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Adenosine level in exhaled breath increases during exercise-induced bronchoconstriction

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ABSTRACT: **In asthmatic patients, airway obstruction provoked by exercise challenge is accompanied by an increase in plasma adenosine level. In this study, the current authors investigated if exercise-induced bronchoconstriction was associated with local changes of adenosine concentration in the airways.**

Oral exhaled breath condensate (EBC) collection (5-min duration) and forced expiratory volume in one second (FEV_1) measurements were performed at rest (baseline) and 4–8 times after treadmill exercise challenge in healthy and asthmatic subjects. Adenosine concentration in EBC was determined by HPLC.

Observations indicated that physical exercise results in bronchoconstriction together with a significant increase of adenosine level in EBC in asthmatic patients (mean±SD maximal fall in FEV_1 27±13%; associated increase in adenosine 110±76% as compared to baseline), but not in healthy control subjects. Exercise-induced changes in adenosine concentration correlated significantly with the fall in FEV_1 values in asthmatic patients.

In conclusion, the observed increase in adenosine concentration of oral exhaled breath condensate most probably reflects changes in the airways during exercise-induced bronchoconstriction. Due to its known bronchoconstrictor property in asthma, adenosine may contribute to the development of bronchospasm.

KEYWORDS: **Adenosine, airway inflammation, asthma, exercise-induced bronchoconstriction, exhaled breath condensate**

Exercise-induced bronchoconstriction (EIB) is a common feature of asthma, and its severity is likely to be associated with airway inflammation [1]. The mechanism of EIB is not completely understood, but it has been widely accepted that exercise-induced hyperpnoea plays a substantial role as an initiating stimulus [2] through cooling (heat-loss theory) and dehydration (water-loss theory) of the airway [3, 4]. In line with the water-loss theory suggesting that dehydration stimulates inflammatory cells in the airways to release bronchoconstrictor mediators [5, 6], pharmacological studies provide evidence for the contribution of histamine [7], cysteinyl-leukotrienes [8] and bronchoconstrictor prostanoids [9] to the development of EIB.

A number of *in vitro* and *in vivo* observations suggest that the bronchoconstrictor mechanisms involved in exercise and adenosine challenges may be interrelated. Both challenges cause mast cell activation, resulting in the release of various bronchoconstrictor mediators, including histamine and cys-leukotrienes [5, 10, 11]. Indeed, inhibition of the 5-lipoxygenase pathway in asthmatic patients significantly attenuates EIB and adenosine-induced bronchoconstriction, indicating that leukotrienes are important mediators in both challenges [12]. Furthermore, a similarity between hyperresponsiveness to exercise and adenosine 5′-monophosphate was demonstrated in children with asthma [13].

Based on the previously mentioned lines of evidence, it is plausible that, during exercise, adenosine released by adenosine triphospate mobilisation and metabolisation could participate in the development of exercise-induced bronchospasm. Previously, it was demonstrated that, during EIB, plasma adenosine concentration increased and this change was related to the degree of airway obstruction [14]. In the present study, the aim was to determine if EIB was associated with local changes of adenosine concentration in the airways of asthmatic patients by

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comparing the time course of changes in mediator level and lung function following exercise.

Collection of exhaled breath condensate (EBC) is a simple, noninvasive mode of obtaining samples from the airways. This type of sample collection can be repeated in short intervals without side-effects and, therefore, it provides an opportunity to follow the changes in mediator levels in the airways, although possible confounding effects of the mouth and the nasal cavities cannot be completely excluded. The level of adenosine in EBC can be determined with good reproducibility [15]. Adenosine concentrations of oral and tracheal EBC samples do not differ significantly [16]. Furthermore, the mode of inhalation (oral or nasal) has no influence on EBC adenosine concentration in subjects without upper airway disease [16].

In this study, EBC adenosine concentrations were determined at rest and several times during the development of EIB in asthmatic patients, and these were compared to those found in samples collected from healthy volunteers before and after exercise challenge. To study the direct effect of bronchoconstriction by itself, the EBC adenosine level was determined after methacholine (MCh) provocation in asthmatic patients.

METHODS

Subjects

Eight nonatopic healthy volunteers (aged 28±7 yrs; five males) and 10 patients with mild asthma (aged 32±11 yrs; four males) participated in the study. All patients met the American Thoracic Society (ATS) diagnostic criteria for bronchial asthma [17] and had elevated levels of exhaled nitric oxide (NO). Seven of the asthmatic patients had a history of EIB and, in addition, seven of them were atopic, as demonstrated by at least two positive results to skin tests with the following common aeroallergens: house dust mite, cat dander, dog dander, grass pollen and *Aspergillus fumigatus* (Soluprick; ALK Abello, Copenhagen, Denmark). Treatment of patients consisted of inhalation of short-acting β_2-agonists as needed.

The protocol was approved by the local ethics committee, and written informed consent was obtained from each subject before the study. All subjects were nonsmokers, and were instructed to avoid consuming caffeinated drinks and foods, and refrain from exercise for ⩾12 h before the study. β_2-Agonists were withdrawn ⩾12 h before the exercise and MCh challenges. No subjects were studied within 6 weeks of having an upper respiratory tract infection or any chronic upper airway/sinus disease.

Study design

Effect of methacholine challenge

When preparing the study, a control experiment was performed in six patients with mild asthma to establish the effect of bronchoconstriction on EBC adenosine concentration (table 1). In this pre-study, EBC samples were collected for adenosine measurement from patients twice before and 3–6 times after MCh inhalation, which caused >20% decrease in forced expiratory volume in one second (FEV_1).

Effect of exercise challenge

Seven asthmatic patients with a history of EIB (table 2) and eight nonatopic healthy volunteers performed an exercise test. The scheme of the test is shown in figure 1. First, EBC was collected twice for 5 min with a minimal pause between samplings to change condensing tubes. This was followed by the measurement of exhaled NO and FEV_1 in each study participant. Participants were then subjected to a standardised treadmill exercise test. At the completion of exercise challenge, minute ventilation was determined. Next, a cycle of FEV_1 measurement, followed immediately by EBC collection for 5 min, was performed. This cycle was repeated four times in healthy volunteers and 4–8 times in asthmatic patients until FEV_1 had returned to baseline either spontaneously or following inhalation of short-acting β_2-agonists as required by the patient. FEV_1 measurement and replacement of condensing tubes lasted 2 min each time. This study protocol was performed between 07:00 and 08:00 h in all subjects.

Collection of exhaled breath condensate

EBC was collected using the EcoScreen condenser (Jaeger, Hoechberg, Germany), as reported previously [15]. At rest, subjects breathed at a normal frequency and tidal volume. EBC collection was performed without wearing a nose-clip (inhaling through the nose and/or mouth and exhaling orally). Subjects were instructed not to alter their breathing pattern,

TABLE 1 Data from asthmatic patients provoked by methacholine (MCh)

Sex	Atopy	Age yrs	F_{ENO} ppb	Baseline FEV_1 % pred	PC_{20} MCh mg·mL^{-1}	Baseline ADO nM	Post-MCh ADO nM	ΔADO %
F	+	17	8.3	97	5.0	8	10	25
F	+	28	40.0	76	0.6	5	4	-20
M		40	50.0	97	7.9	12	7	-42
F#		32	25.2	76	1.3	13	4	-69
M#	+	24	28.5	100	0.6	8	8	0
M#	+	56	10.6	90	5.0	8	7	-13
Mean±SD		33±14	27.1±16.3	89±11	2.1¶	9±3	7±2	-20±33

F_{ENO}: exhaled nitric oxide; baseline FEV_1: the highest of three pre-exercise measurements of forced expiratory volume in one second (FEV_1); PC_{20} MCh: concentration of MCh causing >20% fall in baseline FEV_1; baseline ADO: mean of two adenosine concentrations in exhaled breath condensate (EBC) samples collected consecutively before MCh challenge; post-MCh ADO: adenosine in EBC samples collected after a >20% fall in FEV_1 occurred; ΔADO %: percentage change in EBC adenosine (100 × (post-MCh value–baseline value)/baseline value); F: female; M: male; +: present. #: patients also took part in the exercise study; ¶: geometric mean.

TABLE 2 Data from asthmatic patients who performed an exercise test

Sex	Atopy	Age yrs	F_{ENO} ppb	Baseline FEV_1 % pred	PE min. FEV_1 % pred	ΔFEV_1 max. %	Baseline ADO nM	PE ADO nM	ΔADO %
F	+	26	24.0	93	75	-19	18.5	21	14
F	+	31	41.0	103	80	-22	21	33	57
M		41	10.8	80	37	-54	7	20	186
F	+	28	28.6	122	100	-18	20	26	30
F#		32	36.6	75	53	-29	7	18	157
M#	+	24	30.0	101	85	-15	8	18	125
M#	+	56	14.7	102	69	-31	4	12	200
Mean±SD		34±11	26.5±11.0	97±16	71±21***	-27±13	12±7	21±7**	110±76

F_{ENO}: exhaled nitric oxide; baseline FEV_1: the highest of three pre-exercise measurements of forced expiratory volume in one second (FEV_1); % pred: % predicted; PE min. FEV_1: minimal post-exercise (PE) FEV_1 values; ΔFEV_1 max. %: maximal percentage change in FEV_1; baseline ADO: mean of two adenosine concentrations in exhaled breath condensate (EBC) samples collected consecutively before exercise; PE ADO: adenosine in EBC samples collected before PE min. FEV_1; ΔADO %: percentage increase in EBC adenosine (100×(PE ADO–baseline ADO)/baseline ADO). #: patients who took part in the methacholine study. **: $p<0.01$, difference in EBC adenosine between baseline and PE values; ***: $p<0.001$, difference in FEV_1 between baseline and PE values.

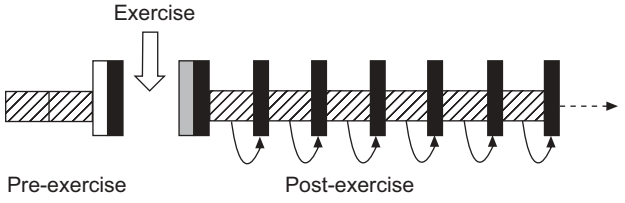

FIGURE 1. Schematic diagram of the study protocol. ▨: collection of exhaled breath condensate (EBC); □: measurement of exhaled nitric oxide; ■: measurement of forced expiratory volume in one second (FEV_1); ■: measurement of minute ventilation; →: paired EBC samples and FEV_1 measurements to study the effect of exercise-induced changes in EBC adenosine in the development of airway obstruction; dashed arrow: the cycles of measurement were repeated more times in some cases.

volume and the route of inhalation (oral/nasal) during EBC collection. Collected samples were stored frozen (at -20℃) until analysis (EBC samples collected from each subject were analysed within 60 min after the end of the visit).

Lung function test and exhaled NO measurement

FEV_1 was measured by means of an electronic spirometer (MS-11; MEDICOR, Budapest, Hungary) according to the ATS guidelines [18]. At rest, three technically acceptable manoeuvres were performed and the highest of them was used in the analysis. After exercise, only single acceptable manoeuvres were performed.

Exhaled NO was measured by a chemiluminescence analyser (Model LR2000; Logan Research, Rochester, UK), sensitive to NO from 1–5,000 ppb by volume, and with a resolution of 0.3 ppb [19].

Methacholine challenge

MCh bronchial challenge was performed using a standardised dosimetric method, as previously described [20]. Briefly, after an initial 0.9% sodium chloride inhalation, patients were exposed to doubling concentrations of MCh chloride (Sigma Chemical Co., St Louis, MO, USA) as five breaths from a breath-actuated dosimeter (MEFAR MB-3; Mefar S.P.A., Bovezzo, Italy). FEV_1 was measured 2 min after each inhalation, and the challenge was discontinued when the fall in FEV_1 was $\geqslant$20% from the post-saline value.

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 set to raise the subjects' heart rate to 90% of the age-adjusted maximum target level. Each subject exercised for 6 min under these target conditions. At the end of the challenge, minute ventilation was not significantly different between healthy subjects (mean±SD 26.2±7.2 $L \cdot min^{-1}$) and asthmatic patients (31.2±15.6 $L \cdot min^{-1}$).

Determination of adenosine

EBC adenosine concentrations were determined by reverse-phased isocratic HPLC [15, 21]. Separation was performed on a 3-μm Microspher C18 (100×4.6 mm internal diameter; Chrompack, Middelburg, The Netherlands) analytical column with a 5-μm Chromsep C18 guard column (10×3 mm internal diameter; Chrompack) by a mixture of 0.05 M Na_2HPO_4 buffer (pH 3.8) and acetonitrile (97.5:2.5 v/v), as a mobile phase. Detection of adenosine was at 260 nm with a dual wavelength ultraviolet detector (LKB 2141; Pharmacia, Uppsala, Sweden). EBC adenosine identity was verified by retention time (6–8 min, depending on flow rate) and/or coelution with an aqueous adenosine standard. When needed, an aliquot of the sample was treated with adenosine deaminase to differentiate the adenosine peak from possible coeluting peaks. To obtain a calibration curve, aliquots of pooled EBC samples were spiked with aqueous adenosine standards to give 3-, 6-, 12-, 24- and 48-nM adenosine concentrations above the original value. The chromatogram of the unspiked, original sample was subtracted from the chromatogram of spiked samples. By plotting adenosine peak areas against adenosine concentrations, a line was calculated, which served for determination of adenosine levels. The calibration line was checked at two concentrations before each set of adenosine measurements. The stability of

adenosine in EBC at room temperature, plus both the within-run precision and the day-to-day repeatability of EBC adenosine measurement, was determined previously [15]. In this study, an agreement between EBC adenosine measurements was also examined by determining the adenosine concentration in two consecutive samples collected before exercise challenge.

Statistical analysis

Results are expressed as mean±SD. The Bland and Altman test was used to assess repeatability of adenosine measurement in EBC at rest [22]. Repeated-measures ANOVA, followed by Newman-Keuls *post hoc* comparison testing, was performed to compare baseline and post-exercise data from healthy volunteers and asthmatic subjects within and between groups. Linear regression analysis was performed to determine the correlation between exercise-induced changes in EBC adenosine concentrations and the decrease in FEV_1 values. Areas under the curves (AUC; for ΔFEV_1 and ΔEBC adenosine) were calculated by trapezoid integration (GraphPad Prism Version 3; GraphPad, San Diego, CA, USA).

RESULTS

Baseline values

Determination of EBC adenosine showed good repeatability. Adenosine concentrations in two consecutive samples collected at rest were 11±6 and 11±5 nM, respectively (n=21, all subjects participating in exercise and/or MCh challenges). The mean difference with coefficient of repeatability was -0.2±2.6 nM, and all differences were within ±2SD.

In healthy volunteers, the baseline values of FEV_1 and EBC adenosine were 98±9% and 11±4 nM, respectively. There was no significant difference either in baseline FEV_1 values or in EBC adenosine between healthy subjects and asthmatic patients (baseline characteristics of asthmatic patients in tables 1 and 2).

Effect of methacholine provocation and exercise challenge

In asthmatic patients provoked by MCh, no significant change was observed in EBC adenosine concentration in association with a 26±6% mean of maximal fall in FEV_1 caused by MCh inhalation (table 1, fig. 2).

Exercise did not induce significant changes either in FEV_1 or EBC adenosine in healthy volunteers, but it caused a significant decrease in FEV_1, accompanied by a significant increase in EBC adenosine in asthmatic patients (table 2). The time course of changes both in post-exercise FEV_1 and EBC adenosine showed wide individual variability, but there was a significant difference in the time course of changes both in EBC adenosine concentrations and in FEV_1 values between healthy and asthmatic subjects (fig. 3a and b).

Relationship between post-exercise FEV_1 values and exercise-induced changes in EBC adenosine

In asthmatic patients, the relationship between the exercise-induced fall in FEV_1 and changes in EBC adenosine concentration were analysed to uncover a possible effect of increased adenosine level in the development of airway obstruction (fig. 4a). Each post-exercise EBC adenosine measurement was matched with subsequent FEV_1 measurements (as outlined in fig. 1). Individual AUC values of percentage changes in FEV_1

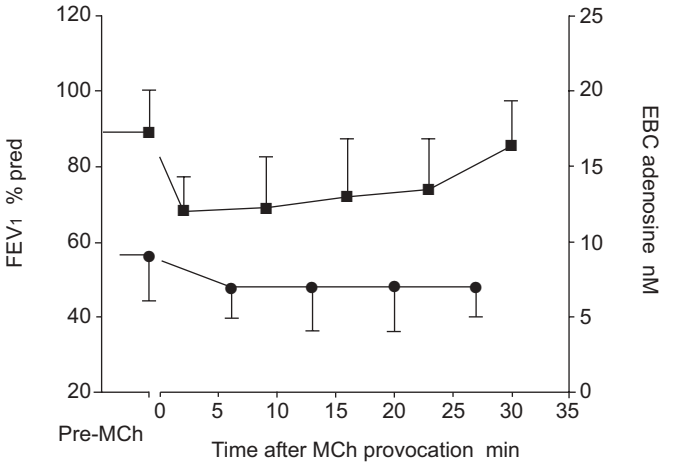

FIGURE 2. Time course of changes in forced expiratory volume in one second (FEV_1; ■) and in exhaled breath condensate (EBC) adenosine (●) in six asthmatic patients after >20% airway obstruction provoked by methacholine (MCh). Data are presented as mean±SD for n≥5 subjects.

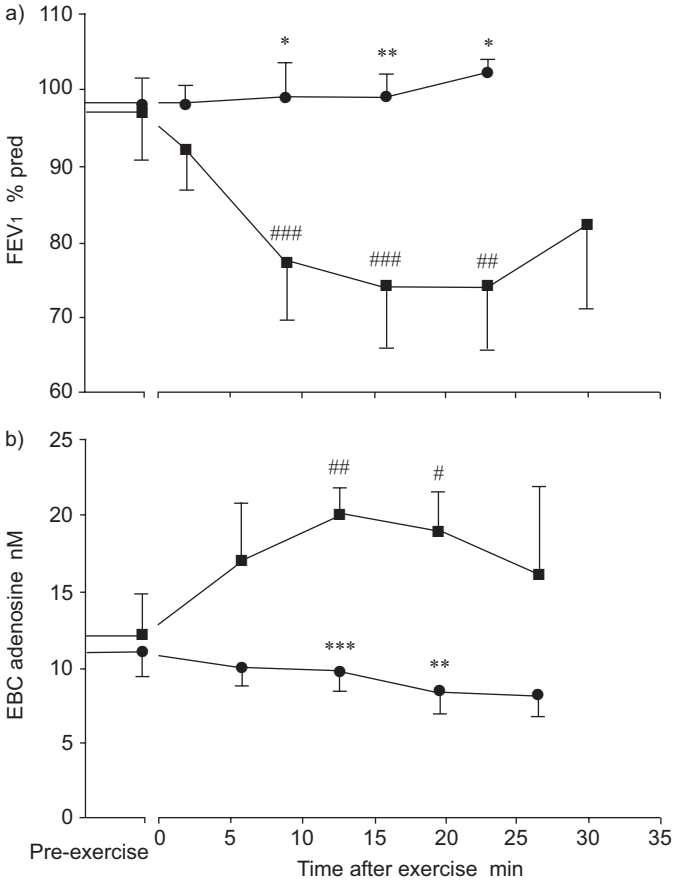

FIGURE 3. Time course of changes in post-exercise forced expiratory volume in one second (FEV_1; a) and in exhaled breath condensate (EBC) adenosine (b) in healthy control volunteers (●) and asthmatic patients (■). Data are presented as mean±SD for n≥6 subjects. Significances are expressed as the difference between controls and asthmatic patients (*) and the difference between baseline and post-exercise values (#). One symbol: $p<0.05$; two symbols: $p<0.01$; three symbols: $p<0.001$.

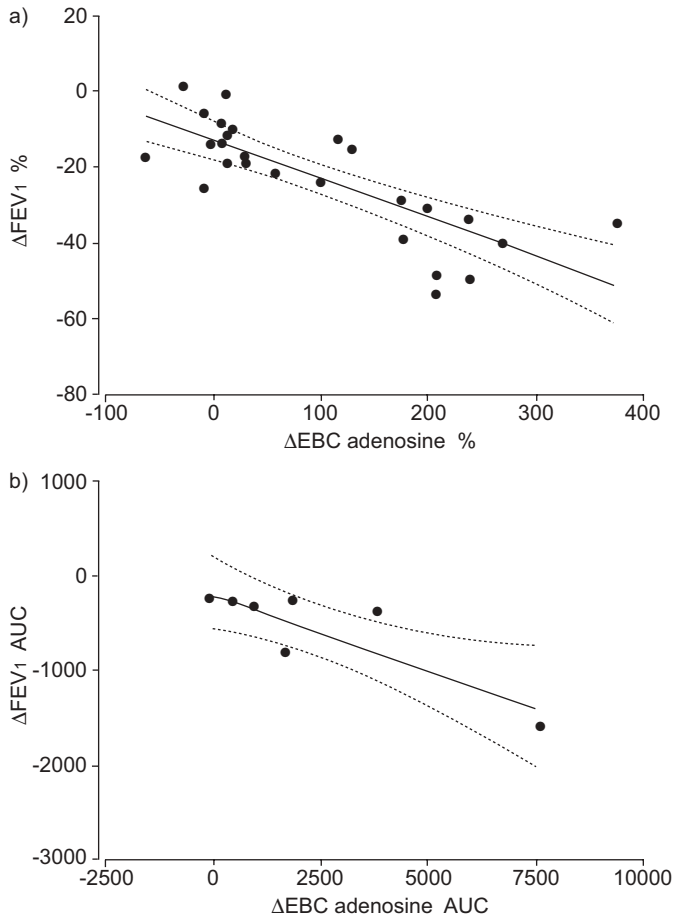

FIGURE 4. a) Relationship between post-exercise changes (Δ) in exhaled breath condensate (EBC) adenosine and fall in forced expiratory volume in one second (FEV_1) values in asthmatic patients ($r^2=0.595$; $p<0.001$). ΔFEV_1 is plotted against changes in adenosine concentrations found in EBC samples collected before the actual FEV_1 measurements. ΔFEV_1 % and ΔEBC adenosine were calculated as: (100 × (actual values–baseline values/baseline values)). b) Relationship between integral values of changes in post-exercise EBC adenosine and FEV_1 ($r^2=0.724$; $p=0.015$). ΔFEV_1 AUC: area under the curve of percentage change in FEV_1 of asthmatic patients after exercise; ΔEBC adenosine AUC: area under the curve of percentage change in EBC adenosine in asthmatic patients after exercise. ·····: 95% confidence interval; ——: linear regression.

were also plotted against that of percentage changes in EBC adenosine (fig. 4b).

DISCUSSION

In the current study, the exercise-induced changes of EBC adenosine levels in asthmatic patients with EIB and healthy volunteers were investigated. While EBC adenosine did not change following exercise in healthy subjects, there was a pronounced increase in its level during EIB in asthmatic patients, and this increase was related to the degree of bronchospasm. The present observations suggest that adenosine is present in the airways in a considerable concentration and provide further evidence for its contribution to the development of EIB.

Orally collected EBC was used to sample the airways in the current study, with the presumption that it would be suitable to reflect lower airway adenosine concentration. This is because no difference in adenosine concentrations had been found between orally collected EBC samples and those obtained through tracheostomies [16]. Furthermore, taking its dilution factor (5,000–10,000-fold) into account, the concentration of airway adenosine calculated from the current EBC values was similar to that proposed by others in bronchoalveolar lavage [23, 24]. Both calculations showed that the airway concentration of adenosine is ~50–200 μM. This local concentration is >1,000 higher than what is found in plasma (50–100 nM) [14] and is in the range of having biological activity [24]. The adenosine concentration in oral EBC samples mainly reflects adenosine arising from the lower airways/alveoli, but adenosine produced in the nasal cavities and/or the mouth may also contribute to it. It had been previously found that the adenosine concentration in saliva is approximately 10-fold higher than in orally collected EBC samples [15]. There was no difference between healthy and asthmatic subjects, and no relationship between saliva adenosine and EBC adenosine in subject-matched samples. In the absence of upper airway inflammation, the adenosine concentration of oral EBC samples remains constant, regardless of the mode of inhalation (*via* the nose or mouth) [16]. Although the previously mentioned pieces of evidence are only indirect, suggesting that oral EBC adenosine arises from the lower airways, and the possibility of contribution of the nasal cavities and the mouth cannot be excluded completely, the data warrant further investigation of adenosine as a mediator participating in airway regulation.

Previously, it was found that EBC adenosine levels were elevated in steroid naïve atopic asthmatic patients as compared with healthy volunteers, with some overlap between the two groups [15]. The baseline adenosine values of the patients selected for the present study represented the whole range of adenosine levels found in asthmatic patients, from normal low values to elevated ones without a significant difference between asthmatic and healthy subjects. The mean concentrations of adenosine were equal in healthy and asthmatic subjects (with higher variability in the patients). This finding is probably attributable to the relatively low number of patients involved in the study and the mild nature of their disease. In the group of healthy volunteers, no change in EBC adenosine concentration was observed, in contrast to the increase found in asthmatic patients after exercise. Therefore, the current authors do not think that potential changes in EBC formation/collection due to exercise have caused any important bias to the results. One may assume that an increase in EBC adenosine observed during EIB is caused by changes in airway calibre *per se*. This is unlikely, however, because the MCh challenge did not cause an elevation in EBC adenosine. Similarly, others did not find changes in EBC histamine and leukotriene levels during bronchospasm caused by MCh [25].

The exact source of adenosine during EIB was not examined. The potential source of increased adenosine release might include structural cells of the airway wall (smooth muscle cells, injured epithelial cells), inflammatory cells, including mast cells, and overactivated nerve endings [26, 27].

Although only an association was shown between adenosine and airway obstruction, not a direct causal relationship,

previous studies have suggested that whatever is the source of adenosine in the airways, an increase in its level would potentiate the airway obstruction produced by other bronchoconstrictor stimuli [28, 29].

In conclusion, the current data show that exercise-induced airway obstruction is associated with a pronounced increase in adenosine concentration in exhaled breath condensate. The time course of exhaled breath condensate adenosine tracked the time course of forced expiratory volume in one second after exercise, and the post-exercise changes in airway function and adenosine concentrations were related. These findings suggest a possible contribution of adenosine in exercise-induced airway obstruction, but a causal relationship between adenosine and exercise-induced bronchoconstriction could only be assessed by using specific adenosine receptor antagonists.

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