# L-Arginine deficiency causes airway hyperresponsiveness after the late asthmatic reaction

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**ABSTRACT**

Peroxynitrite has been shown to be crucially involved in airway hyperresponsiveness (AHR) after the late asthmatic reaction (LAR). Peroxynitrite production may result from simultaneous synthesis of NO and superoxide by inducible NO-synthase (iNOS) at low L-arginine concentrations. L-Arginine availability to iNOS is regulated by its cellular uptake, which can be inhibited by eosinophil-derived polycations, and by arginase, which competes with iNOS for the common substrate.

Using a guinea pig model of allergic asthma, we investigated whether aberrant L-arginine homeostasis could underlie peroxynitrite-mediated AHR after the LAR.

After the LAR, arginase activity in the airways and eosinophil peroxidase release from bronchoalveolar lavage cells were increased. These changes were associated with a 2.0-fold AHR to methacholine as measured in isolated perfused tracheal preparations. AHR was reduced by exogenous L-arginine administration. Moreover, both the arginase inhibitor nor-NOHA and the polycation antagonist heparin normalized airway responsiveness. These effects were reversed by the NOS inhibitor L-NAME, indicating that both agents reduced AHR by restoring bronchodilating NO production.

In conclusion, in allergen-challenged guinea pigs the AHR after the LAR is caused by arginase- and polycation-induced attenuation of L-arginine availability to iNOS, which may switch the enzyme to simultaneous production of superoxide and NO, and, consequently, peroxynitrite.

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**INTRODUCTION**

Allergic asthma is characterized by airway hyperresponsiveness (AHR) to non-specific stimuli, which may be increased after acute allergic asthmatic reactions. Thus, in allergic asthmatics, as well as in sensitized guinea pigs, increased airway responsiveness to histamine and methacholine develops both after the early and the late asthmatic reaction (LAR) [1,2].

During the allergen-induced LAR, inducible nitric oxide (NO) synthase (iNOS) is induced in human [3] as well as in guinea pig [4] airways. Significant correlations have been observed in asthmatics between exhaled (iNOS-derived) NO, AHR and airway eosinophilia, which are all reduced after glucocorticosteroid treatment [5-8]. Based on these observations, iNOS-derived NO is considered as a marker of airway inflammation. Importantly, increased levels of exhaled NO also correlate with increased nitrotyrosine staining – a marker of peroxynitrite – in exhaled breath condensate of mild asthmatics [9]. In addition, increased nitrotyrosine staining of the airway epithelium and inflammatory cells in bronchial biopsies of asthmatic patients correlates with iNOS expression, AHR and airway inflammation [8], whereas glucocorticosteroids decreased the observed nitrotyrosine staining [8,9]. Collectively, these findings indicate that peroxynitrite, the reaction product of NO and superoxide anion, is importantly involved in iNOS-related asthmatic airway inflammation and hyperresponsiveness.

In an *in vivo* guinea pig model for allergic asthma, we have previously demonstrated that iNOS may have both beneficial and detrimental effects on allergen-induced AHR to histamine after the LAR, by functional antagonism of the histamine-induced bronchoconstriction and  promoting airway inflammation and epithelial damage, respectively  [10]. In addition, in perfused guinea pig airways *ex vivo*, the AHR after the LAR was reversed by the nonselective NOS inhibitor $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME) as well as by the superoxide anion scavenger superoxide dismutase,

indicating that peroxynitrite is strongly involved in the development of AHR after the LAR [11]. It has been well established that formation of peroxynitrite may be effectively induced by simultaneous production of both NO and superoxide anion by iNOS when the L-arginine substrate availability to the enzyme is low [12]. Increasing the concentration of L-arginine promotes the production of NO, while the generation of superoxide anion, and hence peroxynitrite, is reduced [12].

In line with above mentioned observations, studies have indicated that both under physiological and pathophysiological conditions NO production and airway responsiveness are tightly controlled by the availability of L-arginine to NOS [13-15]. L-Arginine is a semi-essential amino acid, which is taken up by the diet and may be endogenously produced from L-citrulline by the intestinal mucosa as well as by some NO producing cells [15]. L-Arginine requirements in most cells are met via uptake of L-arginine by specific cationic amino acid transporters (CATs) [16]. Thus, the availability of L-arginine for NOS to produce NO in the airways is importantly regulated by cellular transport. Indeed, inhibition of CATs by eosinophil-derived cationic proteins as well as by poly-L-arginine causes decreased NO synthesis in rat alveolar macrophages and tracheal epithelial cells [17], while poly-L-arginine causes AHR to methacholine in perfused guinea pig tracheal tube preparations by inducing a deficiency of constitutive NOS (cNOS)-derived NO in response to this agonist [18].

Substrate availability to NOS is also regulated by arginase, which hydrolyzes L-arginine into L-ornithine and urea, and thus competes with NOS for the common substrate [14,15]. Although classically considered as an enzyme of the urea cycle, arginase is also present in extrahepatic cells and tissues, including various cell types in the airways [14,19-24]. The functional role of arginase in the airways has been established in isolated guinea pig tracheal preparations. Thus, constitutive arginase activity in these preparations attenuates both agonist-induced, epithelium-derived [20] and

inhibitory nonadrenergic noncholinergic nerve-mediated [23] NO production, thereby promoting airway responsiveness to neural and nonneural stimuli.

The role of altered L-arginine homeostasis in the (peroxynitrite-mediated) development of AHR after the LAR is currently unknown. Therefore, in the present study, we examined the roles of arginase, endogenous polycations and L-arginine availability in the development of AHR after the LAR, using perfused tracheal preparations from ovalbumin-sensitized guinea pigs obtained at 24 h after ovalbumin-challenge. In addition, arginase activity was assessed in tracheal and bronchoalveolar lavage (BAL) cell homogenates, while eosinophil number and release of the polycation eosinophil peroxidase (EPO) were determined in BAL cells.

## METHODS

### Animals

Outbred specified pathogen free guinea pigs (Harlan, Heathfield, United Kingdom), weighing 700 – 900 g, were used in this study. Animals were actively IgE-sensitized to ovalbumin (Sigma, St. Louis, MO, USA) using aluminum hydroxide (Sigma) as an adjuvant [25] and used experimentally 4 to 8 weeks later. All protocols described in this study were approved by the University of Groningen Committee for Animal Experimentation.

### Allergen provocation

Ovalbumin challenges were performed as described previously, using a specially designed provocation cage in which the guinea-pigs could move freely [26]. The provocations were performed by inhalation of an aerosol concentration of 0.5 mg/ml ovalbumin in saline and were discontinued when the first signs of respiratory distress were observed. The animals were sacrificed 24 h after ovalbumin challenge, *i.e.* after the LAR [2]. Nonchallenged sensitized animals were used as controls.

### Tracheal perfusion

Tracheae were mounted in a perfusion setup as described previously [20,27,28] and placed in an organ bath (37°C) containing 20 ml of gassed Krebs-Henseleit medium (serosal or extraluminal (EL) compartment). The lumen was perfused with recirculating Krebs-Henseleit solution from a separate 20 ml bath (mucosal or intraluminal (IL) compartment) at constant flow (12 ml/min). Hydrostatic pressure was measured at the distal and proximal ends of the trachealis and the differential pressure ($\Delta$P), which

is a function of the airway diameter and reflects the resistance of the tracheal segment to perfusion, was recorded.

After equilibration, isoproterenol (Sigma) was added to assess basal tone. After washout, the trachea was extraluminally exposed to 40 mM KCl to obtain a receptor-independent reference response and washed again. Subsequently, a cumulative concentration response curve was constructed with intraluminally administered methacholine. When used, the specific arginase inhibitor $N^{\omega}$-hydroxy-nor-L-arginine (nor-NOHA; 5.0 μM; kindly provided by Dr J.-L. Boucher, Université Paris V, Paris, France) and L-NAME (1.0 mM; Sigma) were applied to the IL reservoir, while heparin (grade IA from porcine intestinal mucosa; 250 units/ml; Sigma) was applied to both the IL and EL reservoirs; all 40 min prior to agonist-addition. L-Arginine (5.0 mM; Sigma) was applied to the EL reservoir 30 min prior to agonist addition.

**Bronchoalveolar lavage**

Animals were anaesthetized and the lungs were gently lavaged with saline via a tracheal canula, using 5 ml of sterile saline at $37^{o}C$, followed by three subsequent 8-ml aliquots of saline. After centrifugation (200g; 10 min, 4°C), BAL cells were combined and resuspended in PBS, and total cell numbers were determined. For cytological examination, cytospin-preparations were stained with May-Grünwald and Giemsa (both Sigma) and a differential cell count was performed.

**Arginase assay**

Tracheal homogenates were prepared as described previously [27]. BAL cells were homogenized in 1.0 ml 20 mM Tris-HCl, 2 μM phenylmethylsulphonyl fluoride (Sigma), pH 7.4 using a polytron homogenizer (Kinematica GmbH, Luzern, Switzerland). The homogenate was centrifuged (20,000g;

30 min; 4°C) and the supernatant was used experimentally. Arginase activity was determined by

measuring the conversion of L-[guanido-$^{14}$C]arginine (specific activity 51.5 mCi/mmol; New

England Nuclear Life Science Products, Boston, MA, USA) to [$^{14}$C]urea, using a modified protocol

as described by Custot et al. [29]. In short, homogenate aliquots (50 μl) were incubated in a final

volume of 150 μl, containing 25 mM Tris-HCl, 0.67 mM MnCl$_2$ (Sigma), 1.66 mM L-arginine and 1 μl

of L-[guanido-$^{14}$C]arginine (51.5 mCi/mmol), pH 7.4 for 20 minutes at 37°C. Reactions were

terminated by adding 450 μl of ice-cold stop buffer, containing 7 M urea (Sigma), 0.25 M acetic acid,

10 mM L-arginine, pH 3.6. Samples were applied to vials containing 400 μl Dowex AG 50W-X8 (H$^+$

form; 1 g/ml; Bio-Rad Laboratories, Hercules, CA, USA) and rotated for 2 min. Vials were

centrifuged at 750 $g$ for 1 min and resulting supernatants were centrifuged again for 1 min. Final

supernatants (150 μl) were counted in triplicate in 4 ml Ultima Gold scintillation fluid (Packard

Bioscience, Groningen, The Netherlands) using a Beckman LS 1701 liquid scintillation counter. The

specificity of the assay in measuring arginase activity was confirmed by the inhibitory effect of nor-

NOHA.


**Eosinophil peroxidase assay**

BAL cells were centrifuged and resuspended in Hanks balanced salt solution to a final density of

$2.5 \times 10^6$ cells/ml and incubated for 30 min at 37$^o$C. Cell incubation was stopped by placing the

samples on ice, followed by immediate centrifugation and subsequent decantation of the supernatant

for measurement of released EPO activity. Cells were lysed, centrifuged and the supernatant was

collected to measure the remaining intracellular EPO content. EPO activity was analysed according

to the kinetic assay described by White et al. [30], which is based on the oxidation of O-

phenylenediamine (Sigma) by EPO in the presence of hydrogen peroxide.

**Data analysis**

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 $E_{max}$. Using this $E_{max}$, the sensitivity to methacholine was evaluated as $pEC_{50}$-value [20,27,28].

Arginase activity was expressed as pmol urea produced per mg protein per min. EPO activity was expressed as a percent of total amount of EPO (amount in supernatant of stimulated cells + amount in supernatant of lysed cells).

Results are expressed as means ± SEM. Statistical analysis was performed using an unpaired Student's t-test, an analysis of variance followed by a Bonferroni post-hoc test, or a Kruskall-Wallis test followed by a Dunn's post-hoc test, as appropriate. Differences were considered statistically significant at $p < 0.05$.

**RESULTS**

Arginase activity was significantly increased after the allergen-induced LAR, both in tracheal homogenates (1.9-fold, $p < 0.05$) and in homogenates of BAL cells (2.9-fold, $p < 0.05$), as compared to unchallenged controls (fig. 1). Total cell number as well as the numbers of eosinophils and neutrophils in the BAL were significantly increased 24 h after ovalbumin challenge (fig. 2a). BAL eosinophils were activated after challenge, as indicated by increased spontaneous release of EPO by these cells ($p < 0.001$; fig. 2b).

Consistent with our previous study [11], a 2.0-fold increase in $E_{max}$ to IL methacholine was observed in perfused tracheal preparations obtained from ovalbumin-challenged guinea pigs after the LAR as compared to unchallenged controls ($p < 0.01$), without a change in the sensitivity ($pEC_{50}$) to the agonist (fig 3; table 1). Remarkably, the increase in $E_{max}$ in these preparations was, reduced by 70% after incubation with L-arginine (5.0 mM; $p < 0.05$), whereas the $pEC_{50}$-value was unaffected (fig. 3, table 1).

Incubation of the preparations from ovalbumin-challenged animals with the arginase inhibitor nor-NOHA (5.0 μM) completely reverted the AHR to methacholine to the level of unchallenged controls ($p < 0.01$), whereas no effect on the sensitivity was observed (fig 4a, table 1). Similar to nor-NOHA, incubation with heparin (250 U/ml) also normalized the airway responsiveness ($p < 0.05$; fig. 4b, table 1). After coincubation with L-NAME (1.0 mM), the normalized responsiveness, both by nor-NOHA and by heparin, was completely reversed to the AHR of untreated ovalbumin-challenged airways ($p < 0.05$, both; figs 4a and 4b, table 1). L-NAME, nor-NOHA, heparin and L-arginine had no effect on basal airway tone (not shown), which is in line with previous studies [20,27,28].

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**DISCUSSION**

The present study demonstrates for the first time that a deficiency – rather than an excess - of (presumably iNOS-derived) NO is a prime cause of the development of AHR after the LAR. This NO deficiency is due to reduced bioavailability of L-arginine in the airways, which may be caused by two distinct mechanisms: increased arginase activity, competing with iNOS for the common substrate, and enhanced release of eosinophil-derived polycations, which inhibit L-arginine transport into the NO producing cells.

Using a guinea pig model of acute allergic asthma, we have previously demonstrated that both NO, derived from iNOS which is induced during the LAR [4,10] and superoxide are involved the development of AHR after the LAR, presumably by the formation of peroxynitrite [11]. Thus, incubation of perfused tracheal preparations with L-NAME as well as superoxide dismutase fully reverted the AHR after the LAR in these preparations [11]. Remarkably, we now demonstrated that L-arginine, as a precursor of NO, did not worsen but even significantly decreased the AHR after the LAR. This may well be explained by the finding of Xia et al. [12] that at low L-arginine concentrations iNOS produces both NO by the oxygenase moiety of the enzyme and superoxide anion by its reductase moiety, leading to a highly efficient formation of peroxynitrite. Increasing the concentration of L-arginine promotes the production of NO, while the generation of superoxide anion, and hence peroxynitrite, is reduced [12]. Therefore, allergen-induced limitation of L-arginine to iNOS caused by increased arginase activity and by polycations may be importantly involved in the development of AHR after the LAR, by promoting the generation of peroxynitrite. Notably, a role for arginase-induced L-arginine limitation in superoxide anion production by NOS has also been found in activated cultured cerebellar granule neurons [31]. Moreover, L-arginine limitation to iNOS has also

been implicated in increased nitrotyrosine immunoreactivity associated with traumatic brain injury in rats [32].

Evidence is rising that most, if not all of the deleterious effects induced by iNOS-derived NO in the airways may proceed via increased formation of peroxynitrite [8,33,34]. Generation of peroxynitrite has been indicated by enhanced nitrotyrosine immunostaining in the airways of allergen-challenged guinea pigs and of asthmatic patients [8,35]. In asthmatic patients increased nitrotyrosine staining was observed in the airway epithelium and inflammatory cells, which correlated with iNOS expression, AHR and airway inflammation [8]. In addition, iNOS expression and nitrotyrosine staining were closely colocalized in bronchial biopsies of these patients [8]. Interestingly, arginase I expression in asthmatic patients is particularly located to these same cells [22,36]. Furthermore, increased nitrotyrosine was found in exhaled breath condensate of asthmatic patients, which correlated with levels of exhaled NO [9]. Peroxynitrite has both procontractile and proinflammatory actions, which may contribute to the development of AHR [8,11,33-35,37]. The procontractile action of peroxynitrite may involve oxidative inactivation of $K_{Ca}$ channels and sarco/endoplasmatic reticulum $Ca^{2+}$-ATPase type 2 [11,38-41]. In addition, peroxynitrite has been shown to induce degranulation of eosinophils, epithelial damage and AHR [33,34], as well as airway microvascular hyperpermeability [35] in guinea pigs. Collectively, the data indicate that not iNOS-derived NO itself, but rather its reaction with (iNOS-derived) superoxide anion to peroxynitrite importantly accounts for the detrimental effects of increased iNOS expression after the LAR. Moreover, increased scavenging of NO by superoxide anion to form peroxynitrite also contributes to the AHR by causing a deficiency of authentic, bronchodilating NO.

That iNOS-derived NO may have beneficial effects on airway responsiveness was nicely demonstrated by a study of Hjoberg et al. [42], using iNOS overexpressing mice. As in asthmatics,

levels of exhaled NO are increased in these mice as compared to wild type. Interestingly, iNOS-derived NO per se had no proinflammatory effects in the airways and even decreased airway responsiveness to methacholine [42]. By *in vivo* studies in our guinea pig model of allergic asthma we have previously demonstrated that iNOS-derived NO - apart from promoting airway inflammation and epithelial damage – also had some beneficial effect on allergen-induced AHR after the LAR, by partially attenuating the hyperresponsiveness due to its bronchodilating effect [10]. Remarkably, treatment with a selective iNOS inhibitor did not affect allergen-induced AHR in asthmatic patients, although levels of exhaled NO were markedly reduced [43].

One mechanism underlying L-arginine limitation after the LAR is increased arginase activity in the airways, which was found in tracheal homogenates as well as in BAL cells, indicating that inflammatory cells may be involved. The AHR after the LAR was completely normalized by the arginase inhibitor nor-NOHA, demonstrating a prominent role for arginase in the development of L-arginine limitation and AHR. Coincubation with L-NAME completely restored the AHR, indicating that arginase inhibition restores bronchodilating NO production. Using guinea pig tracheal preparations, we have previously demonstrated that arginase activity in the airways is already increased after the early asthmatic reaction and contributes to AHR after this reaction by causing a marked deficiency of both neural and agonist-induced nonneural NO, by limiting the availability of L-arginine to cNOS [27,44]. Thus, L-arginine limitation due to increased arginase activity is importantly involved in the development of AHR both after the early and late asthmatic reaction. Very recently, these *ex vivo* observations were confirmed by *in vivo* studies using the same guinea pig model [45]. Inhalation of the specific arginase inhibitor 2(S)-amino-6-boronohexanoic acid (ABH) acutely reversed the allergen-induced AHR after the EAR and LAR, while pretreatment with ABH protected against the development of the AHR after both reactions. Quite remarkably, ABH

also reduced the airway sensitivity to the inhaled allergen and protected against the allergen-induced bronchial obstructive reactions and airway inflammation [46]. Accordingly, intraperitoneal treatment with nor-NOHA significantly decreased allergen-induced AHR as well as inflammatory cell infiltration in the lungs of C57BL/6 mice, following two weeks of ovalbumin challenge [47]. In contrast, oropharyngeal aspiration of the arginase inhibitor S-(2-boronoethyl)-L-cysteine (BEC) 2 h *after* the last of three daily ovalbumin challenges did not affect allergen-induced inflammatory cell profiles or levels of 21 cytokines in the BAL fluid of BALB/c mice, with the exception of IL-4, which was reduced [48]. However, allergen-induced peribronchiolar and perivascular inflammation and protein nitration were further enhanced by BEC treatment [48]. Remarkably, although the allergen-induced increase in peripheral airway responsiveness at 40 sec after methacholine inhalation appeared to be reduced, the peak-response to methacholine was not inhibited, but rather accelerated by BEC [48]. Furthermore, no effect of BEC on allergen-induced hyperresponsiveness of the central airways was observed [48]. An explanation for these unexpected observations is presently not yet at hand. However, a recent study of acetylcholine-induced vasorelaxation suggested that BEC may also have other cellular targets than arginase [49].

The importance of increased arginase expression and activity in allergic asthma has been confirmed in different animal models as well as in asthmatic patients [50]. Thus, in two mouse models of allergic asthma, induced by sensitization with ovalbumin and *Aspergillus fumigatus*, respectively, pulmonary arginase activity was increased after allergen challenge [22]. Microarray analysis of gene expression in these models revealed that genes related to L-arginine metabolism, including the cytosolic isoform arginase I and the mitochondrial isoform arginase II, belonged to the most prominently overexpressed genes [22]. Increased arginase expression and activity in mouse lung has also been found in other studies, after challenge with either ovalbumin [51-53],

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*Nippostrongylus brasiliensis* [53], *Schistosoma mansoni* eggs [54], *Dermatophagoides farinae* [55] or trimellic anhydride [52]. In addition, ovalbumin increased arginase activity in the lungs of sensitized rats [56]. Moreover, mouse lung arginase activity and mRNA expression of both arginase I and II were strongly induced by the Th2 cytokines IL-4 and IL-13, known to be involved in allergic airway inflammation [22,53,57]. Similar observations were made in cultured rat airway fibroblasts [21].

In support of our findings, protein expression of arginase I was increased in BAL cells (presumably alveolar macrophages) of asthmatic patients [22]. In addition, enhanced mRNA expression of arginase I was observed in the airway epithelium and in inflammatory cells in bronchial biopsies of these patients [22], strongly indicating that arginase is involved in the pathophysiology of human asthma. Interestingly, increased serum arginase activity and decreased plasma levels of L-arginine have been observed in asthmatic patients during exacerbation [58]. Moreover, in another study serum arginase activity in severe asthmatics was inversely correlated to $FEV_1$ and $FEV_1/FVC$, whereas lung function was positively correlated to plasma L-arginine availability in these patients [59]. Since immunohistological detection of guinea pig arginase is presently not yet possible, the cellular localization of the allergen-induced increased arginase expression in these animals remains to be established.

A second important mechanism regulating cellular L-arginine availability to NOS is the uptake of the amino acid by CATs. Using the polyanion heparin, acting as a polycation antagonist, we previously demonstrated that endogenous polycations contribute to the NO deficiency and AHR after the early asthmatic reaction, presumably by inhibiting cellular L-arginine uptake [28]. Indeed, eosinophil-derived polycations as well as poly-L-arginine are known to inhibit L-arginine uptake [17] and to cause AHR by affecting airway epithelial function [60] and inducing NO deficiency [18].

Interestingly, we now demonstrated that heparin completely normalized the AHR after the LAR, indicating that endogenous polycations are involved in this process as well. Since the effect of heparin was reversed by L-NAME, it can be concluded that heparin normalizes the AHR by restoring the production of bronchodilating NO. The similar effects of arginase inhibition and polycation scavenging on AHR suggest a common pathophysiological pathway, which could implicate an increased effectiveness of arginase under conditions of polycation-induced substrate limitation.

In line with our hypothesis that eosinophil-derived polycations are involved in the development of AHR, we demonstrated that both the number and activation state of eosinophils in the BAL were markedly increased after allergen challenge. The increased EPO release by the BAL cells found in the present study corresponds to our previous finding of increased EPO activity in the BAL fluid at 24 hours after ovalbumin challenge, which correlated with AHR *in vivo* [61]. Previous studies have indicated that treatment with inhaled heparin before allergen challenge significantly reduced allergen-induced pulmonary eosinophilia in sensitized guinea pigs [62]. In addition, in asthmatic patients and in allergen-challenged sheep and guinea pigs (pretreatment with) inhaled heparin was found to inhibit AHR to various contractile agonists [63-67]. Similar to the arginase inhibitor ABH [68], pretreatment with inhaled heparin also reduced allergen-induced bronchial obstructive reactions in allergic sheep and guinea pigs [65,67,69]. Furthermore, both exercise- and allergen-induced asthmatic reactions in asthmatic patients were reduced by inhaled heparin as well [70-72]. However, inhalation of the heparin-derivative IVX-0142 did not significantly affect allergen-induced early and late asthmatic responses, AHR and exhaled NO in patients with mild asthma, which may have been due to underdosing of the drug [73].

In conclusion, using a well defined guinea pig model of allergic asthma we have demonstrated that a deficiency of bronchodilating NO in the airway wall, due to arginase- and

polycation-induced attenuation of L-arginine availability, is involved in the development of AHR

after the LAR. This process may involve simultaneous production of NO and superoxide anion by

iNOS at the low L-arginine concentration, leading to very efficient formation of the proinflammatory

and procontractile nitrogen species peroxynitrite as well as reduced levels of bronchodilating NO

(fig. 5). Since the AHR is reduced by both nor-NOHA and heparin, arginase inhibitors and polycation

antagonists may have therapeutic potential in allergic asthma.


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**TABLE 1**: Effects of nor-NOHA and heparin, in the absence and presence of L-NAME, and L-arginine on the responsiveness to intraluminally administered methacholine of intact perfused tracheae from ovalbumin-challenged guinea pigs.

| | $E_{max}$ (% KCl) | $pEC_{50}$ (-log M) | $n$ |
|---|---|---|---|
| **Unchallenged** | 51.6 ± 1.2 | 3.12 ± 0.20 | 6 |
| **Ovalbumin-challenged** | 105.1 ± 10.8[**] | 3.28 ± 0.13 | 8 |
| **+ nor-NOHA (5.0 μM)** | 52.8 ± 5.2[††] | 3.41 ± 0.15 | 8 |
| **+ nor-NOHA + L-NAME (1.0 mM)** | 104.8 ± 11.1[‡] | 3.48 ± 0.16 | 4 |
| **+ heparin (250 U/ml)** | 54.2 ± 11.0[†] | 2.84 ± 0.19 | 6 |
| **+ heparine + L-NAME (1.0 mM)** | 108.9 ± 6.5 [§] | 3.25 ± 0.08 | 4 |
| **+ L-arginine (5.0 mM)** | 67.6 ± 11.0[†] | 3.16 ± 0.15 | 8 |

Values are means ± SEM of $n$ experiments. Emax: maximal effect obtained in the presence of 10 mM methacholine; $pEC_{50}$: sensitivity towards methacholine. [**]$p<0.01$ compared with unchallenged control group; [†]$p<0.05$ and [††]$p<0.01$ compared with ovalbumin-challenged controls; [‡]$p<0.05$ compared with nor-NOHA; [§]$p<0.05$ compared with heparin.

**Figure legends**

**Figure 1**

Arginase activity in tracheal and BAL cell homogenates from unchallenged and ovalbumin (OA)-challenged guinea pigs. Results are means ± SEM of 4-8 experiments. [*]p<0.05 compared to unchallenged control.
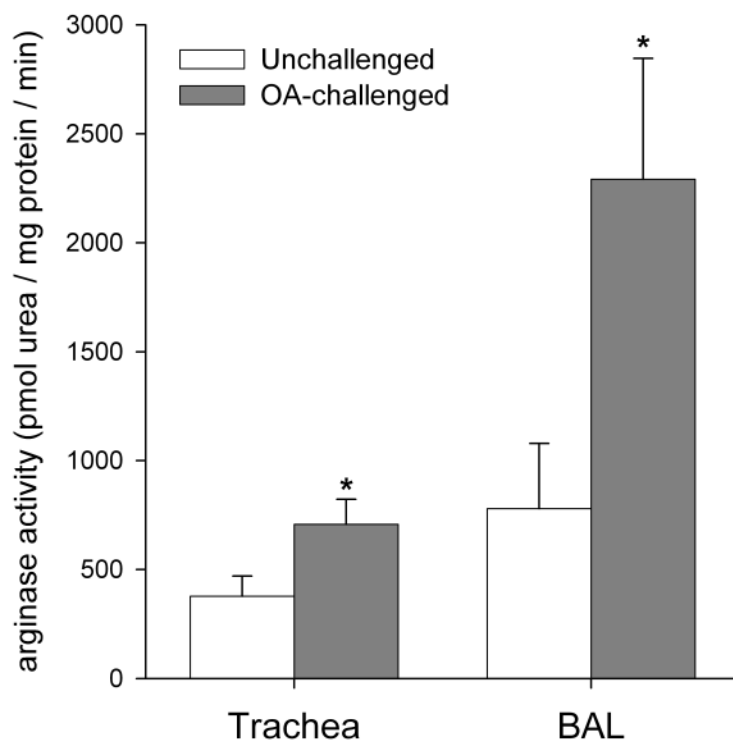


**Figure 2**

Total and differential cell count (A) and EPO release from BAL eosinophils (B) obtained from unchallenged and ovalbumin (OA) challenged guinea pigs. EPO release is expressed as % of total cellular EPO content and 100 % represents 19.3 ± 3.9 and 10.4 ± 1.3 ng/ml EPO/$10^6$ cells for

unchallenged and OA challenged animals, respectively. Results are means ± SEM of 4-8

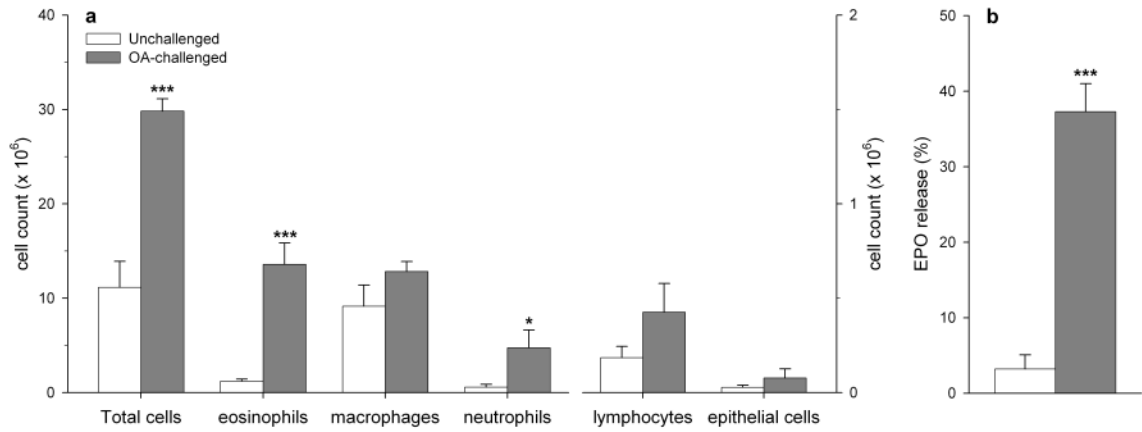experiments. *p<0.05 and ***p<0.001 compared to unchallenged control.



**Figure 3**

Methacholine (MCh)-induced constriction of intact perfused tracheae from unchallenged and

ovalbumin (OA)-challenged guinea pigs, in the absence and presence of 5.0 mM L-arginine. Results
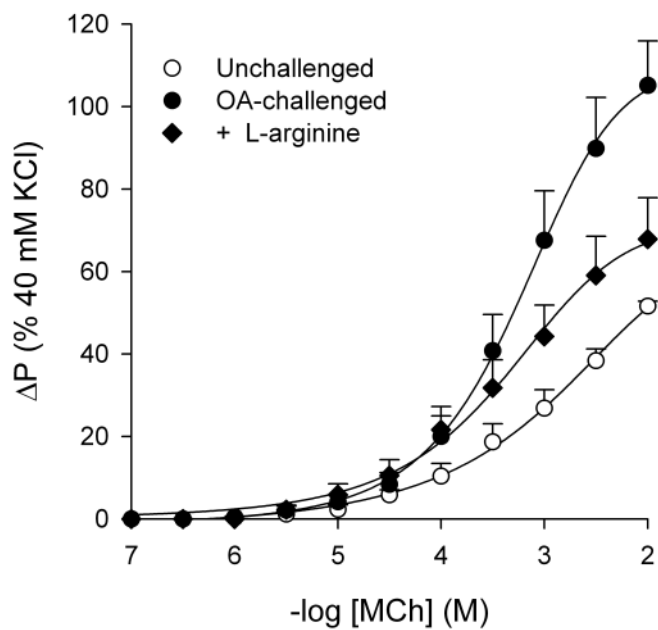
are means ± SEM of 6-8 experiments.

**Figure 4**

Methacholine (MCh)-induced constriction of intact perfused tracheae from unchallenged and ovalbumin (OA)-challenged guinea pigs, in the absence and presence of (A) 5.0 μM nor-NOHA or (B) 250 U/ml heparin alone or in combination with 1.0 mM L-NAME. Results are means ± SEM of 4-9 experiments.
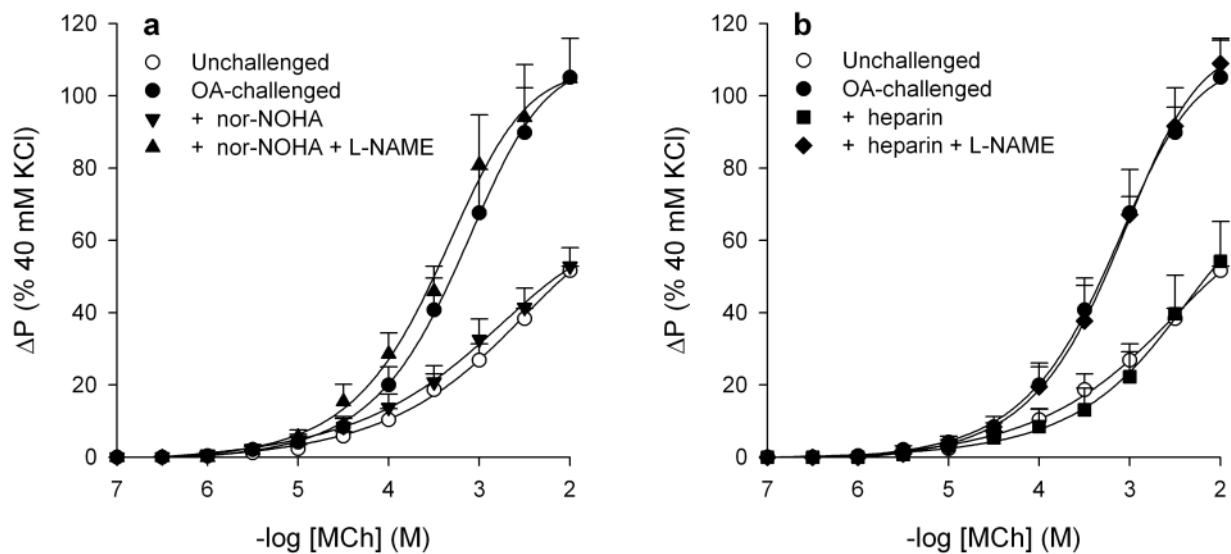
**Figure 5:**

Postulated role of L-arginine homeostasis in the development of airway hyperresponsiveness (AHR) after the late asthmatic reaction (LAR). Increased arginase activity, which may be induced by Th2 cytokines in the airway epithelium and in inflammatory cells (not shown), as well as increased release of eosinophil-derived polycations result in a decreased availability of L-arginine to inducible nitric oxide synthase (iNOS), which is induced in the same cells during the LAR. The reduced availability of L-arginine to iNOS promotes the production of superoxide anion ($O_2^-$) by the reductase moiety of the enzyme. NO, produced by the oxygenase moiety, and $O_2^-$ rapidly react to form the highly reactive nitrogen species peroxynitrite ($ONOO^-$), which has procontractile and proinflammatory actions in the airways and contributes to the allergen-induced AHR. Moreover, the formation of $ONOO^-$ causes a relative deficiency of bronchodilating NO, similarly contributing to the AHR. Increasing the L-arginine availability, by inhibition of arginase activity or restoring cellular transport of the amino acid, diminishes the AHR by inhibiting $O_2^-$ production and stimulating the

production of authentic, bronchodilating NO. CAM: calmodulin, CAT: cationic amino acid transporter, e⁻, electron, EPO: eosinophil peroxidase, FAD: flavin adenine dinucleotide, FMN: flavin mononucleotide, IFN: interferon, IL; interleukin, $K_{Ca}$: $Ca^{2+}$-dependent potassium channel, MBP: major basic protein, NADPH: reduced nicotinamide adenine dinucleotide phosphate, TNF: tumor necrosis factor.