The effect of 1 ppm nitrogen dioxide on bronchoalveolar lavage cells and inflammatory mediators in normal and asthmatic subjects

R. Jörres*, D. Nowak*, F. Grimminger**, W. Seeger**, M. Oldigs*, H. Magnussen*

The effect of 1 ppm nitrogen dioxide on bronchoalveolar lavage cells and inflammatory mediators in normal and asthmatic subjects. R. Jörres, D. Nowak, F. Grimminger, W. Seeger, M. Oldigs, H. Magnussen. ©ERS Journals Ltd 1995.
ABSTRACT: Several studies have suggested that patients with bronchial asthma are more susceptible to the potential effects of nitrogen dioxide (NO_2) than healthy subjects, with respect to airway responsiveness and lung function. We investigated whether these differences are paralleled by differences in the cellular and biochemical response within the airway lumen.

Twelve subjects with mild extrinsic asthma and eight normal subjects breathed either filtered air or 1 ppm NO_2 in a single-blind manner during intermittent exercise for 3 h. Bronchoscopy with bronchoalveolar lavage (BAL) was performed one hour after each exposure, and on a third day without exposure (baseline day). Prostanoids, leukotrienes and histamine were analysed in BAL fluid, and the cellular composition of BAL fluid was assessed.

In the asthmatic subjects, NO_2 induced a small mean drop in forced expiratory volume in one second (FEV_1). Differential cell counts in BAL fluid did not reveal significant effects of NO_2. Levels of 6-keto-prostaglandin$_{1\alpha}$ (6-keto-PGF$_{1\alpha}$) were decreased, and levels of thromboxane B_2 (TxB$_2$) and prostaglandin D_2 (PGD$_2$) in BAL fluid were increased after NO_2 compared to filtered air exposure; whereas, prostaglandin E_2 (PGE$_2$), prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$), histamine and leukotriene levels did not change significantly. The normal subjects showed no change in lung function parameters and a small increase in TxB$_2$ after breathing NO_2.

We conclude that in subjects with mild asthma NO_2 is capable of inducing an activation of cells, which is compatible with enhancement of airway inflammation, even if lung function parameters and cellular composition of BAL fluid are not markedly affected. The lower susceptibility to NO_2 of normal subjects is also reflected in their lower response regarding the soluble markers of inflammation in BAL.
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*Krankenhaus Grosshansdorf, Zentrum für Pneumologie und Thoraxchirurgie, LVA Freie und Hansestadt Hamburg, Grosshansdorf, Germany. **Medizinische Universitätsklinik, Giessen, Germany.

Correspondence: R. Jörres
Krankenhaus Grosshansdorf
Zentrum für Pneumologie und Thorax-chirurgie
Wöhrendamm 80
D-22927 Grosshansdorf
Federal Republic of Germany

Nitrogen dioxide (NO_2) is a common environmental air pollutant and, therefore, considerable efforts have been made to study its potential hazards for the human respiratory tract.

In experimental exposures of healthy subjects, NO_2 has been found to have no influence on baseline lung function [1], but to enhance airway responsiveness [1] or leave it unchanged [2]. The results of some studies suggest that subjects with bronchial asthma could be more sensitive to NO_2 with respect to these functional parameters [3–5]. In bronchial asthma, airway inflammation and airway hyperresponsiveness are generally considered to be prominent features [6].

We therefore investigated whether the increased susceptibility to NO_2 of asthmatic subjects, as derived from lung function studies, reflects greater inflammatory changes in bronchoalveolar lavage (BAL) cells and eicosanoid mediators compared to healthy subjects. For the experimental protocol, a short-term exposure to 1 part per million (ppm) NO_2 for 3 h during intermittent moderate exercise was chosen. Bronchoscopy was performed 1 h after the end of exposure in order to detect the initial changes induced by NO_2. These changes should correspond to the effects of NO_2 on lung function and airway responsiveness, which have been demonstrated to occur within this period of time.

Methods

Subjects

Twelve nonsmoking subjects with bronchial asthma were enrolled into the study (table 1). Their diagnosis of mild extrinsic bronchial asthma was based on typical history and followed published criteria [7, 8]. They were free of symptoms at the time of the study. All, except

Table 1. – Individual data of the asthmatic subjects

Subject No.	Sex M/F	Age yrs	Height cm	VC l	FEV_1 l	FEV_1 % pred*	$PC_{20}FEV_1$ [+] $mg \cdot ml^{-1}$	Therapy
1	F	23	176	4.4	3.7	98	1.65	β_2
2	F	26	167	3.7	3.3	99	0.073	0
3	F	37	165	2.9	2.3	77	0.65	β_2
4	M	23	173	4.4	3.6	84	6.4	0
5	M	21	182	5.1	4.4	93	0.05	β_2
6	M	35	178	4.4	3.5	84	>8	0
7	M	26	188	5.8	3.9	81	0.25	0
8	M	32	187	6.1	4.3	93	0.23	0
9	F	26	176	4.2	3.5	95	0.91	0
10	M	21	183	5.0	4.2	88	1.0	0
11	M	24	186	4.9	4.2	87	0.28	β_2
12	M	25	178	5.0	3.0	68	0.23	β_2
Mean	-	27	178	4.7	3.7	87	0.58	-
SD	-	5	7	0.9	0.6	9	5.5	-

*: predicted values according to ECSC [18]; [+]: airway responsiveness to methacholine (geometric mean value and standard deviation are given). M: male; F: female; ECSC: European Coal and Steel Community; VC: vital capacity; FEV_1: forced expiratory volume in one second; $PC_{20}FEV_1$: provocative concentration causing a 20% decrease in FEV_1; β_2: inhaled β_2-adrenoceptor agonist; 0: no medication.

one, showed airway hyperresponsiveness to inhaled methacholine as demonstrated by a provocative concentration necessary to decrease forced expiratory volume in one second (FEV_1) by 20% ($PC_{20}FEV_1$) of lower than $8 \ mg \cdot ml^{-1}$. Beta-adrenoceptor agonists, which were inhaled by some subjects *p.r.n.* or regularly, were withheld for at least 8 h prior to each study session. None of the subjects had received inhaled corticosteroids, disodium cromoglycate or theophylline within 3 months before the study. All subjects were judged atopic on the basis of history and a positive skin-prick test to at least one common allergen (Allergopharma, Reinbek, Germany). Within 4 weeks preceding each study day, no subject suffered from an upper respiratory tract infection, experienced an uncommon burden of allergen or reported on any other trigger which may worsen asthma. Therefore, all subjects were considered to be in a stable clinical condition.

The data of the asthmatic subjects were compared with those of 8 nonsmoking healthy control subjects (5 females and 3 males), with mean (range) age of 27 (21–33) yrs and mean (range) FEV_1 of 99 (90–112) % of predicted. None of these subjects showed positive skin prick tests or was hyperresponsive to inhaled methacholine. Within 4 weeks preceding the study, no subject had suffered from an upper respiratory tract infection.

The study was approved by the Ethics Committee of the Medical Board of Schleswig-Holstein. All subjects were instructed about the aim of the study and gave their written informed consent.

Study protocol

Each subject was studied on three separate days, being at least one week apart, with a mean interval of 14.5 days between tests. On two of these days, subjects were exposed to 1 ppm NO_2 or filtered air during intermittent exercise, with subsequent bronchoalveolar lavage.

Exposures started at 09:00 a.m. Subjects alternated rest for 10 min and exercise for 10 min on a bicycle ergometer whilst breathing the test gas. After each of the resting and exercise periods, lung function was measured repeatedly within 5 min. The resting and exercise periods were repeated six times for a total of 3 h. In addition, lung function was assessed 2, 10, 20 and 30 min after the end of exposure. Workload was adjusted individually to the maximum load that could be maintained during 3 h; the same load was chosen both on the NO_2 and filtered air exposure day. Bronchoscopy was performed 1 h after the end of exposure. Symptom scores were assessed immediately, 6 and 24 h after exposures.

On another day, bronchoscopy was performed at the same time of day as on the exposure days but without previous exposure to filtered air or NO_2 (baseline day). The order of the three study sessions was randomized. Exposures and lung function measurements were performed single-blind, in that only the subjects were blinded. Bronchoscopy was performed without knowledge of the type of exposure, and analysis of bronchoalveolar lavage fluid (BALF) was performed entirely blind.

Exposure system

Room air was passed through particle and gas filters and a humidifier. Mean ($\pm$SD) temperature was 24.6 ± 2.9°C and relative humidity 68 ± 8%, with no significant differences between exposures. For NO_2 exposures, 200 ppm NO_2 (Linde AG, Unterschleißheim, Germany) was added to the filtered airstream, and the desired concentration of 1 ppm was maintained within ± 5% by a flow-regulating valve. Mixed air was sampled at the inspiratory part of the mouthpiece and NO_2 was monitored continuously by a chemiluminescence nitrogen oxides analyser (Model 8840, Monitor Labs Inc., San Diego, CA, USA). The analyser was calibrated by a permeation tube

calibrator (Model 8550, Monitor Labs Inc.) whose concentrations had been verified by an approved calibration system (Environmental Protection Agency of Hamburg, Germany).

The subjects were exposed *via* a mouthpiece, while sitting on a bicycle ergometer (Jaeger, Würzburg, Germany). The mouthpiece was attached to a two-way valve and an air delivery bag, the mouth part of the valve being partitioned by a separating wall. Inspiratory flow was measured by means of a pneumotachograph and electronically integrated to give tidal volume and minute ventilation (Jaeger, Würzburg, Germany). Mean expired carbon dioxide and oxygen concentrations were determined using a carbon dioxide analyser and an oxygen analyser (Jaeger, Würzburg, Germany) to aid in controlling workload. Oxygen saturation and heart rate were monitored using an ear lobe oximeter (Radiometer, Copenhagen, Denmark). Tidal volume, minute volume, temperature, humidity, and gas concentrations were recorded continuously on a tape recorder (Rikadenki Electronics, Freiburg, Germany).

Lung function measurement

Forced expiratory volume in one second (FEV_1) was determined by a pneumotachograph (Jaeger, Würzburg, Germany). All measurements were performed at least in duplicate, and for analysis the maximum value was taken [8].

Assessment of symptoms

The severity of symptoms induced by exposure was estimated from a questionnaire on an ordinal scale ranging 0–10. A panel of symptoms comprising throat irritation, cough, chest tightness, shortness of breath, headache, nausea and dizziness was checked.

Fibreoptic bronchoscopy

Bronchoscopy was performed according to the guidelines for their scientific use [9] by one investigator (D.N.) using a flexible bronchoscope (BF20-D, Olympus, Tokyo, Japan). All subjects were premedicated subcutaneously with 0.5 mg atropine 30 min prior to bronchoscopy. Immediately before bronchoscopy, all subjects inhaled 0.1 mg of fenoterol. Topical anaesthesia of the nose and pharynx was achieved by 0.5% lidocaine, and additional anaesthesia of the nose by 2% lidocaine gel. During bronchoscopy, subjects received 100% oxygen at a rate of 4 $l\cdot min^{-1}$ through a nasal cannula. The bronchoscope was introduced transnasally or orally, with additional 0.5% lidocaine applied through the channel to the larynx, vocal cords and trachea. Endobronchial examination was performed, and the results were summarized in macroscopic scores ranging 0–3 and comprising the variables: erythema, oedema, secretions, and friability [10]. For analysis, scores were collapsed into two categories

(0–1 *vs* 2–3). Local anaesthesia of the middle lobe (first and third bronchoscopy) or lingula bronchus (second bronchoscopy) was achieved, and the bronchoscope was wedged into a respective segment. BAL was performed using 5×20 ml aliquots of sterile saline at 37°C. BALF was immediately aspirated into siliconized glassware. BALF was transported on ice to the laboratory; after initial centrifugation (15 min, 4°C, 1,000 rpm) cells were transferred to the cytocentrifuge, and the supernatant was further processed on extraction columns. Immediately before and after, and 2–3 h after bronchoscopy, FEV_1 was determined by means of a wedge spirometer.

Analytical procedures

The analytical procedures followed the same protocol as described previously [11]. We determined leukotrienes (LT) C_4, D_4, E_4 and B_4, and their ω-oxidation products 20-OH-LTB_4 and 20-COOH-LTB_4, which were extracted from BALF by solid-phase extraction columns immediately after removal of cells [12]. Nonmethylated compounds were analysed by reverse-phase (RP)-high performance liquid chromatography (HPLC) [13]. Full spectra (190–600 nm) of eluting compounds allowed correction to be made for possible coeluting material, and further verification was achieved by analysing sampled fractions by post-HPLC radioimmunoassays with anti-LTC_4 and anti-LTB_4 [13]. RP-HPLC of methylated compounds was performed by isocratic elution and a subsequent linear gradient [11], and straight phase HPLC as a modification of the method of POWELL [14]. All data were corrected for recoveries of the overall analytical procedure by the addition of 0.125 µCi of (^{3}H)LTC_4 and 0.05 µCi of (^{3}H)LTB_4 as internal standards in each lavage sample. Recoveries were between 25 and 55%, and detection limits between 0.6 and 0.8 $ng\cdot ml^{-1}$.

Histamine in BALF was analysed by means of a solid phase radioimmunoassay (J-125, Biermann Diagnostica, Bad Nauheim, Germany).

The levels of prostaglandin (PG) D_2, E_2 and $F_{2\alpha}$ in BALF were assessed by commercially available radioimmunoassay kits (Amersham, Braunschweig, Germany). Thromboxane B_2 (TxB_2) and 6-keto-$PGF_{1\alpha}$ were determined by custom radioimmunoassays [15].

Analysis of BAL cells

Cytological examination of 500 BAL cells was carried out on cytocentrifuge slides, which had been stained according to May-Grünwald-Giemsa.

Statistical evaluation

The effects of exposures to NO_2 and filtered air were expressed as percentage changes from baseline values. Mean values and standard deviations (SD) or standard errors of the mean (SEM) were computed for lung function and exercise parameters. For BALF data, median

values and interquartile (25–75%) ranges were calculated. Analysis of variance (ANOVA) and *post hoc* paired t-tests [16] were used to evaluate the changes in lung function parameters during exposures. Group mean values were compared using the independent t-test. In the analysis of BALF data, nonparametric test procedures were applied, in order to reduce the assumptions on data distribution; the Wilcoxon matched-pairs signed-rank test was used to compare exposures within groups and the Mann-Whitney-U-test to compare the groups of healthy and asthmatic subjects with each other [17]. Correlation analysis was performed by Pearson's linear correlation, and by Spearman's rank correlation, if lavage parameters were involved [17]. For analysis of macroscopic scores and for comparison of leukotrienes, Chi-squared contingency table analysis and Fisher's exact test were used [17]. The results of macroscopic scores are presented as mean values. The level of statistical significance was assumed at p=0.05 for each single test, and p-values are given explicitly.

Results

Experimental conditions

During intermittent exercise, minute ventilation and heart rate did not differ significantly between exposures, with mean (±SD) values of 34.4±4.7 $l \cdot min^{-1}$ and 134±13 beats·min⁻¹ with filtered air, and 34.5±6.0 $l \cdot min^{-1}$ and 135±14 beats·min⁻¹ with NO_2. Similarly, these parameters did not show significant differences between the asthmatic and healthy group.

Lung function and symptoms

The asthmatic subjects showed mean (±SEM) FEV_1 of 3.57±0.16 and 3.58±0.12 l before and immediately after breathing filtered air, and 3.51±0.14 and 3.46±0.13 l before and immediately after NO_2 exposure (fig. 1). Whereas baseline values did not differ statistically, the mean percentage fall during NO_2 exposure (2.5%) was significantly greater than during filtered air exposure (1.3%) (p=0.011, ANOVA). This difference was mainly due to subject no. 7, who showed a fall of FEV_1 of 20% during breathing NO_2 and an improvement of 6% during breathing filtered air; and to subject No. 12 who showed an improvement of 6% during filtered air and no change during NO_2 exposure.

After the first but not after the following five exercise periods, significant exercise-induced bronchoconstriction occurred (p=0.007, paired t-test); there was no difference between NO_2 and filtered air results.

In the healthy subjects mean (±SEM) FEV_1 was 3.98±0.15 and 4.07±0.16 l before and immediately after exposure to filtered air, respectively, and 4.03±0.19 and 4.07±0.17 l before and immediately after breathing NO_2, respectively (fig. 1). Neither the FEV_1 values before exposures nor the values during and after exposures were statistically different from each other.
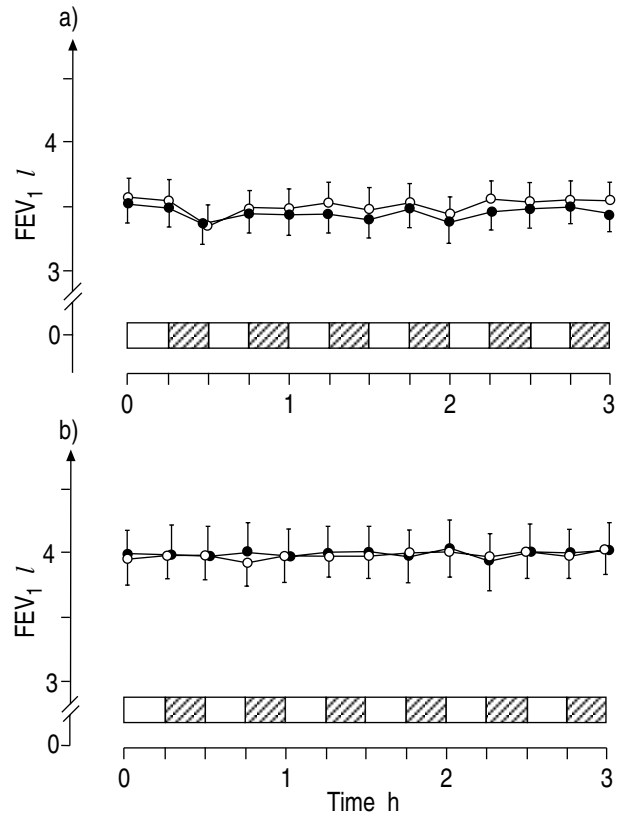

Fig. 1. – Mean (±SEM) FEV_1 before, during and after exposure to filtered air (—○—) and NO_2 (—●—) after periods of rest and exercise (shaded area): a) in the asthmatic patients; b) in the healthy subjects. FEV_1: forced expiratory volume in one second.

Neither the healthy nor the asthmatic subjects reported on symptoms immediately after, and 6 and 24 h after NO_2 or filtered air exposure that were significantly different.

Bronchoscopy

Bronchoscopy on the baseline day and after exposure to filtered air or NO_2 was well-tolerated, both in the asthmatic and healthy subjects. In none was therapeutic intervention necessary. In the asthmatic subjects, mean vital capacity and FEV_1 after bronchoscopy dropped on average by 4.1% (maximum 16) and 2.5% (maximum 14), respectively, and in the healthy subjects by 5.0% (maximum 13) and 5.9% (maximum 16), respectively, as compared to baseline.

Macroscopic scores

In the asthmatic subjects, the macroscopic scores of endobronchial appearance after NO_2 exposure were significantly (p<0.05 each) higher for erythema (1.2 vs 0.6 and 0.3), oedema (1 vs 0.3 and 0.6), and friability (0.6 vs 0.2 and 0.3) compared to filtered air exposure and baseline bronchoscopy, respectively. Secretion was higher (p<0.05) after both exposures compared to baseline bronchoscopy (1.7 vs 1.1 and 0.6). The healthy subjects did not show significant differences between bronchoscopies.

Table 2. – Median values and interquartile range of cell numbers in BALF of the asthmatic subjects and the healthy subjects

	Baseline day		Filtered air		NO$_2$	
Bronchoalveolar lavage fluid of asthmatic patients						
Recovery ml	72	(64–75)	61	(55–68)	69	(57–72)
Total cell number ×10^6	5.5	(3.8–9.0)	4.3	(2.3–6.1)	4.0	(2.6–6.0)
Macrophages %	89.3	(87.7–92.2)	93.2	(89.1–95.3)	92.4	(84.9–95.2)
Lymphocytes %	3.1	(1.35–6.0)	4.0	(1.85–5.55)	2.85	(1.75–6.0)
Neutrophils %	0.15	(0.0–1.15)	0.5	(0.25–0.75)	0.4	(0.3–0.7)
Eosinophils %	1.0	(0.4–1.45)[†]	0.3	(0.2–0.55)*	0.3	(0.0–0.95)*
Epithelial cells %	3.15	(1.5–5.9)	1.0	(0.75–3.3)	2.35	(1.4–7.95)
Mast cells %	0.0	(0.0–0.0)	0.0	(0.0–0.0)	0.0	(0.0–0.0)
Bronchoalveolar lavage fluid of healthy subjects						
Recovery ml	70	(67–78)	71	(67–80)	70	(63–77)
Total cell number ×10^6	5.5	(3.0–9.0)	5.6	(3.9–8.4)	4.9	(4.4–7.8)
Macrophages %	94.0	(89.3–95.0)	93.1	(92.0–94.2)	92.1	(88.4–95)
Lymphocytes %	3.0	(1.9–5.8)	3.35	(2.8–5.35)	3.75	(2.9–5.85)
Neutrophils %	0.8	(0.6–1.0)	0.55	(0.15–0.8)	0.7	(0.25–1.1)
Eosinophils %	0.0	(0.0–0.3)[†]	0.0	(0.0–0.3)	0.1	(0.0–0.5)
Epithelial cells %	1.6	(0.0–3.8)	1.65	(0.75–2.7)	2.1	(1.25–3.25)
Mast cells %	0.0	(0.0–0.2)	0.0	(0.0–0.0)	0.0	(0.0–0.25)

BALF: bronchoalveolar lavage fluid. [†]: significantly different ($p<0.002$) asthmatic *vs* healthy. *: significantly different ($p<0.05$) from baseline.

Cells in bronchoalveolar lavage fluid

On the baseline day, the number of eosinophils in BALF was significantly greater in the asthmatic than in the healthy subjects ($p=0.002$), (table 2). Similarly, the absolute number of eosinophils from peripheral blood was increased in the asthmatic subjects ($p=0.009$).

The asthmatic subjects showed no significant differences between the three bronchoscopies with respect to BAL cell counts of macrophages, lymphocytes, neutrophils, epithelial cells and mast cells. However, the number of eosinophils was significantly reduced after breathing filtered air ($p=0.005$) or NO$_2$ ($p=0.03$), as compared to baseline lavage (table 2).

In the healthy subjects, the differential cell numbers in BALF did not differ significantly between the baseline lavage and the lavages performed after exposure to filtered air or NO$_2$ (table 2).

Recovery and total number of BAL cells did not differ between lavages for both groups (table 2).

Table 3. – Median values and interquartile range of mediators in BALF in the asthmatic subjects and the healthy subjects.

Parameter	Baseline day		Filtered air		NO$_2$	
Bronchoalveolar lavage fluid of asthmatic patients						
6-keto-PGF$_{1\alpha}$ pg·ml^{-1}	93.1	(64.3–136.6)	97.9	(89.5–106.8)	65.5	(0.0–73.5)*+
PGE$_2$ pg·ml^{-1}	34.0	(20.3–46.6)[†]	29.1	(16.7–44.7)	22.2	(0.0–36.9)
PGD$_2$ pg·ml^{-1}	52.2	(39.6–75.6)	55.6	(46.1–66.4)	75.6	(61.5–120.9)+
PGF$_{2\alpha}$ pg·ml^{-1}	20.5	(0.0–31.8)[†]	17.3	(0.0–26.3)	15.0	(0.0–20.3)
TxB$_2$ pg·ml^{-1}	145.6	(100.5–184.5)	120.5	(90.8–187.3)	189.2	(147.6–227.5)+
Histamine nmol·l^{-1}	1.55	(0.76–6.32)	3.20	(1.57–5.75)	2.93	(1.50–5.88)
LTB$_4$ ng·ml^{-1}	0.0	(0.0–1.12)	0.0	(0.0–0.0)	1.06	(0.0–1.54)*
20-OH-LTB$_4$ ng·ml^{-1}	0.0	(0.0–1.08)	0.0	(0.0–1.79)	1.68	(0.0–2.01)*
20-COOH-LTB$_4$ ng·ml^{-1}	0.0	(0.0–0.0)	0.0	(0.0–0.0)	0.0	(0.0–1.17)
LTC$_4$ ng·ml^{-1}	0.0	(0.0–0.0)	0.0	(0.0–0.0)	0.0	(0.0–0.0)
LTD$_4$ ng·ml^{-1}	0.0	(0.0–0.0)	0.0	(0.0–0.0)	0.0	(0.0–0.65)
LTE$_4$ ng·ml^{-1}	0.0	(0.0–0.0)	0.0	(0.0–0.0)	0.0	(0.0–0.73)
Bronchoalveolar lavage fluid of healthy subjects						
6-keto-PGF$_{1\alpha}$ pg·ml^{-1}	88.2	(55.7–127.9)	98.1	(52.3–139.1)	73.4	(54.3–93.6)
PGE$_2$ pg·ml^{-1}	18.8	(0.0–27.7)[†]	14.3	(0.0–24.3)	8.6	(0.0–21.8)
PGD$_2$ pg·ml^{-1}	34.1	(30.2–70.7)	33.0	(20.6–67.5)	35.3	(20.5–66.3)
PGF$_{2\alpha}$ pg·ml^{-1}	0.0	(0.0–0.0)[†]	0.0	(0.0–0.0)	0.0	(0.0–0.0)
TxB$_2$ pg·ml^{-1}	90.4	(80.3–127.3)	84.0	(71.7–107.1)	119.7	(90.6–156.5)*+
Histamine nmol·l^{-1}	0.51	(0.18–2.10)	0.33	(0.15–0.68)	0.29	(0.18–0.70)

PG: prostaglandin; TxB$_2$: thromboxane B$_2$; LT: leukotriene; BALF: bronchoalveolar lavage fluid. [†]: significantly different ($p<0.05$) asthmatic *vs* healthy. *: significantly different ($p<0.05$) from baseline day; +: significantly different ($p<0.05$) from filtered air exposure. Leukotrienes are not shown for healthy subjects, since they did not exceed detection limits in any of the measurements.
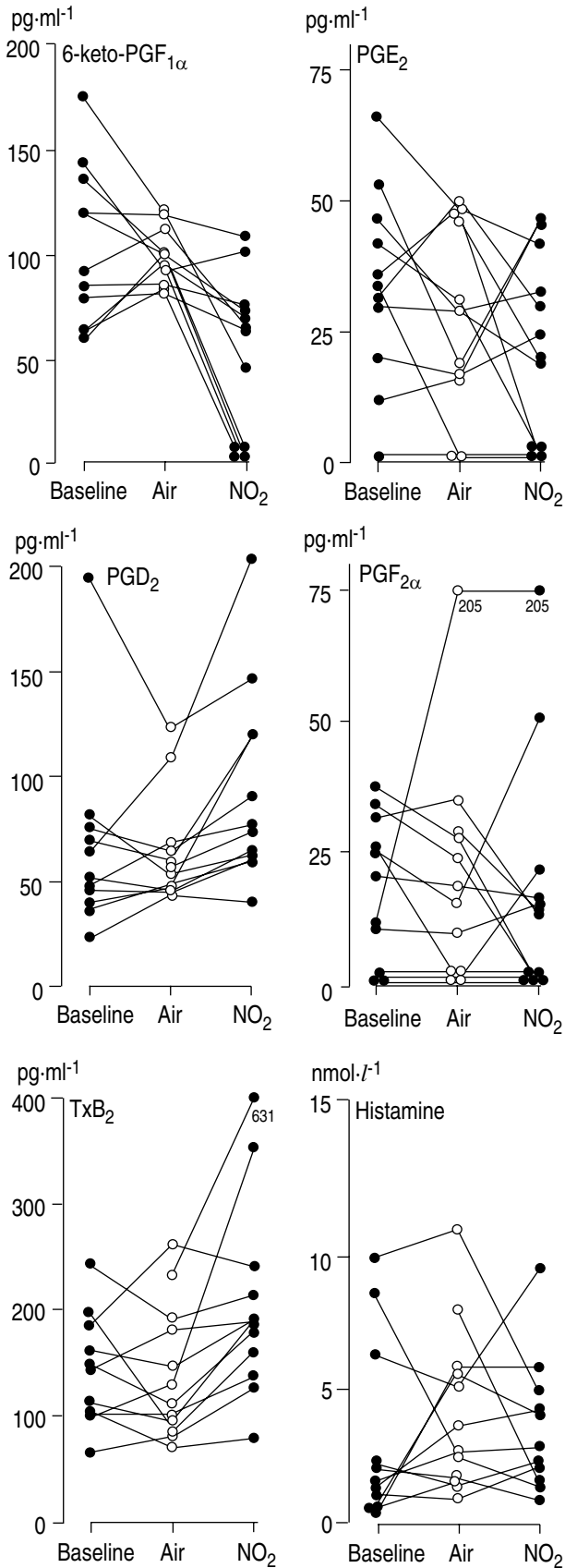
Fig. 2. – Concentrations of prostanoids and histamine in BALF after exposure to filtered air or NO_2, and on the baseline day. BALF: bronchoalveolar lavage fluid; PG: prostaglandin; TxB_2: thromboxane B_2. Data are given for subjects with asthma.

Mediators in bronchoalveolar lavage fluid

In baseline lavages, the levels of $PGF_{2\alpha}$ (p=0.011) and PGE_2 (p=0.033) were significantly greater in the asthmatic subjects compared to the healthy subjects, whereas the concentration of 6-keto-$PGF_{1\alpha}$, TxB_2 (p=0.052), PGD_2, and histamine (p=0.11) in baseline lavages did not differ statistically between groups (table 3). The levels of LTC_4, D_4 and E_4 in baseline lavages did not differ statistically between groups. Similarly, LTB_4 and its metabolites 20-OH-LTB_4 and 20-COOH-LTB_4 were not more often detectable in the asthmatic subjects (p=0.051).

The asthmatic subjects showed no significant differences in $PGF_{2\alpha}$, PGE_2 and histamine between baseline lavage and lavages performed after NO_2 or filtered air exposure. However, the levels of TxB_2 (p=0.0047) and PGD_2 (p=0.003) were significantly increased, and that of 6-keto-$PGF_{1\alpha}$ (p=0.0029) decreased, after NO_2 compared to filtered air exposure (table 3 and fig. 2). Compared to baseline lavages, the respective p-values were 0.051, 0.08 and 0.0044.

No significant differences were found in LTC_4, 20-COOH-LTB_4, LTD_4 and LTE_4 in the asthmatic subjects (table 3 and fig. 3). The levels of LTB_4 (p=0.028) and 20-OH-LTB_4 (p=0.036) after NO_2 exposure were significantly increased compared to baseline lavages; compared with filtered air exposure, differences were not significant (table 3). In the majority of subjects, leukotrienes were below detection limits. However, an occasional increase in LTC_4, D_4 and E_4 was observed after NO_2 exposure (fig. 3).

The healthy subjects showed no significant changes in prostaglandin and histamine levels between baseline lavage and lavages after NO_2 or filtered air exposure (table 3). TxB_2 concentration after NO_2 exposure was significantly higher than after filtered air exposure (p=0.017), or in baseline lavage (p=0.04). None of the leukotrienes in BALF exceeded detection limits.
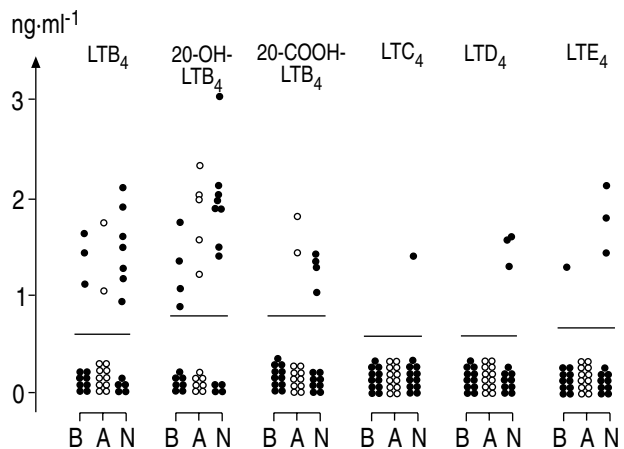

Fig. 3. – Concentrations of leukotrienes (LT) in BALF after exposure to filtered air (A) or nitrogen dioxide (N), and on the baseline day (B). BALF: bronchoalveolar lavage fluid. Data refer to the subjects with asthma. Bars indicate detection levels.

Correlation analysis

In the asthmatic subjects, no significant relationships were found between BAL cell counts and baseline FEV_1 as percentage of predicted values, or between BAL cell counts and baseline airway responsiveness to methacholine as expressed by $PC_{20}FEV_1$.

In addition, no statistically significant correlations were observed between the differences of lung function response and the differences of absolute levels of mediators in BALF between both exposures. For 6-keto-$PGF_{1\alpha}$ *vs* PGD_2 ($r_s=-0.67$) as well as for TxB_2 *vs* PGD_2 ($r_s=0.52$), there were statistically significant ($p<0.05$) correlations between the respective percentage differences of NO_2 and filtered air exposure.

Discussion

In this study, we have demonstrated that in subjects with mild bronchial asthma, short-term exposure to NO_2 produces changes in prostanoid mediator profile in BALF which are indicative of cell activation within the airways. These changes were accompanied by a borderline impairment of lung function. Differential cell counts in BALF were not significantly affected. The changes in BALF after NO_2 exposure were less pronounced in healthy subjects.

We have not observed marked impairment in lung function or respiratory complaints after exposure to 1 ppm NO_2, which is in accordance with the literature [18, 19]. Most of the studies suggest that the adverse functional consequences of NO_2 exposure in asthmatic subjects are better assessed by measuring airway responsiveness to bronchoconstrictive stimuli [3–5, 20–23]. However, some studies were unable to demonstrate effects of NO_2 on airway responsiveness [2, 18, 24, 25] at low concentrations of NO_2. We did not measure airway responsiveness, because, in a previous study, we had found an increase in the amount of LTB_4, its ω-oxidation products, and PGD_2 after methacholine inhalation in asthmatic subjects [11]. This finding suggested that methacholine challenges could interfere with the effects of NO_2. The argument has been corroborated *a posteriori* by the observation that the changes in LTB_4 and PGD_2 after NO_2 exposure were in a similar range as those measured after methacholine inhalation. Omitting methacholine challenges enabled us to perform bronchoscopy within one hour after the end (4 h after the beginning) of exposure, and to measure the initial changes induced by NO_2. The functional effects of NO_2 do occur within one hour after exposure [1, 20, 22, 23].

There was only a small exercise-induced fall in FEV_1 after the first exercise period, which was equal for filtered air and NO_2 exposure and virtually vanished during the repeated exercise periods, presumably due to refractoriness (fig. 1). Since our study was not designed to investigate changes of exercise-induced bronchoconstriction [22, 24], these observations have to be interpreted cautiously. Nevertheless, they suggest that NO_2 did not enhance exercise-induced asthma within the repeated tests or diminish refractoriness.

The results of our baseline lavages in asthmatic and healthy individuals are generally in line with the findings that the asthmatic disease is accompanied by distinct inflammatory processes [6]. The fact that airway inflammation has been demonstrated even in newly diagnosed asthma [26] suggests that an inflammatory cell response is a sensitive indicator of the disease activity. We studied whether short-term exposure to NO_2 could reveal alterations of the inflammatory cell pattern and mediator profile which would fit the higher susceptibility to NO_2 of asthmatic compared to normal subjects.

In the BALF of asthmatic subjects, we found decreased levels of 6-keto-$PGF_{1\alpha}$, (a metabolite of PGI_2) and increased levels of TxB_2 (a metabolite of TxA_2) and PGD_2 after NO_2 compared to filtered air exposure, whereas the concentrations of PGE_2, $PGF_{2\alpha}$ and histamine did not change. There was a tendency for leukotrienes (LTB_4, C_4, D_4, E_4), in single subjects, to show increased levels after NO_2 exposure compared to filtered air exposure and baseline bronchoscopy. These data can be interpreted as an increase in inflammatory mediators, which are associated with bronchoconstriction (PGD_2, TxB_2), and a decrease in a mediator which is associated with bronchodilation (6-keto-$PGF_{1\alpha}$). Since it is known that increased levels of PGD_2 and LTE_4 are related to enhanced bronchial responsiveness, our observations would be consistent with the previous findings that airway responsiveness in humans can be enhanced by NO_2. The slight increase of TxB_2 in the healthy controls suggests that breathing 1 ppm NO_2 (about one fifth of the workplace threshold value) is not completely without effect even in normal subjects. On average, marked bronchoconstriction was not observed in our study. Therefore, the observed changes are attributed to the ability of NO_2 to induce proinflammatory changes, the functional consequences of which could not be detected within our protocol. The changes in mediators indicate an activation of cells and support the idea that asthmatic subjects are more susceptible to NO_2 than normals. In addition, changes in inflammatory mediators seem to precede cellular changes or to be more sensitive to the effects of NO_2 than cell numbers.

Our results are, at least partially, compatible with those from animal *in vivo* and *in vitro* studies. Exposure of rat alveolar macrophages to NO_2 *in vitro* resulted in an increase of LTB_4 production [27]. Rabbits showed a decrease of 6-keto-$PGF_{1\alpha}$ after breathing 10 ppm NO_2, while TxB_2 was elevated after 1 ppm and depressed after 3 and 10 ppm NO_2 [28]. Similarly, in rats, the level of 6-keto-$PGF_{1\alpha}$ decreased [29]. The concentration of TxB_2 was elevated after 5 days of exposure; in contrast, the levels of $PGF_{2\alpha}$ and PGE_2 first decreased, transiently increased, and then decreased again. In sheep, increases in LTB_4 and LTE_4 in BALF between 0.5–24 h after exposure have been demonstrated, although at very high concentrations of NO_2 [30]. These results indicate quite a complicated time course, which depends on exposure levels, and support the view that the effects of prolonged or repeated exposures to NO_2 cannot be derived from short-term exposure results [31–33].

In previous studies, bronchoscopy has been applied in healthy individuals to detect changes in BALF induced

by NO_2. In these studies, investigators focused on parameters which were more likely to reveal an oxidative damage from NO_2 in healthy subjects, and none of them has investigated asthmatic subjects. The activity of the α_1-proteinase inhibitor has been found to be decreased after exposure to 3–4 ppm [34], but not 1.5 ppm NO_2 [35]; the level of the antiprotease (α_2-macroglobulin) increased after exposure to 0.6 ppm NO_2 [36]. In contrast to our results in the healthy control group, an increased number of BAL neutrophils has been observed in the bronchial washing fraction about one day after exposure to 2 ppm NO_2 [37], and the number of mast cells and lymphocytes was elevated in a dose-dependent manner 24 h after breathing 2.25 to 5.5 ppm NO_2 [38], and 4, 8 and 24 h, but not 72 h after exposure to 4 ppm NO_2 [39]. These discrepancies are probably due to the differences in NO_2 concentrations and time intervals between exposure and lavage.

Our data indicate that subjects with asthma as well as healthy subjects do not show significant alterations of BAL cellular composition one hour after the end of NO_2 exposure. Whereas this result is in accordance with some of the studies performed in healthy subjects [34–36], it does not agree with the increase in the number of mast cells and lymphocytes reported by SANDSTRÖM and co-workers [38, 39]. The discrepancy is probably due to the fact that these authors performed lavage 24 h and not one hour after exposure to higher levels of NO_2. The significant decrease in the number of eosinophils in asthmatic subjects, both after filtered air and NO_2 exposure as compared to baseline lavage, has to be attributed to the effects of the prolonged exercise protocol. After exercise-induced bronchoconstriction, elevated numbers of BALF eosinophils have been reported [40]. However, it is unclear whether these results also apply to exercise *per se* in the absence of exercise-induced bronchoconstriction, and to repeated exercise tests.

The macroscopic appearance of the airways is rarely used to describe stimulus-related effects, probably due to the difficulties in quantitating visual impression. Using a simple scoring system [10], we found that NO_2 caused, on average, an increase in erythema, oedema and friability. Clearly, these changes are not only associated with cellular infiltration but also with permeability, water flux and other parameters, which we did not measure.

Most of the functional data suggest that threshold levels of NO_2 which cause "latent" changes in the airways that can only be detected by assessment of airway responsiveness are lower than those levels which induce "apparent" changes, such as impairment of lung function. An increase in airway responsiveness could be interpreted as an indicator (although not unique) of inflammatory changes induced by NO_2. This interpretation is in line with present knowledge on the asthmatic disease [6], and with the parallelism that has been demonstrated between an increase in airways responsiveness and inflammatory changes, for example, by ozone [41].

Although it is not possible to evaluate the clinical implications of the changes in inflammatory mediators which we have observed, their presence and consistency suggest that NO_2 exposure could lead to potential adverse effects at this level. Adverse effects at low levels of NO_2 have been shown by epidemiological studies [42]. The level of nitrogen dioxide exposure chosen in the present study falls within peak exposure limits which can be reached or exceeded under indoor and outdoor exposure conditions, although not regularly encountered [43]. Furthermore, due to mouthpiece breathing, the effective dose of NO_2 may have been slightly higher than in unencumbered breathing at the same ambient concentration. Despite this, data interpretation may benefit from our finding that exposure to a burden of 3 ppm*h of NO_2 is capable of influencing inflammatory mediators in subjects with asthma, without producing obvious changes in lung function or symptoms.

In summary, we have demonstrated that short-term exposure to 1 ppm NO_2 was capable of inducing or enhancing inflammatory changes in the airways of asthmatic but not healthy subjects. These changes were small but consistent. As we failed to reveal consistent NO_2-induced effects on lung function, we would like to suggest that short-term exposure studies could benefit from bronchoscopic techniques, in order not to miss effects on airway cellular function which could be relevant for long-term exposures.

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