ vs. 6.1 ppb /3.6–11.5/, $p < .001$). Calculated FE_{NO50} were also significantly higher in patients with EIB compared with non-EIB asthmatics (36.41 ppb /15.3–65.4/ vs. 15.2 ppb /9.3–27.0/, $p = .02$).

FEV₁ significantly decreased in the EIB group compared with baseline value ($-23\% \pm 6\%$, $p = .004$). In the non-EIB group FEV₁ showed a slight, but nonsignificant, increase ($7 \pm 7\%$, $p = .16$).

There was no difference in the time course of changes in Cys-LT concentrations between the two subgroups ($p = .52$, two-way ANOVA); however, there was a trend toward an increase in the EIB group (EIB group: 130 pg/mL /42–180/ and 150 pg/mL /58–1000/, 0 and 10 minutes after exercise, respectively, $p = .06$; non-EIB group: 141 pg/mL /74–410/ and 190 pg/mL /95–320/, 0 and 10 minutes after exercise, respectively, $p = .47$, repeated measures ANOVA).

The highest post-exercise Cys-LT concentrations were 1000 pg/mL and 410 pg/mL in the EIB and non-EIB groups, respectively. The maximal increase in mediator

concentration was significantly higher in the EIB group than in the non-EIB group ($111 \pm 108\%$ vs. $21 \pm 46\%$, $p = .03$).

DISCUSSION

Cys-LTs have been implicated in the pathogenesis of EIB by indirect evidence, reporting an increase in the mediator level in human samples after exercise testing. In our study, we monitored leukotriene levels in a noninvasive airway sample before and immediately after exercise challenge in asthmatic patients and healthy subjects providing direct evidence on the relationship between airway leukotriene concentration and airway caliber.

We demonstrated that EBC Cys-LT concentration was elevated minutes after the exercise and there was a positive correlation between changes in Cys-LT levels and the baseline exhaled NO concentration in asthmatics whereas no similar findings were observed in healthy volunteers. An increase in Cys-LT levels was observed even in those asthmatics who did not develop EIB, but this change was smaller than in patients with EIB.

Higher baseline Cys-LT values were observed in asthmatics versus healthy subjects, which presumably reflect an increase in airway inflammation in that group. It was previously described that the concentration of Cys-LT is elevated in various biological fluids of asthmatic patients compared with healthy subjects and that their level in EBC tends to increase with the severity of this disease (26–29).

The asthma patients enrolled in this study were ones who had reported dyspneic complaints after exercise. However, only 41 percent of them developed EIB on exercise testing, which was not surprising according to previous data on the sensitivity of the provocation test (1, 25). Our data are in concordance with previous findings that baseline FEV₁ does not predict EIB (2, 12, 30). In addition, we confirmed higher levels of FE_{NO} in the EIB subgroup (3–5).

In this study there were no differences in baseline Cys-LT concentrations between EIB and non-EIB subgroups in contrast to the findings by Carraro et al. (12). The possible explanation for these discrepancies, on the one hand, can be the high individual differences or the different mode of collecting condensate. On the other hand, it suggests that a difference in basal concentration of the measured mediator did not bias the results observed in our study.

Among the previous studies there is a high variability in EBC Cys-LT concentrations even in healthy volunteers; Csoma et al. found 18.5 ± 0.5 pg/mL (31), Pontier et al. showed 76 ± 28 pg/mL (32), whereas Zanconato et al. measured 4.9 ± 0.8 pg/mL Cys-LT concentration (33). Lex et al. reported a mean Cys-LT concentration of 249.1 pg/mL in asthmatic subjects (34). The reasons for these discrepancies are yet unknown, but some studies have described that different methods of EBC collection can influence the Cys-LT levels in the condensate fluid (35, 36). Considering the inter-batch variability of the absolute values of a given metabolite measured by

immunoassays, we also presented our results as a percentage of the baseline values as suggested previously (37). Because of the above reasons together with the lack of reference values in healthy subjects it is not possible to define a “clinically relevant” Cys-LT concentration in EBC as a meaningful cutoff value for detecting asthmatic subjects or to predict EIB in asthmatic patients. At the same time, in our study despite the relatively high variability of EBC Cys-LT readings in repeated samples, the exercise-induced increase (approximately doubling) in the concentration of this biomarker was profoundly larger than the changes observed in patients without EIB. The relationship between the degree of mediator change and the drop in FEV₁ suggests that such a change is clinically relevant.

Further refinement in EBC methodology and LT measurement is required to consider EBC Cys-LT concentration as a potential clinically useful biomarker of asthma.

A recent study showed that the composition of EBC is not affected by changes in airway caliber, minute ventilation, or forceful exhalation, factors that may differed in our study between sampling periods (38). We used a relatively brief sampling time for EBC collection (5 minutes per collection) in our study to enable us to follow rapid changes in EBC leukotrienes. The collected volume, however, was not enough to determine dilution factor in association with Cys-LT measurements. We acknowledge that the assessment of airway lining fluid dilution could have potentially aided the more accurate interpretation of the data as we and others have previously demonstrated for other EBC biomarkers (16).

In our study we enrolled stable, mild asthmatics; therefore it was not surprising that their baseline FE_{NO} levels did not differ from healthy level. However, comparing the EIB and non-EIB subgroups of asthmatics, we found higher FE_{NO} levels in the EIB-positive subgroup. The applicability of baseline FE_{NO} in prediction of EIB is controversial (3–7); some, but not all studies demonstrating higher FE_{NO} in patients with EIB compared with those without exercise-induced bronchospasm. (3–5). One reason for these discrepancies could be that not all of the previous studies have taken account of the presence of atopy in asthmatic patients; however, in our study we enrolled only atopic asthmatics. Our study did not aim and was not powered to answer the clinical potential of baseline FE_{NO} to predict EIB.

We found a significant correlation between baseline FE_{NO} and the maximal change in FEV₁ after exercise confirming previous studies (7, 8, 39). FE_{NO} in asthma is regarded as a marker of eosinophilic airway inflammation (40, 41). Consequently, the physiological changes induced by exercise such as hyperpnoea, drying, and cooling the airways can stimulate inflammatory cells more effectively in patients who present with increased airway inflammation (2, 7, 42, 43). This might also explain the relationship between baseline FE_{NO} and the magnitude of post-exercise change in Cys-LT concentration.

In our study we measured FE_{NO} values at 250 mL/s constant expiratory flow; however, to compare our results

with that of measured at 50 mL/s, we calculated FE_{NO}50 values with a previously validated equation (24). The differences and relationships with these calculated values were similar as obtained with higher flow rates.

In conclusion, exercise triggers an increase in breath condensate Cys-LT concentration in asthmatic patients' minutes after exercise. Although there were significant increases in Cys-LT concentrations, changes in FEV₁ were not significant. This observation strengthens the view that leukotriene formation/release may be at least partly responsible for the development of EIB.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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