British Journal of Pharmacology (1996) 119, 1109–1116

Deficiency of nitric oxide in allergen-induced airway hyperreactivity to contractile agonists after the early asthmatic reaction: an *ex vivo* study

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1 Using a guinea-pig model of allergic asthma, we investigated the role of nitric oxide (NO) in allergen-induced airway hyperreactivity after the early asthmatic reaction, by examining the effects of the NO-synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) on the responsiveness to methacholine and histamine of isolated perfused tracheae from unchallenged (control) animals and from animals 6 h after ovalbumin challenge.

2 All animals developed airway hyperreactivity to inhaled histamine at 6 h after ovalbumin challenge, with a mean 3.11 ± 0.45 fold increase in sensitivity to the agonist ($P < 0.001$).

3 In perfused tracheal preparations from the ovalbumin-challenged guinea-pigs, the maximal responses (E_{max}) to methacholine and histamine were significantly enhanced compared to controls, both after intraluminal (IL) and extraluminal (EL) administration of the contractile agonists. In addition, a small but significant increase in the pD_2 ($-\log_{10} EC_{50}$) for IL and EL methacholine and for IL histamine was observed. As a consequence, the ΔpD_2 (EL − IL) for histamine was slightly decreased from 1.67 ± 0.13 to 1.23 ± 0.14 ($P < 0.05$). However, the ΔpD_2 for methacholine was unchanged (1.85 ± 0.11 and 1.77 ± 0.12, respectively; NS).

4 Incubation of control tracheae with 100 μM L-NAME (IL) significantly enhanced the E_{max} for both IL and EL methacholine and histamine to approximately the same degree as observed after ovalbumin challenge, with no effect on the pD_2 and ΔpD_2 for both agonists. On the contrary, L-NAME had no effect on E_{max} and pD_2 values of tracheal preparations from ovalbumin-challenged guinea-pigs.

5 L-NAME (10 μM – 1 mM) had no effect on methacholine-induced contraction of isolated tracheal strip preparations obtained from control animals, indicating that L-NAME has no antimuscarinic effect on tracheal smooth muscle.

6 Histological examination of the intact tracheal preparations indicated epithelial and subepithelial infiltration of eosinophils after ovalbumin challenge. However, no apparent damage of the airway epithelium was observed in these preparations.

7 The results indicate that a deficiency of NO contributes to allergen-induced airway hyperreactivity after the early asthmatic reaction and that this deficiency appears not to be due to epithelial shedding.

Keywords: Nitric oxide; airway hyperreactivity; allergic guinea-pigs; tracheal perfusion; histamine; methacholine; N^{ω}-nitro-L-arginine methyl ester

Introduction

Allergic asthma is characterized by allergen-induced early (EAR) and late (LAR) asthmatic reactions (Booij-Noord *et al.*, 1971), influx and activation of pro-inflammatory cells, such as eosinophils, neutrophils and lymphocytes (De Monchy *et al.*, 1985; Metzger *et al.*, 1986; Djukanovic *et al.*, 1990) and a concomitant increase in non-specific airway reactivity, which is present both after the EAR and the LAR (Cockcroft *et al.*, 1977; Cartier *et al.*, 1982; Durham *et al.*, 1988; Aalbers *et al.*, 1991). Although the mechanisms underlying allergen-induced airway hyperreactivity are largely unknown, inflammatory processes are considered to play a prominent role (Djukanovic *et al.*, 1990).

One possible mechanism that may be important is epithelial damage by strongly negatively charged proteins, such as major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil peroxidase (EPO), that are derived from activated eosinophils (Gleich *et al.*, 1988). Epithelial damage could contribute to airway hyperreactivity by disrupting the diffusion barrier for inhaled stimuli to nerve endings, mast cells and airway smooth muscle, by causing reduced enzymatic degradation of inhaled or endogenous mediators and neurotransmitters, and by interfering with the production of epithelium-derived relaxing factor(s) (EpDRF) (reviewed by Bertrand & Tschirhart, 1993).

Although the chemical identity of EpDRF has not been fully elucidated, recent investigations have indicated that it may include nitric oxide (NO). Thus, immunohistochemical staining as well as polymerase chain reaction studies have indicated that all three presently known isotypes of NO synthase (NOS), classified as brain NOS (bNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), may be present in the airway epithelium, depending on the cell type within the epithelium and the inflammatory status of the airways: bNOS and eNOS being constitutively expressed and activated Ca^{2+}-dependently by agonists and the Ca^{2+}-independent iNOS being induced by pro-inflammatory cytokine stimulation (Fischer *et al.*, 1992; Kobzik *et al.*, 1993; Hamid *et al.*, 1993; Shaul *et al.*, 1994; Asano *et al.*, 1994).

Functional evidence for epithelium-derived NO was recently presented by Nijkamp *et al.* (1993), who demonstrated that luminal perfusion of guinea-pig tracheal tube preparations with the non-selective NOS inhibitors, N^{ω}-nitro-L-arginine methyl ester (L-NAME) or N^{G}-monomethyl-L-arginine (L-

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NMMA) caused an increased contractile response to intraluminally applied histamine, carbachol and methacholine, which effect was absent after denudation of the airways. In addition, in guinea-pigs it was also demonstrated that inhalation of L-NAME or L-NMMA potentiated histamine-induced bronchoconstriction (Nijkamp *et al.*, 1993), while inhalation of gaseous NO or aerosolized S-nitroso-thiols profoundly inhibited methacholine-induced bronchoconstriction in these animals (Dupuy *et al.*, 1992).

In the present study, we hypothesized that a deficiency of NO, possibly caused by epithelial damage due to inflammation, may contribute to allergen-induced airway hyperreactivity to histamine and methacholine. Using a recently developed guinea-pig model of allergic asthma, characterized by ovalbumin-induced dual asthmatic reactions, airway inflammation and airway hyperreactivity (Santing *et al.*, 1992; 1994a, b, c; Ten Berge *et al.*, 1995), this hypothesis was tested by examining the effect of NOS inhibition on the responsiveness to histamine and methacholine of isolated perfused tracheae obtained from unchallenged control animals and from animals 6 h after ovalbumin challenge (i.e. after the EAR), with enhanced airway reactivity to inhaled histamine. The *in vitro* airway perfusion model, using differential pressure measurements during constant flow perfusion, was used as an excellent model to investigate the role of epithelium in regulating airway tone under physiological and pathophysiological conditions, by allowing separate intraluminal (IL) and extraluminal (EL) administration of stimuli (Munakata *et al.*, 1988; 1989; Fedan *et al.*, 1990; Masaki *et al.*, 1994).

Methods

Animals

Outbred specific pathogen-free guinea-pigs (Charles River SAVO, Kiszlegg, Germany), weighing 500–700 g, were used in this study. All animals were actively IgE-sensitized to ovalbumin at three weeks of age as described by Van Amsterdam *et al.* (1989). In brief, 0.5 ml of an allergen solution containing 100 $\mu g\ ml^{-1}$ ovalbumin and 100 $mg\ ml^{-1}$ $Al(OH)_3$ in saline was injected intraperitoneally, while another 0.5 ml was divided over seven intracutaneous injection sites in the proximity of lymph nodes in the paws, lumbar regions and the neck.

For pleural pressure (P_{pl}) measurement the animals were operated on in week 3 following sensitization and used experimentally in weeks 4 to 8. After the operation the animals were housed in individual cages, in climate controlled animal quarters and given water and food *ad libitum*.

All protocols described in this study were approved by the University of Groningen Animal Health Committee.

Measurement of airway function

Airway function was assessed by on-line measurement of P_{pl} under unrestrained conditions as described by Santing *et al.* (1992). Briefly, a small latex balloon (HSE, Freiburg, Germany), connected to a saline-filled cannula, was surgically implanted inside the thoracic cavity. The cannula was driven subcutaneously to and permanently attached in the neck of the animal. After connection via another saline-filled cannula to a pressure transducer (TC-XX, Viggo-Spectramed B.V., Bilthoven, The Netherlands), P_{pl} was measured in centimetres of H_2O, using an on-line computer system. No postoperative inflammation was observed for at least 5 weeks after operation, and baseline P_{pl} values remained stable during repeated measurements on different days (Santing *et al.*, 1992).

Using a combination of flow measurement with a pneumotachograph, implanted in the trachea, and pressure measurement with the pleural balloon, it was shown that changes in P_{pl} are linearly related to changes in airway resistance and hence can be used as a sensitive index for histamine- and allergen-induced bronchoconstriction (Santing *et al.*, 1992).

Provocation techniques

Ovalbumin and histamine provocations were performed by inhalation of aerosolized solutions. The provocations were performed in a specially designed animal cage, in which the guinea-pigs could move freely (Santing *et al.*, 1992). The volume of the cage was 9 l, which ensured fast replacement of the air inside the cage with aerosol and *vice versa*. A DeVilbiss nebulizer (type 646, DeVilbiss, Somerset, PA, U.S.A.) driven by an airflow of 8 l min^{-1} provided the aerosol required, with an output of 0.33 ml min^{-1}.

Histamine provocations were performed starting with a concentration of 25 $\mu g\ ml^{-1}$ in saline, followed by increasing dosage steps of 25 $\mu g\ ml^{-1}$. The provocations by each concentration lasted 3 min and provocations were separated by 7 min intervals. The animals were challenged until the P_{pl} increased by more than 100% for at least 3 consecutive min during the 10 min period. The provocation concentration causing a 100% increase in P_{pl} (PC_{100}) was derived by linear interpolation.

Allergen provocations were performed by inhalation of increasing aerosol concentrations of 1.0, 3.0, 5.0 and 7.0 $mg\ ml^{-1}$ ovalbumin in saline for 3 min, separated by 7 min intervals. Allergen inhalations were discontinued when an increase in P_{pl} of more than 100% was observed. Under these conditions, no antihistamine was needed to prevent the development of anaphylactic shock. All histamine and ovalbumin provocations were preceded by a period of at least 30 min for adaptation of the animals to the cage, followed by two consecutive inhalations with saline solution, lasting 3 min each and separated by a 7 min interval.

Provocation protocol

On two subsequent days before the experimental protocol, the animals were habituated to the provocation conditions. On the first day, the animals were placed inside the provocation cage unconnected to the transducers. Three consecutive challenges with saline solution were performed lasting 3 min each and separated by 10 min intervals. The next day, this protocol was repeated with the animals connected to the measuring system. On the first day of the experimental protocol, baseline histamine PC_{100} was assessed, which was repeated on the second day. Twenty-four hours later, allergen provocation was performed. At 6 h after allergen provocation (between the early and late asthmatic response; Santing *et al.*, 1992; 1994b) the PC_{100} value for histamine was re-assessed to establish the change in airway reactivity at this time point. Between allergen provocation and the measurement of histamine PC_{100} at 6 h, the animals were removed from the provocation cage and placed in their larger home-cage of 2500 cm^2, where they could eat and drink *ad libitum*.

Tracheal perfusion

After the histamine PC_{100} determination at 6 h after ovalbumin challenge, the guinea-pigs were killed. Non-challenged IgE-sensitized animals were used as controls. The animals were killed by a sharp blow on the head and exsanguinated. The tracheae were rapidly removed and placed in Krebs-Henseleit (KH) solution (37°C) of the following composition (mM): NaCl 117.50, KCl 5.60, $MgSO_4$ 1.18, $CaCl_2$ 2.50, NaH_2PO_4 1.28, $NaHCO_3$ 25.00, D-glucose 5.50; gassed with 5% CO_2 and 95% O_2; pH 7.4.

The tracheae were prepared free of serosal connective tissue and cut into two halves of approx. 17 mm before mounting in a perfusion setup, essentially as described by Munakata *et al.* (1988, 1989). The tracheal preparations were attached at each side to stainless steel perfusion tubes fixed in a Delrin perfusion holder. The holder with the trachea was then placed in a water-jacketed organ bath (37°C) containing 20 ml of gassed KH (the serosal or extraluminal (EL) compartment). The lumen was perfused with recirculating KH from a separate 20 ml bath

(mucosal or intraluminal (IL) compartment) at a constant flow rate of 17 ml min^{-1}. Two axially centred side-hole catheters connected with pressure transducers (TC-XX, Viggo-Spectramed B.V., Bilthoven, The Netherlands) were situated at the distal and proximal ends of the trachealis to measure hydrostatic pressures (P_{outlet} and P_{inlet}, respectively). The signals were fed into a differential amplifier to obtain the difference between the two pressures ($\Delta P = P_{inlet} - P_{outlet}$), which was plotted on a flatbed chart recorder (BD 41, Kipp en Zonen, Delft, The Netherlands). ΔP reflects the resistance of the tracheal segment to perfusion and is a function of the mean diameter of the trachea between the pressure taps (Munakata *et al.*, 1989). The transmural pressure in the trachea was set at 0 cmH_2O. At the perfusion flow rate used, a baseline ΔP of 0.1 to 1.0 cmH_2O was measured, depending on the diameter of the preparation.

After a 45 min equilibration period with three washes with fresh KH (both IL and EL), 1 μM isoprenaline was added to the EL compartment for maximal smooth muscle relaxation to assess basal tone. After three washes during at least 30 min, the trachea was exposed to EL 40 mM KCl in KH to obtain a receptor-independent reference response. Subsequently, the preparation was washed 4 times with KH during 45 min until basal tone was reached. Two consecutive cumulative concentration-response curves (IL followed by EL), were made with histamine or methacholine. When used, L-NAME (100 μM) was applied in the IL reservoir 45 min prior to agonist addition and remained present during the rest of the experiment.

Tracheal strip contraction

Single tracheal open-ring preparations free of serosal connective tissue were mounted in water-jacketed organ baths (37°C) containing 20 ml of gassed KH solution, for isotonic recording with a preload of 300 mg. After a 60 min equilibration period, 1 μM isoprenaline was administered to establish basal tone, followed by a 30 min washout. Subsequently, strips were precontracted twice with methacholine (0.1, 1, 10 and 0.1, 1, 10, 100 μM, respectively, followed by washing periods of 60 min), before the construction of a control cumulative concentration-response curve (CCRC; 10 nM–1 mM). Following a 60 min washing period, a second CCRC was constructed in the absence or presence of various concentrations of L-NAME (0, 10, 100 and 1000 μM; 30 min preincubation).

Histology

Perfused tacheae were fixed with 4% buffered formalin and processed and embedded in paraffin by standard histopathological procedures. The tracheae were cut in two parts and embedded straight up, to allow the study of at least two sections at different levels of the tracheae. Paraffin sections were cut at 4 μm, and stained with haematoxylin-eosin to study general morphology and with Giemsa to identify inflammatory cells.

Each section was examined for epithelial integrity and inflammatory cell (eosinophil) influx.

Data analysis

To correct for differences in baseline ΔP and in ΔP changes in response to contractile stimuli due to variation in internal diameter of the preparations used, IL and EL responses of the tracheal tube preparations to histamine and methacholine were expressed as a percentage of the response induced by EL administration of 40 mM KCl. The sensitivities to these agonists were evaluated as pD_2 ($-\log_{10}$ EC_{50}) values. Methacholine-induced contraction of tracheal open ring preparations was expressed as percentage of the response to 100 μM methacholine in the second precontraction in each experiment. Changes in the *in vivo* airway reactivity to histamine induced by allergen provocation were expressed as the ratio of histamine PC_{100} values obtained 24 h before and 6 h after the allergen provocation, respectively (PC_{100} ratio pre/post allergen challenge).

The results are expressed as means ± s.e.mean. Statistical analysis was performed by Student's *t*-test for unpaired observations. Differences were considered statistically significant at $P<0.05$.

Chemicals

Histamine hydrochloride, ovalbumin (grade III), aluminium hydroxide, (-)-isoprenaline hydrochloride and N^{ω}-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and the methacholine chloride from Aldrich (Milwaukee, WI, U.S.A.).

Results

Airway hyperreactivity

All animals developed airway hyperreactivity to histamine *in vivo* at 6 h after ovalbumin challenge, i.e. after the EAR, with a mean PC_{100} ratio (pre/post allergen challenge) of 3.11 ± 0.45 ($P<0.001$).

In perfused tracheal preparations from unchallenged guinea-pigs and from the ovalbumin-challenged animals, marked differences were observed between the IL and EL responses to both methacholine and histamine (Figure 1a and b). Both the maximal response (E_{max}) and the sensitivity (pD_2) to these agonists were considerably larger upon EL administration than upon IL administration (Table 1). In the ovalbumin-challenged group of animals, the absolute ΔP response to EL KCl (40 mM) was unchanged compared to the control group (8.73 ± 1.03 cmH_2O vs. 10.78 ± 1.93 cmH_2O, respectively, NS). However, the E_{max}-values for both IL and EL methacholine (Figure 1a, Table 1) and histamine (Figure 1b, Table 1) were significantly increased. Also, a small but significant increase in sensitivity to IL and EL methacholine and IL histamine was observed. Six hours after allergen challenge, the ΔpD_2 (EL – IL) was slightly decreased from 1.67 ± 0.13 (control) to 1.23 ± 0.14 ($P<0.05$) for histamine and was unchanged for methacholine, 1.85 ± 0.11 (control) and 1.77 ± 0.12 (6 h; NS), respectively.

Incubation of control tracheae with the NOS inhibitor L-NAME (100 μM, IL) had no effect on the KCl-induced response (9.11 ± 1.00 cmH_2O, NS), nor was there an effect on the basal tone. However, L-NAME significantly enhanced the E_{max} for both IL and EL methacholine (Figure 2a, Table 1) and histamine (Figure 2b, Table 1). The increases in E_{max}-values for methacholine (IL and EL) observed in the presence of L-NAME were in the same order of magnitude as those observed at 6 h after allergen challenge (Figures 1a and 2a and Table 1). For histamine the increases in E_{max}-values (IL and EL) observed in the presence of L-NAME tended to be somewhat smaller than those observed at 6 h after allergen challenge (Figures 1b and 2b and Table 1). Inhibition of NOS activity with L-NAME had no influence on the sensitivity of the tracheal preparations to the IL and EL application of either methacholine or histamine (Table 1).

In tracheae obtained from guinea-pigs at 6 h after ovalbumin challenge, the increased responsiveness was not further potentiated in the presence of 100 μM L-NAME for both agonists (Figure 3a and b, Table 1). As in the control preparations, L-NAME had no effect on basal tone and on the tracheal sensitivity to either agonist in the 6 h preparations (Table 1).

Tracheal strip response to methacholine in vitro

Recent evidence suggested that alkyl esters of L-arginine, including L-NAME, may act as competitive muscarinic receptor antagonists (Buxton *et al.*, 1993). Therefore, we assessed the effects of 10, 100 and 1000 μM L-NAME on the methacholine-

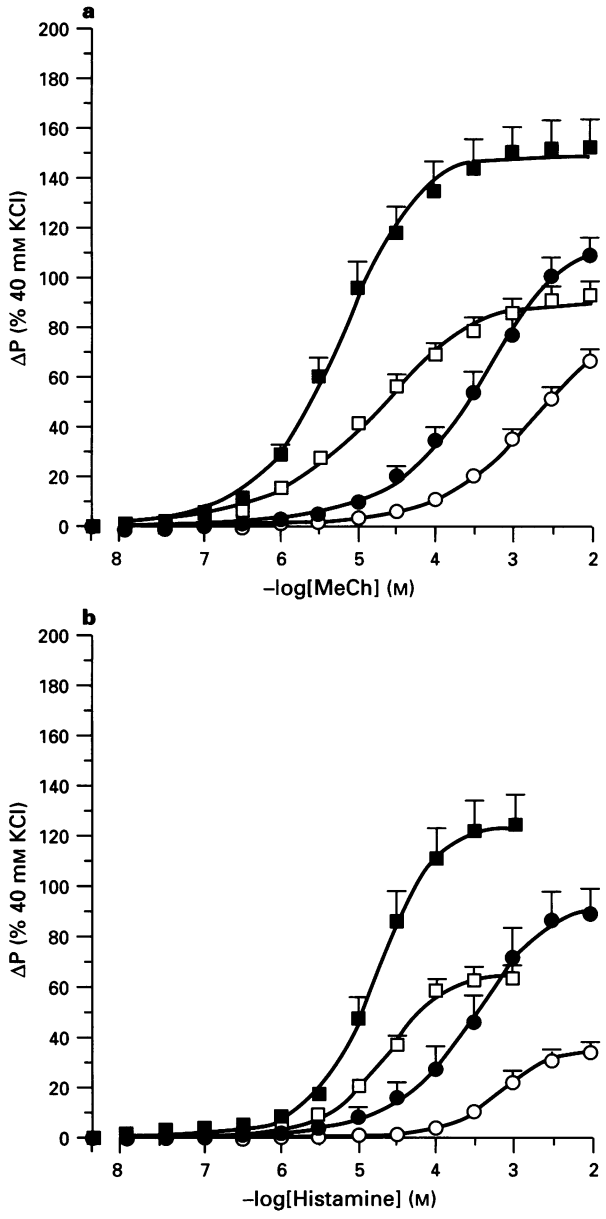

Figure 1 Methacholine (MeCh; a) and histamine (b) IL (circles) and EL (squares) cumulative concentration-response curves of tracheae obtained from ovalbumin-sensitized guinea-pigs without challenge (controls; open symbols) and at 6 h after ovalbumin challenge (solid symbols).

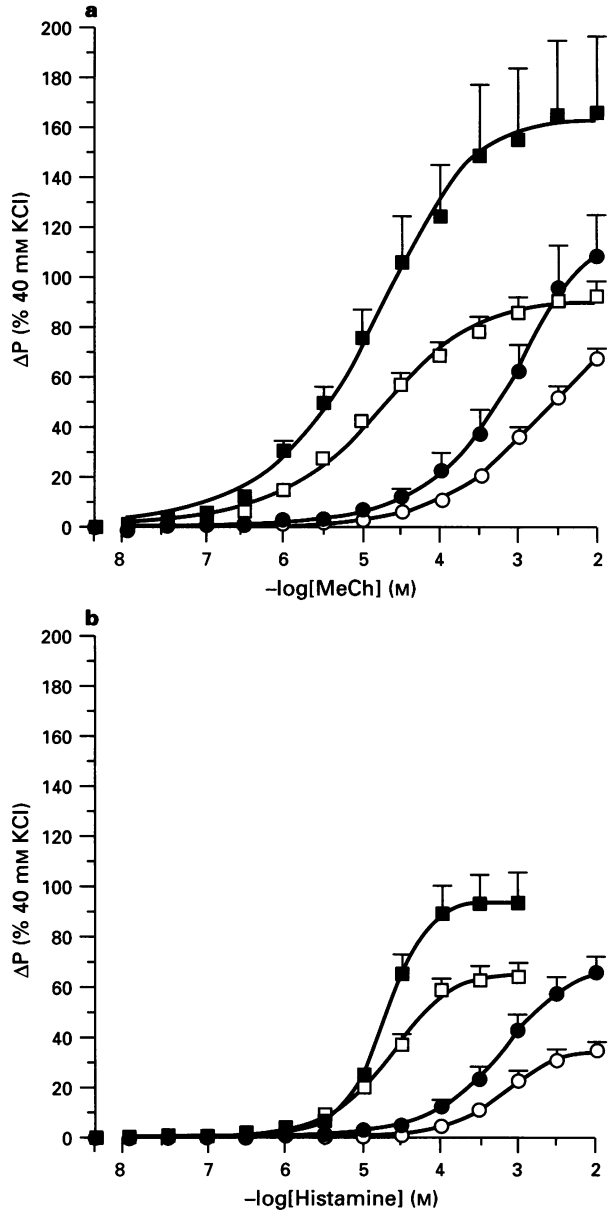

Figure 2 Methacholine (MeCh; a) and histamine (b) IL (circles) and EL (squares) cumulative concentration-response curves of tracheae obtained from unchallenged ovalbumin-sensitized guinea-pigs in the absence (open symbols) and the presence (solid symbols) of intraluminally administered L-NAME (100 μM).

Table 1 Effect of L-NAME (100 μM) on the responsiveness of intact perfused guinea-pig tracheae to intraluminal (IL) and extraluminal (EL) methacholine and histamine

		IL		EL			
Agonist	*Tracheal preparation*	pD_2 (−log M)	E_{max} (% KCl)	pD_2 (−log M)	E_{max} (% KCl)	ΔpD_2 (−log M)	n
Methacholine	Control	3.03±0.09	68.14±4.54	4.89±0.11$^{\#\#\#}$	93.39±6.29$^{\#\#}$	1.85±0.11	18
	+L-NAME	3.20±0.09	108.96±16.81**	5.03±0.21$^{\#\#\#}$	166.36±29.46$^{\#}$/**	1.83±0.22	12
	After ovalbumin	3.47±0.11*	110.58±86.61***	5.24±0.07$^{\#\#\#}$/*	152.41±11.31$^{\#\#}$/***	1.77±0.12	12
	+L-NAME	3.73±0.30†	113.30±8.52	5.00±0.37$^{\#\#}$	161.99±23.08$^{\#}$	1.63±0.29	6
Histamine	Control	3.10±0.09	35.08±4.01	4.77±0.10$^{\#\#\#}$	64.73±5.64$^{\#\#\#}$	1.67±0.13	18
	+L-NAME	3.28±0.14	67.08±5.42***	4.75±0.04$^{\#\#\#}$	94.82±11.83$^{\#}$/*	1.48±0.12	12
	After ovalbumin	3.53±0.15*	90.09±10.76***	4.76±0.07$^{\#\#\#}$	125.50±12.85$^{\#\#}$/***	1.23±0.14*	12
	+L-NAME	3.86±0.14†	95.76±18.00	4.86±0.08$^{\#\#\#}$	132.00±25.04$^{\#}$	1.01±0.11†	6

Results are means±s.e.mean of n experiments. Tracheal preparations were obtained from ovalbumin-sensitized, unchallenged guinea-pigs (Control) and from ovalbumin-sensitized guinea-pigs, 6 h after allergen challenge (After ovalbumin). Statistical analysis: $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ compared to IL application of the same agonist; $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared to Control and †$P<0.05$ compared to Control+L-NAME.

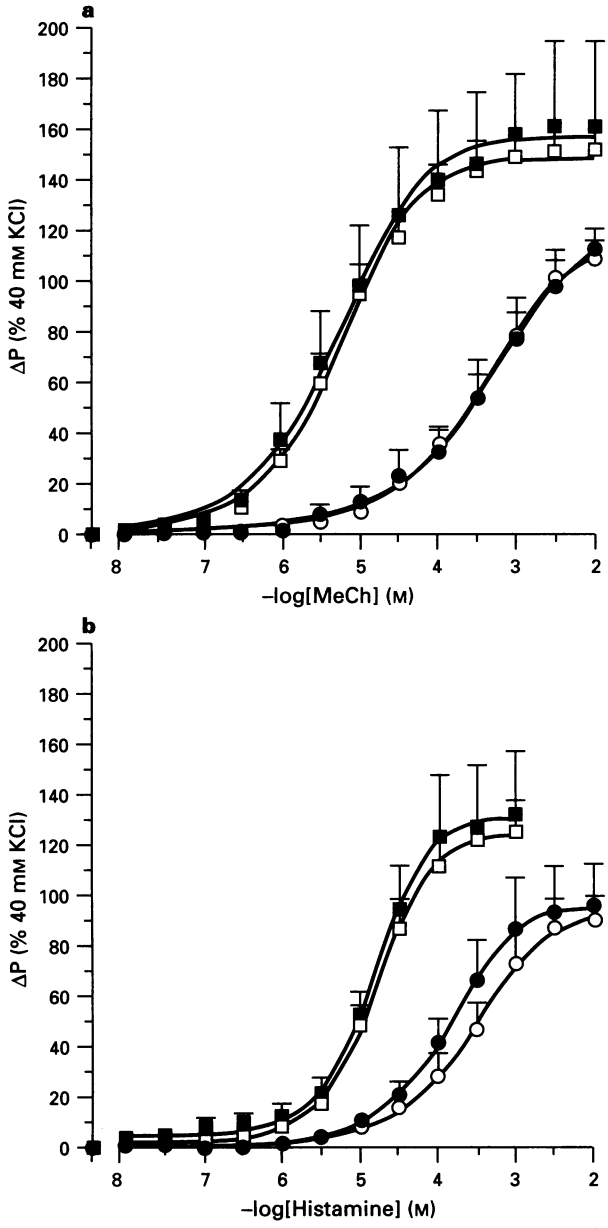

Figure 3 Methacholine (MeCh; a) and histamine (b) IL (circles) and EL (squares) cumulative concentration-response curves of tracheae obtained from ovalbuminin-sensitized guinea-pigs at 6 h after ovalbumin challenge, in the absence (open symbols) and the presence (solid symbols) of intraluminally administered L-NAME (100 μM).

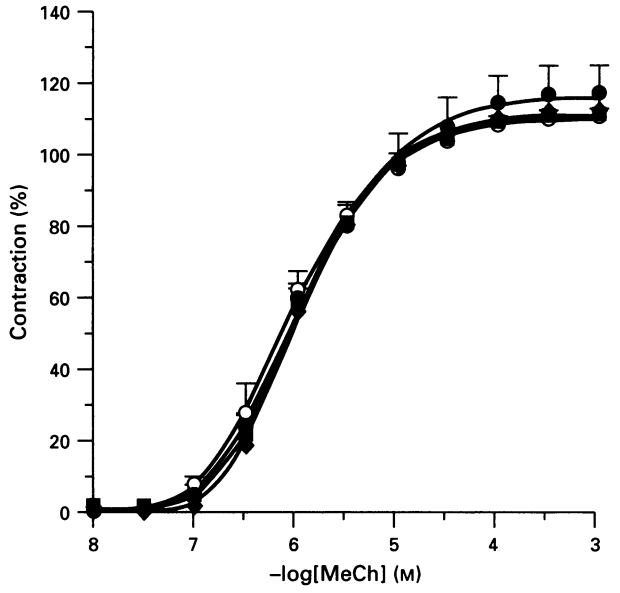

Figure 4 Methacholine (MeCh) cumulative concentration-response curves of tracheal ring preparations obtained from unchallenged ovalbumin-sensitized guinea-pigs in the absence (○) and the presence of 10 μM (●), 100 μM (■) and 1000 μM (◆) L-NAME. Contraction is expressed as percentage of the response to 100 μM methacholine in the second precontraction (see Methods).

induced contraction of strip preparations of single tracheal rings obtained from unchallenged control animals, since contraction of these preparations is mainly determined by direct action of the contractile agonist on the airway smooth muscle. At all concentrations used, L-NAME had no significant effect on either the pD_2 or E_{max} value for methacholine (Figure 4).

Histological examinations

An obvious influx of eosinophils was observed in the epithelial layer and in the submucosa of the perfused tracheae from ovalbumin-challenged guinea-pigs (Figure 5a). Morphological study of both the integrity and the epithelial coverage of the basement membrane of the tracheae showed no differences between ovalbumin-challenged and unchallenged control animals (Figure 5a and b).

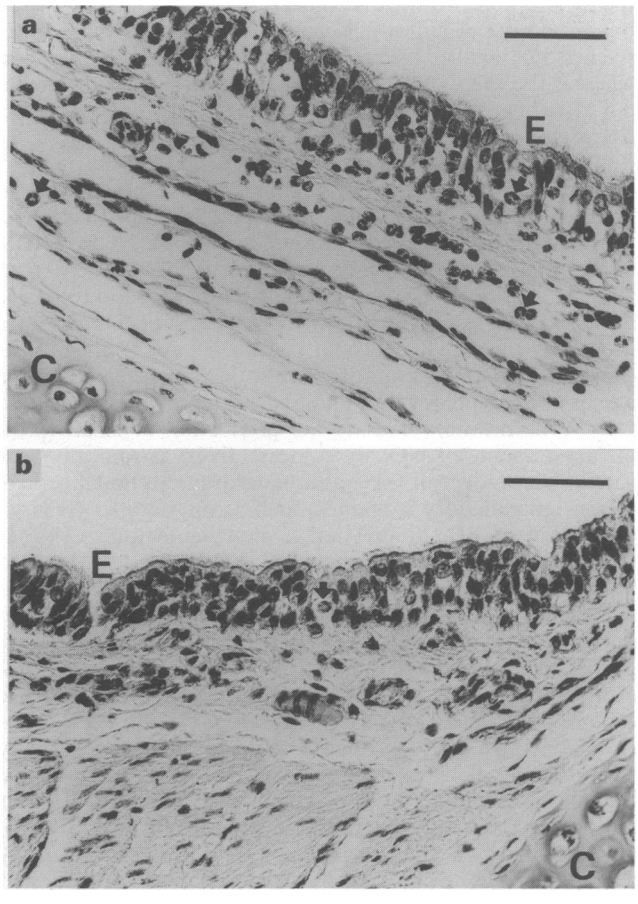

Figure 5 Representative histological details of perfused tracheal preparations from an ovalbumin-challenged (a) and unchallenged (b) guinea-pig. E = epithelium, C = cartilage. Eosinophils are indicated by arrows (haematoxylin-eosin). Calibration bar = 50 μM.

Discussion

Using perfused intact tracheal preparations from ovalbumin-challenged or unchallenged guinea-pigs, we showed that a deficiency of NO may contribute to the allergen-induced airway hyperreactivity observed after the EAR.

In contrast to various previous studies using human or animal isolated airway ring or strip preparations (for review see Meurs & Zaagsma, 1991), including a recent study using tracheal ring preparations from the same guinea-pig model as used in the present study (Santing *et al.*, 1994c), we found that allergen-induced airway hyperreactivity *in vivo* was associated with airway hyperreactivity *in vitro* when using an intact tracheal perfusion system. Since the latter system retains the structural integrity of the airway, including the epithelium, it appears that allergen-induced airway hyperreactivity to contractile agonists is mainly caused by geometric and/or epithelial changes and not by changes of airway smooth muscle function, which is the main determinant of contraction in airway strip or ring preparations.

In the present study, the *in vitro* hyperreactivity was characterized by enhanced E_{max} values for both IL and EL histamine and methacholine, which could point to a change in airway geometry due to thickening of the airway wall (Moreno *et al.*, 1986). However, this possibility seems unlikely, because the constrictive response to EL KCl was unchanged in the hyperreactive airways 6 h after ovalbumin challenge.

Since the observed airway hyperreactivity to IL and EL histamine and methacholine was closely mimicked by the administration of the non-selective NOS inhibitor, L-NAME to control preparations, while, in addition, the hyperreactive airway preparations were unresponsive to this inhibitor, it was concluded that a deficiency of contractile agonist-induced NO in the airways is a major determinant of the observed hyperreactivity. Very recently, this hypothesis was supported by the *in vivo* observation that inhalation of L-NAME by ovalbumin-sensitized guinea-pigs caused increased airway responsiveness to inhaled histamine before inhalation of allergen, whereas the effect of L-NAME had completely disappeared at 6 h after allergen challenge, when the animals are hyperreactive (Schuiling *et al.*, 1995).

The source of contractile agonist-induced NO in our perfused control preparations was not assessed. Although we used the non-selective NOS inhibitor, L-NAME, the NO was most likely produced by a constitutive isotype of NOS (cNOS: bNOS and/or eNOS), since only the agonist-induced constriction and not KCl-induced constriction or basal tone was increased after inhibition of NOS activity. cNOS in the airways is basally expressed in neuronal, endothelial and epithelial cells (Kobzik *et al.*, 1993) and its activation is dependent on the agonist- or depolarization-induced increase in intracellular Ca^{2+} concentration, causing the production of small (picomolar) amounts of NO. By contrast, iNOS is tonically activated, independent of Ca^{2+} and under transcriptional control of pro-inflammatory cytokines, such as interferon γ (IFN γ), interleukin-1β (IL-1β) and tumour necrosis factor α (TNF-α) (Morris & Billiar, 1994). Pulmonary iNOS has been described in endothelial and epithelial cells and in macrophages of inflamed airways (Kobzik *et al.*, 1993) and its induction results in the production of high (micromolar) levels of NO (Barnes & Belvisi, 1993; Morris & Billiar, 1994). Recent studies have indicated that iNOS activity is present in the airways of chronic asthmatics (Hamid *et al.*, 1993; Kharitonov *et al.*, 1994; Yates *et al.*, 1995). In addition, there is evidence that iNOS activity may be induced by allergen challenge of asthmatic patients, leading to enhanced concentrations of NO in exhaled air during the LAR but not the EAR (Kharitonov *et al.*, 1995). Our study would suggest that an enhanced iNOS activity during the LAR is preceded by a reduced cNOS activity and that this reduced activity may contribute to the airway hyperreactivity that is commonly observed after the EAR (Durham *et al.*, 1985; Aalbers *et al.*, 1991; Santing *et al.*, 1994b).

In the present investigation, we did not find any evidence for potential muscarinic receptor antagonism of L-NAME, as previously reported by Buxton *et al.* (1993). Thus, as with histamine, we found that the methacholine-induced constriction of perfused control airways was potentiated with L-NAME and not inhibited, both after IL and EL administration of the agonist. Moreover, L-NAME had no effect on the pD_2 values for both histamine and methacholine. In addition, methacholine-induced contraction of single open tracheal rings, which is mainly determined by direct action of the agonist on the airway smooth muscle due to loss of the epithelial barrier, was not affected by L-NAME, indicating that the NOS inhibitor does not act as an antagonist at the M_3 muscarinic receptor in this preparation. The latter finding also suggests that the airway smooth muscle is not likely to contribute to the regulatory role of NO in the intact airway as observed in the perfused tracheal preparations. Finally, in a previous study using guinea-pig tracheal tube preparations, it was demonstrated that the enhanced histamine-induced airway constriction in the presence of L-NAME is concentration-dependently reversed by L-arginine (Nijkamp *et al.*, 1993), once more indicating that the enhanced agonist-induced contraction of these preparations is due to inhibition of NOS activity.

Since allergen challenge may cause damage of the bronchial epithelium (Laitinen *et al.*, 1985) and the epithelium appears to be an important source of contractile agonist-induced NO production in the trachea (Nijkamp *et al.*, 1993), we hypothesized that the deficiency of NO in the trachea of hyperreactive guinea-pigs was due to epithelial shedding. However, histological examination of the tracheal preparations showed no evidence for morphological damage of the epithelium. Moreover, there was no convincing evidence for loss of the epithelial barrier from the functional studies, since the ΔpD_2 between IL and EL methacholine was unchanged, while there was only a small, but significant, reduction in the ΔpD_2 for histamine. The latter difference between the two agonists is unexplained, but could be related to an (increased) effect of histamine, but not methacholine, on the epithelial permeability (Bhat *et al.*, 1993). A dissociation between tracheal hyperreactivity and epithelial damage was recently also found by Masaki *et al.* (1994).

Nevertheless, eosinophilic inflammation, as observed after the EAR (Figure 5a; see also Santing *et al.*, 1994b), and the subsequent release of MBP and related proteins could lead to reduced tracheal epithelial functions without morphological damage, including reduced release of (a) relaxing factor(s) (Flavahan *et al.*, 1988). In addition, in murine vascular endothelial cells it was demonstrated that cytokines such as IFN γ may inhibit the expression of cNOS (Walter *et al.*, 1994). Since IFN γ may also cause induction of iNOS in these cells, it was hypothesized that inflammatory stimuli trigger the cells to switch from cNOS to iNOS activity (Walter *et al.*, 1994). Evidence for such a switch was recently also reported for human monocytes (Reiling *et al.*, 1994). Alternative mechanisms that may be involved in a deficiency of NO are metabolism of NO to peroxynitrite by superoxide anions released by inflammatory cells (Gaston *et al.*, 1994) or reduced sensitivity of the airway smooth muscle to NO, possibly by desensitization of guanylyl cyclase (De Kimpe *et al.*, 1994).

In conclusion, using a guinea-pig model of allergic asthma, we demonstrated that a deficiency of NO contributes to allergen-induced airway hyperreactivity after the EAR. The mechanism of this deficiency as well as the role of NO in the allergen-induced airway hyperreactivity after the LAR are now under investigation.

We thank Mrs Carolina R.E. Elzinga for performing the tracheal strip experiments. This study was supported financially by Glaxo Nederland B.V.

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(Received June 18, 1996
Accepted August 7, 1996)