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REVIEW

Arginase: a key enzyme in the pathophysiology of allergic asthma opening novel therapeutic perspectives

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Allergic asthma is a chronic inflammatory airways' disease, characterized by allergen-induced early and late bronchial obstructive reactions, airway hyperresponsiveness (AHR), airway inflammation and airway remodelling. Recent *ex vivo* and *in vivo* studies in animal models and asthmatic patients have indicated that arginase may play a central role in all these features. Thus, increased arginase activity in the airways induces reduced bioavailability of L-arginine to constitutive (cNOS) and inducible (iNOS) nitric oxide synthases, causing a deficiency of bronchodilating and anti-inflammatory NO, as well as increased formation of peroxynitrite, which may be involved in allergen-induced airways obstruction, AHR and inflammation. In addition, both via reduced NO production and enhanced synthesis of L-ornithine, increased arginase activity may be involved in airway remodelling by promoting cell proliferation and collagen deposition in the airway wall. Therefore, arginase inhibitors may have therapeutic potential in the treatment of acute and chronic asthma. This review focuses on the pathophysiological role of arginase in allergic asthma and the emerging effectiveness of arginase inhibitors in the treatment of this disease.
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Keywords: allergy; airway hyperresponsiveness; airway remodelling; arginase inhibitors; arginine; collagen; inflammation; nitric oxide; peroxynitrite; polyamines

Abbreviations: ABH, 2(S)-amino-6-boronohexanoic acid; ADMA, asymmetric dimethylarginine; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; BEC, S-(2-boronoethyl)-L-cysteine; CAT; cationic amino acid transporter; EAR, early asthmatic reaction; eIF2α, eukaryotic initiation factor-α; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; HAT, histone acetyltransferase; Hsp47, heath shock protein-47; IκB, inhibitory factor-κB; IL-, interleukin; iNANC, inhibitory nonadrenergic noncholinergic; LAR, late asthmatic reaction; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NF-κB, nuclear factor-κB; NO, nitric oxide; NOHA, N^{ω}-hydroxy-L-arginine; nor-NOHA, N^{ω}-hydroxy-nor-L-arginine; cNOS, constitutive NOS; eNOS, endothelial NOS; iNOS, inducible NOS; nNOS, neuronal NOS; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; SNPs, single nucleotide polymorphisms; STAT6, signal transducer and activator of transcription 6; TGF-β, transforming growth factor-β; V_{max}, maximal enzymatic rate

Introduction

Arginase is the final enzyme of the urea cycle in the liver and is the key enzyme for the removal of highly toxic ammonium ions from the body. To this aim, L-arginine, which is synthesized from L-citrulline by the subsequent actions of argininosuccinate synthase and argininosuccinate lyase, is converted into L-ornithine and urea by the action of arginase. Besides in liver, arginase is also expressed in cells and tissues that lack a complete urea cycle, including the airways and the lung (Jenkinson *et al.*, 1996; Que *et al.*, 1998; Wu and Morris, 1998; Meurs *et al.*, 2000). Two isoenzymes of arginase have been identified: arginase I and arginase II, which are encoded by different genes and differ in cellular location. Arginase I is a cytosolic enzyme, and the gene for human arginase I is assigned to chromosome 6q23, whereas arginase II is a mitochondrial enzyme, with its gene located on chromosome 14q24 in humans (Sparkes *et al.*, 1986; Jenkinson *et al.*, 1996; Gotoh *et al.*, 1997; Wu and Morris, 1998). Arginase I is the

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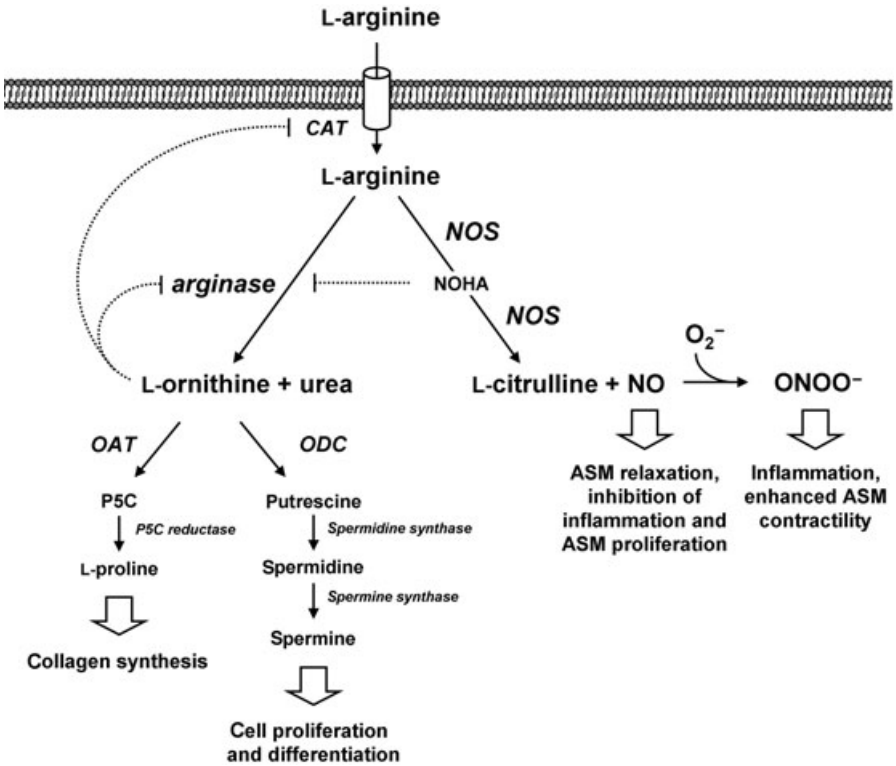

Figure 1 Interactive role of arginase and NOS in regulating airway function. L-arginine is the substrate for both arginase, yielding L-ornithine and urea, and NOS, yielding NO and L-citrulline. Arginase attenuates the production of NO by competing with NOS for their common substrate. On the other hand, arginase activity is inhibited by NOHA, an intermediate in the NO synthesis by NOS. In addition, L-ornithine causes feedback inhibition of arginase and inhibits cationic amino acid transporters involved in cellular uptake of L-arginine. L-Ornithine is also a precursor of the polyamines putrescine, spermidine and spermine, which are involved in cell proliferation and differentiation, and of L-proline, which is required for collagen synthesis. In the respiratory tract, NO induces airway smooth muscle relaxation and inhibits inflammation and airway smooth muscle cell proliferation. Increased arginase activity in allergic asthma compromises these processes by inducing reduced NO production, as well as increased formation of $ONOO^-$, as a consequence of reduced bioavailability of L-arginine to constitutive and inducible NOS isoforms, respectively. Moreover, increased production of L-ornithine by arginase may contribute to airway remodelling in this disease, by enhanced synthesis of polyamines and L-proline. ASM, airway smooth muscle; CAT, cationic amino acid transporter; NO, nitric oxide; NOHA, N^{ω}-hydroxy-L-arginine; NOS, nitric oxide synthase; O_2^-, superoxide anion; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; $ONOO^-$, peroxynitrite; P5C, L-pyrroline-5-carboxylate.

predominant isoform in the liver, but is expressed extrahepatically as well. Although low levels have been detected in the liver (Klasen *et al.*, 2001), arginase II is mainly expressed in extrahepatic tissues (Jenkinson *et al.*, 1996; Wu and Morris, 1998).

Since L-arginine is also the substrate for nitric oxide synthase (NOS), yielding nitric oxide (NO) and L-citrulline (Moncada *et al.*, 1989), one of the biological functions of extrahepatic arginase may be the regulation of NO levels via competition with NOS for the common substrate (Modolell *et al.*, 1995; Wang *et al.*, 1995; Hey *et al.*, 1997; Figure 1). Although the affinity for L-arginine is much (~1000-fold) higher for NOS than for arginase, arginase can still compete with NOS for L-arginine, since the V_{max} (maximal enzymatic rate) of arginase is approximately 1000-fold greater than that of NOS (Reczkowski and Ash, 1994; Griffith and Stuehr, 1995; Wu and Morris, 1998). On the other hand, NOS can inhibit the activity of arginase via the production of N^{ω}-hydroxy-L-arginine (NOHA), an intermediate in the NO synthesis (Boucher *et al.*, 1994; Daghigh *et al.*, 1994; Hecker *et al.*, 1995). In addition, the arginase product L-ornithine competitively inhibits arginase activity (Reczkowski and Ash, 1994; Cox *et al.*, 2001; Maarsingh *et al.*, 2007b), as well as L-arginine transport into NO-producing cells (Deves and Boyd, 1998; Schapira *et al.*, 1998; Messeri Dreissig *et al.*, 2000). These findings indicate a delicate balance between arginase and NOS in L-arginine homeostasis and in the control of NO synthesis (Figure 1).

Arginase may also regulate a number of cellular functions by NO-independent mechanisms (Figure 1). Thus, arginase may be involved in tissue repair processes by the synthesis of L-ornithine, which is the precursor of polyamines and L-proline that are involved in cell proliferation and collagen synthesis, respectively (Shearer *et al.*, 1997; Wu and Morris, 1998; Satriano, 2004).

Both arginase I and II are constitutively expressed in the airways, particularly in epithelial cells, (myo)fibroblasts, endothelial cells and macrophages (Que *et al.*, 1998; Klasen *et al.*, 2001; Lindemann and Racke, 2003), while expression of arginase II has also been observed in alveolar epithelial cells (Que *et al.*, 1998). Moreover, arginase I and II may be conditionally expressed in airway smooth muscle cells (Zuyderduyn *et al.*, 2006; Bergeron *et al.*, 2007). Since arginase regulates a variety of processes involved in the regulation of airway func-

tion, a key role for arginase in the pathophysiology of various airways diseases, including asthma, is emerging (Meurs *et al.*, 2003; Maarsingh *et al.*, 2008a).

Interactive role of arginase and NOS in regulating airway function

NO is generated from L-arginine by a family of NOS isoforms, utilizing oxygen and NADPH as cosubstrates and yielding L-citrulline as a coproduct (Moncada *et al.*, 1989; Boucher *et al.*, 1999). Three isoforms of NOS have been identified: neuronal NOS (nNOS or NOS I) and endothelial NOS (eNOS or NOS III), which are constitutively expressed, and inducible NOS (iNOS or NOS II). All three NOS isoforms are (conditionally) expressed in the airways. The constitutive NOS (cNOS) isoforms are mainly expressed in the airway epithelium (nNOS and eNOS), inhibitory nonadrenergic noncholinergic (iNANC) neurons (nNOS) and endothelial cells from the airway vasculature (eNOS). cNOS isoforms produce relatively low levels of NO in response to increased intracellular calcium concentrations evoked by membrane depolarization or by the action of (contractile) agonists (Ricciardolo *et al.*, 2004). The activity of iNOS, producing large amounts of NO, is regulated at the expressional level, particularly by proinflammatory cytokines during airway inflammation. During airway inflammation, iNOS is predominantly expressed in macrophages and epithelial cells (Nijkamp *et al.*, 1993; De Boer *et al.*, 1996; Ricciardolo *et al.*, 2004).

NO is an important endogenous bronchodilator by inducing airway smooth muscle relaxation (Tucker *et al.*, 1990; Belvisi *et al.*, 1991; Li and Rand, 1991; Belvisi *et al.*, 1992; Ellis and Undem, 1992; Ricciardolo *et al.*, 2004). NO-induced relaxation involves production of cGMP by soluble guanylyl cyclase, as well as activation of calcium-activated potassium channels, collectively leading to decreased intracellular Ca^{2+} concentrations, decreased Ca^{2+} sensitivity and membrane hyperpolarization of the airway smooth muscle cell (Ricciardolo *et al.*, 2004).

In the airways, the availability of L-arginine to NOS isoforms is a limiting factor in NO production, as illustrated by the observation that levels of exhaled NO in healthy subjects are increased after oral or inhalational treatment with L-arginine, without affecting (normal) lung function (Kharitonov *et al.*, 1995; Sapienza *et al.*, 1998). Using guinea pig airway preparations *in vitro*, it has been demonstrated that L-arginine inhibits the airway responsiveness to methacholine (De Boer *et al.*, 1999) and increases iNANC nerve-mediated airway smooth muscle relaxation (Maarsingh *et al.*, 2005), both through increased production of cNOS-derived NO.

That the substrate availability to cNOS in the airways may be attenuated by endogenous arginase activity was first demonstrated in isolated perfused guinea pig tracheal preparations (Meurs *et al.*, 2000) using the specific arginase inhibitor N^{ω}-hydroxy-nor-L-arginine (nor-NOHA), which – in contrast to NOHA – is not a substrate for NOS (Tenu *et al.*, 1999). In these preparations, incubation with nor-NOHA decreased the airway responsiveness to methacholine, which was reversed by the nonselective NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) (Meurs *et al.*, 2000). Moreover, endogenous arginase activity attenuated iNANC nerve-mediated airway smooth muscle relaxation by inhibition of nNOS-derived NO production (Maarsingh *et al.*, 2005). That arginase regulates airway responsiveness by limiting the substrate availability to cNOS was supported by the observation that the effects of nor-NOHA were quantitatively similar to those of L-arginine (De Boer *et al.*, 1999; Meurs *et al.*, 2000; Maarsingh *et al.*, 2005).

cNOS-derived NO and AHR in asthma

Allergic asthma is a chronic inflammatory disorder of the airways, characterized by allergen-induced, IgE-mediated early and late bronchial obstructive reactions, development of acute and transient airway hyperresponsiveness (AHR) after these reactions, and infiltration of inflammatory cells into the airways, particularly eosinophils and Th2 lymphocytes (Bousquet *et al.*, 2000). Chronic inflammation may induce airway remodelling, characterized by structural changes in the airway wall, including thickening of the basement membrane, subepithelial fibrosis and increased airway smooth muscle mass (Bousquet *et al.*, 2000). These structural changes may cause a progressive decline in lung function, as well as persistent AHR (Cockcroft and Davis, 2006; Meurs *et al.*, 2008). Recent evidence suggests that changes in L-arginine homeostasis may importantly contribute to the development of both acute and chronic allergen-induced AHR via altered NO production and synthesis of polyamines and L-proline, respectively (Maarsingh *et al.*, 2008c; Meurs *et al.*, 2008).

The beneficial effect of cNOS-derived, bronchodilating NO on AHR is illustrated by the observation that the allergen-induced AHR to methacholine is compeletely abolished in eNOS-overexpressing mice (Ten Broeke *et al.*, 2006), whereas the AHR is markedly increased in allergen-challenged eNOS knockout mice (Feletou *et al.*, 2001). Loss of bronchoprotective NO after allergen challenge could thus underlie the AHR in allergic asthma. Indeed, various studies in animal models of allergic asthma and in asthmatic patients have indicated that the development of acute allergen-induced AHR may result from a deficiency of cNOS-derived NO in the airways (De Boer *et al.*, 1996; Mehta *et al.*, 1997; Ricciardolo *et al.*, 1997; Schuiling *et al.*, 1998b; Ricciardolo *et al.*, 2001; Samb *et al.*, 2001; Maarsingh *et al.*, 2006). Thus, in a guinea pig model of allergic asthma, in which iNOS is not induced before the late asthmatic reaction (LAR; Yan *et al.*, 1995), L-NAME did not affect the AHR immediately after the early asthmatic reaction (EAR) *ex vivo* (De Boer *et al.*, 1996; Maarsingh *et al.*, 2006) and *in vivo* (Schuiling *et al.*, 1998b). Involvement of attenuated production of cNOS-derived NO in acute allergen-induced AHR was also found in mild asthmatic patients (Ricciardolo *et al.*, 2001). Animal model studies further indicated that reduced substrate bioavailability to cNOS importantly accounts for the NO deficiency after the EAR, as treatment with L-arginine reduced the AHR in perfused tracheal preparations from allergen-challenged guinea pigs *ex vivo* (De Boer *et al.*, 1999) and increased iNANC-mediated airway smooth muscle relaxation in tracheal strip preparations from these animals (Maarsingh *et al.*, 2006).

Interestingly, treatment with inhaled L-arginine also markedly reduced the allergen-induced AHR to histamine after the EAR in guinea pigs *in vivo* (Maarsingh *et al.*, 2008d). In human asthmatics, oral treatment with L-arginine did not significantly affect the AHR to histamine, although the slope of the dose-response curve was slightly reduced (De Gouw *et al.*, 1999). However, since there was not an effect on exhaled NO either, the administered dose of L-arginine could have been too low.

Besides contributing to the development of acute, allergen-induced AHR after the EAR, the deficiency of cNOS-derived NO could also be involved in the induction of the inflammatory response during the late asthmatic reaction (LAR). Thus, it has been shown that eNOS-derived NO inhibits airway inflammation by constitutively suppressing NF-κB activity, thereby inhibiting the expression of inflammatory cytokines, as well as of iNOS (Cirino *et al.*, 2003; Ckless *et al.*, 2007; Thomassen *et al.*, 1997; Marshall and Stamler, 2001; Cook *et al.*, 2003; Ten Broeke *et al.*, 2006). Fully in line with these findings, allergen-induced airway inflammation was markedly reduced in eNOS overexpressing mice as compared with wild type mice (Kobayashi *et al.*, 2006; Ten Broeke *et al.*, 2006).

iNOS-derived NO and AHR in asthma

In inflamed asthmatic airways, particularly during the LAR, the expression of iNOS is markedly increased by proinflammatory cytokines, such as TNF-α and IFN-γ, predominantly in inflammatory cells and in the airway epithelium (Hamid *et al.*, 1993; Asano *et al.*, 1994; Barnes, 1998). Since the NO production by iNOS is more than 1000-fold higher than the NO produced by cNOS, levels of NO in the exhaled air are increased in patients with active and persistent asthma (Kharitonov *et al.*, 1994; Ricciardolo *et al.*, 2004). iNOS-derived NO is generally considered to be detrimental in allergic asthma, as it has been shown to be associated with epithelial damage, infiltration of inflammatory cells, amplification and perpetuation of the Th2-mediated inflammatory response, mucus hypersecretion and vascular hyperpermeability (Kuo *et al.*, 1992; Flak and Goldman, 1996; Schuiling *et al.*, 1998a; Ricciardolo *et al.*, 2004). However, most – if not all – of these detrimental effects in the airways may not be caused by iNOS-derived NO itself, but rather by peroxynitrite, the reaction product of NO and superoxide anions (Sadeghi-Hashjin *et al.*, 1998; Saleh *et al.*, 1998). This highly reactive nitrogen species promotes airway smooth muscle contraction (De Boer *et al.*, 2001) and has proinflammatory actions, including the induction of epithelial damage, eosinophil activation, MUC5AC expression and vascular hyperpermeability (Sadeghi-Hashjin *et al.*, 1996; Sugiura *et al.*, 1999; Fischer and Voynow, 2002; Muijsers *et al.*, 2002). Increased formation of peroxynitrite may importantly contribute to the development of AHR after the LAR in acute and chronic asthma (De Boer *et al.*, 2001; Sadeghi-Hashjin *et al.*, 1996; Sadeghi-Hashjin *et al.*, 1998; Muijsers *et al.*, 2001; Maarsingh *et al.*, 2007a). In support, a marked increase in immunoreactivity for nitrotyrosine – a marker of protein nitration by peroxynitrite – has been observed in airway biopsies of asthmatic patients, particularly in epithelial and inflammatory cells, which correlated with iNOS expression, airway inflammation and AHR (Saleh *et al.*, 1998).

Remarkably, in transgenic mice overexpressing iNOS in the airways, increased levels of exhaled NO were found, while airway resistance and airway responsiveness to methacholine were decreased, and no changes in airway inflammation, mucus secretion, airway fibrosis or nitrotyrosine staining were observed (Hjoberg *et al.*, 2004). In addition, it has been shown that iNOS-derived NO may have a beneficial bronchodilating action in asthmatic airways (De Gouw *et al.*, 1998; Schuiling *et al.*, 1998a). Moreover, ovalbumin-induced AHR and airway inflammation, as well as expression of arginase I and II, were more pronounced in iNOS knockout mice than in wild-type animals (Bratt *et al.*, 2009). However, in other studies, it was shown that the allergen-induced AHR was similar in iNOS knockout compared with wild-type mice (Xiong *et al.*, 1999), and that the inflammation was also unchanged (De Sanctis *et al.*, 1999; Koarai *et al.*, 2002) or even decreased (Xiong *et al.*, 1999; Duguet *et al.*, 2001) in the iNOS knockout mice. For a comprehensive review on the role of the different NOS isoforms in allergic asthma – as studied by using NOS isoform selective inhibitors and transgenic mice – see Mathrani *et al.* (2007).

Taken together, these findings indicate that iNOS-derived NO by itself does not induce, but may even prevent, asthma-like pathology and support the hypothesis that it is increased formation of peroxynitrite from iNOS-derived NO that contributes to AHR and airway inflammation in asthma. Remarkably, increased formation of peroxynitrite may be the result of a decreased bioavailability of L-arginine to NOS. Studies have indicated that at low L-arginine concentrations, iNOS not only produces NO by its oxygenase moiety, but also synthesizes superoxide anions by its reductase moiety, leading to an efficient formation of peroxynitrite (Xia *et al.*, 1998). Increasing the L-arginine concentration in macrophages stimulated NO production and inhibited the formation of superoxide and hence peroxynitrite (Xia and Zweier, 1997).

Interestingly, the *ex vivo* AHR after the LAR in perfused tracheal preparations obtained from guinea pigs after a single (Maarsingh *et al.*, 2009) or repeated allergen challenges (Maarsingh *et al.*, 2007a) was significantly reversed by treatment with exogenous L-arginine. In addition, inhalation of L-arginine also reduced the AHR to histamine after the allergen-induced LAR in guinea pigs *in vivo* (Maarsingh *et al.*, 2008d). These findings indicate that a reduced bioavailability of L-arginine also contributes to the AHR after the late asthmatic response, presumably by inducing an increased formation of peroxynitrite by iNOS.

Arginase and asthma

Since increased L-arginine consumption by arginase could explain the NO deficiency and AHR in allergic asthma, a number of studies have focused on the expression and activity of this enzyme in this disease. In guinea pig tracheal homogenates obtained after the allergen-induced EAR, arginase activity was almost fourfold increased compared with tracheae from unchallenged control animals (Meurs *et al.*,

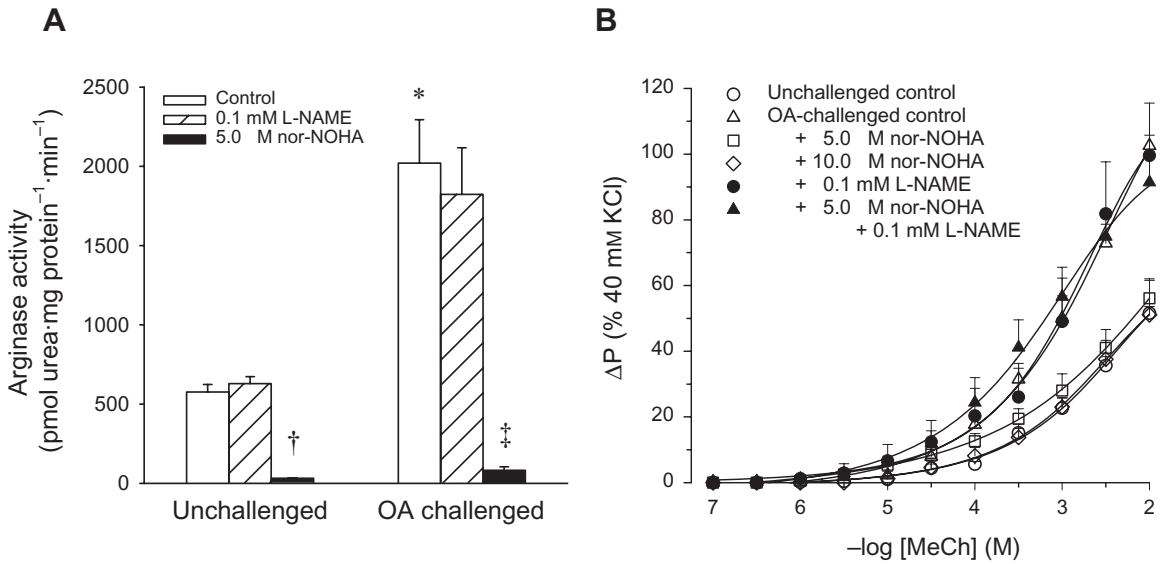

Figure 2 Role of increased arginase activity in allergen-induced AHR after the EAR *ex vivo*. (A) Arginase activity in tracheal homogenates from ovalbumin (OA)-challenged guinea pigs is markedly increased after the EAR as compared with unchallenged controls. The increased arginase activity is fully inhibited by the arginase inhibitor nor-NOHA, but not by the NOS-inhibitor L-NAME. (B) Increased arginase activity contributes to the AHR after the EAR by inducing a deficiency of bronchodilating cNOS-derived NO. Methacholine-induced constriction of intact perfused tracheal preparations from ovalbumin (OA)-challenged guinea pigs in the absence and presence of L-NAME or various concentrations of nor-NOHA, with or without L-NAME. Airway constriction was induced by intraluminal application of increasing concentrations of the agonist and measured as a change in differential pressure (ΔP) at constant flow, normalized to the response induced by extraluminal 40 mM KCl. For comparison, methacholine-induced constriction of control preparations from unchallenged animals is also shown. Data represent means ± SEM of 3–14 experiments. *$P < 0.001$, †$P < 0.0001$ compared with unchallenged control; ‡$P < 0.005$ compared with OA-challenged control. Reproduced with permission from Meurs *et al.* (2002). AHR, airway hyperresponsiveness; EAR, early asthmatic reaction; L-NAME, N$^{\omega}$-nitro-L-arginine methyl ester; NO, nitric oxide; nor-NOHA, N$^{\omega}$-hydroxy-nor-L-arginine; cNOS, constitutive NOS.

2002; Figure 2A). The arginase activity in the airways is still increased after the LAR, both in an acute and chronic guinea pig model of allergic asthma (Maarsingh *et al.*, 2007a; 2009). Increased arginase activity and/or expression has also been demonstrated in other animal models using different antigens in various species and strains (Maarsingh *et al.*, 2008a). Particularly induction of arginase I expression has been observed in these models, while the expression of arginase II was less pronounced or even absent.

In two BALB/c mouse models, arginase activity was increased over 10-fold in animals sensitized to and challenged with either ovalbumin or *Aspergillus fumigatus* (Zimmermann *et al.*, 2003). Microarray analysis of gene expression revealed that both arginase I and II isoforms are induced by these allergens, which was confirmed by Northern blot analysis. Interestingly, arginase I and II belonged to the most predominantly overexpressed genes among the 291 common genes that were induced by these antigens (Zimmermann *et al.*, 2003). Protein expression of arginase I, and, to a lesser extent, of arginase II, was also increased after repeated ovalbumin challenges of BALB/c mice for 2 weeks (Kenyon *et al.*, 2008). Although the induction was lower than in animals challenged with ovalbumin, lung arginase I mRNA expression and arginase activity were also increased in BALB/c mice challenged with trimellitic anhydride (Greene *et al.*, 2005). Marked up-regulation of arginase I after repeated ovalbumin challenge has also been detected by proteomics of lung tissue from C57BL/6 mice (Fajardo *et al.*, 2004). In addition, lung arginase activity, as well as mRNA and protein expression of both arginase I and II, were increased in NC/Nga mice after a single challenge with *Dermatophagoides farinae* (Takemoto *et al.*, 2007). In the allergen-challenged mice, high expression of arginase I has been observed in the airway epithelium and in perivascular and peribronchial pockets of inflammation in the asthmatic lung, particularly in alveolar macrophages and infiltrating cells around the bronchioles (Zimmermann *et al.*, 2003; Takemoto *et al.*, 2007). Increased expression of arginase II has been reported in the distal airways and peripheral lung, including the conducting airways from allergen-challenged mice (Kenyon *et al.*, 2008). Besides in guinea pigs and mice, increased arginase activity in lung homogenates has also been observed in a rat model of allergic asthma after challenge with ovalbumin for three consecutive days (Abe *et al.*, 2006).

Th2 cytokines, like IL-4 and IL-13, have been shown to increase arginase activity in mouse macrophages (Corraliza *et al.*, 1995; Modolell *et al.*, 1995). Although arginase activity in human alveolar macrophages was not induced by IL-4, it greatly enhanced the induction of arginase activity in response to cAMP-elevating agents (Erdely *et al.*, 2005). Since allergic asthma is a Th2 cytokine-mediated disease, increased arginase expression in allergic asthma may be the result of increased release of these cytokines. Interestingly, both arginase isoforms were significantly increased in the lungs of IL-4 overexpressing mice (Zimmermann *et al.*, 2003), animals that display several features of asthma (Rankin *et al.*, 1996). In addition, intratracheal instillation with IL-13 markedly increased arginase I expression in mouse lungs, while the arginase II expression was only slightly or not induced (Zimmermann *et al.*, 2003; Yang *et al.*, 2006). IL-25, a novel member of the IL-17 family, which induces Th2-like airway

inflammation and AHR, also increased arginase I mRNA expression in mouse lung (Sharkhuu *et al.*, 2006). IL-4 also increased the expression of arginase II, but not arginase I, in isolated human airway smooth muscle cells (Zuyderduyn *et al.*, 2006).

Several studies have indicated that Th2 cytokine-induced up-regulation of arginase I expression is regulated by the transcription factors CCAAT-enhancer binding protein (Gray *et al.*, 2005) and STAT6 (Wei *et al.*, 2000; Rutschman *et al.*, 2001; Zimmermann *et al.*, 2003; Gray *et al.*, 2005; Yang *et al.*, 2006). By contrast, arginase II expression is largely STAT6 independent, since IL-4-induced arginase II expression in the lung was hardly affected in STAT6$^{-/-}$ mice (Zimmermann *et al.*, 2003).

The importance of the Th2-response in the induction of arginase expression in allergic asthma was supported by microarray analysis of gene expression in the lung of five different mouse models of Th2 cytokine-mediated inflammation in animals challenged with ovalbumin (BALB/c and C57BL/6 mice), the parasite *Nippostrongylus brasiliensis* (BALB/c) or the fungus *Aspergillus fumigatus* (C57BL/6), and in IL-13 overexpressing mice. Thus, among the 26 characteristic transcript that were commonly expressed in the lungs from these different models, arginase I was strongly increased in all (Lewis *et al.*, 2007). Moreover, in mice sensitized to and challenged with *Schistosoma mansoni* eggs, increased arginase I gene expression was observed in Th2 polarized, but not in Th1 polarized, animals (Sandler *et al.*, 2003). In conclusion, arginase activity and expression are markedly increased in different animal models of asthma, which may involve induction of the enzyme by Th2-cytokines.

Functional consequences of increased arginase activity in asthma

The contribution of increased arginase activity to acute allergen-induced AHR was first studied in perfused tracheal preparations obtained from allergen-challenged guinea pigs after the EAR. Incubation with the arginase inhibitor nor-NOHA completely reversed the allergen-induced AHR by restoring NO production, as illustrated by the reversal of this effect by coincubation with L-NAME (Meurs *et al.*, 2002; Figure 2B). Using the same inhibitor, it was also shown that increased arginase activity after the EAR impairs iNANC nerve-mediated airway smooth muscle relaxation by inducing a deficiency of nNOS-derived NO (Maarsingh *et al.*, 2006). The effects of nor-NOHA were quantitatively similar to the effects of exogenous L-arginine (De Boer *et al.*, 1999; Meurs *et al.*, 2002; Maarsingh *et al.*, 2006), suggesting that increased L-arginine consumption by arginase induces deficiency of both neuronal and non-neuronal NO and AHR. This hypothesis was further supported by a recent study in a mouse model of allergic asthma demonstrating that increased arginase activity in the airways of ovalbumin-challenged mice is associated with reduced levels of L-arginine and L-citrulline in these airways (Kenyon *et al.*, 2008).

As described above, the AHR after the allergen-induced LAR may involve increased synthesis of peroxynitrite due to limitation of L-arginine availability to iNOS (De Boer *et al.*, 2001; Maarsingh *et al.*, 2009). Accordingly, in perfused guinea pig tracheal preparations obtained after the LAR, inhibition of arginase activity by nor-NOHA fully reversed the AHR to methacholine (Maarsingh *et al.*, 2009). Coincubation with L-NAME prevented the effect of nor-NOHA, clearly showing that this normalising effect was due to increased production of bronchodilating NO (Maarsingh *et al.*, 2009). Similar obervations were made in a guinea pig model of chronic asthma (Maarsingh *et al.*, 2007a). Since L-arginine administration similarly reduced the AHR after the LAR (Maarsingh *et al.*, 2007a; 2009), the conclusion seems inevitable that increased arginase causes reduced L-arginine availability to iNOS, subsequently leading to increased formation of peroxynitrite and AHR. In support of this hypothesis, increased nitrotyrosine staining and increased arginase and iNOS expression were observed in *Dermatophagoides farinae*-challenged mice (Takemoto *et al.*, 2007).

In addition to direct substrate competition with NOS isoforms, increased arginase could also contribute to decreased L-arginine bioavailability to these enzymes through the production of L-ornithine. Thus, it has been demonstrated that L-ornithine competes with L-arginine for cellular uptake via cationic amino acid tranporters (CATs) of the y^+ system (Deves and Boyd, 1998; Schapira *et al.*, 1998; Messeri Dreissig *et al.*, 2000). Accordingly, incubation with L-ornithine increased airway responsiveness to methacholine in perfused guinea pig tracheal preparations by inducing a deficiency of cNOS-derived NO (Maarsingh *et al.*, 2007b). Of note, reduced transport of L-arginine into NO-producing cells by inhibition of CATs can also be induced by (eosinophil-derived) polycations (Hammermann *et al.*, 1999), which may also contribute to the NO-deficiency and AHR in asthma (Meurs *et al.*, 1999; Maarsingh *et al.*, 2004; 2008c; 2009).

Taken together, increased arginase activity in the airways contributes to the development of allergen-induced AHR by limiting the L-arginine bioavailability to cNOS and iNOS isozymes, leading to a deficiency of bronchodilating NO and increased formation of procontractile and proinflammatory peroxynitrite.

Effects of arginase inhibition *in vivo*

Thus far, only a few studies have addressed the effectiveness of arginase inhibition on features of allergic asthma *in vivo*. In BALB/c mice, Yang *et al.* (2006) demonstrated that treatment with IL-13 increased lung arginase activity and expression of arginase I, but not of arginase II, whereas NO synthesis was decreased. The increased arginase I expression temporally correlated with the development, persistence, and resolution of IL-13-induced AHR to methacholine. Interestingly, treatment with arginase I RNA interference prevented the IL-13-induced up-regulation of arginase I, as well as the cytokine-induced AHR, supporting the major role of increased arginase activity in the development of AHR in asthma (Yang *et al.*, 2006).

In a guinea pig model of allergic asthma, it was recently demonstrated that inhalation of the specific, isoenzyme-nonselective arginase inhibitor 2(S)-amino-6-boronohexanoic acid (ABH) acutely reversed the allergen-induced AHR after

the EAR and LAR at 6 and 24 h after single allergen challenge, respectively (Maarsingh *et al*., 2008d). This effect was mimicked by inhalation of L-arginine, but not D-arginine, indicating that arginase-induced deficiency of substrate for NOS isoenzymes in the airways is involved. Surprisingly, pre-treatment with ABH 30 min before allergen challenge considerably reduced the sensitivity of the airways to the inhaled allergen, as indicated by a more than 30-fold higher allergen dose needed to induce bronchial obstruction as compared with saline-treated animals (Maarsingh *et al*., 2008d). Moreover, in these animals, pre-treatment with ABH followed by an additional treatment at 8 h after allergen challenge reduced the AHR after the EAR and LAR, indicating that pre-treatment with the arginase inhibitor protects against the development of AHR *irrespective* of its acute anti-allergic effect. Interestingly, in ABH-treated animals challenged with the *same* allergen dose that induced airway obstruction in saline-treated guinea pigs, the AHR after the EAR was even further reduced, whereas the development of AHR after the LAR was fully prevented (Maarsingh *et al*., 2008d; Figure 3). Moreover, the ABH treatment in these animals caused a considerable reduction of the initial allergen-induced bronchial obstruction (Figure 4A,B) and of the magnitude of both the EAR and LAR (Maarsingh *et al*., 2008d; Figure 4A). Similarly, intraperitoneal treatment with nor-NOHA significantly decreased allergen-induced AHR in C57BL/6 mice after 2 weeks of ovalbumin challenge, confirming the therapeutic potential of arginase inhibitors in the treatment of allergic asthma (Bratt *et al*., 2009). However, oropharyngeal aspiration of the arginase inhibitor S-(2-boronoethyl)-L-cysteine (BEC) 2 h after the last of three ovalbumin challenges did not affect allergen-induced AHR of the central airways (Ckless *et al*., 2008). Although treatment with BEC appeared to reduce the allergen-induced increase in peripheral airway responsiveness at 40 s after methacholine inhalation, BEC did not inhibit – but rather accelerated – the peak-response to methacholine (Ckless *et al*., 2008). An explanation for this unexpected discrepancy is presently not yet at hand. However, a recent study of acetylcholine-induced vasorelaxation suggested that BEC may also have cellular targets other than arginase, including guanylyl cyclase (Huynh *et al*., 2009).

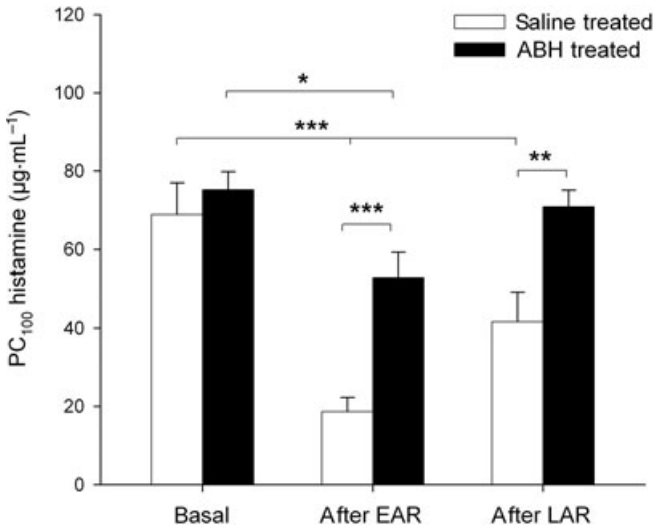

Figure 3 Inhalation of the arginase inhibitor ABH protects against allergen-induced AHR after the EAR and LAR *in vivo*. Inhalation of the arginase inhibitor ABH (25 mM, nebulizer concentration) 0.5 h before and 8 h after allergen challenge protects against the development of AHR to inhaled histamine after the EAR and LAR in a guinea pig model of allergic asthma *in vivo*. Airway responsiveness was determined by assessing the histamine PC_{100}-value, that is, the concentration of histamine causing a 100% increase of pleural pressure. Data represent means ± SEM of seven or eight animals. Reproduced with permission from Maarsingh *et al.* (2008d). ABH, 2(S)-amino-6-boronohexanoic acid; AHR, airway hyperresponsiveness; EAR, early asthmatic reaction; LAR, late asthmatic reaction.

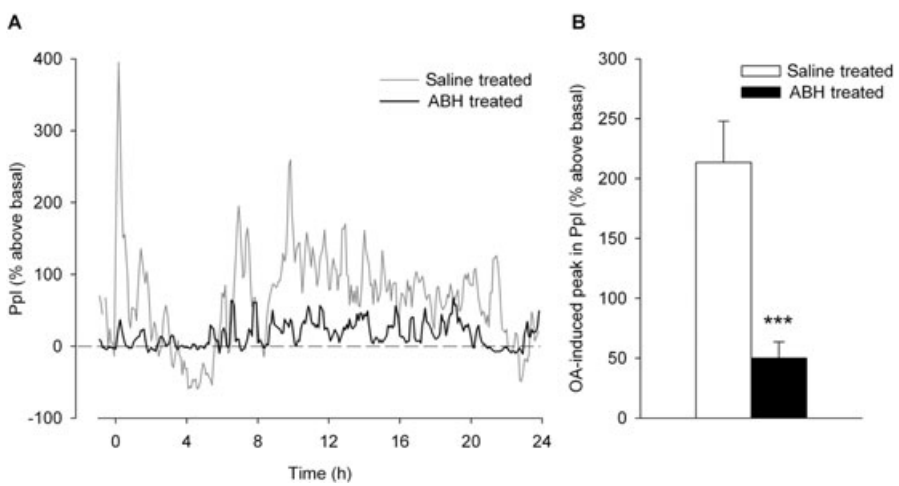

Figure 4 Inhalation of the arginase inhibitor ABH protects against allergen-induced early and late asthmatic reactions. (A) Representative on-line registrations of pleural pressure (P_{pl}) in sensitized, conscious and unrestrained guinea pigs, treated with either saline or ABH (25 mM, nebulizer concentration) 0.5 h before and 8 h after ovalbumin challenge (at $t = 0$ h). Treatment with ABH markedly reduced the magnitude of the allergen-induced EAR and LAR, as indicated by the reduced area under the P_{pl}-time curve. (B) Pre-treatment with ABH greatly reduced the initial ovalbumin (OA)-induced bronchoconstriction, as determined by the peak rise in P_{pl} immediately after allergen challenge. Both treatment groups were exposed to the same allergen dose. Data represent means ± SEM of seven or eight animals. ***$P < 0.005$ compared with saline-treated animals. Reproduced with permission from Maarsingh *et al.* (2008d). ABH, 2(S)-amino-6-boronohexanoic acid; AHR, airway hyperresponsiveness; EAR, early asthmatic reaction; LAR, late asthmatic reaction.

Arginase and airway inflammation

Increased arginase may also be involved in airway inflammation in asthmatics by limiting the production of cNOS-derived NO. As mentioned, cNOS-derived NO has been shown to inhibit inflammation by suppressing the activation of NF-κB, thereby inhibiting the production of inflammatory cytokines, as well as the expression of iNOS (Cirino *et al.*, 2003; Thomassen *et al.*, 1997; Cook *et al.*, 2003; Ten Broeke *et al.*, 2006; Ckless *et al.*, 2007). NO may inhibit the activity of NF-κB in at least two ways: via S-nitrosylation of the DNA-interacting p50 subunit (Marshall and Stamler, 2001) and via suppression of the phosphorylation and degradation of IκB (Chang *et al.*, 2004). Overexpression of arginase I in lung epithelial cells reduced NO production and increased cytokine-induced NF-κB activity, which was associated with decreased S-nitrosylation of p50 (Ckless *et al.*, 2007). Conversely, inhibition of arginase decreased NF-κB activity, whereas the NO synthesis and S-nitrosylation of p50 were increased. These effects of arginase inhibition were prevented by the NOS inhibitor L-NAME (Ckless *et al.*, 2007). Recently, the effect of arginase inhibition on allergen-induced airway inflammation *in vivo* has also been studied. A first report in ovalbumin-challenged guinea pigs indicated that pre-treatment with inhaled ABH significantly reduced total inflammatory cell number, eosinophils and macrophages in the bronchoalveolar lavage (BAL) by approximately 50%, indicating that increased arginase activity in allergic asthma also contributes to airway inflammation (Maarsingh *et al.*, 2008d). This was confirmed in a C57BL/6 mouse model of allergic asthma using intraperitoneally applied nor-NOHA (Bratt *et al.*, 2009). The inhibitory effects of the arginase inhibitors on airway inflammation could indeed involve increased production of NO, as allergen-induced cellular infiltration was higher in iNOS knockout mice than in wild-type animals (Bratt *et al.*, 2009). By contrast, *in vivo* treatment with BEC in BALB/c mice did not reduce allergen-induced inflammatory cell numbers, nor levels of cytokines in the BAL, and even slightly enhanced peribronchiolar and perivascular inflammation in the lung of these animals (Ckless *et al.*, 2008). Remarkably, in the same study, arginase inhibition by BEC also caused further increase in allergen-induced NF-κB activity in the lung, which seems to be at variance with previous findings in cultured epithelial cells (Ckless *et al.*, 2007). Clearly, the role of arginase in allergen-induced airway inflammation in asthma warrants further investigation.

Arginase and airway remodelling in chronic asthma

It has been postulated that increased arginase activity in chronic asthma may contribute to airway remodelling via the synthesis of polyamines and L-proline downstream from L-ornithine, which induce cell proliferation and collagen synthesis, respectively (Meurs *et al.*, 2003; Ricciardolo *et al.*, 2005; Figure 1). The synthesis of polyamines from L-ornithine is initiated by the action of ornithine decarboxylase (ODC), which converts L-ornithine into putrescine. Putrescine is converted into spermidine and subsequently into spermine by spermidine synthase and spermine synthase, respectively (Wu and Morris, 1998). L-Ornithine may also be converted to L-proline, which is a precursor of collagen, in a two-step reaction involving ornithine aminotransferase (OAT) and pyrroline-5-carboxylate reductase (Wu and Morris, 1998). The expression of arginase, ODC as well as OAT, can be increased by growth factors, resulting in increased synthesis of polyamines, L-proline and collagen (Thyberg and Fredholm, 1987a,b; Durante *et al.*, 1998; 2001; Nelin *et al.*, 2005). Growth factor-induced induction of ODC expression and activation has recently also been shown in bovine tracheal smooth muscle (Maarsingh *et al.*, 2008b). Since growth factors are elevated in asthmatics, induction of these enzymes could contribute to airway remodelling.

Polyamines are known to stimulate the expression of genes involved in cell proliferation by promoting histone acetyltransferase (HAT) activity and chromatin hyperacetylation (Hobbs and Gilmour, 2000), and may thus contribute to proliferation of structural cells in the airways (Wu and Morris, 1998; Hoet and Nemery, 2000; Meurs *et al.*, 2003; Ricciardolo *et al.*, 2005). The potential significance of this mechanism in asthma is supported by the observation that HAT activity is increased in bronchial biopsies of asthmatics (Ito *et al.*, 2002). Importantly, elevated levels of putrescine have been observed in mouse lung after allergen challenge (Zimmermann *et al.*, 2003), whereas elevated levels of putrescine, spermidine and spermine were detected in serum of asthmatic patients (Kurosawa *et al.*, 1992).

In support of a potential role for arginase in airway smooth muscle cell proliferation, it has been demonstrated that transfection of vascular smooth muscle cells with arginase I increased polyamine levels and enhanced proliferation of these cells (Wei *et al.*, 2001). Increased arginase activity may also contribute to airway remodelling by reducing NO synthesis. NO-mediated inhibition of ODC has been observed via S-nitrosylation of the enzyme (Bauer *et al.*, 1999). In addition, inhibitory effects of NO on airway smooth muscle proliferation have been demonstrated (Hamad and Knox, 2001).

Subepithelial fibrosis in asthmatic airways may be caused by increased formation of L-proline, and subsequently collagen, from L-ornithine due to increased arginase activity. Interestingly, arginase expression and activity in vascular smooth smooth muscle cells was increased by transforming growth factor-β (TGF-β) (Durante *et al.*, 2001), a mediator that is also important in the development of airway fibrosis in asthma (Howell and McAnulty, 2006). Moreover, the non-specific arginase inhibitor NOHA inhibited TGF-β-induced increase in collagen content in primary mouse fibroblasts in a post-transcriptional manner (Kitowska *et al.*, 2007). In further support of a potential role of arginase in airway fibrosis in asthma, the Th2 cytokines IL-4 and IL-13 increased arginase I and II expression in cultured rat fibroblasts (Lindemann and Racke, 2003).

Increased expression of arginase I and II, as well as of collagen I, have also been reported in a mouse model of bleomycin-induced lung fibrosis (Endo *et al.*, 2003). Recently, it was shown that chronic infusion of the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA) increased

arginase activity, as well as collagen deposition in mouse lung (Wells *et al.*, 2009). Interestingly, ADMA-induced arginase activity and collagen I expression in cultured primary mouse lung fibroblasts were fully reduced by treatment with NOHA, strongly suggesting that increased arginase activity underlies elevated collagen synthesis by these cells (Wells *et al.*, 2009).

In conclusion, evidence for a role of arginase in tissue remodelling is accumulating. However, the involvement of this enzyme in airway remodelling in asthma remains to be established.

Arginase and human asthma

Already in the early eighties, arginase activity was shown to be increased in expectorated sputum of patients with asthma (Kochanski *et al.*, 1980). However, it took more than 20 years to appreciate the potential significance of this finding for the disease (Meurs *et al.*, 2003; Zimmermann *et al.*, 2003). Increased expression of arginase I in the airways of asthmatic patients was first described by Zimmermann *et al.* (2003). Thus, increased numbers of cells expressing arginase I, presumably macrophages, were found in the BAL from these patients. In addition, increased mRNA expression of the enzyme was observed in inflammatory cells, as well as in the airway epithelium in bronchial biopsies obtained from asthmatics (Zimmermann *et al.*, 2003). That increased consumption of L-arginine by arginase may also be involved in the pathophysiology of human asthma, was indicated by Morris *et al.* (2004). Thus, in asthmatics experiencing an exacerbation, a marked increase in arginase activity was found in serum, whereas plasma L-arginine levels were strikingly reduced. Moreover, improvement of asthma symptoms in some of these patients was associated with a decline in arginase activity and an increase in L-arginine concentrations (Morris *et al.*, 2004). In severe asthmatics, it was recently shown that serum arginase activity is inversely correlated to forced expiratory volume in 1 second (FEV_1) and FEV_1/forced vital capacity (FVC), whereas a positive correlation was found between L-arginine bioavailability and lung function (Lara *et al.*, 2008).

Several single nucleotide polymorphisms (SNPs) have been reported for both the arginase I and arginase II gene. A significant association between SNPs in arginase II and increased risk of childhood asthma has been observed (Li *et al.*, 2006). In the same study, evidence was also found for association between arginase I and arginase II SNPs and atopy (Li *et al.*, 2006). Remarkably, screening 844 SNPs from 111 candidate genes for association with inhaled β_2-agonist-induced bronchodilation recently identified arginase I as a potential β_2-agonist response gene (Litonjua *et al.*, 2008).

Arginase as a novel therapeutic target in allergic asthma

In conclusion, research during the last few years has revealed a remarkable causal role for L-arginine homeostasis by arginase in the pathophysiology of allergic asthma. Evidence obtained from animal studies indicates that arginase, particularly arginase I, belongs to the most prominently up-regulated genes in asthma, which may involve Th2 cytokines, as well as growth factors. *Ex vivo* studies in animal models of allergic asthma have shown that a deficiency of bronchodilating NO as well as an increased production of peroxynitrite, induced by reduced L-arginine availability to cNOS and iNOS, may be importantly involved in the development of allergen-induced AHR in this disease. In addition, a potential role for arginase in airway inflammation and airway remodelling by NO-dependent as well as NO-independent mechanisms has also been indicated, suggesting a central role of the enzyme in both acute and chronic asthma.

The functional relevance of arginase in asthmatic patients remains to be established. However, both pulmonary and systemic expression and activity of the enzyme are remarkably increased in these patients, which correlates with symptoms. In addition, genetic studies have indicated associations of gene polymorphisms in both arginase I and II, with increased risk of asthma, atopy and bronchodilator effectiveness in these patients.

Initial animal model studies *in vivo* have indicated the effectiveness of some specific arginase inhibitors in the protection and/or reversal of allergen-induced bronchial obstructive reactions and AHR, which, in addition to acute bronchoprotective effects, may involve both anti-allergic and anti-inflammatory actions. If the animal model studies can be translated to human disease, arginase inhibitors may have great therapeutic potential in the treatment of asthma. Topical application of arginase inhibitors via inhalation would be the preferred route in order to minimize possible unwanted (systemic) side effects, such as inhibition of the urea cycle in the liver, which may lead to hyperammonemia. Administration of L-arginine could also restore the bioavailability of this amino acid to NOS isoforms, and hence increase the production of bronchoprotective NO and reduce the detrimental formation of superoxide and peroxynitrite. However, chronic administration of L-arginine would similarly increase the activity of arginase, leading to enhanced formation of downstream products of L-ornithine that are involved in airway remodelling. Therefore, prolonged treatment of asthmatics with L-arginine may be contraindicated.

Collectively, it can be concluded that arginase represents a drug target that opens exciting novel therapeutic perspectives for the treatment of asthma.

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Statement of conflict of interest

The authors state no conflict of interest.

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