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Modulation of cholinergic airway reactivity and nitric oxide production by endogenous arginase activity

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1 Cholinergic airway constriction is functionally antagonized by agonist-induced constitutive nitric oxide synthase (cNOS)-derived nitric oxide (NO). Since cNOS and arginase, which hydrolyzes L-arginine to L-ornithine and urea, use L-arginine as a common substrate, competition between both enzymes for the substrate could be involved in the regulation of cholinergic airway reactivity. Using a perfused guinea-pig tracheal tube preparation, we investigated the modulation of methacholine-induced airway constriction by the recently developed, potent and specific arginase inhibitor N^{ω}-hydroxy-nor-L-arginine (nor-NOHA).

2 Intraluminal (IL) administration of nor-NOHA caused a concentration-dependent inhibition of the maximal effect (E_{max}) in response to IL methacholine, which was maximal in the presence of 5 μM nor-NOHA ($E_{max} = 31.2 \pm 1.6\%$ of extraluminal (EL) 40 mM KCl-induced constriction *versus* $51.6 \pm 2.1\%$ in controls, $P < 0.001$). In addition, the pEC_{50} ($-\log_{10}$ EC_{50}) was slightly but significantly reduced in the presence of 5 μM nor-NOHA.

3 The inhibition of E_{max} by 5 μM nor-NOHA was concentration-dependently reversed by the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME), reaching an E_{max} of $89.4 \pm 7.7\%$ in the presence of 0.5 mM L-NAME ($P < 0.01$). A similar E_{max} in the presence of 0.5 mM L-NAME was obtained in control preparations ($85.2 \pm 9.7\%$, n.s.).

4 In the presence of excess of exogenously applied L-arginine (5 mM), 5 μM nor-NOHA was ineffective ($E_{max} = 33.1 \pm 5.8$ *versus* $31.1 \pm 7.5\%$ in controls, n.s.).

5 The results indicate that endogenous arginase activity potentiates methacholine-induced airway constriction by inhibition of NO production, presumably by competition with cNOS for the common substrate, L-arginine. This finding may represent an important novel regulation mechanism of airway reactivity.

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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; L-NAME, N^{ω}-nitro-L-arginine methyl ester; L-NMMA, N^{G}-monomethyl-L-arginine; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; nor-NOHA, N^{ω}-hydroxy-nor-L-arginine; 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

Introduction

It is now well established that nitric oxide (NO) is importantly involved in the regulation of airway tone as well as in asthmatic inflammation (Barnes & Belvisi, 1993; Gaston *et al*., 1994; Barnes & Liew, 1995). Thus, NO causes bronchodilation by inducing airway smooth muscle relaxation (Gruetter *et al*., 1989; Dupuy *et al*., 1992), is an important immunomodulator by promoting selective proliferation of Th2 lymphocytes (Barnes & Liew, 1995), and, at high concentrations, has deleterious effects in the airways by causing mucosal swelling, infiltration of inflammatory cells, epithelial damage and airway hyperreactivity (AHR) (Kuo *et al*., 1992; Flak & Goldman, 1996; Schuiling *et al*., 1998a).

NO is synthesized from the semi-essential amino acid L-arginine by the enzyme NO synthase (NOS), of which different isoforms have been identified in the airways. Constitutive NOS (cNOS) isoforms are mainly expressed in inhibitory non-adrenergic noncholinergic nerves (neuronal NOS or nNOS), endothelial cells (endothelial NOS or eNOS) and epithelial cells (nNOS and eNOS) of the airways (Fischer *et al*., 1993; Kobzik *et al*., 1993; Asano *et al*., 1994), and are thought to be primarily involved in the regulation of airway and vascular tone by local production of small amounts of NO in response to neurogenic and non-neurogenic stimuli (Barnes & Belvisi, 1993; Gaston *et al*., 1994). Inducible NOS (iNOS), producing large amounts of NO, is induced by proinflammatory cytokines during airway inflammation, and may be present in macrophages and epithelial cells (Barnes & Belvisi, 1993; Hamid *et al*., 1993; Asano *et al*., 1994). In addition to bronchodilation, high concentrations of iNOS-derived NO may be involved in the detrimental effects described above (Schuiling *et al*., 1998a).

The involvement of endogenous NO in the regulation of airway tone is indicated by observations that NOS inhibitors such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) and N^{G}-monomethyl-L-arginine (L-NMMA) enhanced muscarinic agonist-, histamine- and substance P-induced constriction of

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intact perfused guinea-pig tracheal tube preparations *in vitro* (Nijkamp *et al*., 1993; De Boer *et al*., 1996; Figini *et al*., 1997) as well as bronchoconstriction in response to allergen, methacholine, histamine and bradykinin in guinea-pigs and/or asthmatic patients *in vivo* (Persson *et al*., 1993; Nijkamp *et al*., 1993; Ricciardolo *et al*., 1994; 1996; Mehta *et al*., 1997a, b; Schuiling *et al*., 1998b).

In many cells throughout the body, including the lung (Que *et al*., 1998), L-arginine is also metabolized by arginase to ornithine and urea. Two isoforms of mammalian arginase have been identified (Jenkinson *et al*., 1996). Type I arginase (arginase I) is a cytosolic enzyme, mainly expressed in liver as a component of the urea cycle, whereas type II arginase (arginase II) is a mitochondrial enzyme only expressed in extrahepatic tissue. Because extrahepatic tissues do not possess a complete urea cycle, the biological function of arginase in these tissues is presently unclear. One function of extrahepatic arginase might be the production of L-ornithine, a precursor of polyamines and proline, which are important in cell growth and tissue repair (Jenkinson *et al*., 1996). In addition, since arginase and NOS use L-arginine as a common substrate, it has been suggested that arginase may be important in the regulation of NO production by limiting the availability of intracellular L-arginine. Evidence for this hypothesis has particularly been obtained in activated macrophages or macrophage-like cells, which express high levels of arginase and iNOS (Wang *et al*., 1995; Modolell *et al*., 1995; Hey *et al*., 1997; Morris *et al*., 1998; Chang *et al*., 1998; Boucher *et al*., 1999). Moreover, evidence for reciprocal regulation of arginase and cNOS has recently also been obtained (Baggio *et al*., 1999; Cox *et al*., 1999).

Recent immunohistochemical studies in rat lung have indicated that both arginase I and arginase II are constitutively expressed in the airways, particularly in the bronchial epithelium and in peribronchial connective tissue fibroblasts (Que *et al*., 1998). The expression of both isotypes in the airways was upregulated during hyperoxic lung injury and recovery, and associated with reduced NO production in the lung, which may indicate a role for arginase in inflammatory tissue repair (Que *et al*., 1998).

The role of arginase with respect to airway function is presently unknown. Since both arginase and cNOS are constitutively expressed in the airway epithelium, and activation of cNOS in the airway epithelium by contractile agonists appears to dampen airway constriction in response to these agonists (Nijkamp *et al*., 1993), we hypothesized that competition between both enzymes for their common substrate may be involved in the modulation of cholinergic airway reactivity. Therefore, using a perfused guinea-pig tracheal tube preparation, we investigated the modulation of methacholine-induced airway constriction by the recently developed highly potent and specific arginase inhibitor N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) (Custot *et al*., 1997; Tenu *et al*., 1999). Nor-NOHA is a new analogue of the endogenous intermediate in NO synthesis and arginase inhibitor N^{ω}-hydroxy-L-arginine (NOHA), bearing a shorter lateral side chain with one CH_2, group less (Custot *et al*., 1997).

Methods

Tracheal perfusion

Specified pathogen-free Dunkin Hartley guinea-pigs (Harlan, Heathfield, UK), weighing 600–800 g, were used in this study. The animals were killed by a sharp blow on the head and exsanguinated. The tracheas were rapidly removed and placed in Krebs-Henseleit (KH) solution (37°C) of the following composition (mM): NaCl 117.5, KCl 5.60, $MgSO_4$ 1.18, $CaCl_2$ 2.50, NaH_2PO_4 1.28, $NaHCO_3$ 25.0, D-glucose 5.50; gassed with 5% CO_2 and 95% O_2; pH 7.4.

The tracheas 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). To this aim, 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 gassed KH from a separate 20 ml bath (mucosal or IL compartment) at a constant flow rate of 18 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. 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.

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 four times with KH during 45 min and a cumulative concentration response curve was made with IL methacholine. When used, L-NAME (0.1, 0.5 or 1 mM) and nor-NOHA (1, 5 or 10 μM) were applied to the IL reservoir, 40 min prior to agonist-addition. L-arginine (0.3, 1.0 or 5.0 mM) was added to the EL reservoir, 30 min prior to agonist addition.

Data analysis

To compensate for differences in ΔP due to variation in resting internal diameter of the preparations used, IL responses of the tracheal tube preparations to methacholine were expressed as a percentage of the response induced by EL administration of 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 methacholine was evaluated as pEC_{50} ($-\log_{10}EC_{50}$) value. Data are expressed as means ± s.e.mean. Statistical analysis was performed using the Student's *t*-test for unpaired observations. Differences were considered statistically significant at $P<0.05$.

Chemicals

Methacholine chloride, L-arginine hydrochloride, N^{ω}-nitro-L-arginine methyl ester and L-norvaline were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). N^{ω}-hydroxy-nor-L-arginine was synthesized as described previously (Custot *et al*., 1997).

Results

IL perfusion of the tracheae with the arginase inhibitor nor-NOHA caused a concentration-dependent decrease in metha-

choline-induced airway constriction (Figure 1, Table 1). In the presence of 5 μM nor-NOHA, the E_{max} to methacholine was maximally reduced to approximately 60% of control ($P<0.001$). In addition, the pEC_{50} value for the agonist was slightly reduced in the presence of the arginase inhibitor (Table 1). In the presence of 10 mM of the specific, but less potent arginase inhibitor L-norvaline (Chang *et al*., 1998), an inhibition of methacholine-induced airway constriction was also observed, which was virtually identical to that obtained by 10 μM nor-NOHA (Figure 2).

The reduced responsiveness to methacholine in the presence of 5 μM nor-NOHA was concentration-dependently reversed by the NOS inhibitor L-NAME (Figure 3, Table 1). In the presence of 0.1 mM L-NAME (IL), the methacholine response in the norNOHA-treated airways was normalized to the control response, while a further, maximal increase in responsiveness to about 1.7-fold control was obtained in the presence of 0.5 mM L-NAME ($P<0.001$). In control preparations, the responsiveness to methacholine was also significantly enhanced by the NOS inhibitor; however, the increase in E_{max} was already maximal in the presence of 0.1 mM L-NAME (1.8-fold; Figure 4, Table 1). The responsiveness of the norNOHA-treated airways in the presence of 0.5 mM L-NAME was very similar to that of the control preparations in the presence of 0.1 and 0.5 mM of the NOS inhibitor (Figures 3 and 4, Table 1).

We have previously established that administration of exogenous arginine significantly suppressed the responsiveness to methacholine in a concentration-dependent manner (De Boer *et al*., 1999; Figure 5, Table 1). In the presence of an excess of 5 mM L-arginine, the E_{max} for methacholine was reduced to about 60% of control ($P<0.01$), as in the presence of 5 μM nor-NOHA (Figure 6, Table 1). In the presence of 5 mM L-arginine, 5 μM nor-NOHA had no further effect on the responsiveness to methacholine (Figure 6, Table 1).

Nor-NOHA, L-NAME and L-arginine had no effect on basal airway tone (not shown).

Discussion

Using the recently developed, highly potent arginase inhibitor nor-NOHA, as well as the specific but less potent arginase inhibitor L-norvaline, we demonstrated that constitutive endogenous arginase activity is involved in the regulation of

Table 1 Effects of (combinations of) nor-NOHA, L-norvaline, L-NAME and L-arginine on the responsiveness to methacholine of intact perfused guinea-pig tracheae

	E_{max} (% KCl)	pEC_{50} (−log M)
Control	51.6±2.1	2.91±0.10
Nor-NOHA		
1.0 μM	51.2±0.6	2.78±0.06
5.0 μM	31.2±1.6***	2.43±0.02*
10.0 μM	33.1±3.5***	2.53±0.17
L-norvaline		
10.0 mM	31.1±2.9***	2.82±0.06
L-NAME		
0.1 mM	92.7±8.7***	3.15±0.18
0.5 mM	85.2±9.7***	3.47±0.22*
5.0 μM nor-NOHA +		
0.1 mM L-NAME	58.7±9.8‡	2.74±0.21
0.5 mM L-NAME	89.4±7.7$^{\dagger\dagger/***}$	3.13±0.17†
1.0 mM L-NAME	83.7±7.0$^{\dagger\dagger/***}$	3.26±0.16†
L-arginine§		
0.3 mM	33.0±4.7**	3.33±0.40
1.0 mM	32.9±3.4***	2.86±0.10
5.0 mM	31.1±7.5**	2.68±0.13
5.0 mM L-arginine +		
5.0 μM nor-NOHA	33.1±5.8**	2.73±0.18

Results are means±s.e.mean of 3–10 experiments. §Data derived from De Boer *et al*., 1999. Statistical analysis: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared to Control; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ compared to 5 μM nor-NOHA; $^{\ddagger}P<0.05$ compared to 0.1 mM L-NAME.

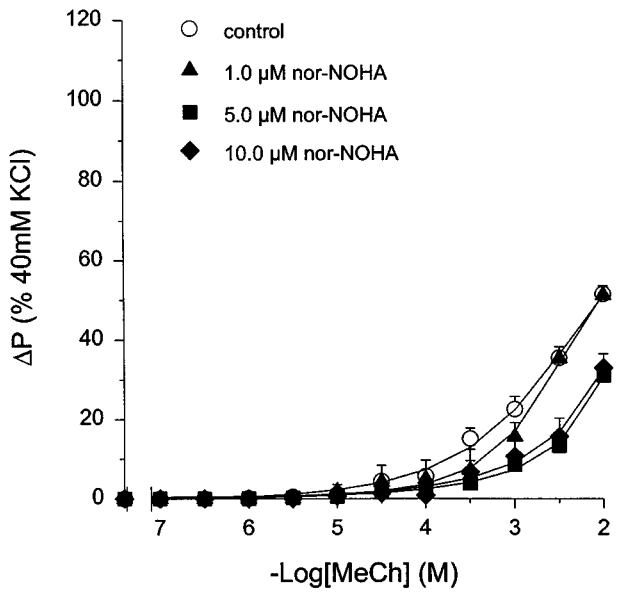

Figure 1 Effect of 1.0, 5.0 and 10.0 μM nor-NOHA (IL) on methacholine (MeCh; IL)-induced constriction of intact perfused guinea-pig tracheae. Results are means±s.e.mean of 3–10 experiments.

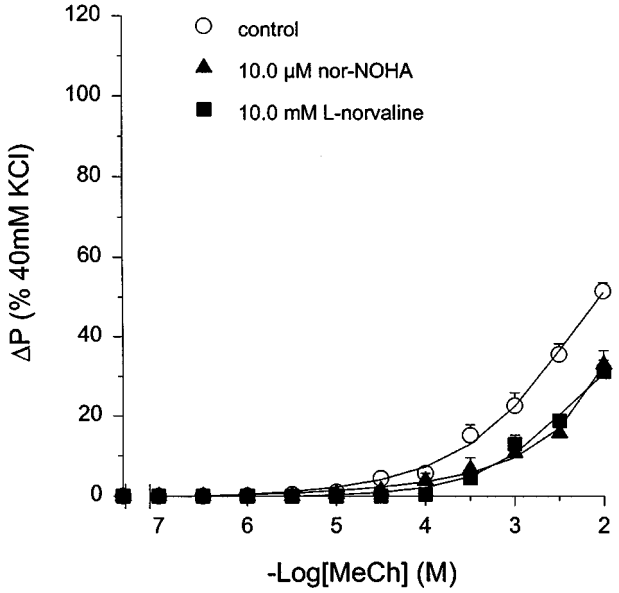

Figure 2 Effect of 10.0 mM L-norvaline (IL) and 10.0 μM nor-NOHA (IL) on methacholine (MeCh; IL)-induced constriction of intact perfused guinea-pig tracheae. Results are means±s.e.mean of 3–10 experiments.

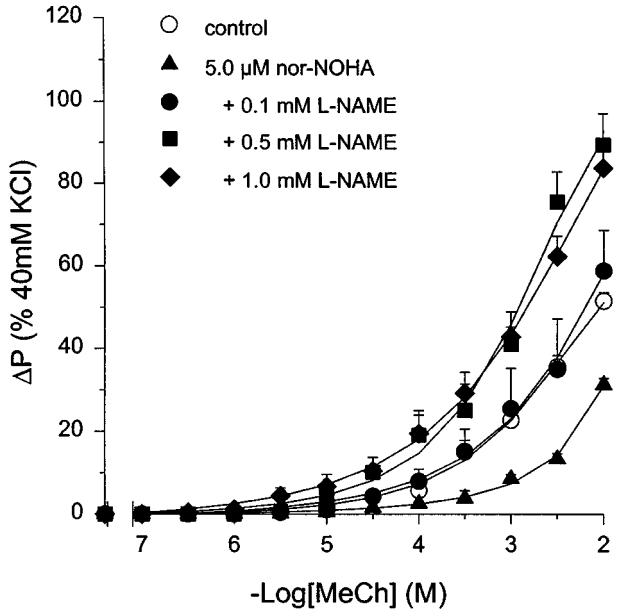

Figure 3 Effect of 5.0 μM nor-NOHA (IL) in the absence and presence of 0.1, 0.5 and 1.0 mM L-NAME (IL) on methacholine (MeCh; IL)-induced constriction of intact perfused guinea-pig tracheae. Results are means$\pm$s.e.mean of 4–10 experiments.

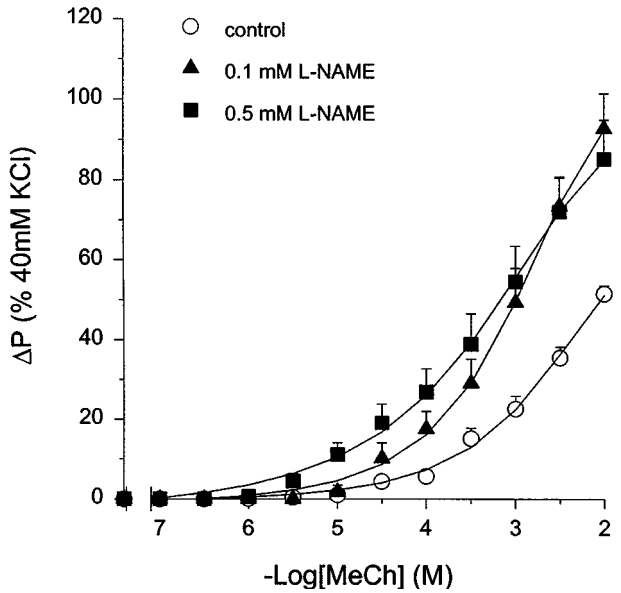

Figure 4 Effect of 0.1 and 0.5 mM L-NAME (IL) on methacholine (MeCh; IL)-induced constriction of intact perfused guinea-pig tracheae. Results are means$\pm$s.e.mean of 4–10 experiments.

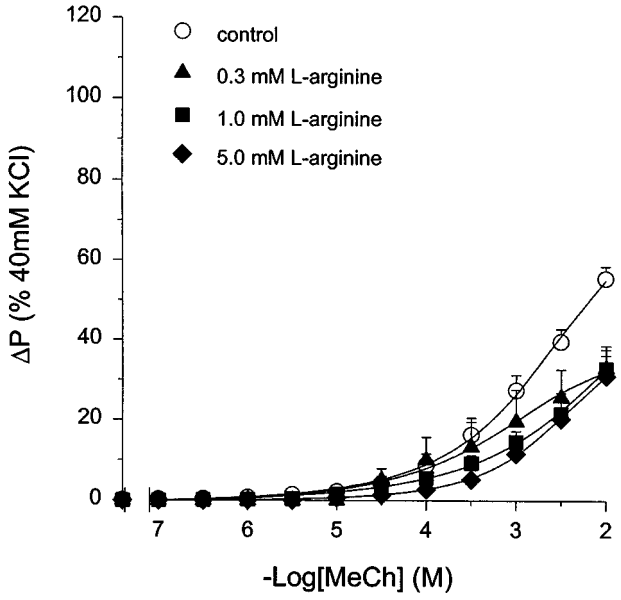

Figure 5 Effect of 0.3, 1.0 and 5.0 mM L-arginine (EL) on methacholine (MeCh; IL)-induced constriction of intact perfused guinea-pig tracheae. Results are means$\pm$s.e.mean of 3–11 experiments. Taken from De Boer *et al*., 1999.

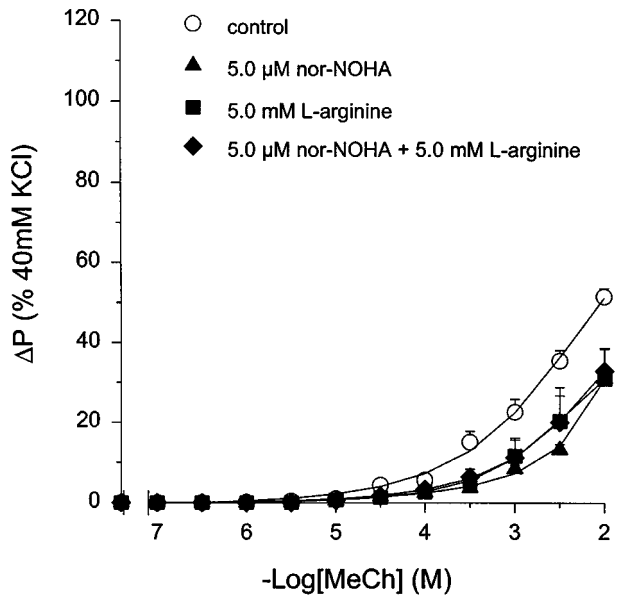

Figure 6 Effect of 5.0 μM nor-NOHA (IL), 5.0 mM L-arginine (EL) and 5.0 μM nor-NOHA (IL) + 5.0 mM L-arginine (EL) on methacholine (MeCh; IL)-induced constriction of intact perfused guinea-pig trachea. Results are means$\pm$s.e.means of 3–10 experiments.

cholinergic airway reactivity. The N$^{\omega}$-hydroxy-L-α-aminoacid nor-NOHA is one of the most potent arginase inhibitors reported so far, inhibiting purified rat liver arginase activity and arginase contained in murine macrophages with IC_{50} values of 2 to 10 μM, respectively (Custot *et al*., 1997; Tenu *et al*., 1999). A maximal effect of the arginase inhibitor on airway reactivity was obtained in the presence of 5 μM nor-NOHA, which is well in the range of these IC_{50} values.

The reduced methacholine-induced airway constriction in the presence of 5 μM nor-NOHA was dose-dependently reversed by the non-selective NOS inhibitor L-NAME, indicating that the reduced airway responsiveness was caused by enhanced production of NO. Similar to previous studies (De Boer *et al*., 1996; 1998; 1999; Meurs *et al*., 1999), 0.1 mM L-NAME caused a 1.8-fold increase in the E_{max} to methacholine in the control airways, demonstrating that methacholine-induced constriction of guinea-pig tracheal tube preparations is functionally antagonized by agonist-induced (cNOS-derived) NO production. In these preparations E_{max} was not further increased by using higher concentrations of the NOS inhibitor. A similar maximal increase in E_{max} to methacholine by L-NAME (to 1.7-fold control) was observed in the nor-NOHA-

treated airways. However, a higher concentration of the NOS inhibitor (0.5 mM) was required to obtain this effect, indicating that L-NAME had to compete with a higher concentration of endogenous L-arginine in these preparations. The higher availability of L-arginine in the nor-NOHA-treated airways and the subsequent NO-dependent reduction of methacholine-induced airway constriction in these preparations may well be explained by reduced metabolism of the substrate due to inhibition of arginase activity. This would imply that under basal conditions L-arginine is a limiting factor for NO production and that the functional antagonism of cholinergic airway tone by NO is governed by competition between arginase and cNOS for their common substrate. The latter hypothesis is strongly supported by the observation that administration of exogenous L-arginine significantly reduced the responsiveness to methacholine (Figure 4; De Boer *et al*., 1999), indicating that the production of agonist-induced NO is enhanced by suppletion of substrate, while, as expected, the nor-NOHA effect was abolished in the presence of a supramaximal concentration (5 mM) of L-arginine (Figure 6). Remarkably, the maximal reduction of airway reactivity induced by nor-NOHA was comparable to that observed in the presence of a maximally effective concentration of L-arginine, indicating that endogenous arginase activity is a major factor in the regulation of airway reactivity.

Although the source of NO in our perfused tracheal tube preparations was not assessed, L-NAME only increased the agonist-induced constriction and not basal tone, both in control and nor-NOHA-treated airways, indicating that the NO was most likely produced by cNOS. In addition, in control preparations, we previously demonstrated that L-NAME also had no effect on airway constriction induced by KCl, when administered in the EL compartment (i.e. directly activating airway smooth muscle), indicating the absence of functional iNOS activity in these preparations (De Boer *et al*., 1996). It has recently been demonstrated that nor-NOHA is not a substrate for nNOS and iNOS (Moali *et al*., 1998), indicating that metabolism of the arginase inhibitor to NO is unlikely to have contributed to the observed effects on airway reactivity. This is also indicated by the observation that L-norvaline, which cannot be a substrate for NOS due to the absence of a guanidino group, caused a similar inhibition of methacholine-induced airway constriction compared to nor-NOHA.

Using a guinea-pig model of allergic asthma, characterized by allergen-induced early and late asthmatic reactions, airway inflammation and AHR to methacholine and histamine after both reactions (Santing *et al*., 1994; 1995), we have recently established 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 observed after the early asthmatic reaction (EAR). A deficiency of cNOS-derived NO modulating airway reactivity was also observed in patients with severe asthma (Ricciardolo *et al*., 1997), which may similarly be induced by allergen challenge (Ricciardolo *et al*., 1999). In perfused tracheal preparations obtained from sensitized guinea-pigs at 6 h after allergen challenge, we subsequently demonstrated that the deficiency of NO after the EAR is due to limitation of L-arginine as substrate for cNOS (De Boer *et al*., 1999). Since it has been reported that the expression of arginase may be enhanced by prostaglandin E_2 and T_H2 lymphocyte-derived cytokines (Corraliza *et al*., 1995; Modolell *et al*., 1995; Waddington *et al*., 1998), which are important mediators of allergic airway inflammation, it is tempting to speculate that induction of arginase after allergen challenge is involved in the development of AHR after the EAR. However, the role of arginase in the modulation of NOS activity, inflammation and airway reactivity in allergic asthma remains to be established.

In conclusion, the present study indicates that endogenous arginase activity potentiates cholinergic airway constriction by inhibition of NO production, presumably by competition with cNOS for the common substrate, L-arginine. This finding represents an important novel regulation mechanism of airway reactivity, which might be disturbed in allergic asthma.

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