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# Increased arginase activity contributes to airway remodelling in chronic allergic asthma

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**Abstract**

Airway remodelling, characterized by increased airway smooth muscle (ASM) mass, subepithelial fibrosis, goblet cell hyperplasia and mucus gland hypertrophy, is a feature of chronic asthma. Increased arginase activity could contribute to these features via increased formation of polyamines and L-proline downstream of the arginase product L-ornithine, and via reduced nitric oxide synthesis.

Using the specific arginase inhitibor 2(S)-amino-6-boronohexanoic acid (ABH), we studied the role of arginase in airway remodelling using a guinea pig model of chronic asthma. Ovalbumin-sensitized guinea pigs were treated with ABH or PBS via inhalation before each of 12 weekly allergen or saline-challenges, and indices of arginase activity and airway remodelling, inflammation and responsiveness were studied 24h after the last challenge. Pulmonary arginase activity of repeatedly allergen-challenged guinea pigs was increased. Allergen challenge also increased ASM mass and maximal contraction of denuded tracheal rings, which were prevented by ABH. ABH also attenuated allergen-induced pulmonary hydroxyproline (fibrosis) and putrescine, mucus gland hypertrophy, goblet cell hyperplasia, airway eosinophilia and IL-13, whereas an increased L-ornithine/L-citrulline ratio in the lung was normalized. Moreover, allergen-induced hyperresponsiveness of perfused tracheae was fully abrogated by ABH.

These findings demonstrate that arginase is prominently involved in allergen-induced airway remodelling, inflammation, and hyperresponsiveness in chronic asthma.

**Key words:**

Airway hyperresponsiveness, airway remodeling, eosiniphils, fibrosis, goblet cells, polyamines

**Introduction:**

Allergic asthma is a chronic inflammatory airways disease, which is characterized by allergen-induced, IgE-mediated early and late bronchial obstructive reactions, acute and transient airway hyperresponsiveness (AHR) after these reactions and infiltration of inflammatory cells - particularly eosinophils and Th2 lymphocytes - into the airways [1,2]. In addition, structural changes in the airway wall, including thickening of the basement membrane, increased airway smooth muscle (ASM) mass, subepithelial fibrosis, goblet cell hyperplasia, submucosal gland hypertrophy and increased vascularization are observed, which may contribute to the progressive decline in lung function and persistent AHR in chronic asthma [2,3].

Recently, arginase, which hydrolyses L-arginine into L-ornithine and urea, has been identified as a key player in the pathophysiology of acute allergic asthma [4]. Two arginase isoforms have been identified, cytosolic arginase I and mitochondrial arginase II [5], both of which are expressed in the airways, particularly in epithelial cells, (myo)fibroblasts, endothelial cells and macrophages [6-8]. Increased expression and/or activity of arginase has been observed in airways and lung tissue obtained from various animal models of allergic asthma [9] as well as from patients [10-12]. Increased consumption of L-arginine by arginase contributes to the development of allergen-induced AHR in acute allergic asthma by limiting the bioavailability of L-arginine to nitric oxide synthase (NOS) isozymes [13-15], which hydrolyze the amino acid into nitric oxide (NO) and L-citrulline. This leads to a deficiency of bronchodilating NO and increased formation of the highly reactive proinflammatory and procontractile NO metabolite peroxynitrite, both of which underlie AHR in animal models of allergic asthma [13-18]. In a guinea pig model of allergic asthma, inhalation of the specific arginase inhibitor (2)S-amino-boronohexanoic acid (ABH) acutely reversed the allergen-induced AHR *in vivo* both after the early and late asthmatic reaction, to a similar extent as

treatment with inhaled L-arginine [19]. Moreover, treatment with inhaled ABH 30 min prior to and 8 h after allergen challenge protected against the development of the early and late obstructive reactions, AHR after both reactions and airway inflammation [19].

Reduced NO synthesis due to increased arginase activity could also contribute to airway remodelling in chronic asthma, since NO inhibits ASM proliferation [20-23] as well as fibrosis [24]. In addition, increased arginase activity could induce airway remodelling via increased synthesis of polyamines (putrescine, spermidine and spermine) and L-proline downstream of L-ornithine [5,9,25]. Polyamines are involved in cell proliferation and differentiation [5] and increased polyamine levels have been demonstrated in allergen-challenged mice [10] and asthmatic patients [26]. L-Proline is a precursor of collagen [5] and could thus play a role in allergen-induced fibrosis. This is supported by the observation that inhibition of arginase activity reduced TGF-β-induced collagen synthesis in primary mouse lung fibroblasts [27]. Therefore, we hypothesize that, besides in acute asthma, increased arginase activity also plays a key role in the pathophysiology of chronic asthma. In support, increased arginase expression and activity have been observed in animal models of chronic allergic asthma [11,28].

In the present study, we investigated the role of arginase in chronic asthma by studying the effects of inhaled ABH on allergen-induced airway remodelling, inflammation and AHR in repeatedly allergen-challenged guinea pigs.

## Methods

*Animals and sensitization procedure*

Outbred male specified pathogen-free Dunkin Hartley guinea pigs (Harlan Hillcrest, UK) weighing 200-250 g were actively IgE-sensitized to ovalbumin (OVA) as described previously [29]. The animals were group-housed in individual cages in climate-controlled animal quarters and given water and food ad libitum, while a 12-h on/12-h off light cycle was maintained.

All protocols described in this study were approved by the University of Groningen Committee for Animal Experimentation.

*Experimental protocol*

Four weeks after sensitisation, the animals were challenged once weekly with either saline (control group) or allergen (OVA) for 12 weeks. The saline and allergen provocations were performed by inhalation of aerosolized solutions, using a DeVilbiss nebulizer (type 646), in a specially designed animal cage, in which the guinea pigs could move freely [29]. OVA challenges were performed by inhalation of increasing concentrations of OVA in saline (0.05%, 0.1%, 0.3%, 0.5% and 0.7% w/v) for 3 min each, with 7 min intervals as previously described [29-31] Allergen inhalations were discontinued when the first signs of respiratory distress were observed. No anti-histaminic was needed to prevent the development of anaphylactic shock. Control animals were weekly challenged with saline for 3 min.

The animals were treated with inhaled ABH in PBS (25 mM nebulizer concentration, 15 min) or PBS (15 min) 0.5 h before and 8 h after each allergen or saline challenge. 2(S)-amino-6-boronohexanoic acid was synthesized as described previously [32].

*Tissue acquisition*

At 24 h after the last challenge, guinea pigs were sacrificed by experimental concussion followed by rapid exsanguination. The lungs were immediately resected and kept on ice for further processing. The trachea was rapidly removed and transferred to a Krebs–Henseleit solution (37°C; pH 7.4), pregassed with 5% $CO_2$/95% $O_2$.

*Isometric tension measurements*

Isometric contraction experiments were performed as described previousl [30]. Briefly, the trachea was prepared free of serous connective tissue. Epithelium-denuded single open-ring preparations were mounted for isometric recording in organ baths containing KH-solution (37°C, pH 7.4), gassed with 5% $CO_2$/95% $O_2$. After equilibration, the resting tension was adjusted to 0.5 g, followed by pre-contractions using 20 and 40 mM KCl. Following wash-outs and another equilibration period of 30 min, cumulative concentration–response curves were constructed using methacholine.

*Tracheal perfusion*

The trachea was prepared free of serosal connective tissue and cut into two halves of approximately 16 mm each. The preparations were attached to a perfusion holder and placed in an organ bath (37°) containing 20 ml of gassed KH-solution (extraluminal (EL) compartment) as described previously [17]. The tracheal lumen was perfused with recirculating KH-solution from a separate 20 ml bath (intraluminal (IL) compartment) at constant flow rate and hydrostatic pressure was measured at the proximal and distal ends of the trachea using axially centred side-hole catheters. The differential pressure (ΔP) was recorded and reflects the resistance of the tracheal segment to perfusion, which is a function of the mean diameter of the trachea between the pressure taps [33].

After a 45 min equilibration period with three washes with fresh KH (both IL and EL), 1 μM isoprenaline was added to the EL compartment to assess basal tone. After three washes (10 min each), the trachea was exposed to 40 mM KCl in KH (EL) to obtain a receptor-independent reference response. After washout with KH during 45 min, a cumulative concentration response curve was made with IL methacholine. 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, to correct for differences in baseline ΔP and in ΔP changes in response to contractile stimuli, due to variation in internal diameter of the preparations used.

*Arginase activity*

Arginase activity was determined in lung homogenates, by measuring the conversion of [$^{14}$C]-L-arginine to [$^{14}$C]-urea as previously described [15]. Arginase activity was expressed as pmol urea produced per mg protein per min.

*IL-13 measurement*

Levels of IL-13 were determined in lung homogenates by ELISA, using anti-guinea pig IL-13 (Cusabio) according to the manufacturer's protocol.

*Histochemistry*

Transverse cryostat cross-sections (4 μm) of the main bronchi from both left and right lung lobes were used for morphometric analyses. Sections were stained for smooth-muscle-specific myosin heavy chain (*sm*-MHC; Neomarkers) and mucin 5 subtypes A and C (MUC5AC; Neomarkers). Primary antibodies were visualized using horseradish-peroxidase-linked secondary antibodies and diaminobenzidine. In addition, haematoxylin-stained nuclei

within the ASM bundle were counted. Eosinophils were identified in haematoxylin- and eosin-stained lung sections and mucus-producing cells were stained using periodic acid-Schiff (PAS). Airways within sections were digitally photographed and subclassified as cartilaginous or non-cartilaginous. All immunohistochemical measurements were carried out digitally using quantification software (ImageJ). For all studied parameters, 3 - 6 airways of each classification per animal were analysed.

*Analysis of amino acids and putrescine*

Lung homogenates (50 µL) were precipitated by adding equal volumes of cold acetonitrile. After centrifugation (5 min at 16.000 x*g*) 30 µL of clear supernatant was mixed with 4 µL deuterated internal standard solution ($10^{-5}$ M) and used directly for the analysis of the amino acids, L-arginine, L-ornithine and L-citrulline, and of putrescine.

Quantitative analysis was carried out by liquid chromatography-mass spectrometry using an LC-20 chromatography platform (Shimadzu, Kyoto, Japan) coupled to an API-4000 triple quadrupole mass spectrometer with Turbo spray ESI interface (AB Sciex, Concord, Canada). Samples were derivatized with 40 µL SymDAQ reagent (Brainlink, Groningen, The Netherlands) immediately prior to analysis by using automated reagent addition on a SIL-10 autosampler (Shimadzu, Kyoto, Japan) and resolved on a 100x3 mm Synergi Max-RP (2.5 µm particle size) column (Phenomenex, Torrance, CA, USA) by a linear gradient of acetonitrile in water, each containing 0.1 % (v/v) formic acid. Eight-point calibration curves (0.1-50 µM for putrescine, 0.01-25 µM for amino acids) were used for quantition. The concentrations of the amino acids and putrescine in the lung homogenates were expressed as µmol per mg protein.

*Hydroxyproline assay*

The hydroxyproline content of lung tissue was determined as an estimate of collagen as decribed previously [34]. The lower right lung lobe was pulverized under liquid nitrogen and sonificated in 10 ml PBS. Homogenates were incubated on ice with 1.25 ml 5% TCA for 20 min. Samples were centrifuged and the pellet was resuspended in 10 ml 12 N hydrochloric acid and heated at 110 °C until charred and dry. The dried pellets were dissolved in 2 ml ultrapure water by incubating at room temperature for 72 h with intermittent vortexing. Five µl of each sample was transferred to a 96-wells plate and 100 µl chloramine T solution (1.4% chloramine T in 0.5 M sodium acetate/10% isopropanol) was added. After 30 min, 100 µl Ehrlich's solution (1.0 M 4-dimethylaminobenzaldehyde in 70% isopropanol/30% perchloric acid) was added and samples were incubated at 65°C for 30 min. Samples were cooled to room temperature and the amount of hydroxyproline was quantified by colorimetric measurement (OD at 550 nm, Biorad 680 plate reader).

*Data analysis*

Both for the isometric contraction and the perfusion experiments, the maximal contractile response to methacholine was defined as $E_{max}$. Using this $E_{max}$, the sensitivity ($pEC_{50}$) towards methacholine was evaluated.

All data represent means ± SEM from *n* separate experiments. Statistical significance of differences was evaluated using one-way ANOVA, followed by a Bonferoni post hoc test. Differences were considered statistically significant at $P<0.05$.

**Results:**

The arginase activity in lung homogenates from repeatedly allergen-challenged guinea pigs was 1.6-fold increased compared to saline-challenged controls ($P<0.01$; Figure 1A). *In vivo* treatment with inhaled ABH completely prevented the increased arginase activity in the multiple challenged animals ($P<0.05$), but did not affect arginase activity in the saline-challenged controls (Figure 1A).

To gain insight into the possible mechanisms underlying these changes in arginase activity, we determined lung levels of IL-13, a Th2-cytokine known to induce arginase activity in the lung [35]. As shown in Figure 1B, IL-13 levels in the lung paralleled the changes in arginase activity. Thus, chronic allergen challenge resulted in a 1.7-fold increase in IL-13 compared to saline-challenged controls ($P<0.01$; Figure 1B). Treatment with ABH normalized the allergen-induced increase in IL-13 ($P<0.05$), leaving basal IL-13 level unaffected (Figure 1B).

Pulmonary L-arginine as well as L-citrulline levels were decreased after repeated allergen challenge ($P<0.01$ and $P<0.05$, respectively, Table 1), whereas L-ornithine was not significantly changed and the polyamine putrescine was increased ($P<0.01$; Table 1, Figure 2A). Accordingly, the putrescine/L-arginine, L-ornithine/L-arginine, and L-ornithine/L-citrulline ratio's were significantly increased after the allergen challenges (Figures 2B, 2C and 2D), the latter ratio indicating that increased arginase activity in the lung competes with NOS activity and reduces NO production. In line, all allergen-induced changes were attenuated by ABH (Figure 2, Table 1). ABH did not affect amino acid and polyamine levels in the saline-challenged animals (Figure 2, Table 1).

Repeated allergen challenge resulted in a trend towards an increased ASM mass in the cartilaginous airways as compared to saline-challenged controls ($P=0.08$; Figure 3A), whereas a marked increase in ASM mass was observed in the noncartilaginous airways

($P$<0.001; Figure 3B). This increase was largely prevented by treatment with inhaled ABH ($P$<0.001; Figure 3B). By contrast, treatment with ABH did not affect ASM mass in the saline-challenged animals. To determine whether allergen-induced changes in ASM content were associated with changes in cell number and/or changes in cell size, the numbers of nuclei within the ASM layer were counted and expressed relative to total ASM area. From this data and the thickness of all sections, the apparent cell volume of the ASM cells was also calculated. OVA challenge did not change the number of nuclei per $mm^2$ of smooth muscle area (Figure 3C) or ASM cell volume (Figure 3D), indicating that the cell size is unchanged and the allergen-induced increase in ASM mass is caused by an increased cell number. The ASM cell size was not affected by treatment with ABH either (Figures 3C and 3D).

In line with previous studies [30,31], repeated allergen challenge induced a 1.7-fold increase in maximal contraction of epithelium-denuded tracheal open-ring preparations to methacholine as compared to saline-challenged controls ($P$<0.01; Figure 4A; Table S1 in the online supplementary material), without an effect on the sensitivity ($pEC_{50}$) towards the agonist. This indicates the development of a hypercontractile ASM phenotype, due to changes distal to the receptor as described previously [30,31]. Treatment with ABH completely prevented the allergen-induced hypercontractility ($P$<0.01), whereas ASM contractility of the saline-challenged animals was not affected (Figure 4A; Table S1 in the online supplementary material).

In addition to intrinsic changes in the ASM, allergen challenge may also affect regulatory processes in the airway wall determining ASM tone, including (epithelial) NO homeostasis [16,17,28]. Therefore, we also determined the responsiveness of intact perfused tracheal preparations to methacholine. The responsiveness of these preparation is expressed proportional to KCl-induced airway constriction, and therefore the induction of a hypercontractile ASM phenotype due to post-receptor changes as indicated above does not

influence this airway responsiveness. Interestingly, a 1.7-fold increase in maximal airway constriction to methacholine was observed in the perfused tracheal segments obtained from allergen-challenged guinea pigs as compared to those from saline-challenged controls ($P$<0.01), without an effect on the sensitivity to the contractile agonist (Figure 4B, Table S2 in the online supplementary material). The development of AHR in these preparations was prevented by ABH treatment of the allergen-challenged animals ($P$<0.01), whereas ABH did not affect the airway responsiveness of saline-challenged animals (Figure 4B, Table S2 in the online supplementary material).

Increased deposition of extracellular matrix proteins, including collagens, in the airway wall is a characteristic feature of airway remodelling in chronic asthma [36,37]. Since detection of collagens in the guinea pig airways by immunohistochemistry was not feasible with the antibodies available, lungs were analyzed for hydroxyproline content, as an estimate of total collagen. Repeated allergen challenge caused a 1.5-fold increase in hydroxyproline ($P$<0.01), which was fully prevented in the ABH-treated animals ($P$<0.05), whereas ABH did not affect hydroxyproline content in saline-challenged animals (Figure 5).

In the saline-treated animals, repeated allergen challenge induced eosinophil influx in the submucosal, ASM and adventitial compartments of both cartilaginous and noncartilaginous airways, as compared to saline-challenged controls ($P$<0.001 all; Table 2). ABH treatment did not affect airway eosinophil numbers in the saline-challenged animals, but largely prevented the allergen-induced eosinophilia in all compartment of both the cartilaginous and noncartilaginous airways ($P$<0.001 all; Table 2).

In line with a previous study using the same guinea pig model of chronic asthma [31], repeated allergen challenge resulted in goblet cell hyperplasia in the cartilaginous airways ($P$<0.01; Figure 6A and 6B), which was attenuated by the arginase inhibitor ($P$<0.05). In addition, repeated allergen challenge resulted in an increase in mucus gland area in the same

airways ($P$<0.01, Figure 6C). This mucus gland hypertrophy was largely prevented by treatment with ABH ($P$<0.01; Figure 6C). Similarly, ABH attenuated the allergen-induced increase in MUC5AC-positive goblet cells ($P$<0.05; Figure 6D). Treatment with ABH did not affect numbers of mucus-producing cells in saline-challenged animals (Figure 6).

**Discussion**

Using a well-defined guinea pig model of chronic allergic asthma, we have demonstrated that increased arginase activity contributes to allergen-induced airway remodelling, inflammation and AHR. Thus, inhalation of the arginase inhibitor ABH caused considerable protection against allergen-induced ASM hyperplasia and hypercontractility, mucus gland hypertrophy, goblet cell hyperplasia and fibrosis. In addition, *ex vivo* AHR to methacholine and airway eosinophilia induced by repeated allergen challenge were also greatly reduced in the ABH-treated animals.

In our guinea pig model, repeated allergen challenge increased arginase activity in the lung by 1.6-fold, which is in line with a recent finding in a mouse model of chronic asthma [11]. Remarkably, *in vivo* treatment with ABH completely prevented the allergen-induced increase in arginase activity. The changes in arginase activity were reflected by changes in the L-ornithine/L-arginine and putrescine/ L-arginine ratio's in the lung. Since ABH treatment did not affect the arginase activity in the saline-challenged animals, it's effect cannot be ascribed to residing ABH in the lung, suggesting that arginase inhibition prevents induction of the enzyme after allergen challenge. The expression of arginase is highly induced by Th2-cytokines, including IL-13 [35], ABH markedly attenuated the allergen-induced increase in IL-13 in our model, indicating that the increased arginase activity and the inhibitory effect of ABH thereon may result from changes in IL-13. This is in line with a recent finding in a mouse model of acute asthma, showing that intranasal application of the arginase inhibitor nor-NOHA prevents house dust mite-induced increases in Th2-cytokine expression and arginase activity in the lung [38]. It is tempting to speculate that the previously observed anti-allergic effect of inhaled ABH [19] underlies the reduction of Th2-cytokine release and subsequent arginase induction.

Increased ASM mass is a characteristic feature of airway remodelling in asthma, which may result both from ASM cell hyperplasia and hypertrophy [39]. In line with previous studies [30,31], repeated allergen challenge in our model induced an increased ASM mass, particularly in the non-cartilaginous airways and characterized by ASM cell hyperplasia. Treatment with ABH inhibited the allergen-induced ASM cell hyperplasia. This could involve restoration of allergen-induced NO deficiency, since NO inhibits mitogen-induced proliferation of cultured human [20-22] and guinea pig [23] ASM cells. Accordingly, we demonstrated that the L-ornithine/L-citrulline ratio in lung tissue was significantly increased after repeated allergen challenge and normalized by the ABH treatment. A second mechanism by which arginase inhibition may inhibit ASM hyperplasia is via inhibition of L-ornithine production. L-Ornithine is a precursor of polyamines (putrescine, spermidine and spermine), which are involved in cell proliferation [5,9,25,40]. In support, transfection with arginase I increased polyamine levels and cell proliferation in vascular smooth muscle cells [41]. Both arginase and ornithine decarboxylase (ODC), which converts L-ornithine into putrescine, are expressed in airway epithelial cells [42] and induction of both enzymes by growth factors, leading to increased polyamine levels, has been described [4]. Moreover, elevated polyamine levels have been detected in lungs of allergen-challenged mice [10] and in serum of asthmatic patients [26]. We now demonstrate that the allergen-induced increase in arginase activity causes an increased production of the polyamine putrescine, which is completely abolished by ABH. In addition to increased formation of L-ornithine, a deficiency of NO may also contribute to the increased polyamine levels after repeated allergen challenge, as NO inhibits ODC via S-nitrosylation [43].

Repeated allergen challenge also increased maximal contraction of tracheal single ring preparations to methacholine. Previous experiments using the same animal model have demonstrated that this ASM hypercontractility results from increased expression of

contractile proteins [31]. Interestingly, our findings indicate that increased arginase activity contributes to the induction of a hypercontractile ASM phenotype. This could be caused by an increased formation of peroxynitrite following multiple allergen challenges [28], as the expression of contractile proteins in human fetal lung fibroblasts is increased by this nitrogen species [44]. It should be noticed that in addition to a hypercontractile ASM phenotype, allergen-induced changes in stimuli governing ASM tone, including NO, may lead to the induction of AHR in chronic asthma [28]. Thus, previous experiments using the same guinea pig model demonstrated that repeated allergen challenge induced hyperresponsiveness of perfused tracheal segments to methacholine, which was caused by increased synthesis of peroxynitrite due to uncoupling of iNOS as a consequence of increased L-arginine consumption by arginase [28]. The present finding suggests that *in vivo* treatment of repeatedly allergen-challenged guinea pigs with ABH protects against the uncoupling of iNOS and subsequent peroxynitrite formation and AHR.

Airway fibrosis in chronic asthma could also result from increased arginase activity, via increased production of L-proline downstream of L-ornithine. In support, the profibrotic factor TGF-β has been shown to induce arginase activity in mouse lung and fibroblasts [45]. Moreover, increased expression of arginase I and II was closely associated with increased collagen I and hydroxyproline expression in bleomycin-induced lung fibrosis in mice [27,46]. Using inhaled ABH, we now demonstrate that increased arginase activity contributes to allergen-induced fibrosis as well. The antifibrotic effect of ABH could similarly result from inhibiting the arginase-induced deficiency NO, which has an antifibrotic action [24]. Accordingly, allergen-induced collagen deposition was shown to be higher in lungs from $iNOS^{-/-}$ mice as well as in mice chronically treated with the NOS inhibitor L-NAME [47,48].

Another characteristic feature of chronic allergic asthma is airway inflammation, which presumably plays an important role in airway remodelling [2]. Our current study

indicates that increased arginase activity may be involved in this process, since the infiltration of eosinophils into different compartments of the airway wall following repeated allergen challenge was markedly reduced by ABH treatment. This anti-inflammatory effect of ABH corresponds to previous observations in a guinea pig model of acute asthma [19] and could be explained by increased production of NO and reduced formation of proinflammatory peroxynitrite. Thus, allergen-induced inflammatory responses are attenuated in mice that overexpress eNOS [49] and are elevated in $iNOS^{-/-}$ mice [50] as compared to wild types. In line with our observations, treatment with the arginase inhibitor $N^{\omega}$-hydroxy-nor-L-arginine (nor-NOHA) reduced inflammatory cell numbers in the bronchoalveolar lavage (BAL) of allergen-challenged mice [38,50]. However, the arginase inhibitor S-(2-boronoethyl)-L-cysteine (BEC) did not affect inflammatory cell numbers or cytokine levels in BAL and even further enhanced peribronchiolar and perivascular inflammation in another mouse model of acute allergic asthma [51]. Remarkably, arginase I from bone marrow-derived cells appeared not to be required for allergen-induced recruitment of inflammatory cells into the lung [52], which could indicate a role for arginase in structural cells, particularly the airway epithelium, in this process.

Increased mucus secretion, which may result from mucus gland hypertrophy as well as goblet cell hyperplasia, is yet another feature of airway obstruction in chronic asthma [53]. We demonstrated that increased arginase activity also regulates changes in mucus cells and MUC5AC expression in repeatedly allergen-challenged guinea pigs. The increased arginase activity may contribute to these features via increased formation of polyamines, which induce cell proliferation and differentiation [5]. Interestingly, L-arginine treatment enhanced allergen-induced goblet hyperplasia in mice [54], which could be explained by increased L-ornithine synthesis from L-arginine by arginase. The anti-inflammatory and anti-allergic effects of ABH [19] could also affect mucus hypersecretion, as many inflammatory cytokines

and mediators, including IL-13, are involved in mucus secretion, goblet cell hyperplasia and mucin expression in the airways [53]. In addition, NO synthesis has been shown to reduce mucus hypersecretion [55], whereas peroxynitrite strongly induces MUC5AC expression [56].

In conclusion, our findings demonstrate that increased arginase activity has a key role in allergen-induced airway remodelling, inflammation and AHR in chronic asthma. Treatment with inhaled ABH effectively inhibits these features, indicating that arginase inhibitors have therapeutic potential in the treatment of this disease.

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**Table 1:** Levels of L-arginine, L-ornithine, L-citrulline and putrescine in lung homogenates of sensitized guinea pigs following repeated saline or ovalbumin challenge and treatment with either inhaled PBS or ABH.

| | **PBS-treated** | | **ABH-treated** | |
|---|---|---|---|---|
| | *Saline challenged* | *OVA challenged* | *Saline challenged* | *OVA challenged* |
| L-Arginine (μmol/mg protein) | 2.53±0.22 | 1.40±0.29$^{**}$ | 1.93±0.27 | 1.56±0.17 |
| L-Ornithine (μmol/mg protein) | 3.52±0.44 | 3.13±0.28 | 2.83±0.29 | 2.33±0.25$^{\#}$ |
| L-Citrulline (μmol/mg protein) | 1.50±0.09 | 1.06±0.17$^{*}$ | 1.44±0.21 | 1.26±0.15 |
| Putrescine (nmol/mg protein) | 13.0±1.6 | 22.2±2.5$^{**}$ | 15.6±0.8 | 14.5±1.1$^{\#\#}$ |

Data are presented as means ± SEM of 7-8 animals. $^{*}P<0.05$ and $^{**}P<0.01$ compared to saline-challenged controls; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ compared to OVA-challenged controls. ABH, (2)S-amino-boronohexanoic acid; OVA, ovalbumin; PBS, phosphate-buffered saline.

**Table 2:** Infiltration of eosinophils (cells/mm basement membrane) into different compartments of the airway wall of sensitized guinea pigs following repeated saline or ovalbumin challenge and treatment with either inhaled PBS or ABH.

| | **Eosinophils (cells/mm basement membrane)** | | | |
|---|---|---|---|---|
| **Airway compartment** | **PBS-treated** | | **ABH-treated** | |
| | *Saline challenged* | *OVA challenged* | *Saline challenged* | *OVA challenged* |
| **Cartilaginous** | | | | |
| Submucosa | 10.7±1.0 | 50.1±5.1$^{***}$ | 12.9±3.3 | 22.4±3.0$^{*}$ / $^{###}$ |
| Airway smooth muscle | 0.1±0.0 | 1.2±0.2$^{***}$ | 0.2±0.1 | 0.6±0.1$^{*}$ / $^{###}$ |
| Adventitia | 14.9±1.9 | 63.2±6.7$^{***}$ | 20.3±3.4 | 27.7±2.6$^{###}$ |
| **Noncartilaginous** | | | | |
| Submucosa | 7.6±0.8 | 43.2±3.5$^{***}$ | 12.4±3.9 | 21.9±3.6$^{*}$ / $^{###}$ |
| Airway smooth muscle | 0.1±0.1 | 1.5±0.3$^{***}$ | 0.2±0.1 | 0.7±0.2$^{*}$ / $^{###}$ |
| Adventitia | 26.3±3.1 | 113.3±9.9$^{***}$ | 25.3±3.6 | 62.4±6.8$^{***}$ / $^{###}$ |

Data are presented as means ± SEM of 7-8 animals. $^{*}P<0.05$ and $^{***}P<0.001$ compared to saline-challenged controls; $^{###}P<0.001$ compared to OVA-challenged controls. ABH, (2)S-amino-boronohexanoic acid; OVA, ovalbumin; PBS, phosphate-buffered saline.

## Figure legends

### Figure 1:

Arginase activity (A) and IL-13 levels (B) in lung homogenates from repeatedly saline- and allergen (OVA)-challenged guinea pigs, treated with inhaled PBS (control) or ABH. Data are presented as means±SEM of 7-8 experiments. **$P$<0.01 compared to saline-challenged controls; $^{\#}P$<0.05 and $^{\#\#}P$<0.01 compared to OVA-challenged controls.

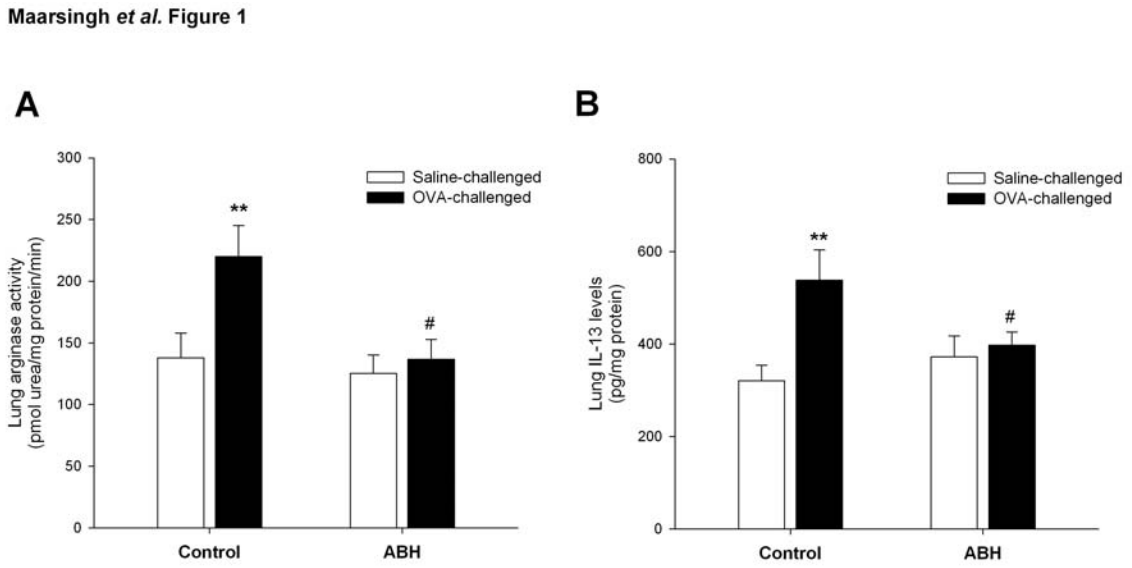

**Figure 2:**

Putrescine levels (A) and putrescine/L-arginine (B), L-ornithine/L-arginine (C) and L-ornithine/L-citrulline (D) ratio's in lung homogenates from repeatedly saline- and allergen (OVA)-challenged guinea pigs, treated with inhaled PBS (control) or ABH. Data are presented as means±SEM of 7-8 experiments. **P*<0.05 and ***P*<0.01 compared to saline-challenged controls; $^{\#}P$<0.05 and $^{\#\#}P$<0.01 compared to OVA-challenged controls.

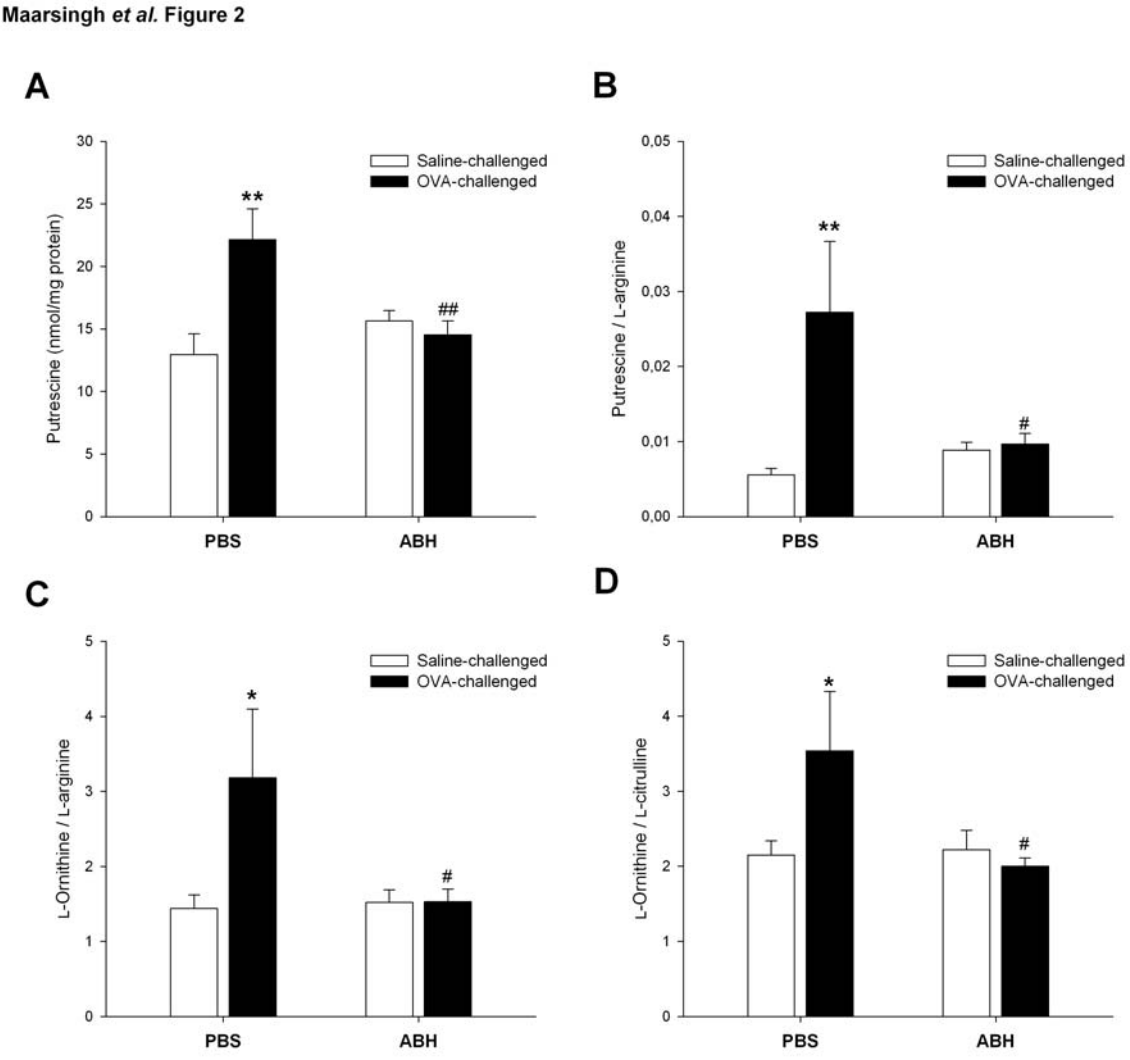

**Figure 3:**

ASM mass in cartilaginous (A) and noncartilaginous (B) airways from repeatedly saline- and allergen (OVA)-challenged guinea-pigs, treated with inhaled PBS (control) or ABH. Number of nuclei per ASM area (C) and apparent ASM cell volume (D) in noncartilaginous airways from above-mentioned animals. Data are presented as means±SEM of 7-8 experiments. ***$P$<0.001 compared to saline-challenged controls; $^{###}P$<0.001 compared to OVA-challenged controls.

**Maarsingh *et al.* Figure 3**

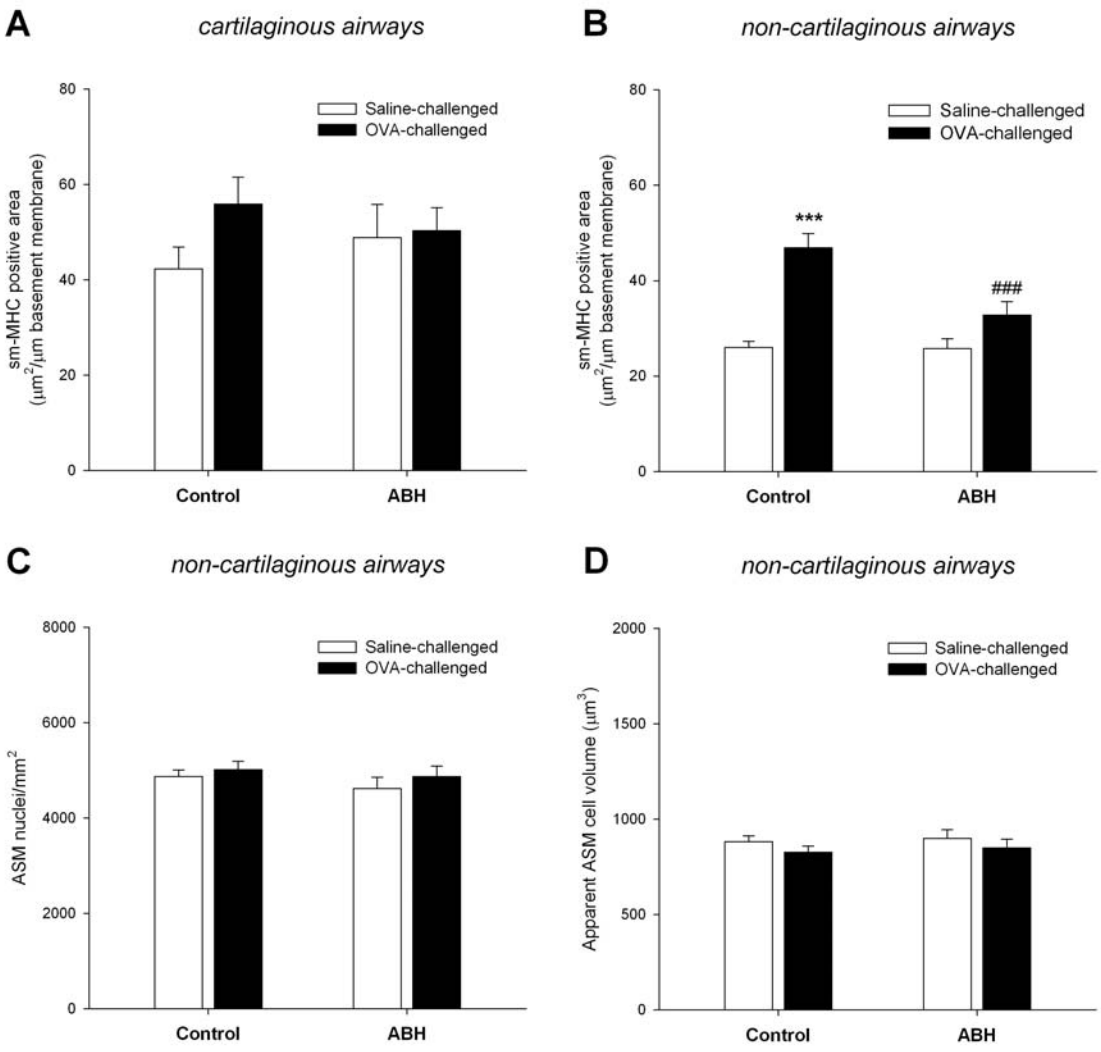

**Figure 4:**

Methacholine-induced isometric contraction of epithelium-denuded tracheal open-ring preparations (A) and constriction of intact perfused guinea pig tracheae (B) from repeatedly saline- and allergen (OVA)-challenged guinea pigs, treated with inhaled PBS (control) or ABH. Data represent means±SEM of 6-8 (A) or 6-10 (B) experiments. $^{**}P<0.01$ compared to saline-challenged controls; $^{\#\#}P< 0.01$ compared to OVA-challenged controls.

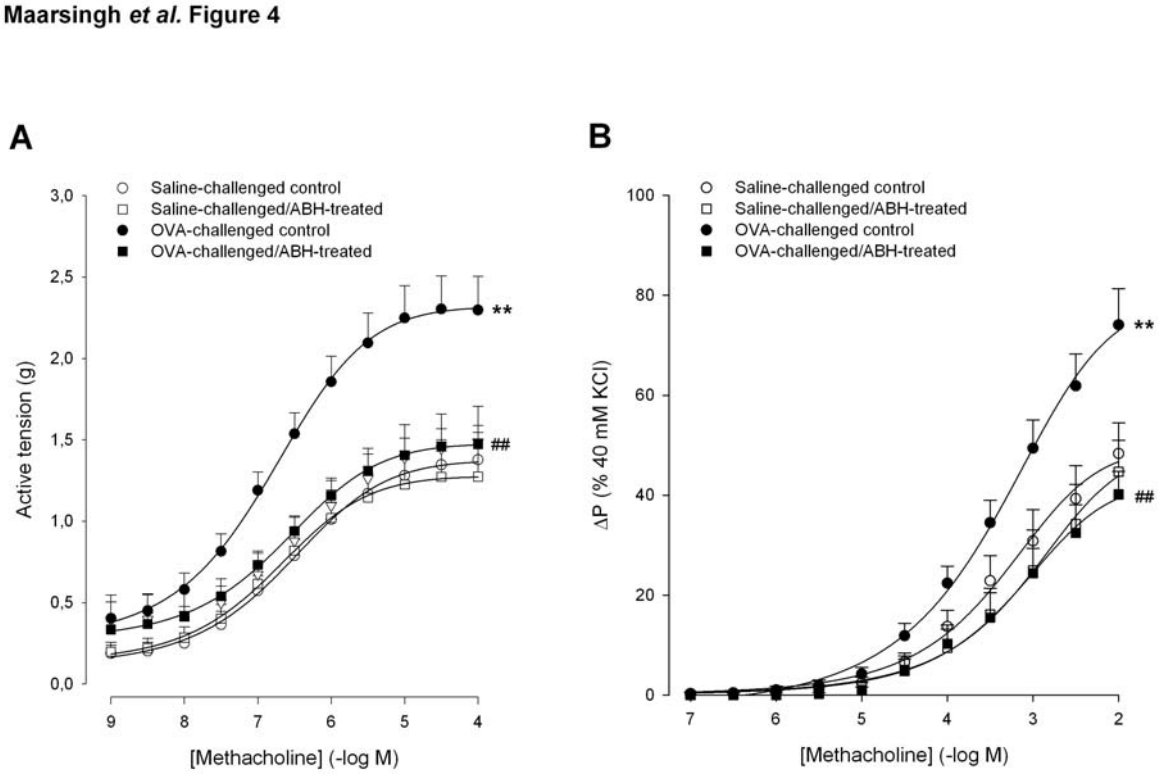

**Figure 5:**

Effect of PBS (control) or ABH treatment *in vivo* on lung hydroxyproline content from multiple saline- or allergen (OVA)-challenged guinea pigs. Results are means±SEM of 4-6 experiments. ***P*<0.01 compared to saline-challenged controls; $^{\#}P$<0.05 compared to OVA-challenged controls.

**Maarsingh *et al.* Figure 5**

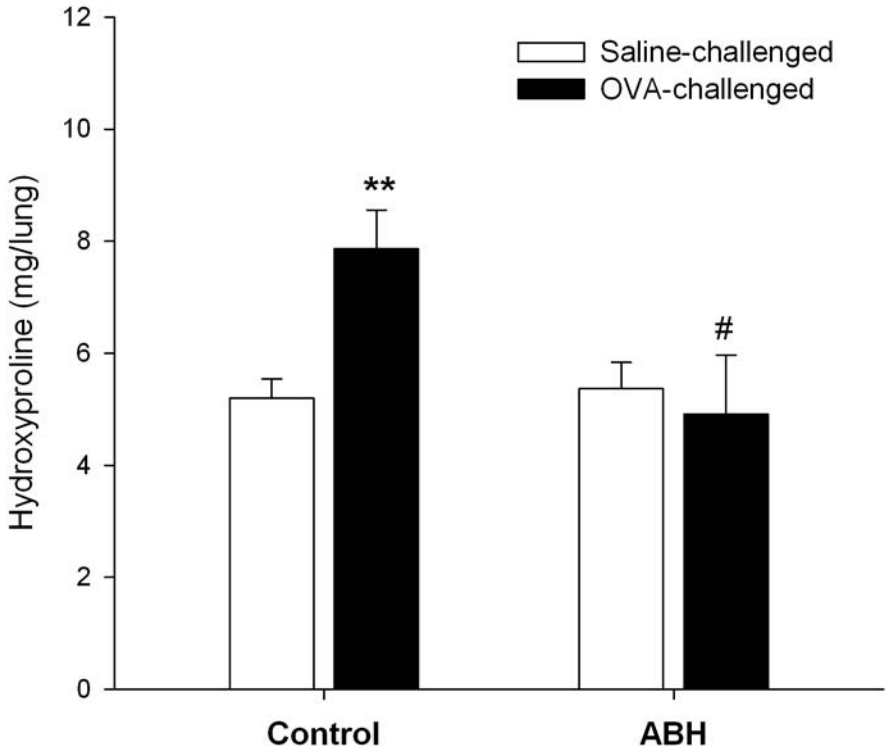

**Figure 6:**

Effects of *in vivo* treatment with PBS (control) or ABH on goblet cell number (B), mucus gland area (C) and MUC5AC-positive goblet cell number (D) in intrapulmonary cartilaginous airways from saline- or ovalbumin (OVA)-challenged guinea pigs. Representative examples of PAS and MUC5AC stainings are shown in panels A and D, respectively. Bars indicate 100 μm. Data are presented as means±SEM of 7-8 experiments. $^{**}P<0.01$ and $^{***}P<0.001$ compared to saline-challenged controls; $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ compared to OVA-challenged controls.

**Maarsingh *et al.* Figure 6**