Chapter 11

Arginase and asthma: novel insights in nitric oxide homeostasis and airway hyperresponsiveness

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Summary
For many years it has been supposed that production of excess of nitric oxide (NO) by inducible NO synthase (iNOS) plays a major role in inflammatory diseases, including asthma. Recent studies, however, have indicated that a deficiency of beneficial, bronchodilating constitutive NOS (cNOS)-derived NO is importantly involved in allergen-induced airway hyperresponsiveness. Amongst various possible mechanisms, reduced substrate availability due to increased arginase activity as well as decreased cellular uptake of L-arginine appears to play a key role in the reduced cNOS activity. Remarkably, recent evidence suggests that also iNOS-induced pathophysiological effects involve substrate deficiency. Thus, at low L-arginine concentrations iNOS is able to produce both NO and superoxide anions, resulting in increased synthesis of the highly reactive and detrimental oxidant peroxynitrite. Based on these observations, we propose that a (relative) deficiency of NO due to increased arginase activity and altered L-arginine homeostasis is a major causative factor in the pathology of asthma.

Introduction
Allergic asthma is a chronic inflammatory disease of the airways. Characteristic features of this disease are allergen-induced early and late bronchial obstructive reactions, associated with infiltration and activation of inflammatory cells - particularly Th2 lymphocytes and eosinophils - in the airways and the development of airway hyperresponsiveness to a variety of stimuli, including allergens, chemical irritants, cold air and pharmacological agents like histamine and methacholine [1]. Alterations in the neurogenic and non-neurogenic control of airway smooth muscle function, as well as airway remodelling, including thickening of the basement membrane, subepithelial fibrosis and increased airway smooth muscle mass, are importantly involved in the development of airway hyperresponsiveness [1]. All these changes can be induced by a cascade of inflammatory reactions involving various mediators, including nitric oxide (NO) [1,2]. In asthmatic airways, production of NO is greatly increased by enhanced expression of inducible NO synthase (iNOS) by proinflammatory cytokines, leading to increased levels of NO in exhaled air [2]. Significant correlations between exhaled NO, airway eosinophilia, and airway hyperresponsiveness have been observed in asthmatics, while all these parameters are reduced after glucocorticosteroid treatment [2,3]. Based on these observations, the NO concentration in exhaled air has been considered as a marker of airway inflammation [2]. Therefore, it is not surprising that much of the literature regarding NO in asthma has been focussed on iNOS and is heavily biased to a harmful, pro-inflammatory role for NO. However, despite clear evidence of increased exhaled NO levels in asthma, the role of NO in the airway wall as a potentially proinflammatory cytotoxic as well as protective bronchodilatory and even anti-inflammatory agent is still unresolved. In this contribution, we highlight recent findings suggesting a
prominent role for constitutive NOS (cNOS) in the pathophysiology of asthma and indicating that airway hyperresponsiveness and inflammation could be importantly due to a deficiency of both cNOS- and iNOS-derived NO to exert bronchodilatory and anti-inflammatory actions. We will focus on the major role of arginase, as a novel modulator of NOS activity and airway responsiveness in asthma.

**Nitric oxide synthases in the airways**

NO is a gaseous free radical produced by a family of NOS isoforms, which utilize the semi-essential amino acid L-arginine, oxygen and NADPH as substrates to synthesise NO and L-citrulline [4]. In the airways, cNOS isoforms are mainly expressed in inhibitory nonadrenergic noncholinergic (iNANC) nerves (neuronal nNOS), endothelial cells (endothelial eNOS) and airway epithelium (nNOS and eNOS) [2]. These isoforms are activated by depolarization or agonist-induced intracellular Ca\(^{2+}\) changes to generate small (picomolar) amounts of NO. This activation is short-lived, and the NO produced serves as a diffusible signalling molecule mediating various processes, including airway smooth muscle relaxation [2] (Figure 1).

iNOS is distinguished from the cNOS isoforms by its prolonged Ca\(^{2+}\)-independent production of relatively large (nanomolar) amounts of NO. Unlike cNOS, its expression can be induced by proinflammatory cytokines, including interferon-\(\gamma\), interleukin (IL)-1\(\beta\) and tumour necrosis factor-\(\alpha\), and is inhibited by glucocorticosteroids [2,3,5]. Expression of iNOS has been observed in normal human airway epithelium [6]. In asthmatic airways, iNOS is markedly upregulated and mainly expressed in epithelial cells and inflammatory cells like macrophages, eosinophils and neutrophils [2,3]. High concentrations of iNOS-derived NO do not only cause smooth muscle relaxation, but also contribute to increased vascular permeability, mucus hypersecretion, inflammatory cell infiltration, epithelial cell damage and perpetuation of the Th2 lymphocyte-mediated inflammatory response in the airways [2,7]. Most, if not all of the deleterious effects induced by iNOS-derived NO might proceed via formation of peroxynitrite (ONOO\(^{-}\)), a highly reactive oxidant synthesised by the rapid reaction of NO with superoxide anion (O\(_2^{-}\)) generated in the inflamed airways [3,8].
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Figure 1: Role of nitric oxide (NO) in airway reactivity and inflammation. Under basal conditions, small amounts of NO are being synthesised by constitutive NO synthase (cNOS) isozymes present in the airway epithelium (endothelial eNOS and neuronal nNOS) and in inhibitory nonadrenergic noncholinergic (iNANC) nerves (nNOS). Activation of the cNOS isozymes by contractile agonists (epithelium) or depolarization (iNANC nerves) causes airway smooth muscle relaxation by increased production of cGMP and/or opening of Ca^{2+}-activated K^+ channels, thereby reducing airway responsiveness to contractile stimuli. Inducible NOS (iNOS) can be induced in the epithelium by inflammatory cytokines and produces large amounts of NO, which can be both beneficial and detrimental, by airway smooth muscle relaxation and by proinflammatory and cytotoxic actions, respectively.

Deficiency of cNOS-derived NO in allergen-induced airway hyperresponsiveness and inflammation

Both in animal models and in mild asthmatics it has been demonstrated that endogenous, presumably epithelially derived NO is importantly involved in the regulation of airway reactivity to bronchoconstrictor stimuli, including muscarinic agonists, histamine and bradykinin [9-13]. Many studies focussing on the role of NO in airway hyperresponsiveness have been performed in experimental models of allergic asthma. Since beneficial and detrimental actions of NO as well as the differential involvement of NOS isozymes in these actions seem to intervene in chronic asthma, and since subtype selectivity of cNOS and iNOS inhibitors is often limited, models of acute allergen-induced asthma, studying the effects of a single
allergen challenge after sensitization, have been found useful. Thus, iNOS activity in these inflammation-naïve models is not detected before the late asthmatic reaction [14], making it possible to investigate the distinct role of cNOS in the early asthmatic reaction and the airway hyperresponsiveness immediately after this reaction. In a guinea pig model of acute allergic asthma, NO in exhaled air was increased immediately after allergen inhalation, which paralleled the degree of bronchial obstruction. However, the increase in NO was transient and decreased below control levels after 15 min, while bronchoconstriction was still present [15]. The immediate increase in exhaled NO presumably represents mediator-induced and/or neurally formed NO, acting as a feedback mechanism against airway obstruction [16]. The subsequent decrease of exhaled NO could represent a reduced bioavailability of NO, contributing to the early asthmatic reaction as well as to the airway hyperresponsiveness after this reaction. Indeed, the responsiveness of isolated perfused guinea pig tracheal preparations to methacholine and histamine was considerably enhanced after the early asthmatic reaction [10]. This effect was closely mimicked by administration of the non-selective NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) to unchallenged control preparations, while, in addition, the challenged preparations were no longer responsive to L-NAME, indicating that a deficiency of agonist-induced production of airway dilating NO is a major determinant of airway hyperresponsiveness [10]. This was confirmed by the in vivo observations that inhalation of L-NAME by sensitized guinea pigs caused hyperresponsiveness to inhaled histamine before allergen challenge, whereas L-NAME was fully ineffective after the allergen-induced early asthmatic reaction [11].

A deficiency of NO contributing to airway hyperresponsiveness was also found after repeated allergen challenge of guinea pigs [17-19], which could possibly involve a reduced activity of iNANC nerve-derived NO [19]. Moreover, reduced cNOS-derived NO importantly contributed to the airway hyperresponsiveness to bradykinin in severe, glucocorticoid-treated asthmatics [20], which could similarly be induced by allergen exposition [21].

In addition to reduced bronchodilation, deficiency of cNOS-derived NO could induce airway hyperresponsiveness by promoting airway inflammation. Thus, cNOS-derived NO suppresses the activation of nuclear factor (NF)-kB, thereby inhibiting the expression of iNOS as well as the production of inflammatory cytokines [22,23]. Accordingly, increased pulmonary iNOS expression has been observed in eNOS null mice [24]. Moreover, down-regulation of eNOS activity causes increased endothelial adhesion and extravasation of leukocytes [25], while overexpression of eNOS inhibits allergen-induced airway inflammation and hyperresponsiveness [26]. Indeed, in asthmatic patients significant correlations were observed between allergen-induced airway hyperresponsiveness and reduced eNOS expression as well as increased iNOS expression [21].
Mechanisms of cNOS deficiency
Several mechanisms have been implicated in the allergen-induced NO deficiency. Reduced nNOS or eNOS protein expression were observed after repeated allergen challenge of guinea pigs [18] and mild asthmatic patients [21], respectively. The stimulus causing downregulation of the cNOS isoforms is as yet unknown, but might involve interferon-γ, acting as a molecular switch between cNOS and iNOS expression [27]. In addition, enhanced O$_2^-$ production in repeatedly challenged guinea pig airways could scavenge iNANC nerve-derived NO, since impaired iNANC relaxation in these airways was restored by superoxide dismutase (SOD) [19].

By contrast, the deficiency of nonneural cNOS-derived NO after single allergen challenge appeared not to result from scavenging of NO by O$_2^-$ [28]. In perfused guinea pig tracheal preparations it was demonstrated that L-arginine is a rate limiting factor for cNOS activity in normal airways and that the hyperresponsiveness to methacholine after the early asthmatic reaction was completely reversed by exogenous administration of L-arginine, indicating that limitation of substrate underlies the deficiency of NO and subsequent airway hyperresponsiveness [29]. Similarly, cNOS-derived NO deficiency due to reduced L-arginine availability was also observed in virally infected hyperresponsive animals [30].

Role of L-arginine homeostasis and arginase in allergen-induced cNOS deficiency
At least two mechanisms could account for reduced L-arginine availability for cNOS after allergen challenge. First, observations in rat alveolar macrophages and tracheal epithelial cells have indicated that polycationic proteins, including eosinophil-derived major basic protein (MBP), cause inhibition of cellular uptake of L-arginine by specific cationic amino acid y$^+$ transporters [31]. In perfused guinea pig tracheae, the polycationic MBP analogue poly-L-arginine indeed caused NO deficiency and airway hyperresponsiveness to methacholine, which was reversed by the polyanion heparin [32]; Moreover, heparin, acting as a polycation antagonist, reduced both the allergen-induced NO deficiency and airway hyperresponsiveness after the early asthmatic reaction [33].

A second mechanism that might be of crucial importance is reduced bioavailability of L-arginine due to increased activity of arginase, which hydrolyzes L-arginine to L-ornithine and urea (Figure 2). Arginase, classically an enzyme of the urea cycle in liver, is also found in many other cells or tissues that do not express a complete urea cycle, including the lung [34,35]. Two distinct isoforms of mammalian arginase have been identified, which are encoded by different genes and differ with respect to their cellular distribution and mode of regulation. Type I arginase is a cytosolic enzyme, mainly expressed in liver, whereas type II arginase is a mitochondrial enzyme only expressed in extrahepatic tissue [35]. The biological function of extrahepatic arginase is largely unclear, but has been implicated in the regulation of NO synthesis and cell growth in inflammatory conditions. For example,
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in activated macrophages, arginase activity limits the utilization of L-arginine by iNOS and suppresses cytotoxicity [35-37], while concomitant polyamine and proline synthesis from L-ornithine causes cell proliferation and repair of inflammatory lesions [35]. Both arginase I and arginase II are constitutively expressed in the airways, particularly in the bronchial epithelium and in peribronchial connective tissue fibroblasts [34]. Using the novel, potent and specific arginase inhibitor Nω-hydroxy-nor-L-arginine (nor-NOHA), it was demonstrated that arginase is also involved in the modulation of airway responsiveness, by limiting cNOS-derived bronchodilating NO production [38]. Interestingly, the expression of arginase I can be increased by Th2 lymphocyte-derived cytokines such as IL-4, IL-10 and IL-13 and by cAMP-elevating stimuli such as PGE2 [39,40], which are involved in asthmatic airway inflammation. Indeed, in guinea pig tracheal preparations it was recently demonstrated that allergen challenge causes a considerable increase in arginase activity in the airways, which was shown to contribute substantially to the deficiency of cNOS-derived NO and airway hyperresponsiveness after the early asthmatic reaction [41]. Moreover, like MBP and poly-L-arginine, the arginase product ornithine inhibits L-arginine transport [42]. NOS activity appears to be more sensitive to reduced substrate availability than arginase [31], which tips the balance to arginase activation.

Very remarkably, as early as two decades ago enhanced arginase activity was found in expectorated sputum of asthmatic patients [43]. The underlying mechanism and its functional relevance have thus far not been investigated, but could well be related to the allergen-induced increase in arginase activity and airway hyperresponsiveness described above. Moreover, elevated levels of polyamines have been found in peripheral blood of asthmatic patients [44].

L-Arginine availability and iNOS-derived reactive nitrogen species
As mentioned, iNOS-derived NO can have both beneficial, bronchodilating, and deleterious, pro-inflammatory, effects. This was nicely illustrated in our guinea pig model of acute allergic asthma, demonstrating that iNOS-derived NO, through its bronchodilatory action, partially reduced the allergen-induced airway hyperresponsiveness after the late asthmatic reaction, while concomitantly it promoted airway hyperresponsiveness by promoting infiltration of inflammatory cells and epithelial damage during the late reaction [7]. By using NOS inhibitors and O2− scavengers it has been demonstrated that the formation of ONOO− is of crucial importance in the development of allergen-induced airway hyperresponsiveness after the late asthmatic reaction or after repeated allergen challenge [45,46]. This would be in line with enhanced 3-nitrotyrosine immunostaining in airways of allergen-challenged guinea pigs and asthmatic patients [3,47], which was correlated with airway hyperresponsiveness and airway inflammation [3], although ONOO−-independent mechanisms of 3-nitrotyrosine formation by granulocyte peroxidases have also been described [48]. Moreover, exogenously applied ONOO− induced
eosinophil degranulation, epithelial damage and airway hyperresponsiveness in guinea pigs [8]. In addition, endogenous ONOO⁻ was shown to have a procontractile action [45].

**Figure 2:** Postulated pathways of L-arginine metabolism by constitutive and inducible nitric oxide syntases (cNOS and iNOS) in relation to airway responsiveness, inflammation and remodelling. NO is synthesised from L-arginine and O₂ by cNOS and iNOS, and has beneficial bronchodilating and anti-inflammatory actions. L-Arginine is also metabolized by arginase activity, which converts L-arginine into L-ornithine and urea. Induction of increased arginase activity by Th2 cytokines reduces L-arginine availability to cNOS and iNOS, thereby reducing the production of NO and inducing the production of superoxide anion (O₂⁻) by these enzymes. By the rapid reaction of NO with O₂⁻, peroxynitrite (ONOO⁻) is being formed, which has proinflammatory, cytotoxic and procontractile actions in the airways. Furthermore, as a consequence of increased synthesis of arginase-derived L-ornithine as a precursor for polyamines (putrescine, spermidine and spermine) and L-proline, arginase might also be involved in inflammation-induced airway remodelling, by stimulation of cell growth and collagen synthesis, respectively.
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Remarkably, studies in macrophages have indicated that iNOS can effectively produce both NO and $\text{O}_2^-$ when L-arginine concentrations are low, leading to a very efficient synthesis of ONOO$^-$ and enhanced cytotoxic action of the cells [49] (Figure 2). Very recently, the relevance of this mechanism for asthmatic airways was indicated by the observation that in perfused guinea pig tracheal preparations obtained after the late asthmatic reaction the responsiveness to methacholine was not only diminished by L-NAME and SOD [45], but also by heparin, nor-NOHA and L-arginine, indicating that reduced L-arginine availability due to impaired cellular uptake or increased arginase activity is involved in iNOS-induced ONOO$^-$ production and airway hyperresponsiveness (Maarsingh et al., unpublished). Similarly, nNOS has also been reported to generate NO and $\text{O}_2^-$ at low L-arginine levels [50], which might contribute to the airway hyperresponsiveness after the late but not after the early asthmatic reaction, since SOD was ineffective at this time point [28].

Concluding remarks

Accumulating evidence suggests that the simplified view that the low levels of cNOS-derived NO are involved in physiological events, whereas the excess of iNOS-derived NO causes detrimental processes in the airways, needs refinement by the contention that a (relative) deficiency of both cNOS and iNOS-derived NO, particularly due to altered L-arginine homeostasis, appears to be an important causative factor in the pathology of asthma. In this regard, the recent discovery of increased expression and activity of arginase by nonhepatic cells in response to Th2 cytokines might be of great importance with regard to the development of airway hyperresponsiveness and inflammation in the airways. This might not only refer to the reduced availability of L-arginine, necessary to produce adequate amounts of NO and to restrict $\text{O}_2^-$ production, but also to the increased production of L-ornithine as a precursor of polyamines and proline, which could be involved in inflammation-induced airway remodelling in chronic asthma by promoting proliferation of structural subepithelial cells (fibroblasts and smooth muscle cells) and collagen deposition (Figure 2). Polyamines can stimulate gene expression implicated in cell proliferation by promoting histone acetyltransferase (HAT) activity resulting in chromatin hyperacetylation [51], and increased HAT activity has recently been demonstrated in biopsies of asthmatic patients indeed [52]. However, the possible role of arginase in asthmatic airway inflammation has just emerged and needs further verification in the near future. Of note, increased arginase activity has recently been associated with several other inflammatory and non-inflammatory conditions, indicating a more widespread role of this enzyme in pathology (Table 1).
Table 1: Pathological conditions associated with increased arginase activity

<table>
<thead>
<tr>
<th>Pathological condition</th>
<th>Species</th>
<th>Refs</th>
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<tbody>
<tr>
<td>Asthma</td>
<td>Guinea pig, human</td>
<td>[41,43]</td>
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<tr>
<td>Hyperoxic lung injury</td>
<td>Rat</td>
<td>[34]</td>
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<tr>
<td>Arthritis</td>
<td>Human</td>
<td>[53]</td>
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<tr>
<td>Psoriasis</td>
<td>Human</td>
<td>[54]</td>
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<td>Diabetic complications,</td>
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<td>[55]</td>
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<td>including erectile dysfunction</td>
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<tr>
<td>Glomerulonephritis</td>
<td>Rat</td>
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<tr>
<td>Trauma</td>
<td>Mouse</td>
<td>[57]</td>
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<tr>
<td>Infectious diseases,</td>
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<td>[58]</td>
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<td>including parasitic diseases</td>
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<tr>
<td>Cancerous diseases,</td>
<td>Mouse</td>
<td>[59]</td>
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<td>including breast cancer</td>
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A role for L-arginine deficiency in airway hyperresponsiveness would predict a beneficial effect of L-arginine administration to these patients. Indeed, compared to a nonasthmatic control group, a pronounced dose-dependent effect of inhaled L-arginine on exhaled NO was observed in patients with mild asthma, which could point to substrate limitation in these patients [60], although the effect of L-arginine on airway responsiveness was not measured in this study. Alternatively, local administration of specific and selective arginase inhibitors could be a novel therapeutic strategy in the treatment of acute and chronic asthma, which warrants further investigation.

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