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Total nitrite/nitrate in expired breath condensate of patients with asthma

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Abstract Production of nitric oxide (NO) is generally increased during inflammatory diseases including asthma. The eventual fate of NO is oxidation to nitrite (NO_2) and nitrate (NO_3), both of which are end-products of NO metabolism. Hydrogen Peroxide (H_2O_2) is increased in exhaled breath condensate of asthmatic subjects and may be used as a non-invasive marker of oxidative stress. NO has in some cases been shown to attenuate oxidant-induced lung injury. Total NO_2/NO_3 concentration and H_2O_2 levels were measured in expired breath condensate in 50 clinically stable asthmatics [all males, all atopics, mean age 22 (3) SD yrs, forced expiratory volume in 1 sec (FEV_1) 91 (10)% predicted, PD_{20} to histamine 0·262 (0·16) mg 20 on inhaled steroids, 20 smokers, all steroid-naive] and in 10 normal, non-atopic subjects [all males, age 23 (4) yrs, FEV_1 101 (14)% predicted, PD_{20} to histamine 1·3 (0·55) mg]. NO_2/NO_3 levels were significantly higher in patients with asthma than in normal subjects (1·08, 95% CI 0·86–1·3 μM *vs.* 0·6; 95% CI 0·46–0·8, $P < 0.001$). Patients who were on inhaled steroids had significantly lower values compared to steroid-naive (0·71, 95% CI 0·55–0·87 μM *vs.* 133, 95% CI 1–1·65 μM, $P < 0.001$). Similar results were observed between smokers and non-smokers (1·11, 95% CI 0·74–1·47 μM *vs.* 1·77, 95% CI 1·1–2·4 μM, $P < 0.0001$). There was a significant positive correlation between NO_2/NO_3 levels and H_2O_2 concentration in expired breath condensate ($r = 0.48$, $P < 0.0001$). No correlation was observed between NO_2/NO_3 levels, airway obstruction and bronchial hyper-reactivity as assessed by PD_{20} to histamine. Total NO_2/NO_3 levels in expired breath condensate are raised in patients with stable asthma and are significantly related to oxidative stress as assessed by H_2O_2 concentration. Measurement of expired breath NO_2/NO_3 and H_2O_2 levels may be clinically useful in the management of oxidation and inflammation mediated lung injury.

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Keywords nitric oxide metabolites; hydrogen peroxide; asthma; inflammation.

INTRODUCTION

Asthma is a chronic lung disorder with the cardinal features of reversible airway obstruction, airway inflammation and airway hyper-responsiveness. Nitric oxide (NO) levels have been found to be raised in the exhaled breath of asthmatic patients and are significantly lower in patients who are treated with inhaled corticosteroids (ICS) (1). NO has in some cases been shown to attenuate oxidant-induced lung injury (2) and to have protective effects against oxidant-induced cytotoxicity (3). NO is a highly reactive molecule with multiple oxidation states and does not persist as a NO gaseous moiety. In an aqueous environment such as the air–fluid interface of the airways, NO generation may be indicated by the formation of stable end-products of NO metabolism, nitrite (NO_2) and nitrate (NO_3) (4).

Oxidative stress defined as an increased exposure to oxidants and/or decreased antioxidant capacities is implicated in airway inflammatory diseases including asthma (5). Inflammatory cells especially eosinophils were found to release several reactive oxygen and nitrogen derived species (6). O_2 and NO combine to form peroxynitrite ($ONOO^-$), which is highly reactive and may damage airway epithelium. O_2^- is rapidly metabolized to form hydrogen peroxide (H_2O_2), which diffuses into the airway lumen and may evaporate into exhaled air. H_2O_2 is increased in expired breath condensate and may be used as a non-invasive marker of oxidative stress.

Based on previous data where NO end products reflect the NO production (7) in exhaled air and by using a non-invasive method such as expired breath condensate, we investigated whether total NO_2/NO_3 concentration is raised in clinically stable asthmatics and

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whether it is related to airway obstruction as assessed by forced expiratory volume in 1 sec (FEV_1)% predicted and to bronchial hyper-responsiveness as assessed by PD_{20} to histamine. Additionally we attempted to show whether a relation between total NO_2/NO_3 and H_2O_2 exists. To account for possible confounding effects of inhaled corticosteroids (ICS) as well as of smoking, further analysis was conducted after stratifying the patients accordingly.

METHODS

Patients

Fifty asthmatics (20 on ICS, 20 smokers all steroid-haive) and 10 healthy non-atopic non-smoking control subjects (Table 1) were recruited from the out-patients clinic and from the hospital staff. All asthmatics were atopic as judged by the positive skin prick tests to six common aeroallergens. Diagnosis of asthma was established by using the American Thoracic Society criteria (8). Patients were included in the study only if they were clinically stable and had no evidence of acute exacerbation for at least 4 weeks prior to the study. None was on inhaled or oral mucolytics and none was receiving oxygen therapy. Twenty received β_2-agonists as a relief medication and 15 were on long-acting β_2-agonists. Normal subjects had a negative history of allergy (negative skin prick tests to common allergens), normal spirometry and normal bronchial reactivity as assessed by the provocative concentration of histamine causing a 20% fall in FEV_1 (PD_{20}) $>0{\cdot}800$ mg in all subjects (Table 1).

The study protocol was approved by the Ethics Committee of our hospital and all subjects gave written informed consent.

Analysis for the effects of corticosteroids and smoking habit

Patients were divided into two subgroups according to a subanalysis performed to account for inhaled corticosteroids (ICS) use: those who received inhaled corticosteroids ($n=20$) and those who were steroid-naive ($n=30$). Similar subanalysis was performed for smoking habit: 20 patients ($n=20$) were current smokers (all steroid-naive) while the remaining 30 ($n=30$) showed negative smoking history.

Lung function

FEV_1 was measured using a dry spirometer (Vica-test, Mijnhardt, Holland). The best value of three manoeuvres was expressed as a percentage of the predicted value. Airway responsiveness was measured in all subjects (controls and asthmatics) by histamine provocation challenge. The PD_{20} was calculated by interpolation of the semi-logarithmic dose–response curve.

Collection of expired breath condensate with total nitrite/nitrate and hydrogen peroxide measurement

Expired breath condensate was collected in the morning. A heat exchanger unit (RHES, Jaeger) was used to produce cold air of $-15^{\circ}C$ to $-18^{\circ}C$ at an airflow of $>80\,l\,min^{-1}$. A double-jacketed glass tube of 45 cm length, internal/external diameter of 4/7 cm, was specifically adapted to the cold air system and a Hans Rudolf two-way unidirectional valve was connected to the proximal end of the tube in order to separate inspiration from

Table 1. Subjects characteristics

	Asthmatics	Asthmatics ICS-treated	Asthmatics ICS-naïve	Normals
n	50	20	30	10
Age	22 (3) (19–30)	23 (3) (19–29)	21 (3) (19–30)	23 (4) (21–32)
FEV_1%, predicted	91 (10) (70–111)	89 (9) (75–109)	92 (10) (70–111)	101 (14) (89–117)
PD_{20} histamine	0·26 (0·16) (0·06–0·65)	0·35 (0·16) (0·12–0·65)	0·2 (0·13) (0·06–0·53)	1·3 (0·55) (0·9–1·86)
Smoking (*n*) (pack-years)	20 (3)	0	20 (3)	0

Data are expressed as mean (SD) with ranges in parentheses.
FEV_1: forced expiratory volume in 1 sec; ICS: inhaled corticosteroids.

expiration. After rinsing their mouth, subjects were comfortably seated in a chair wearing nose clips and breathed in a relaxed manner (tidal breathing) for 10 min. The breath condensate was collected at the distal end of the tube and was immediately stored at $-70^{\circ}C$ for later analysis. According to this design, salivary contamination was highly unlikely and was easily observed, as the proximal cold air connection was 25 cm from the mouthpiece. Approximately 1 ml of breath condensate was collected in a 2 ml sterile plastic tube. Measurements of total NO_2/NO_3 and H_2O_2 in each group were performed the same day. To examine the repeatability of repeated NO_2/NO_3 and H_2O_2 measurements within subjects, condensate was collected from five normal subjects and 10 patients (five on ICS) on two consecutive days. To assess the stability of both NO_2/NO_3 and H_2O_2 in the frozen condensate, 4 ml of condensate was collected from 10 subjects (five patients). The above concentration was divided into 1 ml aliquots, in which NO_2/NO_3 and H_2O_2 concentrations were determined after 2 days, 1 week, 2 weeks and 3 weeks of storage (maximum time of measurement in the whole samples). Repeatability of both NO_2/NO_3 and H_2O_2 measurements and stability of the frozen samples were estimated as previously described (9).

All condensate samples were tested for salivary contamination by determination of amylase activity. Amylase activity was carried out spectrophotometrically (kinetic method) using a commercial reagent kit (KONE Instr. Finland). In this procedure a-amylase of the sample and the enzyme a-glucosidase hydrolyses the substrate p-nitrophenyl-a-D-maltoheptaoside to glucose and p-nitrophenol. The liberation of p-nitrophenol is followed at 405 nm ($37^{\circ}C$) for 2 min. Two samples were spiked with saliva to ensure that this can be detectable by our method. In all the samples tested for saliva, no amylase was detected, suggesting no contamination of breath condensate. The samples, which were spiked with saliva, showed levels of more than 5000 IU salivary amylase.

Nitrate was measured as nitrite after enzymatic conversion by nitrate reductase (10). The total NO_2/NO_3 (converted nitrate plus nitrite) was measured by using the Griess reaction (11). Enzymatic conversion was carried out as following: 60 μl of breath condensate was mixed with 10 μl NADPH 0·5 mM and 10 μl mixture of the nitrate reductase (2000 μl^{-1}) and FAD (50 mol l^{-1}). Samples were incubated for 30 min at $37^{\circ}C$ and then mixed with 10 μl LDH (100 mg l^{-1}) and 10 μl sodium pyruvate (100 mmol l^{-1}). Samples were further incubated for 10 min at $37^{\circ}C$ to oxidize the excessive amounts of NADPH. After the enzymatic conversion of nitrate to nitrite the sample was assayed with 100 μl Griess reagent (Sulfanilamide 1 g^{-1}N–1 naphthylethylenediamine 0·1 $g\,l^{-1}$, H_3PO_4 25 $g\,l^{-1}$). After 10 min of colour development at room temperature the absorbance was measured at 550 nm.

H_2O_2 concentration was determined by an enzymatic assay as previously described (12,13). Briefly, 250 μl of 420 μM 3′, 3, 5, 5′ tetramethylbenzidine (dissolved in 0·42 M citrate buffer, pH 3·8) and 10 μl of 52·5 U ml^{-1} of horseradish peroxidase (HRP, Sigma Chemicals, St Louis, MO, U.S.A.) were reacted with 250 μl of the condensate for 20 min at room temperature. Subsequently, the mixture was acidified to a pH of 1 with 10 μl of 18 N sulphuric acid. The reaction product was quantitated at the absorbency of 450 nm using a double beam spectrophotometer (Uvicon 940, Kontron Instr.). A standard curve was performed for both the assays with a limit of determination at 0·1 μM.

Statistical analysis

Data concerning the subjects characteristics are shown as mean SD (range). Data concerning the comparisons between the various parameters in the study groups are given as mean with 95% confidence intervals (CI). Parameters from patients and controls as well as between the subgroups were compared using the student's t-test. Pearson's correlation coefficient was used to investigate the relation between the various parameters. A P-value < 0.05 was considered significant.

RESULTS

Repeated measurements of NO_2/NO_3 on two consecutive days showed a mean within-subject difference of 0·07 μM (0·04) for patients and 0·03 μM (0·02) for normals. The stability of total NO_2/NO_3 in frozen samples showed no significant difference among the four measurements [0·62 (0·41) μM after 2 days, 0·64 (0·4) μM after 1 week, 0·6 (0·4) μM after 2 weeks, 0·64 (0·42) μM after 3 weeks, (P=0·55)]. Repeated measurements of H_2O_2 on two consecutive days showed a mean within-subject difference of 0·04 μM (0·02) for patients and 0·07 μM (0·03) for normals. The stability of total H_2O_2 in frozen samples showed no significant difference among the four measurements [0·55 (0·21) μM after 2 days, 0·58 (0·33) μM after 1 week, 0·6 (0·3) μM after 2 weeks, 0·63 (0·46) μM after 3 weeks, ($P = 0·76$)].

Mean (95% CI) total NO_2/NO_3 levels were significantly higher in patients with asthma compared to normal subjects [1·08, 0·86–1·33 μM *vs*. 0·63, 0·20–0·41 μM, respectively, $P < 0·001$, Fig. 1(a)] Mean (95% CI) H_2O_2 concentration in expired breath condensate was significantly higher in patients with asthma compared to normal subjects [0·6, 0·48–0·71 μM *vs*. 0·3, 0·2–0·4 μM respectively, $P < 0·005$; Fig. 1(b)]. Patients treated with ICS exhibited significantly lower levels of NO_2/NO_3 in expired breath condensate—similar to normals—compared to those who were steroid-naive [0·71, 95% CI 0·55–0·87 μM *vs*. 1·33, 95% CI 1–1·65 μM, $P < 0·001$,

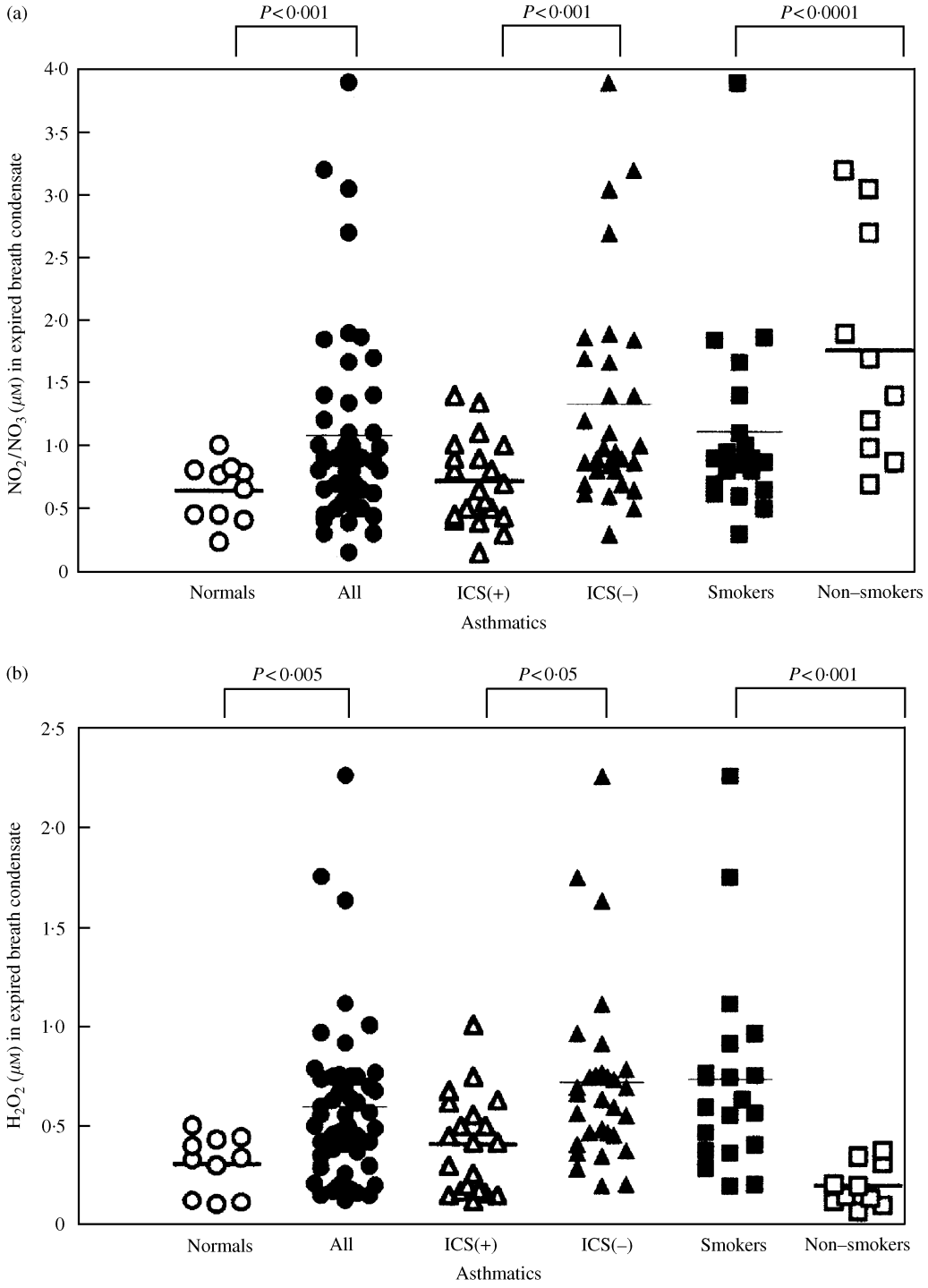

Fig. 1. (a) Total nitrate/nitrite (NO_2/NO_3) concentration in expired breath condensate of patients with asthma [all $n = 50$ (●), inhaled corticosteroid-treated (ICS+) $n = 20$ (△), inhaled corticosteroid-naive (ICS −) $n = 30$ (▲), asthmatics (ICS −) smokers $n = 20$ (■) asthmatics (ICS −) non-smokers $n = 10$ (□)] and normal subjects, $n = 10$ (○)]. Each symbol represents one individual. Concentration of total NO_2/NO_3 was significantly higher in patients with asthma compared to normal subjects, $P < 0{\cdot}001$. Patients treated with inhaled corticosteroids (ICS) had significantly lower values compared to steroid-naïve (ICS −), $P < 0{\cdot}001$. Asthmatic smokers had significantly lower values compared to non-smokers, $P < 0{\cdot}0001$. Horizontal bar represent mean values. (b) Hydrogen peroxide (H_2O_2) concentration in expired breath condensate of patients with asthma [all $n = 50$ (●), inhaled corticosteroid-treated (ICS+) $n = 20$ (△), inhaled corticosteroid-naive (ICS −) n=30 (▲), asthmatics smokers $n = 20$ (■), asthmatics non-smokers $n = 10$ (□)] and normal subjects [$n = 10$ (○)]. Each symbol represents one individual. Concentration of total H_2O_2 was significantly higher in patients with asthma compared to normal subjects, $P < 0{\cdot}005$. Patients treated with inhaled corticosteroids (ICS +) had significantly lower values compared to steroid naïve (ICS −), $P < 0{\cdot}05$. Asthmatic smokers had significantly higher values compared to non-smokers, $P < 0{\cdot}001$. The horizontal bars represent mean values.

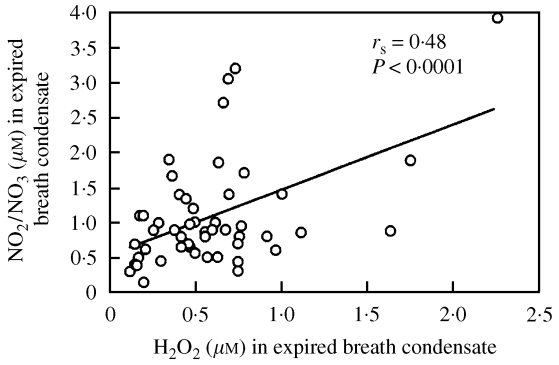

Fig. 2. Correlation between total nitrate/nitrite (NO_2/NO_3) and hydrogen peroxide (H_2O_2) in expired breath condensate of patients with asthma (r_s=0·48, $P<0{\cdot}0001$). Individual data points (○).

Fig. 1(a)]. Similar results were observed for H_2O_2 levels [0·41, 95% CI 0·29–0·52 μM *vs.* 0·72, 95% CI 0·55–0·89 μM, $P<0{\cdot}05$, Fig. 1(b)]. Mean (95% CI) total NO_2/NO_3 levels were significantly lower in asthmatic smokers compared to non-smokers [1·11, 95% CI 0·74–1·47 μM *vs.* 1·77, 95% CI 1·1–2·4 μM, $P<0{\cdot}0001$, Fig. 1(a)]. On the other hand, asthmatic smokers had significant higher H_2O_2 levels compared to non-smokers [0·75, 95% CI 0·5–0·97 μM *vs.* 0·2, 95% CI 0·12–0·28 μM, $P<0{\cdot}001$, Fig. 1(b). Mean (95% CI) total NO_2/NO_3 levels in expired breath condensate of steroid-naïve non-smokers asthmatics were significantly higher compared to normal subjects (1·77, 1·11–2·42 μM *vs.* 0·63, 0·46–0·80 μM, respectively, $P<0{\cdot}005$). There was a significant positive correlation between total NO_2/NO_3 and H_2O_2 concentration in expired breath condensate ($r=0{\cdot}48$, $P<0{\cdot}0001$, Fig. 2). No correlation was observed between NO_2/NO_3 and either the PD_{20} to histamine or the FEV_1% predicted ($r=0{\cdot}04$, $P=0{\cdot}77$, $r=0{\cdot}1$, $P=0{\cdot}3$, respectively).

DISCUSSION

We have found that levels of total NO_2/NO_3 in expired breath condensate were elevated in mild asthmatic patients compared to normal control subjects and were significantly affected by both ICS and smoking habit. They were not related to airway obstruction as well as to bronchial hyper-reactivity as assessed by PD_{20} to histamine. We also found that end products of NO metabolism were significantly correlated with H_2O_2 concentration. Expired breath condensates have been used recently as tools to measure airway inflammation. They are non-invasive, simple to perform, highly repeatable and reflect abnormalities noted in specimens obtained bronchoscopically (14,15), in sputum (7) and exhaled air (16). Our findings are compatible with previous observations regarding the exhaled NO levels in asthmatics. We used a standardized cold air methodology to get the condensate in exhaled breath since the volume collected is of high amount with no saliva contamination and rapid to perform. It is known that NO is unstable and the reaction speed to NO_2/NO_3 is rapid and is influenced by airway inflammation (4). Since NO_2/NO_3 is not volatile, its presence in breath condensate comes from tiny droplets of epithelial lining fluid as the velocity of the air stream through the bronchi and bronchioles forms them. The increased levels of NO derivatives in expired breath condensate may be due to induction of iNOS by cytokines and other inflammatory mediators released into asthmatic airways. This is partially supported by previous observations where inhaled corticosteroids prevent the induction of iNOS by cytokines in epithelial cells (17). The low concentration of NO derivatives in smokers is similar to those previously reported for exhaled NO and may be due to down-regulation of iNOS caused by the high concentration of NO in cigarette smoke (18).

The cellular source of total NO_2/NO_3 in expired breath condensate is unknown. They are probably derived from iNOS activity in the respiratory tract. Immunohistochemical studies suggest that it may be derived largely from epithelial cells and there is little evidence for iNOS expression in airway macrophages. However, due to the origin of expired breath condensate, total NO_2/NO_3 may also derived from infiltrating inflammatory cells within the respiratory tract. Similar results to ours were also observed in another study where induced sputum was used for measuring concentration of nitrate/nitrites (7).

In our study we did not find any significant correlation between NO derivatives and airway obstruction as assessed by FEV_1% predicted, but most of our patients had mild asthma with normal values as for FEV_1% predicted. The lack of significance between end-products of NO metabolism and bronchial hyper-responsiveness is similar to those reported previously between nitrate/nitrite concentration in induced sputum and bronchial hyper-reactivity to metacholine (7). This might be due to the fact that end-products of exhaled NO only reflect the ongoing inflammation and the short-term reaction. On the other hand, bronchial hyper-responsiveness reflect both the ongoing inflammation and the continuous process of injury and healing remodelling (19). Moreover, there is no agreement in most of the studies for the role of exhaled NO in predicting the degree of bronchial hyper-responsiveness (20,21).

The role of NO in inflammation and oxidative stress is complex and depends on NO and O_2 balance. Our findings support previous observations where NO has been shown to attenuate oxidant-induced lung injury (2). Previous observations showed that increased production of endogenous NO has a cytotoxic effect on lung cell and tissue by altering cell membrane permeability (22,23) or by priming lung macrophages (24) to release reactive

nitrogen and oxygen species which are capable of causing tissue injury in (25) and DNA damage (26). However, in a previous study, there was no correlation between NO concentration on exhaled air and H_2O_2 concentration in expired breath condensate (27). This might be explained by the theory that is uncertain what proportion of total NO production is reflected by exhaled gaseous NO (28) and furthermore it is more reasonable to compare compounds generated from the same specimen. Further studies are needed in this respect, since NO reacts with hydrogen peroxide to form peroxynitrite and then nitrotyrosine. Measurement of the latter might be the link between the two substances.

In summary, we have shown that high concentrations of NO derivatives are detected in expired breath condensate of steroid-naïve asthmatic patients. Measurement of expired breath NO_2/NO_3 and H_2O_2 levels may be clinically useful in the management of oxidation- and inflammation-mediated lung injury.

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