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Effect of short-term NO_2 exposure on induced sputum

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Effect of short-term NO_2 exposure on induced sputum in normal, asthmatic and COPD subjects. B. Vagaggini, P.L. Paggiaro, D. Giannini, A. Di Franco, S. Cianchetti, S. Carnevali, M. Taccola, E. Bacci, L. Bancalari, F.L. Dente, C. Giuntini. ©ERS Journals Ltd 1996.

ABSTRACT: The aim of this study was to assess the effects of short-term exposure to low levels of nitrogen dioxide (NO_2) on airway inflammation.

We studied seven normal, eight mild asthmatic and seven chronic obstructive pulmonary disease (COPD) subjects. All subjects were exposed to air or to 0.3 parts per million (ppm) NO_2 for 1 h, with moderate intermittent exercise, on different days and in random order. Before and 2 h after exposure, symptom score and results of pulmonary function tests (PFTs) were assessed. All subjects performed nasal lavage and hypertonic saline (HS) inhalation to collect sputum 2 h after both exposures.

Asthmatic subjects had a higher percentage of eosinophils than normal and COPD subjects in HS-induced sputum after air (asthmatics: median 13 (range 0.4–37)%; normals: 0 (range 0–2)%; COPD 1.8 (range 0.1–19)%), whilst COPD patients showed a higher percentage of neutrophils than the two others groups. No significant differences in PFT values or percentages of inflammatory cells were observed in nasal lavage and in HS-induced sputum in normal, asthmatic and COPD subjects after NO_2 exposure compared to air exposure, except for a mild decrease in forced expiratory volume in one second (FEV_1) 2 h after NO_2 exposure in COPD patients. Symptom score showed a mild increase after NO_2 exposure both in normal subjects and in COPD patients.

We conclude that short-term exposure to 0.3 ppm nitrogen dioxide does not induce an early detectable acute inflammation in proximal airways of normal subjects or of patients with asthma or chronic obstructive pulmonary disease.

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Nitrogen dioxide (NO_2) is a serious air pollutant in urban areas and in some industrial workplaces. Animal studies have shown that NO_2 causes loss of cilia both in central and peripheral airways, inflammation in major and terminal bronchi, and stenosis of respiratory bronchioles [1] through different biochemical mechanisms, such as oxidation of polyunsaturated fatty acids in cell membranes [2].

An acute short-term exposure to NO_2 can decrease lung function in healthy subjects only at concentrations exceeding 1 part per million (ppm), and increases airway responsiveness [3, 4]. Most clinical studies in asthmatic patients have shown that NO_2 exposure at concentrations within the environmental range do not affect lung function, but can slightly enhance airway responsiveness to different stimuli [5–7]. In patients with chronic obstructive pulmonary disease (COPD), progressive decrements in forced vital capacity (FVC) and forced expiratory volume in one second (FEV_1) were observed after 4 h exposure to 0.3 ppm NO_2 [8].

Lymphocytosis and mastocytosis have been found in bronchoalveolar lavage (BAL) fluid collected 4–24 h after single acute exposure to NO_2 in healthy subjects [9], and significant reductions in the total number and percentage of T-cytotoxic-suppressor cells have been found in BAL fluid after repeated short-term exposures to 1.5 ppm [10]. Recently, hypertonic saline (HS)-induced sputum examination has been introduced as a noninvasive tool to evaluate airway inflammation in proximal airways [11]. So far, no clinical studies have evaluated the inflammatory response to NO_2 in the airways using HS-induced sputum collected after a single exposure to NO_2.

This study has been performed to assess whether short-term exposure to low level NO_2 can determine inflammatory changes in the airways detected by HS-induced sputum rather than BAL.

Subjects and methods

Subjects

Three groups of subjects were studied: seven normals (healthy nonsmoking volunteers with no history of asthma

Table 1. – Anthropometric and functional data of the subjects examined

Subjects	n	Age yrs	Sex M/F	FEV_1 % pred	Smoking habit (S/NS/ES)	Height cm	Weight kg
Normals	7	34±5	7/0	100±11	0/7/0	175±4	79±9
Asthmatic	8	29±14	4/4	97±10	0/8/0	167±8	66±13
COPD	7	58±12	7/0	62±25	0/1/6	170±4	78±10

Values are presented as mean±SD. M: male; F: female; FEV_1: forced expiratory volume in one second; COPD: chronic obstructive pulmonary disease; S: smokers; NS: nonsmokers; ES: ex-smokers; % pred: percentage of predicted value.

or COPD), eight asthmatics and seven patients with COPD (table 1). Diagnosis of asthma and COPD was performed according to the American Thoracic Society (ATS) criteria [12]; in particular: 1) asthma was defined in the presence of a history of recurrent attacks of reversible dyspnoea with wheezing, and bronchial hyperreactivity to methacholine; 2) COPD was defined in the presence of chronic bronchial hypersecretion and airway obstruction that did not change markedly over periods of several months observation.

All normal and asthmatic subjects were lifetime nonsmokers, while 6 of the 7 COPD patients were ex-smokers (39.6±17.5 pack-yrs).

Normal and asthmatic subjects had normal baseline FEV_1 and had been free from respiratory symptoms for at least six weeks before the study. COPD patients had a FEV_1/FVC% ratio lower than 70% (mean±SD 60±7), and were in a stable condition at the time of the study.

Bronchial hyperreactivity to methacholine was measured using a dosimetric technique described previously [13]. Briefly, methacholine (Sigma, St. Louis, MO, USA) was delivered by a Devilbiss 646 jet nebulizer; phosphate-buffered saline was inhaled first, followed every 2 min by methacholine inhalation in stepwise cumulative doses from 0.04 to 3.2 mg. FEV_1 was measured 2 min after each step. The test was stopped when FEV_1 fell by 20% or more below the postdiluent value, and the cumulative provocative dose producing a 20% fall in FEV_1 ($PD_{20}FEV_1$) was computed. According to epidemiological studies using the same technique [14], a $PD_{20}FEV_1$ value lower than 1 mg of methacholine was able to distinguish normal from asthmatic subjects, and it was considered as positive for nonspecific bronchial hyperreactivity.

Four of the eight asthmatic subjects were receiving regular treatment with inhaled steroids (beclomethasone dipropionate, 500–1,000 $\mu g \cdot day^{-1}$), which were withdrawn 48 h before each exposure. The remaining asthmatic subjects used short-acting β_2-agonists as required, which were withdrawn 8 h before each exposure. All COPD patients were receiving regular treatment with low-dose inhaled steroids (beclomethasone dipropionate, 500 $\mu g \cdot day^{-1}$) and long-acting β_2-agonists (salmeterol, 50 µg *b.i.d.*), and four of them used oral theophylline; this treatment was withdrawn 48 h before each exposure. All subjects were free from upper respiratory infections for at least 6 weeks before the study. The study was approved by an ethical committee and each subject gave written informed consent.

Study protocol

Exposures were performed in a single-blind manner. All subjects were randomly exposed to NO_2 (0.3 ppm) or filtered air (sham) on two different days, 1 week apart, whilst exercising on a cycle ergometer at a workload predetermined to induce a ventilation rate of approximately 25 $L \cdot min^{-1}$. Before and after exposure they performed pulmonary function tests (PFT) with a computerized water-sealed bell spirometer (Biomedin, Padova, Italy). Before and after each exposure, a symptom questionnaire was filled out by the subject. The subjects were asked to grade from 0 (=no symptom) up to 10 (=worst symptom) the severity of each of the following symptoms: production of tears, burning of the eyes, nausea, nose and throat irritation, cough, shortness of breath, headache, dizziness. A total symptom score (SS) was computed for each subject as the sum of all single symptom scores. Two hours after the end of both chamber exposures, PFT, nasal lavage, and HS-sputum induction were performed.

Methods

Challenge chamber. The subjects were exposed for 60 min in a 9 m^3 static challenge chamber made of glass and aluminium [15], whilst exercising on a stationary cycle ergometer for 10 min every 15 min. Mean air temperature was 21±1°C and the relative humidity was 45±5%. An NO_2 analyser (8840, Monitor labs, Rancom Instruments, Milano, Italy), connected to the chamber at one end and to a tank containing 0.1% NO_2 at the other end by a tubing circuit, continuously monitored gas concentration in the chamber. NO_2 out-put into the chamber was 3 $L \cdot min^{-1}$. An electronic valve automatically closed or opened the circuit when the NO_2 concentration in the challenge chamber was higher or lower than 10%, respectively, of the required concentration, in order to maintain a steady gas level during exposure. A fan in the chamber ensured adequate gas mixing and circulation. Mean (±SD) NO_2 concentration was 0.31±0.03 ppm for normal, asthmatic and COPD subjects.

HS inhalation test and sputum processing. HS solution was nebulized with an ultrasonic nebulizer (2.8 $mL \cdot min^{-1}$ output; Sirius, Technomed, Firenze, Italy) and was inhaled for 5 min periods for up to 30 min. The concentration of NaCl was increased at 10 min intervals from 3 to 4 to 5%. Every 5 min after the start of nebulization, patients were asked to rinse their mouth and throat carefully and to try to cough sputum into a clean container; FEV_1 was then measured. Nebulization was stopped after 30 min or when FEV_1 fell by 20% or more from baseline. The functional response to inhalation of HS was expressed as maximum FEV_1 fall (ΔFEV_1%) after HS inhalation.

Sputum samples were diluted with an equal volume

of 0.1% dithiothreitol (Sputasol; Unipath Ltd, Basingstoke, UK). Samples were incubated in a shaking bath at 37°C for 20 min, and then pipetted to further dissolve the mucous plugs. An aliquot of sputum sample was cytocentrifuged for 5 min at 1,300 rpm (Cytospin; Shandon Scientific, Sewickley, PA, USA) and stained with Diff-Quik (Baxter Scientific Products, Miami, FL, USA). Two investigators, blinded to the subject's history, each first counted at least 500 cells on each sputum slide in order to obtain the squamous cell percentage as an indicator of saliva contamination. Cytospin slides with a large number of squamous cells in which 500 nonsquamous cells could not be counted were considered unsatisfactory and discarded. Thus, at least 500 nonsquamous cells were counted on satisfactory slides. All cell percentages were averaged to give the final values reported. Percentages of macrophages, lymphocytes, neutrophils and eosinophils were, thus, expressed as a percentage of the total inflammatory cells, excluding squamous cells.

In our laboratory, this method gives a reasonably good reproducibility of inflammatory cell counts in sputum induced by HS. The reproducibility has been evaluated in 20 subjects (four normal and 16 asthmatic subjects) who repeated HS inhalation on two different days, a week apart, in stable condition. The intraclass correlation coefficient between two measurements was: +0.90 for % macrophages, +0.23 for % lymphocytes, +0.88 for % neutrophils, + 0.82 for % eosinophils [16].

Nasal lavage. Five millilitres of normal saline at ambient temperature were instilled twice into each nostril, while the neck was extended at about 30°, without the subject breathing or swallowing. After 10 s the subject discharged the solution into a clean container. Nasal lavage fluid was processed in the same way as the sputum, but without adding dithiotreithol.

Statistical analysis

FEV_1 values, maximum FEV_1 fall after HS inhalation and symptom score are expressed as mean±SD. Sputum and nasal cell percentages are expressed as medians (range). Paired t-test was used to compare FEV_1 value pre- and postexposure to air or NO_2. Kruskall-Wallis test was used to compare the difference in cell counts between groups, and Wilcoxon signed-rank test was used to compare cell counts, symptom score and maximum FEV_1 fall after HS inhalation between the value obtained after NO_2 and after air exposure in each group. A p-value lower than 0.05 was considered as significant [17].

Results

Clinical and functional evaluation

FEV_1 did not change after NO_2 or air exposure in any of the three groups of subjects examined. There was a significant difference in FEV_1 between air and NO_2 2 h after exposure in COPD patients (table 2).

In normal subjects and in COPD patients, an increase in symptom score was observed after NO_2 exposure with respect to pre-exposure value (normals: 1.5±1.5 *vs* 0.1± 0.4; p=0.04; COPD 13.6±17 *vs* 5.2±6.8; p=0.04). In asthmatic patients the increase in symptom score was not statistically significant (3.0±4.5 *vs* 0.4±0.7; p=0.1). Symptom score did not change after air exposure.

Bronchial response to HS inhalation was not different between NO_2 and air in all groups of subjects examined.

Cells counts in induced sputum and in nasal lavage fluid

Asthmatic subjects had higher percentages of eosinophil than normal and COPD subjects in the sputum collected after air exposure (asthmatics 13 (0.4–37.4)%, normals 0 (0–2)%, COPD 1.8 (0.1–19)%; Kruskall-Wallis test: H=8.42, p=0.01). COPD patients showed higher percentages of neutrophil after air (78.7 (43.9–95.1)%) compared with normal and asthmatic subjects. Similar differences were observed between the different groups when the percentages of inflammatory cells in HS-induced sputum after NO_2 exposure were considered.

NO_2 exposure did not induce any change in sputum cell percentages with respect to the values measured after air in all groups of subjects examined (fig 1). When the difference in sputum cell percentages between NO_2 and air was computed, the groups of patients were not statistically different (*e.g.* for eosinophils: normals = 0.8 (-1.3–2)%, asthmatics = -7.0 (-21–11.6)%, COPD = 0 (-8.5-3.3)%; Kruskall-Wallis test: H = 3.07, NS). None of the subjects examined showed any difference in nasal lavage cell percentages between air and NO_2 exposure.

Discussion

This study has shown that 60 min exposure to 0.3 ppm NO_2 in a challenge chamber does not affect pulmonary function in normal subjects and in patients with asthma, whereas it elicits a mild decrease in FEV_1 in COPD

Table 2. – FEV_1 and symptom score (SS) measured in normal subjects and in patients with asthma or COPD, before and after exposure to NO_2 or air

	Normals		Asthmatics		COPD	
	NO_2	Air	NO_2	Air	NO_2	Air
FEV_1 pre-exposure L	4.42±0.53	4.42±0.57	3.41±0.51	3.45±0.55	2.00±0.92	2.03±0.71
FEV_1 postexposure L	4.36±0.51	4.38±0.54	3.48±0.56	3.38±0.51	1.98±0.91	2.14±0.76
FEV_1 2 h postexposure L	4.37±0.53	4.37±0.53	3.43±0.48	3.38±0.51	1.94±0.85	2.14±0.79§
SS pre-exposure	0.1±0.4	0.3±0.7	0.4±0.7	0.4±0.7	5.2±6.8	8.8±16.4
SS postexposure	1.5±1.5*	1.0±1.7	3.0±4.5	0.8±1.3	13.6±17*	18.8±31
ΔFEV_1-HS %	4.6±4	1.6±2.9	27±12	29±19	11±9	19±16

Values are presented as mean±SD. The response to HS inhalation (ΔFEV_1-HS) was measured 2 h after exposure to NO_2 or air. For definitions see legend to table 1. *: p<0.05 between pre- and postexposure by Wilcoxon test; §: p<0.05 between NO_2 and air exposure by paired t-test. HS: hypertonic saline. For further definitions see table 1.

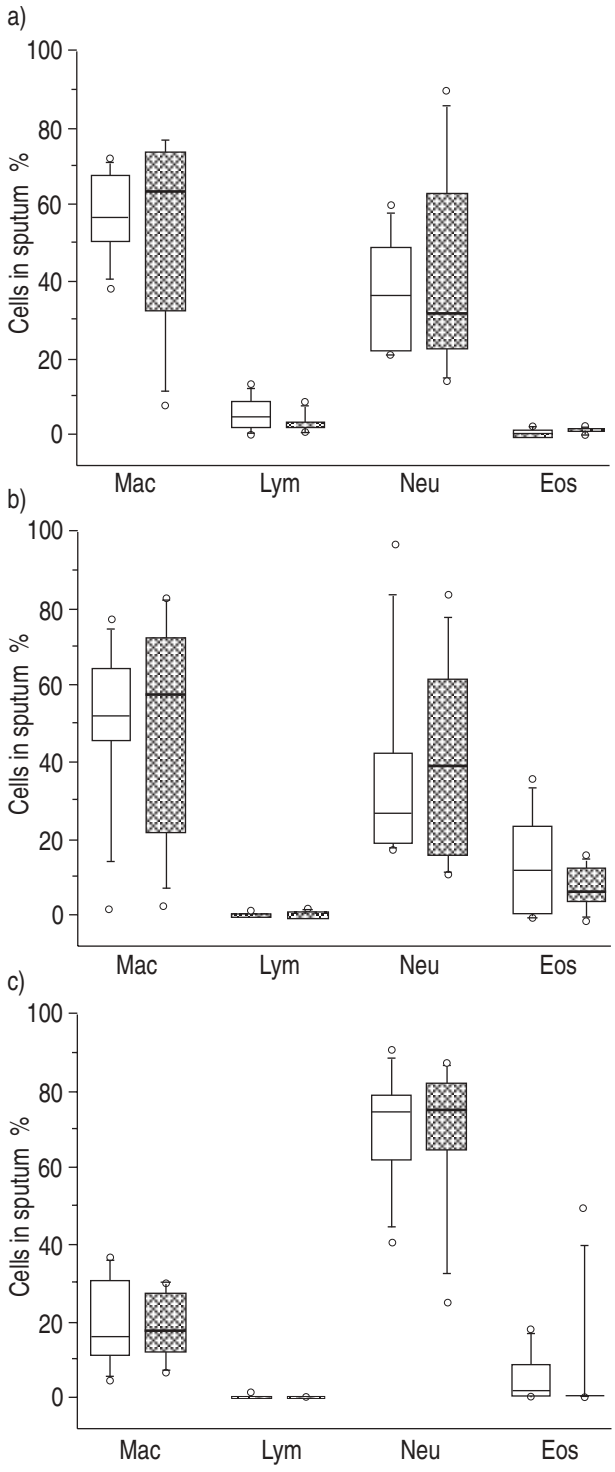

Fig. 1. – Median values, interquartiles, 90th percentiles and range of sputum percentages in a) normal, b) asthmatic and c) chronic obstructive pulmonary disease (COPD) patients after air (▭) and NO_2 exposure (▬). Mac: macrophages; Lym: lymphocytes; Neu: neutrophils; Eos: eosinophils.

patients, with respect to the values obtained after air exposure. A significant increase in symptom score was observed in normal subjects and in COPD patients after NO_2 exposure but not after air, suggesting a possible acute effect of NO_2 exposure on the airways. By contrast, this study has shown no change in inflammatory cell percentages in nasal lavage fluid and in HS-induced sputum after NO_2 exposure compared with the values obtained after air.

Although the analysis of HS-induced sputum is a sensitive technique in detecting inflammation in proximal airways [11], the mild clinical effects observed on symptoms in normal subjects, and on symptoms and FEV_1 change in COPD patients are not reflected by cellular change of airway inflammation.

Several epidemiological studies have emphasized the role of outdoor and indoor NO_2 concentrations on the incidence and duration of acute respiratory symptoms, especially in preschool children [18–20]. However, clinical studies have shown controversial results with respect to the acute effects of NO_2 exposure on pulmonary function and bronchial responsiveness in healthy and asthmatic subjects [3–7, 21]. Although the NO_2 concentrations used in this study were similar to the peak levels measured in the outdoor environment, bronchial responsiveness to HS inhalation showed no significant increase after NO_2 exposure.

Few data obtained using bronchoalveolar lavage (BAL) are available on the acute airway inflammatory response after NO_2 exposure [9, 10]. BAL recovers cells mainly from peripheral airways, which have been supposed to be the target area for NO_2 in animal experimental studies [22]. The use of these models has shown that NO_2 damage occurs to a similar extent between the trachea and the respiratory zone, and peaks at the terminal bronchioles. On the other hand, some authors have shown in animals that the peripheral airways are not initially more susceptible to injury than the central airways, but that repair occurs more promptly and completely in the central airways [1]. Therefore, an acute effect on proximal airways could be expected. We chose to use HS-induced sputum to evaluate the effect of NO_2 on proximal airways because it is a noninvasive, well-tolerated method that has been successfully used to examine changes in airway inflammation after allergen exposure or treatment [23, 24] in asthmatic subjects. A previous study compared cell percentages obtained by BAL, bronchial wash (BW) and induced sputum, showing a concordance between the three different methods [25]. Induced sputum samples more proximally than BAL and BW, but it is not known exactly which portion of the proximal airways is investigated by this tool.

In our laboratory, the reproducibility of HS-induced sputum was good for most cell types recovered in the sputum (*e.g.* macrophages, neutrophils and eosinophils), and similar to that obtained by other authors [11]. In fact, an intraclass correlation coefficient higher than 0.75, which has been considered acceptable [16], was observed for all cell types, except lymphocytes, due to their very low percentages in HS-induced sputum. The difference in the reproducibility of counts of the different cell types in HS-induced sputum makes it possible to draw a definite conclusion about the effect of NO_2 exposure on polymorphonuclear cells but not on lymphocytes. Furthermore, the reproducibility of cell counts in HS-induced sputum is not very different from that of BAL [26].

HS-induced sputum has been assumed to represent the cell composition of the airway lining fluid [11]. Some data have indicated that HS inhalation releases mediators from mast cells and from sensory nerve endings,

suggesting that inflammatory cell counts in the sputum can be a reaction to the hypertonicity. We showed that cell percentages in induced sputum were not different when hypertonic saline or isotonic saline (a non-pro-inflammatory stimulus) were used to collect sputum [27], suggesting that inflammatory cells in HS-induced sputum are probably pre-existing and not acutely recruited in the airways by the hypertonic stimulus.

There are several possible explanations for the lack of significant results concerning cell counts in induced sputum in the present study. Firstly, NO_2 concentrations were lower than those used in previous studies showing changes in airway cell counts [9, 10]. Nevertheless, the small clinical and functional changes that were observed in addition to the data reported by other authors in asthmatic subjects with doses of NO_2 similar to ours, may suggest that cellular changes could occur in the sputum cell percentage [4, 5]. Furthermore, we chose 0.3 ppm NO_2 because this concentration represents the peak value of NO_2 measurable in the ambient air of the major towns in Italy during the rush hours. Secondly, the duration of exposure may be too short. Experimental studies on cultured human bronchial epithelial cells exposed to 0.4 ppm NO_2 for up to 5 h have shown the release of several cytokines potentially responsible for inflammatory cell recruitment in the airways [28, 29]. We do not know whether a longer exposure than we used *in vivo* could induce the same effects as observed *in vitro*. Thirdly, sputum may have been collected too soonafter NO_2 exposure. Despite the fact that changes in pulmonary function have been reported as early as few minutes after the end of exposure [3–5], it cannot be excluded that a longer period between the end of exposure and sputum collection may give different results. Furthermore, 4 of the 8 asthmatics and all of the COPD patients were regularly using inhaled steroids. Although a 48 h withdrawal is usually recommended in challenge tests [30], we cannot exclude the possibility that a longer withdrawal of anti-inflammatory treatment could result in a different cell count after NO_2 exposure. Finally, the limited number of subjects examined could result in a low power for this study.

In conclusion, this is the first study using hypertonic saline induced sputum as a technique to evaluate the airway response to nitrogen dioxide. Using this technique, we confirmed that, 2 h after a 1 hour exposure to nitrogen dioxide at a concentration of 0.3 ppm in a challenge chamber, no acute inflammation could be observed in proximal airways in normal subjects, and in asthmatic and chronic obstructive pulmonary disease patients.

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