

Concentrations of exhaled nitric oxide in asthmatics and subjects with allergic rhinitis sensitized to the same pollen allergen¹

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Summary

Background Some studies have reported that the levels of exhaled nitric oxide (ENO) in asthmatics are similar to those in subjects with allergic rhinitis, and it has been postulated that atopic status might be the determinant of enhanced nitric oxide production in asthma.

Objectives The aim of this study was to determine differences in ENO levels between asthmatics and subjects with allergic rhinitis sensitized to the same allergen, and to correlate these levels with airway responsiveness.

Methods Nineteen patients with asthma and 18 subjects with allergic rhinitis monosensitized to *Parietaria* pollen were enrolled in the study. ENO values and airway responsiveness to methacholine and adenosine 5'-monophosphate (AMP) were measured during the pollen season. The response to each bronchoconstrictor agent was measured by the provocative concentration required to produce a 20% fall in FEV₁ (PC₂₀). ENO was measured with the single-exhalation method.

Results The geometric mean (95% confidence interval) ENO values were significantly higher in asthmatics than in subjects with allergic rhinitis: 72.4 p.p.b. (54.9–93.3 p.p.b.) vs. 44.7 p.p.b. (30.9–64.6 p.p.b., $P = 0.03$). In asthmatics, a significant correlation was found between ENO and PC₂₀ AMP values ($\rho = -0.57$, $P = 0.02$), whereas no correlation was detected between ENO and PC₂₀ methacholine ($\rho = -0.35$, $P = 0.14$).

Conclusions Our results suggest that atopy is not the only determinant of increased ENO levels detected in subjects with asthma, and that responsiveness to AMP may be a more sensitive marker for assessing airway inflammation in asthma compared to methacholine.

Keywords nitric oxide, adenosine 5'-monophosphate, methacholine, airway responsiveness, asthma, allergic rhinitis

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Introduction

Airway hyper-responsiveness is one of the hallmarks of asthma and is often used as an indicator of asthma severity. The degree of airway responsiveness can be assessed with a variety of inhaled bronchoconstrictor agents, such as histamine, methacholine or adenosine 5'-monophosphate (AMP). Methacholine induces airway constriction via direct stimulation of the muscarinic receptors on airway smooth muscle cells. AMP, on the other hand, acts indirectly, causing primed mast cell degranulation and the release of histamine and other mediators with subsequent smooth muscle contraction [1–3]. Since mast cells are believed to play a predominant role in atopic asthma, the bronchial response to AMP may be a more direct marker of allergic airway inflammation than direct bronchoconstrictors.

Nitric oxide is a messenger molecule generated from L-arginine by the action of several distinct enzymes called nitric

oxide synthases (NOS). These enzymes are found in many cells of the lung, including epithelial cells, alveolar macrophages, smooth muscle cells, fibroblast, and neutrophils [4]. It has been suggested that the increased exhaled nitric oxide (ENO) levels found in asthmatic subjects result from an increase in the expression of inducible nitric oxide synthase (iNOS) in the respiratory tract induced by the action of proinflammatory cytokines [5]. Even though iNOS activity seems to be the principal cause of the increased ENO levels found in asthmatic subjects, there are additional determinants of ENO production such as airway acidification [6] and S-nitrosothiol breakdown [7].

ENO levels are known to be increased in asthma [8, 9], to increase during an exacerbation [10], to decrease with corticosteroid therapy [11], and to rise as the dose of inhaled steroids is reduced [12]. In addition, ENO levels are correlated with eosinophils in induced sputum [13, 14]. Therefore, ENO has been proposed as a non-invasive marker of airway inflammation [15], a primary process in the pathogenesis of asthma. However, ENO levels are increased in atopic but not in non-atopic subjects with asthma, when compared with healthy controls [16–18]. Furthermore, it has been demonstrated that ENO concentrations are significantly higher in subjects with allergic

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rhinitis compared with non-allergic rhinitis [19]. In addition, ENO levels are similar in subjects without asthma but with allergic rhinitis, and subjects with allergic asthma [19, 20]. All the above evidence supports the contention that atopic status may be the determinant of enhanced nitric oxide production [21]. These findings were, nevertheless, obtained in patients sensitized to different allergens, and the extent to which this is also the case for subjects sensitized to the same allergen remain thus far unclear.

The aim of the present study was to determine differences in ENO levels between subjects with allergic rhinitis with and without asthma sensitized to the same allergen, and to correlate these levels with airway responsiveness. Therefore, we selected subjects monosensitized to *Parietaria* pollen, and investigated differences in ENO levels and bronchial responsiveness to both methacholine and AMP between patients with allergic rhinitis and those with asthma.

Subjects and methods

Subjects

Thirty-seven adult subjects (18 with allergic rhinitis and 19 with allergic rhinitis plus asthma) were studied, having met the following inclusion criteria: a history of seasonal rhinitis or seasonal mild asthma [22] for at least 2 years; an FEV₁ greater than 80% of the predicted value; a positive skin prick test (≥ 3 mm weal diameter) to *Parietaria judaica* and no skin sensitization for other allergens tested, namely house dust mites, grass pollen, *olea europea*, *platanus orientalis*, *Alternaria*, *Aspergillus*, *Penicillium*, *Cladosporium*, and cat and dog dander (ALK-Abello, Madrid, Spain). Asthma was identified by the presence of symptoms of wheeze, breathlessness, or cough plus methacholine airway hyper-responsiveness with a PC₂₀ (provocative concentration required to produce a 20% fall in FEV₁) of less than 8 mg/mL if the FEV₁/FVC was 70% or greater or an improvement of the FEV₁ from predicted of 15% or greater after 200 µg of inhaled salbutamol if the FEV₁/FVC was less than 70%. Subjects with allergic rhinitis were defined as those individuals with a characteristic history of seasonal rhinitis (rhinorrhea, sneezing, obstruction, and pruritus) during the *Parietaria* pollen season and no history of asthma.

All asthmatic patients had mild asthma and none were receiving any regular medication for asthma other than occasional short acting inhaled β_2 -agonists. All 37 subjects were life-long non-smokers, and none had history of chronic bronchitis, emphysema or respiratory tract infections during the four weeks before the study. Current or ex-smokers, pregnant women, and patients with significant renal, hepatic or cardiovascular disease were specifically excluded. Some subjects with allergic rhinitis ($n = 10$) were selected from participants in a previous study [23]. The study protocol was approved by the local ethics committee, and written informed consent was obtained from all participants.

Study design

The study was performed during the *Parietaria* pollen season in Valencia (April to June). Subjects attended the laboratory on three visits. On the first day, all subjects were evaluated for suitability and spirometry was performed. On each of the next

two visits (at least 7 days but not > 9 days apart), spirometry and concentration-response studies with either methacholine or AMP were performed. Methacholine and AMP challenges were conducted on separate days with the order of challenge randomised. At the second visit, ENO was measured before spirometry and challenge test.

Pulmonary function

Spirometry was performed with a calibrated dry rolling seal spirometer (model 2130, Sensormedics Co., Yorba Linda, CA, USA), according to standardized guidelines [24]. Reference values were those of the European Community for Coal and Steel [25].

Inhalation challenge test

Inhalation provocation tests were performed using the method described by Chai et al. [26] with some modifications [27]. Subjects were instructed to withhold their treatment for at least 8 h (inhaled short-acting β_2 -agonists), 4 weeks (nasal topical corticosteroids and nasal topical cromoglycate) and 3 days (antihistamines) prior to each challenge. Methacholine and AMP (Sigma Chemical Co., St Louis, MO, USA) were dissolved freshly in 0.9% saline. Each solution was administered from a jet nebulizer attached to a breath activated dosimeter (Mefar MB3, Brescia, Italy) at a nebulization time of one second and pause time six seconds. Subjects inhaled the aerosolized methacholine and AMP solutions in five inhalations from functional residual capacity to total lung capacity through a mouthpiece with the subject's nose clipped. Normal saline was inhaled initially, followed by five breaths of doubling concentrations of methacholine or AMP at two- to three-minute intervals. Due to the effect of a deep inspiration on subsequent airway tone [28], single measurements of FEV₁ were made 60–90 s after inhalation of each concentration, unless the forced expiratory manoeuvre was judged to be technically unsatisfactory. The starting concentrations of methacholine were 0.39 mg/mL for subjects with allergic rhinitis and 0.095 mg/mL for asthmatic patients. The starting concentrations of AMP were 1.56 mg/mL for subjects with allergic rhinitis and 0.39 mg/mL for asthmatic patients. The test was interrupted when a 20% decrease in FEV₁ from the post-saline value was recorded or when the highest concentration (25 mg/mL for methacholine and 400 mg/mL for AMP) had been administered. The provocative concentration of agonist required to produce a 20% fall in FEV₁ (PC₂₀) was calculated using a formula given by Cockcroft et al. [29].

Exhaled nitric oxide measurement technique

ENO concentration was measured online by the restricted breath analysis according to the recommendations of the American Thoracic Society [30] using a chemiluminescence analyser (NiOx, Aerocrine, Solna, Sweden) with a sensitivity of 1.5 parts per billion (p.p.b.), a detection range of 0–200 p.p.b., and a response time < 0.8 s. Briefly, the subject was instructed to inhale through the mouth to total lung capacity and immediately exhale through the mouthpiece against a fixed resistance. Subjects inhaled ambient air that was passed through a filter to reduce inhaled nitric oxide concentrations to < 5 p.p.b. Maintenance of a constant flow was achieved using a visual feedback display on the computer screen. A flow rate of 45 mL/s was

used. An exhalation of 10 s with a plateau of at least 3 s duration was required to calculate the NO plateau. Participants repeated the manoeuvre until three acceptable tests were performed. The average of the three plateau values was recorded.

Data analysis

All PC₂₀ and ENO values were log-transformed before analysis and presented as geometric means with 95% confidence intervals (95% CI). All other numerical variables are reported as arithmetic means with 95% CI. Numerical variables were analysed with unpaired *t*-test, whereas categorical variables were compared with the Fisher's exact test. Correlations between variables were calculated using the Spearman rank (ρ) correlation test. Probability (*P*) values are two-sided and values < 0.05 were considered statistically significant. Data were analysed with the statistical package InStat (GraphPad Software Inc., San Diego, CA, USA) for Windows version 3.00.

Results

The clinical and pulmonary function data for asthmatics and subjects with allergic rhinitis are presented in Table 1. The two groups were similar with regard to age, sex and duration of symptoms. The mean lung function value (FEV₁ and FEV₁/FVC) for the group with asthma was significantly lower than the mean value for the allergic rhinitis subjects. As expected, both the PC₂₀ methacholine and the PC₂₀ AMP were also significantly lower in asthmatics than in subjects with allergic rhinitis.

The geometric mean (95% CI) ENO values were significantly higher (Fig. 1) in asthmatics than in subjects with allergic rhinitis: 72.4 p.p.b. (54.9–93.3 p.p.b.) vs. 44.7 p.p.b. (30.9–64.6 p.p.b.; *P* = 0.03).

In asthmatics, no correlation was found between ENO and PC₂₀ methacholine (ρ = -0.35, *P* = 0.14). However, a significant correlation was found (Fig. 2) between ENO and PC₂₀ AMP values (ρ = -0.57, *P* = 0.02).

In subjects with allergic rhinitis, no correlation was found between ENO and either PC₂₀ of methacholine (*n* = 7, ρ = 0.68, *P* = 0.11) or PC₂₀ of AMP (*n* = 8, ρ = -0.21, *P* = 0.62), when it was measurable.

Discussion

In a group of subjects sensitized to the same allergen, we demonstrated that subjects with asthma had significantly higher ENO concentrations than non-asthmatic subjects with allergic rhinitis. Furthermore, in subjects with asthma, there was a significant correlation between ENO values and AMP responsiveness, but not between ENO values and methacholine responsiveness.

To our knowledge, this is the first study to demonstrate differences in the concentration of ENO between asthmatic and allergic rhinitis subjects sensitized to the same allergen. If ENO levels reflect the presence of airway inflammation, our results suggest that bronchial inflammation is greater in asthma than in allergic rhinitis. However, although ENO has been proposed as a marker of airway inflammation [15] in asthma, the correlations between ENO and direct measures of airway inflammation have been of relatively small magnitude [13, 14]. Therefore, the precise mechanism(s) that link(s) nitric oxide with airway inflammation, and whether elevated ENO concentrations are caused by enhanced activity of inflammatory cells or by enhanced diffusion through the airway wall due to structural damage, remain to be elucidated.

Alternatively, increased ENO levels might be the consequence of an independent effect of atopy on nitric oxide production. Several studies have reported that atopic subjects with asthma had higher ENO levels than did nonatopic subjects with asthma [16–18, 31, 32]. Furthermore, no difference was found in ENO levels between non-atopic subjects with asthma and healthy controls [16–18]. In other studies, ENO levels were significantly higher in subjects with allergic rhinitis compared with subjects with non-allergic rhinitis [19]. In addition, ENO was elevated in asymptomatic subjects sensitized to inhaled allergens [33, 34]. These study results indicate that atopic status might be the most important determinant of enhanced nitric oxide production. It is legitimate to question therefore whether airways inflammation is the cause of increased ENO levels in asthmatics.

Our study is not able to answer the question of whether increased ENO is a reflection of atopy or of airway inflammation. However, despite the fact that our subjects were sensitized to the same allergen, we found that subjects with asthma had

Table 1. Subject characteristics, pulmonary function and airway responsiveness to methacholine and adenosine 5'-monophosphate (AMP) in the two groups

	Asthma	Allergic rhinitis	<i>P</i>
Subjects, <i>n</i>	19	18	
Age, years	39.6 (34.5–44.7)	35.9 (30.7–41.2)	0.30
Male/female, <i>n</i>	6/13	8/10	0.51
Duration of symptoms, years	9.8 (6.4–13.2)	9.1 (5.9–12.2)	0.74
FEV ₁ , % predicted	101.6 (96.5–106.6)	112.4 (105.0–119.8)	0.02
FEV ₁ /FVC, %	77.7 (75.4–79.9)	86.3 (83.7–88.8)	< 0.001
PC ₂₀ , mg/mL			
Methacholine*	3.2 (1.7–5.7)	14.4 (9.1–22.4)	0.002
AMP*	36.3 (17.8–74.1)	165.9 (75.8–363.1)	0.005

Data are displayed as mean (and 95% confidence intervals); *: data presented as geometric mean (and 95% confidence intervals); FEV₁: forced expiratory volume in one second; FVC: forced vital capacity; PC₂₀: provocative concentration of agonist required to produce a 20% fall in FEV₁.

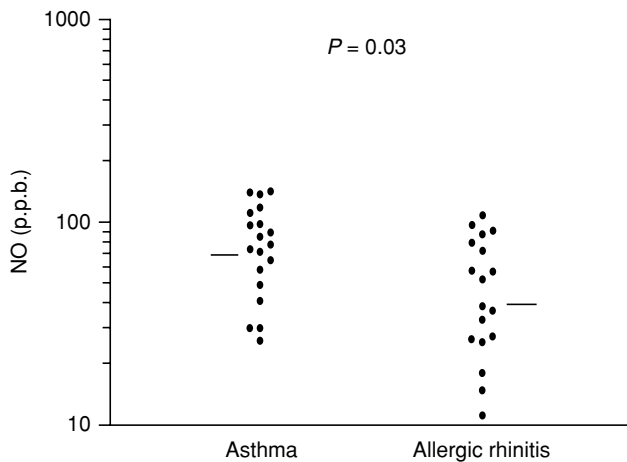


Fig. 1. Individual values for exhaled nitric oxide (NO) concentrations in asthmatics and subjects with allergic rhinitis; geometric means are represented by horizontal lines.

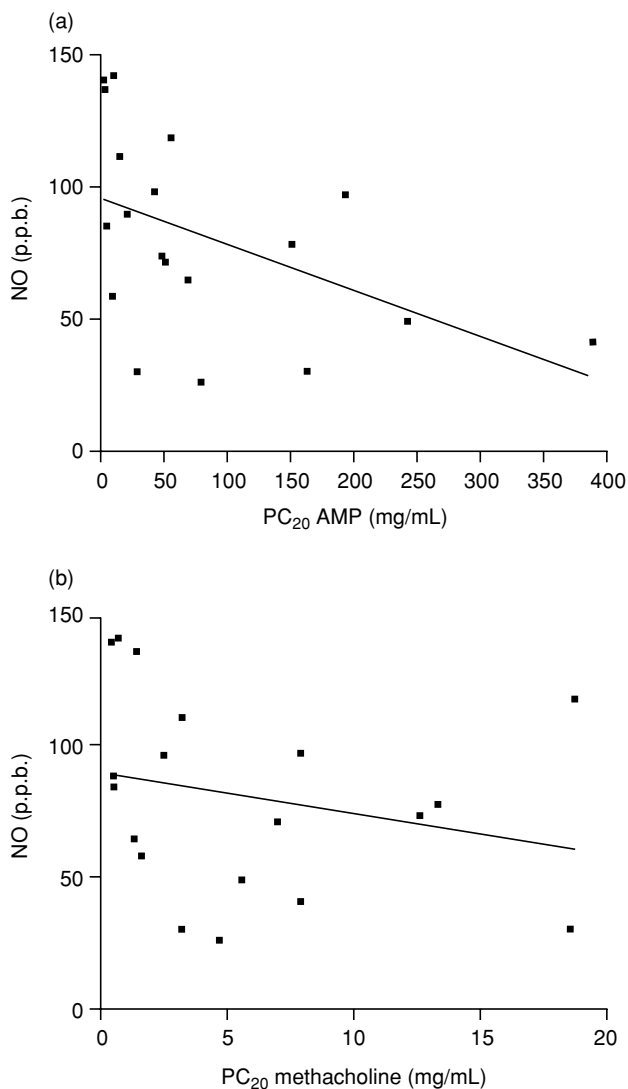


Fig. 2. Correlation between exhaled nitric oxide (NO) concentrations and airway responsiveness to (a) AMP and (b) methacholine in subjects with asthma ($\rho = -0.57$, $P = 0.02$ for AMP and $\rho = -0.35$, $P = 0.14$ for methacholine). PC_{20} = provocative concentration causing a 20% fall in FEV_1 .

significantly elevated ENO levels, as compared with ENO levels from subjects with allergic rhinitis. Thus, the results of the present study indicate that the presence of lower airways disease is an important determinant of increased ENO levels. Previous studies on differences in ENO levels between asthmatics and subjects with allergic rhinitis reached different conclusions. Gratziau and colleagues [19] reported that the levels of ENO in asthmatic patients were similar to those in subjects with allergic rhinitis (14.3 ± 1.3 p.p.b. and 13.3 ± 1.3 p.p.b., respectively). There are several possible explanations for this apparent contradiction. In the study by Gratziau et al. [19], the expiratory flow was 250 mL/s, whereas the expiratory flow used in the present study was 45 mL/s. The concentrations of ENO are inversely related to expiratory flow rate [35] and therefore their lower values than ours for ENO are likely attributable to methodological differences. Furthermore, subjects in the study by Gratziau et al. [19] were sensitized to different allergens, whereas our subjects were sensitized to the same allergen. This is important because some studies have shown an association between the pattern of allergen sensitization and ENO concentrations [32, 36]. In another study from the same group, no difference was found in ENO levels between patients with seasonal asthma and subjects with seasonal allergic rhinitis [20]. The reasons for such discrepancies might be related to differences in patients characteristics, to diversity in the disease activity in the subjects studied, or to important differences in methodology.

Allergic rhinitis and asthma are closely related diseases, and several reports have shown that subjects with allergic rhinitis may have bronchial inflammation in induced sputum [37], BAL fluid [38] and bronchial biopsy specimens [39]. If ENO levels reflect the presence of airway inflammation, the higher concentration of ENO in our subjects with asthma may reflect the higher degree of lower airways inflammation. However, the correlations between ENO and direct measures of airway inflammation in asthma have been of relatively small magnitude [13, 14]. In addition, the relationship between ENO levels and histologic markers of bronchial inflammation has not been studied in subjects with allergic rhinitis. Until these questions are answered the mechanism responsible for the reported differences will remain elusive. However, it is important to clarify that our pollen-sensitive subjects with asthma and/or rhinitis were tested during a period of natural pollen exposure. Since there is convincing evidence that, in sensitized subjects with asthma [40] or allergic rhinitis [20, 41, 42], natural allergen exposure during a pollen season results in increased concentrations of ENO, it may be speculated that natural allergen exposure may promote a greater effect on ENO concentrations in asthmatics than in subjects with allergic rhinitis. Although this needs to be examined in careful prospective studies, it would be of interest to repeat this type of study out of the pollen season.

We do not believe that our findings can be explained by measurement errors, since the results were obtained after carefully addressing such aspects of methodology as study design and measurement procedures. Firstly, none of the subjects was being treated with medications that could have affected ENO levels. Secondly, contamination of the exhaled air by NO-rich air from the nasal cavity is unlikely, as the soft palate elevates when expiration is performed against a resistance [35]. Finally, other factors known to influence the concentration of ENO,

such as current smoking and acute upper and chronic lower respiratory tract infections were absent in the subjects studied.

In subjects with allergic rhinitis, we were not able to show a significant relationship between ENO values and either PC₂₀ of methacholine or PC₂₀ of AMP. These results confirm previous observations [41, 42]. However, methacholine and AMP PC₂₀ values were measurable in only seven and eight subjects, respectively; therefore, it is likely that because of the low number of subjects, there was not enough statistical power to demonstrate such a relationship and the negative finding could be a type II statistical error.

In keeping with previous reports [43], we have detected that, in subjects with pollen-induced asthma, there is a significant correlation between ENO levels and AMP responsiveness, but not between ENO levels and methacholine responsiveness. This could be due to the fact that the two bronchoconstrictors are identifying different alterations in the lower airways. Although the critical features that determine the presence of increased responsiveness to direct and indirect bronchoconstrictors are not yet identified, some studies have demonstrated that AMP caused mast cell degranulation in the lower airways [3]. Since mast cells are believed to play a predominant role in asthmatic airway inflammation, the response to AMP may reflect acute inflammatory changes in the bronchial mucosa, whereas methacholine responsiveness might predominantly be dependent on structural changes of the airways [44]. If ENO levels reflect the presence of airway inflammation, the findings of this study support the hypothesis that responsiveness to AMP may be a more sensitive marker for assessing airway inflammation in asthma compared to methacholine.

In subjects sensitized to the same allergen, we have found that patients with asthma have higher concentrations of ENO than those with allergic rhinitis. In asthmatics, there is a significant correlation between ENO levels and AMP responsiveness but not between ENO levels and methacholine responsiveness. These results suggest that atopy is not the only determinant of increased ENO levels detected in subjects with asthma, and that airway responsiveness to AMP may be a more sensitive marker for assessing airway inflammation in asthma compared to methacholine.

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