RANTES in Exhaled Breath Condensate of Allergic Asthma Patients with Exercise-Induced Bronchoconstriction

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Key Words

Airway inflammation • Asthma • Exercise-induced bronchoconstriction • Exhaled breath condensate • RANTES

Abstract

Background: The response of asthmatics to exercise differs from that of healthy subjects, and the mechanisms responsible for exercise-induced bronchoconstriction (EIB) remain to be elucidated. **Objectives:** The aim of this study was to evaluate changes in RANTES levels in exhaled breath condensate (EBC) following intensive exercise in allergic asthmatics. **Methods:** The study was conducted in a group of 19 asthmatics (11 with EIB and 8 without EIB) and 7 healthy volunteers. Changes in the concentrations of RANTES in EBC induced during the 24 h after intensive exercise were determined. Moreover, these measurements were tested for possible correlations with the results of other tests commonly associated with asthma as well as with changes in airway inflammation after exercise. **Results:** In contrast to asthmatic patients without EIB and healthy controls, in asthmatics with EIB RANTES concentrations were statistically significantly increased in EBC collected during the first 24 h after an exercise test. There was a statistically significant correlation between the maximum increase in RANTES concentrations in

EBC after exercise and either baseline exhaled nitric oxide (F_{ENO}) or bronchial hyperreactivity to histamine and an increase in serum eosinophil cationic protein or F_{ENO} 24 h after exercise in the EIB asthmatics. **Conclusions:** The increase in RANTES in asthmatic airways, promoting the migration and activation of inflammatory cells including eosinophils, may play an important role in the upregulation of airway inflammation after EIB in asthmatic patients.

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Introduction

Evidence is accumulating that exercise-induced bronchoconstriction (EIB) is associated with eosinophilic airway inflammation, bronchial hyperreactivity (BHR), atopy and airway obstruction [1]. However, results related to the participation of inflammatory mediators in either the maintenance or induction of bronchoconstriction provoked by exercise are still conflicting [2].

Eosinophilic granulocytes are attracted and activated by chemokines, proteins of the cytokine family. Eosinophils initiate tissue damage via the release of cytotoxic substances like major basic protein, eosinophil cationic protein (ECP), eosinophil peroxidase and the autocrine production of chemokines, which cause a self-sustained

inflammatory process and chronic disease [3]. Eosinophils are the key effector cells in asthma [4], but their role in EIB is less clear and controversial.

Chemokines are a family of cytokines that are believed to be involved in the pathogenesis of asthma, possibly by recruiting leukocytes to the site of inflammation [5]. CCL chemokines, e.g. RANTES and eotaxin, have been implicated in the allergic inflammation in asthma by promoting the migration and activation of inflammatory cells, including eosinophils [6].

In asthmatic patients, increased expression of local and systemic CCL chemokines has been reported [7, 8]. In vitro studies have demonstrated that hyperosmolar stimuli induce the release of chemotactic mediators from human epithelial cells [9, 10]. Eosinophil and neutrophil counts were also raised in bronchoalveolar lavage fluid in the late phase of EIB [11]. Yoshikawa et al. [12] found a relationship between the severity of EIB and the percentage of eosinophils in sputum as well as the level of ECP in adult asthmatics, suggesting that the presence of sputum eosinophilia predicts airway responsiveness provoked by exercise. EIB, which is stimulated by the release of mediators mainly by mast cells, aggravates eosinophilic inflammation [1]. These findings suggest that eosinophils may play a major role in the severity of EIB in patients with asthma.

In our previous studies in asthmatic patients, plasma concentrations of RANTES were increased following EIB, possibly due to platelet activation [13]. In the present study, we aimed to determine the role of RANTES expression in the airways in the pathogenesis of EIB in asthmatics.

The study was performed on exhaled breath condensate (EBC). EBC samples were collected by cooling exhaled air – a noninvasive, easily performed and effort-independent, rapid procedure to obtain samples from the lower respiratory tract [14]. In earlier studies, Matsunaga et al. [15] reported that RANTES expression was upregulated in EBC of asthmatic airways.

The aim of this study was to evaluate RANTES levels and their changes following EIB in EBC of asthmatic patients, and to establish possible correlations of these measurements with parameters of airway inflammation.

Patients and Methods

Patients

The study involved a group of 19 mild allergic asthma patients. Asthma was diagnosed according to the criteria recommended by the Global Initiative for Asthma updated in 2002 [16]. The diag-

nosis of allergic asthma was based on positive skin prick tests and a history of asthma and symptoms of allergic rhinitis following exposure to sensitizing allergens. All patients had been in a stable condition, and free from acute exacerbations and respiratory tract infections the previous 2 months. Patients with other factors possibly affecting exhaled nitric oxide (F_{ENO}) levels (except for asthma and features of atopy or allergic rhinitis) were not included. In all the patients, tests were performed outside the pollen season. Prior to study entry, patients were allowed to take short-acting β_2 -agonists. Asthmatic patients who had been treated with drugs other than β_2 -agonists (e.g. inhaled steroids or anti-leukotrienes) in the past 3 months were excluded from the study. Before qualifying a patient for the exercise test, F_{ENO} and total IgE levels were assessed, and skin prick tests to commonly encountered aeroallergens (e.g. house dust mites, trees, weeds, grasses, cat, *Alternaria* and *Cladosporium*), flow/volume spirometry and a bronchial provocation test (BPT) with histamine were performed in each asthmatic patient.

Seven healthy volunteers were also recruited for the study as a negative control. All of them underwent F_{ENO} , flow/volume spirometry and skin prick tests to common aeroallergens. Their forced expiratory volume in 1 s (FEV_1) was >80% of predicted. They were free of respiratory tract infections during the 2 months before study entry and from other significant illnesses known to affect F_{ENO} levels. Asthma patients and healthy volunteers were non-smokers and had not been passive smokers during the last year.

All asthmatic patients and healthy volunteers performed an exercise test on the bicycle ergometer.

Study Protocol

BPT with histamine was performed 24 h before and 24 h after exercise. EBC was collected before and 30 min after BPT with histamine, and before and after (10 and 30 min, and 1, 6 and 24 h) exercise. F_{ENO} was measured before BPT with histamine, and before and 24 h after exercise. Serum ECP and peripheral blood eosinophilia were also assessed before and 24 h after exercise.

The study protocol was approved by the Ethics Research Committee of the Medical University of Białystok (agreement No. R-I-003/80/2006). Informed consent was obtained from each patient entered into the study.

Measurements

F_{ENO} was determined using the chemiluminescence technique (280i NO Analyzer; Sievers, Boulder, Colo., USA) at an expiratory flow of 50 ml/s according to recommendations of the American Thoracic Society (ATS) for online measurement of F_{ENO} in adults [17].

Baseline spirometry was performed using a MasterScreen Pneumo PC spirometer (Jaeger, Hoechberg, Germany) according to ATS standards [18]. FEV_1 was determined. Before the examination, the patients did not take any medication possibly affecting spirometry results.

A nonspecific BPT with histamine was carried out according to the method described by Ryan et al. [19]. Provocation was performed using a DeVilbiss nebulizer 646 (Viasys Healthcare, Hoechberg, Germany) at an air pressure of 0.15 MPa linked to a dosimeter (Rosenthal-French, Baltimore, Md., USA). The results were presented as $PC_{20} FEV_1$ – the concentration of histamine which causes a decrease in FEV_1 of exactly 20% in comparison to initial values.

Table 1. Characteristics of asthmatic patient and healthy control groups

Characteristics	Asthmatic patients			Healthy volunteers
	with EIB	without EIB	p value	
Patients, n	11	8		7
Sex, females/males	7/4	5/3		4/3
Age, years	27.36 ± 7.50	31.63 ± 5.40	0.19	28.40 ± 4.90
Duration of symptoms, years	3.70 ± 4.63	4.12 ± 3.54	0.33	
Baseline FEV ₁ , % of predicted	95.63 ± 18.54	92.25 ± 8.61	0.63	106.85 ± 9.73
Maximum decrease in FEV ₁ after exercise, %	25.8 ± 13.5	3.6 ± 1.9	0.0003	0.71 ± 3.2*, **
log PC ₂₀ histamine FEV ₁ , mg/ml	-0.59 ± 1.16	-0.05 ± 0.55	0.24	
Blood eosinophil count, cells/mm ³	239 ± 138	157 ± 66	0.14	51 ± 26*, **
Serum ECP, µg/l	10.88 ± 5.18	7.64 ± 1.63	0.04	3.94 ± 1.05*, **
Serum total IgE, kU/l	358 ± 322	171 ± 69	0.12	65 ± 31*, **
Baseline F _{ENO} , ppB	98.90 ± 55.37	66.62 ± 23.05	0.21	18.00 ± 5.59*, **
Baseline RANTES, pg/ml	8.76 ± 0.93	8.08 ± 0.73	0.11	3.56 ± 0.76*
Positive skin prick test, n of patients	11	8		0
Mite/cat/molds	9/3/2	7/2/2		
Seasonal	4	3		

Data are presented as means ± SD. PC₂₀ histamine FEV₁ = provocative concentration of histamine that caused a 20% fall in FEV₁. * p < 0.05 vs. patients with EIB, ** p < 0.05 vs. patients without EIB.

Exercise was performed on a bicycle ergometer for 9 min with a fixed workload adjusted to increase the heart rate to 85% of the maximum predicted for the age of each patient [20]. Basic spirometric parameters were recorded before and immediately after exercise, and 1, 5, 10, 15, 20, 60 min and 24 h after completion of exercise. Patients with maximum decrease in FEV₁ >15% were considered to have EIB.

EBC was collected using a commercially available condenser (EcoScreen; Jaeger) according to the current ATS/European Respiratory Society guidelines [21]. All measurements were performed at the same time (between 8.00–10.00 a.m.) to avoid possible effects of the circadian rhythm on mediator concentrations in EBC. All patients were asked to refrain from eating and drinking before EBC sampling.

Exhaled air entered and left the chamber through one-way valves (inlet and outlet), thus keeping the chamber closed. Due to the low temperature inside the condensing chamber throughout collection, the expired air is cooled down and condenses. The temperature of collection was around 0°C [14]. Patients were instructed to breathe tidally for 10 min with a nose clip. The respiratory rate ranged from 15 to 20 breaths/min. Patients were asked to swallow their saliva periodically and to temporarily discontinue collection if they needed to cough. At the end of collection 1.5- to 3.5-ml aliquots of condensate were transferred to Eppendorf tubes and immediately frozen. Exhaled breath was collected before, and 10, 30, and 60 min, and 6 and 24 h after exercise. Samples were stored at -80°C for ≤3 months [14]. The samples were not concentrated prior to measurement. Measurements were performed in a blinded fashion, and samples were run in duplicate. Our preliminary study indicated that RANTES in EBC stored at -80°C remains stable for ≥3 months with good reproducibility.

Serum total IgE and ECP concentrations were measured using ImmunoCAP™ Technology (Pharmacia Diagnostics, Uppsala, Sweden). Blood eosinophils were counted using a hematologic analyzer (Coulter Electronics, Miami, Fla., USA). Concentrations of RANTES (R&D Systems, Wiesbaden-Nordenstadt, Germany) in EBC were determined using an enzyme-linked immunosorbent assay. The minimum dose detectable was 2.0 pg/ml.

Analysis

Statistical significance was analyzed by analysis of variance followed by Bonferroni's t test post hoc to determine statistical differences. All values were expressed as means ± SD; p < 0.05 was considered significant. PC₂₀ values were logarithmically transformed for analysis. Associations among the parameters studied were assessed using Pearson's linear correlation coefficient.

Results

Characteristics of the asthmatic and healthy volunteer groups are shown in table 1. Eleven patients of the asthmatic group had a positive and 8 had a negative exercise test. In none of the healthy volunteers were spirometric parameters worse after exercise.

Blood eosinophilia, serum ECP, baseline F_{ENO} and total IgE were statistically significantly higher in both asthmatic subgroups than in healthy volunteers. In the subgroup of patients with EIB, serum ECP concentrations

Fig. 1. Concentrations of RANTES in EBC at rest and concentration changes observed during the 24 h after exercise in asthmatic patients and healthy volunteers.

were significantly raised compared to patients without EIB; blood eosinophil counts, serum levels of total IgE and baseline F_{ENO} were also higher, but these differences were not statistically significant.

RANTES in EBC was statistically significantly elevated in all asthmatic patients compared with healthy controls (8.47 ± 0.90 vs. 3.56 ± 0.76 pg/ml, $p < 0.001$). There was no statistically significant difference in the concentration of RANTES in EBC before exercise between asthmatics with and without EIB (8.76 ± 0.93 vs. 8.08 ± 0.73 pg/ml, $p = 0.24$). In the group of healthy controls, RANTES levels in the EBC were statistically significantly decreased compared with asthmatics (asthma with EIB vs. healthy volunteers: 3.56 ± 0.76 pg/ml, $p < 0.001$; asthma without EIB vs. healthy volunteers: $p < 0.001$).

A statistically significant increase in the concentration of RANTES in asthmatic patients with EIB was noted

($p < 0.001$; 10 min after exercise: 9.69 ± 1.37 pg/ml, $p = 0.016$; 30 min after exercise: 10.82 ± 1.21 pg/ml, $p = 0.004$; 60 min after exercise: 11.26 ± 1.12 pg/ml, $p = 0.002$; 6 h after exercise: 10.72 ± 1.03 pg/ml, $p = 0.001$; 24 h after exercise: 10.37 ± 0.99 pg/ml, $p = 0.003$). Exercise did not affect the concentrations of RANTES in EBC in either asthmatic patients without EIB ($p = 0.99$) or healthy volunteers ($p = 0.99$; fig. 1).

The changes in RANTES in EBC were absent after BPT with histamine (patients with EIB: before BPT with histamine 8.89 ± 0.81 pg/ml, 60 min after 8.67 ± 0.91 pg/ml, 24 h after 8.76 ± 0.93 pg/ml; patients without EIB: before BPT with histamine 7.96 ± 0.81 pg/ml, 60 min after 7.81 ± 0.93 pg/ml, 24 h after 8.08 ± 0.73 pg/ml).

The maximum increase in RANTES after exercise significantly correlates with the baseline level of RANTES in EBC ($r = 0.5$, $p = 0.01$). There were no statistically sig-

Fig. 2. Changes in F_{ENO} levels 24 h after exercise in asthmatic patients.

Fig. 3. Changes in BHR to histamine expressed as the histamine $\log PC_{20}$ 24 h after exercise in asthmatic patients.

nificant correlations between the baseline concentrations of RANTES in EBC and other study parameters in either subgroup of asthmatic patients or in the healthy volunteers and the decrease in FEV_1 after exercise in asthmatics with EIB.

In the asthmatics with EIB, F_{ENO} (before exercise: 98.90 ± 55.37 parts per billion, ppB; 24 h after exercise: 119.18 ± 64.39 ppB; $p = 0.034$) and BHR with histamine

($\log PC_{20} FEV_1$ before exercise: -0.59 ± 1.16 pg/ml; 24 h after exercise: -0.95 ± 1.03 pg/ml; $p < 0.001$) were statistically significantly increased 24 h after exercise but not in the asthmatics without EIB (F_{ENO} before exercise: 66.62 ± 23.05 ppB; 24 h after exercise: 67.87 ± 23.03 ppB; $p = 0.25$; $\log PC_{20} FEV_1$ before exercise: -0.053 ± 0.55 mg/ml; 24 h after exercise: $-0.0511.62 \pm 0.59$ mg/ml; $p = 0.99$; fig. 2, 3).

Fig. 4. Changes in serum ECP levels 24 h after exercise in asthmatic patients.

Fig. 5. Correlations between the maximum increase in RANTES in EBC and either baseline F_{ENO} and baseline BHR to histamine or changes in F_{ENO} and serum ECP 24 h after exercise in the asthmatics with EIB.

In the asthmatics with EIB, there was a statistically significant increase in serum ECP concentrations 24 h after exercise (before exercise: $10.88 \pm 5.18 \mu\text{g/l}$; 24 h after exercise: $14.35 \pm 4.52 \mu\text{g/l}$; $p = 0.02$), which was absent in the asthmatics without EIB (before exercise: $7.64 \pm 1.63 \mu\text{g/l}$; 24 h after exercise: $7.72 \pm 1.57 \mu\text{g/l}$; $p = 0.48$; fig. 4).

Peripheral blood eosinophilia was not significantly different 24 h after exercise. Significant changes in FEV_1 were also not detected in the asthmatic subgroups 24 h after exercise.

A statistically significant correlation between the maximum increase in RANTES concentrations in EBC after exercise and either baseline F_{ENO} ($r = 0.66$, $p = 0.025$) or baseline BHR to histamine (expressed as $\log \text{PC}_{20} \text{FEV}_1$; $r = -0.58$, $p = 0.048$) and the increase in F_{ENO} ($r = 0.75$, $p = 0.007$) or serum ECP ($r = 0.75$, $p = 0.007$) 24 h after exercise was found in the subgroup of asthmatics with EIB (fig. 5).

Discussion

The pathogenesis of EIB in asthma and the role of chemokines in this process are incompletely understood. It has been proposed that the release of mediators such as histamine, leukotrienes and prostanoids from mast and epithelial cells in response to a hyperosmolar stimulus may be responsible for the bronchospasm [22]. The results of studies concerning the role of inflammatory mediators in EIB are conflicting. However, it has been suggested that inflammatory processes are involved in the pathogenesis of EIB [23].

Results of our previous studies indicate that platelet activation, and the increase in RANTES release probably related to this activation, could be one of the factors triggering increased airway inflammation following EIB in asthmatic patients [13].

Eosinophils are the key inflammatory cells in asthma, but their role in EIB remains to be elucidated [4, 24]. In a study by Gauvreau et al. [25], exercise had no effect on inflammatory cells assessed in the blood or sputum. Koh and Choi [26] reported that the severity of EIB correlates with peripheral blood eosinophilia, and that the blood eosinophil count was a significant predictor of EIB. Venge et al. [27] have shown that serum ECP reflects the degree of EIB in asthma patients. The role of eosinophils in EIB was described by Kivity et al. [28], who demonstrated significantly increased sputum eosinophil counts in EIB patients, but not in those without EIB or after BPT with methacholine.

RANTES, a CCL chemokine, is produced by endothelial cells, fibroblasts, T lymphocytes, eosinophils, platelets, bronchial epithelial cells, mast cells and other cells [29]. It is a powerful chemoattractant of inflammatory cells, particularly eosinophils and T lymphocytes [29], which also activates these immune cells and induces the exocytosis of bronchoconstrictive mediators [29]. Therefore, RANTES might be involved in inflammatory cell recruitment and the induction of bronchoconstrictive mediator release from cells resulting in airflow limitation.

In a study by Tahan et al. [30] in children, chemokine profiles were not significantly different following EIB. However, they pointed out some limitations of their study. The chemokine measurements were performed in the systemic circulation and therefore may not reflect changes occurring in the bronchial epithelium after exercise. The authors also suggested that these changes could not be excluded in case of more pronounced exercise responses than those observed in their patients, since previous studies demonstrated that systemic chemokine levels may change after marathon running [8].

In our study, we aimed to assess changes in RANTES concentrations in EBC which may result from the increased expression of this cytokine in asthmatic airways following intensive exercise in asthmatics and to investigate possible correlations between this parameter and the results of other measurements commonly associated with asthmatic inflammation.

The lining fluid of the respiratory tract contains various nonvolatile and >200 volatile substances. EBC is a means to collect respiratory secretions. EBC analysis is a noninvasive tool to determine mediator levels in the airways. EBC is simple to perform, rapid and effort independent, even in children and patients with asthma exacerbation. This type of sampling can be repeated in short intervals without side effects. It provides an opportunity to follow changes in mediator levels in the airways during exercise challenge. EBC collection does not alter airway function or inflammation. Mediator levels in EBC are more variable than EBC volume, but good reproducibility has been reported for adenosine, aldehyde, glutathione, leukotriene and pH levels in EBC [21].

In previous studies on asthmatic patients, inflammatory mediators such as adenosine and Cys-LT were elevated in EBC during exercise. Csoma et al. [31] revealed a pronounced increase in adenosine levels in EBC during EIB in asthmatic patients, and a relationship between this increase and the degree of bronchospasm was observed. Carraro et al. [32] noted higher EBC Cys-LT levels in

asthmatic children with EIB, which correlated with the decrease in FEV₁ after exercise. We also found a significant increase in endothelin-1 levels in EBC collected between 10 min and 6 h after exercise [33].

In our study, the highest baseline concentration of RANTES was observed in asthmatic patients with EIB. However, statistically significant changes in the levels of this parameter were demonstrated only in comparison with the group of healthy volunteers. Only the subgroup of patients with EIB had a statistically significant increase in RANTES levels in EBC collected between 10 min and 24 h after exercise. The maximum increase in RANTES levels was correlated with baseline BHR to histamine and baseline F_{ENO} levels – which has become a more and more noticeable criterion for the evaluation of eosinophilic inflammation in the airways [34] – as well as with the increase in F_{ENO} or serum ECP levels 24 h after exercise.

It cannot be excluded that RANTES in EBC originates from the circulating blood. However, the correlation of RANTES levels in EBC with the changes in the other parameters and the relationship between this cytokine and the inflammatory process, involving RANTES release by several cell types, possibly imply that fluctuations in the concentrations of this cytokine after exercise may be a consequence of increased RANTES expression in asthmatic airways. A possible limitation of our study could be

the small number of asthmatics and healthy volunteers; therefore, the results of this study have to be confirmed by future studies including larger patient cohorts.

Conclusion

This study was performed to assess the possible role of RANTES in the pathogenesis of EIB, particularly during the inflammatory process. EIB results in increased RANTES levels in EBC. Consequently, increased RANTES expression in asthmatic airways, via inflammatory cell recruitment and bronchoconstrictive mediator release, may play an important role in the upregulation of airway inflammation following EIB in asthmatic patients, but not in asthmatics without EIB. Appropriate anti-inflammatory treatment could protect asthmatic patients from EIB and, as a consequence, from increasing airway inflammation.

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