

## Original article

# Effect of inhalation aspirin challenge on exhaled nitric oxide in patients with aspirin-inducible asthma

**Background:** A complex relationship between arachidonic acid metabolites and nitric oxide (NO) synthesis has been reported in asthma. The effects of inhaled aspirin on fractional exhaled NO (FENO) in patients with aspirin-tolerant (ATA) and aspirin-inducible (AIA) asthma compared with normal controls have been investigated.

**Methods:** The FENO was measured baseline, after saline and lysine-aspirin (L-ASA) bronchial challenge in 10 patients with ATA and in 10 patients with AIA [mean (PD<sub>20</sub>FEV<sub>1</sub> L-ASA): 14.7 ± 12.7 mg], who had comparable age and baseline FEV<sub>1</sub>. Ten healthy subjects served as controls. Sputum eosinophils were counted after saline and after L-ASA challenge in the two groups of asthmatics.

**Results:** Asthmatic patients had baseline FENO significantly higher than controls (29.7 ± 6.8 vs 9.8 ± 2.05 p.p.b. respectively,  $P < 0.0001$ ). No difference was observed in methacholine PD<sub>20</sub>FEV<sub>1</sub> and baseline FENO between ATA and AIA patients. After L-ASA inhalation, FENO increased significantly only in patients with AIA, reaching the peak value 4 h after bronchoconstriction (from 31.1 ± 6 to 43 ± 4.8 p.p.b.,  $P < 0.001$ ), while no change was observed in patients with ATA and in controls. Sputum eosinophils increased significantly after L-ASA inhalation only in patients with AIA (from 8.1 ± 2.7 to 11.1 ± 2.8%,  $P < 0.005$ ) and there was a significant relationship between the increase in sputum eosinophils and the increase in FENO after ASA challenge.

**Conclusion:** Exhaled NO may indicate eosinophilic airway inflammation during ASA exposure in patients with ASA inducible asthma.

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Key words: aspirin inducible asthma; exhaled nitric oxide; sputum eosinophils.

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Products of the cyclo-oxygenase (COX) pathway of arachidonic acid have an important role in the inflammatory cascade and in the pathophysiology of asthma. Thromboxane A<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2</sub>α not only cause bronchoconstriction, but also increase airway vascular permeability and reactivity (1, 2). On the contrary, PGE<sub>2</sub> causes a small degree of bronchodilatation and inhibits exercise- and allergen-induced bronchoconstriction (3). Fractional NO concentration in the exhaled air (FENO) is increased in asthmatic patients compared with normal subjects (4, 5) and the increased FENO is thought to depend on airway inflammation (6, 7). The relationship between NO and arachidonic acid metabolites is quite complex and experimental observations gave controversial results. Both positive and negative effects of NO on COX expression have been encountered in experimental systems, depending on the cell type studied (8). In pancreatic islet inflammation, NO has been shown to activate COX leading to overproduction of prostaglandins, which in turn inhibit the production of NO (9). On the contrary, recent evidence suggests that an anti-inflammatory effect of COX inhibitors lies in the inhibi-

tion of the inducible form of NO synthase (iNOS) (10). Both in normal and in asthmatic patients inhaled PGE<sub>2</sub> and PGF<sub>2</sub>α rapidly reduce FENO, particularly in asthmatic patients, independently of changes in airway calibre (11). We wondered whether inhaled aspirin (ASA) may affect FENO, by its inhibitory effect on prostaglandin production. The effect of ASA on FENO could be different in normal subjects and in aspirin-tolerant asthmatic (ATA) patients, compared with patients with aspirin-induced asthma (AIA). Aspirin administration to patients with AIA have been reported to increase the production of leukotrienes (12), which may increase NO production either directly or through eosinophils recruitment (13, 14).

To investigate the effect of ASA on exhaled NO we measured FENO during inhaled ASA challenge in a group of ATA and AIA patients, compared with normal subjects. We reasoned that inhaled ASA could increase FENO by removing the tonic inhibitory effect of prostaglandins on NO production and, in AIA patients, by promoting a further increase in leukotrienes production.

## Methods

### Patients

We studied 10 patients with clinical history of AIA and 10 patients with ATA, recruited from our asthma clinic. The patients with AIA had previously undergone an ASA inhalation challenge, which clearly established their sensitivity to ASA. Within 2 months preceding the study, all patients had inhaled provocative tests with lysine-aspirin (L-ASA) performed and, in those sensitive to aspirin, the dose producing a 20% fall in a baseline forced expiratory volume in 1 s ( $FEV_1$ ) was established. At the time of the study, patients with AIA performed the bronchial challenge using a single dose of L-ASA, corresponding to the last cumulative dose administered in the preliminary test. All the patients were nonsmoking, in stable clinical conditions, had a baseline  $FEV_1$  above 80% of predicted, and were free of respiratory infection for at least 6 weeks. All the patients received a methacholine bronchial challenge at the time of enrolment. All the patients were steroid-naïve and treated only with inhaled  $\beta_2$ -agonists on demand, and five in each group with intranasal steroids for perennial rhinitis (fluticasone propionate 200  $\mu$ g and mometasone furoate 200  $\mu$ g per day, in seven and three patients respectively). Prior to the study, any inhaled adrenergic agents was stopped for at least 8 h. Three patients with AIA and four with ATA were atopic. Patients allergic to pollens were studied outside the pollen season. Ten healthy nonsmoking subjects of comparable age ( $43 \pm 9.8$  years, four female) served as controls. The study was approved by the Institutional Ethical Committee and written informed consent was obtained from each patient.

### Study design

The patients and controls attended the laboratory on two different days, with an interval of 7–10 days, in order to receive the inhalation challenge with normal saline in 1 day and with L-ASA in the other. Obviously, a double-blind design was possible only for controls and patients with ATA. Patients with AIA received the saline challenge first and a single dose of L-ASA corresponding to the last cumulative dose that caused a decrease in  $FEV_1$  of 20% compared with baseline in the preliminary test (15). In controls and in patients with ATA a single 128 mg dose of L-ASA was used for the L-ASA inhalation challenge. The FENO was measured baseline and 0.5, 1, 2, 4, 6 and 24 h after inhalation challenges. Six hours after saline and L-ASA challenges sputum was induced in asthmatic patients.

### Lysine-ASA bronchial challenge

The L-ASA challenge was performed according to a standardized method (16). Briefly, 1800 mg of L-ASA (Flectadol, Maggioni-Winthrop, Italy) as a powder containing 200 mg of glycine was made-up freshly on each challenge day with 5 ml of sterile water to produce a L-ASA solution containing 360 mg/ml, which was equivalent to 200 mg/ml of ASA. Increasing doses of ASA, starting from 0.5 mg, were administered by means of a breath-activated dosimeter (Mefar MB3; Markos, Brescia, Italy) set to deliver 10  $\mu$ l of solution during a deep breath until a decrease in  $FEV_1$  of at least 20% was observed (in patients with AIA) or the maximal cumulative dose of 128 mg was reached.  $PD_{20}FEV_1$  (the dose required to produce a 20% decrease in  $FEV_1$ ) was determined by linear interpolation between points on the log dose–response curve.

The consecutive doses of ASA were inhaled every 30 min and  $FEV_1$  values were obtained at 10, 20 and 30 min after each dose by a computerized water-sealed spirometer (Baires II, Biomedin, Italy).

### Methacholine bronchial challenge

A standard procedure was employed for bronchial challenge with methacholine (17). Briefly, methacholine was inhaled from a breath-activated dosimeter Mefar MB3, Markos, Italy). Doses of methacholine were progressively doubled until  $FEV_1$  had fallen by 20% from baseline or the maximal dose of 1600  $\mu$ g was reached.  $PD_{20}FEV_1$  (the dose required to produce a 20% decrease in  $FEV_1$ ) was determined by linear interpolation between points on the log dose–response curve. The FENO concentrations were measured baseline and at the end of the challenge.

### Measurement of exhaled NO

Exhaled NO was measured by a chemiluminescence analyzer (280 NOA; Sievers Instruments, Boulder, CO), according to ATS/ERS recommendations (18). The analyzer was calibrated daily using air entrained via a NO filter (Sievers Instruments) as a zero gas source, and a NO calibrated gas (100 p.p.b.; Messer Italia, Collegno, Italy) for the upper point calibration. Patients were asked to inhale NO-free air and to perform a slow expiratory vital capacity with a constant flow (50 ml/s), against a resistance (10 mmHg) to exclude nasal NO. Two successive reproducible recordings were measured at 2 min intervals, and the mean values of the plateau (the last part of expiration) of NO concentration (expressed in p.p.b.) were recorded.

### Sputum induction

Sputum induction and processing were performed according to standard method (19). Briefly, subjects inhaled 400  $\mu$ g of salbutamol and then performed three peak-flow manoeuvres by a Mini-Wright peak flow meter. The best peak expiratory flow rate (PEFR) was recorded as the baseline. Sputum was induced by inhalation of hypertonic saline (NaCl 3%). Hypertonic aerosols were generated with a DeVilbiss Ultraneb 2000 ultrasonic nebulizer (DeVilbiss Health Care, Somerset, PA) at maximal output (4 ml/min). At 2-min intervals subjects were instructed to clear their mouth of saliva and expectorate sputum in a cup. After sputum expectoration, PEFR was recorded. Subjects inhaled hypertonic saline for 12 min, unless PEFR fell by 20% of baseline.

### Sputum processing

The expectorated sputum was weighed and diluted 1 : 1 (wt/vol) with 0.1% dithiotriitol (Sputolysin; Behring Diagnostics, Somerville, NJ, USA). The sample was gently vortexed and placed in a shaking water bath at 37°C for 15 min. A 1-ml volume of the suspension was removed for cell count and differential count. Total cell counts were determined with a hemocytometer, and the sample was diluted with normal saline to a final concentration of  $1.6 \times 10^5$  cells/ml for use in the preparation of cytospin slides. Cytocentrifuge slides containing 250  $\mu$ l/slide were spun for 6 min at  $800 \times g$ . Differential counts of eosinophils, lymphocytes, neutrophils, macrophages, epithelial cells, squamous cells were determined on Diff-Quick stained by an investigator who had no knowledge of the source of the specimens. For each sample, 300 nonsquamous cells were counted, and the percentage of each type of cell was expressed as the percentage of the total nonsquamous cells.

### Statistics

Data for exhaled NO during the challenge protocols (are presented as percentage of baseline values unless otherwise

indicated) and were analysed by two-way ANOVA (GraphPad Software, Inc., San Diego, CA). Data for sputum eosinophils and airway responses were analysed with Student's paired *t*-test. Relationships between continuous variables were examined by linear regression.

## Results

The clinical characteristics of the patients are reported in the Tables 1 and 2. No significant differences were observed between AIA and ATA patients for age, baseline spirometric data, PD<sub>20</sub>FEV<sub>1</sub> methacholine and baseline FENO.

In patients with AIA, PD<sub>20</sub>FEV<sub>1</sub> ASA was  $14.7 \pm 12.7$  mg, with a mean decrease of FEV<sub>1</sub> of  $27 \pm 4.2\%$  compared with baseline. All patients tolerated well the challenge, two of them requiring 200 µg metered dose inhaler (MDI) salbutamol.

Asthmatic patients had baseline FENO significantly higher than controls ( $29.7 \pm 6.8$  vs  $9.8 \pm 2.05$  p.p.b. respectively,  $P < 0.0001$ ) (Fig. 1).

NO response following bronchoprovocation

Baseline FEV<sub>1</sub> was similar on the study-days both in AIA and ATA patients. After saline inhalation no significant change was observed in FEV<sub>1</sub> and in FENO in both groups of patients and in controls. After L-ASA inhalation no significant change in FENO was observed in patients with ATA and in controls (Fig. 2). On the contrary, patients with AIA showed a significant increase in FENO, starting 2 h after ASA inhalation, reaching a peak value at 4 h ( $43 \pm 4.8$  p.p.b.,  $P < 0.001$  compared with baseline) and returning to baseline values 24 h after (Fig. 2), whereas FEV<sub>1</sub> returned to the baseline values between 2 and 4 h after ASA challenge (Fig. 3). Changes in FENO were not related neither to the maximum decrease of FEV<sub>1</sub> after ASA nor to the ASA threshold.

Sputum eosinophils

Sputum eosinophils were slightly, but not significantly, higher in patients with AIA compared with patients with ATA ( $8.1 \pm 2.7$  and  $6.7 \pm 2.2\%$  respectively). No

Table 1. Characteristics of patients with aspirin-tolerant asthma

| Number | M/F | Age (years) | Atopy (Y/N) | VC (% pr) | FEV <sub>1</sub> (% pr) | FEV <sub>1</sub> /VC × 100 | PD <sub>20</sub> FEV <sub>1</sub> methacholine (µg) | FENO baseline (p.p.b.) |
|--------|-----|-------------|-------------|-----------|-------------------------|----------------------------|-----------------------------------------------------|------------------------|
| 1      | F   | 26          | Y           | 98        | 93                      | 82                         | 125                                                 | 42                     |
| 2      | F   | 45          | N           | 104       | 95                      | 77                         | 450                                                 | 28                     |
| 3      | M   | 47          | Y           | 97        | 89                      | 75                         | 430                                                 | 20                     |
| 4      | M   | 48          | N           | 111       | 110                     | 77                         | 580                                                 | 20                     |
| 5      | F   | 38          | N           | 94        | 90                      | 75                         | 560                                                 | 23                     |
| 6      | M   | 54          | Y           | 92        | 89                      | 75                         | 180                                                 | 24                     |
| 7      | F   | 57          | N           | 97        | 88                      | 71                         | 130                                                 | 26                     |
| 8      | F   | 39          | Y           | 93        | 85                      | 71                         | 85                                                  | 38                     |
| 9      | M   | 21          | N           | 98        | 92                      | 77                         | 325                                                 | 35                     |
| 10     | M   | 29          | N           | 99        | 93                      | 78                         | 295                                                 | 33                     |
| Mean   |     | 40.4        |             | 98.3      | 92.4                    | 75.8                       | 316                                                 | 28.9                   |
| SD     |     | 12          |             | 5.6       | 6.8                     | 3.2                        | 183                                                 | 7.7                    |

VC, vital capacity; FEV<sub>1</sub>, forced expiratory volume at 1 s; FENO, fractional exhaled nitric oxide; pr, predicted.

Table 2. Characteristics of patients with aspirin inducible asthma

| Number | M/F | Age (years) | Atopy (Y/N) | VC (% pr) | FEV <sub>1</sub> (% pr) | FEV <sub>1</sub> /VC × 100 | PD <sub>20</sub> FEV <sub>1</sub> methacholine (µg) | FENO baseline (p.p.b.) | PD <sub>20</sub> FEV <sub>1</sub> ASA (mg) |
|--------|-----|-------------|-------------|-----------|-------------------------|----------------------------|-----------------------------------------------------|------------------------|--------------------------------------------|
| 1      | M   | 73          | N           | 93        | 94                      | 77                         | 530                                                 | 35                     | 31                                         |
| 2      | M   | 61          | N           | 101       | 84                      | 64                         | 250                                                 | 34                     | 2                                          |
| 3      | F   | 33          | N           | 95        | 90                      | 76                         | 420                                                 | 30                     | 4                                          |
| 4      | M   | 45          | Y           | 98        | 96                      | 76                         | 510                                                 | 23                     | 2                                          |
| 5      | F   | 50          | Y           | 95        | 93                      | 76                         | 520                                                 | 22                     | 32                                         |
| 6      | M   | 55          | N           | 86        | 84                      | 71                         | 150                                                 | 35                     | 15                                         |
| 7      | F   | 28          | N           | 107       | 105                     | 78                         | 210                                                 | 39                     | 8                                          |
| 8      | M   | 27          | Y           | 101       | 98                      | 78                         | 520                                                 | 25                     | 24                                         |
| 9      | F   | 30          | N           | 95        | 88                      | 75                         | 250                                                 | 35                     | 22                                         |
| 10     | F   | 42          | N           | 101       | 90                      | 76                         | 390                                                 | 33                     | 17                                         |
| Mean   |     | 44.4        |             | 97.2      | 92.2                    | 74.7                       | 375                                                 | 31.1                   | 15.7                                       |
| SD     |     | 15.4        |             | 5.7       | 6.5                     | 4.2                        | 147                                                 | 6.0                    | 11.4                                       |

VC, vital capacity; FEV<sub>1</sub>, forced expiratory volume at 1 s; FENO, fractional exhaled nitric oxide; pr, predicted.

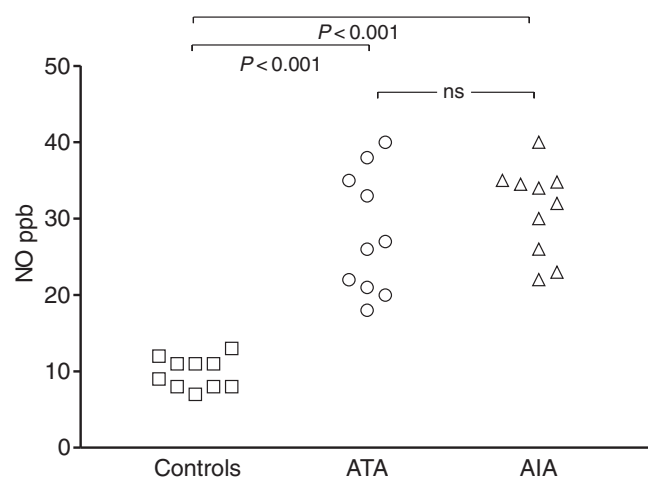


Figure 1. Fractional exhaled nitric oxide (FENO, in p.p.b.) in control subjects, in patients with aspirin-tolerant (ATA) and in patients with aspirin inducible (AIA) asthma.

correlation was found between sputum eosinophils and baseline FENO in all the asthmatics. A significant increase in sputum eosinophils was observed in AIA patients after inhalation challenge with L-ASA, from  $8.1 \pm 2.7$  to  $11.1 \pm 2.8$  ( $P < 0.005$ ), while no significant change in sputum eosinophils was observed in patients with ATA after L-ASA inhalation (from  $6.7 \pm 2.2$  to  $7.2 \pm 1.7\%$ ). A mild correlation was observed between the increase in sputum eosinophils and the increase in FENO ( $r = 0.64$ ,  $P < 0.05$ ) (Fig. 4).

## Discussion

Aspirin inhalation caused a significant increase in exhaled NO only in patients with AIA. Both normal controls and asthmatic patients with ATA did not show any significant change in FENO after ASA challenge, according to a previous observation (20). The time course of the increase in FENO, which started to increase 2 h after ASA-induced bronchoconstriction and reached the peak value after 4 h, suggests that eosinophils recruitment into the airway is clearly involved. Actually there was a significant correlation between the increase in sputum eosinophils and the increase in FENO in patients with AIA after ASA challenge. Aspirin challenge has been reported to increase the number of eosinophils in BAL fluid (21), possibly because of the brisk increase in cysteinyl-leukotrienes, which immediately follows ASA administration to aspirin-sensitive patients (12). Previous studies have demonstrated a relationship between exhaled NO and the number of eosinophils in induced sputum, both in stable asthmatics maintained on  $\beta_2$  agonists alone (14), and in a group of asthmatics at time of mild exacerbation of asthma induced by reducing the dose of inhaled maintenance steroids (22). In our patients we did not observe a

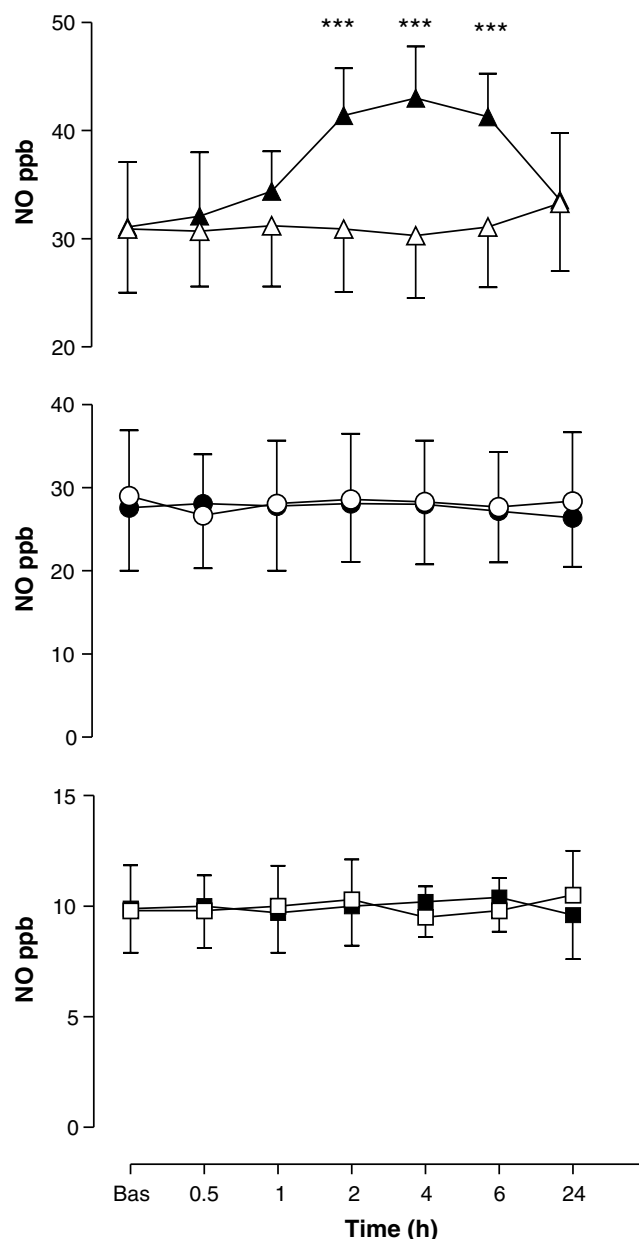


Figure 2. Mean values of fractional exhaled nitric oxide (FENO, in p.p.b.) in control subjects (square), in patients with aspirin-tolerant (ATA, circles) and in patients with aspirin-induced (AIA, triangles) asthma during placebo (open symbols) and lysine-aspirin (L-ASA, closed symbols) inhalation challenge. \*\*\* $P < 0.001$  compared with baseline.

significant correlation between sputum eosinophils and exhaled NO in baseline condition, but some of our patients were receiving intranasal corticosteroids as maintenance therapy at the time of study enrolment. Leukotrienes are proinflammatory molecules which are thought to be related to exhaled NO, as suggested by reports demonstrating that administration of a cysteinyl leukotriene antagonist reduces FENO and prevents increase in

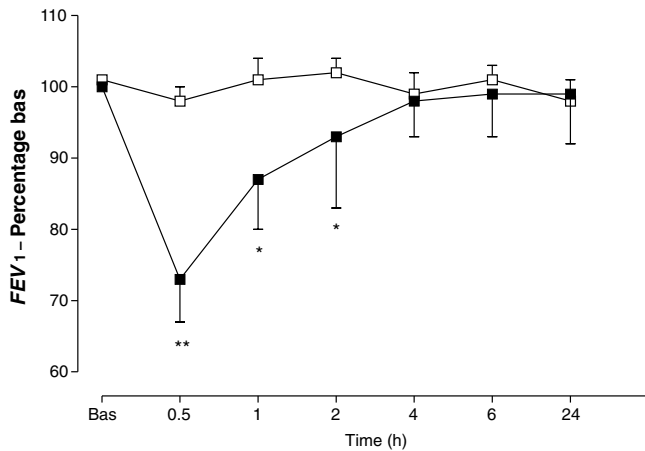


Figure 3. Changes in  $FEV_1$  after aspirin (ASA) inhalation challenge in patients with aspirin-induced (AIA) asthma. \*\* $P < 0.001$ , \* $P < 0.01$ .

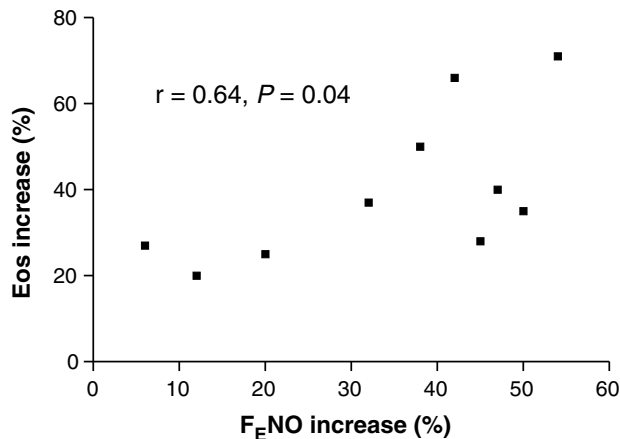


Figure 4. Relationship between the increase in sputum eosinophils (percentage above baseline) and the increase in fractional exhaled nitric oxide (FENO, percentage above baseline) after aspirin (ASA) inhalation challenge in patients with aspirin inducible asthma.

FENO during reduction of an inhaled corticosteroid dose (13). The delay in the rise of FENO we observed in our patients is more consistent with the airway eosinophilic inflammation caused by ASA inhalation, than with a

direct effect of leukotrienes on NO synthesis and release. In agreement with this hypothesis is the recent observation that leukotriene E<sub>4</sub> inhalation does not affect exhaled NO in patients with asthma (23). Theoretically, the delay in the increase in FENO might be partially explained by the bronchoconstriction induced by ASA inhalation, which could have blunted the early increase in exhaled NO. Exhaled NO has been reported to be reduced shortly after bronchoconstriction both to some direct stimuli, such as histamine, and to some indirect stimuli, such as adenosine and hypertonic saline in asthma (24). The possibility that the increase in FENO after ASA was due to the removal of a braking effect by prostaglandins on NO synthesis (9) seems to be unlikely. Prostaglandins are continuously produced and released by many cells of respiratory tract in response to many stimuli, both inflammatory and mechanic. Prostaglandin levels in BAL of asthmatic patients, irrespective of ASA sensitivity, have been reported to be higher than in normal controls (25). Despite inhaled prostaglandins may decrease exhaled NO both in normal and in patients with ATA (11), we do not think that blocking prostaglandin synthesis, as far as it is attainable by ASA inhalation, may have substantially contributed to the increase in exhaled NO in our patients with AIA, as an increase in FENO after ASA was not observed neither in controls nor in patients with ATA.

In conclusion, airway challenge with ASA increases exhaled NO levels only in patients with AIA. The pattern of the delayed increase in exhaled NO after ASA challenge is quite similar to the pattern observed after allergen challenge (26), where exhaled NO has been reported to increase at the time of late response. Eosinophils recruitment into the airway seems to be the best explanation for exhaled NO increase in asthmatic patients with AIA. The clinical implications of our findings are that exhaled NO may indicate airway inflammation during ASA exposure and the fact that exhaled NO increased and remained still higher than baseline, when spirometry had been reversed to normal, makes it a useful and noninvasive method for assessing airway inflammation.

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