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Exhaled nitric oxide during acute changes of airways calibre in asthma

P. Garnier, I. Fajac, J.F. Dessanges, J. Dall'Ava-Santucci, A. Lockhart, A.T. Dinh-Xuan

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ABSTRACT: It has been shown that endogenous nitric oxide (NO), measured in exhaled air, is increased in asthmatic subjects and after allergen challenge in sensitized animals. NO is also a paracrine molecule with some, though weak, bronchodilator effects. However, whether the amount of endogenous NO that originates in the lungs can reflect the degree of bronchial tone and airways calibre in asthmatic subjects has not yet been investigated. The aim of this study was, therefore, to determine whether NO production could be modified by acute changes of airways calibre in mild, nonatopic, asthmatic subjects.

NO output was measured in the exhaled air of 14 steroid-free asthmatics, 8 steroid-treated asthmatics and 21 control subjects. In seven steroid-free asthmatics, exhaled NO was measured after methacholine challenge, and then after salbutamol-induced bronchial dilatation. Exhaled tidal breathing was collected for 30 s and NO in the exhaled air was measured with a chemiluminescence analyser.

Both NO concentration and its output were significantly higher in the steroid-free asthmatic patients (15.6±1.5 parts per billion (ppb) and 6.3±0.7 nmol·min^{-1}, respectively) as compared with the control subjects (8.9±1.0 ppb and 3.5±0.3 nmol·min^{-1}, respectively; $p<0.001$ for both) and with the steroid-treated asthmatic patients (11.3±3.3 ppb and 3.7±0.9 nmol·min^{-1}, respectively; $p<0.05$ for both). Neither methacholine-induced bronchial obstruction nor salbutamol-induced bronchial dilatation caused a significant change in exhaled NO.

We conclude that NO production is higher in steroid-free than in steroid-treated asthmatics and in control subjects. Furthermore, NO production is not affected by acute pharmacologically-induced changes of airways calibre in asthmatic subjects. Our results suggest that NO production is a marker of airways inflammation rather than an endogenous modulator of bronchial tone in asthma.
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Service de Physiologie-Explorations Fonctionnelles, C.H.U. Cochin Port-Royal, Université René Descartes, Paris, France.

Correspondence: A.T. Dinh-Xuan
Service de Physiologie-Explorations Fonctionnelles
Hôpital Cochin
27, rue du faubourg Saint-Jacques
75679 Paris cedex 14
France

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The free radical gas nitric oxide (NO) is a potent pulmonary vasodilator [1], which is synthesized from the amino acid, L-arginine, and molecular oxygen through the action of constitutive and inducible NO synthase (NOS) isoforms [2]. Inducible NOS (iNOS) is expressed in various resident respiratory and inflammatory cells [3–7]. Its expression is enhanced after exposure to certain cytokines [7], and is inhibited by corticosteroids [8].

There is a growing body of evidence that NO might play a role in asthma [9, 10]. Exogenous NO, given by inhalation, reverses methacholine-induced bronchoconstriction in animals [11–13] and in humans, including healthy subjects [14] and asthmatic patients [15]. NO is a potent bronchial vasodilator in animal airways [16], and may either increase [17] or tonically suppress [18] airways plasma exudation in animals. Various investigators have found that the amounts of NO measured in the exhaled air [19, 20] are higher in asthmatic patients as compared with healthy control subjects [21–23]. Furthermore, expired NO output is increased both after allergen- and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-induced bronchoconstriction in sensitized guinea-pigs [24].

The mechanism of the increase in NO output has not yet been fully investigated, and changes in bronchial tone might, by themselves, modify NO production and output in such experiments. Our aim was, therefore, to study the effects on expired NO of pharmacological agents with no known proinflammatory effects, namely methacholine and salbutamol, in order to separate the effects of inflammation, on the one hand, and changes in bronchial tone, on the other hand, on NO production in asthma. Thus, we firstly determined the level of NO in the exhaled air of steroid-free asthmatic patients and compared it with that of steroid-treated asthmatic patients and control subjects. Secondly, we measured NO production before and after methacholine- and salbutamol-induced acute changes in airways calibre in asthmatic patients.

Subjects and methods

Subjects

NO output was measured in the exhaled air of 22 asthmatic patients and 21 healthy subjects. The asthmatic patients (10 males and 12 females; (mean±SEM) age 41±4 yrs) had a history of atopic asthma graded as mild. All of them were nonsmokers. Fourteen patients received either no regular treatment or inhaled β_2-agonists alone. The remaining eight patients were treated with inhaled steroids (beclomethasone dipropionate or budesonide) on a regular basis. The steroid-treated patients also received inhaled β_2-agonists, but only one of them did so on a regular basis (two puffs of salbutamol, four times a day), whereas the remaining seven took β_2-agonists only on an as-needed basis. The group average value (±SEM) of forced expiratory volume in one second (FEV_1) for the steroid-free and the steroid-treated patients was 92±4 and 73±7% of predicted values, respectively.

The 21 nonatopic controls (11 males and 10 females; (mean±SEM) age 34±3 yrs) were nonsmokers. None of them had a history of respiratory or cardiovascular disease or was receiving long-term medication. Their FEV_1 was 95±2% pred. The study was approved by the Ethics Committee of our hospital. Each subject signed an informed consent form.

Study design

Lung function parameters and NO in the exhaled air were measured in all subjects at baseline. In seven of the steroid-free asthmatic subjects whose FEV_1 was normal, NO in the exhaled air was also measured 2, 5, 10 and 20 min after completion of a bronchial challenge, with methacholine having caused a fall in FEV_1 ≥15% of baseline. NO in the exhaled air was measured again, 10 min after inhalation of salbutamol (400 µg), when FEV_1 had returned to the prechallenge value.

Technical details

Lung function measurements. FEV_1 was measured with an automated electronic spirometer (Autospiro AS 500; Minato, Medical Science Co., Osaka, Japan), whose accuracy was regularly verified with a calibrated 3 L syringe.

NO measurements. NO was measured on a chemilumiscence analyser (CLD 700 AL; Eco Physics) with a detection threshold of 1 part per billion (ppb). Before each study, the analyser was calibrated using NO-free air and a certified gas cylinder of 700 ppb of NO. The subjects with the nose occluded were asked to breathe at tidal volume for 30 s through a mouthpiece connected to a Hans Rudoph valve. Inspired air was delivered from a tank containing 21% O_2 balanced in 79% N_2. Even in the ppb range, no trace of NO was detectable in the inspired air. Expired air was collected during the 30 s in a Douglas bag, from which gas was sampled through the NO analyser at a known flow-rate during a period of time whose duration was precisely measured with a stopwatch. The volume of gas remaining in the Douglas bag was measured with a water-sealed spirometer, and was added to the volume of the gas sample.

Methacholine bronchial challenge. Methacholine bronchial challenge was performed according to standard procedures. Briefly, methacholine was administered with a breath-activated dosimeter (Rosenthal-French, model D-2A; Laboratory of Applied Immunology, Baltimore, MD, USA) and a nebulizer (No. 646; DeVilbiss Co., Somerset, PA, USA). The aerosol was produced by an oxygen flow at a pressure of 1.38 kPa, and was inhaled during tidal breathing with the patient's nose occluded. Each activation of the dosimeter delivered a preset quantity of nebulized solution (output 120 µL) in order to deliver the required amount of methacholine. Volume history was standardized by having each subject inhale from functional residual capacity (FRC) to total lung capacity (TLC). Methacholine was diluted in NaCl 0.09%. The first dose of methacholine delivered was 100 µg. Thereafter, one to four serially doubling doses (2–16 times the initial dose) were administered at intervals of 5 min, until a fall in FEV_1 ≥15% from prechallenge FEV_1 was reached.

Statistical analysis

Comparisons between groups were made by analysis of variance (ANOVA) and Scheffe's test. Simple linear regression was used to correlate the percentage changes in exhaled NO from baseline levels with percentage changes of FEV_1 induced by methacholine and salbutamol. Results are expressed as mean±SEM. A p-value of less than 5% was considered significant.

Results

NO at baseline

NO concentration and NO output in the exhaled air were significantly higher in the steroid-free asthmatic patients (15.6±1.5 ppb and 6.3±0.7 nmol·min^{-1}, respectively), as compared both to the steroid-treated asthmatic patients (11.3±3.3 ppb and 3.7±0.9 nmol·min^{-1}, respectively) ($p<0.05$ for both) and to the control subjects (8.9±1.0 ppb and 3.5±0.3 nmol·min^{-1}, respectively) ($p<0.001$ for both) (fig. 1). Neither NO concentration nor NO output of steroid-treated patients differed from those of control subjects (fig. 1).

NO measurements after methacholine challenge and salbutamol

After bronchial methacholine challenge, the mean fall of FEV_1 was 19±2%. NO production was lower after methacholine provocation in five patients and higher in the remaining two (fig. 2a). For the group as a whole, no significant change in NO production was observed after methacholine (4.2±0.7 nmol·min^{-1} before and 3.3±

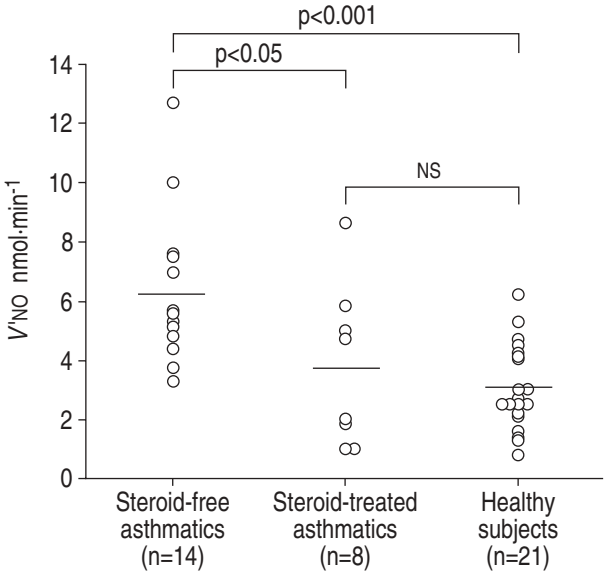

Fig. 1. – Individual values of NO output (V'_{NO}) in steroid-free, steroid-treated asthmatic patients and healthy nonsmoking subjects. Horizontal bars represent mean values for each group.

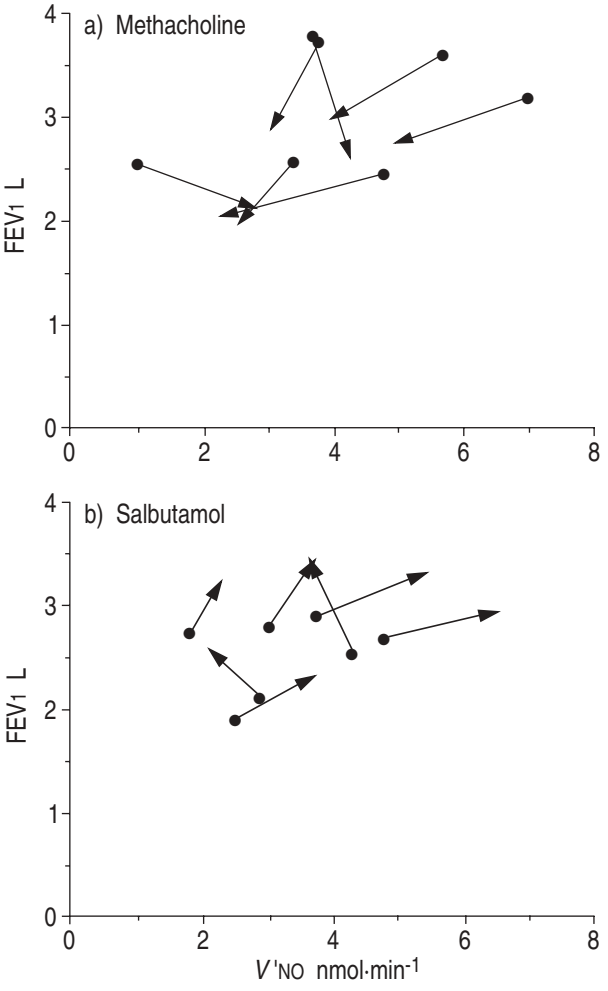

Fig. 2. – Individual changes in NO output (V'_{NO}) in asthmatic patients a) after bronchial obstruction induced by methacholine. V'_{NO} does not significantly change with methacholine-induced fall in forced expiratory volume in one second (FEV1). b) after bronchial dilatatation induced by salbutamol. V'_{NO} does not significantly change with salbutamol-induced increase in FEV1.

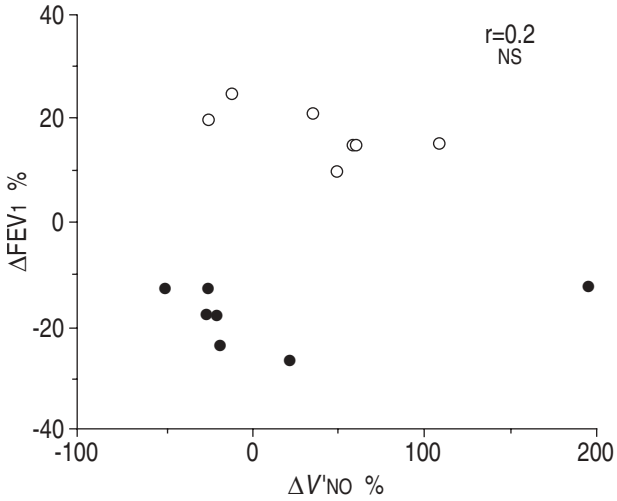

Fig. 3. – Lack of relationship between percentage changes of NO output ($\Delta V'_{NO}$) and acute changes of airway calibre induced by methacholine (closed symbols) and salbutamol (open symbols). ΔFEV1: change in forced expiratory volume in one second.

0.4 nmol·min^{-1} after; NS). In addition, there was no correlation between the methacholine-induced fall in FEV1 and the variation of NO output before and after methacholine challenge (r=0.11; NS).

Inhalation of salbutamol induced a mean increase in FEV1 of 16±2%. NO production increased in 5 out of 7 patients (fig. 2b). However, no significant change in the group-average NO output was observed after salbutamol-induced bronchodilatation (3.3±0.4 nmol·min^{-1} before and 4.3±0.6 nmol·min^{-1} after; NS). Similarly, there was no correlation between the increase in FEV1 and the variation between pre- or post-salbutamol NO output (r=0.18; NS).

No correlation was found either when considering the changes in airway calibre, as shown by an increase or a decrease in FEV1 from baseline FEV1 with respect to the changes in NO output from baseline levels (r=0.2; NS) (fig. 3).

Discussion

Our study shows that NO output in the exhaled air is increased in steroid-free asthmatic patients as compared with control subjects, whereas NO output of steroid-treated asthmatic patients does not differ from control subjects. Furthermore, NO output is not affected by acute pharmacologically-induced changes of airway calibre in asthmatic patients.

The increased NO production in the steroid-free asthmatic patients as compared to control subjects is consistent with previous studies [21–23]. It is likely that NO is predominantly synthesized in the upper respiratory tract [21, 25, 26], which probably accounts for most of the increase of exhaled NO during acute viral infections in healthy subjects [27].

NO is formed from the amino acid, L-arginine, by the action of an enzyme NOS, which exists as constitutive and inducible forms [2]. In animals, iNOS is expressed in various respiratory cells, such as bronchial epithelial cells [3], alveolar macrophages [4], lung fibroblasts [5], and pulmonary vascular smooth muscle [7]. In humans,

both constitutive and inducible isoforms of NOS are expressed, and have recently been localized by immunocytochemical and immunohistochemical techniques, in lung tissue [28, 29]. Transcription of the genes encoding for constitutive neuronal and inducible NOS has also been demonstrated by reverse transcription-polymerase chain reaction in normal human lung epithelial cells in culture [30]. Furthermore, recent evidence suggests that an inducible-like NOS is constitutively expressed in human paranasal sinuses [31], and that NO is continuously synthesized by iNOS in normal human airway epithelium *in vivo* [32]. However, expression of iNOS seems to depend upon the conditions that are present in the airway of normal individuals, as removal of the epithelial cells from the airway environment leads to rapid loss of iNOS expression [32]. It is likely that the many inflammatory processes which occur within the airway might account for the increased expression of iNOS in bronchial epithelium [33], hence increased NO production in disease such as asthma [21–23], or bronchiectasis [34].

In asthma, many cytokines are upregulated [35, 36], including tumour necrosis factor-α, interleukin-1β, and interferon-γ, which are known to induce NOS [37]. It is also known that the latter is inhibited by corticosteroids [8]. Therefore, cytokine-induced upregulation of NOS may well account for the increased output of NO in steroid-free asthmatic subjects. On the other hand, the normal NO output that we found in steroid-treated asthmatics is compatible with the downregulation of iNOS, which is consistent with a previous report [22]. NO is a potent vasodilator both for the pulmonary [1] and bronchial [16] circulations. Furthermore, it increases plasma exudation from airways vessels [17]. Thus, the high level of NO production that we observed in asthmatic patients may reflect the harmful effects that NO might exert, as a proinflammatory mediator, in asthma. However, further studies are needed to relate the level of NO production and the extent of inflammation in asthma.

We found no significant change in NO production after acute pharmacologically-induced variations of airways calibre. Therefore, the immediate and short-lasting increase in NO output that has been described both after antigen-induced bronchoconstriction and administration of the smooth muscle constricting agent $PGF_{2\alpha}$ in guinea-pig [24] cannot be ascribed to the changes in bronchomotor tone themselves. It seems, therefore, unlikely that the amounts of exhaled NO could be used as a marker to either reflect bronchial tone or quantify airways calibre in asthma.

NO is also a neurotransmitter released from inhibitory nonadrenergic-noncholinergic (iNANC) nerves in human airways [38–42]. Furthermore, endogenous NO released from iNANC nerves inhibits cholinergic neural responses *via* functional antagonism of acetylcholine at the airways smooth muscle [42]. *In vivo*, inhaled NO reverses methacholine-induced bronchoconstriction in animals [11–13], and both in healthy [14] and asthmatic [15] subjects. Furthermore, an inhibitory effect of endogenous NO on antigen-induced bronchoconstriction has been reported in the guinea-pig [43]. However, in the present study, we found no correlation between NO production in exhaled air before methacholine provocation and the fall in FEV_1 induced by methacholine. This makes it unlikely that endogenous NO may somehow modulate methacholine-induced bronchoconstriction in asthmatic subjects.

In summary, we have shown that NO production is higher in steroid-free asthmatic than in steroid-treated asthmatic patients and in control subjects, and that NO output is not affected by acute pharmacologically-induced changes of airway calibre in asthmatic patients. Our results, therefore, provide circumstantial evidence that the increased NO output observed in asthma is best explained by upregulation of inducible NO synthase in airways of asthmatic subjects. Furthermore, it seems likely that exhaled NO should be regarded as a marker of airways inflammation rather than as an endogenous modulator of bronchial tone in asthma.

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