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Original Articles

In-vitro exposure of guinea pig main bronchi to 2·5 ppm of nitrogen dioxide does not alter airway smooth muscle response

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In order to investigate whether the oxidant airborne pollutant nitrogen dioxide (NO_2) affects airway smooth muscle responsiveness, the contractile response of guinea pig main bronchi after *in vitro* exposure to 2·5 ppm of nitrogen dioxide was studied. Main bronchi were cannulated and exposed for 2 or 4 h to a constant flow of either NO_2 or air. After exposure, bronchial rings were obtained and placed in a 37°C jacketed organ bath filled with Krebs–Henseleit solution. Concentration–response curves were performed for acetylcholine (10^{-9}–10^{-3} M), substance P (10^{-9}–10^{-4} M), and neurokinin A (10^{-10}–10^{-5} M), and voltage–response curves (12–28 V) were performed for electrical field stimulation. There was no significant difference in either the smooth muscle maximal contractile response, or sensitivity between the bronchi exposed to NO_2 and those exposed to air. We conclude that *in vitro* exposure to 2·5 ppm of NO_2 does not alter airway smooth muscle responsiveness in guinea pigs.

Introduction

Nitrogen dioxide (NO_2) is a common oxidant airborne pollutant generated by processes involving high temperatures. It is found in motor vehicle and power plant emissions, and is produced by cigarette smoking and the combustion of natural gas and kerosene, which are commonly used for cooking and heating. Its environmental concentrations in urban areas may peak up to 0·5 ppm, both outdoors and indoors, while it reaches higher levels in the workplace, particularly in the proximity of welding arcs or cutting torches, where values up to 500 ppm have been reported during operation (1–4). Present recommended occupational exposure limits vary, in different countries, between 2–5 ppm time-weighted average and between 5–10 ppm short-term exposure limit (5).

Nitrogen dioxide exerts several acute and chronic effects on the structure, function and biochemistry of the respiratory system, which have been described both in humans and in animal models (3,4,6–9). Impaired lung respiratory function with increased airway responsiveness has been reported, but it has only been observed in some of the exposed subjects (8–12), it has not always been reproducible in humans (8,9,13–16), and in animals, it has only been reported in guinea pigs (17–20). These inconsistencies may depend on differences between species or subpopulations, possibly because of a different balance among the several components which constitute the response *in vivo*, e.g. lipid peroxidation, antioxidant protective system, inflammation vascular permeability and metabolite production (3,4,6,7). Moreover, increased airway responsiveness *in vivo* may or may not be dependent on smooth muscle hyper-responsiveness. Therefore, to study whether the effects of NO_2 on airway responsiveness involve alterations of smooth muscle contractility, the smooth muscle contractile response of guinea pig main bronchi exposed *in vitro* to 2·5 ppm of NO_2 was investigated.

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Materials and Methods

ANIMALS

The experimental procedure and specific protocols were approved by the Committee on Animal Care of the University of Padua. Male Hartley guinea-pigs

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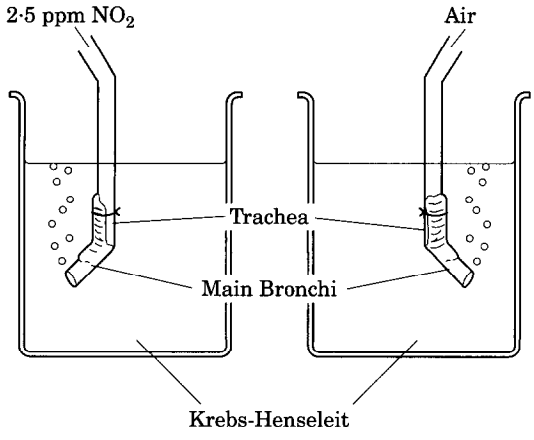

Fig. 1 Schematic representation of the method utilized for intraluminal exposure of guinea pig main bronchi to air or NO_2.

(Rodentia Laboratories, Torre Pallavicina, Bergamo, Italy) weighing 300–400 g were anaesthetized with pentobarbital sodium (100 mg kg^{-1} intraperitoneally). Trachea and 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.

EXPOSURE TO NO_2

The trachea and main bronchi were dissected free of loose connective tissue, under a stereo-microscope Technival 2 (aus JENA). After cutting the trachea along its longitudinal axis, the two main bronchi were cannulated; then, one bronchus was exposed to air and the other bronchus to NO_2 in air, using a constant intraluminal flow of 1 ml s^{-1}, as shown by the schematic representation in Fig. 1. Exposure to NO_2 was performed at 2·5 ppm for 2 or 4 h. This concentration of NO_2 was chosen as it represents a level often reached for short periods in the workplace, where this pollutant is generated.

IN VITRO STUDY

After exposure, a ring was prepared from the portion of each main bronchus which was not cannulated, and mounted in a double jacketed organ bath, filled with Krebs–Henseleit solution at 37°C and continuously aerated with a 95% O_2 and 5% CO_2 gas mixture, which produced a pH of 7·4. The bronchial rings were connected to force displacement transducers (Grass FTO3) for continuous recording of isometric tension. The rings were allowed to equilibrate for 90 min and resting tension was adjusted to 500 mg, which had previously been found to keep this preparation close to its optimal length. During equilibration, the rings were washed with fresh Krebs–Henseleit solution every 20 min.

EXPERIMENTAL PROTOCOLS

Using the procedure described above, two rings were obtained from each animal, one ring was exposed to NO_2 and a paired control ring was exposed to air, in the following experiments.

In the first set of experiments, after equilibration, concentration–response curves for acetylcholine (ACh) (10^{-9}–10^{-3} M) were performed in rings exposed to 2·5 ppm NO_2 or air for 2 h. Since smooth muscle responsiveness to ACh was not affected by exposure to NO_2, administration of 1 mM ACh, performed after equilibration, was used as a standard stimulus in subsequent experiments. The rings were then washed with fresh Krebs–Henseleit solution until the tension returned to resting values, and the experiments completed.

In rings exposed to 2·5 ppm NO_2 or air for 2 h, either voltage–response curves (12–28 V) for electrical field stimulation (EFS), or concentration–response curves for substance P (SP) (10^{-9}–10^{-4} M) or neurokinin A (NKA) (10^{-10}–10^{-5} M) were performed. The electrical stimulus (60 Hz, 8 ms for 10 s) was delivered by means of two wire platinum electrodes placed at the top and bottom of the organ bath, and connected to a Grass S88 stimulator.

The response to NKA was also studied after exposure to 2·5 ppm NO_2 or air for 4 h. Moreover, in paired rings from the same animal exposed to 2·5 ppm NO_2 for 4 h, the response to NKA was studied in the presence or absence of phosphoramidon (10 μM, contact time 15 min). These last experiments were performed to see whether a higher dose of NO_2 could increase the response to NKA, through inhibition of the neutral endopeptidase, and whether phosphoramidon, which inhibits the neutral endopeptidase, could potentiate the response to exogenous NKA after exposure to NO_2.

DRUGS AND CHEMICALS

Acetylcholine and neurokinin A were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.); substance P from Peninsula Lab. (St Helens, U.K.); phosphoramidon from Boehringer Mannheim Italia SpA (Milan, Italy) and cylinders containing 2·5 ppm NO_2 in air were obtained from SIAD (Camin, Padua, Italy).

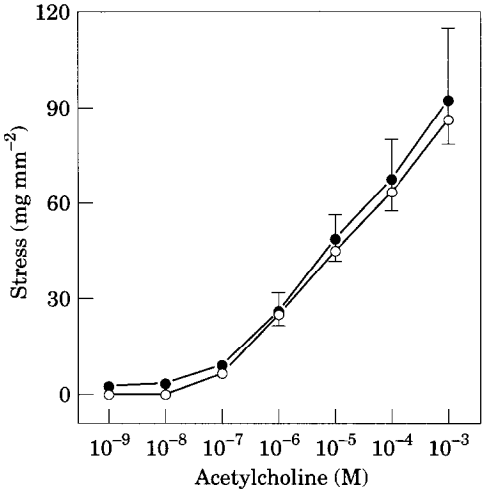

Fig. 2 Concentration–response curves for acetylcholine in guinea pig main bronchi, exposed to air (○) or 2·5 ppm of NO_2 (●) for 2 h. Each point is mean ± SEM, $n=6$.

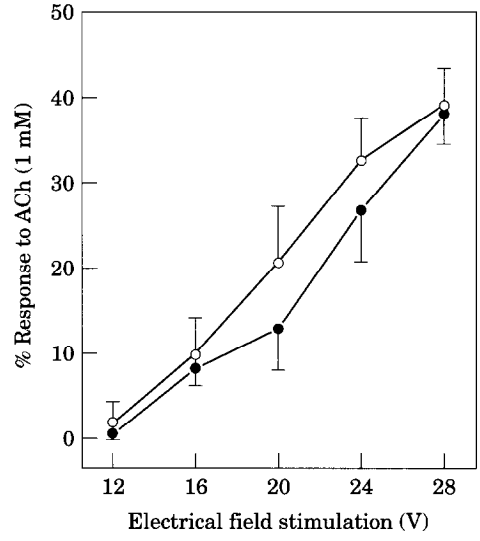

Fig. 3 Voltage–response curves for electrical field stimulation in guinea pig main bronchi, exposed to air (○) or 2·5 ppm of NO_2 (●) for 2 h. Each point is mean ± SEM, $n=6$.

ANALYSIS

Values are given as means ± standard error of the mean (SEM), except for the concentration eliciting 50% of the maximum response (EC_{50}) which is expressed as geometric mean (GM) and geometric standard error of the mean (GSEM).

The smooth muscle response during ACh concentration–response curves was expressed as tissue stress (mg mm^{-2}), whereas responses to other stimuli were expressed as a percentage of the active tension obtained in response to 1 mM ACh. Tissue stress was calculated by dividing tension by tissue cross-sectional area (CSA) of each preparation (21); CSA was calculated using fresh weight (w) and length (l) of the preparation according to the formula $CSA = w/(l \times D)$, where w was measured at the end of the experiments, l represents the ring diameter and D (density of the tissue) is considered equal to one.

Comparisons were performed by analysis of variance (ANOVA) for repeated measures, and by two-tailed Student's t-test for paired data using the StatView II statistical package (Abacus Concepts, Inc., Berkeley, CA, U.S.A.). Values of $P<0{\cdot}05$ were considered significant.

Results

The concentration–response curves for ACh in bronchi exposed to air or 2·5 ppm of NO_2 for 2 h is shown in Fig. 2. No significant difference was found between stress developed by rings exposed to air or to NO_2.

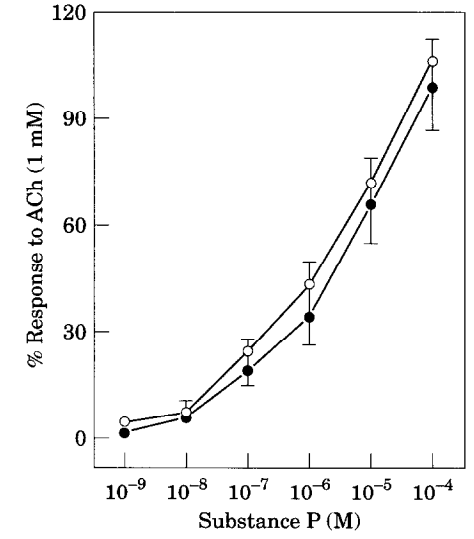

Fig. 4 Concentration–response curves for substance P in guinea pig main bronchi, exposed to air (○) or 2·5 ppm of NO_2 (●) for 2 h. Each point is mean ± SEM, $n=6$.

Figure 3 shows the voltage–response curves for EFS, and Fig. 4 shows the concentration–response curves for SP. There was no significant difference in the response to EFS and to SP between bronchi exposed to air or to 2·5 ppm NO_2.

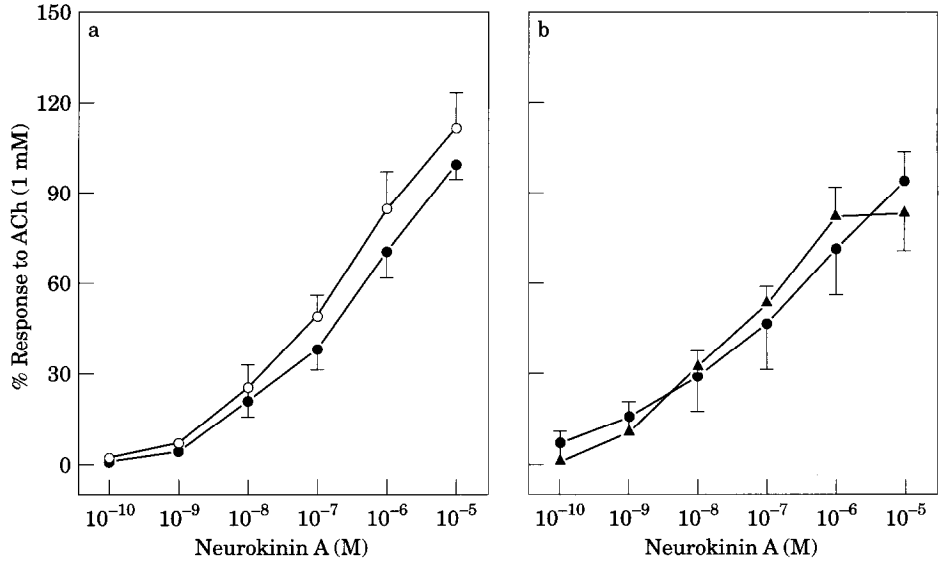

Fig. 5 Concentration–response curves for neurokinin A in guinea pig main bronchi. (a) response obtained from paired bronchi exposed to air (○) or 2·5 ppm of NO_2 (●) for 4 h, each point is mean ± SEM, $n=6$; (b) response obtained in the absence (●) and presence (▲) of 10 μM phosphoramidon from paired bronchi exposed to 2·5 ppm of NO_2 for 4 h, each point is mean ± SEM, $n=5$.

Bronchial smooth muscle response to NKA was studied after exposure to air or 2·5 ppm NO_2 for either 2 or 4 h, even in tissue incubated with phosphoramidon, and no significant difference was revealed. Figure 5 shows the concentration–response curves for NKA obtained from paired rings of bronchi exposed for 4 h to air or NO_2 (a), and curves performed in the presence or absence of phosporamidon using paired rings from bronchi exposed for 4 h to NO_2 (b).

The sensitivity to each contractile agent is reported in Table 1. No significant difference was found between exposed and control rings for this parameter.

Table 1 Smooth muscle sensitivity in guinea pig bronchi exposed *in vitro* to 2·5 ppm NO_2

	Treated	Control
ACh (M)	$1{\cdot}0 \times 10^{-5}$ (1·3)	$8{\cdot}4 \times 10^{-6}$ (1·2)
SP (M)	$2{\cdot}4 \times 10^{-6}$ (1·4)	$2{\cdot}1 \times 10^{-6}$ (1·3)
NKA (M)	$2{\cdot}3 \times 10^{-7}$ (1·4)	$1{\cdot}3 \times 10^{-7}$ (1·9)
EFS (V)	21·1 ± 1·1	19·7 ± 1·1

Sensitivity to acetylcholine (ACh), substance P (SP), and neurokinin A (NKA) is expressed as the concentration eliciting 50% of the maximum response (geometric mean and geometric SEM). Sensitivity to electrical field stimulation (EFS) is expressed as the voltage eliciting 50% of the maximum response (mean ± SEM).

Discussion

To determine whether the effects of NO_2 on airway responsiveness involve alterations of smooth muscle contractility, the contractile response in guinea pig main bronchi exposed *in vitro* to 2·5 ppm of NO_2 was investigated. No significant differences were found between air- and NO_2-exposed tissue, either in terms of smooth muscle maximal response or in terms of sensitivity to ACh, EFS, SP and NKA. Phosphoramidon did not affect the response to exogeneous NKA in rings exposed to NO_2.

Airway hyper-responsiveness in humans, induced by NO_2 has been reported by several authors (8–12), but other investigators could not reproduce these results (8,9,13–16). Moreover, in some of the studies in which a reduced lung function or increased responsiveness was observed, only some of the subjects developed symptoms (10–12). It has therefore been suggested that differences in susceptibility exist in the exposed populations, and that a group of responders and a group of non-responders may be present.

The same controversy also surrounds animals exposed *in vivo* to NO_2, since development of airway

hyper-responsiveness has been shown in guinea pigs (17,18) but not in rats (19,20).

Exposure to ozone (O_3), which has potent oxidant properties and many effects on the respiratory system, as does NO_2 (22–26), consistently induces *in vivo* hyper-responsiveness in several animal species (27,28). These differences may be due to dissimilarity of the mechanism of action of the two oxidants. Indeed some observations, which we quote as examples, may explain both the different effects of O_3 and NO_2 and the existence of non-responder species or subjects. Firstly, nitric oxide, which is a well-known relaxant of smooth muscle, has been identified as a metabolic product of NO_2 exposure (29) and could counteract an increase in responsiveness. Secondly, quantitative differences in lung lipid peroxidation and anti-oxidative protective system activity after exposure to NO_2 have been observed between rats and guinea pigs (24), suggesting that a different effect between species or subjects could be linked to a different biochemical response. Finally, it has been demonstrated that tolerance may develop after repeated exposure to oxidant agents (30), so one could speculate that a different level or mechanism of adaptation to NO_2 or O_3 may lead to differences in development of hyper-responsiveness.

Exposure to NO_2 has also been shown to consistently produce an airway inflammatory response (3,4,6–9). The link between inflammation and bronchial hyper-responsiveness is still a controversial topic, but inflammatory mediators may constitute a trigger or a potentiating factor of smooth muscle hyper-responsiveness. Therefore, inflammatory infiltrate provoked by NO_2 may be the primary event which leads to development of airway hyper-responsiveness, through release of mediators. The relationship between inflammation and hyper-responsiveness has been widely studied in animals exposed to O_3. The experiments with O_3 suggest that these two phenomena are independent and that non-inflammatory cells are the most likely candidates for either the production or release of mediators which determine the increase in airway responsiveness (31). Our study *in vitro* may be the first suggestion that NO_2 does not directly cause an increase in bronchial smooth muscle responsiveness. However, the approach employed does not allow for obtaining information about events linked to inflammation or delayed effects, both of which may occur *in vivo* due to the interrelationship between different phenomena, possibly sequences of events. Therefore, extension of the conclusions relative to O_3 to NO_2 needs to be confirmed by studies with exposure to NO_2 *in vivo*.

This study showed that bronchial smooth muscle exposed to 2·5 ppm of NO_2 for 2 or 4 h does not develop hyper-responsiveness to tachykinins. Since oxidants have been shown to affect neutral endopeptidase activity (32), concentration–response curves were performed for NKA in the presence of phosphoramidon, which failed to potentiate the response to exogenous NKA in rings exposed to NO_2. Therefore, we conclude that neutral endopeptidase may be inhibited by NO_2, but that if this is so, some other counteracting factor abolishes the consequent expected increase in the response to tachykinins.

Airway smooth muscle responsiveness may also be influenced by factors such as airway mechanical determinants (33), previous infections (34) or different airway sites (35). In our experiments, using bronchial rings with intact epithelium, one factor may be particularly relevant to the results obtained — the influence which the epithelium exerts on smooth muscle contractility. NO_2 has been shown to affect epithelial permeability and to damage target cells (3,6,7). It may then cause alteration, maybe potentiation, of the ability of the epithelium to release nitric oxide. Finally, synergistic potentiation between NO_2 and other pollutants has been shown, which could be relevant to development of hyper-responsiveness (24,25). Therefore, it remains possible, as a consequence of exposure to NO_2, that an altered smooth muscle responsiveness to contractile agents may develop in conditions or species different from the ones used. For these reasons, we believe that further studies need to be performed. However, we consider our findings to be the first step towards clarification of mechanisms underlying the conflicting reports on NO_2-induced hyper-responsiveness in man and animals, as it suggests that NO_2 does not act by directly altering airway smooth muscle contractility.

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