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ORIGINAL ARTICLE

Biological effects of inhaled nitrogen dioxide in healthy human subjects

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Abstract
Purpose Several epidemiological studies indicate that inhaled nitrogen dioxide (NO_2) at low concentrations have been statistically associated with adverse health effects. However, these results are not reflected by exposure studies in humans. The aim of the study was to assess the acute functional and cellular responses to different NO_2 concentrations in healthy human subjects with various techniques.
Methods Twenty-five subjects were exposed for 3 h to NO_2 concentrations 0, 0.1, 0.5, and 1.5 ppm in a randomized crossover study design during 4 consecutive weeks. In each subject, lung function, diffusion capacity and exhaled nitric oxide were measured and inflammation markers were assessed in blood, nasal secretions, induced sputum and exhaled breath condensate.
Results From all lung function indices under consideration, only intrathoracic gas volume was borderline significantly increased after 0.5 ppm ($p = 0.048$) compared to 0.1 ppm NO_2. Regarding the cellular effect parameters, the macrophage concentration in induced sputum decreased with increasing NO_2 concentration, although these changes were only borderline significant ($p = 0.05$).
Conclusion These results do not suggest a considerable acute adverse response in human subjects after 3 h of exposure to NO_2 in the NO_2 concentration range investigated in this study.

Keywords Nitrogen dioxide · Exposure · Human subjects · Topic effects · Systemic effects

Introduction

Among all nitrogen oxides, nitrogen dioxide (NO_2) is the most toxic one. It is supposed to have a considerable health impact particularly in workplaces, as, e.g., professional drivers or welders can be exposed to high concentrations of NO_2 (Antonini et al. 2003; Morfeld et al. 2010; Spiegel-Ciobanu 2009). While it is also considered as relevant in the ambient air, the impact of long-term low concentration exposure on human health is, however, still not clear. Several epidemiological studies indicated that NO_2 exposure may be responsible for respiratory symptoms and emergency room visits in humans (Peel et al. 2005; Tolbert et al. 2007), but these studies are discussed controversially (Hesterberg et al. 2009; Latza et al. 2009; US Environmental Protection Agency 2008), and it is speculated that NO_2 may only be a surrogate marker for other components of the complex mixture of environmental air pollutants.

Similarly, experimental exposure studies with human subjects did not yield a consistent view of NO_2 toxicity, despite the fact that most studies employed NO_2 levels much higher than commonly observed in the ambient air. Despite the epidemiological findings on respiratory symptoms (Peel et al. 2005; Tolbert et al. 2007), the measurable

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effects of NO_2 on lung function in exposure studies were small or negligible (Jorres and Magnussen 1990, 1991) and occurred only at high concentrations (Linn et al. 1985) or in subjects with preexisting functional impairment (Morrow et al. 1992; von Nieding and Wagner 1979). In the range of ambient air concentrations (about 0.1 ppm), no effects have been reported (Hazucha et al. 1983). Several studies indicated that NO_2 may not directly affect lung function but increase the nonspecific sensitivity of the airways against agonists such as methacholine, histamine or carbachol (Orehek et al. 1976). This seems to occur mostly in asthmatic and not in healthy subjects (Folinsbee 1992). However, this effect is small, as well as that on allergen responses which has been reported in some studies (Strand et al. 1997, 1998) but questioned in others. After exposure to high NO_2 concentrations (at least 1 ppm), various cellular and/or biochemical changes in the bronchoalveolar lavage fluid (BALF of human subjects have been observed (Devlin et al. 1999; Helleday et al. 1994; Sandström et al. 1991, 1992a, b; Solomon et al. 2000), whereas at lower concentrations (0.6 ppm) no responses were detectable (Rubinstein et al. 1991). Thus, the question of mechanisms underlying potential adverse health effects of inhaled NO_2 is unresolved.

The present exposure study was designed to readdress the issue of biological effects of inhaled NO_2, especially at low concentrations, by including a fairly large, homogeneous study population, by comparing different NO_2 levels and by using advanced detection methods. A number of previous studies have been performed with small and sometimes heterogeneous populations, and some of them used rather short (20 min–1 h) exposure times. In contrast, the present study comprised 25 young, healthy subjects, four different exposure levels between 0 and 1.5 ppm in a randomized, single-blinded, crossover design, and exposure times of 3 h. Apart from lung function measurements [spirometry, body plethysmography, diffusing capacity for inhaled carbon monoxide (CO) and nitric oxide (NO)], cell biological and biochemical endpoints were assessed using samples of induced sputum (topical effects, i.e., effects located in the airways), nasal secretions (topical effects) and blood (systemic effects, i.e., effects in the whole body). The induced sputum technique was used instead of bronchoalveolar lavage as it is much less invasive and more suitable for studies with repeated assessments. The assessments additionally comprised the collection of exhaled breath condensate (EBC) for the measurement of biochemical compounds, the determination of endogenous bronchial and alveolar nitrogen oxide (eNO), and the detection of volatile organic compounds (VOC) in exhaled air by ion mobility spectrometry (data not shown).

Table 1 Anthropometric and baseline lung function data of the study population

Parameter	Unit	Mean	SD	Min	Max
Gender	19 males/6 females				
Age	years	24	3	18	33
Height	cm	178	10	150	196
Weight	kg	80	13	58	102
VC	L	5.60	1.02	2.74	7.05
FEV_1	L	4.41	0.74	2.36	5.42

SD standard deviation, *VC* vital capacity, FEV_1 forced expiratory volume in 1 s

Methods

Subjects

Twenty-five healthy subjects, mostly students, participated in this study (19 males, 6 females). They were nonsmokers since at least 12 months (lifetime cigarette consumption <10 pack years) and had no history of asthma or any other lung or cardiac disease and if their basic lung function was normal. Table 1 shows their anthropometric and baseline lung function data. The study protocol was approved by the Ethics Committee of the Medical Faculty of RWTH Aachen University. Informed written consent was obtained from each subject prior to inclusion.

Study design

The study followed a randomized, single-blinded, fourfold crossover design with three different NO_2 concentrations and one sham exposure (0 ppm):

- 0.0 ppm (control).
- 0.1 ppm (environmentally relevant value).
- 0.5 ppm NO_2 (current German MAK value (MAK-Commission 2012)).
- 1.5 ppm NO_2 (30 % of the German MAK value before 2009).

Each subject was exposed for 3 h once a week on the same weekday with one of the above NO_2 concentrations. The study was performed during four consecutive weeks, with an exposure of five subjects per day.

During the exposures, the subjects cycled for 10 min per hour on a bicycle ergometer at a work load of 80 W and were at rest otherwise. In all subjects, the time interval between two consecutive exposures was 1 week. Measurements were taken before and immediately after each exposure, using the following assessments:

Before exposure:

1. Measurement of exhaled nitric oxide (eNO) at four different flow rates.
2. Collection of exhaled breath condensate.
3. Body plethysmography.
4. Measurement of CO and NO diffusing capacity.
5. Sampling of nasal secretions fluid.
6. Blood sample.

After exposure:

1. Measurement of exhaled nitric oxide (eNO) at four different flow rates.
2. Collection of exhaled breath condensate.
3. Body plethysmography.
4. Measurement of CO and NO diffusing capacity.
5. Sampling of nasal secretions.
6. Blood sample.
7. Collection of induced sputum.

Furthermore, before and after exposure, but prior to sputum collection, exhaled air was analyzed using ion mobility spectrometry. Because of the more tentative nature of this nonstandard method and the complexity of the data evaluation, the results will be presented in a separate manuscript.

Exposure technique

Exposures were performed within the Aachen Workplace Simulation Lab which was initially designed for welding fume exposure (Brand et al. 2013b). This laboratory comprises a 41-m^3 room where the study subjects were situated around a central table. The room was ventilated with clean conditioned air via four vortex air inlets that assure complete and fast mixing. The facility was modified for the purpose of the present study. NO_2 was provided in a 40-L bottle at a pressure of 145 bar (0.2 % NO_2 in synthetic air). A small stream of this NO_2 was introduced into the air entering the exposure room (225 m^3/h). The NO_2 stream was adjusted by a needle valve in order to obtain the desired NO_2 concentration within the exposure room. The NO_2 level in the room was measured by a chemiluminescence NO/NO_x analyzer (Eco Physics, model CLD 700 AL, Dürnten, Switzerland) which measures NO and NO_2 simultaneously. Prior to the start of the study, it had been demonstrated that the NO_2 concentration profile in the exposure room was homogeneous.

Lung function: spirometry and body plethysmography

Spirometry and body plethysmography were performed using a MasterScreen Device (CareFusion, Höchberg, Germany). Airway resistance was measured during tidal breathing. Subsequently, spirometric parameters were assessed during a standard sequence of deep expiration, deep inspiration and forced full expiration. This maneuver was performed according to the ATS criteria (ATS 1995). The following endpoints were assessed: slow inspiratory vital capacity (VC), forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), peak expiratory flow (PEF), maximal expiratory flow at 75 % of vital capacity (MEF_{75}), maximal expiratory flow at 50 % vital capacity (MEF_{50}), maximal expiratory flow at 25 % vital capacity (MEF_{25}), effective airway resistance (R_{eff}), intrathoracic gas volume (ITGV), expiratory reserve volume (ERV), total lung capacity (TLC) and residual volume (RV).

Lung function: diffusing capacity

Diffusing capacities for inhaled CO and NO were assessed using the PFT-Device (Carefusion, Höchberg, Germany) and a test gas mixture consisting of CO (0.3 %), CH_4 (0.3 %), C_2H_2 (0.3 %), He, O_2 and air. After a deep expiration test gas was inhaled up to total lung capacity, followed by a breath hold of over 10 s and a deep but not forced expiration. The following parameters were assessed: total lung capacity (TLC_{He}), diffusing capacity for CO (DLCO) and alveolar volume (VA) as derived from helium dilution. For the assessment of NO diffusion capacity (DLNO), a test gas mixture was used consisting of NO (50 ppm), CO (0.3 %), N_2 and O_2. After deep exhalation and inhalation of test gas, a breath hold of 3 s was performed before deep exhalation.

Topical effects: exhaled nitric oxide (eNO)

Exhaled NO was measured using a Sievers NO-Analyzer (NOA280, Sievers, Boulder, Co, USA). In each subject, measurements were taken at four different flow rates, which were obtained by using different airflow resistances in the exhalation tube (43.3, 148.3, 228.3, 300 ml/s). For each measurement, subjects performed a deep inhalation, followed by a deep exhalation at the target flow during which the NO plateau concentration was recorded. The air pressure within the mouthpiece was visualized to the subjects in order to help them to keep it constantly at 12 mbar, with the aim of velum closure and achieving a constant flow rate. The eNO level at 43.3 mL/s was taken as bronchial eNO. From the eNO values obtained at the other flow rates, the alveolar concentration of NO was calculated utilizing the flow dependency of the NO signal via a mathematical model (Tsoukias and George 1998).

Topical effects: exhaled breath condensate

Exhaled breath condensate (EBC) was collected using the ECoScreen condenser (CareFusion, Höchberg, Germany).

The subjects were instructed to breathe tidally for 15 min through a mouthpiece connected to a condenser. The exhaled air entered and left the condensing tube (cooled to -20 °C) through one-way valves. The EBC samples were stored in aliquots at -80 °C until analysis (Brand et al. 2010, 2013a; Gube et al. 2010). Nitrite and nitrate in EBC were determined photometrically (Multiskan Ascent, Thermo Fisher Scientific, Waltham, USA) via the Griess reaction using a quantification kit from Roche Diagnostics. Tyrosine was measured for normalizing the results and assessed by hydrophilic interaction liquid chromatography (HILIC), coupled to tandem mass spectrometry (API 3000, AB SCIEX, Framingham, USA) according to Conventz (Conventz et al. 2007).

Topical effects: nasal secretions

As described by Chawes et al. (2010), a 2.5-cm-long synthetic filter paper stripe (Leukosorb, Pall) was placed unilaterally under anterior rhinoscopy into the inferior nasal meatus, removed after 45 s and transferred into a 0,22-µm filter spinning tube (Corning Costar®, Lowell, MA, US). In total, 300 µl of DPBS 1× buffered solution (Dulbecco´s Phosphate-Buffered Saline, Gibco by Life technologies, USA) was added. After centrifugation at 4° C and 3000 rounds per minute for 10 min, the filter was removed together with the filter stripe and the supernatant was stored at −80 °C until analysis.

Topical effects: induced sputum

Sputum induction was performed as previously described (Holz et al. 1998). Briefly, subjects inhaled 4.4 % hypertonic saline solution in two consecutive 10-min inhalation periods. After each period, subjects were asked to produce sputum. Sputum plugs were selected, pooled and homogenized with Sputolysin® (Calbiochem, Darmstadt, Germany). PBS (Sigma Life Science, St. Louis, USA) was added, and samples were homogenized and filtrated through a 70-µm cell filter (BD Falcon). After centrifugation, supernatants were collected and stored at −80 °C for the analysis of biomarkers. Cytospins were prepared on microscope slides (Thermo Fisher Scientific, Superfrost Plus, Waltham, USA) after a resuspension of the cell pellet with PBS, and a differential cell count was performed by counting at least 400 nonsquamous cells.

Biochemical analysis

Biomarkers in sputum supernatants and nasal secretions were analyzed by ELISA (myeloperoxidase, R&D, Wiesbaden-Nordenstadt, Germany) or by an ECL (electrochemiluminescence)-based technique [V- Plex human Cytokine Kits: (1) GM-CSF, IL-5, (2) IFN-y, IL-1ß, IL-2, IL-6, IL-8, IL-13 TNFa, (3) IL1ß, IL-6, IL-8; Meso Scale Diagnostics, Rockville, USA].

Systemic effects: blood

The following endpoint parameters in the collected blood samples were assessed: leukocyte number, neutrophil number and lymphocyte number. Interleukin-6 and interleukin-8 were measured using an electrochemiluminescence immunoassay (ECLIA; Cobas, Elecsys 2010), and the CD4/CD8 ratio was measured using flow cytometry (FACSCalibur, Becton Dickenson, Heidelberg, Germany). Endothelin-1 in blood plasma was determined using a commercially available ELISA Kit (Quantikine ELISA kit cat. no. DET100 R&D Systems).

Statistical analysis

For all endpoint variables except those measured in induced sputum, values before and after exposure were available. For each endpoint variable Y_i the difference between the value after 3 h of exposure, $Y_{i,3h}$, and the value before exposure, $Y_{i,0h}$, was calculated:

$$Y_{i,3-0} = Y_{i,3h} - Y_{i,0h}.$$

The differences $\Delta Y_{i,3-0}$ were used to test whether there were differences between the four exposures. As the data showed heterogeneous distributions, both an analysis of variance (assuming normally distributed data) and a non-parametric Kruskal–Wallis test (not assuming normally distributed data but less power) were performed. Results were considered statistically significant if $p < 0.05$ in both tests, i.e., data did not critically depend on the assumptions. In case of differences between the analysis of variance and the Kruskal–Wallis test, a Shapiro–Wilks test for normality was performed to select the appropriate test method for final evaluation. In case of a significant overall difference, post hoc comparisons with Tuckey alpha adjustment were performed, in order to assess which of the post–pre differences observed for the different NO_2 concentrations significantly differed from the others.

Analysis of variance was chosen instead of regression analysis since regression analysis is restricted by the assumption of a linear dose effect relationship which is not mandatory.

For the data obtained from induced sputum, the analysis of variance and a Kruskal–Wallis test were performed, using the data obtained after exposure, $Y_{i,3h}$.

All statistical calculations were performed using the SPSS Software (version 21, IBM, USA).

Results

Exposure

The target NO_2 concentrations in the exposure room could be achieved and kept constantly over time with good accuracy. As an example, the time course of the NO_2 concentration on an exposure day with 0.5 ppm is shown in Fig. 1.

The overall average values (SD) of NO_2 during the four exposures with the respective target NO_2 concentrations were as follows: control: 0.009 ± 0.0007 ppm; 0.1 ppm: 0.101 ± 0.006 ppm; 0.5 ppm: 0.49 ± 0.02 ppm; and 1.5 ppm: 1.50 ± 0.02 ppm. NO concentration throughout the study was close to zero.

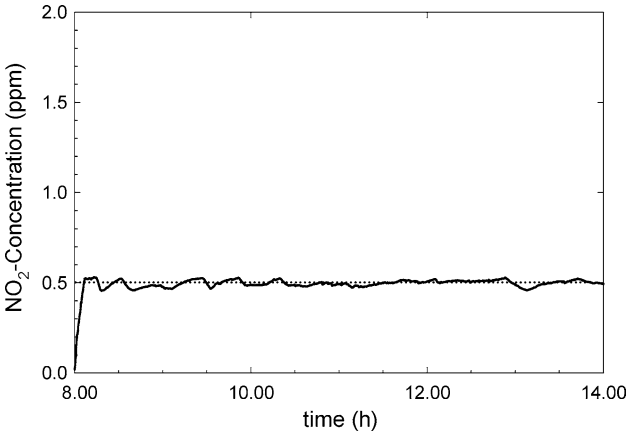

Fig. 1 Time course of the measured NO_2 concentration on an exposure day with a concentration of 0.5 ppm

Lung function: body plethysmography

For the intrathoracic gas volume (ITGV), the Kruskal–Wallis test revealed a statistically significant dependence on the exposure concentration ($p = 0.048$), whereas the analysis of variance did not confirm this ($p = 0.17$; online supplement Table 2). However, the Shapiro–Wilks test revealed that the data were not normally distributed ($p < 0.001$), so that a borderline significant p of 0.048 has to be assumed. Post hoc pairwise comparisons revealed that the post–pre difference observed for 0.5 ppm NO_2 was slightly higher than the value for 0.1 ppm (Fig. 2). For vital capacity (VC), a nonsignificant dependency on exposure concentration was found in the analysis of variance ($p = 0.08$) and in the Kruskal–Wallis test ($p = 0.09$; Fig. 3).

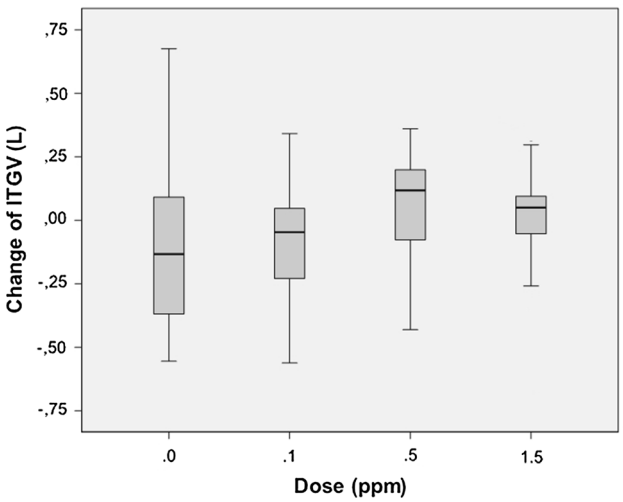

Fig. 2 Changes in ITGV for the four different exposure conditions

Lung function: diffusing capacity

For none of the endpoint variables regarding this assessment, a statistically significant dependence on the exposure concentration was observed (online supplement Table 3).

Topical effects: exhaled nitric oxide (eNO)

For none of these endpoint variables, a statistically significant dependence on the exposure concentration was observed (online supplement Table 4).

Topical effects: exhaled breath condensate

For none of these endpoint variables, a statistically significant dependency on the exposure concentration was observed (online supplement Table 5).

Topical effects: nasal secretions

For TNF_α, the analysis of variance (online supplement Table 6, Fig. 4) indicated a borderline significant dependency on the exposure concentration ($p = 0.05$), while the Kruskal–Wallis test yielded a level of $p = 0.072$. Since the data were not normally distributed, the dependency has to be considered as not significant.

Topical effects: induced sputum

The percentage of macrophages in the induced sputum showed a borderline significant dependence on the exposure condition (online supplement Table 7; Fig. 5, analysis of variance: $p = 0.050$, Kruskal–Wallis test: $p = 0.052$). Post hoc analysis revealed that the macrophage percentage was significantly lower after exposure to 1.5 ppm compared to 0.1 ppm ($p = 0.027$).

For none of the soluble sputum markers, a statistically significant dependence on the exposure condition was observed (online supplement Table 8).

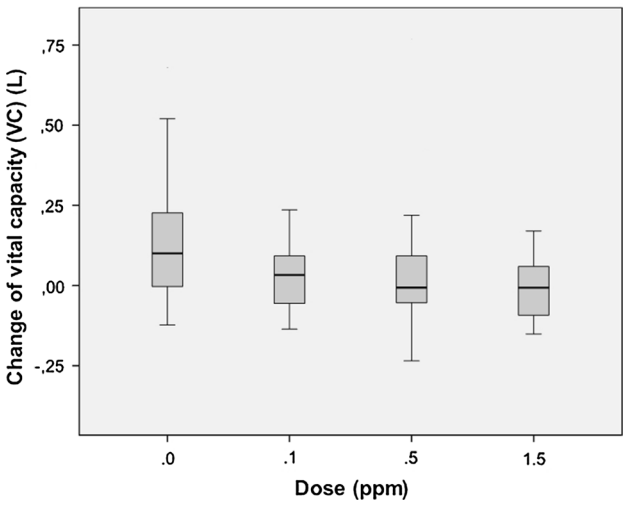

Fig. 3 Changes in vital capacity (VC) for the four different exposure conditions

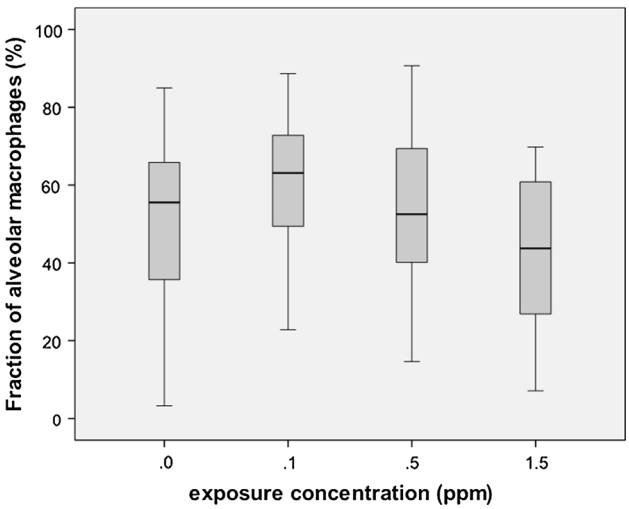

Fig. 5 Changes in macrophage concentration for the four different exposure levels

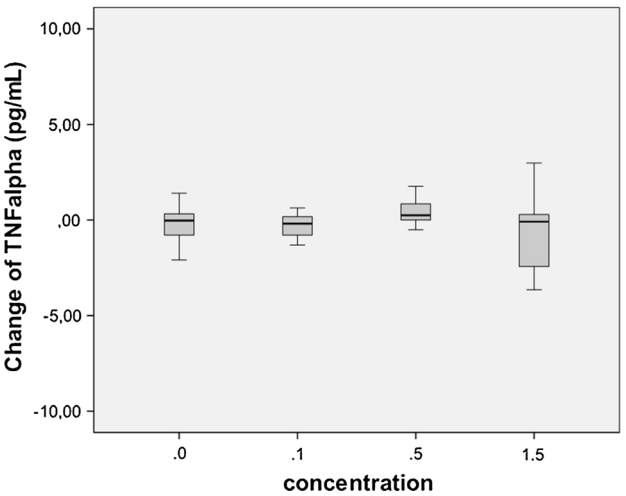

Fig. 4 Changes in TNF_{alpha} concentration in nasal secretions for the four different exposure levels

Systemic effects: blood

For none of the investigated endpoint variables in peripheral blood, a statistically significant dependence on the exposure condition was observed (online supplement Table 9).

Discussion

In this study, 25 young healthy human subjects were exposed to three different NO_2 concentrations ranging from 0.1 ppm to 1.5 ppm, as well as clean air, serving as control for 3 h. A panel of lung function indices including spirometry, body plethysmography and diffusing capacity of the lung for CO and NO was assessed before and after exposures. In parallel, biological effect markers were measured in the blood, in nasal secretions, in exhaled air in terms of exhaled nitric oxide and in exhaled breath condensate. Moreover, induced sputum analysis was performed after exposures.

Only one of the 12 measured functional indices showed a statistically borderline significant dependence on the NO_2 level. Intrathoracic gas volume (ITGV) tended to an increase after 0.5 ppm compared to 0.1 ppm NO_2 (Fig. 2). However, due to the large number of lung function parameters measured it is most probable that this finding is only due to multiple testing and after alpha adjustment, this difference will no longer be significant (Bonferroni correction for 12 parameters results in a p value for significance of 0.004).

Regarding the cellular effect parameters, we found that the percentage of macrophages in induced sputum tended to a decrease with increasing the NO_2 concentration (Fig. 5), although, after correction for multiple testing, these results were statistically not significant. In the nasal secretions, the concentration of TNF_{α} was slightly increased after 0.5 ppm NO_2 but decreased after 1.5 ppm NO_2. Both results, however, were not statistically significant.

Several studies have assessed biochemical or cell biological responses in the BALF in human subjects, but they most often used rather high NO_2 concentrations $\geq$1.5 ppm (Blomberg et al. 1997; Devlin et al. 1999; Helleday et al. 1994; Sandström et al. 1991, 1992a, b; Solomon et al. 2000). Few studies reported a significant response at lower concentrations (Barck et al. 2005; Barck et al. 2002; Frampton et al. 2002). Noteworthy enough, most results

were obtained in subjects with asthma who are often supposed to be more susceptible to inhaled irritants. Furthermore, in the present study the level of exercise and thus the increase in minute ventilation were relatively low, compared to other studies (Devlin et al. 1999) which resulted in a lower NO_2 burden. To which extent the exercise levels used in different studies adequately model real-world exposure conditions in the majority of subjects, remains unclear.

To summarize the findings of the present study, considering possible errors due to multiple testing, no changes in parameters of lung function, induced sputum and nasal secretions were observed. Therefore, with our study design, we were not able to identify significant inflammatory reactions that could explain the epidemiological findings (Peel et al. 2005; Tolbert et al. 2007), although it was attempted to cover a broad spectrum of potential effects. However, in the present study only acute effects of NO_2 exposure were investigated and it cannot be excluded that chronic exposure may result in different reactions, which may be responsible for the published epidemiological findings. Furthermore, it may well be that in epidemiological studies NO_2 was more a surrogate parameter for other environmental pollutants (Latza et al. 2009). On the other hand, there might be cellular and functional responses that were not assessed in the present study, which are responsible for the observed health impact of NO_2. For example, it might be speculated that NO_2, or ambient air nitrogen oxides in general, may interfere with the NO-mediated regulation of the vascular tone and even the immune system. It is known that inhaled NO_2 can be transformed to NO within the body (Davidson et al. 1996). NO plays an important role in the regulation of the vascular system and is linked to eosinophilic inflammation (Barnes 1995). We did not observe changes in exhaled endogenous NO, neither of bronchial nor of alveolar origin, but it might be that the NO derived from inhaled NO_2 is depleted by biochemical reactions and not visible in the exhaled NO. In fact, the exhaled NO was considered primarily as a marker of diffusion and potential oxidative stress than a marker of potential NO_2 conversion. Despite these limitations, the NO pathway appears interesting, and one might expect effects especially in subjects with preexisting cardiovascular disease who are also known to be prone to adverse effects of inhaled particulate matter. To study such subjects experimentally remains, however, a very difficult ethical issue.

Conclusions

The 3-h exposure of 25 young healthy subjects to three concentrations of NO_2 (0.1, 0.5, 1.5 ppm) elicited no significant effects on lung function and cellular or biochemical effect markers. These results do not suggest a considerable acute adverse response to NO_2 inhalation.

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Compliance with ethical standards

Conflict of interest None of the authors have a conflict of interest to declare in relation to this work.

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