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**Oxidative stress and airway inflammation after allergen challenge evaluated by exhaled breath condensate analysis.**

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**Background** Allergen exposure may increase airway oxidative stress, which causes lipid membrane peroxidation and an increased formation of 8-isoprostane.

**Objective** The aim of the study was to investigate oxidative stress induced by allergen challenge in mild asthmatics, by measuring 8-isoprostane in exhaled breath condensate (EBC), and to examine their relationship with mediators derived from arachidonic acid.

**Methods** 8-isoprostane, cysteinyl leukotrienes (cys-LTs) and prostaglandin E2 (PGE2) concentrations in EBC were measured at baseline and after allergen challenge in 12 patients with mild allergic asthma sensitized to cat allergen.

**Results** At 24 h after allergen challenge, compared with baseline values, EBC 8-isoprostane increased [48.64 pg/mL (44.14–53.61) vs. 21.56 pg/mL (19.92, 23.35), *P*<0.001], cys-LTs increased [27.37 pg/mL (24.09–31.10) vs. 13.28 pg/mL (11.32, 15.57), *P*<0.001] and PGE2 decreased [18.69 pg/mL (12.26, 28.50) vs. 39.95 pg/mL (34.37, 46.43), *P*<0.001]. The trend of increasing 8-isoprostane after allergen challenge was significantly correlated with the trend of increasing cys-LTs (R²=0.85, *P*<0.001) whereas the trend of decreasing PGE2 after allergen challenge was significantly correlated with the trend of increasing cys-LTs (R²=0.52, *P*=0.001).

**Conclusions and Clinical Relevance** The increase in EBC 8-isoprostane observed after allergen challenge indicates that allergen exposure increases airway oxidative stress in allergic asthma. The strict correlation between cys-LTs and 8-isoprostane underlines the relationship between allergic inflammation and oxidative stress. A shift of arachidonic acid metabolism towards lipoxygenase pathway is induced by the allergen challenge. Airway oxidative stress occurs after allergen challenge even in patients with mild intermittent allergic asthma.

**Keywords**: 8-isoprostane, allergen challenge, asthma, exhaled breath condensate

**Introduction**

Allergen exposure may increase airway oxidative stress in allergic asthma, as the major inflammatory cells involved in asthmatic inflammation, such as macrophages, mast cells and eosinophils, produce reactive oxygen species (ROS) when activated [1, 2]. F2-isoprostanes (F2-IsopPs) are arachidonate products formed on membrane phospholipids by the action of ROS and thereby represent a quantitative measure of oxidant stress in vivo [3]. Particular attention has focused on 8-isoprostane, which, due to its stability, specificity for lipid peroxidation and relative abundance in biological fluids, is a reliable marker of lipid peroxidation and oxidative stress [4]. Exhaled breath condensate (EBC) is a completely non-invasive method for collecting airway secretions and studying the composition of airway lining fluid [5] and it is particularly adequate to be frequently repeated after allergen challenge. 8-isoprostane can be measured in EBC [6], where its concentrations have been reported to be significantly higher in patients with asthma than in healthy controls [7–9]. The concentrations of Cys-LTs, including LTE4 and LTB4, in EBC are elevated in adults and children with asthma [10–14]. In contrast, prostaglandin E2 (PGE2) has been reported not to be elevated in asthmatics [10, 15] and not related to bronchial hyperresponsiveness [15]. EBC is a simple non-invasive technique that seems to be particularly useful to assess airway inflammation after allergen
challenge, where relative contra-indications exist for more invasive techniques. Actually, significant increases in the concentrations of cysteinyl leukotrienes (cys-LTs) and prostaglandin D2 (PGD2) have been reported in EBC following allergen-induced bronchoconstriction in asthmatic patients [13], whereas changes in 8-isoprostane EBC concentration after allergen challenge have not been reported so far. The aim of the study was to investigate oxidative stress induced by allergen challenge in mild asthmatics, by measuring 8-isoprostane in EBC, and to examine its relationship with lipid mediators derived from arachidonic acid, cys-LTs and PGE2.

Methods
Patients
Twelve non-smoking patients, sensitized to cat, with mild intermittent asthma and forced expiratory volume in 1 s (FEV1)X80% predicted were recruited, whether they reported to be symptomatic when exposed to cats. All were taking only short-acting b-2-agonists p.r.n., demonstrated a methacholine PD20FEV1 o800 mg and a maximum fall in FEV1X20% to inhaled cat allergen within 0–3 h (early airway response, EAR), followed by a fall in FEV1X10% between 3 and 7 h (late airway response, LAR). Exclusion criteria were respiratory infection or cat exposure for 4 weeks before enrolment and previous desensitization to cat allergen.

Study design
A cat allergen inhalation challenge was conducted. To test the specific change of the concentrations of the biomarkers analysed here, all the patients had been exposed previously to saline. EBC was obtained at baseline, 1, 8 and 24 h after allergen (or saline) challenge and immediately stored at 80 1C for later assessment of 8-isoprostane, cys-LTs and PGE2. Exhaled nitric oxide (FENO) was also measured at baseline, 8 and 24 h after inhalation challenge. Twenty normal subjects matched for age (mean age 32, range 20–56 years) and gender (six male) acted as controls for baseline values. Informed and written consent was obtained from all participating patients and the protocol was approved by the local Ethics Committee.

Allergen inhalation challenge
Serial twofold dilutions were prepared from standardized stock allergen (cat, 100 IR/mL, Stallergenes, Antony, France) and diluted in normal saline. Starting concentration for inhalation was determined by mathematical prediction of allergen PC20 using the skin tests end-point and methacholine PD20FEV1 [16]. Increasing doses of allergen were administered by means of a breathactivated dosimeter (Mefar MB3; Markos, Brescia, Italy) set to deliver 10 mL of solution during a deep breath until a decrease in FEV1 of at least 20% was observed. PD20FEV1 (the dose required to produce a 20% decrease in FEV1) was determined by linear interpolation between points on the log dose–response curve. Two technically acceptable FEV1 manoeuvres were performed 15 min after each inhalation dose. Once the EAR was reached, FEV1 was assessed every hour up to 8 h after allergen inhalation, in order to observe the LAR. Saline challenge was performed 1 week before allergen challenge, by inviting the patients to take 10 deep breaths from the same breath-activated dosimeter. Spirometry, EBC collection and exhaled breath NO were obtained at the same time intervals as after allergen challenge.

Exhaled nitric oxide
FENO was measured using a chemiluminescence analyser (NIOX, Aerocrine AB, Solna, Sweden) calibrated with a “certified NO calibration gas mixture. The online single exhalation technique with an exhalation rate of 50 mL/s and positive expiratory mouth pressure of 10 cm H2O was applied according to the recommendations [17]. The mean FENO of three acceptable last 3 s end-expiratory plateau tracings was calculated.

Collection of exhaled breath condensate
EBC collection was performed using the R Tube™ EBC collection system (Respiratory Research Inc., Charlottesville, VA, USA). EBC collections were obtained after thorough rinsing of mouth with water, at an initial condenser temperature of 20 1C, for 10 min and samples were immediately stored at 80 1C for later assays.
Analysis of exhaled breath condensate

Exhaled 8-isoprostane concentrations were measured in duplicate using a specific enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI, USA) as described previously [7, 18]. The detection limit was 5 pg/mL and the intra-assay and inter-assay variability were 5% and 6%, respectively. LTC4/D4/E4 concentrations (cys-LTs) were determined in duplicate using a specific enzyme immunoassay kit (Amersham Pharmacia Biotech, Amersham, UK), as described previously [19]. The detection limit was 10 pg/mL, whereas the intra-assay and inter-assay variability were 8% and 10%, respectively. PGE2 was measured in duplicate using a specific enzyme immunoassay kit (Cayman Chemicals as described previously [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 10 pg/mL was arbitrarily assigned to it. PGE2 assay shows a detection limit of 2 pg/mL, with a coefficient of variation for intraassay values of 5% and a coefficient of variation for interassay values of 4%.

Statistical analysis

Statistical analysis was performed with the aid of a commercially available statistical package (STATA 10s). In all analysis, a-error of 5% was assumed for significance level. Patients’ characteristics at baseline are expressed as mean ±SEM (Table 1). FE\textsubscript{NO} as well as EBC Cys-LTs, isoprostane and PGE2 are known to have a skewed to the right distribution [20, 21]. Therefore, log transformation was performed and the results regarding these markers are presented as geometrical mean [95\% confidence interval (95\% CI)] in the Results section and Table 2. A two-sample t-test with unequal variances of logtransformed values was used to compare normal controls with asthmatic patients for FENO and EBC markers. Comparisons of parameters obtained before and after allergen inhalation were performed by means of generalized linear model with random-intercept, using log-transformed EBC 8-isoprostane, cys-LTs, PGE2 and FE\textsubscript{NO} as dependent variables and the time intervals (hours) measurements obtained after the challenge as time-fixed within subjects effect. The time effect was considered as a factor. Bootstrap method was used for the variance–covariance matrix estimation. Linear regression analysis was performed with logtransformed values of EBC markers in order to analyse the relation between different EBC markers both at baseline and after challenge (all values pooled). A generalized linear model using data at all time-points and grouping for patients was used also in order to investigate the correlation between the trends of evolution of two different EBC markers after allergen challenge. The R\textsuperscript{2} for within subjects’ effect is presented.

Results

All the patients studied had a mean (±SEM) decrease in FEV\textsubscript{1} of 26.1\% (±2.6\%) during the EAR and a mean decrease in FEV\textsubscript{1} of 17.3\% (±1.9\%) between 3 and 8 h after allergen challenge (LAR). All patients produced adequate EBC for analysis of mediators before and after saline and allergen challenge. Patients’ characteristics are shown in Table 1. Compared with normal controls, asthmatic patients had higher values of FENO (P<0.001), EBC 8-isoprostane (P<0.001), and lower values of cys-LTs (P = 0.04) while no significant differences were observed regarding PGE2 (see Table 2). Saline inhalation did not produce any significant changes in spirometric, FENO and EBC measurements (data not shown). Allergen inhalation produced a significant increase in FENO 24 h after allergen inhalation (81.88 ppb, 95\% CI 56.65–118.35 ppb, P = 0.002) (Fig. 1). EBC 8-isoprostane concentrations increased significantly 8 h (42.90 ppb, 95\% CI 40.00–46.00 pg/mL, P<0.001 compared with baseline and 1 h after challenge) and 24 h after allergen challenge (48.64, 95\% CI 44.14–53.61 pg/mL, P<0.001 both compared with baseline and 1 h after challenge) and 24 h after allergen challenge (48.64, 95\% CI 44.14–53.61 pg/mL, P<0.001 both compared with baseline and 1 h after challenge); EBC cys-LTs concentrations increased significantly after 1 h (15.96, 95\% CI 13.88–18.35 pg/mL, P<0.001 both compared with baseline), 8 h (24.38, 95\% CI 22.11–26.89 pg/mL, P<0.001 both compared with baseline and 1 h after challenge) and 24 h (27.37, 95\% CI 24.09–31.10 pg/mL, P<0.001 both compared with baseline and 1 h after challenge) after allergen challenge. A significant decrease of PGE2 was observed at 8 h (15.88, 95\% CI 11.30–22.31 pg/mL, P<0.001 compared with baseline) and 24 h after allergen inhalation (18.69, 95\% CI 12.26–28.50 pg/mL, P<0.001 compared with baseline).
The changes in EBC cys-LTs, 8-isoprostane and PGE2 are plotted in Fig. 2. There was a significant positive correlation between EBC 8-isoprostane and cys-LTs levels measured during allergen challenge (R = 0.79, Po0.001, Fig. 3) and negative correlations between EBC PGE2 and 8-isoprostane (R =0.61, Po0.001) and Cys-LTs (R =0.46, P = 0.006), respectively. Moreover, the increasing trend of 8-isoprostane and negative correlations between EBC PGE2 and 8-isoprostane (R =0.52, Po0.001). No significant relationships were observed between the changes in FEV1 or FENO and the changes in EBC parameters observed after allergen challenge. Discussion The main finding of our study is that allergen challenge leads to an increase of 8-isoprostane, together with a significant increase in cys-LTs and FENO, while PGE2 concentration decreased. These results demonstrate that oxidant stress occurs with allergic inflammation induced by allergen challenge in mild atopic asthmatics. Moreover, these EBC markers were strongly related to each other, reflecting the close relation between inflammation and oxidative stress in our asthmatic patients after allergen exposure. To our knowledge, this is the first time that EBC has been used to investigate airway oxidative stress, by means of 8-isoprostane, after allergen challenge. Our study is also the first to report a decrease of EBC PGE2 after allergen challenge, simultaneously with an increase of EBC Cys-LTs, suggesting that allergen challenge induces a shift of arachidonic acid metabolism towards lipoxygenase pathway. The increase of EBC 8-isoprostane is in agreement with previous studies reporting an increased excretion of the major urinary metabolite of 15-F2-Isoprostanes (8-isopGF2a) following inhaled allergen challenge in patients with mild atopic asthma [22, 23]. The increase of Cys-LTs after allergen challenge was also in agreement with previous reports of increase in Cys-LTs, measured both in BAL fluid [24], sputum [25] and EBC [13] following allergen challenge in atopic asthmatics, as well following specific challenge in occupational asthma [26]. A significant correlation between baseline EBC levels of 8-isoprostane and cys-LTs has been reported in adult patients with severe asthma [8], as well in inhaled corticosteroids treated children with stable asthma [9]. We did not observe such a correlation in our patients at baseline evaluation, probably because of mild intermittent asthma. Moreover, the asthmatics in our study were not characterized by an increased EBC Cys-LTs at baseline, finding in line with another study reporting the lack of increase of Cys-LTs in EBC in mild asthmatics [8]. On the other hand, we observed a strict correlation between the increase in 8-isoprostane and the increase in cys-LTs after challenge when the airways inflammation is augmented. The correlation between these different markers of airway inflammation suggests a link between inflammation and oxidative stress in the airways of patients with asthma.

Another important result of our study is the decrease of PGE2 concentration in EBC after allergen challenge. There is evidence of increased respiratory tissue damage caused by oxidative stress in a murine model of asthma during ongoing allergic inflammation [27]. The cyclooxygenase product PGE2 is produced by several cells in human airways, including epithelium [28] and smooth muscle [29]; it has inhibitory effects on inflammatory cells and bronchoprotective effects in patients with bronchial asthma. Indeed, PGE2 has been shown to protect against allergen-induced bronchoconstriction [30]. There are a few data in the literature on the effect of allergen challenge on PGE2. Results similar to ours have been reported by Macfarlane et al. [25] who demonstrated an increase in cys-LTs, but not in PGE2, associated with a late asthmatic reaction after allergen challenge in patients with atopic asthma and by Fernandez-Nieto et al. [26] who observed a significant increase in the LTC4/PGE2 ratio in patients after positive inhalation challenge with occupational agents compared with those with negative responses. Taken together, these data suggest that the underproduction of PGE2 and overproduction of cys-LTs may be important factors in the bronchoconstrictive response induced by allergens. Moreover, the correlation between the increase of cys-LTs and decrease of PGE2 suggests that allergen challenge induces a shift of arachidonic acid metabolism towards lipoxygenase pathway.

Exhaled NO levels increased in our study at 24 h after exposure [31, 32] and the magnitude of the increase was similar with previous reports [31]. No measurements of exhaled NO were carried out at
1 h after allergen exposure in the present study, as the effects on FE\textsubscript{NO} during early allergen response were beyond the aims of the study. A decrease of FE\textsubscript{NO} would have been expected at that time-point [31, 33], most probably due to acute bronchoconstriction [34]. Considering the important role oxidative stress may play in allergic diseases, antioxidant supplementation has been investigated as possible therapeutic strategy in allergic diseases with some promising results in animal model studies [35–37].

In conclusion, our study demonstrates an increase of airway oxidative stress in asthmatic subjects after allergen challenge. The close relation between the levels of cys-LTs and 8-isoprostane after allergen exposure underlines the relationship between allergic mediators release and oxidative stress. The relation between the allergen challenge-induced increase of Cys-LTs and decrease of PGE2 in the EBC suggests that a shift of arachidonic acid metabolism towards lipooxygenase pathway is activated by the allergen challenge. The noninvasive technique of EBC appears to be particularly useful to investigate airway inflammatory changes induced by allergen challenge in allergic asthmatics.


34. De Gouw HW, Hendriks J, Wolteman AM, Twiss IM, Sterk PJ. Exhaled nitric oxide (NO) is reduced shortly after bronchoconstriction to direct and indirect stimuli in asthma. *Am J Respir Crit Care Med* 1998; **158**:315–9.


**Table 1.** Patients' characteristics and allergen-induced airway responses

FEV₁, forced expiratory volume in 1 s; EAR, early airway response; LAR, late airway response

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Age</th>
<th>Sex</th>
<th>FEV₁ (% predicted)</th>
<th>EAR (% decrease FEV₁)</th>
<th>LAR (% decrease FEV₁)</th>
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<tbody>
<tr>
<td>1</td>
<td>35</td>
<td>M</td>
<td>105</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>M</td>
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<td>39</td>
<td>22</td>
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<td>111</td>
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<tr>
<td>Mean (SEM)</td>
<td>30 (5)</td>
<td></td>
<td>104 (1)</td>
<td>26.08 (9.1)</td>
<td>17.33 (6.86)</td>
</tr>
</tbody>
</table>
Table 2. Comparison of EBC parameters and exhaled nitric oxide [geometrical mean (95% CI)] obtained in asthmatic patients and in healthy controls

<table>
<thead>
<tr>
<th></th>
<th>Asthmatic patients</th>
<th>Normal controls</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-Isoprostane (pg/mL)</td>
<td>21.56 (19.92, 23.35)</td>
<td>16.43 (15.50, 17.41)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cys-LTs (pg/mL)</td>
<td>13.28 (11.32, 15.57)</td>
<td>15.84 (14.96, 16.77)</td>
<td>0.04</td>
</tr>
<tr>
<td>PGE₂ (pg/mL)</td>
<td>39.95 (34.37, 46.43)</td>
<td>43.20 (41.52, 44.96)</td>
<td>0.29</td>
</tr>
<tr>
<td>FE\textsubscript{NO} (ppb)</td>
<td>47.39 (31.36, 71.63)</td>
<td>15.86 (14.23, 17.67)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Figure 1. Individual values of exhaled nitric oxide (FE\textsubscript{NO}) concentrations at baseline (time=0) and after allergen challenge. *P<0.001 compared with baseline (time 0) values.
Figure 2
Individual values of exhaled breath condensate (EBC), 8-isoprostane, cys-leukotrienes (cys-LTs) and prostaglandin E₂ (PGE₂) concentrations at baseline (time=0) and after allergen challenge. *Significantly different compared with baseline (time 0) values. ¶ Significantly different compared with 1 h values (see text for level of significance).

Figure 3
Correlation between exhaled breath condensate (EBC), 8-isoprostane, and cys-leukotrienes (cys-LTs) levels measured during allergen challenge ($R^2=0.80$, $P<0.001$).