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EXHALED NITRIC OXIDE IS RELATED TO BRONCHIAL EOSINOPHILIA AND AIRWAY HYPERRESPONSIVENESS TO BRADYKININ IN ALLERGEN-INDUCED ASTHMA EXACERBATION

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Summary

Exhaled nitric oxide (FeNO) has been associated with bronchial eosinophilia and with airway hyperresponsiveness (AHR) in mild stable asthma. We previously demonstrated in a large project that allergen exposure is able to raise FeNO and to worsen AHR to bradykinin. We postulated that allergen-induced increase in FeNO could be related to heightened mucosal eosinophils and AHR to bradykinin in atopic asthma. We performed a new immunohistochemical analysis on bronchial biopsies specimens, previously obtained from the same large project, in order to assess the number of mucosal eosinophils (EG-2+ cell) and other inflammatory cells at 48 hours after diluent and allergen exposures. Inflammatory cell counts were related to FeNO and AHR to BK (expressed as logPD20 bradykinin). In 10 atopic mild asthmatics, we found that the numbers of EG-2+ and CD4+ cells in bronchial submucosa were significantly increased after allergen compared to the respective counts after diluent (p < 0.01). EG-2+ cells in the bronchial submucosa were negatively correlated with logPD20 bradykinin only after allergen challenge (rho = -0.709, p = 0.027). We also found a positive strong correlation between EG-2+ cells and FeNO values in atopic asthmatics at 48 hours after both diluent (rho = 0.746, p = 0.017) and allergen (rho = 0.644, p = 0.049) challenge. FeNO values negatively correlated with responsiveness to bradykinin only after allergen challenge (rho = -0.675, p = 0.039). This study indicates that after allergen exposure heightened level of exhaled NO may reflect augmented airway eosinophilic inflammation and airway responsiveness to bradykinin indicating loss of asthma control. Michi, ora è più chiara?

Word count in abstract: 254
INTRODUCTION

A plethora of studies by using bronchial mucosal biopsies demonstrated that bronchial asthma is a chronic inflammatory disease of the airways characterized by the presence of an array of activated inflammatory cells and mediators. Biopsy specimens from proximal bronchi obtained through a fiberoptic bronchoscope are the most direct method of measuring airway inflammation and are considered the “gold standard” for assessment of airway inflammation in asthma against which other methods should be compared (1). Different noninvasive methods have been proposed for monitoring inflammation in asthma, and in particular exhaled nitric oxide [FeNO] has been indicated as surrogate marker of airway inflammation (2).

FeNO is elevated in patients with atopic asthma (2) and is related, before and after steroid treatment, to sputum eosinophilia (3, 4) and bronchoalveolar lavage [BAL] eosinophil counts (5) in adult patients, and to airway eosinophilic inflammation in BAL (6) and biopsies of children patients (7). In addition, in sensitized asthmatics with late asthmatic response [LAR] after allergen exposure FeNO is elevated (8) suggesting that FeNO may reflect allergic inflammation in exacerbated asthmatic airways.

Airway hyperresponsiveness [AHR] to nonspecific bronchoconstrictor stimuli is a key feature of asthma and is associated with the underlying inflammatory process of the disease (9). AHR is regarded as a marker of disease severity and control (9). A previous study showed a significant relationship between AHR to bradykinin, but not to methacholine, and eosinophilic inflammation in a wide range of subjects with mild-to-moderate asthma treated and not treated with corticosteroids (10), proposing AHR to bradykinin as a clinical marker of assessing severity of asthma.

We previously demonstrated in a larger asthma clinical trial that allergen exposure is
able to raise FeNO during LAR (8) and to worsen AHR to bradykinin (11). We postulated that allergen-induced increase in FeNO could be related to heightened mucosal eosinophils and AHR to bradykinin in atopic asthma. For this purpose we performed new immunohistochemical analysis on bronchial biopsies specimens, obtained from the same large project (11) in order to assess the number of mucosal eosinophils and other inflammatory cells at 48 hours after diluent and allergen (house dust mite) exposures. Furthermore, inflammatory cell counts were related to the previously published data (FeNO and AHR to bradykinin).
MATERIAL AND METHODS

**Subjects**

Ten non-smoking house dust mite [HDM]-atopic individuals with mild intermittent asthma participated in the study which was part of a larger project (11). The characteristics of the participants have been previously published (11). Briefly, all participants met the criteria of mild intermittent asthma and were symptom-free at the time of the study; none of them were on regular medications. Their values of baseline FEV1 were >70% of predicted and were hyperresponsive to inhaled histamine (provocative concentration producing a 20% fall from baseline FEV1 [PC20] < 8.0 mg/mL). None of the participants had a history of relevant allergen exposure or respiratory tract infection in the 6 weeks before and during the study. Inhaled short-acting β2-agonists and beverages containing xanthines were withheld for at least 12 hours before testing. The subjects had a documented early asthmatic response [EAR] and LAR to inhaled HDM extract in the screening period. The study was approved by the Medical Ethics Committee of the Leiden University Medical Centre, and all the patients gave written informed consent.

**Study design**

The study had a randomized, placebo-controlled and cross-over design. FeNO measurements, bradykinin challenges (to measure AHR to bradykinin) and bronchoscopy were performed in each patient 2 days after either allergen or diluent exposure (11). Each exposure was separated by a wash-out interval of at least 2 weeks. Exhaled NO measurements were performed before and after each challenge at 48 hours.
We considered the bronchial biopsies carried out at 2 days after diluent challenge as baseline (11).

**Bradykinin and allergen challenge, FeNO and bronchoscopy**

Bradykinin challenge (expressed as logPD$_{20}$ bradykinin), diluent/allergen challenge, FeNO measurements (ppb values) and fiberoptic bronchoscopy were performed as previously described (8, 11).

**Immunohistochemistry**

Three biopsy samples per subject were formalin-fixed and paraffin embedded. Four micrometer [$\mu$m] thick sections were used for immunohistochemistry. Sections were incubated overnight with mouse primary monoclonal antibodies directed against EG-2 (Pharmacia - Netherlands), CD4 (Novocastra – UK), CD8 (Novocastra, UK) and mast cell tryptase (DAKO, Denmark), and binding of the antibodies was detected as previously described (12).

**Quantitative analysis**

All coded biopsy specimens were examined by one observer, who was blinded to the patient and to the study day on which the biopsy was taken.

EG-2$^+$ and other inflammatory cells were quantified in the area 100 $\mu$m beneath the epithelial basement membrane in several non-overlapping high power fields until all the available area was covered. The final result was expressed as the number of positive cells per square millimetre of submucosa. Light microscopic analysis was performed at a magnification of 400X for quantification of the structural parameters. Morphometric
measurements were performed with a light microscope (Leitz Biomed, Leica, Cambridge, UK) connected to a video recorder linked to a computerized image system (Quantimet 500 Image Processing and Analysis System, Qwin V0200B Software, Leica).

**Statistical analysis**

As the main objective of the study was to evaluate correlations between airway responsiveness to bradykinin and airway inflammatory markers (FeNO and EG2+ or other inflammatory cells), the needed sample size was calculated, assuming that the expected correlation coefficients would be around 0.7, the type I error = 0.05, the power = 0.80 and the t-test on the correlation coefficient (ρ = 0) would be two-sided. With these assumptions the needed sample size should be 13.

All data are reported as median with upper and lower quartiles in parentheses unless otherwise stated. Wilcoxon signed rank test or paired t-test was applied to explore the effect of allergen on different parameters. Correlation analyses were made by Spearman rank correlation test (Rho). Statistical significance was accepted for a p value less than 0.05.
RESULTS

Quantitative immunostainings in bronchial submucosa and correlations between cells

Satisfactory endobronchial biopsies were obtained in all subjects. The mean area of examined submucosa was 0.296 ± 0.02 (mean ± SEM) mm$^2$ for all immunostainings performed after diluent and allergen challenge. Allergen challenge significantly increased the number of eosinophils (EG-2$^+$ cells) and CD4$^+$ cells in submucosa of the bronchial biopsies compared to diluent exposure (EG-2$^+$ cells: 730 (456-1203) cells/mm$^2$ vs 180 (101-268) cells/mm$^2$, $p = 0.004$; CD4$^+$ cells: 501 (444-601) cells/mm$^2$ vs 269 (220-381) cells/mm$^2$, $p = 0.010$) (Figure 1 and Figure 2). The counts of CD8$^+$ cells and mast cells in submucosa after diluent inhalation was not significantly changed by allergen (CD8$^+$ cells: 221 (140-347) cells/mm$^2$ vs 213 (152-309) cells/mm$^2$, $p = 0.846$; mast cells: 198 (143-257) cells/mm$^2$ vs 187 (146-242) cells/mm$^2$, $p = 0.695$) (Figure 2).

The count of EG-2$^+$ cells in the bronchial submucosa of atopic asthmatics were not correlated with CD4$^+$ cells after diluent or allergen exposure (rho = 0.054 and rho = -0.03, respectively) (data not shown). No significant correlations were found among the other inflammatory cells either after diluent or after allergen exposure (data not shown).

Responsiveness to bradykinin and FeNO

As previously described (11), logPD$_{20}$ bradykinin after allergen exposure diminished as compared to that observed after diluent inhalation ($p = 0.019$) (data not shown).
As previously reported (8), baseline FeNO was similar in the 2 study days before placebo and allergen challenges (p = NS). The values of FeNO at 48 hours after allergen are significantly increased from the levels of FeNO at 48 hours after diluent (p = 0.002).

**Correlation between inflammatory cells in bronchial submucosa, responsiveness to bradykinin and FeNO**

The count of EG-2$^+$ cells in the bronchial submucosa of atopic asthmatics were negatively correlated with logPD$_{20}$ bradykinin after allergen challenge (rho = -0.709, p = 0.027) (Figure 3A) but not after diluent exposure (rho = 0.07, p = 0.865) (data not shown). Conversely, the number of CD4$^+$ cells in the bronchial submucosa was not correlated with logPD$_{20}$ bradykinin both after diluent (rho = 0.018, p = 0.973) and allergen (rho = 0.079, p = 0.838) exposure (data not shown).

We also found a positive strong correlation between EG-2$^+$ cells and FeNO values in atopic asthmatics at 48 hours after both diluent (rho = 0.746, p = 0.017) (data not shown) and allergen (rho = 0.644, p = 0.049) challenge (Figure 3B). The number of CD4$^+$ cells in the bronchial submucosa was not correlated with FeNO after diluent (rho = -0.164, p = 0.657) and allergen (rho = -0.152, p = 0.682) exposure (data not shown).

In addition, FeNO values negatively correlated with responsiveness to bradykinin only after allergen challenge (rho = -0.675, p = 0.039) (Figure 4) but not after diluent exposure (rho = 0.079, p = 0.838) (data not shown).
DISCUSSION

At our knowledge, these data showed for the first time that FeNO could be associated with heightened mucosal eosinophils and AHR to bradykinin in atopic asthma at 48 hours after allergen exposure suggesting that measurements of FeNO in atopic asthma during allergic exacerbation may mirror the recruitment of eosinophils in the asthmatic airways in conjunction with elevated AHR.

An enhancement of eosinophils in bronchial mucosa of atopic asthmatics has been previously reported at 24 and 48 hours after allergen (13-15), but we did not find studies exploring the relationship between cellular inflammation on the airway wall of asthmatics and FeNO or airway responsiveness at 48 hours after allergen exposure. Previous studies have shown a significant relationship between airway eosinophilia, either in sputum and BAL of adult mild asthmatics, and FeNO levels in stable conditions (3, 5) or segmental NO at 24 hours after segmental allergen challenge (16). Here, we reported that either eosinophilic count after diluent or increased number of mucosal eosinophils at 48 hours after allergen exposure is clearly related to heightened FeNO values in atopic asthma pointing to this non-invasive method as a good and useful clinical marker for monitoring activation of eosinophilic inflammation either in stable condition (as phenotypic expression of asthma activity per se) or in the delayed phase of allergen-induced asthma exacerbation.

AHR to bradykinin, an indirect stimulus, is observed only in asthmatics (11, 17), whilst methacholine, a direct agonist, may induce bronchoconstriction also in normal subjects (18), indicating that airway sensitivity to bradykinin is due to functional airway abnormalities which are present specifically in asthma (10). In a previous study Roisman et al. found that AHR to bradykinin, but not to methacholine, in stable mild-to-
moderate asthma is correlated with eosinophil count in BAL, in the epithelium, in the lamina propria and in total submucosa (10) demonstrating that AHR to bradykinin is dependent on the eosinophilic inflammatory status of the airways. The present study is in line with the latter observation showing that in subjects with atopic mild intermittent asthma after allergen exposure heightened AHR to bradykinin is related to increased mucosal eosinophilia suggesting a main role for eosinophils in augmented airway reactivity to bradykinin during allergen-induced asthma exacerbation. The lack of correlation between eosinophil counts in bronchial mucosa and logPD_{20} bradykinin after diluent challenge in the present study is probably due to the lower level of airway inflammatory activity in our “asymptomatic mild intermittent asthmatics” at baseline compared to the “mild-to-moderate asthmatics” (in part treated with inhaled steroids) showed by Rosiman et al. (10).

In previous reports controversial correlations between FeNO and methacholine or histamine (direct stimuli) responsiveness in mild stable asthma have been shown (19, 20). More recently, it has been demonstrated an association between increased FeNO values and the degree of AHR to the indirect stimulus mannitol (R=0.48) or, to a lesser extent, to the direct stimulus methacholine suggesting that the responsiveness to indirect stimulus might be more reflective of asthma with ongoing airway inflammation (21). In this study we found that after allergen challenge elevated FeNO is related to AHR to bradykinin (indirect stimulus) in sensitized asthmatics. We previously demonstrated that NO derived from the constitutive NO synthase [cNOS] isoform is involved in the regulation of bronchomotor tone in asthma (11), whilst NO derived from the inducible NO synthase [iNOS] isoform is a pro-inflammatory marker and the most important component of FeNO in asthma (22, 23). In the past we showed that allergen-induced
increase in AHR to bradykinin is dependent on the impairment of bronchoprotective cNOS in airway epithelium of atopic asthmatics (11). On the other hand, in the same larger asthma clinical trial it has been reported a dramatic increase in FeNO after allergen in conjunction with upregulation of iNOS in airway epithelium (8, 11).

Thus, we may suggest that elevated FeNO, marker of asthmatic inflammatory activity, could indirectly reflect a potentiation of AHR to bradykinin in allergen-induced asthma exacerbation.

Finally, even though we analyzed data of a limited population (10 patients), the study was only a little underpowered for the correlations between airway responsiveness to bradykinin and FeNO or EG2⁺ cells or between FeNO and EG2⁺ cells but the needed sample size was very close to the used one. Regarding the other correlations, these were clearly not present (correlation coefficient close to zero) and a reasonable increase in the sample size would not have changed the conclusion of no relationship. In addition, we were not able to increase the number of patients to reach the needed sample size since we performed new immunohistochemical analysis on bronchial biopsies specimens that had been obtained during a previous study (11).

The present study showed, for the first time, a relationship between FeNO values, eosinophilic inflammation and AHR to bradykinin during allergen induced exacerbation. We may point out that after allergen exposure in sensitized asthmatic airways eosinophilic inflammation with the subsequent release of pro-inflammatory mediators is able to increase inducible NO synthase-derived NO production (2), to directly liberate bradykinin (24) and to heighten AHR to bradykinin (11).

In conclusion, we demonstrated that elevated FeNO seems to be directly associated with an increased number of eosinophils and with a potentiated airway responsiveness
to bradykinin in atopic asthmatics after allergen exposure. All of these data indicate that eosinophilic inflammation in the airway mucosa of asthmatics after allergen exposure could specifically be reflected by FeNO levels. Finally, the novel message of the present study derives also from the evidence of a link between exhaled NO and airway motor response to bradykinin in atopic asthmatics after allergen exposure supporting a role for the non-invasive monitoring of exhaled NO and airway responsiveness to bradykinin in asthma control. In future work, the relationships between oxidative stress (Montuschi P, Currò D, Ragazzoni E, Preziosi P, Ciabattoni G. Anaphylaxis increases 8-isoprostaglandin F2alpha release from guinea-pig lung in vitro Eur J Pharmacol. 1999 Jan 15;365(1):59-64) and different markers of eosinophilic airway inflammation (Montuschi P LC/MS/MS analysis of leukotriene B4 and other eicosanoids in exhaled breath condensate for assessing lung inflammation. J Chromatogr B Analyt Technol Biomed Life Sci. 2009 May 1;877(13):1272-80; Montuschi P, Santonico M, Mondino C, Pennazza G, Mantini G, Martinelli E, Capuano R, Ciabattoni G, Paolesse R, Di Natale C, Barnes PJ, D'Amico A. Diagnostic performance of an electronic nose, fractional exhaled nitric oxide, and lung function testing in asthma.; Chest. 2010 Apr;137(4):790-6) or AHR after allergen exposure in patients with atopic asthma should be studied. Whether leukotriene receptor antagonists, which reduce FENO concentrations in patients with atopic asthma (Montuschi P, Mondino C, Koch P, Ciabattoni G, Barnes PJ, Baviera G. Effects of montelukast treatment and withdrawal on fractional exhaled nitric oxide and lung function in children with asthma. Chest. 2007 Dec;132(6):1876-81), also decrease airway eosinophils and AHR should be clarified. MI SEMBRA DI AVER CAPITO CHE IL REVISORI SI CHIAM.................MONTUSCHI.....Chissà come mai!!!????
ACKNOWLEDGMENTS

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LEGENDS TO THE FIGURES

**Fig. 1.** Immunoreactivity for EG-2⁺ and CD4⁺ cells in the mucosa of bronchial biopsies of atopic asthmatics 48 hours after diluent (panels A and C) and after allergen (panels B and D) challenge. Original magnification: X 400.

**Fig. 2.** Changes in the numbers of EG-2⁺, CD4⁺, CD8⁺ and mast cells in the mucosa of atopic asthmatics at 48 h after diluent [Dil] and allergen [All] challenge. Each bar indicates the median values in the respective group and boxes represent lower and upper quartiles.

**Fig. 3.** Correlation between the numbers of EG-2⁺ cells in the mucosa of the bronchial biopsies and logPD₂₀ bradykinin (Panel A) or FeNO levels (Panel B) in atopic asthmatics after allergen challenge. rho: Spearman rank correlation coefficient.

**Fig. 4.** Correlation between FeNO levels and logPD₂₀ bradykinin in atopic asthmatics after allergen challenge. rho: Spearman rank correlation coefficient.