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In vivo exposure to nitrogen dioxide (NO_2) induces a decrease in calcitonin gene-related peptide (CGRP) and tachykinin immunoreactivity in guinea-pig peripheral airways

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In vivo *exposure to nitrogen dioxide (NO_2) induces a decrease in calcitonin gene-related peptide (CGRP) and tachykinin immunoreactivity in guinea-pig peripheral airways. R.E. Lucchini, D.R. Springall, P. Chitano, L.M. Fabbri, J.M. Polak, C.E. Mapp. ©ERS Journals Ltd 1996.*

ABSTRACT: The mammalian respiratory tract is densely innervated by sensory and autonomic fibres. Subsets of the nerves contain bioactive regulatory peptides, such as substance P, calcitonin gene-related peptide (CGRP), and neurokinins. The sensory nervous system responds to inhaled irritants, resulting in a release of neuropeptides and, thus, a decrease in the peptide immunoreactivity of the fibres.

We examined the effects of inhaled nitrogen dioxide (NO_2), a well-known indoor and outdoor air pollutant, on pulmonary sensory neuropeptides. Guinea-pigs were exposed for 4 h to 18 parts per million (ppm) NO_2 or to air (n=5 each). At the end of the exposure, they were killed with urethane and their lungs were fixed in 1% paraformaldehyde in phosphate-buffered saline. Cryostat sections were stained with antisera to an anatomical nerve marker, protein gene product (PGP) 9.5, and to CGRP and tachykinins, utilizing the avidin-biotinylated peroxidase method.

In the noncartilaginous airways (diameter <250 μm) of NO_2-exposed animals, less tachykinin- and CGRP-immunoreactive nerve fibres were found compared with controls. No change was seen in the total nerve fibre distribution (PGP 9.5).

It is concluded that the peptidergic nerves of guinea-pig peripheral airways are a sensitive indicator of exposure to nitrogen dioxide.

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Air pollution has been shown to exert adverse respiratory health effects, *e.g.* to alter respiratory function, to induce inflammatory response, and to facilitate infection of the lower respiratory tract [1, 2]. Several studies have shown that NO_2 and/or O_3, two major air pollutants, affect pulmonary function and alter lung morphology [3–5]. Pulmonary function changes include airflow limitation, altered breathing pattern, air trapping and airway hyperresponsiveness [3–7]. Morphological changes include damage to Clara, ciliated, and type I epithelial cells in the bronchioloalveolar region [8, 9]. The mechanisms of action of these pollutants not well-known, but might be similar, as both gases are powerful oxidizing agents [10, 11].

The mammalian respiratory tract is densely innervated by sensory and autonomic fibres [12]. Subsets of these nerves contain bioactive regulatory peptides that can act locally as hormones or possibly as neurotransmitters [13]. Of the neuropeptides in the respiratory tract, those predominant in the sensory nervous system are calcitonin gene-related peptide (CGRP) and the tachykinins, such as substance P (SP), neurokinin A (NKA) and neurokinin B (NKB). Overall innervation can be visualized by immunocytochemistry with antiserum to an anatomical nerve marker called protein gene product (PGP) 9.5 [14], which stains all nerve fibres in tissues including the lung [15], whilst antisera to specific neuropeptides can stain sensory nerve fibres.

The sensory nervous system is known to respond to inhaled irritants with the release of neuropeptides and, therefore, with a decrease in the peptide immunoreactivity of the fibres [16–18]. Ozone has been shown to exhibit sensory irritating effects in humans [19, 20], in dogs [21, 22], and in guinea-pigs [23]. NO_2 is known as a "deep" lung irritant, since it mostly affects the distal small airways [24, 25], but there is no evidence of its effect on sensory nerves.

Therefore, by means of quantitative immunohistochemical techniques, the effect of short-term exposure to NO_2 was examined in guinea-pig peripheral airways using antisera to CGRP and to SP (which cross-reacts with other tachykinins). The aim was to establish whether the mechanism of NO_2 action in the lung could be partly mediated by sensory nerves by causing changes in the innervation of the respiratory tract.

Material and methods

Animal exposure and lung fixation

The experimental procedure and specific protocols were approved by the committee on animal care of the University of Padova. Male Dunkin Hartley guinea-pigs (350–400 g body weight; Morini, S. Polo D'Enza, Italy)

were anaesthetized with urethane (1.5 g·kg^{-1} *i.p.*). The trachea was cannulated with a Y-shaped tube: one branch was connected to a cylinder which delivered 150–200 mL·min^{-1} of gas (standard air or 18 parts per million (ppm) NO_2 stabilized in standard air); and the other branch was connected to a "water valve" to prevent air inlet into this branch during the inspiration. With this exposure apparatus, gas was inhaled during spontaneous inspiration and exhaled, bubbling into water just under the surface, during spontaneous expiration. The exposure was carried out for 4 h.

Five animals were exposed to NO_2, and five control animals were exposed to air alone. Supplemental anaesthesia was administered (0.4 g·kg^{-1} *i.p.*) every 90 min. At the end of the exposure, the animals were killed with an additional urethane injection. Lungs were inflated with 5 mL of 1% paraformaldehyde in phosphate-buffered saline (PBS) (0.01 M phosphate buffer in 0.15 M NaCl; pH 7.4). The thorax was then opened, the respiratory tract dissected out and immersed in the same fixative. After 6 h, the trachea and the lungs were transferred to PBS containing 0.45 M sucrose and 0.01% sodium azide.

Immunohistochemistry

For each animal, five cryostat lung blocks were prepared by immersion in melting dichlorodifluoromethane cooled with liquid nitrogen. Frozen sections (10 μm thick) were taken up on poly-L-lysine-coated microscope slides and stained with rabbit antisera, utilizing the avidin-biotinylated complex (ABC) peroxidase method. Antiserum to PGP 9.5 and antisera raised to the neuropeptides CGRP and SP were used. The antiserum to SP was used as a marker of nerve fibres containing tachykinins, since it cross-reacts with other tachykinins. One section per block was stained for each antigen. In brief, endogenous peroxidase activity was blocked by immersing slides in 0.03% hydrogen peroxide in methanol for 1 h. After washing in PBS (3×10 min), nonspecific binding was blocked by incubating in 3% normal goat serum in PBS containing 0.05% bovine serum albumin (BSA) and 0.1% sodium azide (BSA solution) for 30 min. The sections were incubated overnight at 4°C with primary antisera in BSA solution at predetermined optimal dilutions (PGP 9.5, 1:10,000; CGRP, 1:4,000; SP, 1:4,000).

Negative controls were performed by omission of the primary antibody. Moreover, preabsorption with a specific antigen was performed for antisera to neuropeptides. Antiserum to CGRP was preabsorbed with a specific peptide, and antiserum to tachykinins was preabsorbed with antigens SP and NKA. Partial inhibition of immunostaining was obtained using NKA and negative staining using either SP or both together (1 nmol·mL^{-1} antigen in diluted antiserum). After washing in PBS (3×10 min), the sections were incubated successively for 30 min with biotinylated goat anti-rabbit immunoglobulin G (IgG) (1:100) and ABC reagent for 60 min. The sections were rinsed first in PBS (3×10 min), then in acetate buffer (0.1 M, pH 6.0). The peroxidase activity was revealed using the nickel enhancement method [26]. Sections were dehydrated, mounted in Pertex and examined with a light microscope (Olympus BH-2).

Quantitative and statistical analysis

Quantitative analysis of immunoreactive nerve fibres was performed in noncartilaginous airways with diameter less than 250 μm, using the method described by COWEN [27]. Images of appropriate airways were captured by an operator who was unaware of the identity of the specimens, and were digitized by a Seescan Symphony image analysis system (Seescan, Cambridge, UK) *via* a video camera (Sony XC 77CE) attached to a microscope (model BH-2; Olympus, London, UK). Digital images were 512×512 pixels (picture points), each pixel represented by a grey value from 0 to 255 equivalent to the intensity of the light in the corresponding area of the microscopic image. The area of the airway wall (A_W) was measured in the image by interactive delineation. The boundaries comprised by the luminal (surface of the epithelium) and the adventitial surfaces were traced using the computer mouse. The number of pixels contained within the boundaries was converted to the tissue area using a factor determined by calibrating the image analyser with a series of area standards viewed with the same microscope and objective used for the study.

The area of immunostained nerves (A_N) within the airway wall boundaries was determined by the same method but using a different technique for delineation: interactive thresholding. For this, the image is segmented to separate nerves from tissue that is not nerves by selecting a threshold grey value that is intermediate between the intensities of each. The image analyser highlights in an artificial colour the pixels having a grey value smaller than that of the threshold, altered until all the nerves are selected and their area is then calculated.

The results were expressed as the area density of nerves, *i.e.* A_N/A_W. For each peptide, 25 airways from each animal were measured (five airways for each section). Each reported value is mean±SEM. Wall area data in exposed and control animals were normally distributed and were compared with the Student's t-test for unpaired data, while nerve density data were compared with the Mann-Whitney U-test as the data were not normally distributed.

Materials

Urethane was obtained from Riedel de Haen SpA (Hanover, Germany); cylinders containing standard air or 18 ppm NO_2 stabilized in standard air from SIAD (Camin, Italy) dichlorodifluoromethane (Arcton) from ICI (Cheshire, UK); antibody PGP 9.5 from Ultraclone (Isle of Wight, UK); antisera to neuropeptides were raised in rabbits at the Royal Postgraduate Medical School, Hammersmith Hospital (London, UK); ABC reagents purchased as a Vectastain/Elite kit, and normal goat serum were obtained from Vector Laboratories (Peterborough, UK); bovine serum albumin (BSA), sodium azide, 3,3'-diaminobenzidine tetrahydrochloride dihydrate, and glucose oxidase from Sigma Chemical Co. (Poole, UK).

Results

Airway wall

The bronchiolar wall area was similar in NO_2-exposed animals compared to controls, with means (±SEM) of 0.0194 (±0.0006) and 0.0186 (±0.0010), respectively.

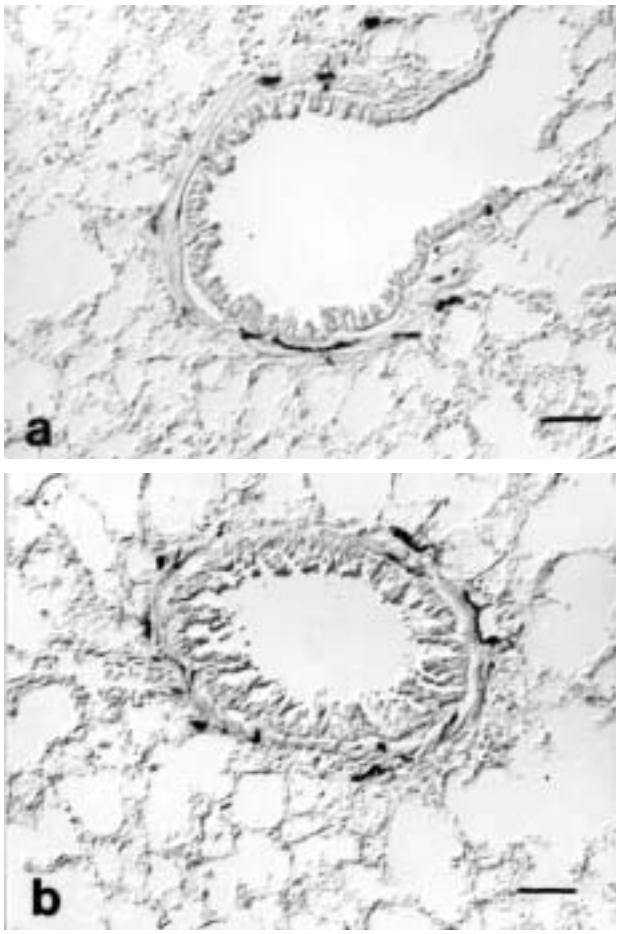

Fig. 1. – Immunostaining of airways with diameter <250 μm for protein gene product 9.5 (PGP 9.5): a) in air-exposed; and b) in nitrogen dioxide (NO_2)-exposed guinea-pigs. (Internal scale bar=50 μm).

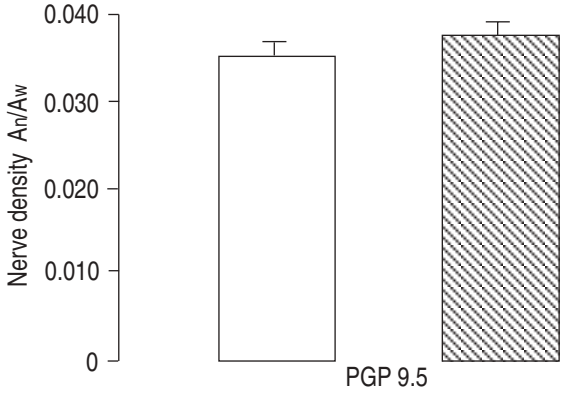

Fig. 2. – Protein gene product 9.5 (PGP 9.5) nerve density in airways with diameter <250 μm in air-exposed (▭) and in nitrogen dioxide (NO_2)-exposed guinea-pigs (▨). Values are presented as mean±SEM. A_n: area of immunostained nerves; A_w: area of the airway wall.

PGP 9.5

Immunoreactive nerve fibres were abundant and they were seen in the epithelium, smooth muscle of airways and around blood vessels in the lung. Scattered fibres were also seen in the lung parenchyma. The quantitative analysis, performed in airways with diameter less than 250 μm, showed no changes in the innervation after NO_2 exposure (figs. 1 and 2).

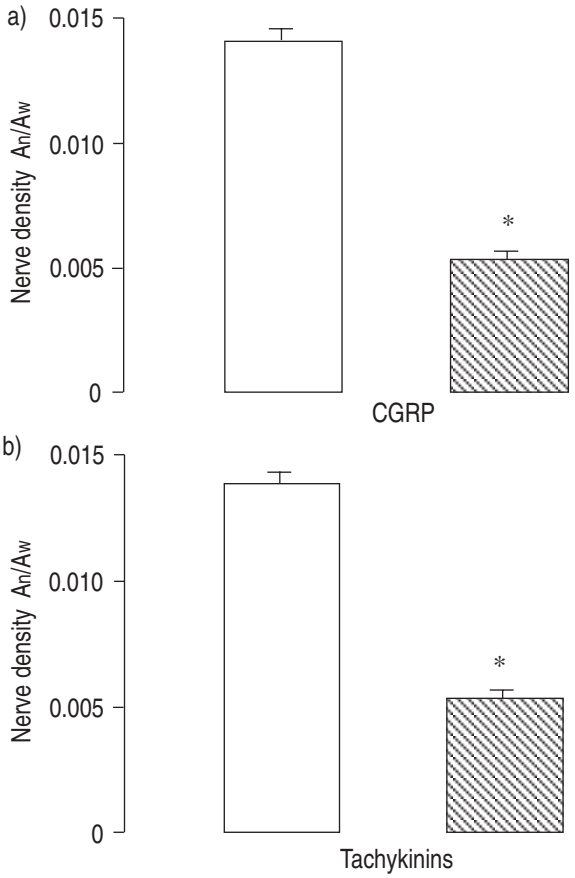

Fig. 3. – Nerve density in airways with diameter <250 μm in air-exposed (▭) and in nitrogen dioxide (NO_2)-exposed guinea-pigs (▨). a) CGRP nerve density. b) tachykinin nerve density. Values are presented as mean±SEM. *: $p<0.05$; CGRP: calcitonin gene-related peptide; A_n: area of immunostained nerves; A_w: area of the airway wall.

Tachykinins and CGRP

The distribution of nerves immunoreactive for these neuropeptides and their response to NO_2 were similar. Immunoreactive fibres were seen in the epithelium and smooth muscle of intrapulmonary airways and around blood vessels in the lung. These fibres represented just a small part of the innervation stained with PGP 9.5. The number of CGRP and tachykinin immunoreactive fibres in airways with diameter less than 250 μm was significantly lower ($p<0.05$) in NO_2-exposed animals compared to controls (fig. 3).

Discussion

This study has shown that short-term exposure to NO_2 results in a decrease in the number of nerves immunoreactive for tachykinins and CGRP in guinea pig-airways, suggesting an effect of NO_2 on sensory nerves. The decrease of the staining of tachykinins and CGRP could be due to sensory neuropeptide release or to nerve damage. Since the overall innervation in the lung tissue was similar in control and NO_2-exposed animals, as suggested by the unchanged immunoreactivity for PGP 9.5, and since fixation of tissue was performed shortly after the

end of the brief (4 h) exposure to the pollutant, it is conceivable that the change in neuropeptides was not due to damage to the nerves. PGP 9.5, a general neural antigen, rapidly disappears following a nerve lesion [14]. Such an interpretation is supported by the finding that capsaicin administration causes a release of sensory neuropeptides, as shown by their increased concentration in the fluid outflow from isolated lung preparations and by a concomitant decrease in neural peptide immunostaining in tissues analogous to those shown here [28].

Similar experiments have shown changes in the rate of neuropeptide release with decreased nerve staining in the lung tissue following administration of the sensory irritant sulphur dioxide [29]. Release of tachykinins is also implied by the SP-mediated vasodilation and plasma extravasation caused by the gas phase of cigarette smoke, which itself has an appreciable concentration of NO_2 [30].

The mechanism through which capsaicin produces neuropeptide depletion in the sensory neurons is not completely understood. It has been shown that capsaicin in μM concentrations releases substance P-like immunoreactivity (SP-LI) from the rat urinary bladder without any detectable change in the SP-LI tissue content; and that only when the bladders were incubated with capsaicin for 3 h, was a marked decrease in SP-LI observed [31]. The authors concluded that the early phase of tissue depletion of sensory neuropeptides following systemic capsaicin desensitization may involve a peripheral site of action. However, 3 h is a prolonged exposure for capsaicin, whereas for pollutants such as nitrogen dioxide, 3–4 h is considered a short-term exposure.

Both tachykinins and CGRP appear to be involved in the neural response to NO_2. This finding is not surprising since SP, other tachykinins and CGRP are known to be co-localized in some sensory nerves [32, 33].

The concentrations of NO_2 used in this study were relatively high, compared with the time-weighted average (TWA) value of 3 ppm and the short-term exposure level (STEL) value of 5 ppm accepted in several countries [34]. However, short-term exposure to NO_2 was used in this study, and concentrations of 18 ppm can easily be reached in the workplace in certain conditions. In oxyacetylene torch shrinking operations and in the ship-building industry, NO_2 can reach concentrations of 196–480 ppm in unventilated compartments and concentrations of 4–89 ppm in ventilated compartments [35]. It would be interesting to determine whether longer exposure at lower concentrations of NO_2 would have similar effects on sensory nerves. NO_2, as a relatively insoluble gas, is not absorbed to a great degree in the upper airways and a large proportion of inhaled NO_2 descends deep into the lungs [36]. It is likely that with the system of exposure used in the present study, NO_2 could reach peripheral airways at concentrations close to the initial value.

The rate of absorption of inhaled NO_2 from pulmonary air spaces is limited by the reaction with the first compartment that is contacted, *i.e.* the epithelial lining fluid, rather than by its solubility. This observation suggests that the univalent reduction of absorbed NO_2 to NO_2^- is quantitative and occurs near the air space surface [37]. Interaction of inspired NO_2 with water in the vapour or liquid phase forms nitrous acid or nitrite, and secondarily nitrate [38]. It is possible that the action of nitrous acid or its ion may be opposed by the neutralizing capacity of the mucous layer in the central airways, but this mechanism may be insufficient to protect small airways and alveoli [39]. Since absorbed NO_2 does not penetrate through the lung without reacting, the possibility that the effect on sensory nerves described in this study may be due to a product of reaction of NO_2, and not to NO_2 itself, cannot be excluded.

In conclusion, in the guinea-pig, the target tissue of nitrogen dioxide pollution seems to be that of the peripheral airways. At this level, nitrogen dioxide, or some chemical intermediates which form in the lung after a short-term exposure to this pollutant, cause a reduction in calcitonin gene-related peptide and tachykinin immunoreactivity. The reduced immunoreactivity could be due to a release of sensory neuropeptides that may play a role in the pulmonary response to nitrogen dioxide.

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