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Bronchial and alveolar nitric oxide in exercise-induced bronchoconstriction in asthmatic children

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Abbreviation list

NO: nitric oxide

FeNO₅₀: fractional exhaled nitric oxide at the flow of 50 mL/s

J_{NO}: bronchial flux of NO

C_{alv}: alveolar concentration of NO

EIB: exercise-induced bronchoconstriction

FEF₂₅₋₇₅: Forced Expiratory Flow between 25% and 75% of Forced Vital Capacity

FEV₁: Forced Expiratory Volume in the first second

FVC: Forced Vital capacity

ΔFEF₂₅₋₇₅: fall in FEF₂₅₋₇₅ induced by exercise

ΔFEV₁: fall in FEV₁ induced by exercise

Summary

Background: Epidemiological studies have shown an association between the severity of exercise-induced bronchoconstriction (EIB) and fractional exhaled nitric oxide at the flow of 50 mL/s (FeNO₅₀). However, no study has assessed the correlation between alveolar production (C_{alv}) and bronchial flux (J_{NO}) of nitric oxide (NO) and EIB in asthmatic children.

Objective: To identify the relationship between severity of EIB and bronchial or alveolar nitric oxide.

Methods: Our group included 36 allergic children with intermittent asthma. EIB was determined by a standard exercise challenge and the severity was expressed as the maximum change in percentage from the baseline value of lung function (ΔFEV₁%, ΔFEF₂₅₋₇₅%) after exercising. A chemiluminescence analyzer at multiple flows was used to calculate FeNO₅₀, J_{NO} and C_{alv}, which reflect large airways, bronchial flux and alveolar concentration of NO, respectively.

Results: Sixteen (44.4%) children presented a $\Delta FEV_1 \geq 10\%$, eight (22.2%) had $\Delta FEV_1 \geq 15\%$ and nine children had a $\Delta FEF_{25-75} \geq 26\%$. A significant correlation was observed between severity of EIB and $FeNO_{50}$, J_{NO} and C_{alv} . EIB was significantly more severe in children sensitive to indoor allergens as compared to outdoor allergens only ($p=0.014$); those children showed also higher levels of C_{alv} ($p=0.003$) and of J_{NO} ($p=0.044$).

Conclusions and Clinical Relevance: Our results suggest that inflammation is present in the central and peripheral airways and that it is associated with the severity of EIB.

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Introduction

Several studies in asthmatics with exercise-induced bronchoconstriction (EIB) have demonstrated a heterogeneous inflammatory response at the site of the airways, suggesting that bronchoconstriction can be mirrored by several parameters, directly or indirectly reflecting the level of airway inflammation (1,2) Fractional exhaled nitric oxide at the flow of 50 mL/s ($FeNO_{50}$) is considered a surrogate marker of inflammation and oxygen stress in the lung (3), and has been studied in several asthmatic conditions including EIB.

Firstly, it has been shown that children with EIB have significantly higher baseline $FeNO_{50}$ values than healthy controls, and that baseline exhaled nitric oxide values correlate with the degree of post-exercise bronchoconstriction, suggesting that $FeNO_{50}$ may be a predictor of airway hyper-responsiveness to exercise (4). Subsequently, it was suggested that $FeNO_{50}$ measurements could possibly be used as a predictor of EIB since no subjects with pre-exercise $FeNO_{50}$ levels < 12 parts per billion (ppb) showed bronchial hyper-responsiveness to exercise, thus obviating the need for broncho-provocation testing in patients who complain of exertional dyspnea (5). This finding was later confirmed in a large group of asthmatic children. In fact, EIB could be excluded in asthmatic children with a probability of 90% for $FeNO_{50}$ levels < 20 ppb, in subjects not currently using inhaled corticosteroids, and < 12 ppb in those currently using inhaled corticosteroids (6).

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Recently it has been reported that FeNO₅₀ levels correlated better with an exercise-induced fall in FEF₂₅₋₇₅ (Δ FEF₂₅₋₇₅) than with a fall in FEV₁ (Δ FEV₁) suggesting a closer relationship between this exhaled marker of inflammation and the early stages of airflow limitation (7). In the same study, the authors also suggested that the improved performance of FEF₂₅₋₇₅ (percentage fall) might reflect a concentration of nitric oxide in peripheral airways; but this was not duly demonstrated by the measurement of exhaled nitric oxide (NO) at multiple exhalation flow rates.

The aim of the study was to evaluate the relationship between exhaled NO at different flows (8,9) response to exercise challenge, assessed at different degrees of reactivity, in order to better address the role of central and peripheral airway inflammation in EIB.

METHODS

Study Population

We recruited thirty-six Italian children diagnosed with asthma according to the American Thoracic Society guidelines (10) and followed at the out-patient clinic of the Department of Paediatrics of the University of Verona Hospital. In order to avoid the effect of therapy on nitric oxide (11), only children with intermittent asthma, and therefore not on regular medication with either inhaled corticosteroids or leukotriene receptor antagonists, were included in our study. Each child had a written therapeutic plan prescribing salbutamol and fluticasone propionate (FP) by metered dose inhaler and spacer as well as oral prednisone in case of severe exacerbation.

In order to reduce confounding factors, only atopic patients were included in the study group, since it has been previously suggested that FeNO₅₀ is significantly associated with EIB in atopic, but not in non-atopic steroid-naïve patients with asthmatic symptoms (12).

Children with upper respiratory infections, actual or in the previous month, were excluded.

Both parents and children both gave informed, written consent. The study was approved by the Verona Research Ethics Committee.

Study design

Spirometry: We recorded measurements of lung function- best of three blows according to ATS criteria (13) from full-forced vital capacity manoeuvres using an electronic spirometer (Jaeger, Master Screen IOS, Germany), calibrated before the arrival of each subject, using a 3-litre syringe (Cardinal Health Germany 234 GmbH). The FVC manoeuvres were carried out with the child standing and wearing a nose clip. Subjects were instructed to avoid the use of short-acting bronchodilators for at least 6 hours before undergoing testing.

Exercise challenge test: We used a standardized exercise test protocol (14,15). After a baseline spirometry, the children ran on an electrically driven treadmill (Technogym Run med, Gambettola, Italy) for 6 minutes. The work load required to achieve 85% of the maximum predicted value for heart rate $[220 - \text{age (y)}]$ within the first minute of exercising was obtained by adjusting the inclination and speed of the treadmill. Stable environmental conditions were maintained by laboratory air conditioning (19–21°C; relative humidity <50%) during all exercise tests. Nose clips were applied to ensure breathing through the mouth. FEV₁ and FEF₂₅₋₇₅ were measured at 1, 3, 5, 10, 15, 20 and 30 min after concluding the exercise tests (single blows). The severity of EIA was expressed as the maximum change in percentage from the baseline value of lung function ($\Delta\text{FEV}_1\%$, $\Delta\text{FEF}_{25-75}\%$) after exercising. Children whose FEV₁ fell by 10% or more were classified as having EIB (13,14). However, a more stringent cut-off level (*i.e.* a $\Delta\text{FEV}_1\% \geq 15\%$) was also used, as previously suggested in studies that evaluated the relationship between EIB and FeNO₅₀ (4,5). A positive response to the challenge evaluated by FEF₂₅₋₇₅ was defined by a $\Delta\text{FEF}_{25-75} \geq 26\%$ as has been previously suggested (16).

The thresholds for EIB severity were defined before any statistical evaluation of the data.

Exhaled NO measurement: Exhaled NO was measured immediately before the baseline spirometry and the exercise challenge with a chemiluminescence analyzer (NIOX system; Aerocrine, Stockholm, Sweden) using a single-breath on-line method according to the ERS/ATS guidelines (17). The children were instructed to exhale at multiple flows (10, 50, 100, 200 ml/s) (18); they

were aided by visual feedback starting from a flow rate of 10 ml/s and increasing to 200 ml/s, always in the same order. The linearity of the curve was automatically verified by the software of the analyzer (r^2). At each expiratory flow ($\dot{V}_E$), expressed in ml/s, J_{NO} was automatically calculated as $J_{NO} = FeNO_{50} \times \dot{V}_E$. As shown by Tsoukias and colleagues (8), the slope of this regression line is C_{alv} and the intercept is J_{NO} .

Background NO was measured and was always less than 100 ppb.

Allergy testing: All the subjects were given prick tests for common allergens (indoor: mites, cat, dog; outdoor: grass pollen, *Parietaria*, tree pollens, molds). A wheal diameter of 3 mm or more over the negative control value was considered a positive result.

Statistical Analysis

The sample size was calculated for testing the difference in C_{alv} levels for subjects with $\Delta FEV_1\% > 10\%$ using the method of Rahardja and Zhao (19) and considering an effect size $\pi = 0.75$, a significance level $\alpha = 5\%$ and a power $1-\beta = 80\%$.

Since data were not normally distributed they are summarised by medians and interquartile ranges (1st to 3rd quartiles) and analysed by nonparametric methods.

Wilcoxon rank-sum test was used to test the equality of distributions of a continuous variable between two groups (Table 2 and 3).

The degree of association between $FeNO_{50}$, C_{alv} , J_{NO} and pulmonary function after removing the effect of potential confounders (gender, age, type of sensitivity - indoor and outdoor vs. only outdoor) was quantified using partial correlations (20). These association were also measured by Spearman's rank correlation. The effect of outliers and influential observations on estimated correlations was evaluated first identifying critical sample units by the leverage vs. squared-residual plot and then re-estimating correlations without these units.

Calculation of the area below the Receiver Operating Characteristic (ROC) curve was done by nonparametric ROC analysis and the hypothesis of equal areas below two ROC curves from the same sample was tested using the method described by DeLong et al. (21).

Two-sided p-values < 0.05 were considered statistically significant.

All analyses were done using Stata software (StataCorp. 2007. Stata Statistical Software: Release 10. College Station, TX, USA: StataCorp LP).

RESULTS

Thirty-six asthmatic children (median age = 10, range = 5 - 15 years) were enrolled. Personal characteristics and baseline spirometry values are reported in Table 1. No child had received FP in the previous four weeks or systemic steroids in the previous six months.

For all the children who participated in our study, the curve of NO output at the different flow rates fit a linear model with a mean ($\pm$ SD) correlation $r^2 = 0.95 \pm 0.05$.

The number of subjects with a positive exercise challenge, medians and interquartile ranges of nitric oxide measurements (FeNO_{50} , J_{NO} , and C_{alv}) and their associations with response to exercise testing are reported in Table 2. As one can see, with the loose cut-off level for a positive response to exercise challenge (*i.e.* $\Delta\text{FEV}_1 \geq 10\%$), responders compared to non-responders showed significantly higher values of C_{alv} and J_{NO} , while FeNO_{50} just failed to be significantly higher in responders. When the more stringent cut-off level (*i.e.* $\Delta\text{FEV}_1 \geq 15\%$) was considered, FeNO_{50} and J_{NO} were significantly higher, whereas C_{alv} just failed to be so. When a $\Delta\text{FEF}_{25-75} \geq 26\%$ was used as the cut-off for a positive response, all the different nitric oxide parameters were significantly higher in patients with a positive exercise challenge. All the subjects with a $\Delta\text{FEV}_1 \geq 15\%$ also had a $\Delta\text{FEF}_{25-75} \geq 26\%$ and only one child with $\Delta\text{FEF}_{25-75} \geq 26\%$ showed a $\Delta\text{FEV}_1 = 13.3\%$.

Figure 1 shows ROC curves of FeNO_{50} , J_{NO} and C_{alv} for two EIB cut-offs ($\Delta\text{FEV}_1 \geq 10\%$, $\Delta\text{FEV}_1 \geq 15\%$), that is sensitivity (true positive rate) vs. 1-specificity (false positive rate). The predictive power of FeNO_{50} , J_{NO} and C_{alv} was estimated using areas under ROC curves. For C_{alv} the

estimated areas are 0.77 (95% CI = 0.61 to 0.93) and 0.73 (95% CI = 0.51 to 0.95), for J_{NO} areas are 0.78 (0.60 to 0.95) and 0.82 (0.68 to 0.96) and for $FeNO_{50}$ are 0.69 (0.50 to 0.88) and 0.78 (0.59 to 0.97). No statistical differences between the predictive power of these variables were found. These results show that $FeNO_{50}$, J_{NO} and C_{alv} have a fairly/good ability to discriminate positive response to exercise challenge.

The relationships between $\log_{10}(C_{alv})$ and exercise challenge [$\log_{10}(10+\Delta FEV_1)$ and $\log_{10}(10+\Delta FEF_{25-75})$] are shown in Figures 2 a) and b). A positive, moderate, statistically significant correlation between $\log_{10}(10+\Delta FEV_1)$ and $\log_{10}(C_{alv})$ was found: Spearman's $\rho = 0.38$, 95% CI = 0.04 to 0.64, $p = 0.031$ and partial correlation $r = 0.49$, $p = 0.006$. A similar result was found for the relationship between $\log_{10}(10+\Delta FEF_{25-75})$ and $\log_{10}(C_{alv})$: $\rho = 0.46$, 95% CI = 0.14 to 0.69, $p = 0.030$ and $r = 0.44$, $p = 0.011$.

The relationships between exercise challenge and $\log_{10}(J_{NO})$ are shown in Figures 2 c) and d). A positive, moderate, statistically significant correlation between $\log_{10}(10+\Delta FEV_1)$ and $\log_{10}(J_{NO})$ was estimated: $\rho = 0.48$, 95% CI = 0.14 to 0.71, $p = 0.007$ and $r = 0.54$, $p = 0.003$. A comparable result was found when investigating the relationship between $\log_{10}(10+\Delta FEF_{25-75})$ and $\log_{10}(J_{NO})$: $\rho = 0.43$, 95% CI = 0.10 to 0.67, $p = 0.013$ and $r = 0.40$, $p = 0.027$.

The estimated partial correlation between $\log_{10}(FeNO_{50})$ and $\log_{10}(10+\Delta FEV_1)$ was $r = 0.41$, $p = 0.017$, with Spearman's $\rho = 0.36$, 95% CI = 0.04 to 0.62, $p = 0.031$, while between $\log_{10}(FeNO_{50})$ and $\log_{10}(10+\Delta FEF_{25-75})$ partial correlation was $r = 0.34$, $p = 0.048$, with Spearman's $\rho = 0.35$, 95% CI = 0.02 to 0.61, $p = 0.037$. See figures 2 e) and f).

The above correlations were not significantly influenced by outliers and values at the extremes of the covariates range.

According to admission criteria, all the children were sensitive to one or more allergens. Six subjects (16.7%) had SPT positive responses for indoor and outdoor allergens, while the remaining children were sensitive only to outdoor allergens. A comparison between type of sensitivity (indoor and outdoor vs. only outdoor) and the different study parameters are shown in Table 3. As one can

see, EIB was significantly more severe in subjects who were sensitive to indoor and outdoor allergens.

DISCUSSION

In the present study we confirmed the findings of previous studies that have shown a significant correlation between FeNO_{50} and the severity of EIB (4-6). In addition, we observed a significant relationship between EIB, J_{NO} and C_{alv} .

An analysis of the different NO parameters measured when the children were grouped according to a cut-off value of $\Delta\text{FEV}_1 \geq 10\%$ suggested that J_{NO} and C_{alv} could be slightly more discriminative than FeNO_{50} . This result is in keeping with the fact that the sample size of this study was calculated for testing the difference in C_{alv} levels for subjects with $\Delta\text{FEV}_1\% > 10\%$.

When children were grouped according to the $\Delta\text{FEV}_1 \geq 15\%$ cut-off level, only FeNO_{50} values were higher than those observed for the lower cut-off point, while patients with $\Delta\text{FEV}_1 \geq 10\%$ or $\Delta\text{FEV}_1 \geq 15\%$ had similar levels of J_{NO} and C_{alv} . The same was true for $\Delta\text{FEF}_{25-75} \geq 26\%$. These findings suggest that peripheral lung inflammation may facilitate the onset of EIB.

J_{NO} and C_{alv} values observed in our patients with EIB are higher than those reported in healthy children (18), further supporting the concept that NO could be a stimulatory factor during exercise (22) and that it exerts its action both in proximal and distal airways. In fact, we also found a moderate relationship between FeNO_{50} and C_{alv} (Spearman's $\rho = 0.54$, 95% CI = 0.26 – 0.74, $p < 0.001$), which may confirm that inflammation is distributed throughout lung parenchyma also in children with intermittent asthma. On the other hand, it may simply reflect a back diffusion of NO from airways to alveolar compartments (23). Whether distally produced or back diffused, the significant correlation of C_{alv} with ΔFEV_1 supports the finding that nitric oxide is more closely related to the prolonged airway narrowing phase, measured by the area below the curve of the percentage fall in FEV_1 , rather than to maximal airway narrowing (ΔFEV_1) evoked by exercise (24). In this regard, it has been shown that excessive production of NO is related to increased

airway vascular permeability proportional to the severity of EIB (25). Thus airway narrowing after exercise in asthmatic subjects is considered to be, at least in part, due to bronchial micro-vascular phenomena, such as vascular engorgement and plasma leakage. When this occurs in the distal airways, it may have more prolonged consequences in relation to some physiological difference in the liquid lining the small and large airways. In fact, only in the small airways does this liquid have the characteristics of a surfactant that lowers surface tension and protects the small airways from closing at low lung volumes on expiration (26). If the normal surfactant is replaced by serum or mucus, which have higher surface tensions, the small airways could become unstable and collapse (27).

Finally, the results of our study confirm that ΔFEV_{25-75} can be used to evaluate the response to exercise and that it reflects the severity of the response and has the best correlation with eosinophilic inflammation as reflected by NO output. This is in agreement with the findings that a low FEF_{25-75} value is a risk factor for the persistence of respiratory symptoms in asthmatics (28) and that wheezing is associated more often with abnormal FEF_{25-75} values than with abnormal FEV_1 or PEF in asthmatic children (29).

To avoid the potential interference of asthma medication, our study involved a specially selected study population - atopic children with intermittent asthma. These patients have been shown to represent a particular subgroup of asthmatic patients, particularly those with intermittent but potentially severe asthma, especially when sensitive to indoor allergens (30). A positive reaction to indoor allergens has been shown to be significantly associated with the occurrence of more severe asthma (31,32), and this was clearly the case in our patients. More severe EIB and a trend towards higher levels of different nitric oxide parameters, particularly for J_{NO} and C_{alv} , have been in fact found in subjects sensitive to perennial allergens. A previous study on a large number of adult asthmatics showed higher levels of nitric oxide parameters in patients allergic to cats (33).

Our results suggest that NO production is high throughout the lungs and, though eosinophilia is the not the only determinant of variations in $FeNO_{50}$ values, eosinophilic inflammation is commonly

considered to be the major contributor. Indeed, the children who participated in our study were steroid naive, and it is actually in this group that eosinophilic inflammation is most closely related to FeNO₅₀. However, given the young age of the population, it was not ethical to include any pathological study to support this.

In atopic children with asthma, the response to montelukast as assessed by FENO concentrations is affected by allergen exposure (34). The relationships between LTRA response in children with EIB and baseline FENO should be established. It is also worth studying the relationships between EIB severity and respiratory inflammation using other techniques including measurement of leukotriene B4 in exhaled breath condensate (35, 36) and electronic nose (37).

In conclusion, our results allow us to posit that inflammation of both peripheral and central airways, as documented by exhaled NO at different flows, is associated with the severity of EIB also in patients with intermittent asthma, particularly those sensitive to indoor allergens.

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The authors have no conflict of interest to declare.

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Tables

Table 1. Age, sex and baseline lung function test results for subjects enrolled in the study: median (IQR).

Characteristics	Data
Number of subjects	36
Age, y	10 (8 - 11)
Sex (n. female, %)	9 (25%)
Baseline FeNO ₅₀	16.2 (8.8 – 27.3)
FVC (L)	2.4 (2 – 2.9)
FVC (%)	103.5 (91.8 – 113.8)
FEV ₁ (L)	1.9 (1.7 – 2.4)
FEV ₁ (%)	102.9 (90.0 – 110.7)
FEV ₁ /FVC (%)	98.5 (90.9 – 104.0)
FEF ₂₅₋₇₅ (L)	1.9 (1.5 -2.5)
FEF ₂₅₋₇₅ (%)	78.0 (59.8 - 87.9)

Table 2. Association between response to exercise testing and baseline levels of NO output: median, interquartile range (1st to 3rd quartile) and p-value of independence test (Wilcoxon rank-sum test).

	ΔFEV_1			ΔFEV_1			ΔFEF_{25-75}		
	$\geq 10\%$	$< 10\%$	<i>p</i>	$\geq 15\%$	$< 15\%$	<i>p</i>	$\geq 26\%$	$< 26\%$	<i>p</i>
Subjects (n., %)	16 (44.4)	20 (55.6)		8 (22.2)	28 (77.8)		9 (25.0)	27 (75.0)	
FeNO₅₀ (ppb)	24.4 (11.4 - 46.4)	14.6 (8.0 - 19.6)	0.052	31.8 (18.4 - 53.8)	14.6 (7.3 - 23.9)	0.018	42.8 (19.9 - 59.2)	14.6 (7.1 - 22.8)	0.004
J_{NO} (nl/min)	56.9 (41.9 - 95.3)	28.5 (20.4 - 38.5)	0.005	56.9 (52.9 - 116.2)	29.2 (20.4 - 53.7)	0.006	58.4 (53.5 - 97.2)	28.5 (20.3 - 52.3)	0.002
C_{alv} (ppb)	6.8 (3.2 - 10.0)	3.7 (1.9 - 4.4)	0.006	6.8 (3.2 - 11.5)	3.9 (2.0 - 4.7)	0.052	6.4 (3.4 - 10.9)	3.8 (2.0 - 4.8)	0.042

Abbreviations: FeNO₅₀= fraction of exhaled NO; J_{NO}= bronchial flux of NO; C_{alv}= alveolar concentration.

Table 3. Comparison between type of sensitivity (indoor and outdoor vs. only outdoor), severity of EIB and levels of nitric oxide parameters: median, interquartile range (1st to 3rd quartile) and p-value of independence test (Wilcoxon rank-sum test).

	Allergen: type of sensitivity		
	Indoor and outdoor	Only outdoor	<i>p</i> -values
Subjects (n., %)	6 (16.7)	30 (83.3)	
ΔFEV₁	13.5 (10.0 – 36.6)	7.1 (2.1 – 12.1)	0.014
ΔFEF₂₅₋₇₅	30 (7.0 – 49.2)	13.7 (3.8 – 24.9)	0.047
FeNO₅₀ (ppb)	23.9 (16.1 - 48.3)	14.9 (8.5 - 25.0)	0.269
J_{NO} (nl/min)	74.8 (38.0 - 103.1)	31.9 (20.4 - 55.4)	0.044
C_{alv} (ppb)	8.2 (6.4 - 12.0)	3.7 (2.0 - 4.6)	0.003

Abbreviations: FeNO₅₀= fraction of exhaled NO; J_{NO}= bronchial flux of NO; C_{alv}= alveolar concentration.

Figure legends

Figure 1: ROC curves investigating the predictive power of C_{alv} [graphs a) and b)], J_{NO} [graphs c) and d)] and FeNO_{50} [graphs e) and f)] for two EIB cutoffs ($\Delta\text{FEV}_1 \geq 10\%$ and $\Delta\text{FEV}_1 \geq 15$). Estimated areas under ROC curves (AU-ROC) are reported on graphs, together with 95% confidence intervals. Cutoff values of C_{alv} , J_{NO} and FeNO_{50} are plotted on corners of ROC curves.

Figure 2: Scatter plots showing the relationships between logarithmic values of C_{alv} [graphs a) and b)], J_{NO} [graphs c) and d)], FeNO_{50} [graphs e) and f)] and $\log_{10}(\Delta\text{FEV}_1)$, $\log_{10}(\Delta\text{FEF}_{25-75})$. Partial correlation r and statistical significance p are shown.



