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The promotion of patent airways and inhibition of antigen-induced bronchial obstruction by endogenous nitric oxide

Magnus G. Persson, Sten G. Friberg, Lars E. Gustafsson & Per Hedqvist

Department of Physiology and Pharmacology, and Institute of Environmental Medicine, Karolinska Institute, S-171 77 Stockholm, Sweden

1 The aim of the present study was to investigate the role of nitric oxide (NO), histamine and leukotrienes in bronchial obstruction. For this, guinea-pigs immunised against ovalbumin were studied under anaesthesia during challenge with antigen or agonists.

2 Challenge with nebulised antigen (0.1 – 1 mg) elicited dose-dependent increases in insufflation pressure which were abolished by combined administration of histamine and leukotriene antagonists.

3 Challenge with nebulised antigen (0.1 – 1 mg) also elicited dose-dependent increases in the concentration of endogenous nitric oxide in the exhaled air. After an initial peak, exhaled NO concentrations returned to pre-challenge levels.

4 The increase in insufflation pressure and in exhaled NO caused by ovalbumin challenge was inhibited by combined administration of histamine and leukotriene antagonists.

5 In non-immunised guinea-pigs, challenge of the airways with nebulised histamine (10 – 1000 nmol) or leukotriene C_4 (LTC_4, 30 – 300 pmol) elicited dose-dependent increases in insufflation pressure and in concentrations of endogenous NO in exhaled air.

6 The increase in exhaled NO correlated with the increase in insufflation pressure in response to ovalbumin, histamine and LTC_4. An inhibitor of endogenous NO synthesis, N^{ω}-nitro-L-arginine methylester (L-NAME, 30 mg kg^{-1} i.v.) abolished NO exhalation, and markedly augmented the airway responses to ovalbumin, histamine, or LTC_4.

7 The potentiation by L-NAME of the increase in insufflation pressure in response to ovalbumin or histamine was prevented by exogenous NO (20 p.p.m.) in the inhaled air.

8 The results indicate that endogenous NO has an inhibitory effect on bronchial obstruction. Increased NO release during allergen challenge is likely to be due to actions of histamine and leukotrienes.

Keywords: Nitric oxide; asthma; leukotrienes; histamine; airway smooth muscle

Introduction

There is a growing body of evidence indicating that endogenous nitric oxide (NO) has a key role in normal pulmonary vascular regulation (Archer *et al.*, 1989; Persson *et al.*, 1990; Sprague *et al.*, 1992). NO is formed from L-arginine and may be produced in a variety of tissues within the respiratory system, including the epithelium (Fischer *et al.*, 1993; Kobzik *et al.*, 1993), endothelium (Moncada, 1992), and nerves (Belvisi *et al.*, 1992). Furthermore, endogenously produced NO can be detected in exhaled air (Gustafsson *et al.*, 1991). The concentration of NO in exhaled air is stable at rest but increases after challenge with nebulised antigen (Persson & Gustafsson, 1993), implying that endogenous NO may also have a role in the regulation of bronchial tone in pathophysiological conditions. In line with this view, higher concentrations of nitric oxide have been observed in the exhaled air of asthmatics than in that of healthy subjects (Alving *et al.*, 1993; Persson *et al.*, 1994b; Kharitonov *et al.*, 1994). In addition, inhaled NO has been shown to counteract agonist-induced bronchial obstruction in animal models (Dupuy *et al.*, 1992; Högman *et al.*, 1993a) as well as in man (Högman *et al.*, 1993b). Further evidence for a bronchodilating role of endogenously formed NO is provided by the observation that histamine- (Nijkamp *et al.*, 1993) and antigen-induced (Persson *et al.*, 1993) bronchial obstruction is aggravated by inhibitors of NO-synthesis, and that this effect is reversed by inhaled NO (Persson *et al.*, 1993).

The aim of the present study was to investigate the role of NO, histamine, and leukotrienes in bronchial regulation by studying (1) whether antigen- or agonist-induced bronchial obstruction is affected by inhibition of NO synthesis and/or by inhaled NO, (2) whether agonist-induced bronchial obstruction is associated with increased concentrations of NO in exhaled air, and (3) whether such increased concentrations of NO correlate with the degree of bronchial obstruction.

Methods

Dunkin-Hartley guinea-pigs were sensitized with ovalbumin (100 mg i.p. and 100 mg s.c.) six weeks before the experiments. Sensitized or nonsensitized guinea-pigs (400 – 600 g) were anaesthetized with pentobarbitone sodium (50 mg kg^{-1} i.p.). Catheters were inserted in a carotid artery and a jugular vein for blood pressure recordings and for infusion of supplementary fluid and anaesthetic, respectively. Tracheal cannulation was performed to assure that any measured NO was of lower airway rather than nasopharyngeal origin (see Gerlach *et al.*, 1994). The animals were ventilated with a rodent ventilator (Harvard Apparatus 683, South Natick, MA, U.S.A.) set at 250 ml kg^{-1} min^{-1} at 40 breaths per minute, $FiO_2 = 0.21$ (NO-free air, see below). A solution containing glucose (3.6 g 100 ml^{-1}), dextran-70 (3 g 100 ml^{-1}), $NaHCO_3$ (0.7 g 100 ml^{-1}) and pentobarbitone sodium (300 mg 100 ml^{-1}) was continuously infused at a rate of 5 ml kg^{-1} h^{-1} by means of an infusion pump (Terumo STC-521, Terumo Corp., Tokyo, Japan). All animals received atropine sulphate (2 mg kg^{-1}) and pancuronium bromide (2 mg kg^{-1}) i.v. prior to antigen challenge. The rectal temperature of the animal was maintained at

[1] Author for correspondence.

38–38.5°C by a heating pad connected to a thermostat (Heraeus-Wittman, Heidelberg, Germany). Insufflation pressure was measured by a pressure transducer connected to a sidearm of the ventilator tubing. An ultrasonic nebuliser (NB108, LKB Medical, Stockholm, Sweden) was connected to the inlet of the ventilator and was used for aerosolising substances. At the beginning of all experiments, the animals were exposed to nebulised saline (0.9%, 0.5 ml), which was found to be without effect on NO concentration and insufflation pressure. Drugs, in 0.5 ml of saline, were nebulised over a 3 min period. Care was taken to minimize condensation of aerosol by warming the tubing with lamps. Each animal was exposed to only one agent (agonist or antigen). For dose response curves, histamine or leukotriene C_4 (LTC_4) was in most cases administered at one concentration per animal. In some instances, however, histamine or LTC_4 was applied at two different concentrations in one animal. In these cases, a 60 min resting period was allowed between the two provocations. The amounts of nebulised agents presented throughout this paper represent the amounts nebulised into the inhaled air as it entered the ventilator circuit.

For a limited series of experiments (data in Figure 2), another nebuliser was used (DeVilbiss Ultra-Neb 2000, DeVilbiss Health Care, Somerset, PA, U.S.A.). In this case 5 ml of 0.9% NaCl (with or without ovalbumin 0.1–10 mg ml^{-1}) was administered into the nebuliser and nebulisation was applied for 5 min. Thereby, each animal was exposed to 0.5 ml fluid during the nebulisation period, as above. The exact amount of fluid retained by the animal was not determined.

Nitric oxide concentrations in exhaled air were monitored on-line by a Sievers 270 chemiluminescence analyser (Sievers Research Inc. Boulder, CO, U.S.A.) set for an integration time of 0.12 s and calibrated at 1, 3, 10, 30, 100, and 300 p.p.b. The calibration gas was diluted in synthetic air, by two mass flow meters, connected to a control unit (Bronkhorst, Ruurlo, Holland), and a certified standard NO gas (2 p.p.m. in N_2; AGA Specialgas, Lidingö, Sweden). Dilution from a stock is necessary since concentrations of NO below 1 p.p.m. are not stable during storage. The principles for chemiluminescence assay have recently been reviewed (Archer, 1993). Briefly, the sample gas (60 ml min^{-1}, continuously withdrawn at ambient pressure from a mixing tube at the exhaust of the ventilator) is carried by suction into a chamber under vacuum (7×10^{-3} bar) where it reacts with a flow of O_3 in O_2. The reaction leads to the formation of NO_2 and O_2 and to the emission of photons, which are quantified by a photomultiplier tube (Archer, 1993). The detection limit of the NO measurement was below 1 p.p.b and the precision was better than 5%. The analysis of a standard gas diluted to 13 p.p.b. was unaffected by admixture of 5% CO_2. Ethanol and water vapour did not give signals in the NO analyser, and the signal is not due to exhaled CO (Gustafsson *et al.*, unpublished observations). An organonitrite, i.e. ethyl nitrite, did not evoke a signal when evaporated in small amounts into the inlet of the analyser. Thus, the analysis was considered specific for NO.

In order to obtain precise measurements of the endogenously formed NO, the air supply for the ventilator was filtered through a 1.5 m long charcoal filter (inner diameter 12 cm). This procedure reduces NO concentrations in inhaled air to below 1 p.p.b., thus eliminating the possible influence of variable NO concentrations in the environment.

In some experiments, NO was administered at 20 p.p.m. in the inhaled air by means of precision flow meters, and nitric oxide concentrations were monitored by a Monitor Labs 8840 nitrogen oxides analyser (Lear Siegler Instr. Co., Englewood, CO, U.S.A.) calibrated as above, using appropriate dilutions of a 200 p.p.m. NO standard. Administration of NO was commenced 5 min before challenge of airways with antigen or agonist.

Statistics

Data are expressed as mean ± s.e.mean. Differences between groups were compared by ANOVA (Student-Newman-Keul's test), and correlation coefficients were calculated as Pearson correlation coefficients during least squares regression analysis.

Drugs

Pentobarbitone sodium (Mebumal vet., Nordvacc, Sweden); pancuronium bromide (Pavulon, Organon Teknika, Oss, Holland); dextran-70 (Macrodex, Pharmacia, Uppsala, Sweden); heparin (Kabi, Stockholm, Sweden); atropine sulphate, ovalbumin, N^{ω}-nitro-L-arginine (L-NAME), chlorpheniramine maleate (Sigma Chemical Co, St Louis, MO, U.S.A.). ICI 198,615 ((1-((2-methoxy-4-(((phenylsulphonyl) amino) carbonyl) phenyl) methyl)-1H-indazol-6-yl) carbamic acid cyclopentylester) was from Stuart Pharm. (Wilmington, Del, U.S.A.) and was dissolved in dimethylsulphoxide and Tris and diluted in saline.

Results

Insufflation pressure (Table 1), mean arterial blood pressure (67 ± 4 mmHg), heart rate (245 ± 11 min^{-1}) and the endogenous NO concentration (18 ± 6 p.p.b.) in exhaled air

Table 1 Effects of agonists and antigen in pentobarbitone-anaesthetized and artificially ventilated guinea-pigs on insufflation pressure, and on concentrations of exhaled NO

Agent	*Dose*	*Insufflation pressure* (peak, cmH_2O)	*Exhaled NO* (peak, p.p.b.)
Histamine (nmol)	0	16 ± 2	12 ± 3
	10	34 ± 11**	29 ± 21*
	100	70 ± 9**	99 ± 39**
LTC_4 (pmol)	0	15 ± 2	29 ± 8
	30	17 ± 4	40 ± 17
	100	40 ± 8*	57 ± 22*
	300	55 ± 5*	84 ± 21*
Ovalbumin (mg)	0	8 ± 0.4	15 ± 3
	0.1	40 ± 17**	81 ± 43**
	1	79 ± 13**	111 ± 57**
ICI + chlorphenir: OA (mg)	1	22 ± 6	18 ± 2

Shown are dose-dependent responses to nebulised histamine or leukotriene C_4 (LTC_4) in non-sensitized animals, and to ovalbumin (OA) in sensitized animals. ICI + chlorphenir denotes that a group of sensitized animals had received the combination of ICI 198,615 (5 mg kg^{-1}) and chlorpheniramine (3 mg kg^{-1}) intravenously 20 min before ovalbumin challenge. Asterisks denote significant difference compared to controls, $*P < 0.05$, $**P < 0.01$, means ± s.e.mean, $n = 3–8$ for each group.

measured at the beginning of the experiments remained stable during a 30 min control period. There were no significant differences in the measured parameters between normal animals ($n=28$) and animals which had been immunised with ovalbumin ($n=20$).

Challenge with nebulised antigen (0.1–10 mg) in immunised animals induced a dose-dependent and sustained increase in insufflation pressure, and a rapid and short-lasting increase in the concentration of NO in exhaled air (Figure 1 and Table 1), whereas nebulised vehicle (0.9% NaCl) had no effect. The blood pressure response to inhaled ovalbumin was small and somewhat variable; usually there was a transient increase, followed by oscillations around the blood pressure level noted during the control period before challenge. Heart rate was little affected.

In nonsensitized animals, challenge with nebulised histamine (10–1000 nmol) or leukotriene C_4 (30–300 pmol) induced a dose-dependent increase both in insufflation pressure and in the concentration of NO in exhaled air (Table 1). There was a positive correlation between the increase in insufflation pressure and the peak concentration of exhaled NO during all three types of challenge (Figure 2).

Systemic pretreatment with the leukotriene receptor antagonist ICI 198,615 (5 mg kg^{-1}) (Snyder *et al.*, 1987) or the histamine H_1-receptor antagonist chlorpheniramine (3 mg kg^{-1}), attenuated the increase in insufflation pressure induced by ovalbumin in sensitized animals. Furthermore, the insufflation pressure response to ovalbumin was abolished by the combined pretreatment with ICI 198,615 and chlorpheniramine (Figure 3), suggesting that histamine and cysteinyl leukotrienes were major mediators of the reaction. The increase in exhaled NO was also abolished by the combined treatment with the antagonists, leading to an exhaled NO concentration of 18 ± 2 p.p.b. ($n=3$) 10 min after ovalbumin, a concentration not significantly different from the concentration before ovalbumin (Table 1).

Administration of L-NAME (30 mg kg^{-1} i.v.) before challenge virtually abolished the presence of NO in exhaled air (reduced to less than 1 p.p.b.), and increased mean arterial blood pressure (from 67 ± 5 to 92 ± 9 mmHg, $P<0.001$, $n=28$). However, the drug did not affect insufflation pressure. L-NAME also abolished the increase in exhaled NO concentration in response to nebulised ovalbumin in sensitized animals, and in response to histamine or leukotriene C_4 in nonsensitized animals. In animals pretreated with L-NAME, the increase in insufflation pressure in response to challenge with nebulised ovalbumin, LTC_4, or histamine was substantially augmented (Figures 4 and 5 and Table 2).

When NO (20 p.p.m.) was included in the inhaled air of animals pretreated with L-NAME (30 mg kg^{-1}) the increases in insufflation pressure in response to ovalbumin (0.1 mg) and

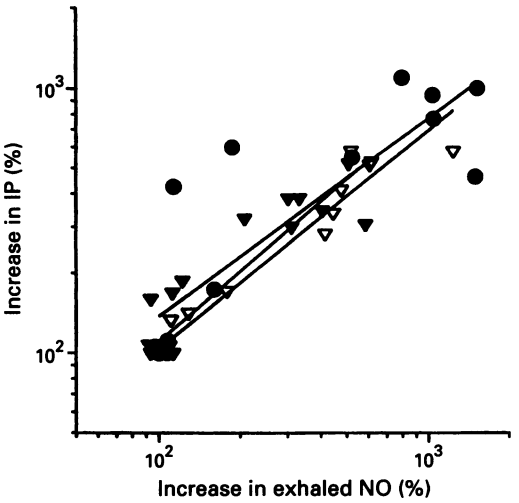


Figure 2 Correlation between relative increases in insufflation pressure and peak concentrations of NO in exhaled air in pentobarbitone-anaesthetized and artificially ventilated guinea-pigs. Shown are data from challenge of airways with nebulised histamine (▽; 10–1000 nmol, $n=12$) or leukotriene C_4 (▼, 30–300 pmol, $n=13$) in normal animals. Also shown are data from challenge with ovalbumin (●; 0.1–1 mg, $n=14$) in ovalbumin-sensitized (6 weeks previously) animals. $r=0.752$, $P<0.001$; $r=0.904$, $P<0.001$; $r=0.873$, $P<0.001$ for LTC_4, histamine and ovalbumin, respectively.

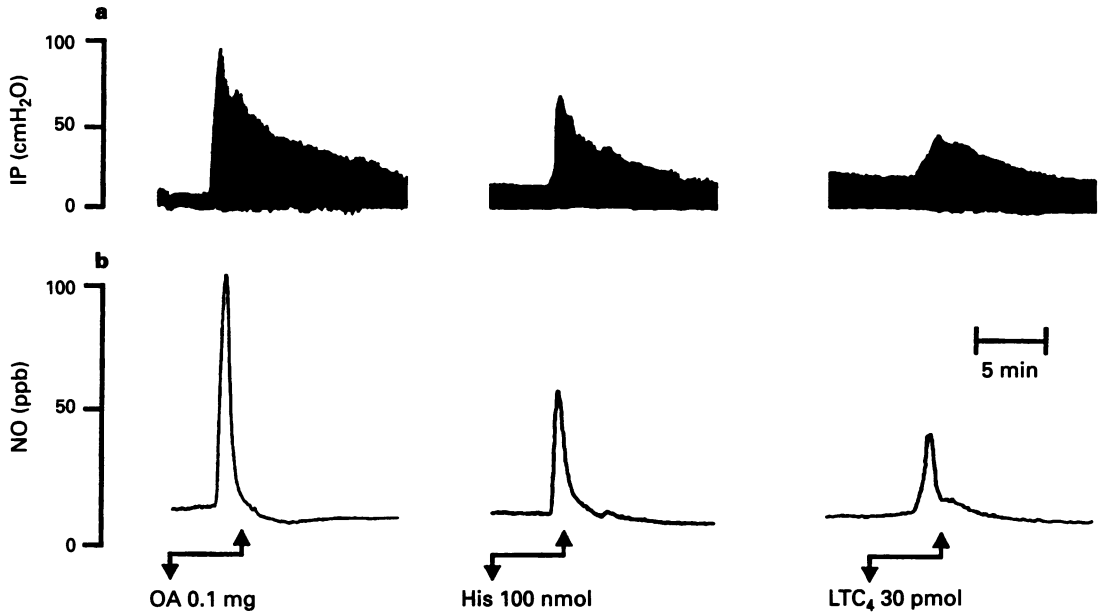


Figure 1 Insufflation pressure (IP, a), and endogenous NO in exhaled air (NO, b) in pentobarbitone-anaesthetized and artificially ventilated guinea-pigs. Shown are original recordings from animals sensitized to ovalbumin (6 weeks before the experiments) and challenged with ovalbumin (OA, 0.1 mg, administered over a 5 min period), and from normal animals challenged with histamine (His, 100 nmol), or leukotriene C_4 (LTC_4, 30 pmol).

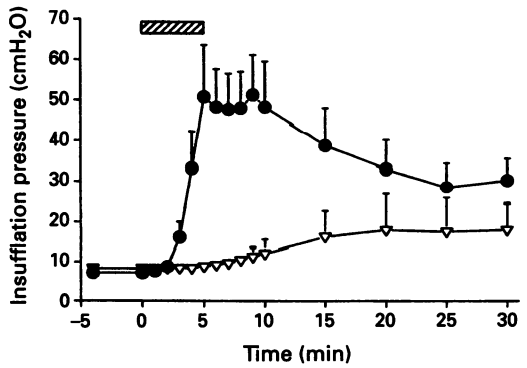


Figure 3 Insufflation pressure responses to challenge with nebulised ovalbumin (1 mg ml^{-1}, 0.5 ml administered during a 5 min period as indicated by horizontal bar) in pentobarbitone-anaesthetized and artificially ventilated guinea-pigs sensitized to ovalbumin 6 weeks before the experiment. Responses in the absence (●) and presence (▽) of the combination of ICI 198,615 (5 mg kg^{-1}) and chlorpheniramine (3 mg kg^{-1}) ($n=3$, mean values; vertical bars denote + s.e.mean.

histamine (100 nmol) were significantly reduced. The response to LTC_4 (30–300 pmol) was delayed but not reduced (Figures 4 and 5).

Discussion

The present study indicates that endogenous NO can counteract bronchial obstruction induced in the guinea-pig by both specific antigen and bronchoconstrictor agonists. The antigen-induced bronchial obstruction evidently involved histamine and leukotrienes as major mediators, since combined pretreatment with histamine- and leukotriene-receptor antagonists abolished the response. This is in accord with observations in other animal models and in man (Chung & Barnes, 1992; Björck & Dahlén, 1993).

It has been shown that the levels of endogenous NO in exhaled air change in a biphasic manner in response to inhaled antigen. This involves a rapid increase, which peaks within 5 min, followed by a slow decrease to levels below those observed during the control period (Persson & Gustafsson, 1993). In the present study, the increase in exhaled NO concentration was abolished by combined inhibition of histamine and leukotriene receptors, indicating that airway release of NO in response to antigen occurs subsequent to activation of these receptors. The exact mechanism responsible for the increased NO release is not known but it has been observed that, in

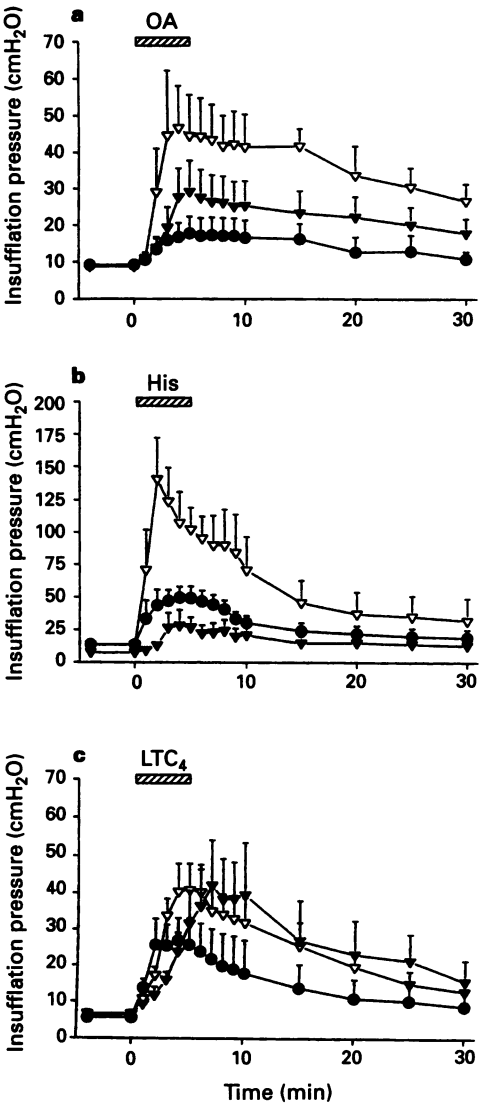
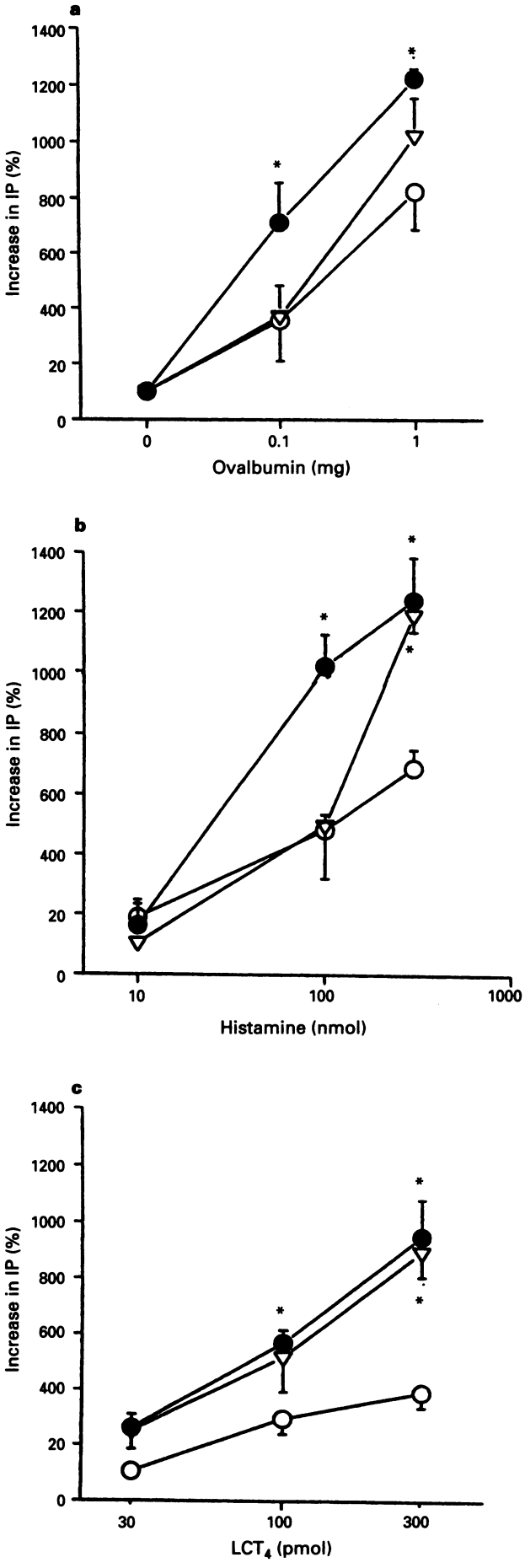


Figure 4 Time courses for insufflation pressure responses to (a) ovalbumin (OA, 0.1 mg, administered during a 5 min period as indicated by horizontal bar) in ovalbumin-sensitized guinea-pigs, and (b) histamine (His, 100 nmol), or (c) leukotriene C_4 (LTC_4, 100 pmol) in normal guinea-pigs. Shown are responses in the absence of any inhibitor of NO formation (●), and in animals pre-treated with L-NAME (30 mg kg^{-1}, ▽). Also shown are the responses in animals pretreated with L-NAME (30 mg kg^{-1}) which received NO (20 p.p.m.) in the inhaled air from 5 min before airway challenge with ovalbumin, histamine or leukotriene C_4 (▼). Points are means and vertical lines show s.e.mean, $n=4-8$.

Table 2 Insufflation pressure responses, in pentobarbitone-anaesthetized and artificially ventilated guinea-pigs, measured as area under curve above baseline (0–20 min) and expressed as surface units (cmH_2O × min)

	Insufflation pressure, area under curve		
Agent	*Control*	*L-NAME*	*L-NAME+NO*
None	6±1	7±1	6±1
OA (0.1 mg)	61±38	375±88*	193±75
His (100 nmol)	234±77	695±152*	98±28
LTC_4 (100 pmol)	182±74	294±61*	278±138

Shown are responses to nebulised ovalbumin in sensitized animals, and to histamine (His) or LTC_4 in non-sensitized animals. Experiments were performed in the absence of any inhibitor of NO formation (control), in the presence of the NO synthase inhibitor L-NAME (30 mg kg^{-1} b.w., administered i.v. 20 min before challenge), and in the presence of L-NAME (30 mg kg^{-1} b.w., administered i.v. 20 min before challenge) and with exogenous NO (20 p.p.m.) present in the inhaled air from 5 min before challenge. Asterisks denote significant difference ($P<0.05$) compared to control group, means ± s.e.mean, $n=4-8$ for each group. For key to abbreviations used see legend of Table 1.



addition to bronchoconstriction, inhalation of the smooth muscle activator prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) causes dose-dependent increases in the concentration of NO in the exhalate (Persson & Gustafsson, 1993). This would seem to indicate that increased NO release is due to induced bronchoconstriction. Furthermore, the peak concentrations in exhaled NO were observed during the development of increased bronchial tone; thereafter the concentration of NO in exhaled air returned to values close to those observed during the control period before challenge, although insufflation pressure remained elevated. Thus, it may be speculated that increased NO release is caused by dynamic changes in bronchial tone rather than being related to an altered setpoint of airway resistance. This does not exclude the possibility that NO formation in the airways may also occur as a direct consequence of activation of histamine and leukotriene receptors, as has been shown for endothelial cells (Moncada, 1992). It might be speculated that endothelial cells or other microvascular elements known to be involved in asthma (Greiff *et al.*, 1993) contribute to the increased NO concentrations in response to antigen or agonist. However, this does not seem likely, since the passage of NO from the luminal to the abluminal site of pulmonary vessels is negligible (Persson *et al.*, 1994c). In this context, it must be emphasized that the present model of allergic bronchial obstruction may differ from the pathophysiological course of events in human asthma. In human asthmatics without acute symptoms, the increased NO concentrations in exhaled air do not correlate with airway resistance (Persson *et al.*, 1993) and might, to some extent, mirror the inflammatory state of the airways (Kharitonov *et al.*, 1994; Barnes & Liew 1995).

Inhibition of NO synthesis augmented the bronchial obstruction in response to antigen in sensitized guinea-pigs, as well as the bronchial obstruction induced by histamine or LTC_4 in non-sensitized animals. This strongly indicates that NO plays a role in the regulation of bronchial tone in the present experimental model. Furthermore, the increased airway obstruction in response to antigen or histamine after treatment with L-NAME was reversed by exogenous NO. However, the increased bronchial obstruction in response to LTC_4 in the presence of L-NAME, although slightly delayed, was essentially unaffected by exogenous NO. This might indicate that NO acts selectively or also possibly that insufficient concentrations of exogenous NO were used in the present study. In this context it is worth noting that a 15 fold higher concentration of NO (300 p.p.m.) was required to reverse completely methacholine-induced bronchial obstruction in guinea-pigs (Dupuy *et al.*, 1992). The concentration of inhaled NO used in the present study was chosen on the basis of dose-response curves showing that, in rabbits, 20 p.p.m. is required to mimic the effects of endogenous NO on pulmonary vasculature (Persson *et al.*, 1994a), suggesting that this corresponds to endogenous concentrations. Also in favour of this concentration is that it has been used successfully in clinical trials (Frostell *et al.*, 1993) and is considered relatively non-toxic (Fratacci *et al.*, 1991).

The observed inhibitory effect of exogenous NO on antigen-induced bronchial obstruction after inhibition of endogenous

Figure 5 Change in insufflation pressure (IP) induced by nebulised agents in pentobarbitone-anaesthetized and artificially ventilated guinea-pigs. Dose-response curves for (a) ovalbumin given to ovalbumin-sensitized (6 weeks previously) animals ($n=16$), and for (b) histamine ($n=14$), or (c) leukotriene C_4 ($n=12$) in normal animals. Shown are responses in animals in the absence of any inhibitor of NO formation (○), in animals pretreated with the NO synthase inhibitor L-NAME (30 mg kg^{-1}, ●), and in the presence of L-NAME (30 mg kg^{-1}) during concomitant administration of NO (20 p.p.m. in the inhaled air, ▽). Asterisks denote significant difference compared to animals not pretreated with L-NAME (*$P<0.05$, $n=4-6$ in each group). Points shown are means and vertical lines represent s.e.mean.

NO synthesis suggests that exogenous NO may substitute for endogenous NO, and extends the findings of Dupuy *et al.* (1992) that exogenous NO can counteract agonist-induced bronchial obstruction.

In conclusion, the present observations demonstrate an inhibitory role of endogenous NO on antigen- as well as on agonist-induced bronchial obstruction and support a role for NO-mechanisms in bronchial asthma.

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