Effect of subchronic in vivo exposure to nitrogen dioxide on lung tissue inflammation, airway microvascular leakage, and in vitro bronchial muscle responsiveness in rats

Pasquale Chitano, Vanda Rado, Antonino Di Stefano, Alberto Papi, Anna Boniotti, Gianna Zancuoghi, Piera Boschetto, Maria Romano, Mario Saliena, Adalberto Ciaccia, Leonardo Michele Fabbri, Cristina Elisabetta Mapp

Abstract

Objectives—In a previous study on bronchoalveolar lavage fluid from rats exposed in vivo for seven days to 10 ppm nitrogen dioxide (NO₂), it has been shown that there is an influx of macrophages into the airways. The present study investigated the effect of seven day exposure to 10 ppm NO₂ on: (a) lung tissue inflammation and morphology; (b) airway microvascular leakage; (c) in vitro contractile response of main bronchi.

Methods—Lung tissue was studied by light microscopy, after fixing the lungs by inflation with 4% formalin at a pressure of 20 cm H₂O. Microvascular leakage was measured by extravasation of Evans blue dye in the larynx, trachea, main bronchi, and intrapulmonary airways. Smooth muscle responsiveness was evaluated by concentration-response curves to acetylcholine (10⁻⁸–10⁻³ M), serotonin (10⁻⁶–10⁻⁴ M), and voltage-response curves (12–28 V) to electrical field stimulation.

Results—Histology showed an increased total inflammation at the level of respiratory bronchioles and alveoli. No influx of inflammatory cells was found in the main bronchi. A loss of cilia in the epithelium of small airways and ectasia of alveolar capillaries was also found. By contrast, no alterations to microvascular permeability or modification of bronchial smooth muscle responsiveness was found.

Conclusions—Subchronic exposure to 10 ppm NO₂ causes airway inflammation and structural damage, but does not cause any persistent alteration to microvascular permeability or bronchial smooth muscle responsiveness in rats.

Keywords: airway smooth muscle; lung histology; vascular permeability

Nitrogen dioxide (NO₂) is a common component of environmental air pollution produced by processes involving high temperatures. Its general environmental concentration may reach values of 0.5 ppm in urban areas, but its concentration at the workplace may reach values of up to 500 ppm in those specific zones where welding arcs and blow torches are used.

Many studies have reported injurious effects of exposure to NO₂ on the structure, function, and biochemistry of the respiratory system. In particular, exposure to NO₂ causes pulmonary inflammation, lung oedema, and airway hyperresponsiveness.

Airway inflammation is a consistent finding both in animals and in humans exposed to NO₂. It is more pronounced in peripheral airways and alveoli. In animals, it is mainly characterised by neutrophil and macrophage infiltration, whereas in humans an increased number of mast cells and lymphocytes have been reported in bronchoalveolar lavage fluid.

Lung oedema has been shown in animals to be associated with cellular infiltration into the airways. It occurs mainly during early exposure to NO₂ and is correlated with the degree of exposure, but it has also been reported after chronic exposures.

Airway hyperresponsiveness caused by NO₂ has been reported in healthy, bronchitic, and asthmatic subjects, although several inconsistencies exist in these studies. Whether the NO₂ effect on airway responsiveness involves alterations in bronchial smooth muscle responsiveness has not yet been elucidated. Indeed, contradictory results have also been reported in the few studies performed on responsiveness of bronchial smooth muscle exposed in vitro.

We have recently established an animal model of inflammation induced by NO₂ in which we showed an increase in the number of macrophages in bronchoalveolar lavage fluid from rats exposed for seven days to 10 ppm NO₂. In the present study we wanted to investigate whether the inflammatory response produced by NO₂ in this model was associated with structural alterations of the lung tissue, with persistent increase of microvascular leakage and of bronchial smooth muscle responsiveness. Indeed, the contemporary induction by NO₂ of pulmonary inflammation, airway microvascular leakage, and airway smooth muscle hyperresponsiveness, which are the three most relevant features of asthma, would supply a suitable animal model to study pathological mechanisms possibly involved in asthma. We therefore studied: (a) the inflammatory response in lung tissue and the morphological alterations of airway and...
parenchimal structural components by lung histology; (b) the microvascular leakage in the airways by direct measurement; (c) the in vitro bronchial smooth muscle contractile response.

Materials and methods

**ANIMALS AND EXPOSURE TO NO**

Sprague-Dawley CD rats weighing 200–300 g (Charles River) were used in this study. The procedures involving animal care and exposure were conducted conforming with our institutional guidelines, which comply with national and international laws and policies (EEC Council directive 86/609; NIH guide for care and use of laboratory animals, NIH Publ No 85–23, 1985). Specific protocols and procedures were approved by the committee on animal care of the Universities of Ferrara or Padova.

Twenty rats were exposed to 10 ppm NO for seven days, and 24 control animals were exposed to filtered air for the same period. The exposure was carried out as previously described. Briefly, rats were exposed in a stainless steel chamber (3.96 m³), with conditioned air at a mean (SD) of 21 (1) °C and 50% (10%) humidity. Cylinders containing 1% NO in N₂ (SIO, Milan, Italy) were used as the source of NO. The NO concentration in the chamber was measured by a Monitor Labs nitrogen oxides analyser 8840 (Denver, CA, USA), connected to an Olivetti PCS 86 computer system (Ivrea, Italy), and to a control module MPS 2000 Micros ( Treviso, Italy). This computerised system recorded and kept constant the NO concentration in the chamber for the duration of the exposure, with a closed loop feedback and thus regulated the flow of NO, and air from the cylinders to the exposure chamber. Gas concentrations varied less than 1% from the nominal value.

Rats were killed 24 hours after the end of the exposure to investigate any persistent effect. At the end of the exposure, the animals underwent one of the following experimental protocols.

**LUNG HISTOLOGY**

For histological studies, the animals were killed and the heart and lungs were gently extracted from the chest. The lungs were then inflated and fixed by endotracheal instillation of 4% formaldehyde in sodium phosphate buffer for six hours, at a constant pressure of 20 cm H₂O at room temperature. After fixation, the left lung was cut into two halves (medial and lateral) along its midsagittal plane. These two preparations were then dehydrated in ethanol, passed through xylene, and embedded in paraffin. A section 6 μm thick was obtained from the surface of each block, and stained with haematoxylin-eosin for analysis by light microscopy, with a Jenamed 30G0040 microscope.

Neutrophils were assessed in the main lobar bronchus (in an area 20 μm beneath the epithelial basement membrane), and in the small airways (in the whole wall). The cells were counted at a magnification of × 780 in contiguous non-overlapping high power fields until all the available area was covered. About seven high power fields were analysed for each section and the final cell count relative to each animal was expressed as the average number of cells per high power field counted in the two sections.

Total inflammation was assessed at a magnification of × 400 according to the method of Cosio et al. and studied in the main lobar bronchus, in small airways, and at the level of respiratory bronchioles and alveoli—that is, bronchioles with a discontinuous airway wall and the surrounding alveolar regions. This method allows for a semiquantitative assessment, in histological preparations, of a given event that produces alterations at tissue level. The occurrence of an event X under examination is classified according to a discrete scale—that is, the range of all possible intensities of X is divided into classes which score from 0 (absence of X) to N (maximum intensity of X). Then X is measured several times and each measurement is assigned to a given class of intensity. We investigated the intensity of total inflammation in the lung tissue with a subdivision into four classes: 0, absence of inflammatory cell infiltrate; 1, presence of a few cells (low inflammation); 2, presence of several cells (moderate inflammation); and 3, massive cellular infiltration (high inflammation). Therefore, an appropriate score from 0 to 3 was assigned to each airway or bronchiolar-alveolar area examined. The scores of each area were then summed and the results were expressed as the percentage of the maximum possible score for each case (maximum cellular infiltration).

The same method (score from 0 to 3) was used to assess loss of cilia by the epithelial cells in the small airways and results were expressed as a percentage of the maximum damage (no presence of cilia).

In the lung parenchyma, as an index of alveolar structural damage, we measured the mean linear intercept (Lm), defined as the mean linear distance between alveolar septa. To do this we used a microscope with a × 10 objective and a × 10 eyepiece according to the modified method of Thurlbeck.

**AIRWAY MICROVASCULAR LEAKAGE**

To assess vascular permeability, animals were premedicated with diazepam (3 mg/kg) by intraperitoneal injection and anaesthetised with 0.5 ml of Leptofen (containing 1 mg droperidol and 0.032 mg fentanyl citrate) by intramuscular injection. Then the jugular veins were exposed and 30 mg/kg of Evans blue dye (30 mg/ml in 0.9% NaCl, filtered through a 0.22 μm Millipore filter) were injected intravenously as a tracer. Five minutes later, the chest was opened and a cannula was inserted into the ascending aorta through the left ventricle to perfuse the animal’s vascular system. Perfusion was carried on for two minutes under a pressure of 100 mm Hg with 100 ml of 0.9% NaCl solution (pH 5.5) at room temperature. Blood and saline were drained through an incision made in the right
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atrium. Thereafter, the larynx, trachea, main bronchi, and lungs were removed and separated from each other. The intrapulmonary airways were stripped of the parenchyma by gently scraping with a razor blade. Excess fluid was removed by squeezing between filter papers and the tissue wet weight was measured.

Evans blue dye was extracted from the tissues by incubating each sample overnight in 2 ml of formamide at 50°C. The concentration of Evans blue dye was measured by light absorbance against a formamide blank at a wavelength of 620 nm with a DU-40 spectrophotometer (Bacilman Instruments, Irvine, CA, USA), and by interpolation on a standard curve of dye in formamide in the range 0.5–10 µg/ml. Final results were expressed as ng of Evans blue dye per mg of wet weight tissue.

BRONCHIAL SMOOTH MUSCLE RESPONSE IN VITRO

To study smooth muscle response in vitro, animals were killed and the trachea and lungs were rapidly removed and immersed in oxygenated Krebs-Henseleit solution containing the following (mM): NaCl 118.3, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25.0, CaCl2 2.5, and d-(-)-glucose 11.1. The two main bronchi were dissected free of loose connective tissue and a ring was prepared from each. The rings were mounted in double jacketed glass organ baths filled with Krebs-Henseleit solution, that was maintained at 37°C and aerated continuously by bubbling it with a mixture of 95% O2 and 5% CO2, which produced a pH of 7.4. As it has been reported that altered smooth muscle contractility may consist of increased shortening not associated with any alteration in the ability to develop tension, this study was performed both isometrically and isotonically, the two techniques which allow investigation of these two types of muscle response. The rings were therefore connected to a force displacement transducer (Grass FTO3) or to an isotonic transducer (Basile 7006), and allowed to equilibrate for 90 minutes with a resting tension of 300 mg. During equilibration the medium was changed every 20 minutes. We continuously monitored isometric tension or isotonic shortening and recorded the responses on a paper polygraph (Battaglia Rangoni KV380).

For each ring in both isometric and isotonic conditions we performed cumulative concentration-response curves to acetylcholine (ACh) from 10⁻⁹ to 10⁻¹ M, serotonin (5-HT) from 10⁻⁹ to 10⁻⁴ M, and in isometric conditions voltage-response curves (12–28 V) to electrical field stimulation with a 10 second stimulus (60 Hz, 8 ms duration). Electrical stimuli were produced by a Grass S88 stimulator and applied through wire electrodes placed at the top and bottom of the bath chamber. Isometric contractions were expressed as a percentage of the ring length. Isometric contractions in response to ACh were normalised per tissue cross sectional area of each preparation. Cross sectional area (mm²) was calculated by an indirect method, with weight (w) and length (l) of the rings according to the formula:

cross sectional area = w/(l × D)

where 1 (mm) represents the ring diameter measured in the bath after the equilibration period, w (mg) is the ring fresh weight measured at the end of each experiment, and D is the tissue density, considered to be equal to 1 mg/mm³. As the tissue stress generated by ACh did not show differences between bronchial rings from rats exposed to NO2 and controls (see results), we could express the isometric contractions in response to 5-HT and to electrical field stimulation as a percentage of the maximum response to ACh obtained in the same ring, thus reducing the magnitude of the statistical errors inherent in sampling and manipulation.

DRUGS AND CHEMICALS

Diazepam was obtained from Roche Pharmaceuticals (Basel, Switzerland), leptofen from Farmitalia Carlo Erba SpA (Milano, Italy). Evans blue dye, acetylcholine, and serotonin were obtained from Sigma Chemical (St Louis, MO, USA). Stock solutions for the study on bronchial responsiveness were made in water at concentrations 1000-fold greater to minimise changes in bath volume, pH, and ionic strength.

DATA ANALYSIS

Data relative to lung histology and airway microvascular leakage were expressed as medi ans (ranges). Data relative to bronchial smooth muscle response in vitro were expressed as mean (SEM), except for the concentration eliciting 50% of the maximum response (EC50) which was expressed as geometric mean (GM) (GSEM), and 95% confidence intervals (95% CIs).

Results relative to lung histology and airway microvascular leakage were analysed by Mann-Whitney U test. Results relative to bronchial smooth muscle response in vitro were compared by a one way analysis of variance (ANOVA) for repeated measures, and by unpaired Student’s t test. Differences were considered significant when the P value was < 0.05.

RESULTS

LUNG HISTOLOGY

We found a significant increase in total inflammation at the level of respiratory bronchioles and alveoli in rats exposed to 10 ppm NO2 for seven days. Although we did not attempt a precise differential count of inflammatory cells, we found that cellular infiltrate was predominantly composed of mononuclear leukocytes (fig 1). Figure 2 shows the pathological scores for total tissue inflammation. By contrast, no significant difference in neutrophil tissue infiltration was found. The number of neutrophils in rats exposed to 10 ppm NO2 and air were respectively 0.18 (range 0.00–0.50) and 0.00 (range 0.00–0.25) cells/high power field in small airways (z = -1.01, P = 0.31 by Mann-Whitney U test),
Figure 1
Photomicrographs of peripheral airways and lung parenchyma of a rat exposed to (A) air and (B) 10 ppm NO₂. Arrows indicate the mononuclear leucocyte infiltrate in the tissue from the animal exposed to NO₂. Stain: haematoxylin-eosin, light microscopy. Final magnification: × 400.

and 0·10 (range 0·00–0·20) and 0·08 (range 0·00–0·16) cells/high power field in the main bronchus (z = -0·29, P = 0·77 by Mann-Whitney U test).

We also found epithelial damage, with loss of cilia, and ectasia of alveolar capillaries. The scores for loss of cilia in the epithelial cells were 83·5 (range 33–100) and 0·0 (range 0·0–11) in animals exposed to NO₂ and air respectively (z = -2·31, P = 0·02 by Mann-Whitney U test). The distance between alveolar walls, as measured by Lm, was not different in rats exposed to NO₂ and air, which were respectively 50·5 (range 45·0–57·0) μm and 36·0 (range 30·0–50·0 μm, z = -1·59, P = 0·11 by Mann-Whitney U test).

AIRWAY MICROVASCULAR LEAKAGE
Macroscopic observation of the tissue before the extraction of the Evans blue dye was similar for rats exposed to NO₂ and air where only the vessels were notably stained. Measurement of Evans blue dye showed that plasma extravasation did not increase above control values in any site of the airways from rats exposed to NO₂. Table 1 shows the concentrations of
Table 1. Concentration of Evans blue dye in airways from rats exposed for seven days to 10 ppm NO₂ and from control rats.

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<tr>
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<th>Exposed to NO₂</th>
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<tr>
<td>Larynx</td>
<td>11.0 (8.1-14.7)</td>
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</tr>
<tr>
<td>Trachea</td>
<td>25.1 (23.2-39.2)</td>
<td>27.8 (20.8-38.5)</td>
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<tr>
<td>Main bronchi</td>
<td>56.4 (32.8-64.5)</td>
<td>70.7 (42.4-105.8)</td>
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<tr>
<td>IPA</td>
<td>26.9 (24.8-34.6)</td>
<td>35.1 (16.5-53.2)</td>
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Data are medians (ranges) and are expressed as pg per mg of wet weight of tissue, n = 4 for rats exposed to NO₂, n = 8 for controls. Mann-Whitney U test values were: z = 0.73, P = 0.46 for larynx; z = 0.34, P = 0.73 for trachea; z = -0.78, P = 0.40 for main bronchi; z = -1.19, P = 0.23 for intrapulmonary airways (IPA).

Table 2. Smooth muscle sensitivity (EC₅₀) calculated from curves performed in isometric conditions in bronchi from rats exposed for seven days to 10 ppm NO₂ and in control rats.

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<td>Electrical field stimulation (V)</td>
<td>16.6 (1.0)</td>
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The sensitivity of acetylcholine (ACh) and to serotonin (5-HT) is expressed as EC₅₀ (geometric mean and GSEM), whereas sensitivity to electrical field stimulation is expressed as EV₅₀ (mean SEM). Sample sizes for rats exposed to NO₂ and controls were respectively n = 11 and n = 12 for ACh and 5-HT, n = 8 and n = 9 for electrical field stimulation. The test statistical values by Student's unpaired t test were: t = 1.16, P = 0.26 for ACh; t = 1.18, P = 0.25 for 5-HT; t = 0.015, P = 0.99 for electrical field stimulation.

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Evans blue dye in the airways of rats exposed to NO₂ and air at the levels of the larynx, trachea, main bronchi, and intrapulmonary airways.

SMOOTH MUSCLE RESPONSE

Isometric conditions

Acetylcholine caused a concentration dependent isometric contraction of bronchial smooth muscle which was not altered by exposure to 10 ppm NO₂ for seven days (fig 3); for this reason we expressed tension developed by electrical field stimulation and 5-HT as a percentage of the response to 1 mM of ACh. Exposure to NO₂ did not alter the curves in response to 5-HT or electrical field stimulation, nor did it modify the maximum response. Figure 4 shows the isometric voltage-response curves to electrical field stimulation and the concentration-response curves to 5-HT for bronchi obtained from rats exposed to air and NO₂. Table 2 shows the sensitivity to ACh and 5-HT expressed as EC₅₀, and to electrical field stimulation expressed as EV₅₀ obtained from isometric curves.

Isotonic conditions

No change in the shape of the curve and in the maximum response after exposure to NO₂ was found in isotonic experiments. Figure 4 shows the shortening produced isotonically that is dependent upon concentrations of ACh and 5-HT in bronchi from rats exposed to filtered air or 10 ppm NO₂. Table 3 shows the sensitivity to ACh and 5-HT calculated from concentration-response curves performed isotonically.

Discussion

In the present study we found an increased total inflammation at the level of respiratory bronchioli and alveoli after seven days in vivo exposure to 10 ppm NO₂. No influx of inflammatory cells was found in the main bronchi. We also found loss of cilia from the epithelial cells and ectasia of alveolar capillaries. By contrast, we found no enlargement of the airspaces, no alteration of microvascular permeability in the larynx, trachea, main bronchi, and intrapulmonary airways, and no modification of bronchial smooth muscle contractile response to acetylcholine, electrical field stimulation, and serotonin.

In different animal species the inflammatory response induced by NO₂, which is more pronounced in peripheral airways and alveoli, consists of an initial increase in neutrophils, followed by macrophage and lymphocyte infiltration. The influx of alveolar macrophages and lymphocytes reaches a maximum after one week of exposure or more, although it has also been reported at earlier times. 7,22

The results of the present study are in agreement with our previous report from bronchoaveolar lavage, 18 and confirm that the inflammatory response and the structural damage occur distally in the airways and in the alveoli, at least at the concentrations of NO₂ we have used. As NO₂ is a gas with low solubility, it is poorly absorbed by the airway
mucosa. Nevertheless, its uptake in the respiratory system is extremely high (because of its reactivity) and is maximum at the level of the terminal bronchioles.।

Like inflammation, lung oedema induced by NO₂ is mainly reported in small airways and alveolae, is more evident during early exposure, and declines with time.।

In the present study we used a method which has been shown to be particularly sensitive in detecting exudation of plasma into the airway tissue.।

We found no alteration of microvascular permeability after seven days of continuous exposure to NO₂ although we have previously shown that with the same method the concentration of Evans blue dye increases more than twofold over basal levels after giving 1 μg/kg of platelet activating factor.।

These findings may depend on the time course of the alteration of vascular permeability induced by NO₂, which could indeed be back to baseline at the time point we studied. Were this the case, the observed swelling of septal capillaries would represent a late or persistent morphological alteration. Alternatively, we could speculate that the exposure to 10 ppm NO₂ is too low to cause a persistent microvascular leakage and that ectasia of alveolar capillaries is a more suitable index of the effects of NO₂ on vessels.

The considerations we have made about microvascular leakage may also apply to our results on the contractile response of bronchial smooth muscle as the fact that it was not altered by NO₂ could possibly depend on the time course or on the need of a higher level of exposure for this effect to be produced. However, several other factors may possibly be responsible.

The fact that airway hyperresponsiveness induced by NO₂ has been inconsistently reported may be due to either differences in the exposed populations—that is, the possible existence of a group of responders and one of non-responders—or to a different strain or species susceptibility. The recent finding in mice that some inbred strains develop airway hyperresponsiveness to intravenous acetylcholine after exposure to NO₂ whereas some others do not, further supports the possibility of a differential susceptibility to oxidant haz-

Table 3 Smooth muscle sensitivity (EC₅₀) calculated from curves performed in isotonic conditions in bronchi from rats exposed for seven days to 10 ppm NO₂ and in control rats

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<td>1.0.10⁻¹ (1-3)</td>
<td>5.7.10⁻⁴ (4-7)</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.5.10⁻¹ to 2.0.10⁻³</td>
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</tr>
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<td>5-HT (M)</td>
<td>3.5.10⁻³ (1-8)</td>
<td>6.8.10⁻³ (1-3)</td>
</tr>
<tr>
<td>95% CI</td>
<td>0.5.10⁻⁴ to 1.3.10⁻⁶</td>
<td>3.4.10⁻⁴ to 1.4.10⁻⁶</td>
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The sensitivity to acetylcholine (ACh) and to serotonin (5-HT) is expressed as EC₅₀ (geometric mean and GSEM). For each group n = 8. Values were tested by Student’s unpaired t test and were for ACh and 5-HT respectively t = 1.24, P = 0.23 and t = −1.05, P = 0.31.
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ard. Also, a quantitative difference in lung lipid peroxidation and antioxidative protective system activity after exposure to NO₂ has been found between rats and guinea pigs.²⁶

It is possible that our negative results, as well as the inconsistency of data on humans, depend on several factors, which may differ between subjects or species. Firstly, nitric oxide, a potent relaxant of smooth muscle, has been identified as a metabolite product of NO₂ exposure.²⁷

The formation of this compound could prevent development of hyperresponsiveness by reducing the basal tone and therefore the contractility of the smooth muscle. Secondly, it has been shown that tolerance may develop after repeated exposure to oxidant agents,²⁸ so individual history could influence the functional effects. Even though the concept of tolerance refers more properly to repeated exposures, protective compensatory mechanisms may also develop during a single continuous exposure, as shown by the increase of protective enzymatic activity found in rats after about five days of continuous exposure to NO₂.²⁹

Finally, the contemporary or previous exposure to other pollutants may be necessary for the development of airway hyperresponsiveness, as synergism between NO₂ and other pollutant substances has been shown.²⁶

It has been suggested that bronchial hyperresponsiveness may be caused by inflammatory mediators.³⁰ In the present study we found an inflammatory infiltrate in small airways and alveoli, but not in the main bronchi, and we found no alteration of smooth muscle responsiveness in the main bronchi. It is therefore possible that an increase of smooth muscle responsiveness was not detected because it occurs peripherally to the main bronchi. Further studies will be required to elucidate the role of the factors we have discussed and to identify those most influential in the development of hyperresponsiveness as a consequence of exposure to NO₂.

We finally need to consider the complexity of the events leading to airway hyperresponsiveness and the multitude of factors which contribute to its development. In fact, in vivo airway hyperresponsiveness may be affected by factors such as secretions, neural reflexes, and parenchymal elastic recoil, which do not influence smooth muscle response in vitro. Those factors may be responsible for the frequently found absence of correlation between airway responsiveness in vivo and smooth muscle responsiveness in vitro.³¹

In the present study, as for every study on isolated smooth muscle, we intended to reduce the number of components contributing to airway responsiveness, with the aim of investigating the involvement of smooth muscle alterations in the hyperresponsiveness induced by NO₂. Therefore, a possible explanation for our negative results would be that the in vivo airway hyperresponsiveness is affected by alterations of non-muscular factors contributing to the bronchospasm, which would not be detectable by our approach.

In summary, we have found that subchronic exposure to up to 10 ppm NO₂ causes airway inflammation and structural damage but does not alter microvascular permeability and bronchial smooth muscle responsiveness in rats.

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