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Role of nitric oxide in the development and partial reversal of allergen-induced airway hyperreactivity in conscious, unrestrained guinea-pigs

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1 Using a conscious, unrestrained guinea-pig model of allergic asthma, we investigated the role of endogenous nitric oxide (NO) in the regulation of airway (hyper)reactivity to histamine before and after the allergen-induced early and late asthmatic reactions, by examining the effect of inhalation of the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 12 mM, 15 min) on the histamine-induced airway obstruction of ovalbumin-sensitized guinea-pigs before, and at 5.5 h and 23.5 h after allergen challenge.

2 Before allergen challenge, inhaled L-NAME caused a significant 2.02 ± 0.25 fold increase ($P < 0.01$) in airway reactivity to histamine; this effect was reversed within 2.5 to 6 h after administration.

3 After the allergen-induced early asthmatic reaction at 5 h after ovalbumin provocation, a significant 3.73 ± 0.67 fold increase ($P < 0.01$) of the airway reactivity to histamine was observed; subsequent inhalation of L-NAME at 5.5 h had no effect on the airway hyperreactivity, reassessed at 6 h.

4 After the late asthmatic reaction, at 23 h after ovalbumin provocation, a reduced, but still significant airway hyperreactivity to histamine (2.18 ± 0.40 fold; $P < 0.05$) was observed. Subsequent inhalation of L-NAME now significantly potentiated the partially reduced airway hyperreactivity 1.57 ± 0.19 fold ($P < 0.05$) to the level observed after the early asthmatic reaction.

5 When administered 30 min before allergen exposure, L-NAME significantly enhanced the allergen-induced early asthmatic reaction. However, when administered at 5.5 h after allergen provocation, L-NAME did not affect the subsequent late asthmatic reaction.

6 These results indicate that endogenous NO is involved the regulation of histamine- and allergen-induced bronchoconstriction and that a deficiency of cNOS-derived NO contributes to the allergen-induced airway hyperreactivity to histamine after the early asthmatic reaction, while a recovery of NO deficiency may account for the partial reversal of the allergen-induced airway hyperreactivity after the late asthmatic reaction.

Keywords: Nitric oxide; N^{ω}-nitro-L-arginine methyl ester; histamine; airway hyperreactivity; allergic asthma

Introduction

It is now well established that endogenous nitric oxide (NO) plays a pivotal role in various pulmonary functions, including the regulation of vascular and airway smooth muscle tone (Moncada & Higgs, 1993). In addition, NO has been recognized as an important immunomodulatory and cytotoxic mediator of inflammatory responses in the lung (Barnes & Liew, 1995).

NO is synthesized by the enzyme NO synthase (NOS) from the semi-essential amino acid L-arginine, and at least three isoforms of this enzyme have been identified (Forstermann *et al*., 1991). Constitutive isoforms (cNOS) are expressed in nonadrenergic noncholinergic neurones (nNOS), endothelial cells (eNOS) and epithelial cells (nNOS and eNOS) of the airway (Fischer *et al*., 1993; Kobzik *et al*., 1993). These isoforms are basally expressed and their activity is regulated by agonist- or depolarization-induced intracellular calcium changes, causing the production of small (picomolar) levels of NO. By contrast, an inducible isoform (iNOS) is expressed in a variety of cells, including inflammatory, epithelial, endothelial and smooth muscle cells, upon stimulation by pro-inflammatory cytokines or endotoxin (Barnes & Belvisi, 1993). The activity of iNOS is independent of intracellular calcium, and nanomolar levels of NO are being produced.

NO can be detected in the exhaled air of both man and animals (Gustafsson *et al*., 1991) and is enhanced in patients with chronic asthma (Kharitonov *et al*., 1994), presumably due to inflammation-induced expression of iNOS (Hamid *et al*., 1993; Yates *et al*., 1995; 1996).

In the airways, NO has a potent bronchodilator action, presumably by increasing the intracellular level of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) and inducing relaxation of airway smooth muscle (Ahlner *et al*., 1991).

In guinea-pigs (Dupuy *et al*., 1992) and dogs (Brown *et al*., 1994), a concentration-dependent reversal of methacholine and histamine-induced bronchoconstriction was found by inhalation of gaseous NO or aerosols of S-nitrosothiols. The role of endogenous NO in the regulation of airway tone was also indicated by the observation that inhalation of the NOS inhibitors N^{ω}-nitro-L-arginine methyl ester (L-NAME) or N^{G}-monomethyl-L-arginine (L-NMMA) caused enhanced bronchoconstriction to histamine (Nijkamp *et al*., 1993) and bradykinin (Ricciardolo *et al*., 1994) in unsensitized guinea-pigs, while L-NAME increased the immediate allergen-induced airway obstruction of ovalbumin-sensitized guinea-pigs (Persson *et al*., 1993). In addition, luminal perfusion of guinea-pig

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tracheal tube preparations with L-NAME or L-NMMA *in vitro* caused increased contractile responses to both intraluminally (Nijkamp *et al*., 1993; De Boer *et al*., 1996) and extraluminally (De Boer *et al*., 1996) applied histamine and methacholine. Since some of the *in vitro* effects were shown to be dependent on the presence of an intact epithelium, it has been proposed that cNOS-derived NO acts as an epithelium-derived relaxant factor that counteracts agonist-induced airway smooth muscle contraction (Nijkamp *et al*., 1993). Based on this assumption, we have recently hypothesized that a reduced production of NO, possibly related to epithelial damage due to inflammation, may contribute to allergen-induced airway hyperreactivity to histamine and methacholine in asthma (De Boer *et al*., 1996). In an *ex vivo* study, with isolated perfused tracheae from unchallenged and ovalbumin-challenged IgE-sensitized guinea-pigs, it was indeed demonstrated that a deficiency of NO contributes to allergen-induced tracheal hyperreactivity after the early asthmatic reaction (6 h after allergen challenge). However, this appeared not to be due to epithelial shedding of the tracheal preparations (De Boer *et al*., 1996). This observation is remarkably in line with the previous observation by Persson & Gustafsson (1993) that a single allergen challenge of ovalbumin-sensitized guinea-pigs causes an immediate dose-dependent increase of exhaled NO, which drops below baseline while the early asthmatic reaction is still present. On the other hand, both in human asthmatics (Kharitonov *et al*., 1995) and in a guinea-pig model of occupational asthma (Yan *et al*., 1995), evidence was obtained for enhanced NO production during the late asthmatic response, probably due to inflammation-mediated induction of iNOS.

The role of endogenous NO in allergen-induced airway hyperreactivity *in vivo*, both after the early and late asthmatic reaction, is still unknown. Therefore, using a guinea-pig model of acute allergic asthma, characterized by ovalbumin-induced early and late asthmatic reactions, airway inflammation and airway hyperreactivity after these reactions (Santing *et al*., 1994b), we investigated the effect of L-NAME on histamine-induced airway obstruction before, and at 6 and 24 h after aerosol challenge with ovalbumin (i.e. after the early and late asthmatic reaction, respectively).

Methods

Animals

Specific pathogen free guinea-pigs of either sex (Charles River, SAVO, Kiszlegg, Germany) were used in this study. All animals, weighing 200–300 g, were sensitized to ovalbumin at 4 weeks of age as described previously (Van Amsterdam *et al*., 1989). To obtain a shift to IgE class antibodies, an allergen solution containing 100 μg ovalbumin and 100 mg $Al(OH)_3$ ml^{-1} saline was used. The mixture of allergen solution and $A1(OH)_3$ was gently rotated for 60 min to obtain an alu-gel, and 0.5 ml 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 neck (Van Amsterdam *et al*., 1989). Animals were operated on 3 weeks after sensitization and used experimentally 4 to 8 weeks after sensitization. The animals were housed in individual cages in climate-controlled animal quarters and were given water and food *ad libitum*

All protocols described were approved by the University of Groningen Animal Health Committee, which is responsible for the care and proper use of experimental animals.

Measurement of airway function

Airway function was assessed by measurement of pleural pressure (P_{pl}) as described previously (Santing *et al*., 1992). In short, a small saline-filled latex balloon, connected to a saline-filled cannula, was surgically implanted inside the thoracic cavity. The free end of the cannula was driven subcutaneously to the neck of the animal, where it was exposed and attached permanently. Via an external fluid-filled cannula the pleural balloon was connected to a pressure transducer (Ohmeda DTX, SpectraMed, Bilthoven, The Netherlands). P_{pl} (in cmH_2O) was continuously measured by use of an online computer system. P_{pl} data were sampled for 10 s every minute. Breath by breath variation was normally less than 10%, incidental large variations caused by sudden movements or deep sights were rejected from the calculation.

We have previously shown that changes in P_{pl} are linearly correlated to changes in airway resistance and hence can be used as a sensitive index for allergic and non-allergic bronchoconstriction (Santing *et al*., 1992).

During the experimental protocol (1–5 weeks after surgery) baseline P_{pl}-measurements remained stable and no signs of inflammation were observed at the sites of surgery. Airway function can be monitored repeatedly and continuously for periods of at least 24 h.

Provocation procedures

Ovalbumin and histamine provocations were performed by inhalation of solutions in aerosol form. These provocations were carried out in a specially designed perspex cage of 9 1, in which the guinea-pigs could move freely. A DeVilbiss nebulizer (type 646, DeVilbiss, Somerset, PA, U.S.A.) driven by an airflow of 8 1 min^{-1}, provided the aerosol with an output of 0.33 ml min^{-1}.

The animals were habituated to the experimental conditions and the provocation procedures on two sequential days at least one week after surgery, when preoperative weight was restored. On the first day, the animals were placed in the provocation cage unconnected to the pressure transducer. After an adaptation period of at least 30 min, three consecutive provocations with saline were performed, each provocation lasting 3 min, separated by 7 min intervals. The next day, this procedure was repeated with the animals connected to the measurement system.

On the experimental days following the habituation procedure, allergen and histamine provocations were performed as indicated below. All provocations were preceded by an adaptation period of at least 30 min, followed by two consecutive control provocations with saline as described above. A baseline P_{pl}-value was calculated by averaging the P_{pl}-values from the last 20 min of the adaptation period.

In order to assess the airway reactivity for histamine, provocations with the agonist were performed starting with a 25 μg ml^{-1} solution in saline, followed by increasing dosage steps of 25 μg ml^{-1}. Histamine provocations lasted 3 min and were separated by 7 min intervals. Animals were challenged until P_{pl} was increased by more than 100% above baseline for at least 3 consecutive minutes. The provocation concentration causing a 100% increase of P_{pl} (PC_{100}) was derived by linear intrapolation of the concentration-P_{pl} response curve and was used as an index for airway reactivity towards histamine. P_{pl} returned to baseline within 15 min after the last histamine provocation.

Allergen provocations were performed by inhalation of increasing concentrations of 1.0, 3.0 and 5.0 mg ml^{-1}

ovalbumin in saline for 3 min each, separated by 7 min intervals. Allergen inhalations were discontinued when an increase in P_{pl} of more than 100% was observed. With these conditions, none of the animals developed anaphylactic shock after allergen provocation.

Experimental protocol

Effect of L-NAME on basal airway reactivity to histamine In a first group of animals, we assessed the effect and the duration of action of L-NAME on basal histamine reactivity. Thirty minutes after the assessment of the basal histamine PC_{100}, L-NAME was inhaled for 15 min at a nebulizer concentration of 12 mM, a dose which yielded a maximal increase in histamine reactivity (not shown). After 10 min the next histamine PC_{100}-measurement was started, such that histamine-induced bronchoconstriction occurred approximately 30 min after L-NAME inhalation. L-NAME was dissolved in saline and this vehicle was used as a control.

In order to monitor the time-dependence of the L-NAME effect, in some of the animals subsequent histamine PC_{100}-measurements were performed at 30 min, 2.5 h and 6 h after L-NAME inhalation, as described above.

Following the method by Karlsson *et al*. (1990), it was calculated that the dose of inhaled L-NAME maximally disposed in the airways (0.58 μmol kg^{-1}) was similar to that of Ricciardolo *et al*., (1994) (0.38 μmol kg^{-1}), which was shown to have no effect on blood pressure and heart rate.

Effect of L-NAME on allergen-induced BHR and early and late asthmatic reactions In a second group of animals, baseline histamine PC_{100} was assessed 24 h before an ovalbumin provocation. Thirty minutes later, L-NAME (12 mM, 15 min) was inhaled and a subsequent histamine PC_{100}-measurement was performed to assess the effect of L-NAME on baseline histamine reactivity as indicated above. The next day, at 5 h and 23 h after ovalbumin provocation, histamine PC_{100} was measured again to determine allergen-induced airway hyperreactivity after the early and late asthmatic reaction, respectively. To investigate the role of endogenous NO in the observed histamine reactivity at these time-points, L-NAME was inhaled at 5.5 h and 23.5 h after ovalbumin provocation, and subsequently histamine PC_{100}-values were redetermined at 6 h and 24 h after ovalbumin provocation, respectively.

For the quantitative assessment of the early (between 0 h and 5 h after allergen provocation) and late (between 8 h and 24 h after allergen provocation) phase asthmatic reactions, airway function was continuously measured during the whole procedure. Between the measurements of PC_{100}-values at 6 h and 24 h, the animals were placed in their homecage (0.16 m^2), in which water and food were freely accessible and where they could move around freely. During this transfer the animals remained connected to the measurement system.

To establish the possible effects of the L-NAME inhalation, given at 5.5 h after ovalbumin provocation, on the late asthmatic response as well as on the histamine reactivity after the late response, a separate groups of animals received L-NAME only at 23.5 h after ovalbumin provocation.

The effect of L-NAME inhalation on the early asthmatic response was established in separate experiments. In the first week of a 2 week protocol, animals inhaled saline 30 min before ovalbumin provocation, and the allergen-induced early asthmatic response was subsequently measured. The effect of L-NAME on the early response was established 7 days later, by inhalation of the NOS inhibitor 30 min before the second ovalbumin provocation.

Data analysis

The magnitudes of the allergen-induced early and late asthmatic responses were expressed as the area under the P_{pl} time-response curve (AUC) between 0 h and 5 h after allergen provocation for the early reaction, and between 8 and 23 h after provocation for the late response. P_{pl} was expressed as percentage change from baseline and AUC was calculated by trapezoid integration over discrete (5 min) time-periods. Based on saline control provocations, threshold values of AUC (mean + 2 × s.d.; 99% confidence interval) were defined as 1185% × 5 min for a positive early response and 2790% × 5 min for a positive late response, respectively (Santing *et al*., 1994b). With these criteria, animals were characterized as single early responders and dual responders (i.e. animals expressing an early as well as late asthmatic response). Only dual responders (18 out of 24 animals (75%)) were included in this study. Changes in airway reactivity towards histamine as well as in allergen-induced asthmatic reactions were analysed by Student's t test for paired or unpaired data as indicated. Differences were considered to be statistically significant when $P<0.05$. All data are presented as mean ± s.e.mean.

Chemicals

Histamine hydrochloride, ovalbumin (grade III), and L-NAME were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). $Al(OH)_3$ was obtained for J.T. Baker Chemical Co. (Phillipsburg NJ, U.S.A.).

Results

Effect of L-NAME on basal airway reactivity to histamine

In the whole group of animals a baseline P_{pl} of 5.01 ± 0.40 cmH_2O was found which remained stable throughout the whole experiment.

Aerosol inhalation of L-NAME caused a significant 2.02 ± 0.25 fold increase ($P<0.01$) in airway reactivity to histamine when measured at 30 min after administration of the NOS inhibitor, while the effect was reversed within 2.5 to 6 h after administration (Figure 1). Inhalation of the L-NAME aerosol did not affect baseline P_{pl}. In addition, administration of saline instead of L-NAME did not affect subsequent histamine PC_{100}-values (results not shown).

Effect of L-NAME on allergen-induced airway hyperreactivity to histamine

In the second group of animals, at 24 h before ovalbumin challenge, L-NAME inhalation caused a 1.72 ± 0.16 fold increase ($P<0.05$) in the airway reactivity to histamine (Figure 2). After the early reaction, at 5 h following ovalbumin exposure, a significant 3.73 ± 0.67 fold increase ($P<0.01$) in the airway reactivity to histamine was observed; after subsequent inhalation of L-NAME at 5.5 h, the airway hyperreactivity redetermined at 6 h had not changed (Figure 2). A reduced, but still significant hyperreactivity to histamine (2.18 ± 0.40 fold; $P<0.05$) was present after the late reaction, at 23 h after ovalbumin exposure. Subsequent inhalation of L-NAME at 23.5 h now caused a significant 1.57 ± 0.19 fold increase in the hyperreactivity to histamine as monitored at 24 h ($P<0.05$; Figure 2). Inhalation of L-NAME at 5.5 h after

ovalbumin provocation did not affect the allergen-induced airway hyperreactivity observed at 23 h after provocation, nor did it affect the acute enhancing effect of L-NAME inhalation at 23.5 h (Figure 3).

In an additional group of animals, inhalations of saline instead of L-NAME did not at all affect the airway reactivity to histamine before and the hyperreactivity at 5 h and 23 h after allergen challenge (not shown).

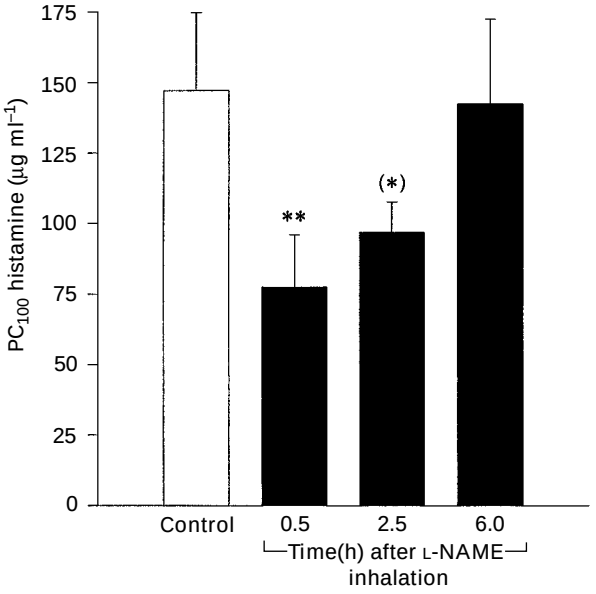

Figure 1 Effect of inhalation of the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 12 mM, 15 min) on the histamine PC_{100}-value at various time points after inhalation. Data represent mean values ± s.e.mean of 5 animals. Statistical analysis: $^{**}P<0.01$, $(*)P=0.06$, Student's paired t test.

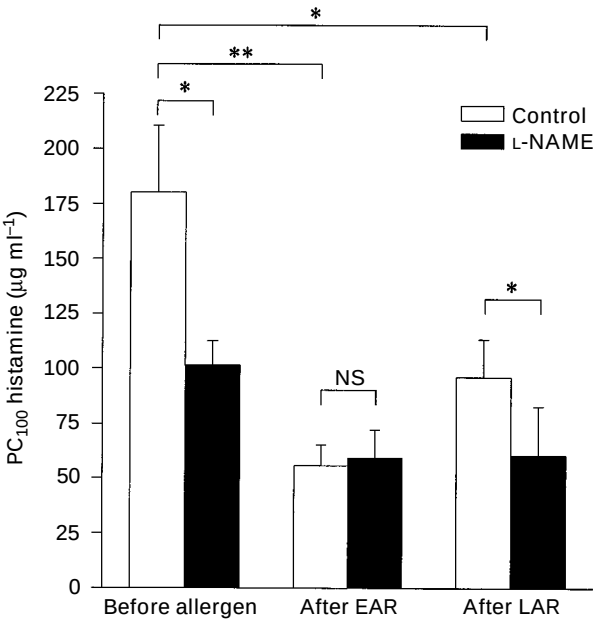

Figure 2 Effect of inhalation of the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 12 mM, 15 min) on the histamine PC_{100}-value 24 h before allergen challenge, and after the allergen-induced early (EAR) and late (LAR) asthmatic reactions. Two subsequent PC_{100}-measurements were performed at 5 h and 6 h, and at 23 h and 24 h after allergen provocation, respectively, with L-NAME being administered 30 min after the preceding PC_{100}-measurement. Data represent mean values ± s.e.mean of 7 animals. Statistical analysis: $^{*}P<0.05$, $^{**}P<0.01$, Student's paired t test.

Effect of L-NAME on allergen-induced early and late asthmatic reactions

Table 1 shows the early and late asthmatic reactions of the three groups of animals (saline inhalations at 5.5 h and 23.5 h after allergen provocation, L-NAME inhalations at 5.5 h and 23.5 h, and L-NAME inhalation only at 23.5 h after allergen provocation) described above. Not only were the early asthmatic reactions comparable between these groups but also no differences were observed in the severity of the late

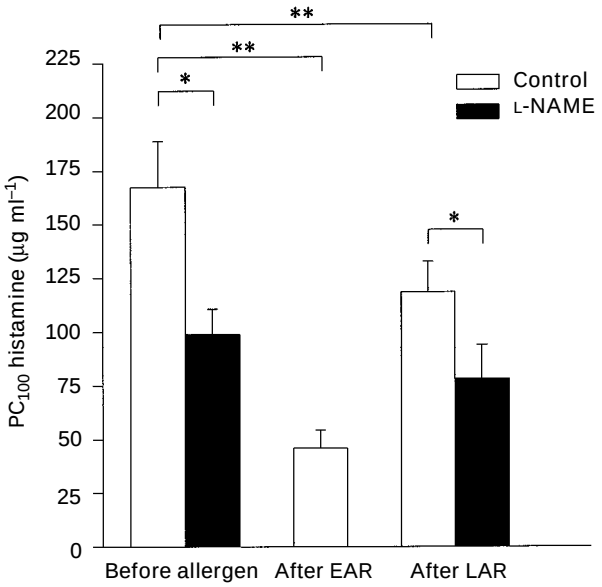

Figure 3 Effect of inhalation of the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 12 mM, 15 min) on the histamine PC_{100}-value 24 h before allergen challenge, and after the allergen-induced late asthmatic reaction (LAR). Subsequent PC_{100}-measurements after LAR were performed at 23 h and 24 h after allergen provocation, with L-NAME being administered 30 min after the preceding PC_{100}-measurement. Data represent mean values ± s.e.mean of 6 animals. Statistical analysis: $^{*}P<0.05$, $^{**}P<0.01$, Student's paired t test.

Table 1 Early and late asthmatic reactions in three groups of dual responding guinea-pigs inhaling saline or the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 12 mM, 15 min) at 5.5 h and 23.5 h after allergen challenge

	Area under curve (% × 5 min)[a]	
Treatment	*Early asthmatic reaction* (n)	*Late asthmatic reaction* (n)
Saline at 5.5 h and 23.5 h	2647 ± 512[b] (5)	5301 ± 775 (5)
L-NAME at 5.5 h and 23.5 h	3278 ± 428 (7)	5958 ± 1116 (7)
L-NAME at 23.5 h	4181 ± 511 (6)	5618 ± 1086 (6)

[a]Data are expressed as area under the % change in P_{pl} time-response curve between 0 h and 5 h after allergen provocation for the early asthmatic reaction and between 8 h and 23 h after allergen provocation for the late asthmatic reaction (see Methods). [b]Results represent mean values ± s.e.mean of n animals. No statistically significant differences were noticed between the different groups (Student's t test for unpaired observations).

asthmatic reaction. The latter indicates that inhalation of L-NAME after the early reaction (at 5.5 h after allergen challenge) had no effect on the subsequent late reaction. By contrast, in a separate group of animals, we observed that L-NAME significantly enhanced the early asthmatic reaction when inhaled 30 min before the allergen challenge, while inhalation of saline was without effect (Table 2).

Discussion

Using an unrestrained guinea-pig model of allergic asthma, we showed that a deficiency of endogenous NO may contribute to the allergen-induced *in vivo* airway hyperreactivity towards histamine after the early asthmatic reaction, while renewed production or effectiveness of NO appears to be involved in the partial reversal of hyperreactivity after the late asthmatic reaction.

Deficiency of NO and airway hyperreactivity after the early asthmatic reaction

The observation that a deficiency of NO is involved in the airway hyperreactivity after the early asthmatic reaction *in vivo* extends the previous observation by De Boer *et al.* (1996), who demonstrated *ex vivo*, by use of perfused airway preparations, that hyperreactivity to contractile agonists after the allergen-induced early asthmatic reaction was associated with a deficiency of NO.

The deficiency of NO presumably reflects a dysfunctional production or effectiveness of cNOS-derived NO. This is supported by the observation that inhalation of the NOS inhibitor L-NAME did not affect the basal pleural pressure (P_{pl}) in unchallenged guinea-pigs. Furthermore, Persson *et al.* (1994) demonstrated that under basal conditions NO in exhaled air of guinea-pigs is formed from L-arginine in a Ca^{2+}-dependent manner, indicating that a constitutive (cNOS) rather than a inducible isoform (iNOS) of NOS is involved.

In a previous study, these authors demonstrated that cNOS-derived NO may already be reduced during the early asthmatic reaction (Persson & Gustafsson, 1993). Thus, it was shown that allergen provocation of ovalbumin-sensitized guinea-pigs resulted in a direct increase in insufflation pressure, which was paralleled by a rapid but transient (cNOS-derived) increase in NO concentration in the exhaled air, which dropped below basal levels while bronchoconstriction was still present (Persson & Gustafsson, 1993). Apparently, in the initial phase of the early asthmatic reaction, the increase in NO acted as a feedback mechanism against bronchoconstriction, since intraperitoneally administered L-NAME led to a clear potentiation of the allergen-induced bronchoconstriction, which was reversed by inhalation of NO (Persson *et al.*, 1993). In the present study, inhalation of L-NAME 30 min before allergen provocation also resulted in a significant, though modest, increase of the magnitude of the subsequent allergen-induced early asthmatic response, confirming this hypothesis.

Inhalation of L-NAME under basal conditions markedly increased the airway reactivity to histamine, implying that endogenous cNOS-derived NO exerts a functional antagonism to the histamine-induced bronchoconstriction. The cellular source of histamine-induced release of NO *in vivo* is unknown. Since histamine-induced bronchoconstriction in guinea-pig is partly mediated by a vagal reflex mechanism (Santing *et al.*, 1995), neurally formed nNOS-derived NO may be involved (Fischer *et al.*, 1993). In addition, *in vitro* studies with luminally perfused guinea-pig tracheal tube preparations, have indicated that histamine-induced cNOS activity in the airway epithelium may be involved (Nijkamp *et al.*, 1993; De Boer *et al.*, 1996).

Different mechanisms may account for the NO deficiency after the allergen-induced early asthmatic reaction. One possible mechanism that could be important is epithelial damage by eosinophil-derived cytotoxic proteins (Gleich *et al.*, 1988). Evidence for infiltration and activation of eosinophils after the early asthmatic reaction in our model was previously demonstrated by Santing *et al.* (1994a,b). However, in guinea-pig isolated tracheal tube preparations, we recently found that tracheal hyperreactivity to contractile agonists and a deficiency of endogenous NO in these preparations after the early asthmatic reaction were not associated with epithelial shedding (De Boer *et al.*, 1996). A dissociation between tracheal hyperreactivity and epithelial damage was also obtained by Masaki *et al.* (1994). Nevertheless, eosinophilic inflammation and the subsequent release of cytotoxic mediators like major basic protein could lead to reduced tracheal epithelial functions without morphological damage (Flavahan *et al.*, 1988). Furthermore, in the present *in vivo* study, histamine-induced NO may be derived mainly from the pulmonary airway epithelium.

A second possible mechanism which could explain the observed NO deficiency after the allergen-induced early asthmatic reaction, is an altered metabolism of NO by superoxide anions (O_2^-), which are produced during the allergic response (Barnes, 1990). Since O_2^- can rapidly react with locally generated NO to form the metabolite peroxynitrite (Huie & Padmaja, 1993), NO could be scavenged, making it unavailable to act as a functional antagonist in the contractile agonist-induced bronchoconstriction. Evidence for such an NO-scavenging role of O_2^- in the regulation of smooth muscle contraction was previously found in the vasculature (Ignarro *et al.*, 1988). In addition, peroxynitrite may cause damage to the airway epithelium and thus contribute to enhanced airway reactivity to histamine (Sadeghi Hashjin *et al.*, 1996).

Table 2 Effect of the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME, 12 mM, 15 min) inhaled 30 min before allergen provocation, on the allergen-induced early asthmatic reaction

	Early asthmatic reaction *Area under curve* (% × 5 min)[a]	
Treatment	*Before treatment* (n)	*After treatment* (n)
Saline	2436 ± 647[b] (4)	2634 ± 374 (4)
L-NAME	3076 ± 662 (6)	3939 ± 907* (6)

[a]Data are presented as area under the % change in P_{pl} time-response curve (AUC) between 0 h and 5 h after allergen provocation (see Methods). [b]Results represent mean values ± s.e.mean of *n* animals. Statistical analysis: $*P < 0.05$, Student's *t* test for paired data.

Role of NO in the partial reversal of airway hyperreactivity after the late asthmatic reaction

After the late asthmatic reaction (i.e. 23 h after allergen challenge), the allergen-induced airway hyperreactivity towards histamine was less pronounced than after the early asthmatic reaction. The partial reversal of the airway hyperreactivity at this time point was accompanied by the ability of L-NAME to enhance airway hyperreactivity again. Moreover, in the presence of L-NAME airway hyperreactivity was similar to that found after the early asthmatic reaction.

This indicates that recovery of production or effectiveness of NO may account for the partially reversed airway hyperreactivity after the late asthmatic reaction. Since we demonstrated that inhibition of NOS by L-NAME lasts less than 6 h, a direct effect of L-NAME that was administered after the early asthmatic reaction can be precluded.

In asthmatic patients, it has been demonstrated that allergen provocation results in an enhanced concentration of NO in the exhaled air during the late, but not during the early asthmatic response (Kharitonov *et al*., 1995). Furthermore, by use of a [^{3}H]-citrulline assay in pulmonary tissue of ovalbumin-sensitized rats, it was found that allergen provocation resulted in the induction of Ca^{2+}-independent NOS activity during the late asthmatic reaction. This induction of NOS activity was inhibited by pretreatment with a corticosteroid, suggesting inflammation-mediated induction of iNOS (Yeadon & Price, 1995). Therefore, induction of iNOS by cytokines during the late asthmatic reaction could be involved in the reversal of the airway hyperreactivity after the late reaction in our model. This seems to be in conflict with the observation that L-NAME administered before the late reaction (at 5.5 h after allergen challenge) had no significant effect on the magnitude of this reaction. However, since we demonstrated that L-NAME causes inhibition of NOS for less than 6 h, such an effect would not be expected.

Finally, it has recently been established that the inflammatory cytokine interferon-γ (IFN-γ), which is released during the allergic asthmatic response (Borish & Joseph, 1992) and which may induce iNOS (Robbins *et al*., 1994), may also cause inhibition of cNOS expression (Walter *et al*., 1994), indicating that a cytokine-induced switch from cNOS to iNOS may be involved in the observed changes in NO and airway hyperreactivity after allergen challenge.

In conclusion, we demonstrated that a deficiency of cNOS-derived NO may contribute to the allergen-induced airway hyperreactivity towards histamine after the early asthmatic reaction. A restoration of NO deficiency may account for the partly recovered allergen-induced airway hyperreactivity after the late asthmatic reaction. The NOS isozyme involved in this recovery remains to be established.

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