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Role of nitric oxide and superoxide in allergen-induced airway hyperreactivity after the late asthmatic reaction in guinea-pigs

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1 In the present study, the roles of nitric oxide (NO) and superoxide anions (O_2^-) in allergen-induced airway hyperreactivity (AHR) after the late asthmatic reaction (LAR) were investigated *ex vivo*, by examining the effects of the NO synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) and superoxide dismutase (SOD) on the responsiveness to methacholine of isolated perfused guinea-pig treacheae from unchallenged (control) animals and from animals 24 h after ovalbumin challenge.

2 At 24 h after allergen challenge, the animals developed AHR *in vivo*, as indicated by a mean 2.63 ± 0.54 fold ($P<0.05$) increase in sensitivity to histamine inhalation.

3 Compared to unchallenged controls, tracheal preparations from the ovalbumin-challenged guinea-pigs displayed a significant 1.8 fold ($P<0.01$) increase in the maximal response (E_{max}) to methacholine, both after intraluminal (IL) and extraluminal (EL) administration of the agonist. No changes were observed in the sensitivity (pEC_{50}) to the agonist. Consequently, the ΔpEC_{50} (EL-IL), as a measure of epithelial integrity, was unchanged.

4 In the presence of L-NAME (100 μM, IL), tracheae from control guinea-pigs showed a 1.6 fold ($P<0.05$) increase in the E_{max} of IL methacholine. By contrast, the E_{max} of IL methacholine was significantly decreased in the presence of 100 u ml^{-1} EL SOD (54% of control, $P<0.01$).

5 Remarkably, the increased responsiveness to IL methacholine at 24 h after allergen challenge was reversed by L-NAME to control ($P<0.01$), and a similar effect was observed with SOD ($P<0.01$).

6 The results indicate that both NO and O_2^- are involved in the tracheal hyperreactivity to methacholine after the LAR, possibly by promoting airway smooth muscle contraction through the formation of peroxynitrite.

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Abbreviations: AHR, airway hyperreactivity; cNOS, constitutive nitric oxide synthase; EAR, early asthmatic reaction; EL, extraluminal; E_{max}, maximal effect; eNOS, endothelial nitric oxide synthase; IL, intraluminal; iNOS, inducible nitric oxide synthase; KH, Krebs-Henseleit; LAR, late asthmatic reaction; L-NAME, N^{ω}-nitro-L-arginine methyl ester; L-NMMA, N^{G}-monomethyl-L-arginine; nNOS, neuronal nitric oxide synthase; ΔP, differential (hydrostatic) pressure; P_{inlet}, (hydrostatic) pressure at the inlet; P_{outlet}, (hydrostatic) pressure at the outlet; pEC_{50}, $-\log_{10}$ of the concentration causing 50% of the effect; SOD, superoxide dismutase

Introduction

Airway hyperreactivity (AHR) to chemical, physical and pharmacological stimuli, including histamine and methacholine, is a hallmark of allergic asthma. In allergic asthmatics as well as in sensitized guinea-pigs, AHR can be developed both after the early asthmatic reaction (EAR) and the late asthmatic reaction (LAR), which presumably involves airway inflammation due to influx of inflammatory cells into the airways (Durham *et al*., 1988; Aalbers *et al*., 1993; Santing *et al*., 1994).

Endogenous nitric oxide (NO) plays a key role in the physiological regulation of airway function and has been implicated in the pathophysiology of inflammatory airway diseases, including bronchial asthma (Barnes & Belvisi, 1993; Moncada & Higgs, 1993; Barnes, 1998).

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Thus, NO has a potent bronchodilator action by inducing relaxation of airway smooth muscle (Gruetter *et al*., 1989; Dupuy *et al*., 1992), is an important immunomodulator by promoting the proliferation of Th2 lymphocytes (Barnes & Liew, 1995) and, at high concentrations, may have deleterious effects in the airways by causing mucosal swelling, infiltration of inflammatory cells and epithelial damage (Kuo *et al*., 1992; Flak & Goldman, 1996; Schuiling *et al*., 1998a).

In the airways, NO is synthesized by constitutive NO synthase isoforms (cNOS) mainly expressed in inhibitory nonadrenergic noncholinergic nerves (neuronal NOS or nNOS), endothelial cells (endothelial NOS or eNOS) and airway epithelium (nNOS and eNOS) (Fischer *et al*., 1993; Kobzik *et al*., 1993; Asano *et al*., 1994), which are primarily involved in the regulation of airway and vascular tone by the local production of small amounts of NO in reponse to physiological stimuli (Barnes & Belvisi, 1993). The inducible

isoform of NOS (iNOS), producing much larger amounts of NO, may be induced by proinflammatory cytokines during airway inflammation, particularly in inflammatory and epithelial cells (Barnes & Belvisi, 1993; Hamid *et al*., 1993; Asano *et al*., 1994), and may be involved in the detrimental effects described above.

Studies *in vitro* and *in vivo* have indicated that endogenous NO is involved in the regulation of airway reactivity to bronchoconstrictor stimuli. Thus, in isolated guinea-pig tracheal tube preparations it was demonstrated that non-selective NOS inhibitors such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) and N^{G}-monomethyl-L-arginine (L-NMMA) cause enhanced muscarinic agonist-, histamine-, substance P- and bradykinin-induced airway constriction *in vitro* (Nijkamp *et al*., 1993; De Boer *et al*., 1996; Figini *et al*., 1996; 1997). *In vivo*, inhaled NO reversed histamine and methacholine-induced bronchoconstriction in mild asthmatics (Kacmarek *et al*., 1996), guinea-pigs (Dupuy *et al*., 1992) and dogs (Brown *et al*., 1994), while administration of L-NAME or L-NMMA caused enhanced bronchoconstriction in response to allergen (Persson *et al*., 1993), histamine (Nijkamp *et al*., 1993; Schuiling *et al*., 1998b), bradykinin (Ricciardolo *et al*., 1994) and NKA (Ricciardolo *et al*., 2000) in the guinea-pig and to methacholine, bradykinin, histamine and adenosine 5′-monophosphate in mild asthmatics (Ricciardolo *et al*., 1997; Taylor *et al*., 1998).

In a guinea-pig model of acute allergic asthma, characterized by allergen-induced early and late asthmatic reactions, airway inflammation and AHR after both reactions (Santing *et al*., 1994), we have previously demonstrated both *ex vivo* (De Boer *et al*., 1996) and *in vivo* (Schuiling *et al*., 1998a,b) that a deficiency of cNOS-derived NO contributes to the AHR after the EAR. *In vivo*, using the same animal model, we have also demonstrated that restoration of NO activity by induction of iNOS during the LAR may have both beneficial – bronchodilating – and detrimental – proinflammatory – effects on the AHR observed after the LAR, indicating the dualistic action of iNOS-derived NO in the airways (Schuiling *et al*., 1998a).

Both in guinea-pigs and in asthmatics there is evidence that at least some of the deleterious effects induced by iNOS-derived NO, including enhanced vascular permeability and epithelial damage, may proceed *via* peroxynitrite ($ONOO^-$), a cytotoxic and more stable oxidant formed by the reaction between NO and superoxide anion (O_2^-) generated by activated inflammatory cells during the allergic reaction (Beckman & Koppenol, 1996; Sadeghi-Hashjin *et al*., 1996; Saleh *et al*., 1998; Sugiura *et al*., 1999). Therefore, in the present study, we investigated the roles of endogenous NO and O_2^- in the allergen-induced AHR after the LAR *ex vivo*, by examining the effects of L-NAME and the O_2^- scavenger superoxide dismutase (SOD) on the responsiveness to methacholine of isolated perfused tracheal tube preparations from animals at 24 h after allergen challenge.

Methods

Animals

Outbred specified 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 3 weeks of age as described previously (Van Amsterdam *et al*., 1989). The animals were operated on 3 weeks after sensitization and used experimentally 4 – 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 in this study were approved by the University of Groningen Committee for Animal Experimentation.

Measurement of airway function

Airway function was assessed by on-line measurement of pleural pressure (P_{pI}) under unrestrained conditions, as described by Santing *et al*. (1992). Briefly, a small latex balloon (HSE, Freiburg, Germany), connected to a saline-filled canula, was surgically implanted inside the thoracic cavity. The free end of the canula was driven subcutaneously to and permanently attached in the neck of the animal. After connection *via* an external saline-filled canula to a pressure transducer (TC-XX, Viggo-Spectramed B.V., Bilthoven, The Netherlands), P_{pI} was measured in centimetres of H_2O, using an on-line computer system.

Provocation techniques

Ovalbumin and histamine provocations were performed by inhalation of aerosolized solutions. The provocations were performed in a specially designed 9 l animal cage, in which the guinea-pigs could move freely (Santing *et al*., 1992). 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 μg ml^{-1} in saline, followed by increasing dosage steps of 25 μg 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 intrapolation of the concentration-P_{pl} response curve.

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 each, separated by 7-min intervals. Allergen inhalations were discontinued when an increase in P_{pl} of more than 100% was observed. No anti-histamine was needed to prevent anaphylactic shock. All 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 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 24 h after allergen provocation (after the LAR; Santing *et al*., 1992; 1994) 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 24 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 24 h after ovalbumine challenge, the guinea-pigs were killed by a sharp blow on the head and exsanguinated. Non-challenged IgE-sensitized animals were used as controls. 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 approximately 17 mm before mounting in a perfusion setup, as described previously (De Boer *et al*., 1996). 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 extraluminal (EL) compartment). The lumen was perfused with recirculating KH from a separate 20 ml bath (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 at these sites. The signals were fed into a differential amplifier to obtain the difference between the two pressures (ΔP), which was plotted on a flatbed chart recorder. 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. The transmural pressure in the trachea was set at 0 cm H_2O. At the perfusion flow rate used, a baseline ΔP of 0.1 to 1.0 cm H_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 to assess basal tone. After three washes (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 four times with KH during 45 min until basal tone was reached and a consecutive cumulative concentration response curve (CCRC) was made with IL methacholine. In some experiments, the CCRC to IL methacholine was followed by a CCRC to EL methacholine. When used, L-NAME (100 μM) was applied in the IL reservoir 45 min prior to agonist addition. SOD (100 u ml^{-1}) was administered to the EL reservoir 30 min prior to agonist addition (De Boer *et al*., 1998).

Data analysis

To compensate for differences in ΔP due to variation in internal diameter of the tracheal preparations used, responses to EL and IL methacholine were expressed as a percentage of the response induced by EL 40 mM KCl. The contractile effect of 10 mM methacholine (highest concentration) was defined as E_{max} (De Boer *et al*., 1996). Using this E_{max} the sensitivity to the agonist was evaluated as pEC_{50} ($-\log_{10} EC_{50}$). 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 24 h after the allergen provocation, respectively (PC_{100} ratio pre/post allergen challenge).

Statistical analysis was performed using the Student's *t*-test for unpaired observations. Differences were considered statistically significant at $P<0.05$.

Chemicals

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

Results

At 24 h after allergen challenge, the guinea-pigs displayed AHR *in vivo*, as indicated by a histamine PC_{100} ratio (pre/post allergen challenge) of 2.63 ± 0.54 ($n=12$; $P<0.05$). Perfused tracheal tube preparations obtained from these animals showed a 1.8 fold increase in E_{max} to both IL and EL methacholine when compared with preparations obtained from unchallenged controls ($P<0.01$; Figure 1 and Table 1), without a change in the absolute reference response to KCl ($\Delta P = 8.86 \pm 2.77$ cmH_2O in the challenged group *vs* 9.37 ± 1.20 cmH_2O in controls). No significant effects were

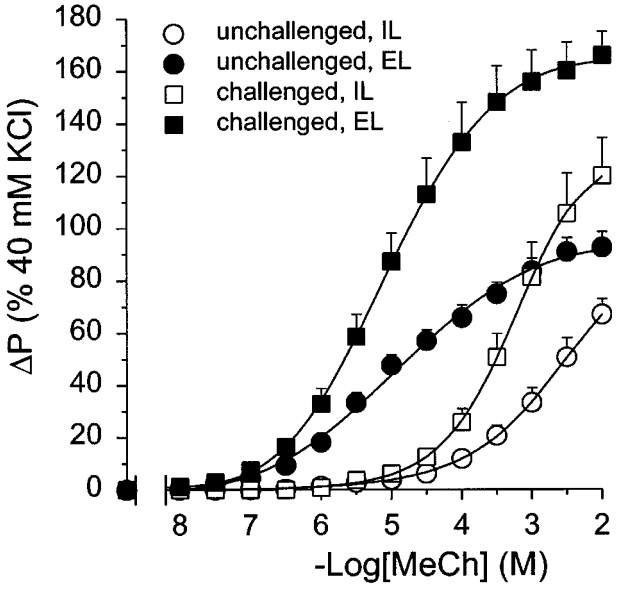

Figure 1 Methacholine (MeCh)-induced constriction of intact perfused tracheae obtained from unchallenged guinea-pigs and from guinea-pigs at 24 h after allergen challenge, after EL and IL administration of the agonist. Results are means ± s.e.mean of nine experiments.

observed on the sensitivity to methacholine (pEC_{50}; Table 1) and on basal tone (not shown). Moreover, allergen challenge did not affect the difference in sensitivity to EL and IL methacholine, the ΔpEC_{50} (EL-IL) amounting to 2.01 ± 0.13 and 1.84 ± 0.19 $\log_{10}$ units for control and challenged airways, respectively.

In perfused tracheal preparations from unchallenged guinea-pigs, IL administration of 100 μM L-NAME caused a significant 1.6 fold increase in the E_{max} of IL methacholine ($P<0.05$), without a significant effect on the pEC_{50} to the agonist (Figure 2, Table 1). By contrast, inactivation of O_2^- with SOD (100 u ml^{-1}, EL) in unchallenged control airways resulted in a significant decrease in the E_{max} to IL methacholine to 54% of control ($P<0.01$), while no significant effect was observed on methacholine pEC_{50} (Table 1).

The enhanced E_{max} to IL methacholine at 24 h after allergen challenge was significantly reduced in the presence of L-NAME (100 μM, IL) by 57% ($P<0.01$), to a level similar to that of unchallenged control preparations (Figure 3 and Table 1). No change in the pEC_{50} to methacholine was observed (Table 1). In the presence of SOD (100 u ml^{-1}, EL) the enhanced responsiveness to IL methacholine was similarly attenuated (by 53%, $P<0.01$), while there was a small but significant decrease in the sensitivity to the agonist (Figure 3 and Table 1). Both in the control preparations and in the preparations from ovalbumin-challenged animals, L-NAME and SOD had no effect on basal tone (not shown).

Discussion

Using perfused tracheal tube preparations from unchallenged and ovalbumin-challenged guinea-pigs, we demonstrated that allergen-induced AHR after the LAR *in vivo* is associated with a significant increase in tracheal responsiveness to both IL and EL methacholine *ex vivo*.

Table 1 IL and EL methacholine responses in the absence or presence of L-NAME and SOD of intact perfused tracheae from unchallenged and ovalbumin-challenged guinea-pigs

	IL methacholine		*EL methacholine*	
	E_{max} (% KCl)	pEC_{50} (−log M)	E_{max} (% KCl)	pEC_{50} (−log M)
Unchallenged				
Vehicle	67.0±5.9	2.95±0.14	92.6±5.9	4.96±0.18
100 μM L-NAME§	109.0±16.8*	3.20±0.09	N.D.	N.D.
100 u ml^{-1} SOD§	36.0±4.5**	2.73±0.06	N.D.	N.D.
24 h after challenge				
Vehicle	120.0±14.5**	3.32±0.12	165.8±9.2***	5.16±0.11
100 μM L-NAME	51.7±6.5††/‡‡	3.09±0.11	N.D.	N.D.
100 u ml^{-1} SOD	56.4±8.8††	2.86±0.05††	N.D.	N.D.

Results are means±s.e.mean of 6–12 experiments. §Data derived from De Boer *et al*., 1998. N.D., not determined. Statistical analysis: *$P<0.05$; **$P<0.01$, ***$P<0.001$ compared to vehicle unchallenged, ††$P<0.01$ compared to vehicle 24 h after challenge, ‡‡$P<0.01$ compared to L-NAME unchallenged.

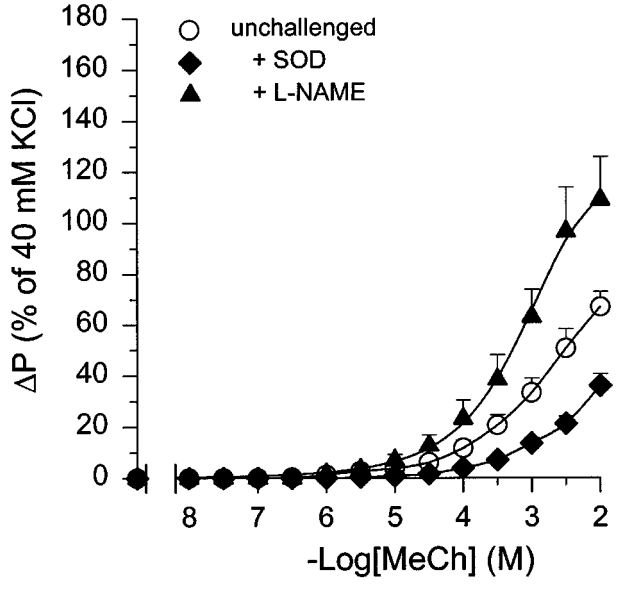

Figure 2 Effect of 100 μM L-NAME (IL) and 100 u ml^{-1} SOD (EL) on methacholine (MeCh; IL)-induced constriction of intact perfused tracheae from unchallenged guinea-pigs. Results are means±s.e.mean of 7–12 experiments.

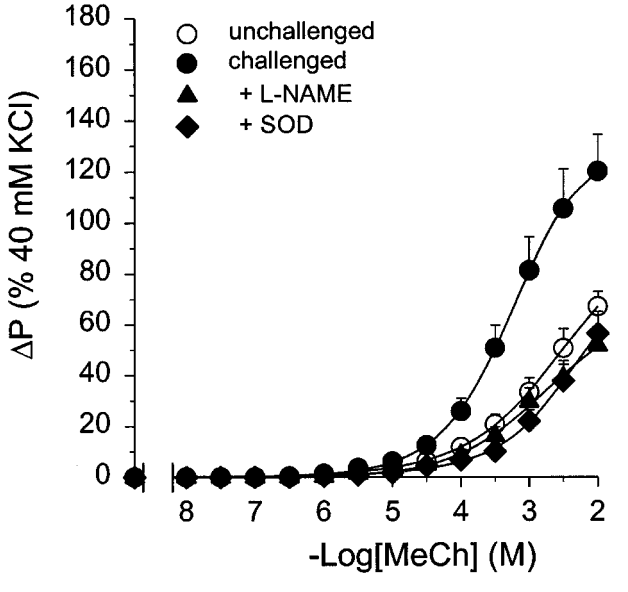

Figure 3 Methacholine (MeCh; IL)-induced constriction of intact perfused tracheae obtained from unchallenged guinea-pigs and from guinea-pigs at 24 h after allergen challenge, in the absence or presence of 100 μM L-NAME (IL) and 100 u ml^{-1} SOD (EL). Results are means±s.e.mean of 6–9 experiments.

Remarkably, the AHR to IL methacholine after the LAR was significantly reduced in the presence of the nonselective NOS inhibitor L-NAME, indicating a role for NO in the enhanced agonist responsiveness. In the presence of the O_2^- scavenger SOD a similar reduction in responsiveness to IL methacholine was observed as with L-NAME, which indicates that O_2^- is also involved, *via* a common pathway. This common pathway could involve the formation of $ONOO^-$, the rapidly formed and highly oxidative reaction product of NO and O_2^- (Huie & Padmaja, 1993; Beckman & Koppenol, 1996), which has been demonstrated to induce AHR in guinea-pigs (Sadeghi-Hashjin *et al*., 1996). The possible involvement of $ONOO^-$ is supported by the recently observed enhanced 3-nitrotyrosine immunostaining in the airways of allergen-challenged guinea-pigs during the LAR (Sugiura *et al*., 1999). $ONOO^-$ leads to nitration of tyrosine residues in proteins at the 3-position adjacent to the hydroxyl group and 3-nitrotyrosine formation may hence provide evidence of local generation of $ONOO^-$. However, tyrosine nitration may also be induced by other reactive nitrogen intermediates generated by eosinophil peroxidase and myeloperoxidase, using the O_2^- metabolite H_2O_2 and the NO metabolite NO_2^- as substrates (Eiserich *et al*., 1998; Wu *et al*., 1999). The presence of nitrotyrosine has recently also been demonstrated in the airways (Saleh *et al*., 1998; Kaminsky *et al*., 1999) and exhaled air (Hanazawa *et al*., 2000) of asthmatics, the amount of nitrotyrosine immunostaining in the epithelium and inflammatory cells showing a significant inverse correlation with the methacholine PC_{20} and FEV_1 (Saleh *et al*., 1998).

The NO in the present study is presumably produced by iNOS, which is induced during the LAR both in asthmatics (Kharitonov *et al*., 1995) and in animal models, including guinea-pigs (Yan *et al*., 1995; Schuiling *et al*., 1998a). In asthmatic airways, iNOS may be present in the epithelium and in some inflammatory cells (Hamid *et al*., 1993; Renzi *et al*., 1997; Saleh *et al*., 1998), while inflammatory cells are also the likely source for the release of O_2^- after allergen challenge (Meltzer *et al*., 1989; Calhoun *et al*., 1992; Cerasoli *et al*., 1991).

The precise mechanism of $ONOO^-$ induced AHR is not known. $ONOO^-$ has cytotoxic actions, which may cause epithelial damage in the airways at relatively high concentrations of the oxidant (Sadeghi-Hashjin *et al*., 1996). However, no significant changes were observed for the pEC_{50} values of methacholine (both IL and EL) as well as for the ΔpEC_{50} (EL-IL), indicating that the enhanced responsiveness is not due to loss of the epithelial barrier. This corresponds to previous observations of enhanced tracheal responsiveness after the EAR, at 6 h after allergen provocation (De Boer *et al*., 1996). Moreover, epithelial damage is unlikely to be restored after the acute inhibition of NO production and scavenging of O_2^- according to our protocol.

Alternatively, several observations have indicated that $ONOO^-$ may also promote smooth muscle contraction by a number of mechanisms, which could be more rapidly reversible. First, it has been demonstrated that $ONOO^-$ reversibly inhibits hyperpolarizing Ca^{2+}-activated K^+ channels in rat cerebral artery smooth muscle cells, leading to enhanced smooth muscle contraction and vasoconstriction (Elliott *et al*., 1998; Brzezinska *et al*., 2000). In addition, in skeletal muscle $ONOO^-$ may cause reversible inhibition of sarco/endoplasmatic reticulum ATPase (SERCA) type 2 (Viner *et al*., 1996a,b), which is also present in airway smooth muscle (Grover & Kahn, 1992). In the vasculature, $ONOO^-$ has been shown to enhance prostaglandin H_2 synthesis, either indirectly by inhibition of PGI_2 synthase, causing enhanced PGH_2 levels to act on the TxA_2/PGH_2 receptor before it is converted to PGE_2 (Zou & Ullrich, 1996; Zou *et al*., 1999), or directly by activating PGH_2 synthase (Upmacis *et al*., 1999). Evidence for PGH_2 synthase activation by $ONOO^-$ has also been observed in cultured, immunostimulated macrophages (Landino *et al*., 1996). In line with these observations, in the dog coronary endothelium evidence was obtained for the generation of an $ONOO^-$-dependent contractile factor after global cardiac ischaemia and reperfusion (Pearson *et al*., 1991).

Remarkably, L-NAME inhibited the enhanced methacholine-induced airway constriction after allergen challenge to a level that is normally observed in unchallenged animals in the absence of a NOS inhibitor, i.e. in the presence of cNOS-derived NO that counteracts methacholine-induced constriction. This indicates that the methacholine-induced airway constriction at 24 h after allergen challenge may be functionally antagonized by one or more L-NAME-insensitive relaxing factors. These relaxing factors could include stable, biologically active S-nitrosothiols, which have been demonstrated in the airways (Gaston *et al*., 1993), and which may be generated by NO and $ONOO^-$ (Stamler *et al*., 1992; Stamler, 1994, Wu *et al*., 1994) that are formed during the LAR. Thus, $ONOO^-$ may have both detrimental and beneficial properties in the airways, as in the cardiovascular system (Ronson *et al*., 1999).

As demonstrated previously (De Boer *et al*., 1998), methacholine-induced tracheal constriction in unchallenged control airways was decreased in the presence of SOD. The SOD-induced decrease in responsiveness can be reversed by L-NAME (De Boer *et al*., 1998), indicating that in normoreactive tracheal preparations smooth muscle relaxation by cNOS-derived NO is limited by its reaction with endogenous O_2^-, as has also been observed in the vasculature (Ignarro *et al*., 1987).

Using the same guinea-pig model of allergic asthma, we have previously demonstrated both *ex vivo* and *in vivo* that a deficiency of (cNOS-derived) NO contributes to the AHR after the EAR, at 6 h after inhalational challenge of the animals with ovalbumin aerosol (De Boer *et al*., 1996; Schuiling *et al*., 1998a, b). In perfused tracheal preparations of these animals, it was demonstrated that this deficiency of NO is not caused by its possible reaction with enhanced levels inflammation-induced superoxide anion (O_2^-) in the airways (De Boer *et al*., 1998), but rather by limitation of L-arginine as a substrate for cNOS (De Boer *et al*., 1999). Recent investigations have indicated that this reduced availability of substrate may be caused by reduced cellular uptake of L-arginine by cationic amino acid transporters induced by eosinophil-derived polycations (Meurs *et al*., 1999; Hammermann *et al*., 1999), which are present in the inflamed airways both at 6 and 24 h after allergen challenge (Santing *et al*., 1994). Another mechanism that could be involved is enhanced competition between cNOS and arginase for the substrate (Meurs *et al*., 2000), possibly by enhanced expression of the latter enzyme by Th2 lymphocyte-derived cytokines (Modolell *et al*., 1995).

In vivo, we demonstrated that the deficiency of NO after the EAR is reversed during the LAR, presumably by the induction of iNOS (Schuiling *et al*., 1998a). In the same study, it was shown that iNOS-derived NO has both detrimental effects on the airway reactivity, by promoting airway inflammation, and beneficial effects by causing bronchodilation. The latter observation seems to be at variance with the results obtained in the present study. However, it is important to note that in the present *ex vivo* study tracheal preparations were used, which may not fully reflect the balance between detrimental and beneficial effects of NO in the entire respiratory tract. This is also indicated by the observation that the AHR *in vivo* is partially reversed after the LAR (Schuiling *et al*., 1998a, b), whereas in the present *ex vivo* study the AHR after the LAR was of similar magnitude as that observed after the EAR (1.8 fold increase in E_{max}, De Boer *et al*., 1996).

In conclusion, in isolated perfused tracheal tube preparations obtained from actively IgE-sensitized guinea-pigs at 24 h after allergen provocation, the responsiveness to methacholine is increased compared to preparations obtained from non-challenged animals. Both the nonselective NOS inhibitor L-NAME and the O_2^- scavanger SOD significantly inhibited the observed hyperresponsiveness to a similar degree, indicating that NO and O_2^- are involved by a common pathway, presumably through the formation of their reaction product, $ONOO^-$. Thus, in contrast to the airway hyperresponsiveness after the early asthmatic reaction in which deficiency of cNOS-derived NO is a major determinant, tracheal hyperresponsiveness after the late asthmatic reaction appears to involve the generation of a NO-derived pro-contractile factor.

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