ELSEVIER

European Journal of Pharmacology 374 (1999) 241–247

ejp

www.elsevier.nl/locate/ejphar

Bronchopulmonary inflammation and airway smooth muscle hyperresponsiveness induced by nitrogen dioxide in guinea pigs

Alberto Papi [a], Silvia Amadesi [b], Pasquale Chitano [c], Piera Boschetto [d], Adalberto Ciaccia [a], Pierangelo Geppetti [b], Leonardo M. Fabbri [a,*], Cristina E. Mapp [e]

[a] *Research Centre on Asthma and COPD, Department of Experimental and Clinical Medicine, University of Ferrara, Ferrara, Italy*
[b] *Section of Pharmacology, Department of Experimental and Clinical Medicine, University of Ferrara, Ferrara, Italy*
[c] *Division of Pulmonary Diseases, Department of Pediatrics, Duke University, Durham, NC, USA*
[d] *Research Centre on Environmental Medicine, Department of Experimental and Clinical Medicine, University of Ferrara, Ferrara, Italy*
[e] *Section of Occupational Medicine, Department of Environmental and Forensic Medicine, University of Padua, Padua, Italy*

Received 10 December 1998; received in revised form 14 April 1999; accepted 22 April 1999

Abstract

We investigated whether acute exposure to nitrogen dioxide (NO_2) causes major inflammatory responses (inflammatory cell recruitment, oedema and smooth muscle hyperresponsiveness) in guinea pig airways. Anaesthetised guinea pigs were exposed to 18 ppm NO_2 or air for 4 h through a tracheal cannula. Bronchoalveolar lavage was performed and airway microvascular permeability and in vitro bronchial smooth muscle responsiveness were measured. Exposure to NO_2 induced a significant increase in eosinophils and neutrophils in bronchoalveolar lavage fluid, microvascular leakage in the trachea and main bronchi (but not in peripheral airways), and a significant in vitro hyperresponsiveness to acetylcholine, electrical field stimulation, and neurokinin A, but not to histamine. Thus, this study shows that in vivo exposure to high concentrations of NO_2 induces major inflammatory responses in guinea pig airways that mimic acute bronchitis induced by exposure to irritant gases in man.

Keywords: Bronchitis; Bronchial provocation test; Oxidant; Bronchoconstriction

1. Introduction

Nitrogen dioxide (NO_2) is an oxidising agent commonly found in both outdoor and indoor ambient air. This pollutant is formed by the oxidation of nitric oxide, generated from oxygen and nitrogen during most operations requiring combustion (United States Department of Health, Education, and Welfare, 1976; Samet et al., 1987; Berglund et al., 1993; Fishbein, 1993). Ambient levels may peak at 0.6 ppm, and exposures of up to 500 ppm have been reported in workplaces during operations involving welding arcs or cutting torches. Present recommendations fix the level of occupational exposure at between 3 ppm time-weighted average and between 5 ppm short-term exposure limit (American Conference of Hygienists, 1998).

A number of studies have suggested that NO_2 exposure may lead to inflammation, microvascular leakage, and hyperresponsiveness of the airways, i.e., the most important characteristic abnormalities present in acute bronchitis, whereas other studies have failed to confirm those findings. Inflammation of bronchial airways and/or lung parenchyma in response to NO_2 exposure has been previously reported (Chitano et al., 1995a). However, different studies have described this response to be characterised by the recruitment of diverse inflammatory cells depending on the concentration, the duration of exposure, and the animal species (United States Department of Health, Education, and Welfare, 1976; De Nicola et al., 1981; Magnussen, 1992; Meulenbelt et al., 1992). Exposure to NO_2 may also cause lung and airway microvascular leakage, as suggested by observations of oedema both in the alveolar spaces (Bhalla and Crocker, 1987; Vassilyadi and Michel, 1988) and in peribronchial and perivascular tissues (Vassilyadi and Michel, 1988; Meulenbelt et al., 1992). Protein levels in bronchoalveolar lavage fluid, a sensitive index of microvascular leakage, have been shown to increase follow-

* Corresponding author. Department of Experimental and Clinical Medicine, Section of Respiratory Diseases, Università degli Studi di Ferrara, Via Savonalrola 9, 44100 Ferrara, Italy. Tel.: +39-532-210420; Fax: +39-532-210297; E-mail: fab@dns.unife.it

0014-2999/99/$ - see front matter
PII: S0014-2999(99)00302-7

ing NO_2 exposure, although not consistently (Sherwin and Carlson, 1973; De Nicola et al., 1981; Bhalla and Crocker, 1987; Glasgow et al., 1987; Vassilyadi and Michel, 1988; Frampton et al., 1989; Magnussen, 1992). In a previous study, we were unable to detect oedema or microvascular leakage in rats exposed to 5–10 ppm NO_2 (Chitano et al., 1996b).

In vivo exposure to low concentration of NO_2 may increase airway responsiveness in healthy subjects as well as in bronchitic and asthmatic subjects (Orehek et al., 1976; Magnussen, 1992). In vitro exposure to NO_2 has been shown to increase the responsiveness of isolated human bronchi in one study (Ben-Jebria et al., 1992), but not in others, (Chitano et al., 1996a). In experimental animal models, in vivo airway hyperresponsiveness has been reported in guinea pigs (Silbaugh et al., 1981; Kobayashi and Shinozaky, 1990), whereas in vitro responsiveness of bronchial rings does not change either in rats after in vivo exposure to 5–10 ppm NO_2 (Chitano et al., 1996b), or in guinea pigs after in vitro exposure to 10 ppm NO_2 (Chitano et al., 1994, 1995b).

In previous studies we were unable to induce acute inflammation and hyperresponsiveness of the airways by exposing guinea pigs to 5–10 ppm of NO_2 for increasing duration of exposure. In this study, we increased the concentration of NO_2 to 18 ppm and the duration of exposure to 4 h, with the aim to induce in guinea pigs a broad spectrum of inflammatory responses similar to those commonly encountered in acute bronchitis, i.e., inflammatory cell recruitment, microvascular leakage and hyperresponsiveness. To do that, we delivered intratracheally a constant flow of NO_2 to spontaneously breathing anaesthetised guinea pigs. By avoiding upper airway removal of inspired gases (Yokoyama and Frank, 1972; Goldstein et al., 1977), this method of exposure should reduce the variability of the amount of NO_2 that reaches central as well as peripheral airways.

2. Materials and methods

2.1. Animals and NO_2 exposure

The experimental procedure was approved by the committee on animal care of the University of Ferrara. Male Hartley guinea pigs (Morini, S. Polo D'Enza, Reggio Emilia, Italy) weighing 350–400 g were anaesthetised with urethane (1.5 g/kg i.p.) and tracheostomised; supplemental anaesthesia was administered (0.4 g/kg i.p.) every 90 min. The trachea was cannulated and a Y-shaped tube was connected to a chamber containing NO_2 at a known concentration, while the other branch was connected to a water valve. With this apparatus, NO_2 was inhaled during spontaneous inspirations and exhaled, bubbling into water, during spontaneous expirations. Exposure to 18 ppm NO_2 was carried out for 4 h at a constant flow of 150–200 ml/min, i.e., the lowest value which produced a bubbling into water during the inspiratory phase. Control animals were exposed with the same apparatus to the air (standard air for chromatography) used to dilute NO_2.

At the end of the exposure, the animals underwent one of the following experimental protocols.

2.2. Bronchoalveolar lavage

Six control and six NO_2-exposed animals were sacrificed with an additional urethane injection, to perform bronchoalveolar lavage fluid. Five milliliter of normal saline at 37°C were instilled into the lungs through a tracheal cannula, which was then clamped to perform a 30-s massage of the thorax before recovering the bronchoalveolar lavage. Subsequently, a further 5.0 ml of saline were instilled into the lungs, the massage procedure was repeated, and the recovered fluid was pooled with the previous one for assessment of cellular content. Total cell count was assessed in a standard haemocytometer and cell viability was evaluated by Trypan blue exclusion. For cytologic examination, cytospin preparations were made by cytocentrifugation at $200 \times g$ for 10 min at room temperature (Universal Hettich, Hettich Zentrifugen, Tuttlingen, Germany). After air drying, the slides were stained with haematoxylin-eosin. A differential count on at least 400 cells was carried out for each slide. Cell counts were performed on coded specimens by two observers who had no knowledge of the coding.

2.3. Airway microvascular leakage

In six control and seven NO_2-exposed guinea pigs, the jugular veins were exposed and Evans blue dye injected (30 mg/kg) as a tracer to assess microvascular permeability. The extent of microvascular leakage was determined by measuring the concentration of Evans blue dye extravasated into the airway tissue after removing intravascular dye. Thirty minutes after Evans blue injection, the chest was opened and the animals perfused through the heart with 100 ml of saline (pH 5.5, 21°C) at 100 mmHg to remove intravascular dye. The trachea, main bronchi, and lungs were then removed and isolated. Intrapulmonary airways were obtained by stripping them from lung parenchyma. Wet weight of each sample was recorded. Evans blue dye was extracted by incubating tissues in 2 ml of formamide (50°C) for 16 h, and its concentration determined by spectrophotometric absorbance at 620 nm wavelength (DU 40 Beckmann Spectrophotometer, UK) and by interpolation on a standard curve of Evans blue dye concentrations (0.5 to 10 mg/ml). Evans blue dye content of each sample was expressed as ng/mg of tissue wet weight.

2.4. In vitro airway responsiveness

The animals used in the experiments for airway smooth muscle responsiveness were sacrificed with an additional

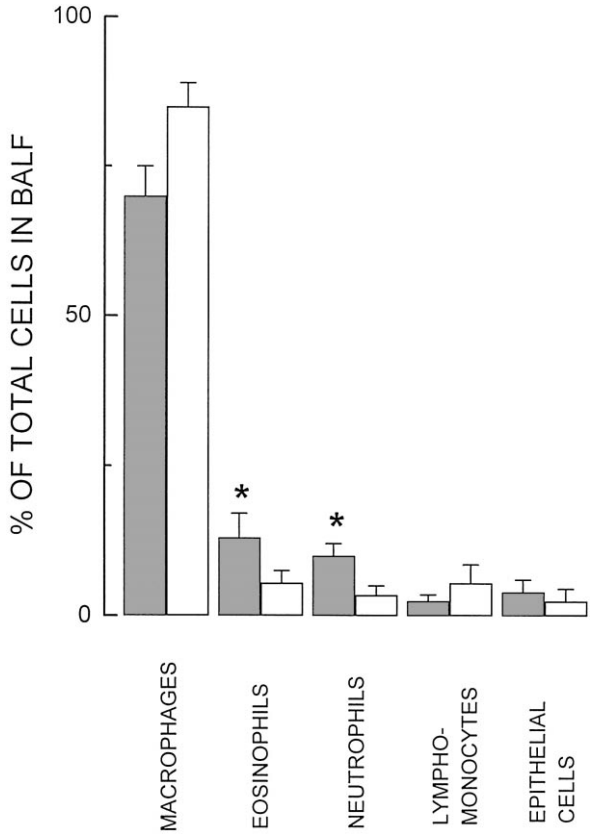

Fig. 1. Differential cell count in bronchoalveolar lavage fluid (BALF) from guinea pigs exposed for 4 h to 18 ppm NO_2 (filled columns) or to air (open columns). Values are expressed as mean ± S.E.M. percent of total cell counts, $^{*}P < 0.05$ between NO_2 and air exposed groups.

urethane injection. The chest wall was opened and the trachea and the lungs were quickly removed and immersed in oxygenated Krebs–Henseleit solution containing: 118.3 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25.0 mM $NaHCO_3$, 2.5 mM $CaCl_2$, and 11.1 mM D-(+)-glucose. The two main bronchi were dissected free of loose connective tissue and a ring was prepared from each of them. The rings were mounted in glass chambers filled with 15 ml of Krebs–Henseleit solution which was maintained at 37°C and aerated continuously by bubbling it with a mixture of 95% O_2 and 5% CO_2, which produced a pH of 7.4. Isometric tension was measured continuously by connecting the rings to a force displacement transducer (Grass FTO3) and the responses were recorded on a paper polygraph (Battaglia Rangoni KO380, Bologna, Italy). The tissues were allowed to equilibrate for 90 min at a resting tension of 0.5 g. During equilibration, the medium was changed every 20 min. After the equilibration period, a response to 10^{-3} M acetylcholine was elicited and recorded.

Bronchial rings were then rinsed until tension returned to resting values. Either a voltage–response curve (8–48 V) to electrical field stimulation and a concentration–response curve to acetylcholine (10^{-9}–10^{-2}), or a concentration–response curve to histamine (10^{-9}–10^{-3}) and neurokinin A (10^{-11}–10^{-4}) were performed in each individual ring. In each case, the second curve was performed after restoring baseline resting tension by repeated washing. The electrical stimulus (60 Hz, 8 ms for 10 s) was delivered by means of two rectangular platinum plate electrodes placed at the two sides of the preparation and connected to a Grass S88 stimulator.

To reduce the magnitude of the statistical error due to sampling and manipulation, isometric contractions in response to the different stimuli were expressed as a percentage of the maximal response to 10^{-3} M acetylcholine (single dose) obtained in the same ring.

2.5. Drugs and chemicals

Drugs and chemicals were obtained from the following sources: urethane from Riedel-de Haen (Hannover, Germany); cylinders containing 18 ppm NO_2 or air from SIAD (Camin, Padova, Italy); Evans blue dye, formamide, acetylcholine, neurokinin A and histamine from Sigma (St. Louis, MO, USA). Evans blue dye, 30 mg/ml in saline, was filtered using a 0.22-mm micropore membrane prior to use. Acetylcholine, neurokinin A and histamine were diluted in water. Stock solutions were made at concentrations 1000-fold greater than the final bath concentrations to minimise changes in isolated organ bath volume, pH and ionic strength.

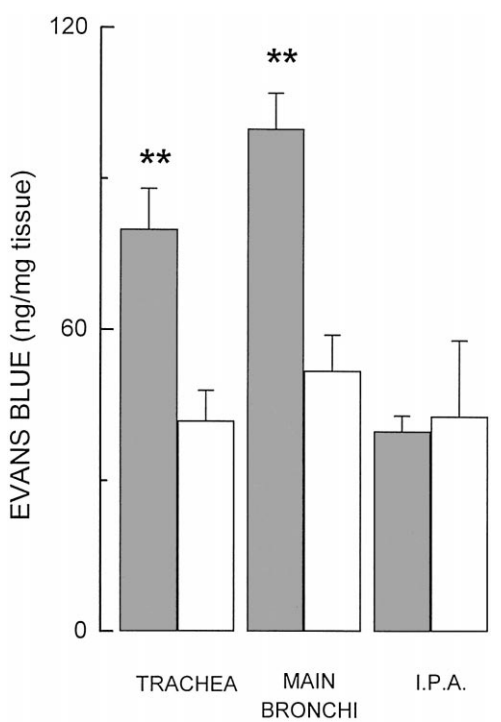

Fig. 2. Effect of 4 h of exposure to 18 ppm NO_2 (filled columns) or air (open columns) on airway plasma extravasation. Values shown are means ± S.E.M., $^{**}P < 0.01$ between NO_2 and air exposed groups. I.P.A., intrapulmonary airways.

Table 1
EC_{50}–EV_{50} and maximal tension in bronchi from guinea pigs exposed to 18 ppm NO_2 or air

Stimulus	EC_{50}^a, NO_2 exposed	EC_{50}^a, air exposed	EV_{50}^b, NO_2 exposed	EV_{50}^b, air exposed	*P* value	Maximal tension (%), NO_2 exposed	Maximal tension (%), air exposed	*P* value
ACh	$7.96 \cdot 10^{-6}$ M (1.36)	$2.83 \cdot 10^{-5}$ M (1.30)			< 0.02	115.8 ± 3.9	113.5 ± 6.4	NS
NKA	$2.50 \cdot 10^{-8}$ M (1.42)	$7.20 \cdot 10^{-8}$ M (1.73)			< 0.05	115.4 ± 3.0	111.2 ± 4.0	NS
Histamine	$3.13 \cdot 10^{-6}$ M (1.87)	$3.47 \cdot 10^{-6}$ M (1.68)			NS	110.8 ± 5.4	107.3 ± 4.8	NS
EFS			23.0 ± 1.6	24.1 ± 2.1	NS	54.1 ± 4.1	32.9 ± 8.4	< 0.05

The sensitivity to acetylcholine (ACh), neurokinin A (NKA) and histamine is expressed as EC_{50} (geometric mean and G.S.E.M.), whereas the sensitivity to electrical field stimulation (EFS) is expressed as EV_{50} (mean ± S.E.M.). The maximal response to electrical field stimulation, acetylcholine, histamine, neurokinin A is expressed as percent of the response to 1 mM acetylcholine (means ± S.E.M.).
[a] EC_{50} = effective concentration evocative of 50% of the maximum response.
[b] EV_{50} = effective voltage evocative of 50% of the maximum response.

2.6. Data analysis

Results are given as means ± S.E.M., except EC_{50}, that is expressed as geometric mean (G.M.) and geometric standard error of the mean (G.S.E.M.). Comparisons were performed by the two-tailed Student's *t*-test for unpaired data.

Values of $P < 0.05$ were considered significant. Software employed was the Statview II (Abacus Concepts, Berkeley, Los Angels, CA, USA).

The smooth muscle response to 10^{-3} M acetylcholine was expressed as tissue stress (mg/mm^2). Tissue stress was obtained by normalising active tension per tissue cross-sectional area (CSA) of each preparation. CSA was calculated according to the formula $CSA = wt/(lD)$, where l represents the ring diameter (mm) measured in the bath after the equilibration period, wt is the ring fresh weight (mg) measured at the end of each experiment, and D is the tissue density, assumed equal to 1 mg/mm^3 (Herlihy and Murphy, 1973).

3. Results

3.1. Bronchoalveolar lavage

There was no significant difference between air-exposed ($n = 6$) and NO_2-exposed ($n = 6$) guinea pigs in the amount of bronchoalveolar lavage fluid recovered (7.8 ± 0.3 ml vs. 7.8 ± 0.6 ml), the total number of broncho-

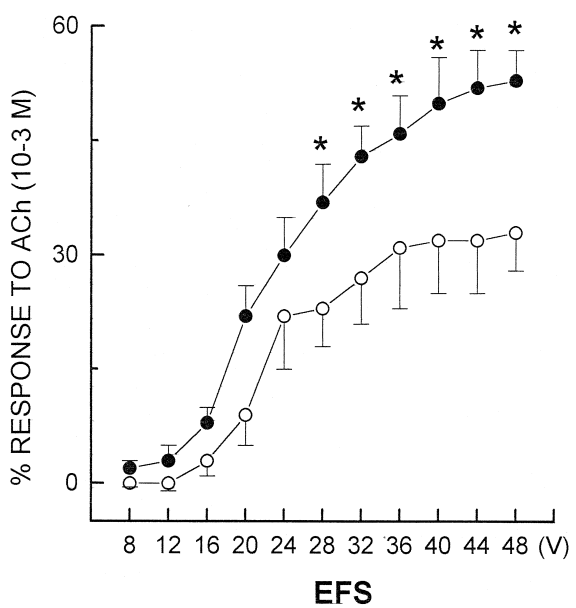

Fig. 3. Voltage–response curves to EFS in guinea pig main bronchi from animals exposed for 4 h to air (open circles) or 18 ppm NO_2 (closed circles). Each point is mean ± S.E.M., $^*P < 0.05$ vs. air treated controls.

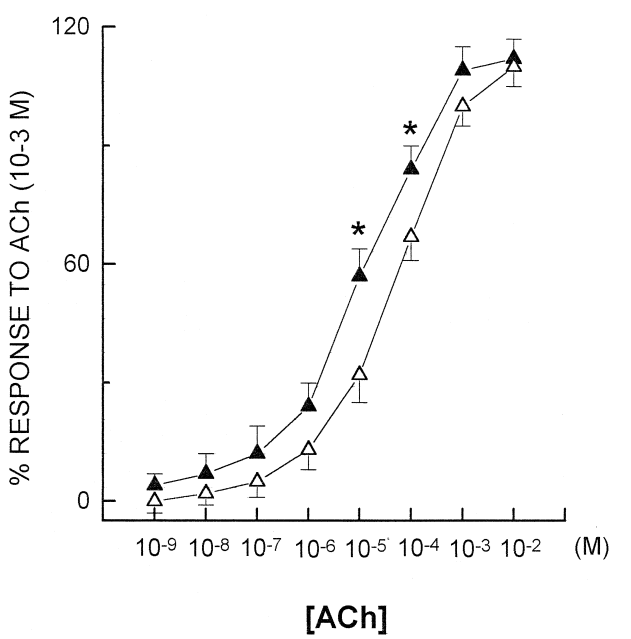

Fig. 4. Concentration–response curves to acetylcholine (ACh) in guinea pig main bronchi from animals exposed for 4 h to air (open triangles) or 18 ppm NO_2 (closed triangles). Each point is mean ± S.E.M., $^*P < 0.05$ vs. air treated controls.

alveolar lavage cells ($13.5 \pm 3.1 \times 10^6$ cells vs. $14.9 \pm 1.9 \times 10^6$ cells), and the number of cells per ml of bronchoalveolar lavage fluid ($1.80 \pm 0.48 \times 10^6$ cells/ml vs. $1.95 \pm 0.27 \times 10^6$ cells/ml). Neutrophils and eosinophils were significantly increased in bronchoalveolar lavage fluid from NO_2-exposed guinea pigs (Fig. 1). There was no significant difference in the other cell type counts between the two groups.

3.2. Airway microvascular leakage

Baseline Evans blue dye extravasation was significantly higher in the trachea and in the main bronchi obtained from guinea pigs exposed to NO_2 ($n = 7$) than in tissues taken from control animals ($n = 6$) (Fig. 2). In contrast, no difference in the Evans blue dye extravasation was found in the intrapulmonary airways between the two groups.

3.3. In vitro airway responsiveness

There was no significant difference in tissue stress generated by 10^{-3} M acetylcholine in bronchial rings obtained from animals exposed to NO_2 (193.7 ± 25.5 mg/mm^2, $n = 12$) and controls (185.9 ± 24.6 mg/mm^2, $n = 12$). In bronchial rings, electrically stimulated force generation was significantly increased at the highest voltages (including the maximal response, Table 1), in NO_2-exposed ($n = 6$) compared to control ($n = 5$) guinea pigs (Fig. 3)). No difference was found in EV_{50} between rings obtained from exposed and control animals (Table 1). NO_2 exposure significantly increased the isometric response to 10^{-5} and 10^{-4} M acetylcholine (Fig. 4, $n = 6$ for both groups), and this resulted in EC_{50} values significantly different between the two treatments (Table 1). However, the maximal response to acetylcholine was not changed (Table 1).

Contractile response to 10^{-7} to 10^{-4} M neurokinin A was significantly increased in bronchial rings from NO_2-exposed guinea pigs (Fig. 5). The maximal response to neurokinin A was similar in both groups ($n = 6$), while EC_{50} value was significantly higher in NO_2-exposed animals (Table 1).

NO_2 exposure did not affect isometric contraction in response to histamine.

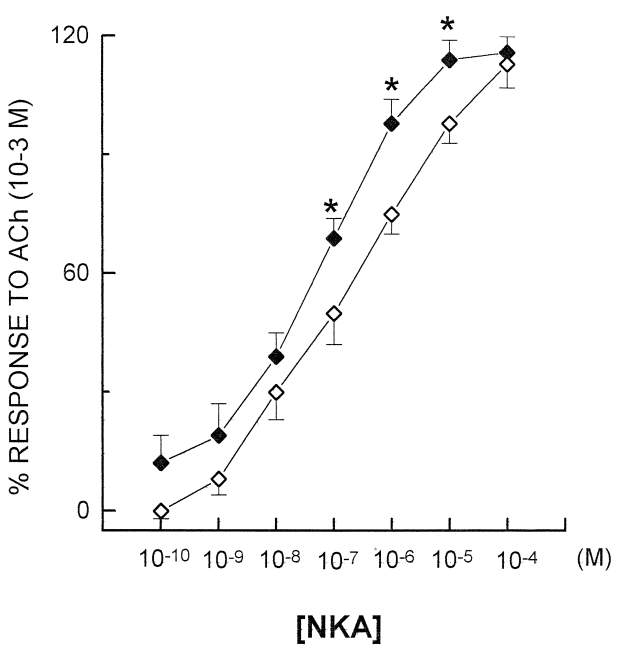

Fig. 5. Concentration–response curves to neurokinin A (NKA) in guinea pig main bronchi from animals exposed for 4 h to air (open rhombi) or 18 ppm NO_2 (closed rhombi). Each point is mean ± S.E.M., $^*P < 0.05$ vs. air treated controls.

4. Discussion

In the present study, we observed that in guinea pigs, 4 h in vivo exposure to 18 ppm NO_2 through a tracheal cannula causes intraluminal airway inflammation characterised by influx of both eosinophils and neutrophils, airway microvascular leakage, and airway smooth muscle hyperresponsiveness to electrical stimulation, acetylcholine, and neurokinin A. The results of our study confirm and extend the results of previous studies. Inflammation of the airways and of the lung parenchyma has been previously reported after exposure to NO_2 (De Nicola et al., 1981; Kawakami et al., 1989; Magnussen, 1992; Meulenbelt et al., 1992; Mochitate et al., 1992) with different characteristics according to the concentration of NO_2, duration of exposure, and animal species used. Bronchoalveolar lavage neutrophilia has been found in different animal species as one of the most sensitive indicators of lung damage after exposure to high concentrations of NO_2 (De Nicola et al., 1981; Meulenbelt et al., 1992), and neutrophilia was found to persist when exposure to NO_2 lasts for 3–6 weeks (Glasgow et al., 1987). In hamsters, a time course study showed that the increased number of neutrophils in bronchoalveolar lavage fluid was maximal after 5 days, but it was already present after 2 days of exposure to 30 ppm NO_2 (Kleinerman and Soresen, 1982). In comparison to these studies, we found a smaller increase of bronchoalveolar lavage neutrophils, possibly because of the lower total dose of NO_2 delivered. Interestingly, at variance with previous studies, we also observed a significant bronchoalveolar lavage eosinophilia after exposure to NO_2, possibly due, at least in part, to the animal species used in our study. Indeed, guinea pigs have more eosinophils in their lung in baseline conditions, and tend to have a mixed neutrophilic and eosinophilic bronchopulmonary response to irritant gases (Chitano et al., 1995a).

In the present study, exposure to NO_2 induced airway microvascular leakage. This observation confirms previous findings which suggested the development of airway microvascular leakage by showing increased proteins in bron-

choalveolar lavage fluid after exposure to NO_2 (Sherwin and Carlson, 1973; De Nicola et al., 1981; Bhalla et al., 1987; Man et al., 1990; Meulenbelt et al., 1992). In addition, our results show that airway microvascular leakage occurs in the trachea and in the main bronchi, but not in peripheral airways. The reason for this selective proinflammatory action of NO_2 in the central airways is not known. However, there is evidence for differential distribution on neural and cellular structures and mediators in the central vs. peripheral airways. For instance, a different distribution of sensory nerves in the central and peripheral airways of guinea pigs (Manzini et al., 1989) might justify the present different response after 4 h exposure to NO_2.

In the present study, exposure to NO_2 induced an increased responsiveness of isolated bronchial rings. Previous reports showed hyperresponsiveness to individual agonists after exposure to NO_2 in experimental animals (Silbaugh et al., 1981; Kobayashi and Shinozaky, 1990). To our knowledge, this is the first study in which hyperresponsiveness to several stimuli (acetylcholine, neurokinin A and electrical field stimulation) has been documented in isolated bronchial rings obtained from animal exposed to NO_2 in vivo. Airway hyperresponsiveness induced by exposure to NO_2 has been inconsistently described in healthy, asthmatic, and bronchitic subjects (Orehek et al., 1976; Hazucha et al., 1983; Linn et al., 1985; Jorres and Magnussen, 1991; Ben-Jebria et al., 1992; Magnussen, 1992; Morrow et al., 1992). A different susceptibility among the exposed populations has been proposed to justify such contradictory results. The in vivo studies do not allow to understand the mechanisms underlying the development of airway hyperresponsiveness, and particularly whether airway smooth muscle itself becomes hyperresponsive or whether this hyperresponsiveness depends on other factors. Interestingly, both microvascular leakage and hyperresponsiveness occur in central airways, suggesting that the central airways are the main site of NO_2 induced airway injury. NO_2 is known to cause damage of the epithelium (Chitano et al., 1995a) and damaged epithelium is associated with bronchial smooth muscle hyperresponsiveness (Folkerts and Nijkamp, 1998). Thus, the hypothesis may be advanced that damage of the epithelium occurs in our present model and is the underlying factor of hyperresponsiveness.

Oxidants have been suggested to inactivate both cholinesterase (Ohrui et al., 1991) and neutral endopeptidase (Murlas et al., 1990), which are the enzymes responsible for acetylcholine and neuropeptide cleavage, respectively. It has also been suggested that the muscarinic M_2 receptors may be inactivated by oxidants (Barnes, 1990). Therefore, an oxidant-mediated mechanisms could sustain the airway hyperreactivity to acetylcholine, electrical field stimulation, and neurokinin A observed in our model. We did not find any change in the response to histamine, although in vivo transient airway hyperresponsiveness to histamine has been reported in guinea pigs after exposure to NO_2 (Silbaugh et al., 1981; Kobayashi and Shinozaky, 1990). It is possible that any transitory effect might be over at the time when in vitro experiments have been performed in our protocol. It has been shown that voltage dependent calcium channel are activated during stimulation of smooth muscle by histamine but not by cholinergic stimuli (Black et al., 1988). Accordingly, it is possible that the signalling pathway, mediating contraction by histamine is different from those activated by other stimuli and is responsible for the absence of hyperresponsiveness to histamine after exposure to NO_2.

In conclusion, the present method of in vivo exposure that we have developed enabled us to demonstrate that exposure to NO_2 can induce in guinea pigs three important inflammatory responses: inflammatory cell recruitment, microvascular airway leakage and airway hyperresponsiveness. Thus, exposure to 18 ppm of NO_2 for 4 h is a relatively simple method that produces reliably an inflammatory status in guinea pigs. The present method may be used either as a model for inflammation induced by a common and important air pollutant, and as a model for environmental-induced acute bronchitis.

Acknowledgements

This work was funded in part by the NRC-ENEL Project-Interactions of Energy Systems with Human Health and Environment-Roma, NRC FATMA Project 93.00650.PF41, MURST, European Commission (Biomedical and Health Research Program BMH 1, CT 94-1281), A.S.T.M.P. Padova, and CNR Rome, grant 97.04620.CT13.

References

American Conference of Hygienists, 1998. TCV_5 and BEI_5. A.C.G.H., Cincinnati, OH, p. 52.

Barnes, P.J., 1990. Reactive oxygen species and airway inflammation. Free Radical Biol. Med. 9, 235–243.

Ben-Jebria, A., Marthan, R., Savineau, J.P., 1992. Effect of in vitro nitrogen dioxide exposure on human bronchial smooth muscle response. Am. Rev. Respir. Dis. 146, 376.

Berglund, M., Bostrom, C.E., Bylin, G., Ewetz, L., Gustafsson, L., Moldeus, P., Norberg, S., Pershagen, G., Victorin, K., 1993. Health risk evaluation of nitrogen oxides. Exposure Scand. J. Work Environ. Health 19, 14–20, suppl. 2.

Bhalla, D.K., Crocker, T.T., 1987. Pulmonary epithelial permeability in rats exposed to O_3. J. Toxicol. Environ. Health 21, 73–87.

Bhalla, D.K.R., Mannix, R.C., Lavan, S.F., Phalen, R.F., Kleinmann, M.T., Crocker, T.T., 1987. Tracheal and bronchoalveolar permeability changes in rats inhaling oxidant atmospheres during rest or exercise. J. Toxicol. Environ. Health 22, 417–437.

Black, J., Morthan, R., Armour, C., Johnson, P., 1988. Is airway hyperresponsiveness a smooth muscle abnormality? In: Armour, C.L., Black, J.L. (Eds.), Mechanisms in Asthma. Pharmacology, Physiology and Management. Alan R. Liss, New York, NY, pp. 267–279.

Chitano, P., Coser, E., Lucchini, R.E., Papi, A., Saetta, M., Maestrelli, P.,

Faggiani, D., Plebani, M., Ciaccia, A., Fabbri, L.M., Mapp, C.E., 1994. In vitro exposure to nitrogen dioxide (NO_2) does not alter bronchial smooth muscle responsiveness in ovalbumin-sensitized guinea-pigs. Pulm. Pharmacol. 7, 251–257.

Chitano, P., Hosselet, J.J., Mapp, C.E., Fabbri, L.M., 1995a. Effect of oxidant air pollutants on the respiratory system: insights from experimental animal research. Eur. Respir. J. 8, 1357–1371.

Chitano, P., Lucchini, R.E., Coser, E., Papi, A., Saetta, M., Maestrelli, P., Ciaccia, A., Fabbri, L.M., Mapp, C.E., 1995b. In-vitro exposure of guinea pig main bronchi to 2.5 ppm of nitrogen dioxide does not alter airway smooth muscle response. Respir. Med. 89, 323–328.

Chitano, P., Lucchini, R.E., Calabrò, F., Saetta, M., Maestrelli, P., Fabbri, L.M., Mapp, C.E., 1996a. Isotonic smooth muscle response in human bronchi exposed in vitro to nitrogen dioxide. Eur. Respir. J. 9, 2294–2297.

Chitano, P., Rado, V., Di Stefano, A., Papi, A., Boniotti, A., Zancuoghi, G., Boschetto, P., Romano, M., Salmona, M., Ciaccia, A., Fabbri, L.M., Mapp, C.E., 1996b. Effect of subchronic in vivo exposure to nitrogen dioxide on lung tissue inflammation, airway microvascular leakage, and in vitro bronchial smooth muscle responsiveness in rats. Occup. Environ. Med. 53, 379–386.

De Nicola, D.B., Rebar, A.H., Henderson, R.F., 1981. Early damage indicators in the lung: V. Biochemical and cytological response to NO_2 inhalation. Toxicol. Appl. Pharmacol. 60, 301–312.

Fishbein, L., 1993. Sources, nature and levels of air pollutants. In: Tomatis, L. (Ed.), Indoor and Outdoor Air Pollution and Human Cancer. Springer-Verlag, Berlin, Germany, pp. 17–66.

Folkerts, G., Nijkamp, F.P., 1998. Airway epithelium: more than just a barrier!. Trends Pharmacol. Sci. 19, 334–341.

Frampton, M.W., Finkelstein, J.N., Roberts, N.J., Smeglin, A.M., Morrow, P.E., Utell, M.J., 1989. Effects of nitrogen dioxide on bronchoalveolar lavage proteins in humans. Am. J. Respir. Cell Mol. Biol. 1, 499–505.

Glasgow, J.E., Pietra, G.G., Abrams, W.R., Blank, J., Oppenheim, D.M., Weinbaum, G., 1987. Neutrophil recruitment and degranulation during induction of emphysema in the rat by nitrogen dioxide. Am. Rev. Respir. Dis. 135, 1136–1136.

Goldstein, E., Peek, N.F., Parks, N.J., Hines, H.H., Steffey, E.P., Tarkington, B., 1977. Fate and distribution of inhaled nitrogen dioxide in rhesus monkeys. Am. Rev. Respir. Dis. 115, 403–412.

Hazucha, M.J., Ginsberg, J.F., McDonnel, W.F., Haak, E.D. Jr., Pimmel, R.L., Salaam, S.A., House, D.E., Bromberg, P.A., 1983. Effects of 0.1 ppm nitrogen dioxide on airways of normal and asthmatic subjects. J. Appl. Physiol. 54, 730–739.

Herlihy, J.T., Murphy, R.A., 1973. Length–tension relationship of smooth muscle of the hog carotid artery. Circ. Res. 33, 275–283.

Jorres, R., Magnussen, H., 1991. Effects of 0.25 ppm nitrogen dioxide on the airway response to methacholine in asymptomatic asthmatic patients. Lung 169, 77–85.

Kawakami, M., Yasui, S., Yamawaki, I., Katayama, M., Nagai, A., Takizawa, T., 1989. Structural changes in airways of rats exposed to nitrogen dioxide intermittently for seven days. Comparison between major bronchi and terminal bronchioles. Am. Rev. Respir. Dis. 140, 1754–1762.

Kleinerman, J., Soresen, J., 1982. Nitrogen dioxide exposure and alveolar macrophage elastase in hamsters. Am. Rev. Respir. Dis. 125, 203–207.

Kobayashi, T., Shinozaky, Y., 1990. Effect of subacute exposure to nitrogen dioxide on the airway responsiveness of guinea pig. Agents Actions Suppl. 31, 71–74.

Linn, W.S., Solomon, J.C., Trim, S.C., Spier, C.E., Shamoo, D.A., Venet, T.G., Avol, E.L., Hackney, J.D., 1985. Effects of exposure to 4 ppm nitrogen dioxide in healthy and asthmatic volunteers. Arch. Environ. Health 40, 234–239.

Magnussen, H., 1992. Experimental exposure to nitrogen dioxide. Eur. Respir. J. 5, 1040–1042.

Man, S.F.P., Williams, D.J., Amy, R.A., Mam, G.C.W., Lien, D.C., 1990. Sequential changes in canine pulmonary epithelial and endothelial cell functions after nitrogen dioxide. Am. Rev. Respir. Dis. 142, 199–205.

Manzini, S., Conti, S., Maggi, C.A., Abelli, L., Somma, V., Del Bianco, E., Geppetti, P., 1989. Regional differences in the motor and inflammatory responses to capsaicin in guinea pig airways. Correlation with content and release of substance P-like immunoreactivity. Am. Rev. Respir. Dis. 140, 936–941.

Meulenbelt, J., Dormans, J.A.M.A., Marra, M., Rombout, P.J.A., Sangster, B., 1992. Rat model to investigate the treatment of acute nitrogen dioxide intoxication. Hum. Exp. Toxicol. 11, 179–187.

Mochitate, K., Ishida, K., Ohsumi, T., Miura, T., 1992. Long-term effects of ozone and nitrogen dioxide on the metabolism and population of alveolar macrophages. J. Toxicol. Environ. Health. 35, 247–260.

Morrow, P.E., Utell, M.J., Bauer, M.A., Smeglin, A.M., Frampton, M.W., Cox, C., Speers, D.M., Gibb, F.R., 1992. Pulmonary performances of elderly normal subjects and subjects with chronic obstructive pulmonary disease exposed to 0.3 ppm nitrogen dioxide. Am. Rev. Respir. Dis. 145, 291–300.

Murlas, C.G., Murphy, T.P., Lang, Z., 1990. HOCl causes airway substance P hyperresponsiveness and neutral endopeptidase hypoactivity. Am. J. Physiol. 258, L361–L361.

Ohrui, T., Sekizawa, K., Yamauchi, Y., Ohkawara, Y., Nakazawa, H., Aikawa, T., Sasaki, H., Takishima, T., 1991. Chemical oxidant potentiates electrically and acetylcholine-induced contraction in rat trachea: possible involvement of cholinesterase inhibition. J. Pharmacol. Exp. Ther. 259, 371–376.

Orehek, J., Massari, J.P., Gaynard, P., Grimaud, C., Charpin, J., 1976. Effect of short-term, low-level of nitrogen dioxide exposure on bronchial sensitivity of asthmatic patients. J. Clin. Invest. 57, 301–307.

Samet, J.M., Marbury, M.C., Spengler, J.D., 1987. Health effects and sources of indoor air pollution, Part I. Am. Rev. Respir. Dis. 136, 1486–1508.

Sherwin, R.P., Carlson, D.A., 1973. Protein content of lung lavage fluid of guinea pigs exposed to 0.4 ppm nitrogen dioxide. Arch. Environ. Health 27, 90–93.

Silbaugh, S.A., Mauderly, J.L., Macken, C.A., 1981. Effects of sulfuric acid and nitrogen dioxide on airway responsiveness of the guinea pig. J. Toxicol. Environ. Health 8, 31–45.

United States Department of Health, Education, and Welfare, 1976. Criteria for a recommended standard…occupational exposure to oxides of nitrogen (nitrogen dioxide and nitric oxide). HEW publication N. (NIOSH), USA, pp. 76–149.

Vassilyadi, M., Michel, R.P., 1988. Pattern of fluid accumulation in NO_2-induced pulmonary edema in dogs. A morphometric study. Am. J. Pathol. 130, 10–21.

Yokoyama, E., Frank, R., 1972. Respiratory uptake of ozone in dogs. Arch. Environ. Health 25, 132–138.