

Original article

Similar levels of nitric oxide in exhaled air in non-asthmatic rhinitis and asthma after bronchial allergen challenge

Background: Nitric oxide in exhaled air (eNO) is elevated in allergic asthma compared with healthy subjects and has been proposed as a marker of bronchial inflammation. However, eNO is elevated to a lesser extent in allergic non-asthmatic rhinitis as well. Considering the distinctive clinical appearances of both allergic diseases, differences in eNO are expected to persist after allergen exposure. The aim of the study was to compare allergen-induced changes in eNO in house dust mite sensitized patients with asthma and patients with perennial rhinitis without asthma symptoms.

Methods: Bronchial allergen challenge was performed in 52 patients sensitized to house dust mite (*Dermatophagoides pteronyssinus*), of whom 26 had non-asthmatic rhinitis and 26 had asthma. Levels of eNO were measured before and 1 h, 1 day and 1 week after challenge.

Results: At baseline eNO was significantly lower in non-asthmatic rhinitis compared with asthma (geometric mean eNO (SEM): 121 (1.1) in non-asthmatic rhinitis vs 197 (1.1) nl/min in asthma, $P < 0.006$). However, the increase in eNO after bronchial allergen challenge in non-asthmatic rhinitis, in particular in those patients with a dual asthmatic response, significantly exceeded the increase in asthma resulting in similar levels of eNO after challenge (geometric mean eNO (SEM) at 24 h postchallenge 204 (1.1) in non-asthmatic rhinitis vs 244 (1.1) nl/min in asthma, $P = 0.3$).

Conclusion: The difference in eNO between non-asthmatic rhinitis and asthma at baseline is abolished after allergen exposure due to a significantly greater increase in eNO in non-asthmatic rhinitis.

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Nitric oxide in exhaled air (eNO) is elevated in asthma (1) and to a lesser extent in non-asthmatic rhinitis as well (2, 3). Measuring eNO has been proposed as a diagnostic tool for asthma (1), both in adults and in children (4). In addition, eNO is advocated to be a valuable mean of monitoring asthmatic patients (5), as eNO seems to be related to the degree of bronchial inflammation in asthma. On the one hand eNO decreases during treatment with inhaled steroids (6) and on the other eNO increases after allergen challenge (7).

Apart from the potential clinical usefulness of eNO in asthma, nitric oxide (NO) is an interesting molecule in the pathophysiology of asthma and rhinitis because of its possible immunologic effects. Allergic diseases are generally assumed to be characterized by a shift in the balance between Th1 and Th2 cells toward Th2 cells. Nitric oxide causes prolonged suppression of Interferon (IFN)- γ production by helper T (Th) cells *in vitro* (8). As IFN γ

is one of the key cytokines in the differentiation of Th cells into Th1 and Th2 subsets and inhibits the proliferation of the Th2 cells, NO might indirectly enhance the Th2-mediated inflammatory response in allergic asthma and allergic rhinitis by the suppression of IFN- γ .

The increased level of eNO is considered to be caused by upregulation of the inducible form of NO synthase (NOS) particularly in bronchial epithelial cells (9). Recently, upregulation of inducible NOS (iNOS) within the airways was demonstrated after bronchial allergen challenge (10), confirming the association between upregulation of iNOS and increase in bronchial inflammation. The inducible isoform of NOS is an enzyme that is capable of producing large amounts of NO and is expressed only after induction by immunologic and inflammatory stimuli (11). By contrast, the constitutive isoform of NOS (cNOS) produces only small amounts of NO. The large quantities of NO that are produced by iNOS possibly are deleterious in allergic airway disease. In a study in ovalbumin sensitized iNOS-knock out mice there was less eosinophil accumulation in the lungs after ovalbumin challenge compared

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with controls (11). However, the precise role of the different isoforms of NOS is still a matter of controversy.

The previously reported elevated levels of eNO in rhinitics without asthma symptoms (2) are in keeping with the finding that in addition to the upper airways, the lower airways in non-asthmatic rhinitics are also characterized by some degree of eosinophilic inflammation (12). In rhinitics without symptoms of the lower airways an early asthmatic response (EAR) can be elicited by bronchial allergen challenge (13). The late asthmatic response (LAR), which develops in some sensitized patients a few hours after bronchial allergen challenge, is accompanied by an increase in bronchial inflammation and is therefore considered a model for the bronchial inflammation in chronic asthma. A LAR can also be found in rhinitics without asthma, although less frequently than in asthmatics. However, considering the distinctive clinical appearances of both allergic diseases, differences in either the allergic inflammatory process, including eNO, or in characteristics of the bronchial tissue are expected.

The aim of the present prospective, experimental study was to compare allergen-induced changes in eNO, as an indirect marker for changes in bronchial inflammation, in patients sensitized to house dust mite (HDM, *Dermatophagoides pteronyssinus*) who had either asthma or perennial rhinitis without any clinical evidence of asthma. To our knowledge, eNO in rhinitics without asthma has only been studied at baseline (2, 3), but not after bronchial allergen challenge. In addition, no comparative challenge studies between asthma and non-asthmatic rhinitis, with respect to eNO, have been carried out. In this paper we describe the results of eNO measurements before and after a standardized bronchial allergen challenge with HDM extract.

Materials and methods

Study population

Fifty-two patients sensitized to HDM (*D. pteronyssinus*), as determined by skin prick test (SPT) and radioallergosorbent test (RAST), were investigated of whom 26 had asthma and 26 had perennial rhinitis without current or past history of asthma symptoms. Asthma was diagnosed according to the American Thoracic Society (ATS) criteria and included a documented history of recurrent episodes of wheezing, chest tightness and dyspnea and a normal lung function between asthmatic attacks (14). The majority of asthmatics (14/26) used inhaled corticosteroids on a regular basis before inclusion and all used inhaled short-acting β_2 agonists as needed. Perennial rhinitis was characterized by episodes of sneezing, watery rhinorrhoe, pruritus in the nose and nasal obstruction without any current or past asthmatic symptoms (15). The participating patients were recruited via the outpatient departments of Pulmonology and Otorhinolaryngology at the Academic Medical Center.

The following inclusion criteria were used for all: (a) $FEV_1 \geq 70\%$ of predicted value, (b) IgE to *D. pteronyssinus* > 0.5 IU/ml, (c) able to stop short acting β_2 -adrenoceptor agonists for at least 8 h, oral antihistamines for 2 weeks and inhaled corticosteroids for 6 weeks prior to the start of the study, and (d) no significant change in

allergen exposure caused by allergen avoidance during at least 6 months. Whenever co-sensitization to pollen was present the study was performed outside the relevant pollen season. Exclusion criteria were: (a) immunotherapy in the history, (b) respiratory tract infection within 6 weeks prior to the study, (c) immunosuppressive medication, and (d) smoking. The AMC Medical Ethical Committee approved the study and all subjects gave written informed consent.

Study design

The study had a prospective experimental design. After screening, medication by corticosteroids and antihistamines was ceased 6 weeks and 2 weeks prior to the study period, respectively. Baseline levels of eNO, forced expiratory volume in 1 second, (FEV_1) and histamine threshold (PC_{20} histamine) were determined on the first day of the study period. Subsequently, FEV_1 was measured hourly for establishing diurnal variation. Bronchial allergen challenge was performed on the next day. Twenty-four hours and 1 week after bronchial allergen challenge eNO, FEV_1 and PC_{20} histamine were repeated.

Allergen extract

A standardized *D. pteronyssinus* extract (1 mg/ml) (ALK, Houten, the Netherlands) containing 10^6 6 SQ units/ml (representing 50 000 BU/ml) was kept at -20°C in small aliquots. This allergen batch was used for all assays, and contained 85.1 μg Der p1 and 9.7 μg Der p2/mg protein. Two-fold dilutions were made freshly from the stock immediately before allergen challenge in phosphate-buffered saline (PBS), 0.03% human serum albumin, 0.5% phenol (ALK).

Bronchial allergen challenge

Bronchial allergen challenge was performed by using a reservoir aerosol delivery system according to the method described by Sterk et al. (16) with modifications (17). A collapsible reservoir of approximately 30 l, made of static field dissipative material (RCAS 1206, Richmond Redlands, CA, USA) and filled with dry air, was connected to a nebulizer (Mallinckrodt Diagnostica, Petten, the Netherlands) producing allergen aerosols from a 0.5-ml sample. The entire volume of the reservoir was inhaled by tidal breathing through a three-way valve system with the nose clipped. Previous studies demonstrated a 70% recovery of nebulized allergen. The starting allergen dose was calculated from the SPT threshold and the PC_{20} histamine according to the method of Cockcroft et al. (18). FEV_1 was measured on a dry rolling-seal spirometer (SensorMedics BV, Bilthoven, the Netherlands). Drop in FEV_1 (mean of two measurements) relative to baseline values (median of three measurements within 5%) was measured 0.5 and 10 min after inhalation of allergen. With intervals of 10 min doubling doses of allergen were inhaled from the reservoir, until a decline of 20% in FEV_1 was reached. Subsequently, FEV_1 was measured hourly for at least 10 h. The bronchial response to allergen was expressed as the percentage change from baseline and was corrected for the diurnal variation. Both EAR and LAR were defined as $\geq 20\%$ drop in FEV_1 relative to the baseline value.

Bronchial histamine challenge

Bronchial histamine challenge was performed according to the 2-min tidal breathing method as described by Sterk et al. (16). Bronchial responsiveness to histamine was defined as the concentration of histamine diphosphate in PBS (range: 0.015–32 mg/ml) causing a drop of 20% in FEV_1 as calculated by log-linear interpolation.

NO measurements

Nitric oxide was measured in exhaled air using a chemiluminescence NO/NO_x analyzer (CLD 700 AL med ECO Physics, Dürnten, Switzerland, range 1–100 ppb, update frequency 100 msec, T-90 1 s). The subjects inhaled NO-free air through a mouthpiece in a sitting position with the nose clipped. During tidal breathing the expired air was collected for 2 min in a NO-inert Douglas bag via a T-valve with an expiratory resistance of 5 cmH₂O. Simultaneously, respiratory flow and volume data were collected via a pneumotachograph. The NO concentration in the sample bag was measured immediately after this procedure. The NO-output per minute (eNO) was calculated from the NO-concentration in the sample bag and expiratory volume. In addition to the eNO measurements, real time NO concentrations were measured during 2 min of tidal breathing by direct sampling from the mouthpiece via a Teflon tube. The mean plateau expiratory NO concentration was then calculated. There were no significant differences in tidal volume and frequency of breathing, neither between non-asthmatic rhinitis and asthma nor before and after allergen challenge (results not shown). Significant correlations between eNO and NO expiratory plateau values were found at all time points (at baseline: $R_s = 0.54$, $P < 0.0001$; after EAR: $R_s = 0.55$, $P < 0.0001$; 24 h after allergen challenge: $R_s = 0.50$, $P < 0.0001$; and 1 week after allergen challenge: $R_s = 0.34$, $P < 0.03$).

Statistical methods

SPSS version 8.0.2 Statistics UK (Chicago, IL, USA) was used for statistical analyses. Within group comparisons were made with Wilcoxon test, between group differences were analyzed with Mann–Whitney test. The Spearman rank test was used to determine correlations. All P -values were two-tailed and P -levels < 0.05 were considered significant.

Results

Baseline characteristics, lung function and bronchial allergen challenge

Baseline characteristics and lung function parameters in non-asthmatic rhinitis and asthma are shown in Table 1.

All patients tolerated the bronchial allergen challenge without complications. Short acting β_2 -agonists were not used 8 h before and 12 h after allergen challenge. All asthmatic patients had an EAR, of whom 19 had a dual response, i.e. both an EAR and LAR. Twenty-one of the non-asthmatic rhinitis patients experienced an EAR, 11 had a LAR. The EAR was similar in non-asthmatic rhinitis and in asthma (median maximal decrease in FEV₁ –29% in non-asthmatic rhinitis vs –32% in asthma, $P = 0.1$), but the late asthmatic reaction was more pronounced in asthmatics (–21% in non-asthmatic rhinitis vs –34% in asthma, $P = 0.02$).

Rhinitis and asthmatics were divided on the basis of their late phase response to allergen into single and dual responders. Baseline characteristics and lung function parameters of non-asthmatic rhinitis and asthmatic single and dual responders are shown in Table 1. Two non-asthmatic rhinitis patients had a LAR, in the absence of a definitive EAR, but with an early decrease in FEV₁ between 15 and 20%. They were included in the group of dual responders.

eNO measurements

Values of eNO before and after bronchial allergen challenge in non-asthmatic rhinitis and asthma are shown in Fig. 1A. The eNO in asthmatic and rhinitic single and dual responders before and after allergen challenge is depicted in Fig. 1B. At baseline eNO was significantly higher in asthma than in non-asthmatic rhinitis [geometric mean eNO (SEM): 197 (1.1) in asthma vs 121 (1.1) nl/min in non-asthmatic rhinitis, $P = 0.006$].

Allergen-induced changes in eNO: non-asthmatic rhinitis vs asthma

In both non-asthmatic rhinitis and asthma there was an increase in eNO at 24 h after allergen challenge which was highly significant in non-asthmatic rhinitis

Table 1. Baseline characteristics and lung function parameters in non-asthmatic patients with rhinitis and patients with asthma

	Non-asthmatic rhinitis			Asthma		
	<i>n</i> = 26	Dual responders <i>n</i> = 11	Single responders <i>n</i> = 15	<i>n</i> = 26	Dual responders <i>n</i> = 19	Single responders <i>n</i> = 7
Age (years)#	27 (5)			27 (6)		
Sex (male/female)	6/20			6/20		
splgE (KU/l)§	13 (3.4)	20.2 (2.1)	9.47 (4.1)	27 (2.8)†	29.9 (2.7)	19.5 (3.2)
Total IgE (KU/l)§	126 (3.8)	160 (3.1)	106 (4.4)	243 (3.8)	202 (3.8)	404 (3.9)
FEV ₁ (% pred)#	103 (13)	103 (15)	103 (11)	95 (1.5)†	92 (15)	101 (10)
PC ₂₀ hist (mg/ml)§	9.6 (2.7)	6.4 (3.2)	12.9 (2.1)*	1.2 (4.0)†††	0.8 (3.6)	3.6 (3.1)‡
PC ₂₀ hist, 24 h (mg/ml)§	2.1 (1.5)	1.0 (1.7)	3.7 (1.6)**	0.4 (1.4)††	0.22 (1.4)	1.5 (1.4)‡‡
EAR Δ FEV _{1,max} (%)¶	–29.1 (12.0)	–29.1 (9)	–29.1 (13)	–31.7 (11.5)	–32.9 (14)	–30.6 (8)
LAR Δ FEV _{1,max} (%)¶	–18.9 (21.1)	–31.5 (15)	–11.1 (10)***	–27.6 (34.1)†	–40.8 (30)	–9.6 (6)‡‡‡

Mean and SD, § geometric mean and GSD, ¶ median value and IQR, splgE: specific immunoglobulin E, PC₂₀ hist, 24 h: PC₂₀ histamine at 24 h after allergen challenge, EAR: early asthmatic reaction, LAR: late asthmatic reaction, Δ FEV_{1,max}: maximal decline in FEV₁ after allergen challenge.

Non-asthmatic rhinitic dual vs non-asthmatic rhinitic single responders: * $P = 0.07$, ** $P = 0.08$, *** $P < 0.0001$.

Non-asthmatic rhinitis vs asthma: † $P < 0.05$, †† $P = 0.001$, ††† $P < 0.0001$.

Asthmatic dual vs asthmatic single responders: ‡ $P < 0.05$, ‡‡ $P < 0.01$, ‡‡‡ $P < 0.0001$.

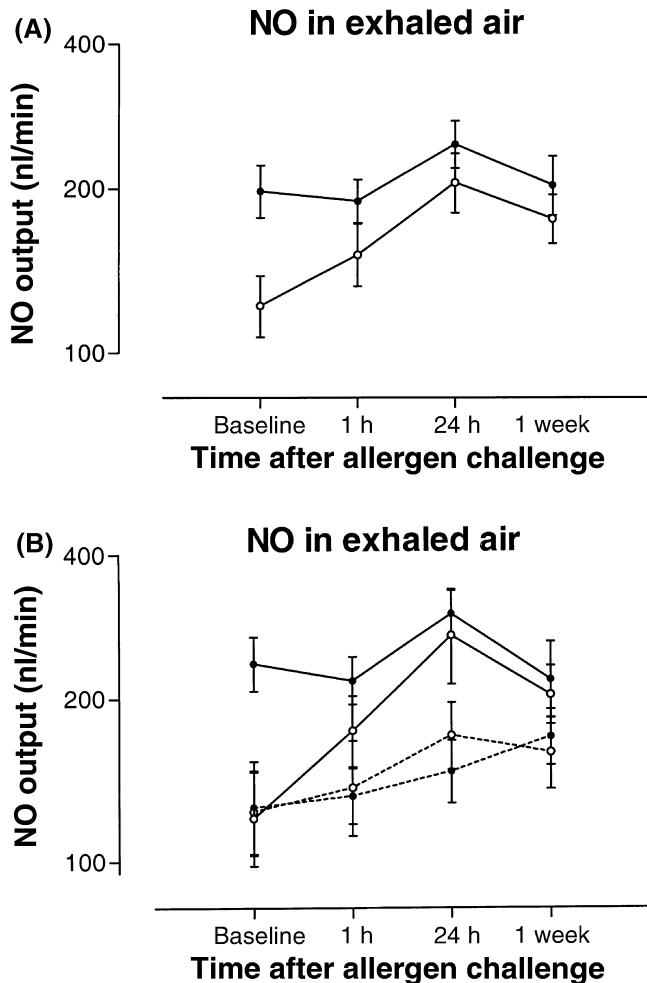


Figure 1. (A) eNO after bronchial allergen challenge in non-asthmatic rhinitis (○) and asthma (●). Geometric mean values $\pm$ SEM are shown. At 24 h eNO increased significantly in both groups. Differences between groups: non-asthmatic rhinitis vs asthma: $P < 0.006$ at baseline, $P > 0.3$ at 24 h and 1 week after challenge. (B) eNO after bronchial allergen challenge in single responders (dotted line) and dual responders (solid line) with non-asthmatic rhinitis (○) or asthma (●). Within-group statistics: at 24 h there was a significant rise in all groups ($P < 0.05$) except for asthmatic single responders. Between-group statistics (Kruskal–Wallis test) showed significant differences at baseline (asthmatic dual responders vs other groups) and at 24 h (dual responders vs single responders).

($P < 0.0001$), but also significant in asthma ($P < 0.05$) (Fig. 1A). At 24 h after challenge levels of eNO did not differ significantly between asthma and non-asthmatic rhinitis [geometric mean eNO (SEM) 244 (1.1) in asthma vs 204 (1.1) nl/min in non-asthmatic rhinitis, $P = 0.3$]. One week after challenge there was still a significant increase in eNO in non-asthmatic rhinitis ($P < 0.05$ relative to baseline), but not in asthmatics. The relative change in eNO at 24 h after allergen challenge was

significantly greater in non-asthmatic rhinitis compared with asthmatics ($P < 0.05$).

Allergen-induced changes in eNO in single and dual responders

The increase in eNO at 24 h after challenge was significant in rhinitic dual responders ($P < 0.01$), in rhinitic single responders ($P < 0.01$) and in asthmatic dual responders ($P < 0.05$), but not in asthmatic single responders (Fig. 1B). The increases in eNO at 24 h postchallenge in the four subgroups were significantly different (Kruskal–Wallis $P = 0.01$). The increase in eNO relative to baseline was most pronounced in rhinitic dual responders ($P < 0.01$) compared with rhinitic single responders and asthmatic dual responders.

Correlations with changes in lung function

There was a significant correlation at baseline between eNO and PC₂₀ histamine ($R_s = -0.31$, $P = 0.03$). Values of eNO at baseline correlated significantly with the magnitude of the LAR ($R_s = -0.31$, $P < 0.05$). There was also a significant correlation between eNO at 24 h after challenge and the magnitude of the LAR ($R_s = -0.46$, $P = 0.001$).

Discussion

This is the first study to demonstrate an increase in eNO after bronchial allergen challenge in rhinitics without asthma symptoms. The increase in eNO in this group exceeded the increase in eNO in asthmatics, resulting in similar levels in non-asthmatic rhinitis and asthma 24 h after allergen challenge. These findings underline the similarities in bronchial inflammatory changes in HDM allergic patients with and without asthma.

It seems unlikely that methodological aspects of the NO measurements can explain the findings in the present study. In spite of the fact that eNO was sampled during tidal breathing with the nose clipped and patients exhaled against an expiratory resistance, some contamination with NO by diffusion from the nasal cavity cannot be ruled out completely. However, it is unlikely that contamination with nasal NO has influenced the main findings of the study. The low baseline values of eNO are in agreement with levels reported by others (2). Moreover, the difference between non-asthmatic rhinitis and asthmatics at baseline and the significant increase in asthmatics after specific bronchial allergen challenge are very similar to the data reported earlier (2, 7). The significant correlations between on line measured plateau expiratory NO concentrations and eNO argue against a significant contribution of nasal NO. We like others found that nasal NO increases after nasal allergen challenge (19) and eNO increases after bronchial allergen

challenge (7), but we are not aware of any report of an increase in nasal NO after specific bronchial allergen challenge. Moreover, in a pilot study in four patients we did not detect an increase in nasal NO after bronchial allergen challenge. So, the increase in eNO after the bronchial allergen challenge in the present study is almost certainly derived from the lower airways. The significant differences in eNO between bronchial single and dual responders confirms a bronchial origin of the bulk of the eNO. Finally, the majority of asthmatic patients also had symptoms of perennial rhinitis and all of them had signs of an allergic inflammatory reaction in the nose as detected by increased levels of eosinophil cationic protein (ECP) in nasal washings which were indistinguishable from levels in rhinitics (data not shown). Combined with the fact that all patients underwent the same reproducible procedure a difference in contamination with nasal NO, if any, between both groups is highly unlikely.

The cause of the higher increase in eNO in non-asthmatic rhinitics compared with asthmatics, with disappearance of the difference that was present before challenge, remains unclear. Possibly, a plateau in the expression of iNOS, which will be reached earlier in asthma due to a higher initial value, explains the smaller increase in eNO in asthmatics.

Nitric oxide in exhaled air has been proposed as a marker of bronchial inflammation. Monitoring the inflammatory process in asthma may be important for the management of asthma (5). Some studies reported significant associations between eNO and sputum eosinophil numbers or bronchial responsiveness (20), but this was not confirmed in other studies (21). Moreover, no relationship could be demonstrated between eNO and mucosal eosinophils in bronchial biopsies (22). Likewise, we did not find significant associations at baseline between eNO and parameters for bronchial inflammation in induced sputum (inflammatory cells, eosinophil cationic protein and myeloperoxidase; Clin. Exp. Allergy, *in press*). This may suggest that eNO reflects other aspects of the bronchial inflammatory process. The absence of correlations between steroid-induced changes in levels of eNO and changes in eosinophils in sputum (6) is in keeping with this hypothesis.

Both eNO and PC₂₀ histamine differed significantly between asthma and non-asthmatic rhinitis at baseline. In contrast, we did not find a significant difference in baseline percentage and numbers of sputum eosinophils between both groups (Clin. Exp. Allergy, *in press*). These findings suggest that eNO and PC₂₀ histamine surpass sputum eosinophils in discriminating between non-asthmatic rhinitis and asthma. After bronchial allergen challenge PC₂₀ histamine decreased to a similar extent in non-asthmatic rhinitis and asthma and the difference between both groups remained unchanged. By contrast, the difference in eNO disappeared after allergen exposure indicating that the diagnostic value of the PC₂₀ histamine is probably superior to discriminate between both groups.

The division of patients on the basis of their late phase bronchoconstriction to allergen showed low eNO values in asthmatic single responders, with levels very similar to eNO levels in non-asthmatic rhinitis. This finding demonstrates that a low level eNO at baseline does not exclude the presence of asthmatic symptoms. It is unlikely that increased eNO in these patients was obscured by anti-inflammatory treatment as in this study inhaled corticosteroids were not used during the 6 weeks before eNO measurements.

The larger increase in eNO in non-asthmatic rhinitis as compared with asthma appeared to be due to a significantly larger increase in eNO in rhinitic dual responders as opposed to the other subgroups. In spite of the similar levels of baseline eNO, eosinophilic bronchial inflammation at baseline was significantly greater in rhinitic dual responders than in rhinitic single responders, as concluded from the difference in eosinophils in induced sputum (median percentage of eosinophils in induced sputum at baseline 8.1 in rhinitic dual responders vs 0.7 in rhinitic single responders). Rhinitics without asthma symptoms but with a dual bronchial response to allergen apparently have a considerable degree of asymptomatic bronchial inflammation and can be triggered more easily to produce large amounts of NO. It is tempting to speculate that these patients represent a subgroup of rhinitics which is at risk of developing asthma symptoms. It has been suggested previously that elevated levels of eNO in atopic asymptomatic subjects (23), or in atopic asthmatics in clinical remission (24), predict the onset or relapse of asthmatic symptoms. However, a long-term follow-up study is necessary to settle this point. Bronchial hyperresponsiveness also has been proposed as a useful parameter to identify patients at risk of developing asthma (25, 26). The value of PC₂₀ histamine in this respect is underlined in our study as rhinitic dual responders had increased bronchial hyperresponsiveness at baseline, compared with rhinitic single responders.

We conclude that the difference in eNO at baseline between HDM allergic patients with rhinitis but without asthma and patients with asthma disappeared after bronchial allergen challenge. The increase in eNO after allergen challenge was most pronounced in the subgroup of rhinitics that exhibited a dual asthmatic response after challenge. These findings underline the similarities in bronchial changes in allergic patients with and without asthma and may help to identify patients at risk of developing asthma.

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