

Increased Ornithine-Derived Polyamines Cause Airway Hyperresponsiveness in a Mouse Model of Asthma

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Up-regulation of arginase contributes to airways hyperresponsiveness (AHR) in asthma by reducing L-arginine bioavailability for the nitric oxide (NO) synthase isozymes. The product of arginase activity, L-ornithine, can be metabolized into polyamines by ornithine decarboxylase. We tested the hypothesis that increases in L-ornithine-derived polyamines contribute to AHR in mouse models of allergic airways inflammation. After measuring significantly increased polyamine levels in sputum samples from human subjects with asthma after allergen challenge, we used acute and subacute ovalbumin sensitization and challenge mouse models of allergic airways inflammation and naive mice to investigate the relationship of AHR to methacholine and polyamines in the lung. We found that spermine levels were elevated significantly in lungs from the acute model, which exhibits robust AHR, but not in the subacute murine model of asthma, which does not develop AHR. Intratracheal administration of spermine significantly augmented airways responsiveness to methacholine in both naive mice and mice with subacute airways inflammation, and reduced nitrite/nitrate levels in lung homogenates, suggesting that the AHR developed as a consequence of inhibition of constitutive NO production in the airways. Chronic inhibition of polyamine synthesis using an ornithine decarboxylase inhibitor significantly reduced polyamine levels, restored nitrite/nitrate levels to normal, and abrogated the AHR to methacholine in the acute model of allergic airways inflammation. We demonstrate that spermine increases airways responsiveness to methacholine, likely through inhibition of constitutive NO synthesis. Thus, inhibition of polyamine production may represent a new therapeutic target to treat airway obstruction in allergic asthma.

Keywords: polyamines; arginase; airways hyperresponsiveness; α -difluoromethylornithine; spermine

L-arginine plays an important role in the maintenance of airways tone, and imbalances in L-arginine metabolism can contribute to airway obstruction (1–3). Two pathways of L-arginine breakdown are known to be important in asthma: the nitric

CLINICAL RELEVANCE

L-arginine metabolism plays an important role in the maintenance of airways tone. Shunting of L-arginine from the nitric oxide synthase pathway to the arginase pathway can increase airways responsiveness in asthma. We report that downstream metabolites of the arginase pathway, specifically, increased production of L-ornithine-derived polyamines, can contribute to airways hyperresponsiveness in a mouse model of allergic airways inflammation, and that pharmacological inhibition of this pathway can ameliorate the disease pathogenesis.

oxide (NO) synthase (NOS) pathway, to form NO and L-citrulline, and the arginase pathway, which produces L-ornithine and urea (Figure 1). NO is a nonadrenergic, noncholinergic neurotransmitter that can cause bronchodilation through direct effects on airway smooth muscle cells. Constitutive NO (cNO) production in the airways is important for the maintenance of bronchomotor tone. We, and others, have shown that arginase expression and activity are increased in humans with asthma, and contribute to airways hyperresponsiveness (AHR) in animal models of asthma (4–6). L-ornithine, the product of arginase activity, is converted into the polyamine, putrescine, by ornithine decarboxylase (ODC) (7). Putrescine is then further metabolized into the higher-order polyamines, spermidine and spermine, by spermidine synthase and spermine synthase, respectively (2, 8). Arginase has been identified as a potential therapeutic target in asthma to restore the availability of L-arginine for NOS and, subsequently, cNO production (9), and pharmacologic inhibition of arginase attenuates the allergen-induced increase in airways responsiveness to methacholine in mouse models (1, 6, 10–12). Although this effect is thought to be cNO mediated, the potential roles of the L-ornithine-derived polyamines in airway physiology and pathophysiology have not yet been investigated.

Although it has recently been proposed that AHR in asthma develops, in part, as a result of increased substrate competition between the arginase and NOS enzyme families, leading to reduced production of cNO (1–3), it remains unknown: (1) whether increased arginase activity leads to increased production of L-ornithine-derived polyamines; and (2) whether the increased polyamine concentrations would also contribute to airways obstruction. Polyamines are known to accumulate in the epithelium via active transport, and thus may play an important role in lung and airway biology (13). Putrescine has been shown to be elevated in mouse and guinea pig models of asthma, concurrent with increased arginase activity (4, 14); however, levels of the higher-order

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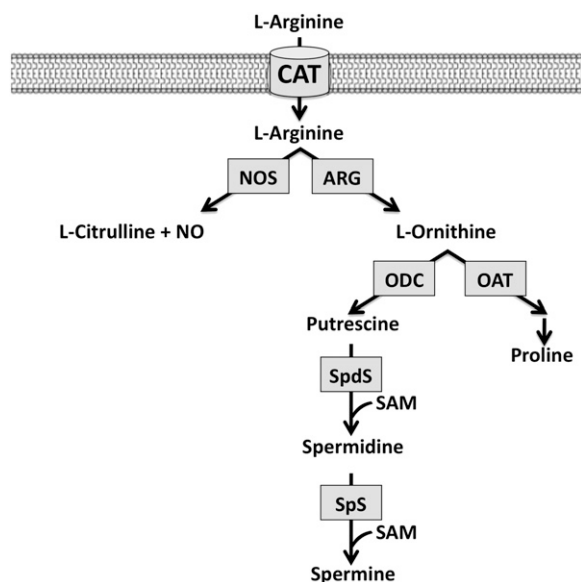


Figure 1. Pathways involved in L-arginine metabolism and polyamine synthesis. ARG, arginase; CAT, cationic amino acid transporter; NO, nitric oxide; NOS, NO synthase; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; SAM, S-adenosyl methionine; SpdS, spermidine synthase; SpS, spermine synthase.

polyamines, spermidine and spermine, and their contribution to airways reactivity have not been well studied in asthma. Furthermore, the effect of inhibition of polyamine biosynthesis on airways responsiveness has not been explored.

We tested the hypothesis that increased levels of polyamines in the airways contribute to increased airways responsiveness to methacholine. First, we report that L-ornithine–derived polyamine levels are increased in human subjects with allergic asthma after an allergen challenge. We then demonstrate that endogenous levels of the L-ornithine–derived polyamines are also significantly elevated in lungs from an acute murine model of ovalbumin (OVA)-induced allergic inflammation. We show that nebulization of physiologically relevant concentrations of spermine into the airways increases airways responsiveness both in naive mice and in mice with pre-existing allergic inflammation, but no prior AHR, and reduces lung accumulation of NO metabolites. Finally, we show that pharmacological inhibition of ODC with α -difluoromethylornithine (DFMO), significantly improved lung total nitrate plus nitrite (NO_x^-) levels and airways responsiveness in the acute murine model.

MATERIALS AND METHODS

Additional experimental details are available in the online supplement.

Allergen Challenge and Induced Sputum

Written informed consent was obtained from five male and five female nonsmokers with clinically stable asthma (FEV_1 % predicted: 100% at baseline) and an average age of 37 years (range, 18–61 yr). After skin-prick testing to make the determination as to which allergen(s) the volunteers were sensitive, subjects were exposed to allergen (i.e., cat, house dust mite, ragweed) in a controlled manner, as described previously (15). All subjects exhibited a reduction in FEV_1 % predicted during both the early and late phase after challenge (ΔFEV_1 % predicted, -33 and -22% , respectively), as well as reductions in the provocative concentration of methacholine causing a 20% fall in FEV_1 (PC_{20}) 24 hours after the allergen challenge. Induced sputum samples were obtained before and at 7 and 24 hours after challenge (16).

Polyamine Levels in Human Sputum and Mouse Lung

Human sputum and mouse lungs were prepared as described previously (17, 18). Quantitative analysis of polyamines was performed using liquid chromatography–tandem mass spectrometry (LCMS) (19). All measurements were standardized to protein concentration, which were determined using the Bradford assay (Bio-Rad, Hercules, CA).

Mouse Models of Allergic Airways Inflammation

To investigate lung polyamine production in murine models of airways inflammation, we employed subacute and acute OVA sensitization and challenge models, which have been described previously (6, 20). At 24 hours after the final OVA or PBS challenge, pulmonary function and methacholine responsiveness testing was performed via forced oscillation with the flexiVent system and the constant-phase model (Scireq Inc., Montreal, PQ, Canada) (6, 21). Lungs were harvested for determination of polyamine and NO_x^- levels, histological assessment, and cytokine and protein analyses. In randomly selected subacute model mice, spermine (12 ± 0.1 nmol/g body weight) was administered by nebulization 15 minutes before methacholine challenge. To investigate the effects of putrescine, spermidine, and spermine on airways responsiveness in naive mice, individual polyamines were delivered by nebulization 15 minutes before methacholine challenge.

Inhibition of ODC in the Acute Model of Allergic Airways Inflammation

To investigate the role of polyamine synthesis in the development of AHR, mice were sensitized to OVA on Days 0 and 7, and administered the ODC inhibitor, DFMO, *ad libitum* in drinking water (1 mg/ml) (22) beginning on Day 14, concomitant with daily OVA or PBS challenges (Days 14–20). On Day 21, lung function and methacholine responsiveness were assessed. Lungs were subsequently harvested, and stained with hematoxylin and eosin or picrosirius red for the quantification of collagen staining under polarized light, as described previously (23).

NO_x^- Measurement in Lung Homogenates

NO_x^- levels were measured in lung homogenates using the Griess assay (Cayman, Ann Arbor, MI).

Statistical Analysis

All data are expressed as the mean ($\pm$ SE). Dose–response curves were compared using the F test and two-way ANOVA with Bonferroni's *post hoc* test. Data were analyzed using either *t* test or one-way ANOVA with Bonferroni's multiple comparison *post hoc* test, where appropriate. *P* values less than 0.05 were considered significant. All statistical analyses were performed using GraphPad Prism 4.0c (GraphPad Software, LaJolla, CA).

RESULTS

L-Ornithine–Derived Polyamines Are Elevated in Acute Airways Inflammation

To investigate whether polyamines were altered in asthma, we determined putrescine, spermidine, and spermine levels in sputum samples obtained from 10 subjects with asthma before and 7 and 24 hours after allergen challenge (15). Although spermidine levels were unaltered after allergen challenge, putrescine and spermine were significantly increased at 7 and 24 hours, respectively, after challenge ($P < 0.05$ to baseline; Figure 2).

Putrescine, spermidine, spermine, and total polyamine concentrations in lung homogenates from mice subjected to the acute OVA sensitization and challenge (OVA/OVA) protocol, which elicits robust AHR to methacholine (6), were increased significantly compared with OVA-sensitized and PBS-challenged controls (OVA/PBS) (Figure 3A). The concentration of cadaverine,

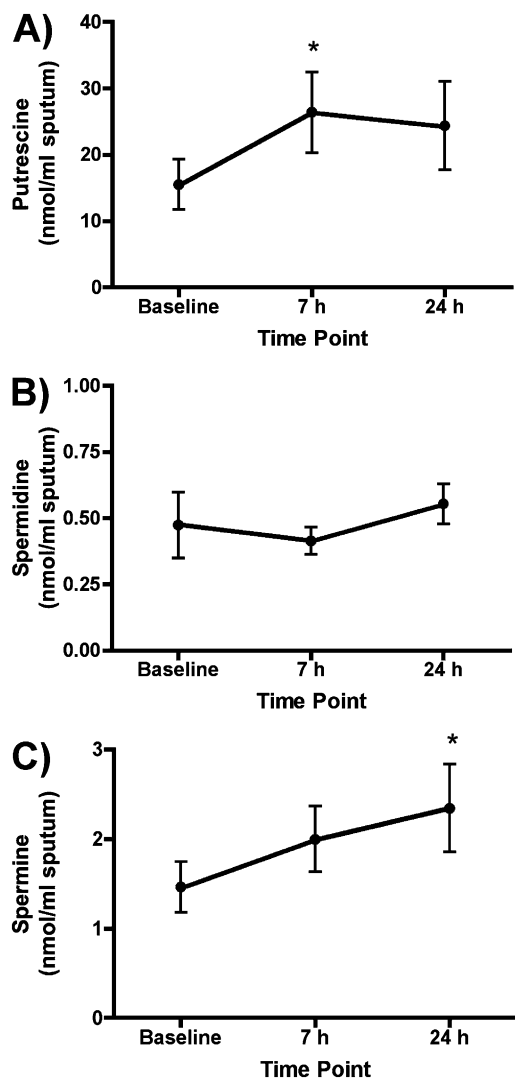


Figure 2. Polyamine levels are increased in sputum samples obtained from asthma patients after allergen challenge. Putrescine (A), spermidine (B), and spermine (C) concentrations were determined in sputum samples obtained before (baseline) and up to 24 hours after allergen challenge. * $P < 0.05$ to baseline ($n = 10$ subjects).

a polyamine unrelated to L-ornithine metabolism, was not different between groups (3.9 ± 0.8 versus 4.2 ± 0.4 pmol/mg protein for OVA/PBS versus OVA/OVA lungs, respectively; $n = 7$ /group), suggesting that the increase was specific to L-ornithine-derived polyamines. ODC protein levels were not significantly different between groups, consistent with our previous report (6) (see Figure E1A in the online supplement). However, the increased arginase activity levels in the OVA/OVA mice correlated significantly with total L-ornithine-derived polyamine levels (Figure 3B). Increased arginase 1 protein expression (Figure E1B) also correlated significantly with L-ornithine-derived polyamine levels (Spearman $r_s = 0.78$; $P < 0.01$; Figure E1C).

Nebulized Spermine Increases Airways Responsiveness to Methacholine

The effects of exogenous spermine were examined in mice subjected to the subacute OVA-sensitization and -challenge protocol, which we have previously demonstrated to be characterized by airways inflammation in the absence of AHR (20). In this model, putrescine and spermidine levels were significantly

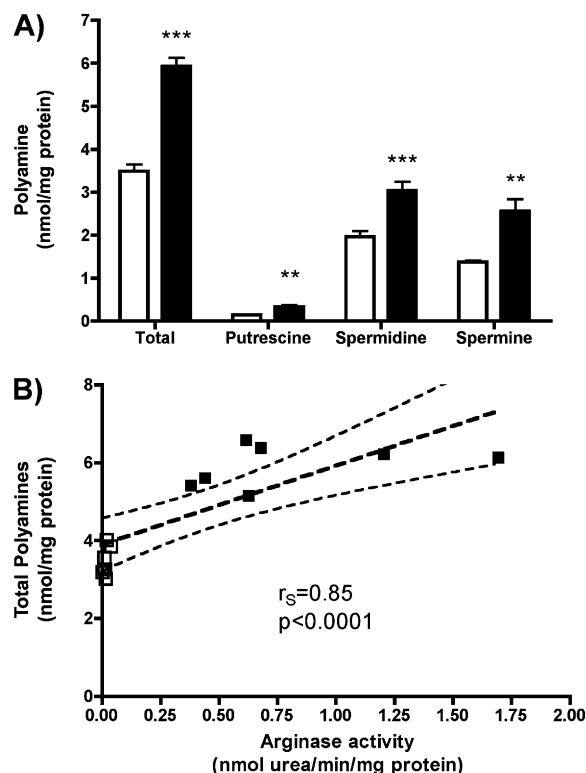


Figure 3. Polyamine levels are increased in the acute mouse model of allergic lung inflammation. (A) Putrescine, spermidine, spermine, and total L-ornithine-derived polyamines in ovalbumin (OVA)/PBS (open bars) and OVA/OVA (closed bars) mice. (B) Total levels of L-ornithine-derived polyamines correlate with arginase activity in OVA/PBS (open squares) and OVA/OVA (closed squares) mice (Spearman $r_s = 0.85$; $P < 0.001$). ** $P < 0.01$, *** $P < 0.0001$ versus OVA/PBS controls ($n = 7$ per group).

elevated in OVA/OVA mice (0.58 ± 0.08 and 2.9 ± 0.3 nmol/mg protein, respectively, compared with 0.20 ± 0.02 and 1.8 ± 0.3 nmol/mg protein, respectively, in OVA/PBS controls), whereas spermine levels were not (2.8 ± 0.2 versus 2.1 ± 0.3 nmol/mg protein for OVA/OVA and OVA/PBS lungs, respectively). Administration of nebulized spermine to the subacute OVA/OVA mice did not affect baseline resistance before methacholine challenge (see Table E1 in the online supplement). However, spermine pretreatment resulted in AHR to methacholine compared with OVA/OVA mice pretreated with PBS of equal isotonic strength (Table E1), which was employed as a control, as polyamine analogs have been shown to induce catabolic enzymes (24, 25). Responsiveness of the peripheral tissue parameters, G (tissue damping) and H (tissue elastance), did not change significantly with spermine pretreatment (Table E2). After nebulization of spermine, concentrations in lung homogenates from a subset of the subacute animals were greater than those measured in the acute-model OVA/OVA lungs (3.6 ± 0.2 versus 2.6 ± 0.3 nmol/mg protein for the subacute model with spermine versus the acute model lungs; $P = 0.012$; $n = 6$ and 7 for the subacute and acute models, respectively). Central airways Newtonian responsiveness (R_N) to methacholine correlated directly with the levels of spermine measured in these lung specimens (Table E1), suggesting that spermine augmented methacholine responsiveness in allergically inflamed airways.

We then determined whether administration of polyamines by nebulization would also affect airways responsiveness in naive mice. Responsiveness to methacholine (maximum central airways

responsiveness [$R_{N \text{ Max}}$]) was not altered after nebulized administration of putrescine or spermidine (0.95 ± 0.07 , and 1.03 ± 0.08 cm $\text{H}_2\text{O} \cdot \text{s/ml}$, respectively) compared with PBS (0.88 ± 0.07 cm $\text{H}_2\text{O} \cdot \text{s/ml}$). However, nebulized spermine resulted in a significant increase in $R_{N \text{ Max}}$ (Figure 4A) and reactivity to methacholine challenge, as indicated by the increased Hill slope of the methacholine dose–response relationship (Figure 4B) in these mice. Peripheral tissue parameters, G and H, were not significantly affected by polyamine nebulization before methacholine challenge (Table E2). To determine whether exogenous polyamines affected NO production, NO_x^- levels were measured in lung homogenates from these mice. Spermine administration before methacholine challenge resulted in a significant (80%) reduction in total lung NO_x^- levels compared with PBS controls (Figure 4C).

Inhibition of Polyamine Synthesis Prevents the Development of AHR

We then investigated whether blockade of the polyamine synthetic pathway would improve airways dysfunction in mice. DFMO, an irreversible inhibitor of ODC, was administered in the drinking water throughout the 7-day challenge period. R_N to methacholine in the OVA/OVA mice was completely abrogated by DFMO treatment (Figures 5A and 5B); DFMO treatment did not affect the response of peripheral tissue parameters, G and H, to methacholine (Table E3). Polyamine levels measured in lung homogenates revealed a significant (26%) decrease in total L-ornithine–derived polyamines after DFMO treatment in the OVA/OVA mice (Figure 5C); although decreases were observed in each of the polyamines, only the changes in spermidine showed statistical significance (spermidine, 3.0 ± 0.2 versus 2.1 ± 0.2 nmol/mg protein, $P < 0.01$; spermine, 2.6 ± 0.3 versus 1.9 ± 0.3 nmol/mg protein, $P = 0.17$; and putrescine, 0.33 ± 0.04 versus 0.26 ± 0.04 nmol/mg protein, $P = 0.22$). Cadaverine levels were not altered by DFMO compared with lungs from non-treated mice (4.2 ± 1.5 versus 4.3 ± 0.7 pmol/mg protein for saline- versus DFMO-treated, respectively; $P = 0.98$). The decrease in NO_x^- levels observed in lungs from the OVA/OVA mice compared with OVA/PBS mice was also prevented by the administration of DFMO (Figure 5D).

Inhibition of Polyamine Synthesis Does Not Affect the Inflammatory Response

Histological sections stained with hematoxylin and eosin revealed significant inflammation in lungs from OVA/OVA mice compared with OVA/PBS mice, but no effect of DFMO treatment in either group (Figure 6A). Furthermore, DFMO treatment did not affect the expression of eosinophil peroxidase (Figure 6B), eotaxin, or the T helper type 2 cytokines, IL-4 and IL-5 (Figures 6C–6E, respectively) in lung homogenates from the OVA/OVA mice. In addition, DFMO treatment did not affect either ODC protein expression or the up-regulation of arginase 1 protein in the acute model (Figure E1). Thus, pharmacologic blockade of polyamine synthesis significantly attenuated AHR in this model without affecting airways inflammation or up-regulation of the arginase pathway. However, when collagen deposition was assessed in second-order airways (26) of 250 (± 12) μm diameter, using picosirius red staining, we observed significantly increased staining in the peribronchiolar region in both OVA/PBS and OVA/OVA mice after treatment with DFMO (Figure 7). This suggests shunting of L-ornithine toward the ornithine aminotransferase pathway and, hence, increased production of proline, the precursor of collagen deposition, in these airways.

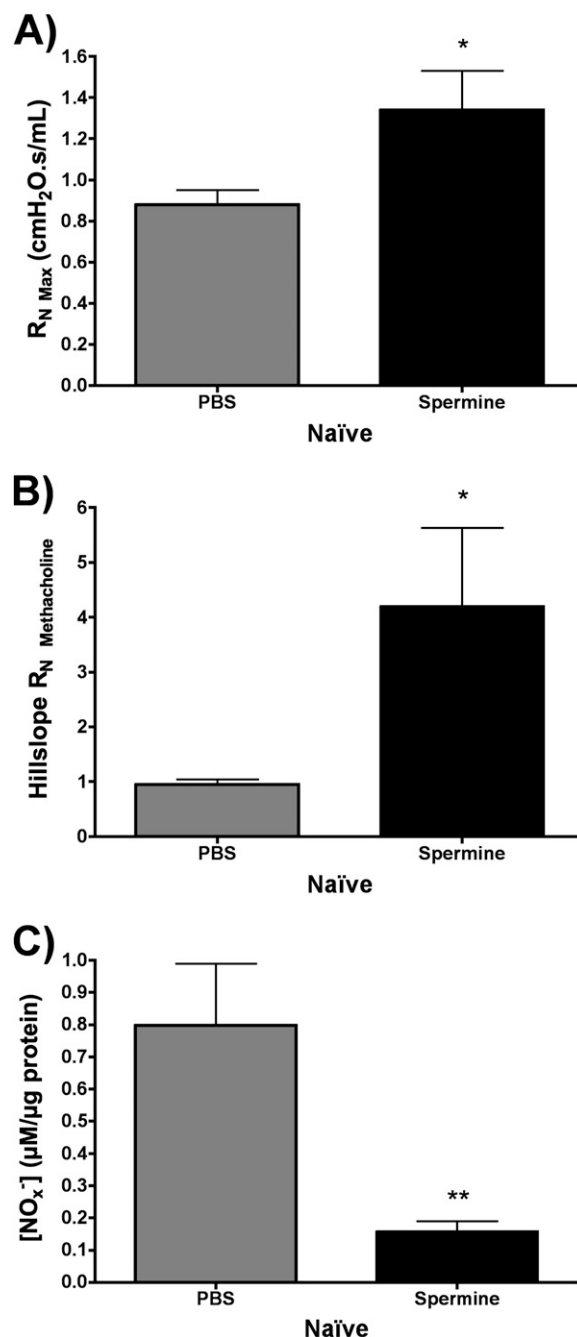


Figure 4. Exogenous spermine increases airways responsiveness to methacholine and reduces lung total nitrate plus nitrite (NO_x^-) levels in naïve mice. Exogenous spermine increased maximum central airways resistance ($R_{N \text{ Max}}$) (A) and reactivity to methacholine (Hill slope of the methacholine dose–response curve) (B) in naïve mice ($n = 10$ –12 per group). (C) NO_x^- levels are reduced in lung homogenates from naïve mice treated with spermine. * $P < 0.05$, ** $P < 0.01$, versus PBS control group ($n = 6$ per group).

DISCUSSION

In this study, we measured polyamines in sputum samples from patients with asthma, and found that putrescine and spermine levels increased after allergen challenge. We also demonstrated a significant increase in the L-ornithine–derived polyamine concentrations in lungs from the acute murine model of OVA-induced allergic airways inflammation and hyperresponsiveness. This increase correlated significantly with the increase in

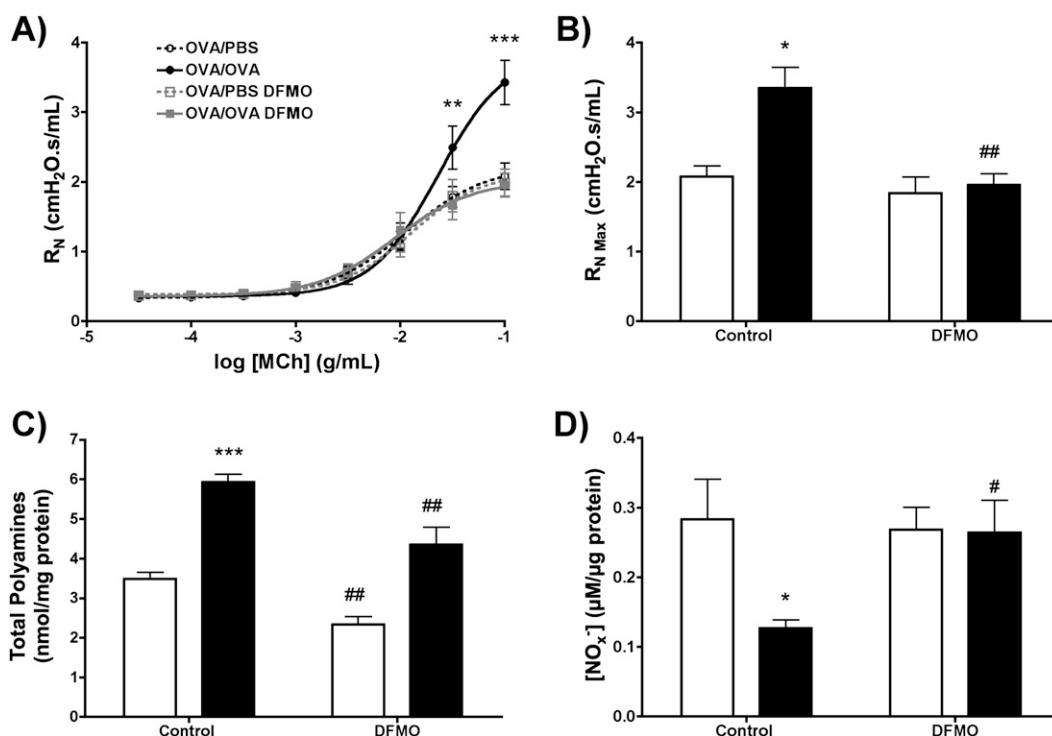


Figure 5. Ornithine decarboxylase inhibition prevents the airways hyperresponsiveness (AHR) to methacholine in the acute model of allergic airways inflammation. Dose-response relationships (A) and maximum response (B) for the central airways responsiveness (R_N) to methacholine in OVA/PBS (open symbols and bars) and OVA/OVA (closed symbols and bars) mice from the acute model treated with α -difluoromethylornithine (DFMO) or vehicle (** $P < 0.01$, *** $P < 0.001$ to OVA/OVA + DFMO by ANOVA; $n = 10$ –12 per group). (C) Total polyamine levels in control and DFMO-treated acute model mice ($n = 6$ –8 mice per group). (D) NO_x^- levels in lung homogenates from control and DFMO-treated acute model mice ($n = 11$ –12 mice per group). * $P < 0.05$, *** $P < 0.001$ versus OVA/PBS; # $P < 0.05$, ## $P < 0.01$ to control-treated mice.

arginase activity that is characteristic of this model and human asthma (6). Although the L-ornithine-derived polyamines, putrescine and spermidine were also increased significantly in the lungs of the subacute model of allergic airways inflammation, which does not exhibit AHR, there was no significant increase in spermine. We further demonstrated that the nebulized administration of spermine, to levels similar to those encountered in the acute model, increased airways responsiveness to methacholine in mice with pre-existing allergic airways inflammation but without AHR and naive mice. We also demonstrated that pharmacologic blockade of the polyamine biosynthesis pathway with the ODC inhibitor, DFMO, significantly attenuated the airways responsiveness and prevented the decrease in lung NO_x^- levels in the acute murine model of allergic airways inflammation. These findings suggest that increased arginase activity in asthma, in addition to competing with NOS for the common substrate, L-arginine, also contributes to AHR by producing spermine that augments airways responsiveness, likely through an inhibitory effect on NOSs in the airway.

Up-Regulation of Arginase and Production of Polyamines in Asthma

The metabolism of L-arginine plays an important role in airway function through regulation of the synthesis of cNO by NOS (27). Arginase competes with NOS for L-arginine as substrate, and it has been well established that up-regulated arginase in human asthma and animal models of asthma can contribute to cNO-deficient bronchoconstriction (1, 3–6, 11, 20, 28–30). Although ODC is considered to perform the rate-limiting step in polyamine synthesis (31), production of L-ornithine by arginase is also rate limiting (4, 7, 31). In the seminal study by Zimmermann and colleagues (4), the authors demonstrated increased levels of putrescine, the product of ODC, in murine models of allergic airways inflammation, concurrent with up-regulation of arginase activity and subsequent production of L-ornithine-derived polyamines. Recently, Maarsingh and colleagues (14) demonstrated

significant increases in putrescine levels in a model of chronic asthma in the guinea pig, which was prevented by pharmacological blockade of arginase. It is important to note that the increased putrescine levels were considered by Maarsingh and colleagues to contribute to the proliferation and differentiation of the remodeled airway, not directly to AHR. In this study, we demonstrated that, in addition to putrescine, levels of the higher-order polyamines, spermine and spermidine, are also elevated in lungs from the acute murine model of allergic airways inflammation, and that these increases in polyamine levels correlated directly with arginase activity and with airways responsiveness to methacholine.

Polyamines and Airways Responsiveness

Polyamines have been suggested to play an important role in lung physiology (13), as they are selectively concentrated in the lung epithelium. However, the few studies in airway physiology have revealed contrasting results. Chideckel and colleagues (32, 33) found that spermidine and spermine induced transient relaxation of guinea pig tracheal smooth muscle, and that subsequent exposures resulted in diminished responses, suggesting tachyphylaxis and possibly neurogenic mechanisms. In contrast, Bueb and colleagues (34) demonstrated contractile effects of spermidine and spermine on epithelial-denuded rat tracheal segments. Sward and colleagues (35) demonstrated increased Ca^{+2} -activated force generation and shortening velocity in guinea pig ileum in the presence of spermine. More recently, Chen and colleagues (36) demonstrated that spermine restored contractility of isolated tracheal rings to normal after they had been made hyporesponsive through deletion of cationic amino acid transporter 2 gene, encoding a high-affinity L-arginine transporter. Thus, although there have been reports of spermine affecting airway smooth muscle cell contractility *ex vivo*, to date there have been no reports of spermine playing a role in the AHR of asthma.

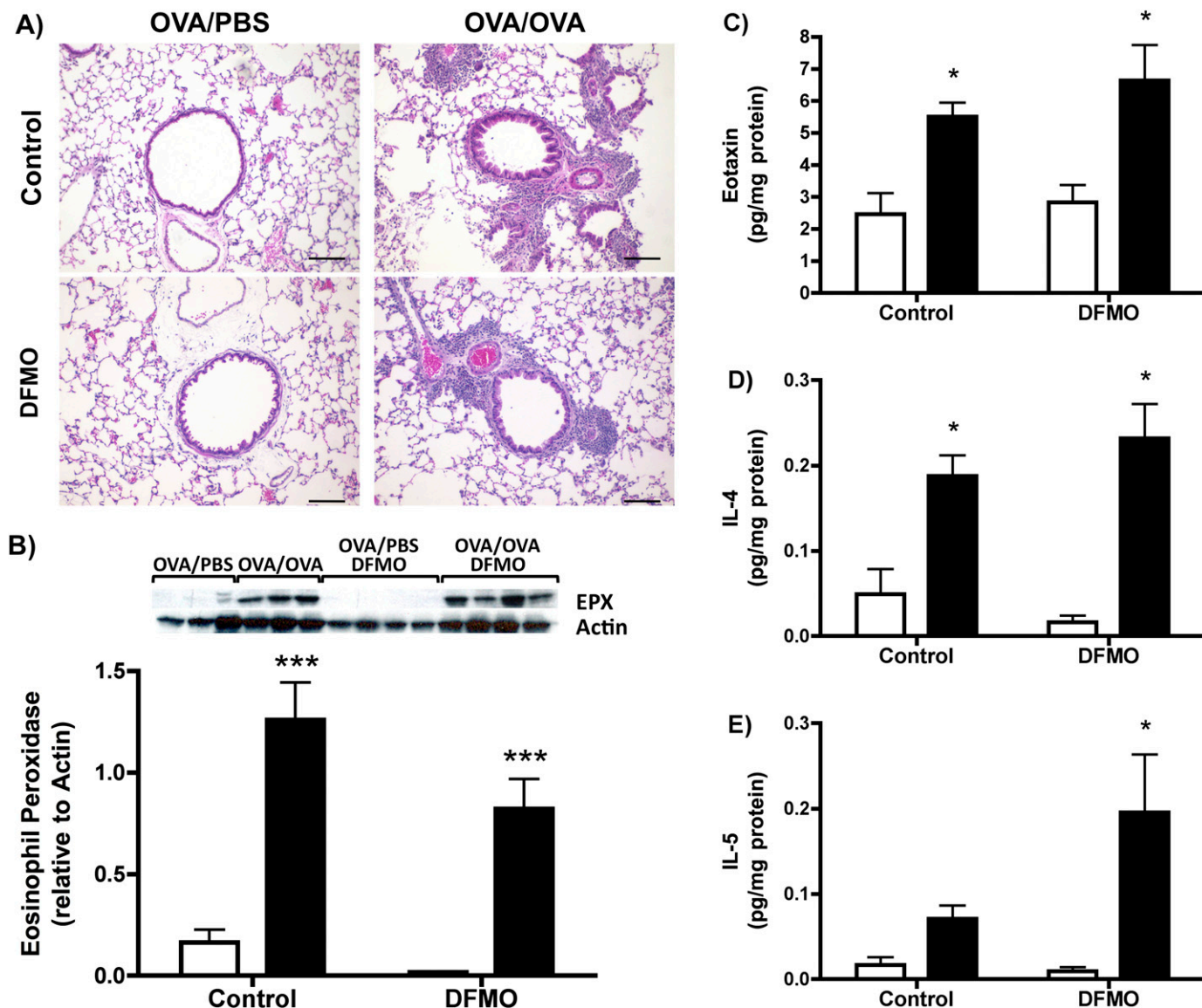


Figure 6. Ornithine decarboxylase inhibition does not affect pulmonary inflammation or cytokine release. (A) Representative micrographs of hematoxylin and eosin–stained histological sections of lungs from DFMO-treated and untreated OVA/PBS and OVA/OVA mice (representative of $n = 8$ per group). Scale bar indicates 100 μ m; 200 $\times$ magnification. (B) Western blot and relative expression of eosinophil peroxidase in lung homogenates from DFMO-treated and untreated OVA/PBS (open bars) and OVA/OVA (closed bars) mice. (C) Eotaxin, (D) IL-4, and (E) IL-5 levels in lung homogenates from DFMO-treated and untreated OVA/PBS (open bars) and OVA/OVA (closed bars) mice. * $P < 0.05$, *** $P < 0.001$ to OVA/PBS ($n = 8$ per group).

As many of the studies to date have been performed in *ex vivo* sections from multiple species, as opposed to whole animals, or in isolated transformed cell lines, it may be difficult to compare the previous observations with the present findings. In addition, the removal of the epithelia in many previous studies may be important, as lung epithelial cells are known to possess a particularly efficient polyamine uptake system (13). In this study, we demonstrated that, in the subacute model, which exhibits inflammation in the absence of AHR, putrescine and spermidine levels were significantly elevated, whereas spermine was not. Administration of spermine to subacute-model OVA/OVA mice increased airways responsiveness to methacholine, and pulmonary spermine levels correlated with $R_{N\text{ Max}}$. This is suggestive of spermine playing a major role in the development of AHR compared with the other polyamines; furthermore, the effect of spermine on airways reactivity to methacholine was also confirmed in naive mice.

However, when looking at the alleviating effects of polyamine synthesis inhibition on AHR in acute-model OVA/OVA mice, where a decrease in all polyamines was observed, only the decrease in spermidine was statistically significant. Although these data may suggest a role of spermidine, not spermine, for AHR, looking at polyamine concentrations in lung homogenates does have limitations, as this does not allow differentiation between cell types or tissues in the homogenate. Similarly, there may be tissue-specific differences in enzymatic conversion and back-conversion of polyamines (37, 38). Thus the changes in polyamine concentrations in lung homogenates after 7 days of ODC inhibition may not necessarily reflect the polyamine concentrations in the cell types most relevant to AHR (i.e., airway epithelial and/or smooth muscle cells).

Polyamines play many complex and sometimes conflicting roles in airways biology and biochemistry that may contribute to airways responsiveness. Ha and colleagues (39) demonstrated

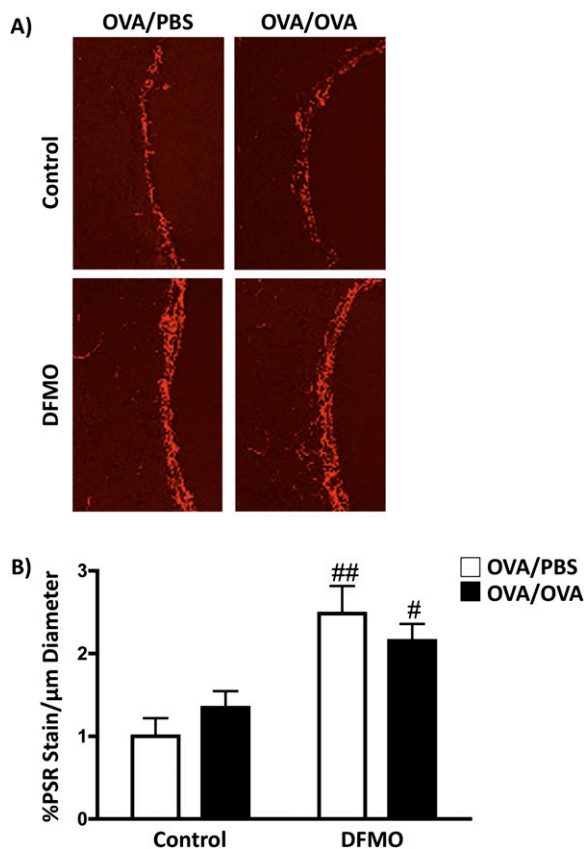


Figure 7. Collagen deposition is increased in airways from DFMO-treated mice. (A) Representative polarized light micrographs of picosirius red-stained histological sections of lungs from DFMO-treated and untreated OVA/PBS and OVA/OVA mice (representative of $n = 8$ per group). Scale bar indicates 100 μm ; magnification, 200 $\times$. (B) Bar graph representing the quantification of the peribronchiolar collagen levels in DFMO-treated and untreated OVA/PBS (open bars) and OVA/OVA (closed bars) mice. $^{\#}P < 0.05$, $^{##}P < 0.01$ to control-treated mice ($n = 8$ per group).

that spermine can act a free radical scavenger *in vitro*, protecting DNA from oxidative damage. By contrast, in cytosol, back-conversion of spermine to spermidine and of spermidine to putrescine generates hydrogen peroxide (40). It has been proposed that, under normal conditions, low levels of polyamine back-conversion do not result in appreciable oxidative stress. However, under conditions of inflammation or tissue damage, excessive polyamine production and breakdown could contribute to cellular injury (41). As hydrogen peroxide has been shown to induce AHR (42), potential enhancement of oxidative stress in the airways due to polyamine metabolism/catabolism represents an alternative pathway by which polyamines contribute to the AHR observed herein.

Polyamines and NOS Inhibition

Mechanisms contributing to reduced formation of bronchodilator NO in asthma include increased arginase activity and the production of asymmetric dimethylarginine, an endogenous competitive NOS inhibitor (43) that is increased in murine models of allergic airways inflammation (17, 44) and in human asthma lung and airway secretions (17). In this study, we observed a significant decrease in lung NO_x^- levels after short-term administration of spermine to naive mice, suggesting a spermine-related reduction of NO formation in this model. Polyamines have been previously

reported to affect the production of NO by the NOS isoenzymes in various cell types. Hu and colleagues (45) examined the inhibitory effects of polyamines on the conversion of [^3H]L-arginine to [^3H]L-citrulline in rat cerebellar lysates (i.e., NOS1-enriched samples). The authors demonstrated that this effect was dependent on L-arginine levels, suggesting a competitive mechanism, and determined an IC_{50} (half-maximum inhibitory concentration) value of 56 μM for spermine (45). Blachier and colleagues (46) employed a similar design, but examined lysates from endotoxemic rat liver containing NOS2, and reported an IC_{50} of 500 μM . The single log-order difference in IC_{50} values reported by Hu and Blachier may be due to differential susceptibilities between the isozymes to competitive inhibition by spermine. In our experiments, the endogenous concentrations of spermine in control mice and those with allergic inflammation in the acute model were well within the range at which spermine could be expected to have a physiologic effect. The recent work of Chaturvedi and colleagues (47) also suggests that spermine can inhibit NO synthesis in macrophages by inhibiting L-arginine uptake via cationic amino acid transporter 2. The inhibition of NOS by spermine, whether direct or indirect, is consistent with our finding of suppression of NO metabolite production in naive mice, and subsequent normalization of nitrate/nitrite concentrations in mice with allergic airways inflammation that were treated with DFMO. One alternative explanation for the effect of DFMO could be the inhibition of arginase by DFMO treatment. However, the inhibition constant of DFMO for the reversible inhibition of arginase is several orders of magnitude greater than that for the inhibition of ODC (48, 49).

Although we observed effects of spermine on the NOS pathway, as noted previously here, polyamines have also been reported to affect numerous other biochemical and physiological processes. While the data presented here suggest that NO inhibition by polyamines plays a role in AHR, this does not preclude the involvement of polyamines in the pathogenesis of asthma through NO-independent mechanisms.

Clinical Relevance

The clinical relevance of NOS inhibition has been studied in clinical trials using various inhaled NOS inhibitors and challenge protocols. Taylor and colleagues (50) observed that NOS inhibition had no effect on the early and late response to allergen challenge, measured by the fall in $\text{FEV}_{1\text{L}}$, in subjects with mild allergic asthma. However, the same group also demonstrated that NOS inhibition was able to augment airways responsiveness to histamine and adenosine-5'-monophosphate (51). Furthermore, Ricciardolo and colleagues (52) reported increased airways responsiveness after NOS inhibition and challenge with bradykinin and methacholine, the same provocative agent used in our study. Thus, the effects of NOS inhibition on airways function may be highly dependent on the challenge procedure employed to measure its effects, as well as the cell types and isoforms affected in the airways, and the ability of the inhibitor to reach the compartment of relevance in the airways.

Polyamines have been implicated in the pathology of lung diseases, including cystic fibrosis, pulmonary arterial hypertension, and lung cancer (53–55). Interestingly, increases in arginase expression and activity have also been demonstrated in the same clinical conditions (2, 56, 57). Consistent with our current observation of increased sputum spermine levels after allergen challenge of subjects with allergic asthma, increased polyamine levels have been reported in peripheral blood from humans during spontaneous asthma attacks (58). Kurosawa and colleagues (58) examined polyamine levels in serial peripheral blood samples after an asthma attack, demonstrating an elevation

at presentation, followed by a decrease to normal levels in parallel with clinical improvement. This is similar to the time course of increased arginase activity during asthma exacerbation reported by Morris and colleagues (5). Thus, it has been established that alterations in L-arginine metabolism occur in asthma, and we now report, for the first time, that increased production of L-ornithine-derived polyamines, downstream of arginase, are increased in human asthma and also play a functional role in airways responsiveness in mice, regardless of the presence or absence of inflammation. Therefore, arginase inhibition in asthma may not only improve AHR by improving L-arginine bioavailability and, subsequently, NO production, but also by reducing the production of the L-ornithine-derived polyamine spermine. Our data suggest that, although inhibition of ODC reduces the production of spermine and restores the NO deficiency-induced AHR, it may result in increased collagen deposition, and may therefore contribute to airways remodeling, an effect that would not occur with arginase inhibition. Although we observed increases in polyamines in human subjects with asthma after an allergen challenge, these changes did not correlate with the reduction in PC₂₀ or reduction of the FEV₁ % predicted at 24 hours. This is likely reflective of the compartments being investigated (i.e., the sputum concentrations of these metabolites do not reflect the intracellular concentrations at that time point). Our functional correlations in terms of spermine concentrations, airways responsiveness, and NO were all obtained in our mouse model. Thus, these pathways must be confirmed in human airway tissues and additional subjects in future investigations.

Conclusions

This study demonstrates, for the first time, that the L-ornithine-derived polyamine, spermine, is elevated in asthma lungs, and increases airways responsiveness to methacholine in the presence or absence of allergic airways inflammation in mice. Pharmacologic blockade of polyamine synthesis through ODC, concurrent with OVA challenge, attenuates AHR in a murine model of allergic airways inflammation and prevents the reduction in pulmonary NO formation observed in vehicle-treated mice. These findings further support that imbalances of L-arginine metabolism are important in the pathogenesis of asthma, and illustrate the therapeutic potential of arginase inhibition in the treatment of asthma airways obstruction.

Author disclosures are available with the text of this article at www.atsjournals.org.

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INCREASED POLYAMINE LEVELS CAUSE AIRWAYS HYPERRESPONSIVENESS IN A MOUSE MODEL OF ASTHMA.

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ONLINE SUPPLEMENTAL METHODS

METHODS

Animal and Human Study Approval

All animal studies were conducted in accordance with the guidelines of the Canadian Council of Animal Care and were approved by the University of Toronto, Faculty of Medicine Animal Care Committee. All human studies were conducted in accordance with the Declaration of Helsinki after approval by the Institutional Review Board of McMaster University. Patients provided written informed consent prior to enrolment in the study.

Polyamine levels in Human asthma

Ten subjects (5 male; 5 female), with clinically stable asthma (FEV1 % predicted: 100% at baseline) and an average age of 37 years (range: 18-61 years) were recruited for an allergen-challenge study and provided written informed consent. After skin-prick testing to determine which allergen(s) the volunteers were sensitive to, subjects were exposed to allergen (i.e., cat, house dust mite, ragweed), in a controlled manner, as described previously (1). All subjects exhibited a reduction in both the FEV1% predicted during the early and late phase after challenge (Δ FEV1% -33 and -22%, respectively), as well as reductions in the PC₂₀ to methacholine 24 hours after the allergen challenge. Induced sputum samples were obtained before and at 7 and 24 hours post allergen challenge (2). Polyamines were then measured in sputum supernatants using LC tandem mass spectrometry, as described below.

Mouse models of allergic airways inflammation

To investigate lung polyamine production in murine models of airways inflammation and hyperresponsiveness we employed subacute and acute ovalbumin (OVA) sensitization and challenge models, which have both been described previously (3, 4). Briefly, female BALB/c mice (6-8 weeks of age; Charles River Laboratories, Saint-Constant, PQ) were sensitized to ovalbumin (OVA; intraperitoneal injection of 25 μ g in 0.2 ml PBS with 1 mg Al(OH)₃; Sigma Aldrich, Mississauga, ON) on days 0 and 7, followed by exposure to aerosolized PBS (OVA/PBS) or 6% OVA in phosphate buffered saline (PBS) (OVA/OVA) for 25 minutes/day from days 14-16 (3 consecutive days; *sub-acute model*) or days 14-20 (7 consecutive days; *acute model*) (3, 4). Twenty-four hours after the final OVA challenge, mice were anaesthetized for

pulmonary function testing and methacholine challenge using the flexiVent® system (Scireq Inc., Montreal, PQ), as described below.

Pulmonary function and airways responsiveness testing

Mice were anesthetized with ketamine (50 mg/kg, Bioniche, Belleville, ON)/xylazine (10 mg/kg, Bayer Inc., Toronto, ON) and intubated for *in vivo*, ventilator-based assessment of methacholine responsiveness using the forced oscillation technique. The ventilator delivered 150 breaths/minute, with a tidal volume of 10 ml/kg, and maintained a positive end expiratory pressure (PEEP) of 3 cmH₂O. We used the constant phase model, which utilizes forced oscillation over a range of frequencies to determine respiratory input impedance and differentiate between airways Newtonian resistance (R_N) and peripheral tissue parameters (G, tissue damping and H, tissue elastance) (5). After baseline was established, mice were challenged with increasing nebulized doses of methacholine (*i.t.*, 0-100 mg/mL; Sigma), as described previously (3). All data were collected using the flexiVent® software (Version 5.2, Scireq) and analyzed using Excel (Microsoft Corporation, Redmond, WA). Following pulmonary function testing, the mice were euthanized with an overdose of ketamine/xylazine and the lungs were harvested for determination of polyamine and nitrite/nitrate (NO_x) levels, histological assessment, and protein analyses.

Determination of polyamine levels in mouse lung and human sputum samples

Each lung was homogenized in 1 ml of buffer containing 0.1% Triton X-100, 0.8% glycerol, 0.7 µg/ml pepstatin A (Bioshop, Burlington, ON, Canada), 0.7 µg/ml antipain (Bioshop), 50mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Invitrogen, Carlsbad, CA) and 2 mM ethylenediaminetetraacetic acid (Bioshop Canada, Burlington, ON), pH 7.3. Sputum samples were prepared for analysis as described previously (6, 7). Quantitative analysis of the L-arginine derived polyamines, putrescine, spermidine and spermine, was performed using liquid chromatography–tandem mass spectrometry (LCMS), according to the method described by Byun *et al.* (10). Briefly, the polyamines were carbamylated with isobutyl chloroformate, extracted with diethyl ether, and separated using HPLC (Agilent Infinity 1290), with an Agilent Eclipse XDB-C18 column, followed by mass spectrometry analysis (Sciex 5500-QTrap). As an internal standard, 1,6-diaminohexane was added to all experimental and reference samples. As

an additional control, cadaverine, a polyamine product of the decarboxylation of lysine, similar in structure to putrescine, was also quantified in all samples. All measurements were standardized to the protein concentration of the original homogenate, which was determined using the Bradford assay (BioRad, Hercules, CA).

Intratracheal administration of spermine to OVA-sensitized and -challenged mice

To investigate whether exogenous spermine, the largest and most biologically available L-ornithine-derived polyamine, would augment airways responsiveness to methacholine in mice with allergically inflamed lungs, we used the sub-acute model of OVA-induced allergic inflammation (4). In this model, mice exhibit airways inflammation, but do not exhibit significant AHR, compared to the OVA/PBS controls (4). Twenty-four hours after the final challenge, mice were anaesthetized and ventilated, as described above. After baseline determination of airways Newtonian resistance (R_N), spermine (12 ± 0.1 nmol/g body weight, *i.t.*) was administered by nebulization (AeroNebLab nebulizer), and mice were challenged with increasing concentrations of methacholine 15 minutes later, as described above. Spermine did not affect baseline resistance parameters prior to methacholine challenge (data not shown).

Intratracheal administration of polyamines to naïve mice

To investigate the effects of administration of all three of the L-ornithine-derived polyamines on airways responsiveness, naïve mice were anesthetized and ventilated using the flexiVent, as described above. Stock solutions of putrescine, spermidine and spermine were prepared at 0.1 M and pH 7.4. After baseline determination of airway resistance, polyamines (12 ± 0.1 nmol/g body weight) were delivered by nebulization directly into the ventilatory circuit using the AeroNebLab nebulizer, synchronized with inspiration; control mice received an equivalent volume of nebulized PBS directly into the ventilatory circuit. After determination of the R_N 15 minutes after polyamine administration, mice were challenged intratracheally with increasing concentrations of methacholine, as described above.

Inhibition of ornithine decarboxylase in the acute model of allergic airways inflammation

To investigate the role of polyamine synthesis in the development of airways hyperresponsiveness we employed the acute model, described above. The irreversible ornithine

decarboxylase (ODC) inhibitor, difluoromethylornithine (DFMO), was administered *ad libitum* in the drinking water (1 mg/ml), beginning on day 14, to pharmacologically inhibit polyamine synthesis throughout the progression of allergic airways inflammation. On day 21, 24 hours after the final PBS or OVA exposure, mice were anaesthetized, and methacholine responsiveness was determined. DFMO administration did not affect water intake, food intake or body weight, and a mean dose of 0.16 ± 0.04 g/kg/day was consumed by the treated mice. After completion of the pulmonary function testing, lungs were harvested for histological staining, Western blotting, cytokine, NO_x and polyamine measurement.

Assessment of inflammation and Western blot analyses

Lungs were inflation fixed to a pressure of 20 cmH₂O with 10% neutral buffered formalin (Sigma, Mississauga, ON) (11). After at least 24 hours, the right inferior lobe and the left lobe were isolated and bisected into superior and inferior segments. The inferior portion of the left lung was embedded in paraffin with the bisected face down to obtain transverse cross sections of the primary bronchus for large airway morphometry. The superior portions were subjected to a sagittal cut and embedded with the sagittal face down for airway morphometry of airways distal to the primary bronchus. Sections (5 µm) were cut and stained with hematoxylin and eosin for the assessment of inflammatory changes. The degree of peribronchial inflammation was assessed from captured images, centered on airways, at 200× magnification. Ten images from each mouse were semi-quantitatively assessed for peribronchiolar inflammation according to the following scoring system: 0, normal; 1, few cells; 2, a ring of inflammatory cells 1 cell layer deep; 3, a ring of inflammatory cells 2–4 cells deep; 4, a ring of inflammatory cells of >4 cells deep (12). Western blotting for eosinophil peroxidase (EPX; bs-3881R, Bioss, Woburn, MA), arginase 1 (sc-18351, Santa Cruz Biotechnology, Santa Cruz, CA), , arginase 2 (sc-18357, Santa Cruz) and ODC (sc-21516, Santa Cruz) were performed in lung homogenates, and normalized to actin (sc-1616, Santa Cruz), as previously described (3).

Cytokine measurement in mouse lung homogenates.

The cytokines were measured by MILLIPLEX kit (Millipore Corporation, Billerica, MA) according to the manufacturer's protocol. The assay is based on Luminex xMAP technology that allows immunoassays to be performed on the surface of fluorescent-coded beads. These beads

are internally coded with precise concentrations of two fluorescent dyes, each of these beads are also coated with a specific capture antibody. The lung homogenate samples were mixed with the antibodies on a 96-well plate and left overnight at 4°C. The cytokines captured by the antibodies on the beads are then treated with a biotinylated detection antibody. After that, streptavidin conjugated with fluorescent dye (PE) is added to the reaction mixture to allow its binding to the detection antibody. The plate is then run on Luminex 100 wherein the beads or microspheres are passed through a laser that excites the internal dyes, as a result microsphere sets are characterized. Then a second laser excites PE on the streptavidin bound to antibody linked with molecule of interest. The individual microspheres are identified by the digital signal processor and quantified. Standard curves were generated for each cytokine and the resultant concentration normalized to the protein concentration in each aliquot. Protein concentrations were determined using the micro BCA Protein Assay Kit (Cat# 23235, Thermo Scientific, Rockford IL), according to the manufacturer's instructions.

Quantification of picosirius red collagen staining in lung sections

To determine the effects of DFMO on collagen deposition in the murine model, lung sections were stained with Picosirius Red (PSR). Using polarized light microscopy (Olympus BX40; Carsen Group Inc., Markham Ontario), second order airways (according to the Strahler ordering system, which has been suggested to be the most useful ordering system for lung pathology (11)) were randomly sampled. Airways had a mean diameter of $250 \pm 12 \mu\text{m}$, and airways diameters were not significantly different between treatment groups. Airways that satisfied the following criteria were included for analysis: (i) the airway was completely contained in a single microscope field of view ($690 \mu\text{m} \times 520 \mu\text{m}$); (ii) the ratio of the major and minor axes were less than 2 (iii) the airway perimeter needed to be intact (13). The basement membrane was traced using a digital pen and tablet image analysis system (Northern Eclipse, Version 7.0; Empix Imaging Inc., Mississauga, Ontario, Canada) (13). Areas of airway wall associated with connective tissue from neighboring vessels were excluded. The software then calculated the percentage of PSR stain within the region of interest, extending $20 \mu\text{m}$ beneath the basement membrane (14). Measurements from 4 airways were averaged per animal. For visualization of PSR stain, greyscale images were processed using the lookup table red function in ImageJ 1.46 (National Institutes of Health, USA).

NO_x measurement in lung homogenates

To determine the effects of polyamine administration and DFMO treatment on stable oxidation products of NO, measurement of NO_x (total nitrates + nitrites) was carried out using the Griess assay, according to the manufacturer's instructions (Cayman, Ann Arbor, MI).

Statistical Analyses

All data are expressed as the mean±standard error. Dose-response curves (including, baseline, maximum response, EC₅₀ and log EC₅₀ values) were compared using the F-test, with the null hypothesis that the data from all groups could be modeled by the same sigmoidal curve, and using two-way ANOVA with Bonferroni's post-hoc test. Hill slopes were calculated for each animal by fitting a sigmoidal dose-response curve with nonlinear regression in Prism with the following equation:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC}_{50} - X) * \text{HillSlope})})$$

Where X is the logarithm of concentration and Y is the response. Data were analyzed using either t-test or one-way ANOVA with Bonferroni's multiple comparison post-hoc test, where appropriate. P-values < 0.05 were considered significant. All statistical analyses were performed using GraphPad Prism 4.0c (GraphPad Software, Inc, LaJolla, CA).

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Supplemental Figure 1

DFMO treatment does not affect expression of ODC (A) or arginase 1 (B) in lung homogenates from DFMO treated and untreated OVA/PBS (□) and OVA/OVA (■) mice. Polyamine levels in lung homogenates correlate directly with arginase 1 expression in lung homogenates from OVA/PBS (□) and OVA/OVA (■) mice (Spearman $r_s=0.78$; $p<0.05$).

Supplemental Table 1

Methacholine responsiveness and spermine concentration in sub-acute model mice

Parameter	OVA/PBS	OVA/OVA + PBS	OVA/OVA Spermine
Lung [Spermine] nmol/mg	2.1±0.3	2.8±0.2	3.6±0.2**
R _N Baseline cmH ₂ O.s/mL	0.26±0.01	0.26±0.02	0.25±0.02
R _N Max cmH ₂ O.s/mL	1.6±0.2	1.9±0.2	2.5±0.2* [#]
Spearman Correlation	[Spermine] correlated with R _N Max across all groups r _s =0.52, p<0.05		

R_NMax; Maximum central airways Newtonian resistance to methacholine. *p<0.05, **p<0.01 to OVA/PBS, #p<0.05 to OVA/OVA+PBS, n=12 per group. Central airways Newtonian responsiveness to methacholine correlated directly with the levels of spermine measured in these lung specimens (r_s=0.52, P<0.05).

Supplemental Table 2

Peripheral Tissue Parameters in naïve and sub-acute mice administered polyamines or PBS

Parameter	PBS	Putrescine	Spermidine	Spermine
Sub-acute Model OVA/OVA Mice				
G _{Max} cmH ₂ O/mL	30.2±2.8	N/A	N/A	41.79±4.9
H _{Max} cmH ₂ O/mL	110.8±15.2	N/A	N/A	114.8±11.7
Naïve Mice				
G _{Max} cmH ₂ O/mL	10.56±0.84	11.04±0.84	12.23±1.12	15.52±2.24
H _{Max} cmH ₂ O/mL	35.60±2.0	36.97±2.5	33.07±2.7	45.59±6.7

G_{Max}; Maximum peripheral tissue damping, H_{Max}; Maximum tissue elastance. Polyamines (12±0.1 nmol/g body weight) or PBS were delivered to sub-acute model mice and naïve mice by nebulization, and methacholine challenge was initiated 15 minutes later. The peripheral tissue parameters measured were not affected by polyamine administration (n=12/group).

Supplemental Table 3

Peripheral Tissue Parameters in acute model mice administered DFMO

Parameter	OVA/PBS	OVA/OVA	OVA/PBS DFMO	OVA/OVA DFMO
G _{Max} cmH ₂ O/mL	30.5±2.1	45.1±4.5*	31.8±2.6	44.2±4.4
H _{Max} cmH ₂ O/mL	75.3±7.3	153.2±19.6**	73.2±9.9	140.2±20.8

G_{Max}; Maximum peripheral tissue damping, H_{Max}; Maximum tissue elastance. *p<0.05, **p<0.01 to OVA/PBS. Peripheral tissue parameters responseiveness to MCh were upregulated in OVA/OVA relative to OVA/PBS mice, but were not affected by DFMO administration.

Supplemental Figure

