Effect of short-term NO₂ exposure on induced sputum


ABSTRACT: The aim of this study was to assess the effects of short-term exposure to low levels of nitrogen dioxide (NO₂) 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) NO₂ 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 NO₂ exposure compared to air exposure, except for a mild decrease in forced expiratory volume in one second (FEV₁) 2 h after NO₂ exposure in COPD patients.

Symptom score showed a mild increase after NO₂ 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.
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 non-smokers, while 6 of the 7 COPD patients were ex-smokers (39.6±17.5 pack-yrs).

Normal and asthmatic subjects had normal baseline FEV1 and had been free from respiratory symptoms for at least six weeks before the study. COPD patients had a FEV1/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. FEV1 was measured 2 min after each step. The test was stopped when FEV1 fell by 20% or more below the postdiluent value, and the cumulative provocative dose producing a 20% fall in FEV1 (PD20FEV1) was computed. According to epidemiological studies using the same technique [14], a PD20FEV1 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 µg·day−1), which were withdrawn 8 h before each exposure. All COPD patients were receiving regular treatment with low-dose inhaled steroids (beclomethasone dipropionate, 500 µg·day−1) and long-acting β2-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 NO2 (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.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 m3 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 2±1°C and the relative humidity was 45±5%. An NO2 analyser (8840, Monitor labs, Rancom Instruments, Milano, Italy), connected to the chamber at one end and to a tank containing 0.1% NO2, was used. An electronic valve automatically closed or opened the circuit when the NO2 concentration in the chamber was higher or lower than 3 L.min−1. An electronic valve automatically closed or opened the circuit when the NO2 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) NO2 concentration was 0.3±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.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; FEV1 was then measured. Nebulization was stopped after 30 min or when FEV1 fell by 20% or more from baseline. The functional response to inhalation of HS was expressed as maximum FEV1 fall (%FEV1%) after HS inhalation.

Sputum samples were diluted with an equal volume

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

<table>
<thead>
<tr>
<th>Subjects</th>
<th>n</th>
<th>Age ± yrs</th>
<th>Sex M/F</th>
<th>FEV1 % pred</th>
<th>Smoking habit S/NS/ES</th>
<th>Height cm</th>
<th>Weight kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normals</td>
<td>7</td>
<td>34±5</td>
<td>7/0</td>
<td>100±11</td>
<td>0/7/0</td>
<td>175±4</td>
<td>79±19</td>
</tr>
<tr>
<td>Asthmatic</td>
<td>8</td>
<td>29±14</td>
<td>4/4</td>
<td>97±10</td>
<td>0/8/0</td>
<td>167±8</td>
<td>66±13</td>
</tr>
<tr>
<td>COPD</td>
<td>7</td>
<td>58±12</td>
<td>7/0</td>
<td>62±25</td>
<td>0/1/6</td>
<td>170±4</td>
<td>78±10</td>
</tr>
</tbody>
</table>

Values are presented as mean± SD. M: male; F: female; FEV1: forced expiratory volume in one second; COPD: chronic obstructive pulmonary disease; S: smokers; NS: nonsmokers; ES: ex-smokers; % pred: percentage of predicted value.
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 cyto-centrifuged 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 dithiothreitol.

Statistical analysis

FEV1 values, maximum FEV1 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 FEV1 value pre- and postexposure to air or NO2. 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 FEV1 fall after HS inhalation between the value obtained after NO2 and after air exposure in each group. A p-value lower than 0.05 was considered as significant [17].

Results

Clinical and functional evaluation

FEV1 did not change after NO2 or air exposure in any of the three groups of subjects examined. There was a significant difference in FEV1 between air and NO2 2 h after exposure in COPD patients (table 2).

In normal subjects and in COPD patients, an increase in symptom score was observed after NO2 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; 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 NO2 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 NO2 exposure were considered.

NO2 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 NO2 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)%; 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 NO2 exposure.

Discussion

This study has shown that 60 min exposure to 0.3 ppm NO2 in a challenge chamber does not affect pulmonary function in normal subjects and in patients with asthma, whereas it elicits a mild decrease in FEV1 in COPD patients with asthma or COPD.

Table 2. – FEV1 and symptom score (SS) measured in normal subjects and in patients with asthma or COPD, before and after exposure to NO2 or air

<table>
<thead>
<tr>
<th></th>
<th>Normals</th>
<th>Air</th>
<th>Asthmatics</th>
<th>NO2</th>
<th>Air</th>
<th>COPD</th>
<th>NO2</th>
<th>Air</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV1 pre-exposure L</td>
<td>4.42±0.53</td>
<td>4.42±0.57</td>
<td>3.41±0.51</td>
<td>3.45±0.55</td>
<td>2.00±0.92</td>
<td>2.03±0.71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV1 postexposure L</td>
<td>4.36±0.51</td>
<td>4.38±0.54</td>
<td>3.48±0.56</td>
<td>3.38±0.51</td>
<td>1.98±0.91</td>
<td>2.14±0.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV1 2 h postexposure L</td>
<td>4.37±0.53</td>
<td>4.37±0.53</td>
<td>3.43±0.48</td>
<td>3.38±0.51</td>
<td>1.94±0.85</td>
<td>2.14±0.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS pre-exposure</td>
<td>0.1±0.4</td>
<td>0.3±0.7</td>
<td>0.4±0.7</td>
<td>0.4±0.7</td>
<td>5.2±6.8</td>
<td>8.2±16.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS postexposure</td>
<td>1.5±1.5*</td>
<td>1.0±1.7</td>
<td>3.0±4.5</td>
<td>0.8±1.3</td>
<td>13.6±17*</td>
<td>18.8±31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFEV1-HS %</td>
<td>4.6±1.4</td>
<td>1.6±2.2</td>
<td>27±12</td>
<td>29±19</td>
<td>11±9</td>
<td>19±16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are presented as mean±SD. The response to HS inhalation (AFEV1-HS) was measured 2 h after exposure to NO2 or air. For definitions see legend to table 1. *: p<0.05 between pre- and postexposure by Wilcoxon test; #: p<0.05 between NO2 and air exposure by paired t-test. HS: hypertonic saline. For further definitions see table 1.
sputum after NO\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{2} exposure on pulmonary function and bronchial responsiveness in healthy and asthmatic subjects [3–7, 21]. Although the NO\textsubscript{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\textsubscript{2} exposure.

Few data obtained using bronchoalveolar lavage (BAL) are available on the acute airway inflammatory response after NO\textsubscript{2} exposure [9, 10]. BAL recovers cells mainly from peripheral airways, which have been supposed to be the target area for NO\textsubscript{2} in animal experimental studies [22]. The use of these models has shown that NO\textsubscript{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\textsubscript{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\textsubscript{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, NO₂ 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 NO₂ similar to ours, may suggest that cellular changes could occur in the sputum cell percentage [4, 5]. Furthermore, we chose 0.3 ppm NO₂ because this concentration represents the peak value of NO₂ 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 NO₂ 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 \textit{in vivo} could induce the same effects as observed \textit{in vitro}. Thirdly, sputum may have been collected too soon after NO₂ 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 NO₂ 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 h 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.

References


