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Exhaled cysteinyl-leukotrienes and 8-isoprostane in patients with asthma and their relation to clinical severity

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Summary
Background: Collection of exhaled breath condensate (EBC) is a safe, non-invasive method to collect droplets of the airway surface liquid and measure mediators of airway inflammation and oxidative stress, such as cysteinyl-leukotrienes (cys-LTs) and 8-isoprostane.
Objective: The aim of our study was to investigate baseline values of inflammatory lipid mediators in EBC and their relation to asthma severity.
Methods: Nineteen healthy subjects, 16 mild, 12 moderate and 15 severe asthmatics were studied. All subjects attended a clinic visit for spirometry and EBC collection. The concentrations of exhaled cys-LTs and 8-isoprostane were measured by means of specific enzyme immunoassays.
Results: 8-isoprostane levels were significantly increased in mild (49.1 ± 5.2 pg/mL, $p < 0.001$), moderate (49.7 ± 5.2 pg/mL, $p < 0.001$) and severe asthmatics (77.7 ± 7.3 pg/mL, $p < 0.001$), compared to healthy controls (16.4 ± 1.6 pg/mL). Moreover, 8-isoprostane levels were significantly higher in severe compared to mild and moderate asthmatics ($p < 0.01$). Cys-LT levels were significantly higher in moderate (34.6 ± 4.4 pg/mL, $p < 0.05$) and severe asthmatics (47.9 ± 6.0 pg/mL, $p < 0.001$), while no significant difference was found between healthy controls and mild asthmatics. 8-isoprostane levels in EBC of asthmatics strongly correlated with cys-LT levels ($r = 0.61$, $p < 0.0001$).

Abbreviations: Cys-LT, cysteinyl-leukotriene; EBC, exhaled breath condensate; BAL, bronchoalveolar lavage; FEV_1, forced expiratory volume in 1 s; ICS, inhaled corticosteroids; BMI, body mass index.

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Conclusions: 8-isoprostane and cys-LT are detectable in EBC of healthy subjects and their levels progressively increase in asthmatic patients according to disease severity. The correlation found between these two lipid mediators indicating a link between oxidative stress and airway inflammation.

Introduction

Inflammation is the cornerstone of asthma pathophysiology.[1] Hence, monitoring airway inflammation may be useful in the follow up of asthmatic patients and in guiding pharmacological therapy. Different biomarkers might reflect the different aspects of lung inflammation or oxidative stress, which is an important component of inflammation.[2] Quantification of oxidant stress status in vivo may be very important in assessing airway inflammation in patients with asthma; however it has not often been possible due to recognized shortcomings with methods previously available.[3]

Eicosanoids, which include prostaglandins (PGs), leukotrienes (LTs) and thromboxanes, are important inflammatory mediators of asthma[4] that derive via a cascade pathway from arachidonic acid, a cell membrane phospholipid constituent. F2-isoprostanes are PGF2α isomers produced in situ in membrane phospholipids, primarily by free radical-induced peroxidation of arachidonic acid in vivo.[5] Particular attention has focused on 8-isoprostane, which is among the most abundant of the F2-isoprostanes and has several biological effects.[6] Due to its stability, specificity for lipid peroxidation, in vivo production, and relative abundance in biological fluids, it is a reliable marker of lipid peroxidation and oxidative stress.

Cysteinyl-leukotrienes (cys-LTs; LTC4/D4/E4) are also eicosanoids derived from arachidonic acid, via the lipoxygenase enzymatic pathway. Their synthesis is primarily dependent on arachidonic acid release by phospholipases and activation of 5-lipoxygenase.[7] All cys-LTs have the same range of biological effects, with LTE4 being 10-fold less potent than LTD4.[8] Cys-LTs are generated predominantly by mast cells and eosinophils and have been implicated in the pathophysiology of asthma.[9]

Most of the studies investigating the role of eicosanoids in asthma have either used invasive techniques, such as bronchoalveolar lavage,[10] or have measured them in plasma[11] or urine,[12] far from the site of production. Exhaled breath condensate (EBC) consists mainly of condensed water vapor. Only a small fraction of it concerns aerosolized respiratory fluid droplets, which are released from the respiratory epithelial lining fluid and contain traces of non-volatile solutes,[13] such as eicosanoids. These solutes can be recovered in EBC samples. EBC collection does not affect the airway in contrast to bronchial biopsy, bronchoalveolar lavage and induced sputum. It can be obtained with minimal risk and minimal inconvenience for both adults and children, especially in patients with severe asthma, where relative contra-indications exist for more invasive techniques. EBC is, therefore, a simple non-invasive technique that may be used to sample the site of inflammation and measure mediators of airway inflammation even in severe asthmatics.

The aim of this study was to determine baseline values of cys-LTs and 8-isoprostane in exhaled breath condensate of healthy subjects and asthmatic patients. We also investigated possible correlations between these inflammatory markers and disease severity.

Methods

Study population

A total of 62 subjects were enrolled in the study and classified in four groups: 19 healthy non-smokers, 16 mild asthmatics, 12 moderate asthmatics and 15 patients with severe asthma. Subjects' characteristics are summarized in Table 1. Asthma was defined as clear clinical history with current symptoms plus 15% reversibility in FEV_1 after two puffs of $\beta 2-$ agonist and/or positive metacholine challenge. All patients had physician confirmed diagnosis of persistent asthma (mild, moderate or severe), according to GINA guidelines.[14] All asthmatic patients were on ICS therapy at least for 3 months prior to the examination, in doses according to their asthma severity (see Table 1 with demographics). None of the patient received anti-leukotriene treatment such as montelukast. In moderate asthmatics add on treatment with LABA and theophylline was allowed (and in severe asthma oral corticosteroids) and their dose was stable for at least 2 months prior to their participation in the study. None of the participants had upper respiratory tract infection in the previous 4 weeks. Healthy subjects had no history of asthma or any other chronic disease, were not receiving any medication and had normal spirometry. Atopy was assessed by means of skin prick tests for 18 common aeroallergens (HAL Allergy, Benelux). Raised total and specific IgE levels (Pharmacia, Sweden) were also measured.

Study design

The study was cross-sectional. All subjects attended the Sotiria Chest Hospital Asthma Centre Clinic on one occasion for clinical examination, spirometry and EBC collection. Informed and written consent was obtained from all participating subjects and the protocol was approved by the Research Ethics Committee of the Hospital and the National Organization for Medicines.

Pulmonary function testing

Pulmonary function tests were performed on the same day prior to the measurement of exhaled breath condensate.

Table 1 Patient characteristics.[*,@]

	Control subjects	All asthmatics	Mild asthma	Moderate asthma	Severe asthma
Number of patients	19	43	16	12	15
Age, year	47 ± 2.4	49 ± 2.2	44 ± 4.6	50 ± 4.2	54 ± 2.3
Sex, M/F	7/12	14/25	4/8	5/7	5/10
FEV_1% predicted	100 ± 2.5	77 ± 3.3	94 ± 3.7[†,¥]	71 ± 3.1[§]	65 ± 5.4[£]
Atopy	0	29	12	9	8
BMI	29 ± 0.98	28 ± 0.84	26 ± 0.92	28 ± 1.80	31 ± 1.41
ICS[@]	0		415 ± 85	975 ± 130	1850 ± 230
CS p.o.	0		0	0	8

Definition of abbreviation: FEV_1 = forced expiratory volume in 1 s.
[*] Data are expressed as mean ± SEM.
[†] $p < 0.001$ compared with moderate asthmatics.
[¥] $p < 0.001$ compared with severe asthmatics.
[§] $p < 0.001$ compared with healthy controls.
[£] $p < 0.001$ compared with healthy controls.
[@] Beclomethasone equivalent dose.

Forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC) were measured using a dry spirometer (Sensor Medics, Vmax22, CA) and the best value of the three maneuvers was expressed as a percentage of the predicted value.

Collection of exhaled breath condensate

Exhaled breath condensate was collected in the morning (ECoScreen, Jaeger, Hoechberg, Germany) according to the ATS/ERS Task Force recommendations.[15] All subjects breathed in a relaxed manner (tidal breathing) for 10 min not wearing a noseclip. Approximately 1 mL of breath condensate was collected in a 2 mL sterile eppendorf tube and was immediately stored at −80 °C for later analysis. The stability of the frozen samples of EBC for both 8-isoprostane and cys-LTs was tested in 12 randomly selected samples for both patients (8) and controls (4), as previously described.[16] Samples used for stability evaluation were evenly aliquoted and thawed only once for measurement performed on the first, fourth and eighth week after collection (the maximum time between collection and measurement in any sample).

Measurements of exhaled breath condensate

Exhaled 8-isoprostane concentrations were quantified in duplicate using a specific enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI) as previously described.[17] The detection limit was 5 pg/mL and the intraassay and interassay variability were ± 5% and 6%, respectively.

LTC4/D4/E4 concentrations were determined in duplicate using a specific enzyme immunoassay kit (Amersham Pharmacia Biotech, Amersham, UK), as previously described.[18] The detection limit for this assay was 13 pg/mL, while the intraassay and interassay variability were <10%. If cys-LTs were not detected in the breath condensate samples or if they were detected at a concentration lower than the detection limit, then a value of 13 pg/mL was arbitrarily assigned to it.

As suggested by recent data,[19] we coated all plastic surfaces with 0.005% Tween 20 for 30 min, to prevent absorbance of fatty acid derivates (eicosanoids and prostaglandins) to plastic surfaces.

Statistical analysis

Data concerning subject characteristics are expressed as means ± SEM. Statistical analysis was done using Kruskal–Wallis one way analysis of variance accompanied by Dunn's multiple comparison. Spearman's rank correlation coefficient was applied to investigate the relation between different parameters. Uni or multi-variate analysis was applied whenever needed to adjust for age, gender, atopy and BMI as potential confounders. A statistical software package was used for all data analysis and comparisons (SPSS v.13). A p value of less than 0.05 was considered significant.

Results

Stability data

The evaluation of the stability of 8-isoprostane in the frozen EBC samples did not show significant differences among the three measurements performed (1st week, [41.4 ± 3 pg/mL], after 4 weeks, [45.2 ± 3 pg/mL] and after 8 weeks, [46.1 ± 2 pg/mL], $p = 0.19$). Similar results were observed for cys-LTs (1st week, [28.5 ± 1.5 pg/mL], after 4 weeks, [25.2 ± 1.3 pg/mL] and after 8 weeks, [26.4 ± 2 pg/mL], $p = 0.1$). Individual sample variability was not significant for both 8-isoprostane and cys-LTs.

8-isoprostane

8-isoprostane levels in breath condensate were detectable in all healthy subjects (16.4 ± 1.6 pg/mL) and were increased in patients with asthma (59.3 ± 4.0, $p < 0.001$). When compared to healthy controls, 8-isoprostane levels were significantly increased in mild (49.1 ± 5.2 pg/mL, $p < 0.001$), moderate (49.7 ± 5.2 pg/mL, $p < 0.001$) and severe asthmatics (77.7 ± 7.3 pg/mL, $p < 0.0001$). Moreover, 8-isoprostane levels were significantly higher in

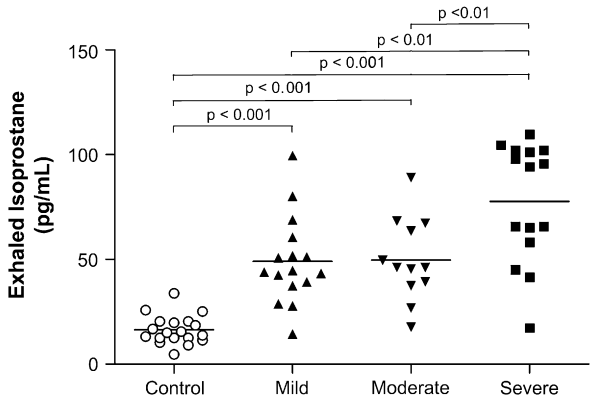

Figure 1 8-isoprostane concentration in exhaled breath condensate of normal subjects and patients with mild, moderate and severe asthma. Data in all figures are expressed as mean + SEM. Mean values are shown by horizontal bars.

subjects with severe as compared with mild and moderate asthma ($p < 0.01$). There was no significant difference found in the 8-isoprostane levels of patients with mild and moderate asthma. Figure 1 shows mean levels of 8-isoprostane in patients with mild, moderate and severe asthma and healthy controls.

Cysteinyl-leukotrienes

Cys-LTs were significantly increased in patients with asthma (35.5 ± 2.9 pg/mL, $p < 0.0001$) compared to healthy controls (17.5 ± 1.2 pg/mL). In seven cases (six controls and one mild asthmatic) where cys-LT levels were undetectable or lower than the detection limit, a value equal to the detection limit was arbitrarily assigned to them. When compared to healthy controls, cys-LT levels were significantly higher in patients with moderate (34.6 ± 4.4 pg/mL, $p < 0.05$) and severe asthma (47.9 ± 6.0 pg/mL, $p < 0.001$). There was no significant difference found between healthy controls and mild asthmatics (24.4 ± 2.2 pg/mL). Cys-LT levels were significantly increased in patients with severe as compared with mild asthma ($p < 0.001$), while there was no difference found between moderate and severe asthmatics. Figure 2 shows mean levels of cys-LTs in healthy controls and patients with mild, moderate and severe asthma.

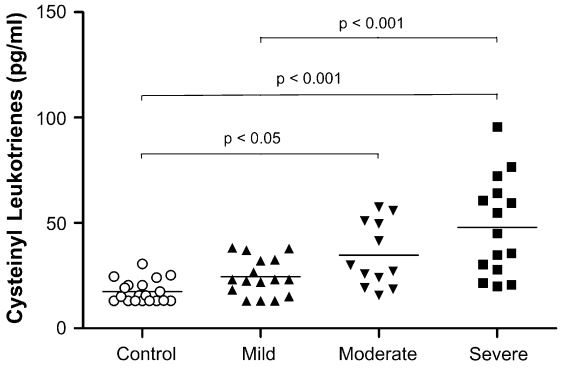

Figure 2 Cys-LTs concentration in exhaled breath condensate of normal subjects and patients with mild, moderate and severe asthma.

Correlations

8-isoprostane levels in breath condensate of patients with asthma strongly correlated with exhaled cys-LT levels ($r = 0.61$, $p < 0.0001$) (Fig. 3). Subgroup analysis showed that this correlation persisted only in severe asthmatics ($r = 0.69$, $p = 0.044$). 8-isoprostane also weakly correlated with BMI of asthmatic patients ($r = 0.37$, $p = 0.015$) and healthy controls ($r = 0.73$, $p = 0.0006$) (Fig. 4). Cys-LT levels inversely correlated with FEV_1 ($r = -0.35$, $p = 0.029$) (Fig. 5). No correlation was found between 8-isoprostane and FEV_1 or between BMI and FEV_1.

Discussion

In the present study we evaluated the levels of 8-isoprostane and cys-LTs concentration in exhaled breath condensate. Our study shows that both 8-isoprostane and cys-LT concentrations in exhaled breath condensate are significantly higher in patients with asthma than in healthy adults. Moreover, 8-isoprostane levels were significantly higher in subjects with severe as compared with mild and moderate asthma, while there was no significant difference found between mild and moderate asthmatics. Cys-LT levels were also significantly higher in patients with asthma, and more so in moderate and severe asthmatic patients than in mild asthmatics. These data are confirmatory to previous studies reporting elevated levels of these two mediators in patients with asthma. We also found a significant correlation between 8-isoprostane and cys-LTs levels in adult asthmatics, which has only been reported in children with asthma.[20,21]

A representative group of patients with persistent asthma according to GINA, matched for gender and age was carefully selected. All patients received treatment according to their asthma severity. All recommendations by the ATS/ERS Task Force[15] were applied during the collection procedure of exhaled breath condensate and all samples were analyzed within 2 months of collection and

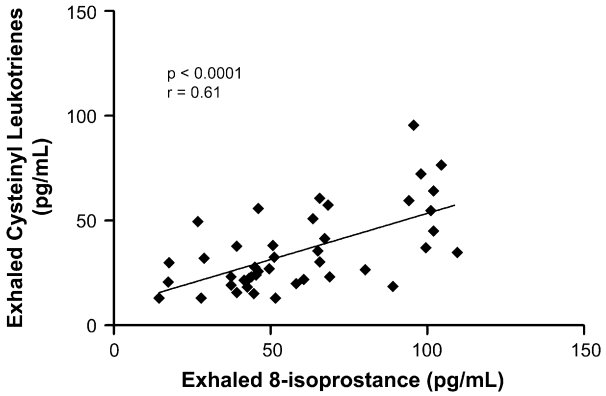

Figure 3 Correlation between cys-LTs and 8-isoprostane concentrations in exhaled breath condensate of subjects with asthma.

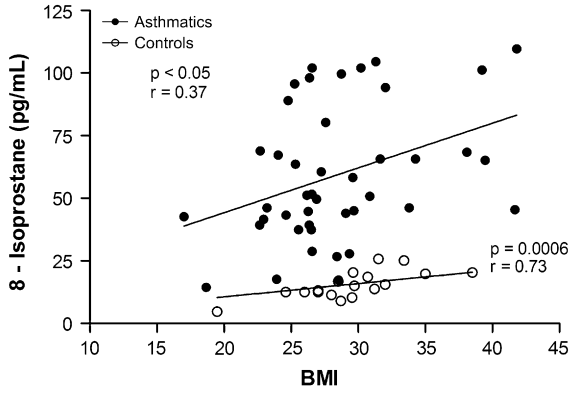

Figure 4 Correlation between 8-isoprostane concentration and BMI in exhaled breath condensate of healthy controls and subjects with asthma.

were measured in duplicates to decrease the possibility of error.

Inflammation and oxidative stress are cardinal features of asthma. Quantification of oxidant stress status can take place by either directly measuring free radicals or by measuring stable byproducts of lipid peroxidation. Several in vitro biomarkers of oxidative stress are available; regrettably most of them are of limited value in vivo because they either lack sensitivity and/or specificity, or require invasive methods.[22] 8-isoprostane is a specific, stable product of lipid peroxidation that is formed in vivo by ROS peroxidation of arachidonic acid.[5] It is present in detectable amounts in all normal tissues and biological fluids,[10–13] thus allowing definition of normal ranges, and is unaffected by the lipid content of the diet.[23] 8-isoprostane is considered an ideal marker for investigating oxidative injury in asthma.[24]

Previous studies have indicated increased 8-isoprostane levels of both systemic[15] and airway concentrations[25] of adults with asthma. We have demonstrated that 8-isoprostane concentration in EBC was higher in adult asthmatic patients than in healthy control subjects, indicating that lipid peroxidation and oxidative stress are increased in the lungs of patients with asthma. Other authors reported

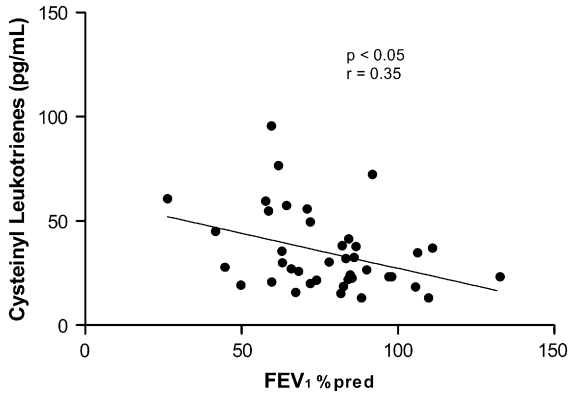

Figure 5 Correlation between cys-LTs concentration in exhaled breath condensate of subjects with asthma and FEV_1% predicted.

a similar increase in 8-isoprostane in EBC of patients with stable moderate asthma who were either steroid-naïve or treated with ICS.[26] Montuschi et al.[27] have also demonstrated a comparable increase of 8-isoprostane in EBC of asthmatic patients. In keeping with this study, we have shown that 8-isoprostane EBC levels increase with disease severity. We also found that in patients with mild and moderate asthma 8-isoprostane levels were almost tripled compared to healthy controls, while severe asthmatics exhibited approximately a 4-fold increase. Subjects with moderate asthma had levels of 8-isoprostane comparable to those observed in subjects with mild asthma, which could be attributed to a controlling effect of ICS, up to a certain extent, in these patients. However, conflicting data have been reported on the effects of corticosteroids on 8-isoprostane; in children with asthma who were either steroid-naïve or treated with ICS, 8-isoprostane EBC levels were elevated compared to those in healthy children, but there was no difference between the two study groups.[28–30] In our study, severe asthmatics had significantly higher 8-isoprostane levels compared to both mild and moderate asthmatics, despite treatment with oral and/or high doses of ICS.

Cys-LTs have been implicated in asthma pathophysiology.[31] Increased LTC4 levels were reported in BAL,[10] induced sputum;[32] and EBC of asthmatics. We showed that cys-LT levels in EBC of asthmatic patients are elevated compared to healthy subjects. Our findings are consistent with the results of previous studies reporting increased cys-LTs EBC concentrations in patients with asthma.[18,33,34] In keeping with Hanazawa et al.,[18] we have demonstrated that EBC cys-LT levels are significantly higher in patients with moderate and severe asthma than in subjects with mild asthma and control subjects, although we found no difference between healthy controls and mild asthmatics. This suggests that, compared to mild asthmatics or healthy controls, patients with moderate and severe asthma has increased baseline production of cys-LTs.

In our study there was a significant correlation between exhaled 8-isoprostane and cys-LT levels in breath condensate of patients with asthma. After performing a subgroup analysis, we found that this correlation persisted only in the severe asthmatics group. Although cys-LTs and 8-isoprostane are thought to reflect different aspects of airway inflammation and their levels could increase independently of each other, this correlation maybe indicative of a link between inflammation and oxidative stress in the airways of patients with asthma. This association seems to be even more prominent in patients with severe asthma, where inflammation and oxidative stress levels are more pronounced. Similar data have been reported in children; Baraldi and coworkers[20] found a significant correlation between 8-isoprostane and cys-LTs in ICS-treated asthmatic children that did not persist after oral treatment with prednisone, suggesting that steroids may affect isoprostanes and leukotrienes differently. Zanconato and coworkers[21] also reported a significant correlation between 8-isoprostane and cys-LTs in EBC of ICS-treated children with stable asthma. In addition, we found a small but significant negative association between cys-LT EBC levels and FEV_1, which could be explained by the bronchoconstrictive effect of cys-LTs.[8] Bronchoconstriction per se

might influence indirectly the concentrations of EBC molecules due to factors such as drying of mucosal membranes, higher viscosity of airway fluid, collection of solutes from more proximal airways, especially in severe asthmatics where bronchoconstriction is more prominent. This could be an alternative explanation of the correlation found between FEV_1 and cys-LT, other than the bronchoconstrictive effect of cys-LT.

We have also found a significant correlation between 8-isoprostane and BMI in both asthmatic patients and healthy controls. Similar results have been reported recently,[35] although this association was not observed in healthy non-asthmatics. Our finding that BMI is associated with higher EBC levels of 8-isoprostane (but not cys-LTs) in both healthy subjects and asthmatics may be attributed to a parallel increase in baseline airway oxidative stress and/or to obesity-related changes in the production of adipokines.[35] Since the association of BMI with 8-isoprostane EBC levels could affect our results, all presented data on 8-isoprostane have been adjusted for BMI as a confounder.

Our study presents certain limitations. In an observational cross-sectional study it is very difficult to determine causation and specific mechanisms of action. In addition, although EBC analysis based on recent guidelines[15] is widely used for investigating airway pathology, it is still not a fully standardized method.[36,37] Another limitation of the present study is that a reference dilution indicator was not used. The high inter-individual variability in the amount of aerosol particles in EBC indicates the need for a dilution marker.[13] Finally, we could have concentrated our samples as suggested[19] to avoid undetectable levels of cys-LTs in EBC of some healthy controls and mild asthmatics.

In conclusion, we have shown that 8-isoprostane and cys-LTs are detectable in breath condensate of healthy subjects and that their levels progressively increase in patients with asthma according to disease severity. This study shows that EBC may be an effective tool in assessing airway inflammation and oxidative stress status in patients with asthma.

Conflict of interest statement

All authors declare no potential conflict of interest related to the article or the research described.

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