

Both inflammation and remodeling influence nitric oxide output in children with refractory asthma

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Background: Exhaled nitric oxide can be used to monitor airway inflammation in asthma. We hypothesized that the strong link between nitric oxide and inflammation may obscure a weaker link with airway remodeling.

Objective: The aim of this study was to determine whether airway remodeling influenced exhaled nitric oxide in 28 asthmatic children (median age [25th-75th], 11 [10-14] years old) with refractory asthma defined as airflow limitation and/or exacerbations despite high-dose inhaled steroids.

Methods: Multiple-flow analysis of exhaled nitric oxide was used to correlate alveolar nitric oxide concentration and maximal conducting airway nitric oxide output to pulmonary function tests, bronchoalveolar lavage, and bronchial biopsy findings.

Results: Nitric oxide measurements were related to inflammation and T_H1/T_H2 balance, that is, subepithelial eosinophilic infiltration and eosinophilic cationic protein and IFN- γ /IL-4 ratio in bronchoalveolar lavage fluids. Nitric oxide measurements were also correlated with several parameters of airway remodeling: alveolar nitric oxide concentration with TGF- β in bronchoalveolar lavage fluid ($r = 0.42$, $P = .03$) and maximal conducting airway nitric oxide output with reticular basement membrane thickness ($r = 0.61$, $P = .0007$) and tissue inhibitor of matrix metalloproteinases 1/matrix metalloproteinase 9 ratio in bronchoalveolar lavage fluid ($r = 0.43$, $P = .04$). Moreover, alveolar nitric oxide concentration was correlated with MEF₂₅₋₇₅ ($r = 0.60$, $P = .02$).

Conclusions: These findings suggest that both subacute inflammation and remodeling influence nitric oxide output in refractory asthma. (*J Allergy Clin Immunol* 2004;113:252-6.)

Key words: Bronchial biopsy, reticular basement membrane, eosinophil, exhaled nitric oxide

Refractory asthma has been defined on the basis of medication requirements, asthma symptoms, frequency of asthma exacerbations, and degree of airflow limitation.¹ The clinical presentation and course of refractory

Abbreviations used

BAL:	Bronchoalveolar lavage
BB:	Bronchial biopsy
ECP:	Eosinophilic cationic protein
FA _{NO} :	Alveolar NO concentrations
FE _{NO} :	Exhaled nitric oxide
MMP-9:	Matrix metalloproteinase 9
NO:	Nitric oxide
Q _{MAX, CA} :	Maximal NO output from the conducting airways
RBM:	Reticular basement membrane
TIMP-1:	Type 1 tissue inhibitor of matrix metalloproteinases

asthma vary across a broad spectrum, extending from the patient with highly labile airways but normal lung function to the patient with partly irreversible obstruction. This heterogeneity has been ascribed to differences in pathologic patterns. Bronchial biopsy specimens in refractory asthma show various combinations of active inflammation, with eosinophilic and occasionally neutrophilic infiltration, and of structural changes, consisting of epithelial damage, reticular basement membrane (RBM) thickening, and muscle hyperplasia. The ability to identify patient subsets among adults and children with refractory asthma might improve patient management.² Pathologic studies cannot be relied on to identify subsets, however, as routine bronchoscopy would not be appropriate in these patients.

An alternative approach may be noninvasive measurement of exhaled nitric oxide (FE_{NO}). Payne et al recently demonstrated a positive relation between FE_{NO} levels and the degree of eosinophilic inflammation on bronchial biopsy specimens in children with refractory asthma.³ In addition, nitric oxide (NO) output from the conducting airways may be associated with the severity of airflow limitation in steroid-treated adult asthmatic patients.⁴ Evidence pointing to a relation between the degree of fixed air flow obstruction and the presence of subepithelial collagen deposition has been reported,⁵ suggesting that NO output may be associated with airway remodeling. Separating conducting-airway NO output from alveolar NO requires multiple-flow analysis of FE_{NO}. Both theoretic⁶ and clinical studies⁷ have demonstrated that multiple-flow analysis of FE_{NO} is reliable

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for separately estimating alveolar NO concentrations (FA_{NO}) and maximal NO output from the conducting airways ($Q_{MAX, CA}$).

The aim of this study was to investigate whether these NO parameters are useful for evaluating both the degree of airway inflammation and the degree of airway remodeling in children with refractory asthma. The reference standard methods were bronchoalveolar lavage (BAL) and bronchial biopsy (BB). Airway inflammation was assessed on the basis of eosinophil counts in BBs and eosinophilic cationic protein (ECP) levels in BAL fluids. The ratio of IFN- γ over IL-4 (IFN- γ /IL-4) was used to evaluate the T_H1 - T_H2 balance. Airway remodeling was evaluated in BBs on the basis of RBM thickness. In BAL fluids, 2 parameters were used to evaluate airway remodeling: levels of TGF- β , which is a profibrotic growth factor,⁸ and the ratio of type 1 tissue inhibitor of matrix metalloproteinases (TIMP-1) over matrix metalloproteinase 9 (MMP-9), which is one of the ratios that characterizes the inhibitor/proteinase balance governing extracellular matrix deposition.

METHODS

Patients

Children with refractory asthma were included prospectively and investigated by fiberoptic bronchoscopy. The diagnosis of asthma was based on clinical symptoms and history of reversible airflow limitation. In accordance with the recent ATS workshop, refractory asthma was defined as presence of the major criterion, for example, need for high-dose inhaled corticosteroids and at least 2 of the following 5 minor criteria: (1) requirement for daily long-acting β -agonist or leukotriene antagonist in addition to inhaled corticosteroids; (2) asthma symptoms requiring short-acting β -agonist use on a daily or nearly daily basis; (3) persistent airway obstruction ($FEV_1 < 80\%$ predicted); (4) 1 or more emergency care visits for asthma per year; (5) 3 or more oral steroid "bursts" per year.¹

Children and their parents underwent a full conventional assessment. BAL and BB were justified to exclude alternative diagnosis and assess airway inflammation to optimize asthma treatment.^{9,10} Children were evaluated after at least 15 days without acute exacerbations to ensure that the explorations reflected their basal status.

Ethics approval

Written consent to participation in the study was obtained from the parents of all the study children. The ethics committee (CCP-PRB) of Necker-Enfants Malades, University hospital, Paris, approved this study.

Pulmonary function tests

Spirometry measurements and flow-volume curves were obtained with the use of a spirometer (MedGraphics, PF/DX 1085D, St Paul, Minn). Three technically acceptable measurements were obtained for each test. The values were expressed as percentages of predicted values.

Fiberoptic bronchoscopy

Flexible bronchoscopy and BAL were performed under deep sedation, as previously described.¹¹ Cupped forceps were used to take 4 to 6 endobronchial biopsy specimens from segmental or subsegmental bronchi of the lower lobes.

Lavage and tissue processing

BAL fluids were immediately placed on ice and centrifuged. Aliquots of centrifuged BALs were stained using eosin methylene blue (RAL, Paris, France). Biopsy specimens were fixed in 10% formaldehyde and embedded in paraffin blocks. Two independent observers blindly assessed inflammatory cells in the epithelium and subepithelial area (the area beneath the epithelial basement membrane). The interobserver variation was 8%. Results were expressed as the number of cells per millimeter of RBM length and number of cells per millimeter squared of submucosal area. RBM thickness was determined as the average of 20 measurements in sections where the epithelium was oriented in such a way that measurement artifacts did not occur.¹²

Evaluation of mediator concentrations

IL-4, IFN- γ (Diacclone, Besançon, France), and total TGF- β , MMP-9, and TIMP-1 (R & D Systems Europe, Lille, France) were measured by means of enzyme-linked immunosorbent assays, as recommended by the manufacturers, and ECP levels were assessed by means of a competitive radioimmunoassay (Pharmacia and Upjohn Diagnostics, Uppsala, Sweden). Concentrations of soluble mediators were run in duplicate.

NO measurements

Technical aspects have been reported previously.⁷ Briefly, 4 to 6 FE_{NO} values were obtained at several expiratory flow rates between 50 and 200 mL/s. FE_{NO} was measured using a chemiluminescent nitric oxide analyzer (EVA4000, Seres, Aix en Provence, France). NO measurements were done as recommended by ATS guidelines.¹³ At each expiratory flow (V), NO output was calculated as $Q_{NO} = FE_{NO} \times V \times 0.06$, where Q_{NO} was expressed in nL/min, FE_{NO} in ppb, and V in mL/s; 0.06 was a unit-correcting factor.

As shown by Tsoukias et al,⁶ the slope of this regression line is the alveolar NO concentration (FA_{NO}) and the intercept to zero flow is the maximal conducting airway NO output ($Q_{MAX, CA}$).

Statistical analysis

Data are expressed as median and 25th and 75th percentiles. Correlations were evaluated by means of a nonparametric test, for example, the Spearman rank correlation coefficient. A multiple regression analysis was performed by using $Q_{MAX, CA}$ as the dependent variable and RBM thickness and subepithelial eosinophils as independent variables.

RESULTS

Patients, pulmonary function testing

Twenty-eight children (16 boys and 12 girls) were enrolled (median age, 11 years; range, 10 to 14 years). All had asthma since early childhood and were atopic, as defined by one or more positive skin tests. All were receiving beclomethasone (1000 μ g/d) or equivalent (major inclusion criterion) and a long-acting β -agonist (22 children) or leukotriene antagonist (6 children) (first minor inclusion criterion). The second minor criteria were (1) persistent airway obstruction defined as $FEV_1 < 80\%$ ($n = 17$), (2) asthma symptoms requiring several oral steroid "bursts" per year and one or more emergency care visits for asthma per year ($n = 11$). Almost all the children had at least peripheral airway obstruction ($MEF_{25-75} < 65\%$). Postbronchodilator FEV_1 did not increase $>10\%$ of baseline value, whatever the inclusion criteria.

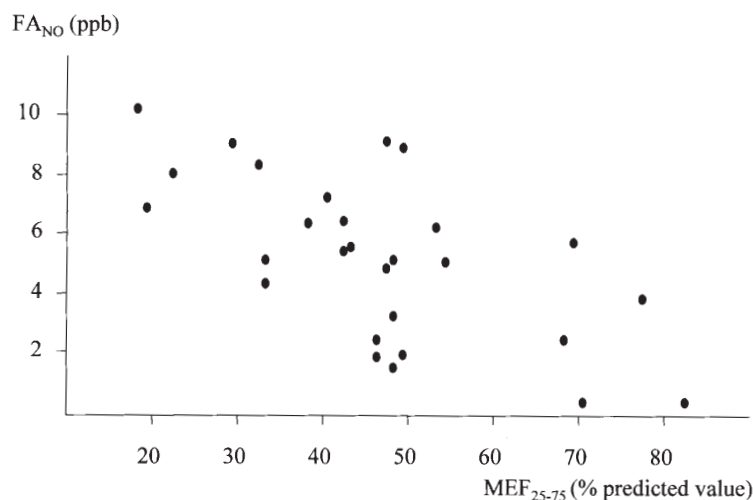


FIG 1. Relation between distal inflammation and airflow limitation. Alveolar NO concentration (FA_{NO}) and percentage of predicted value of MEF_{25-75} were correlated.

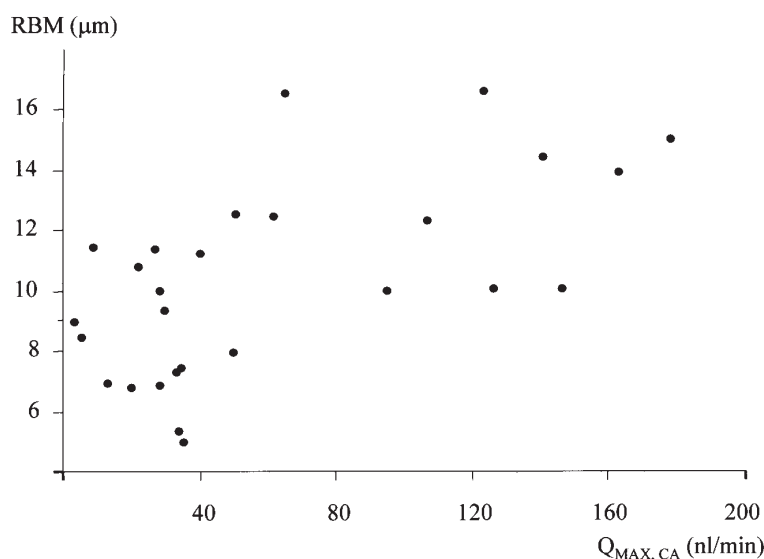


FIG 2. Relation between NO output from bronchial tree and airway remodeling. Maximal NO output from conducting airways ($Q_{MAX, CA}$) was correlated with reticular basement membrane (RBM) thickness ($r = 0.61$, $P = .0007$).

Bronchoalveolar lavage and bronchial biopsy

No bacteria $>10^3$ colony-forming units/mL were identified, and immunofluorescence did not reveal any virus in BAL fluid. BAL and BB findings were not different in regard to the second minor inclusion criteria, that is, low FEV_1 or frequent clinical exacerbations, and in regard to bronchodilator treatment, that is, long-acting bronchodilator or leukotriene antagonist. Table 1 reports the characteristics of the population.

Relation between NO measurements and lung function test

A significant relation ($r = 0.60$, $P = .02$) between FA_{NO} and MEF_{25-75} was observed (Fig 1).

Relation between NO measurements and markers for inflammation

FA_{NO} was positively correlated with ECP ($r = 0.67$, $P = .004$) and negatively correlated with $IFN-\gamma/IL-4$ ($r = -0.40$, $P = .04$). $Q_{MAX, CA}$ was correlated with subepithelial eosinophilic infiltration ($r = 0.40$, $P = .04$).

Relation between NO measurements and remodeling parameters

Both FA_{NO} ($r = 0.42$, $P = .03$) and $Q_{MAX, CA}$ ($r = 0.41$, $P = .04$) were correlated with $TGF-\beta_1$ in BAL fluids. $Q_{MAX, CA}$ was correlated with RBM thickness ($r = 0.61$, $P = .0007$, Fig 2) and TIMP-1/MMP-9 ($r = 0.43$, $P = .04$). To check that the relation between RBM

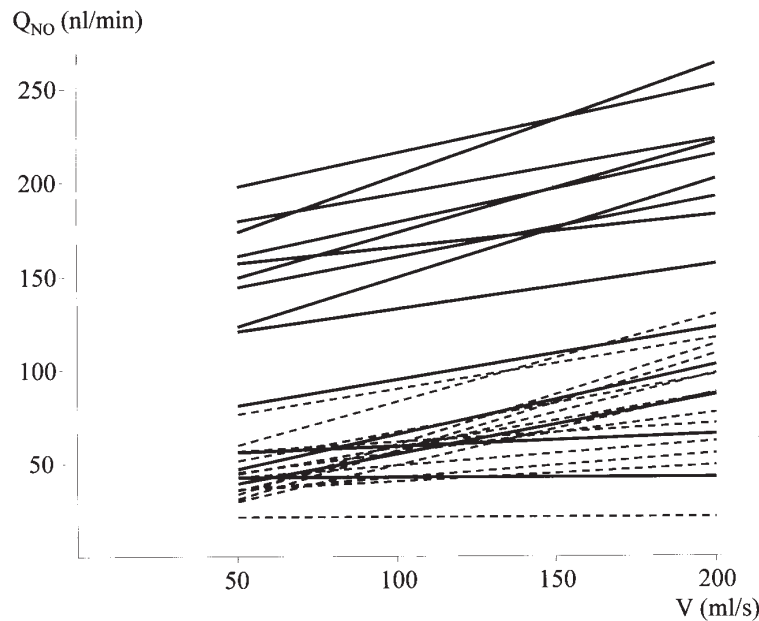


FIG 3. Individual relations between NO output and expiratory flow rate in 28 children. Slope of each individual linear relation represents alveolar NO concentration (FA_{NO}); intercept represents maximal NO output from conducting airways ($Q_{MAX, CA}$). Plain lines represent linear relation in children with RBM $\geq 9.9 \mu m$ (mean $Q_{MAX, CA}$: 47.2 ± 45 nL/min); dashed lines represent linear relation with RBM $< 9.9 \mu m$ (mean $Q_{MAX, CA}$: 110 ± 71 nL/min).

thickness and $Q_{MAX, CA}$ was not explained by an effect of eosinophil infiltration on NO production, we performed a multiple regression analysis. The significant multiple regression ($r = 0.70$, $P < .0005$) between $Q_{MAX, CA}$ as the dependent variable and RBM thickness and subepithelial eosinophils as independent variables allowed us to observe a correlation between RBM thickness and subepithelial eosinophil count. Fig 3 illustrates the 28 individual relations between NO output and expiratory flow rate according to the median of RBM thickness ($9.9 \mu m$). Among the 14 children with RBM thickness $< 9.9 \mu m$, 7 had low FEV_1 ($< 80\%$), and among the 14 children with RBM $> 9.9 \mu m$, 10 had low FEV_1 .

DISCUSSION

In children with asthma, bronchoscopy, and BB are appropriate only to clarify the diagnosis and to assist in treatment decisions for patients with poorly controlled disease. The patients in this study were children with refractory asthma, for example, with frequent symptoms and/or airflow obstruction despite maximal conventional therapy. We examined relations between multiple-flow measurements of exhaled NO and evidence of airway inflammation or remodeling obtained by bronchoscopy and BB. Our objective was to determine whether alveolar NO concentration and conducting airway NO output could be used as surrogate markers for inflammation and remodeling, thus obviating the need for BB and BAL.

The main finding from this study is that NO parameters were significantly correlated with both airway

TABLE I. Characteristics of the population

	Median [25 th -75 th]
Age	11 [9.8-13.7]
Duration of asthma, years	9.6 [7.8-12.8]
FEV_1	76.5 [69.5-84.5]
MEF_{25-75} (%)	50 [44-63]
FA_{NO} (ppb)	4.9 [2.5-6.7]
$Q_{MAX, CA}$ (nL/min)	39 [29-116]
Bronchoalveolar lavage	
Cells/mL	140,000 [100,000-175,000]
Macrophages (%)	67 [31-85]
Neutrophils (%)	3.3 [1.3-9.3]
Eosinophils (%)	1[0.3-3.5]
Lymphocytes (%)	4.4 [3.3-6.5]
Epithelial cells (%)	19 [7-34]
ECP (ng/mL)	4 [1.5-26.5]
TGF- β_1 (pg/mL)	150 [87-180]
IFN γ /IL-4	7 [2-16]
TIMP-1/MMP-9	50 [17-109]
Bronchial biopsy: Subepithelium	
Neutrophils (N/mm ²)	39 [28-60]
Eosinophils (N/mm ²)	10.2[3.4-23.7]
Lymphocytes (N/mm ²)	132[92-186]
RBM (μm)	9.9 [7.4-12.3]

eosinophilic inflammation and remodeling in patients with refractory asthma. The former is in agreement with findings reported by Paynes et al.³ Using multiple flow analysis of exhaled NO allowing distinguishing alveolar and bronchial contributions to exhaled NO, we extend their results by showing that $Q_{MAX, CA}$ was correlated with RBM thickness.

A potential weakness of the study is the absence of a group of healthy children. However, it is unethical to perform bronchoscopy and BB in normal children solely for the purpose of research. Another limitation is that bronchodilator treatment regimens varied across the study patients. However, all patients were taking high-dose inhaled steroids. The important contribution of children with normal FEV₁ is probably related to the age of the population, airflow limitation being a progressive feature in asthma. Accordingly, Paynes et al reported a similar level of patients without airflow limitation in their study of children with refractory asthma (13/31).³

The links between Q_{MAX}, CA and subacute eosinophilic infiltration on the one hand and FA_{NO} and ECP on the other confirm that NO is an appropriate non-invasive marker of persistent inflammation in refractory asthma. We observed a surprising negative relation between FA_{NO} and IFN- γ /IL4 ratio, which is at discrepancy with experimental findings suggesting that these cytokines stimulate inducible NO synthase expression.¹⁴ An original finding from our study is the relation between several parameters associated with remodeling, for example, TGF- β ₁, TIMP-1/MMP-9, and RBM thickness, and the NO output by conducting airways. RBM thickening has been ascribed to subepithelial fibrosis induced by the profibroblastic activity of TGF- β ⁸ and by an imbalance between proteases and protease inhibitors, leading to airway wall thickening and increased bronchial tone. Maximal NO output from conducting airways might reflect trends in concentrations of other nitrogen oxides in the airway relevant to airway remodeling. Along this line, it is known that nitrogen oxides have antiproliferative effects at higher concentrations than those of NO measured in vivo^{15,16} and that S-nitrosothiols inhibit cytokine-induced lung epithelial MMP-9 expression.¹⁷

However, increased NOS 1 expression, decreased diffusion of NO across a thickened RBM, increased airway acidification, and increased S-nitrosothiol breakdown could also affect rate of NO transfer from the epithelium.

Combining NO measurements and pulmonary function testing may prove useful as a noninvasive assessment of uncontrolled eosinophilic inflammation and airway remodeling in children with refractory asthma. The relation between midflows and expired NO has been reported previously; we further demonstrate that this relation is related to an increased NO output from distal airways.¹⁸ Payne et al recently reported a trend between FE_{NO} measured at a single elevated expiratory flow (200 mL/s) and RBM thickness in 29 children.¹⁹ FE_{NO} measured at lower expiratory flows that increase the contribution of conducting airway to exhaled NO or at multiple flows that differentiate the conducting airway from the alveolar compartment would perhaps have yielded a significant relation in these patients. It has been stated by Sippel et al that exhaled NO levels are correlated predominantly with markers of asthma control rather than asthma severity²⁰; perhaps the multiple flow analysis of exhaled NO could help to evaluate these two aspects of asthma. The impact

of NO evaluation on treatment (ie, change in inhaled steroid dosage and/or alternative treatments) needs to be further assessed in prospective studies.

In summary, both inflammation and remodeling influence NO output in children with refractory asthma.

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