

Increased bronchial NO output in severe atopic eczema in children and adolescents

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Atopic children have an increased risk for asthma, which is preceded by bronchial inflammation. Exhaled nitric oxide (NO) measured at multiple exhalation flow rates can be used to assess alveolar NO concentration and bronchial NO flux, which reflect inflammation in lung periphery and central airways, respectively. Exhaled breath condensate is another non-invasive method to measure lung inflammation. The purpose of the present study was to find out if the severity of atopic eczema is associated with lung inflammation that can be observed with these non-invasive tests. We studied 81 patients (7–22 yr old) with atopic eczema and increased wheat-specific IgE (≥ 0.4 kUA/l) and no diagnosis of asthma. Exhaled NO was measured at multiple exhalation flow rates, and bronchial NO flux and alveolar NO concentration were calculated. Cysteinyl-leukotriene concentrations were measured in exhaled breath condensate. The patients were divided into two groups according to the severity of atopic eczema. Patients with severe atopic eczema had enhanced bronchial NO output as compared with patients with mild eczema (2.1 ± 0.5 vs. 0.9 ± 0.1 , $p = 0.003$). No statistically significant differences in alveolar NO concentrations were found between the groups. In the whole group of patients, the bronchial NO output correlated positively with serum eosinophil protein X ($r_s = 0.450$, $p < 0.001$), serum eosinophil cationic protein ($r_s = 0.393$, $p < 0.001$), serum total IgE ($r_s = 0.268$, $p = 0.016$) and with urine eosinophil protein X ($r_s = 0.279$, $p = 0.012$), but not with lung function. Alveolar NO concentration correlated positively with serum eosinophil protein X ($r_s = 0.444$, $p < 0.001$) and with serum eosinophil cationic protein ($r_s = 0.362$, $p = 0.001$). Measurable cysteinyl-leukotriene concentrations in exhaled breath condensate were found only in one-third of the patients, and there were no differences between the two groups. The results show that increased bronchial NO output is associated with eosinophilic inflammation and severe atopic eczema in patients without established asthma.

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Eosinophilic airway inflammation is a hallmark of the pathogenesis of asthma. Bronchial hyper-responsiveness and eosinophilic airway inflammation are found to be more common in atopic than in healthy children. However, most of the children at risk to get allergic symptoms do not develop allergic diseases by school age, and at

this moment it is not possible to predict who will develop asthma at the individual level (1, 2).

Cysteinyl-leukotrienes (Cys-LTs: LTC₄, LTD₄, LTE₄) are important mediators of inflammation and among the most potent bronchoconstrictors in asthmatic patients. Cys-LTs are released from inflammatory cells and cause increased

microvascular permeability, exudation of macromolecules and edema. They induce the influx of eosinophils into the airway mucosa, reduce ciliary motility and are associated with airway remodeling (3).

Nitric oxide (NO) is an important endogenous regulatory molecule in inflammation (4). Although the relationship between NO and the pathogenesis of asthma is not clear, it is evident that increased NO levels in exhaled breath are associated with asthma (5). Patients with atopic asthma have higher levels of exhaled NO than those with non-atopic asthma (6, 7), and exhaled NO is reduced along with therapeutic effects of inhaled glucocorticoids (5). Exhaled NO levels may also be increased before the onset of asthma symptoms as a marker of asthmatic inflammation (8), and there is some evidence suggesting that the severity of atopic disease correlates with exhaled NO concentrations (9, 10).

Exhaled NO has usually been measured at a single exhalation flow rate of 50 ml/s (11). However, the measurement of exhaled NO at multiple exhalation flow rates allows assessment of alveolar/peripheral and bronchial/central NO output separately, and the method is more sensitive to peripheral inflammation than the single exhalation flow rate method (11–13). The multiple flow rate method is based on a mathematical model, which divides the lung into two compartments, namely the alveolar/peripheral and bronchial/central compartments (14). We and others have shown that the multiple exhalation flow rate method can be used to successfully differentiate between bronchial and peripheral inflammation in asthma, COPD and alveolitis (15–18).

The aim of the present study was to investigate if the severity of atopic eczema correlates with lung inflammation measured by bronchial and alveolar NO output and by specific markers in exhaled breath condensate.

Methods

Patients

We recruited 81 atopic children and adolescents (27 girls and 54 boys) who were previously found to have increased serum levels of wheat-specific IgE (≥ 0.4 kUA/l). The children and adolescents were between 7 and 22 yr of age (mean 11.5 yr). Eleven (14%) patients had used bronchodilators and five (6%) inhaled corticosteroids periodically (none of them within 1 month before examination). Twenty-two (27%) had seasonal antihistamine treatment (none of them during the study).

The protocol was approved by the Ethics Committee of Tampere University Hospital. The children and their parents gave written informed consent before inclusion.

Protocol

A thorough pediatric physical examination was performed with special attention to possible focuses of infection, pulmonary auscultation and the state of the skin. One pediatrician (Laura Linkosalo) performed all examinations. The status of the skin was described carefully and classified without knowing laboratory values as nearly normal or dry skin (= mild eczema group) or scratched and severely eczematous skin with or without multiple eczema lesions (= severe eczema group). The examinations were carried out in September and October, within a 6-wk period, out of pollen season and before the cold period. Exhaled breath condensates were collected, and exhaled NO concentrations were measured at multiple exhalation flow rates. Spirometry was measured (Vmax Series Spirometry, SensorMedics, Yorba Linda, CA, USA) before and after salbutamol (0.3 mg if <10 yr old and 0.4 mg if ≥ 10 yr old) inhalation. The results were compared to normal values in Finnish children (19). Blood samples were drawn from antecubital vein without strain and allowed to clot for 60 min at 22°C. Serum was separated by centrifugation (1260 g 10 min at 22°C). The samples were stored at –20°C for total IgE assays, and at –70°C for other measurements. Urine was collected into clean tubes and centrifuged (2700 g 10 min at 22°C), and the samples were stored at –70°C until analysis.

Exhaled breath condensates

Exhaled breath condensates were collected (Eco-Screen Jaeger, Hoechenberg, Germany) for 15 min of tidal breathing with a nose clip. Immediately after collection, the condensate was divided into small aliquots and frozen. The samples were stored at –70°C until analysis for cysteinyl-leukotrienes.

Exhaled NO measurement

Nitric oxide concentrations in exhaled air were measured with Sievers NOA 280 analyzer (Sievers Instruments, Boulder, CO, USA) with a sensitivity of less than 1 ppb. The analyzer was calibrated daily with known NO concentration (103.00 ppm, AGA, Lidingö, Sweden) and before every subject with filtered NO free air

(zero-air filter, Sievers Instruments, Boulder, CO, USA). Exhaled NO concentration was measured at four exhalation flow rates (50, 100, 200 and 300 ml/s). Exhalation flow rates were achieved by using a computer-driven servo-system designed to maintain flow rate at a predetermined level (NOFLA device developed at the University of Tampere). Exhalation flow rate was measured in real-time with a mass flow meter. Based on the measured flow rate value, exhalation resistance was adjusted with a solenoid valve in real-time to keep exhalation flow rate fixed at desired value regardless of changes in exhalation effort. This system was found to have high capability to maintain steady exhalation flow rate (mean flow varied around the target flow ± 1 ml/s or $\pm 0.5\%$, whichever is greater) in children regardless of changes in exhalation effort. NO output (product of exhaled NO concentration and exhalation flow rate) was calculated for each subject at every flow rate. NO output was plotted against exhalation flow rate, and a regression line was set between these variables. Alveolar NO concentration and bronchial NO flux are the slope and intercept of the regression line, respectively (14). Mean correlation coefficients in the regression analysis were 0.97 ± 0.005 and 0.96 ± 0.013 in the mild and severe eczema groups, respectively. Alveolar NO concentration reflects NO dynamics in the peripheral lung (from respiratory bronchioles to alveoli), and bronchial NO flux represents NO dynamics in central conducting airways.

Blood and urine measurements

Serum eosinophil cationic protein (ECP), serum eosinophil protein X (EPX), serum myeloperoxidase (MPO) and urine eosinophil protein X were measured by radioimmunoassay using reagents from Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden. The detection limits were: ECP 1 ng/ml, EPX 1.5 ng/ml and MPO 4 ng/ml. Serum total-immunoglobulin E (total-IgE) values were determined by immunoluminometry (Bayer Corporation, Tarrytown, NY, USA).

Cytokines were measured from plasma. Interleukin-5 (IL-5) was determined with sandwich enzyme immunoassay technique by Quntikine kit, and cytokines IL-8 and IL-12 were measured by enzyme immunoassay using DuoSet ELISA Development kit (R&D Systems, Inc, Minneapolis, USA). The detection limits were 3.9 pg/ml for IL-5, 2.0 pg/ml for IL-8 and 1.4 pg/ml for IL-12. Cytokines IL-6 and IL-10 were measured by enzyme immunoassay by PeliPair reagent set (CLB, Amsterdam, The Netherlands), and the detection limit for IL-6 and IL-10 was 0.6 pg/ml.

The intra- and interassay coefficients of variation were 2.6–6.6%.

Exhaled cysteinyl-leukotrienes

Cys-LTs were measured by leukotriene C₄/D₄/E₄ enzyme immunoassay (EIA) system (Amersham Pharmacia Biotech, Buckinghamshire, England), and the detection limit was 7.5 pg/ml. Cross-reaction for LTC₄ and LTD₄ was 100%, and that for LTE₄ was 70%. The intra- and interassay coefficients of variation were <9%.

Skin prick tests

Skin prick testing was performed by Laura Linkosalo with standard procedure on the volar aspect of the forearm, using commercially available allergens of birch, cat and timothy (ALK, Allergologisk Laboratorium A/S, Hørsholm, Denmark). The positive control was histamine dihydrochloride (10 mg/ml), and physiologic saline was used as the negative control. Reactions were read at 15 min, and regarded positive if the mean diameter of the wheal was at least 3 mm.

Statistics

Data were analyzed with SPSS 10.1 program. In normally distributed data, unpaired *T*-test was used, and if the data were not normally distributed, Mann–Whitney and Kruskal–Wallis tests were applied. Spearman's correlations were calculated and frequencies were compared with chi-square test. *p*-Values less than 0.05 were considered significant.

Results

Eighty-one children and adolescents were examined. Sixty-five (80%) belonged to mild eczema group, and sixteen (20%) to severe eczema group. The allergologic and clinical characteristics of the patients are shown in Table 1.

Bronchial NO flux was higher in the severe eczema group (2.1 ± 0.5 nl/s) than in the mild eczema group (0.9 ± 0.1 nl/s; $p = 0.003$) (Fig. 1). Alveolar NO concentrations were slightly higher in the severe eczema group (3.4 ± 0.4 ppb) than in the mild eczema group (3.1 ± 0.2 ppb), but the difference was not statistically significant ($p = 0.094$) (Fig. 1). Mild eczema group was also divided into two subgroups according to the presence of allergic rhinitis. There was no difference in bronchial NO flux ($p = 0.533$) or alveolar NO concentration ($p = 0.522$) between the two subgroups.

Table 1. Allergologic and clinical data of patients with mild and severe eczema

	Mild eczema (n = 65)	Severe eczema (n = 16)
Total-IgE (mean $\pm$ s.e.m.)	1170 $\pm$ 200	4030 $\pm$ 1790
FEV1% (mean $\pm$ s.e.m.)	96.4 $\pm$ 1.7	97.6 $\pm$ 3.4
FEF ₅₀ % (mean $\pm$ s.e.m.)	90.5 $\pm$ 2.9	89.8 $\pm$ 6.8
FVC% (mean $\pm$ s.e.m.)	97.8 $\pm$ 1.5	101.4 $\pm$ 3.1
VC% (mean $\pm$ s.e.m.)	102 $\pm$ 1.5	104.2 $\pm$ 3.0
Skin prick tests* (%)		
3 positive prick tests	63	94
2 positive prick tests	18	6
1 positive prick tests	16	0
No positive prick tests	3	0
Male/female	42/23	12/4
Height (cm mean $\pm$ s.e.m.)	141.8 $\pm$ 2.1	154.3 $\pm$ 4.7
Age [years mean (range)]	10.7 (7–19)	14.8 (7–22)

*Three common allergens (birch, cat and timothy) were tested.

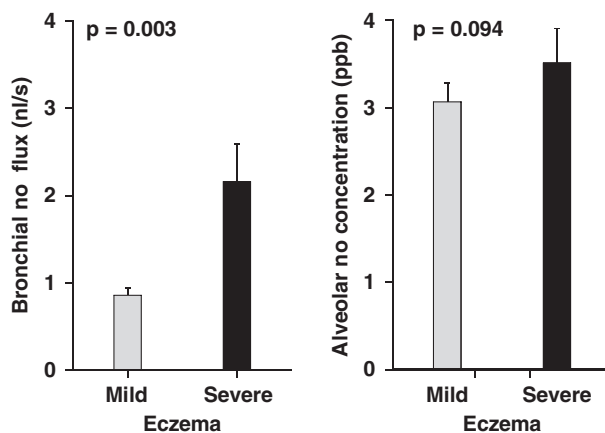


Fig. 1. Bronchial NO flux and alveolar NO concentration in patients with mild and severe eczema. Mean $\pm$ s.e.m., n = 65 in mild eczema group and n = 16 in the severe eczema group.

Exhaled NO concentrations measured at exhalation flow rates of 50, 100, 200 and 300 ml/s in subjects with mild and severe eczema are given in Table 2.

Bronchial NO flux correlated positively with serum concentrations of EPX ($r_s = 0.450$, $p < 0.001$; Fig. 2a), ECP ($r_s = 0.393$,

Table 2. Exhaled NO concentrations at different exhalation flow rates in patients with mild and severe eczema

Exhalation flow rate (ml/s)	Exhaled NO concentration (ppb)		
	Mild eczema (n = 65)	Severe eczema (n = 16)	p-value
50	20.5 $\pm$ 1.8	47.1 $\pm$ 9.3	0.003
100	11.6 $\pm$ 1.0	24.9 $\pm$ 4.6	0.004
200	7.2 $\pm$ 0.6	14.1 $\pm$ 2.4	0.003
300	6.2 $\pm$ 0.4	10.8 $\pm$ 1.7	0.009

Mean $\pm$ s.e.m.

$p < 0.001$; Fig. 2b) and IgE ($r_s = 0.268$, $p = 0.016$), and with urinary excretion of EPX ($r_s = 0.279$, $p = 0.012$). There was no correlation of bronchial NO flux to lung function as measured by spirometry or to cytokine IL-5, IL-6, IL-8, IL-10 or IL-12 concentrations in the plasma. Alveolar NO concentration correlated with serum EPX ($r_s = 0.444$, $p < 0.001$; Fig. 2c) and serum ECP ($r_s = 0.362$, $p = 0.001$; Fig. 2d) but not with urinary excretion of EPX ($r_s = 0.118$, $p = 0.292$).

In the mild eczema group, 22 (34%) children had detectable Cys-LT concentrations (> 7.5 pg/ml) in exhaled breath condensate. The corresponding value in the severe eczema group was eight patients (50%). In chi-square test, the difference between the two groups was not statistically significant ($p = 0.314$).

Spirometry results did not differ between the two groups (Table 3). Serum levels of eosinophil activation markers EPX and ECP were higher in the severe eczema group. The circulating levels of cytokines IL-5, IL-6, IL-8, IL-10 and IL-12, and the serum concentrations of neutrophil activation marker MPO were similar in the two groups (Table 3).

Discussion

In the present study, we measured lung inflammation in atopic children and adolescents without established asthma by using two recently described non-invasive methods, i.e. exhaled NO measurement with multiple exhalation flow rates and exhaled breath condensate. Bronchial NO output was increased in patients with severe atopic eczema along with serum markers of eosinophilic inflammation as compared to subjects with mild eczema. NO parameters did not correlate with lung function as measured by spirometry. Measurable cysteinyl-leukotriene concentrations (> 7.5 pg/ml) in exhaled breath condensate were found only in one-third of the patients.

Exhaled NO increased in patients with asthma and the levels decreased towards normal after treatment with anti-inflammatory steroids (5, 15, 20, 21). Recently, the pioneering long-term studies examining the use of exhaled NO to adjust the dose of inhaled glucocorticoids in the treatment in asthma were published and showed promising results (22, 23). In asthma, high levels of exhaled NO are associated with atopic asthma and correlate with markers of eosinophilia, whereas patients with non-atopic asthma have rather normal exhaled NO levels (15, 24–26).

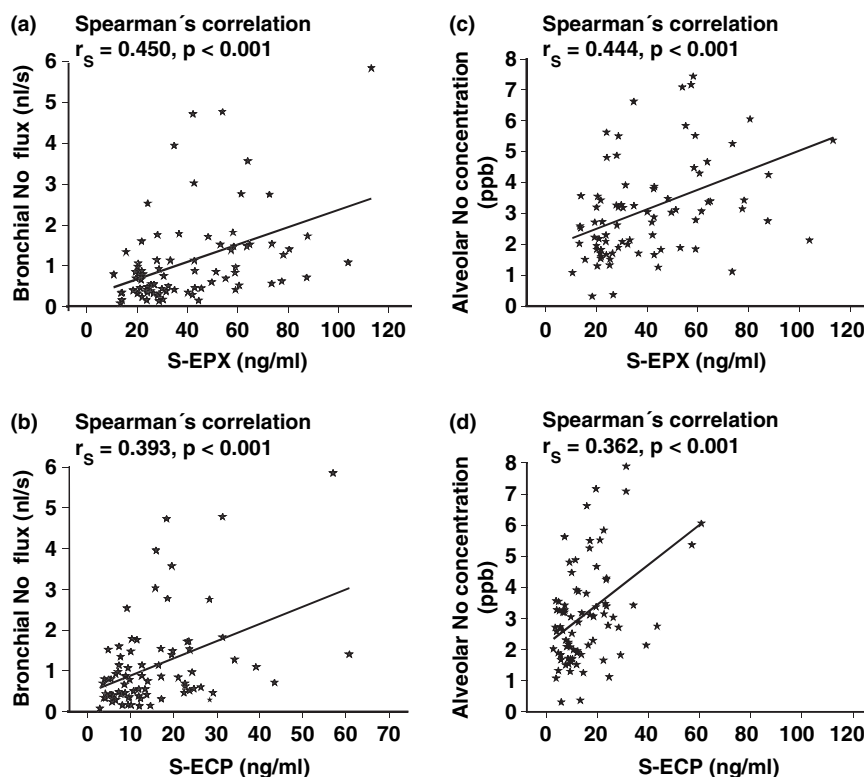


Fig. 2. Correlation between S-EPX and bronchial NO flux (a) or alveolar NO concentration (c), and correlation between S-ECP and bronchial NO flux (b) or alveolar NO concentration (d) in 81 children with atopic eczema.

Table 3. Laboratory measures and spirometry values in patients with mild and severe eczema

	Mild eczema (n = 65)	Severe eczema (n = 16)	p-value
S-EPX (ng/ml)	38.0 ± 2.8	51.0 ± 5.4	0.009
S-ECP (ng/ml)	14.4 ± 1.4	19.4 ± 3.0	0.031
S-MPO (ng/ml)	233.3 ± 11.5	270.7 ± 38.1	0.393
U-EPX (ng/ml)	574.1 ± 67.7	745.1 ± 153.0	0.270
IL-5 (pg/ml)	25.6 ± 3.2	24.5 ± 5.3	0.981
IL-6 (pg/ml)	4.5 ± 0.4	4.3 ± 1.0	0.691
IL-8 (pg/ml)	11.0 ± 4.0	3.0 ± 1.5	0.362
IL-10 (pg/ml)	29.7 ± 5.3	34.4 ± 11.6	0.873
IL-12 (pg/ml)	89.0 ± 26.3	34.2 ± 24.0	0.138

Mean ± s.e.m.

A few studies have applied the multiple exhalation flow rate method to measure exhaled NO in patients with asthma (15–18). In untreated asthmatics, bronchial NO output is increased and alveolar NO concentrations remain close to normal levels. Interestingly, bronchial NO output in patients with steroid-naïve asthma correlates with serum IgE and markers of eosinophil activation (EPX and ECP). In addition, there seems to be some subtypes of asthma in which peripheral NO output is also increased (27, 28).

The present study is one of the first investigations that have applied the multiple exhalation flow rate method to measure exhaled NO in

pediatric patients, and, to our knowledge, it is the first study on atopic patients with various degrees of severity of the disease. In the present study, we found that atopic children with clinical (eczema) and biochemical (IgE and serum markers of eosinophil activation) signs of severe atopic disease had higher bronchial NO output than children with a milder disease. Peripheral NO was at the same level in both eczema groups. In adult patients with atopic dermatitis, the highest prevalence of bronchial hyper-responsiveness and eosinophilic airway inflammation was seen in patients who were skin prick positive and had high serum IgE levels (1). Studies with pediatric patients have shown a correlation of exhaled NO concentrations measured at a single exhalation flow rate to the reactivity in skin prick tests (7, 9, 10) as well as to the standing height (10). In the present study, there was no correlation between bronchial NO flux or alveolar NO concentration and the skin prick test positivity or cumulative size of wheal diameters in the prick tests. The explanation might be that we performed skin prick tests by using only three common allergens (birch, cat and timothy), and most of the patients had positive responses in all the tests. In the present study, we did not find a correlation between NO parameters and standing height, but it may be a contributing factor explaining some variability of the values, and needs further

studies. As compared with earlier findings from our laboratory, the bronchial NO output even in the severe eczema group was somewhat lower than in adult asthmatics before the commencement of treatment with anti-inflammatory steroids (2.5 ± 0.3 nl/s) (15). In addition, peripheral NO concentrations in both eczema groups were higher than in healthy or asthmatic adults (15) or in healthy children (2.0 ± 0.8 ppb) (29) as compared with the values previously measured in our laboratory. In this connection, it is of interest that (in addition to bronchial NO output) alveolar NO concentrations were found to correlate with concentrations of eosinophil markers EPX and ECP. In the present study, all the patients were sensitized to wheat allergens. It remains unknown if sensitization to gastrointestinal allergens (as in the present study) is associated with more peripheral lung/airway inflammation than sensitization to inhaled allergen.

Non-gaseous markers of pulmonary inflammation can be non-invasively sampled by collecting exhaled breath condensate (30). Cysteinyl-leukotrienes are lipid mediators that are associated with the development of allergy and asthma, and their antagonists have been successfully used in the treatment of asthma and allergic rhinitis (3, 31). Elevated levels of cysteinyl-leukotrienes have been found in exhaled breath condensate in patients with asthma (32–34). However, Sandrini et al. (35) did not find measurable levels of cysteinyl-leukotrienes in exhaled breath condensates from patients with mild asthma. In the present study, we found detectable concentrations of cysteinyl-leukotrienes in samples from one-third of the atopic patients. There were no clear differences in cysteinyl-leukotriene concentrations between patients with severe and mild eczema, although eosinophil markers and values of bronchial NO flux separated those groups. We did not find a correlation between cysteinyl-leukotriene concentrations and IgE or markers of eosinophil activation.

At present, we know that atopy is associated with increased risk of developing asthma. In the present study, we found a connection between increased bronchial inflammation and symptomatic atopic eczema in children and adolescents. This supports the clinical impression that children who have the most severe atopic eczema are at the highest risk of developing asthma. The results suggest that bronchial NO flux in atopic individuals might have a helpful predictive value in screening bronchial inflammation and developing asthma.

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