Cytokine profiling in exhaled breath condensate after exercise challenge in asthmatic children with post-exercise symptoms

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Abstract

Introduction: Markers of exhaled breath condensate (EBC) correlate with lung function impairment, airway remodeling and different aspects of the disease such as exercise-induced bronchoconstriction (EIB). Aim of the study was to determine the cytokine profile in EBC of children with asthma after an exercise treadmill challenge in order to obtain clinically useful information about mechanisms of EIB; also, to assess correlations between cytokine concentrations in EBC and clinical characteristics of the patients.

Material and methods: The study population consisted of 25 randomly selected children, aged 8 to 19 years, with asthma and EIB symptoms despite the use of control medications. Patients on the day of the study visit underwent fractional exhaled nitric oxide measurement (FeNO) and baseline spirometry, performed an exercise treadmill challenge (ETC), and EBC samples were obtained at the end of the ETC.

Results: In asthmatic children with positive ETC, monocyte hemotactic protein-1 (MCP-1) and IL-16 adjusted to pre-EBC forced expiratory volume in 1 s (FEV₁) were significantly higher compared to children with negative ETC (p = 0.022 and p = 0.017 respectively). After adjustment to pre-EBC FEV₁ other cytokines (IL-4, IL-5, IL-6, IL-8, MIG, TNF-α) were not related to post-exercise changes in FEV₁.

Conclusions: We observed a specific inflammatory profile in the airways of asthmatic children with bronchoconstriction induced by exercise. The concentration of cytokines in EBC depended on the post-exercise decrease in FEV₁, which was measured by the area under the curve (AUC). MCP-1 and IL-16, adjusted to pre-EBC FEV₁, were significantly higher in children with a positive exercise challenge compared to those with a negative one.

Key words: asthma, children, cytokines, exhaled breath condensate, exercise-induced bronchoconstriction.

Introduction

Physical activity is an important trigger of asthma symptoms for most patients, including children [1–3]. The mechanism of exercise-induced
bronchoconstriction (EIB) may involve changes in airway osmolarity resulting from water loss and/or changes in airway temperature. Both mechanisms could lead to bronchoconstriction and bronchospasm [4, 5]. However, the pathogenesis of EIB is a more complex problem, and the release of mediators from the mast cells or other inflammatory cells of the airways should be considered [5].

There is evidence that parameters obtained from exhaled breath condensate (EBC) reflect changes in the level of airway lining fluid and theoretically allow assessment of the severity of asthma and may provide some guidance for adjustment of drug therapy [6, 7]. Markers of EBC may also correlate with lung function impairment, airway remodeling and EIB in asthmatic patients.

Fractional exhaled nitric oxide (FeNO) has proven to be a reliable noninvasive measure for airway inflammation [8] as well as to have increased levels in asthmatic children with EIB [9, 10].

We aimed to show the cytokine profile in EBC of children with asthma after exercise treadmill challenge in order to obtain clinically useful information about mechanisms of EIB. In addition, we tried to assess correlations between cytokine concentrations in EBC and clinical characteristic of the patients.

Material and methods

Patients

The study population consisted of 25 randomly selected children, aged 8 to 19 years, with asthma and EIB symptoms. Asthma was diagnosed on the basis of the symptoms and an improvement in the prebronchodilator forced expiratory volume in 1 s (FEV1) of ≥12% after administration of salbutamol (400 μg) [11].

Patients on the day of the study visit underwent FeNO and baseline spirometry. They also performed an exercise treadmill challenge (ETC), and EBC samples were obtained at the end of the ETC. The study lasted from February 2013 to June 2013.

Inclusion criteria

Chronic asthma with duration of at least 2 years, currently with symptoms only after exercise despite controlled medications [11].

Exclusion criteria

Exclusion criteria included immunotherapy; active smoking; inability to obtain an appropriate FeNO, spirometry and EBC procedure; diagnosis of a specific respiratory disease, such as cystic fibrosis, primary ciliary dyskinesia, interstitial lung disease, pneumonia; tuberculosis and/or current upper airway infection; other clinically significant pulmonary, hematological, hepatic, gastrointestinal, renal, endocrine, neurological, cardiovascular, and/or psychiatric diseases or malignancies that could influence the results of the study; obesity.

Exhaled breath condensate collection and cytokines analysis

Exhaled breath condensate samples were collected using an EcoScreen-II device (Viasys Healthcare GmbH, Berlin, Germany). Samples of EBC were obtained from children during tidal breathing while wearing a nose clip, as described previously [12]. The two-way non-rebreathing valves and tubing to the condenser served as a saliva trap. After collecting, EBC samples were rapidly frozen in small plastic tubes at −80°C using dry ice and stored at −80°C until analysis.

The material was the exhaled breath condensate of 25 children diagnosed with asthma. The analysis was performed using Quantibody Human Inflammation Array 3 (RayBiotech, Norcross, GA, USA) according to the manufacturer’s instructions. Each standard glass slide consisted of 16 wells of identical cytokine antibody array. All antibodies and positive controls were plated in quadruplicate in each well.

In the first step, the capture antibody was bound to the glass surface of the slide. Next, 100 μl of each patient sample and the array-specific cytokine standards of known concentration were added to each well. After incubation for 2 h at room temperature, the array was washed 5 times with 150 μl of Wash Buffer I and twice with 150 μl of Wash Buffer II, 5 min per wash. Next, the array was incubated for 2 h with 1.4 ml of the biotin-conjugated antibody at the room temperature. Then the washing protocol was repeated before the addition of 80 μl of Cy3 equivalent dye-conjugated streptavidin to each well. After washing the array, the fluorescence signal was detected and quantified with the Axon GenePix 4000B scanner and GenePix Pro 6.0 software (Molecular Devices). The results were analyzed using Q-Analyzer software (RayBiotech, Norcross, GA, USA).

Nitric oxide measurement

The NO measurements were performed according to the European Respiratory Society/American Thoracic Society (ERS/ATS) recommendations [13] with a chemiluminescence analyzer (model 280i nitric oxide analyzer; Sievers, Boulder, CO, USA) and defined in parts per billion. The analyzer provides an on-line continuous measurement of NO in a single exhalation with a detection range of 0.1 to 500 ppb. Environmental NO was measured before and after each test, and it never exceeded 5 ppb. The subjects exhaled at a constant flow
rate (50 ml/s) from total lung capacity to residual volume without breath holding. They maintained a constant mouth pressure (17 cm H2O) by monitoring a visual display in order to eliminate contamination from nasal NO. Dead space and nasal NO (which are reflected by the NO concentration peak during exhalation) and NO from the lower respiratory tract (determined by the plateau value after the peak) were recorded automatically by using the manufacturer’s software. Three FeNO measurements of the plateau phase were obtained, with less than 10% variation. The mean value of 3 successive, reproducible recordings was retained for statistical analysis.

**Standardized exercise treadmill challenge (ETC)**

Exercise-induced bronchoconstriction was tested using a motor-driven treadmill (Kettler, Ense-Parsit, Germany) according to ATS/ERS guidelines [2]. The children were instructed to run for 8 min with a submaximal exercise load [14]. The exercise test consisted of a 2-minute warm-up and 6 min of steady-state running on a treadmill. The slope of the treadmill was 5.5% (3°). Small adjustments in workload (treadmill speed) were made, if necessary, to achieve targeted heart rates. Nasal clips were used during the test, and heart rate was continuously monitored (electronic heart rate scanner; Kettler). The submaximal run on the treadmill was performed at the same speed (exercise load) for each child. The ambient temperature in the air-conditioned laboratory was kept stable at 22°C, and the humidity was stable between 40% and 50% on each day of the study. Differences of 1°C in temperature and 5 mg H2O l−1 of air humidity on the test days were accepted. The FEV1 was measured before running, immediately after, and after 3, 6, 10, 15, 20 and 30 min. Maximum percentage fall in FEV1 after the exercise test was calculated using the following formula: [(pre-exercise FEV1 − lowest post-exercise FEV1)/pre-exercise FEV1] × 100. The FEV1 values were plotted against time for each treatment. The area under the curve (AUC) for the FEV1 values from exercise over the 30-minute period was calculated by using a trapezoidal rule. EIB was defined as a ≥ 15% decrease in FEV1 from baseline.

**Ethics**

The study was approved by the Medical University of Lodz ethics committee. Written consent from the patients and their parents was obtained.

**Statistical analysis**

The differences between positive and negative ETC in cytokine in EBC and FENO levels were assessed by the Mann-Whitney test; for potential confounding, logistic regression analyses were used, and adjusted odds ratio for each cytokine in relation to ETC results were given. In the analyses of differences across quartiles of AUC in EBC cytokines Kruskal-Wallis and Mann-Whitney tests were used. Nominal and categorical variables were compared with the χ² test. Linear correlations were assessed by Spearman’s test. All statistical analyses were performed using StatSoft Statistica for Windows, release 8.0 (StatSoft, Inc., Tulsa, USA). P < 0.05 was used as a definition of statistical significance.

**Results**

Data of 25 children were included in the analysis. Detailed characteristics are shown in Table I.

We observed significant positive correlations between pre-EBC FEV1 and interleukin 4 (r = 0.61), 5 (r = 0.51) and tumor necrosis factor (TNF)-α (r = 0.68) concentrations in EBC. We also assessed the relation between cytokine concentrations in EBC and changes over time in FEV1 (AUC_{pre} of FEV1 in time), AUC above 75 percentiles was associated with higher concentrations of IL-4, IL-5 and TNF-α.

The above observations suggested that higher baseline FEV1 and higher FEV1 during exercise challenge allow higher concentrations of cytokines to accumulate in EBC. We assumed that, in such conditions, reliable assessment of cytokines in EBC (as inflammatory not lung function parameters) during exercise challenge requires adjustment for FEV1. We accepted the following ratio for adjustment of the effect of pre-EBC FEV1 to cytokine concentrations in EBC collected after exercise challenge in our patients: cytokine in EBC/pre-EBC FEV1.

In the next step we analyzed pre-EBC FEV1 adjusted concentrations of cytokines.

**Cytokine comparison between positive and negative exercise treadmill challenge (ETC)**

Among children with a positive exercise treadmill challenge, monocyte chemotactic protein-1 (MCP-1) adjusted to pre-EBC FEV1 (MCP-1/pre-EBC FEV1 ratio) and IL-16 adjusted to pre-EBC FEV1 (IL-16/pre-EBC FEV1 ratio) were significantly higher compared to children with a negative exercise challenge. After adjustment to pre-EBC FEV1, all other cytokines (IL-4, IL-5, IL-6, IL-8, monokine induced by γ-interferon (MIG), TNF-α) were not related to post-exercise changes in FEV1; see Table II and Figure 1. Values below the lower detection limit were excluded. MCP-1 and IL-16 in EBC were independently associated with positive ETC in our patients, since we did not observe a significant effect of the following potential confounders: age, gender, presence of atopy, allergic rhinitis, FeNO level and intensity of current anti-
Table I. Clinical characteristics of study groups

| Parameter                        | EIB negative (n = 15) | EIB positive (n = 10) | P-value |
|----------------------------------|-----------------------|-----------------------|---------|
| Age, mean ± SD [years]           | 14 ±3.1               | 12.5 ±3.2             | 0.160   |
| Male gender, n (%)               | 11 (73.3)             | 4 (40)                | 0.096   |
| BMI, median (interquartile range) [kg/m²] | 21.5 (19.0–25.0)     | 19.4 (18.1–22.3)      | 0.311   |
| Allergic rhinitis, n (%)         | 4 (26.7)              | 3 (30)                | 0.856   |
| Atopy, n (%)                     | 6 (40.0)              | 5 (50.0)              | 0.622   |
| Allergen exposure, n (%)         | 7 (46.7)              | 5 (50.0)              | 0.870   |
| FENO, median (interquartile range) [ppb] | 19.7 (11.7–33.8)    | 24.9 (15.4–54.7)      | 0.558   |
| Current asthma therapy, n (%):   |                      |                       | 0.188   |
| GINA II                          | 10 (66.7)             | 4 (40.0)              |         |
| GINA III                         | 4 (26.7)              | 4 (40.0)              |         |
| GINA IV                          | 1 (6.7)               | 2 (20.0)              |         |
| Maximal fall in FEV₁, median (interquartile range) [%] | 5.0 (0.0–8.0)       | 14.5 (12.0–28.0)      | < 0.001 |
| AUC 0–30, median (interquartile range) [%pred. × min] | 2940 (2804–3402)  | 2778 (2615–3073)      | 0.047   |

Table II. Comparison of cytokine concentrations in exhaled breath condensate unadjusted and adjusted for pre-EBC collection FEV₁. Associations between cytokine concentrations with positive exercise-induced bronchoconstriction (EIB) adjusted for the effects of: age, gender, presence of atopy, allergic rhinitis, FeNO level and intensity of current anti-asthma therapy. Data are presented with median value (interquartile range) and adjusted odds ratios (OR)

| Parameter | EIB negative (n = 15) | EIB positive (n = 10) | ORa | P-value for unadjusted values | P-value for adjusted values |
|-----------|-----------------------|-----------------------|-----|-----------------------------|-----------------------------|
| IL-4      | 25.8 (15.4–77.9)      | 0.11 (0.08–0.39)      | 14.3 (12.1–21.3) | 0.06 (0.05–0.08) | 0.001 | 0.154 | 0.079 |
| IL-5      | 56.8 (37.2–75.6)      | 0.41 (0.15–0.58)      | 47.7 (29.0–74.7) | 0.19 (0.09–0.38) | 0.19 | 0.709 | 0.219 |
| IL-6      | 17.7 (6.9–30.7)       | 0.15 (0.09–0.17)      | 23.3 (18.0–31.1) | 0.12 (0.10–0.16) | 36.3 | 0.285 | 0.815 |
| IL-8      | 6.0 (3.1–7.9)         | 0.03 (0.01–0.03)      | 4.8 (4.5–6.2)    | 0.02 (0.02–0.02) | 23451 | 1.000 | 0.907 |
| IL-13     | 18.7 (3.3–26.5)       | 0.05 (0.02–0.08)      | 21.5 (18.8–25.0) | 0.06 (0.05–0.08) | 11012 | 0.508 | 0.291 |
| IL-15     | 29.8 (6.9–42.8)       | 0.24 (0.16–0.45)      | 46.5 (23.6–54.6) | 0.34 (0.23–0.46) | 4.81 | 0.312 | 0.320 |
| IL-16     | 7.1 (4.6–11.1)        | 0.22 (0.14–0.30)      | 14.2 (8.3–18.8)  | 0.36 (0.24–0.52) | 133651 | 0.074 | 0.017 |
| MCP-1     | 40.7 (17.2–67.8)      | 0.17 (0.11–0.29)      | 84.2 (58.6–103.5)| 0.42 (0.38–0.64) | 325 | 0.042 | 0.022 |
| MIG       | 436 (287–660)         | 1.02 (0.55–1.27)      | 530 (408–579)    | 0.83 (0.50–1.94) | 1.45 | 0.437 | 1.000 |
| TNF-α     | 137 (107–154)         | 0.42 (0.18–0.63)      | 137 (77–190)     | 0.27 (0.20–0.41) | 1.19 | 0.841 | 0.815 |

aOdds ratio for positive EIB (as dependent variable) adjusted for age, gender, presence of atopy, allergic rhinitis, FeNO level and intensity of current anti-asthma therapy.
Figure 1. Comparison of cytokine concentrations in exhaled breath condensate adjusted for pre-EBC collection FEV\textsubscript{1} between negative and positive ETC. Data are presented with the individual value for each participant and the median value (interquartile range) for the group.

Figure 2. MCP-1 and IL-16 concentrations in EBC across four categories of AUC variables (defined according to upper and lower quartile); the \( p \) level for all cytokines > 0.05 in Kruskal-Wallis test.
asthma therapy (adjusted odds ratios are given in Table II). There was no difference between ETC positive and negative patients in FeNO level.

**Correlation between cytokine concentrations and AUC**

We observed a decreasing trend in MCP-1 and IL-16 concentrations in EBC across four categories of AUC variables (defined according to lower and upper quartiles); however, this trend did not reach significance (see Figure 2).

**Discussion**

This study was performed to establish the possible role of airway cytokines in EIB in asthmatic children. We found that higher baseline FEV1 and higher FEV1 during exercise challenge allow higher concentrations of cytokines to accumulate in EBC. Therefore, we postulate that reliable assessment of inflammatory cytokines in EBC during exercise challenge requires adjustment for FEV1. We found that in children with a positive exercise challenge, MCP-1 adjusted to pre-EBC FEV1 and IL-16 adjusted to pre-EBC FEV1 were significantly higher compared to children with a negative exercise challenge. After adjustment to pre-EBC FEV1, none of the other cytokines (IL-4, IL-5, IL-6, IL-8, MIG, TNF-α) were related to post-exercise changes in FEV1.

Monocyte chemotactic protein-1, also referred to as chemokine ligand 2 (CCL2), recruits monocytes, basophiles, memory T cells, and dendritic cells to the sites of inflammation produced by either tissue injury or infection [15]. MCP-1 is primarily secreted by monocytes, macrophages, dendritic cells and epithelium [16]. More importantly, in the context of pathogenesis of EIB in asthmatics, MCP-1 increases IL-4 production by activated T cells and may induce basophile and mastocyte degranulation [15]. Interleukin 16 is a cytokine released by a variety of cells (including lymphocytes and epithelial cells) that has been characterized as a chemoattractant for certain immune cells including eosinophils [17]. We observed higher MCP-1 and higher IL-16 in the EBC, collected 30 min after a positive exercise challenge in comparison to those with a negative exercise challenge. We suspect that those cytokines which were released by injured epithelium in asthmatics may play an important role in the mechanism of EIB in a pathway similar to early (via MCP-1) and late (via IL-16) phases of an allergic reaction. This hypothesis could be explained by interactions between MCP-1 and mast cells together with IL-16 and eosinophils and the role of these cells in EIB [18].

Our study has some limitations. We did not carry out EBC collection before the exercise challenge due to ethical reasons. Repeated EBC collection, together with exercise challenge performed on the same day, could be difficult to accept for our patients. Another weak point of our study is the small group of asthmatic children to assess independence of the association between EBC cytokines and post-exercise changes in lung functions. Also, in studying a heterogeneous group of asthmatics, who have heterogeneous levels of cytokines, the signal-to-noise ratio is not favorable for finding a cytokine footprint that reflects exercise. Possibly, a study that did not include the use of controller medications would have been a better way to examine EBC. Repeatability of the described changes in EBC and their possible effect on airway remodeling should be verified [19, 20]. Finally, pharmacological modulation of the inflammatory response to exercise should be assessed [21].

In conclusion, the study shows a significant inflammatory response of the airways in asthmatic children with bronchoconstriction after exercise, despite the use of control medications. This suggests the need for increased anti-inflammatory treatment in children with chronic asthma with a positive exercise challenge. Based on our findings, specific cytokines are up-regulated in the airways after significant bronchoconstriction, and they may play an important role in the mechanism of EIB, which may justify anti-cytokine therapy (e.g. anti-MCP-1) in the future. The results should be confirmed in widespread investigations incorporating more children with strict homogeneous clinical characteristics and including the evaluation of other important asthma-related chemokines (RANTES, CCL18, MIP-1α) [22, 23]. Another important clinical implication of our study is the need for FEV1 adjustment when measuring cytokine concentrations in EBC after exercise challenge. Despite the weak points of this study, EBC and exercise could prove to be very fruitful in the future.

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The study was approved by the Medical University of Lodz Ethics committee, Poland. Written consent from the patients and their parents was obtained.

**Conflict of interest**

The authors declare no conflict of interest.

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