

Beneficial effects of high dose of L-arginine on airway hyperresponsiveness and airway inflammation in a murine model of asthma

Ulaganathan Mabalirajan, MBBS,^a Tanveer Ahmad, MSc,^a Geeta Devi Leishangthem, MVSc,^b Duraisamy Arul Joseph, MSc,^a Amit Kumar Dinda, MD, PhD,^b Anurag Agrawal, MD, PhD,^a and Balaram Ghosh, PhD^a Delhi and New Delhi, India

Background: Disturbance in the delicate balance between L-arginine-metabolizing enzymes such as nitric oxide synthase (NOS) and arginase may lead to decreased L-arginine availability to constitutive forms of NOS (endothelial NOS), thereby increasing the nitro-oxidative stress and airway hyperresponsiveness (AHR).

Objective: In this study, we investigated the effects of high doses of L-arginine on L-arginine-metabolizing enzymes and subsequent biological effects such as cyclic guanosine monophosphate production, lipid peroxidation, peroxynitrite, AHR, and airway inflammation in a murine model of asthma.

Methods: Different doses of L-arginine were administered to ovalbumin-sensitized and challenged mice. Exhaled nitric oxide, AHR, airway inflammation, T_H2 cytokines, goblet cell metaplasia, nitro-oxidative stress, and expressions of arginase 1, endothelial NOS, and inducible NOS in lung were determined. **Results:** L-arginine significantly reduced AHR and airway inflammation including bronchoalveolar lavage fluid eosinophilia, T_H2 cytokines, TGF- β 1, goblet cell metaplasia, and subepithelial fibrosis. Further, L-arginine increased ENO levels and cyclic guanosine monophosphate in lung and reduced the markers of nitro-oxidative stress such as nitrotyrosine, 8-isoprostane, and 8-hydroxy-2'-deoxyguanosine. This was associated with reduced activity and expression of arginase 1, increased expression of endothelial NOS, and reduction of inducible NOS in bronchial epithelia.

Conclusion: We conclude that L-arginine administration may improve disordered nitric oxide metabolism associated with allergic airway inflammation, and alleviates some features of asthma. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: Allergic airway inflammation, arginase, L-arginine, endothelial nitric oxide synthase, exhaled nitric oxide

The incidence of allergic airway diseases such as asthma is rising in both developed and developing countries. Allergic airway inflammation (AAI) is a central feature of these diseases, and many of the long-term effects have been suggested to be a result of recruited inflammatory cells in the airway.¹ These inflammatory cells affect the structural cells of the airway such as bronchial epithelia, which orchestrate the airway inflammation by interacting with various foreign proteins.² Recently, we have shown that oxidative stress and mitochondrial dysfunction are associated with allergic airway inflammation.^{3,4} AAI is characterized by the infiltration of various inflammatory cells including eosinophils, and increased T_H2 response. In animal models of AAI, allergic sensitization to foreign proteins followed by repeated allergen exposures also leads to structural changes such as subepithelial airway fibrosis and goblet cell metaplasia.⁵ In human AAI, increased exhaled nitric oxide (ENO) has been noted.⁶ Although the role of nitric oxide (NO) in allergic diseases including asthma remains controversial, successful inhibition of airway NO synthesis does not improve asthma.⁷ Although various studies have explored the complex interrelationships between the components of the L-arginine (L-ARG)-NO pathway in the pathogenesis of allergic inflammatory diseases, it remains poorly understood. The multifunctional properties of NO depend on its enzyme source, substrate availability, microenvironment, and its final products after combination with other biomolecules.⁸ NO is produced by NO synthase (NOS), which is of 2 principal types: constitutive (neuronal NOS, endothelial NOS [eNOS]) and inducible NOS (iNOS). One of the crucial limiting factors in NO production is substrate availability.⁶ In normal airways, constitutive NOS consumes L-ARG to maintain airway smooth muscle tone through activation of the cyclic guanosine monophosphate (cGMP) pathway. It has been hypothesized earlier that exogenous administration of L-ARG may increase the bronchodilating effect by constitutive forms of NOS.⁹ Unfortunately, this strategy failed to have a significant effect in human asthma¹⁰ and potentiated the airway inflammation in animal models.¹¹ However, it remains possible that substrate limitations of L-ARG exist in asthmatic airways because of competition by other L-ARG-metabolizing enzymes such as iNOS and arginase, and that the dosage used by previous studies (50 mg/kg) was insufficient, possibly because competition by other enzymes was not considered. Hence, the potentiation of airway inflammation could be a result of consumption of both endogenous and exogenous L-ARG by arginase and iNOS. L-ARG has been used in various clinical conditions

From ^athe Molecular Immunogenetics Laboratory and Centre of Excellence for Translational Research in Asthma and Lung Disease, Institute of Genomics and Integrative Biology, Delhi; and ^bthe Division of Renal Pathology, Department of Pathology, All India Institute of Medical Sciences, New Delhi.

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Reprint requests: Balaram Ghosh, PhD, Molecular Immunogenetics Laboratory, Institute of Genomics and Integrative Biology, Mall Road, Delhi-110007, India. E-mail: bghosh@igib.res.in.

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Abbreviations used

AAI: Allergic airway inflammation
ADMA: Asymmetric dimethyl arginine
AHR: Airway hyperresponsiveness
BAL: Bronchoalveolar lavage
cGMP: Cyclic guanosine monophosphate
ENO: Exhaled nitric oxide
eNOS: Endothelial nitric oxide synthase
iNOS: Inducible nitric oxide synthase
L-ARG: L-arginine
NO: Nitric oxide
NOS: Nitric oxide synthase
OVA: Ovalbumin
Penh: Enhanced pause
sGAW: Specific airway conductance
VEH: Vehicle

up to 30 g/d without any adverse effects.¹² Importantly, in cardiovascular diseases, higher doses were required to obtain sufficient vasodilation and other effects.¹² Also, it was demonstrated that depletion of L-ARG in cytosol triggers superoxide generation in macrophages. This leads to increased formation of peroxynitrite because of balanced synthesis of both superoxide and NO by iNOS with L-ARG depletion.¹³ Peroxynitrite is known for its properties of causing bronchoconstriction and airway inflammation.¹⁴ In this study, we have addressed the possibility that the state of allergic airway inflammation may be associated with relative L-ARG depletion causing increased nitro-oxidative stress and whether it can be overcome by high doses of L-ARG comparable to those used in cardiovascular diseases.

METHODS**Animals**

Male BALB/c mice (8–10 weeks old; National Institute of Nutrition, Hyderabad, India) were acclimatized for a week before starting the experiments. All animals were maintained according to Committee for the Purpose of Control and Supervision of Experiments on Animals guidelines, and protocols were approved by the Institutional Animal Ethics Committee.

Grouping of mice

In pilot experiments, mice were divided into 5 groups, and each ($n = 6$) was named according to sensitization/challenge/treatment: SHAM/PBS/vehicle (VEH; sham controls), ovalbumin (OVA)/OVA/VEH (allergic controls, OVA, chicken egg OVA, Grade V; Sigma, St Louis, Mo), OVA/OVA/L-ARG 25 (low-dose L-ARG, 25 mg/kg; Sigma, St Louis, Mo), OVA/OVA/L-ARG 250, and OVA/OVA/L-ARG 500 (250 mg/kg and 500 mg/kg, respectively). L-ARG was soluble in water (vehicle). From these pilot experiments, 250 mg/kg twice daily dose was selected as the most effective dose for further studies involving NO metabolism, in which there were 3 groups of mice ($n = 6$ in each group): SHAM/PBS/VEH (sham controls), OVA/OVA/VEH (allergic controls), and OVA/OVA/L-ARG (250 mg/kg L-ARG treated).

Sensitization, challenge, and treatment of mice

Mice were sensitized and challenged as described previously.^{3,4} As shown in Fig 1, mice were injected with 50 μ g OVA in 4 mg aluminum hydroxide or only 4 mg aluminum hydroxide by the intraperitoneal route on days 0, 7, and 14. Mice were challenged with 3% OVA in PBS or PBS from day 21 to day 32 (30 minutes per day). Vehicle or L-ARG was given orally from day 19 to 32 once a day in a 30- μ L volume per dose.

Airway hyperresponsiveness measurement

Airway hyperresponsiveness (AHR) to methacholine (Sigma) was determined in unrestrained and restrained conscious mice by single-chamber and double-chamber whole-body plethysmography, respectively (Models PLY 3211 and PLY 3351; Buxco Electronics) as described previously.^{3,4} Also, airway resistance was estimated by invasive measurements on anesthetized mice using the flexiVent system (Scireq), which integrates the computer-controlled mouse ventilator with the measurements of respiratory mechanics as described previously.¹⁵ Because methacholine aerosols have been nebulized directly to the trachea in airway resistance measurements, the methacholine dose-response curve for airway resistance might vary from specific airway conductance (sGAW) obtained from double-chamber plethysmography. Final results were expressed in enhanced pause (Penh) or sGAW or airway resistance with increasing concentrations of methacholine.

ENO measurement

Exhaled NO as a gas was measured as described previously¹⁶ by using a standard clinical ENO analyzer (CLD88sp; Ecomedics, Dürnten, Switzerland) based on photometric determination of chemiluminescence.

Bronchoalveolar lavage and sera separation

On day 33, each mouse was killed bronchoalveolar lavage (BAL) was performed, BAL fluids were processed,^{17,18} and absolute cell count for each cell type was calculated after determining total cell count and differential cell count. Blood was withdrawn by cardiac puncture, and serum was separated as described previously.^{17,18}

Lung histopathology

Formalin-fixed, paraffin-embedded lung tissue sections were stained with hematoxylin and eosin, periodic acid-Schiff, and Masson Trichrome stainings to assess the airway inflammation, goblet cell metaplasia, and subepithelial fibrosis, respectively.^{3,4} Stained sections were observed, and microphotographs were taken with a Nikon microscope with a camera (model YS-100). Inflammation scoring with hematoxylin and eosin-stained slides and quantitative morphometry with periodic acid-Schiff and Masson Trichrome-stained sections were performed as described previously.^{3,4}

Measurements of IL-4, IL-5, TGF- β_1 , IL-13, eotaxin levels in the lung, and OVA-specific immunoglobulins in sera

Lung tissue homogenates in duplicate were used for ELISA of IL-4, IL-5, and TGF- β_1 (BD Pharmingen, San Diego, Calif; the lower detection ranges are 7.5 pg/mL, 15.6 pg/mL, and 125 pg/mL, respectively) and IL-13 and eotaxin ELISA (R&D Systems, Minneapolis, Minn; the sensitivity for IL-13 is 1.5 pg/mL, and the lower detection range for eotaxin is 7.5 pg/mL). Results were expressed in picograms and normalized by protein concentrations. OVA-specific IgE, IgG₁, and IgG_{2a} were measured as described previously.³

Cytosolic separation

After mice were killed, the lung portion below the trachea was removed and processed to separate cytosolic fractions as described previously,³ and protein estimation was done in those fractions by bicinchoninic acid (Sigma) assay.

Arginase activity and Western blot

Arginase activity was measured in lung cytosolic fractions as per the manufacturer's instructions by an indirect method of urea measurement (Bioassay Systems, Hayward, Calif). Briefly, 25 μ g cytosolic fraction protein in 40 μ L volume was mixed with 10 μ L substrate buffer containing arginine, and MnCl₂, and the total mixture was incubated at 37°C for 2 hours. The reaction was terminated by addition of urea reagent and further incubated at

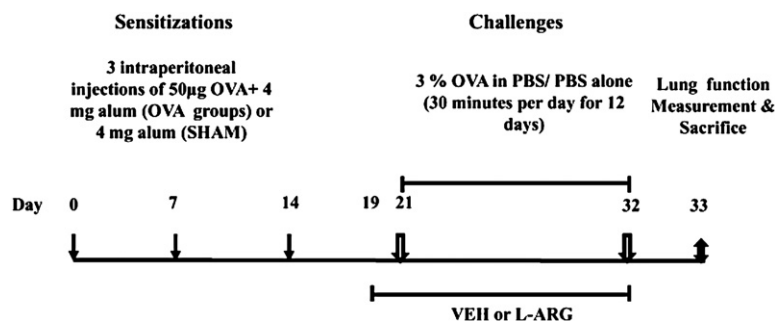


FIG 1. Experimental protocol to induce allergic asthma in mice. Male BALB/c (8-10 weeks old) mice were grouped, sensitized, and challenged. Vehicle (water) or L-ARG had been given orally from days 19 to 32. Measurements of lung function were performed and mice were killed on day 33.

room temperature for 15 minutes and read at 430 nm. Negative control wells were made for every sample in which substrate buffer was not added. Water and 1 mmol/L urea have been taken as a background and standard, respectively.

Western blot was performed for Arginase I in lung cytosolic fraction proteins with Arginase I antibody (1:500; Santa Cruz Biotechnology). α -Tubulin was used as a loading control. Signals were detected by spot densitometry (Alpha EaseFC software from Alpha Innotech).

Immunohistochemistry

Commercial rabbit polyclonal antibodies for iNOS, eNOS, and Arginase I were used as primary antibodies, and respective horseradish peroxidase-conjugated secondary antibodies (Sigma) were used for immunohistochemistry. Either γ -globulin as isotype controls (Jackson ImmunoResearch Laboratories) or omission of primary antibodies was used for negative control experiments. Semiquantitative analysis of eNOS and iNOS-stained slides was performed as described previously¹⁹ with little modification by 2 different investigators who were blind to the experiments.

Measurements of 8-isoprostane, and nitrotyrosine

The levels of 8-isoprostane (a marker of lipid peroxidation), and nitrotyrosine (a marker of peroxynitrite formation) were measured in lung homogenates by competitive ELISA, and results were expressed in picograms per 25 μ g protein (8-isoprostane), or picomoles per 10 μ g protein (nitrotyrosine). The lower detection ranges are 0.8 pg/mL and 13 picomoles/mL for 8-isoprostane, and nitrotyrosine, respectively.

Statistical analysis

Data are expressed as means $\pm$ SEMs. Significant differences for multiple groups were determined by ANOVA followed by *post hoc* testing and Bonferroni correction for multiple comparisons, and significant differences between 2 selected groups were estimated by using an unpaired Student *t* test. Statistical significance was set at $P \leq .05$.

RESULTS

High-dose exogenous L-ARG reduces AHR and airway inflammation

To determine the possible beneficial effects of exogenous L-ARG supplementation in asthma, the effects of 25, 250, and 500 mg/kg L-ARG on Penh and AI were investigated. In dose titration experiments, allergic control mice (OVA/OVA/VEH) developed not only increased Penh (Fig 2, A) but also a dense infiltration of inflammatory cells, predominantly eosinophils and mononuclear cells, in perivascular and peribronchial regions compared with sham control mice (SHAM/PBS/VEH; Fig 2, B and C). Although low-dose L-ARG treatment had no significant effect on airway

inflammation, both higher doses reduced the Penh and infiltration of eosinophil and lymphocytes in bronchovascular regions (Fig 2). Because the dose-dependent effects on Penh and AI plateaued at 250 mg/kg, this dose was selected for further investigation. Airway resistance was increased with increasing doses of methacholine in allergic control mice compared with sham control mice (Fig 3, A). However, L-ARG treatment reduced it significantly. On the other hand, there was an exaggerated methacholine induced fall in sGAW in allergic control mice compared with sham controls, which was restored to near-normal levels by L-ARG treatment (Fig 3, B). Also, L-ARG treatment significantly reduced the absolute eosinophil count in BAL fluid (Fig 3, C), further confirming the previously noted effects on AI.

L-ARG reduces T_H2 cytokines, eotaxin, TGF- β_1 , and ovalbumin-specific IgE

Because L-ARG reduced airway inflammation and T_H2 response is the primary feature of AAI, we determined the effects of L-ARG on T_H2 cytokines by measuring the levels of IL-4, IL-5, IL-13, and TGF- β_1 in lung tissue. As shown in Table I, the levels of all these T_H2 cytokines and TGF- β_1 were increased in allergic controls compared with sham controls, and L-ARG treatment was associated with reduction of all these cytokines. Because eotaxin-induced eosinophil migration is one of the critical events in AAI and L-ARG treatment was associated with reduced eosinophils in BAL, we also measured eotaxin levels in the lung homogenates. Similar to T_H2 cytokines, levels of eotaxin were increased in the lungs of allergic control mice and were reduced by L-ARG treatment (Table I). L-ARG treatment also resulted in significant reduction of OVA-specific IgE without any significant effect on IgG_{2a} (Table I) or OVA-specific IgG₁ (data not shown).

L-ARG reduces subepithelial fibrosis and goblet cell metaplasia

Because L-ARG treatment reduced T_H2 response and TGF- β_1 levels, we next determined the effect of L-ARG on subepithelial fibrosis. As shown in Fig 3, D, a dense accumulation of collagen was found in allergic control mice, especially in subepithelial regions of bronchi and also around vascular regions, compared with sham control mice. L-ARG treatment significantly reduced the collagen deposition in the bronchovascular regions (Fig 3, D). Similarly, goblet cell metaplasia, another important feature of airway remodeling that contributes to airway obstruction,²⁰

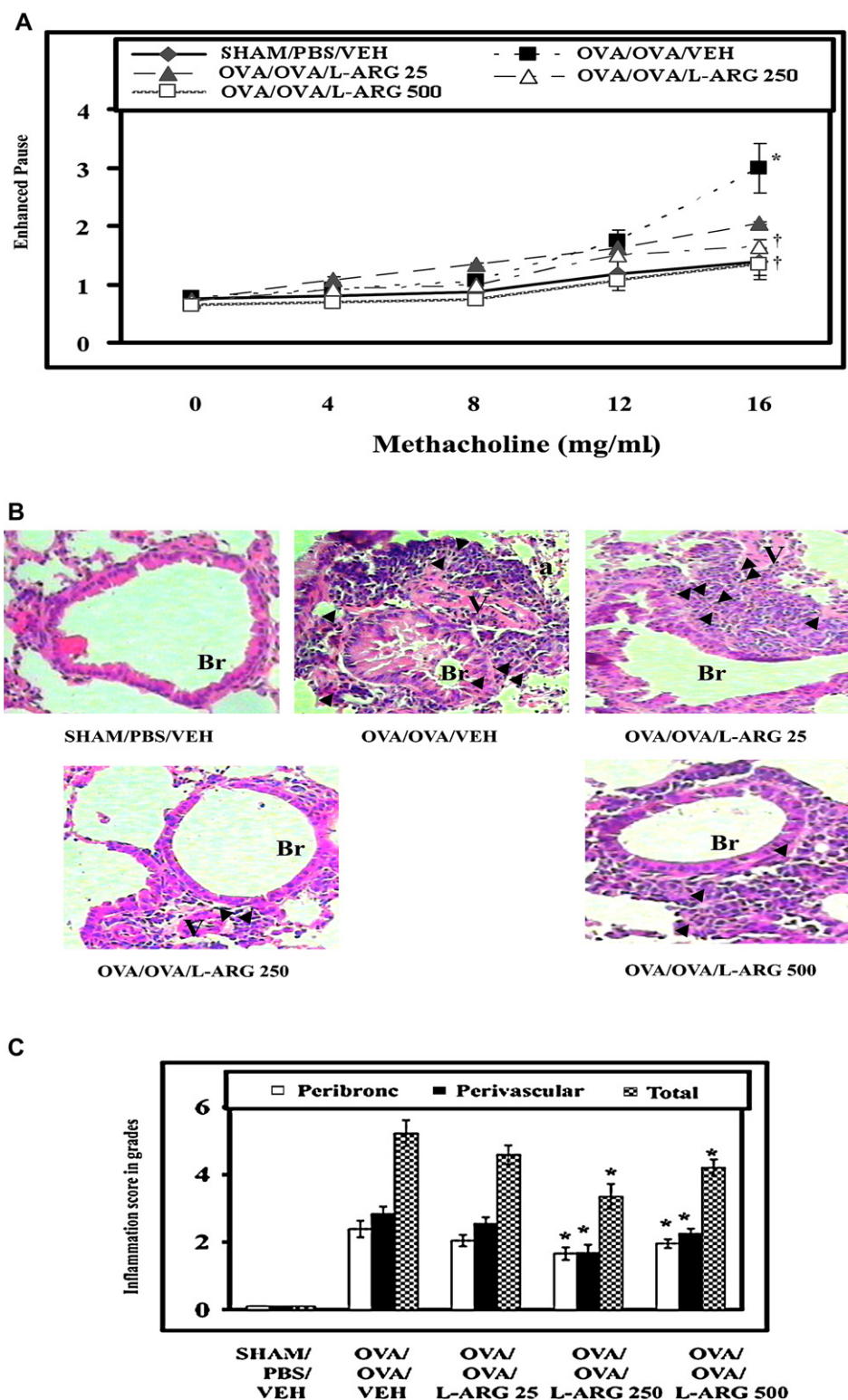


FIG 2. Effect of L-ARG on Penh and airway inflammation. Mice were randomly divided and named as per status of sensitization/challenge/treatment: SHAM/PBS/VEH, OVA/OVA/VEH, OVA/OVA/L-ARG 25, OVA/OVA/L-ARG 250, and OVA/OVA/L-ARG 500. * $P < .05$ vs SHAM/PBS/VEH; † $P < .05$ vs OVA/OVA/VEH. Data were means $\pm$ SEMs of 3 independent experiments. **A**, Penh. **B**, Representative photographs ($\times 10$ magnification) of hematoxylin and eosin staining. *a*, Alveolus; *Br*, bronchus; *V*, vessel. Black arrows indicate the eosinophils. **C**, Inflammation score. Hematoxylin and eosin-stained slides were evaluated by 2 different investigators blindly. Total inflammation score was calculated by addition of both peribronchial and perivascular inflammation scores. The sham group was given a value of 0.1 to make the graph. * $P < .05$ vs OVA/OVA/VEH.

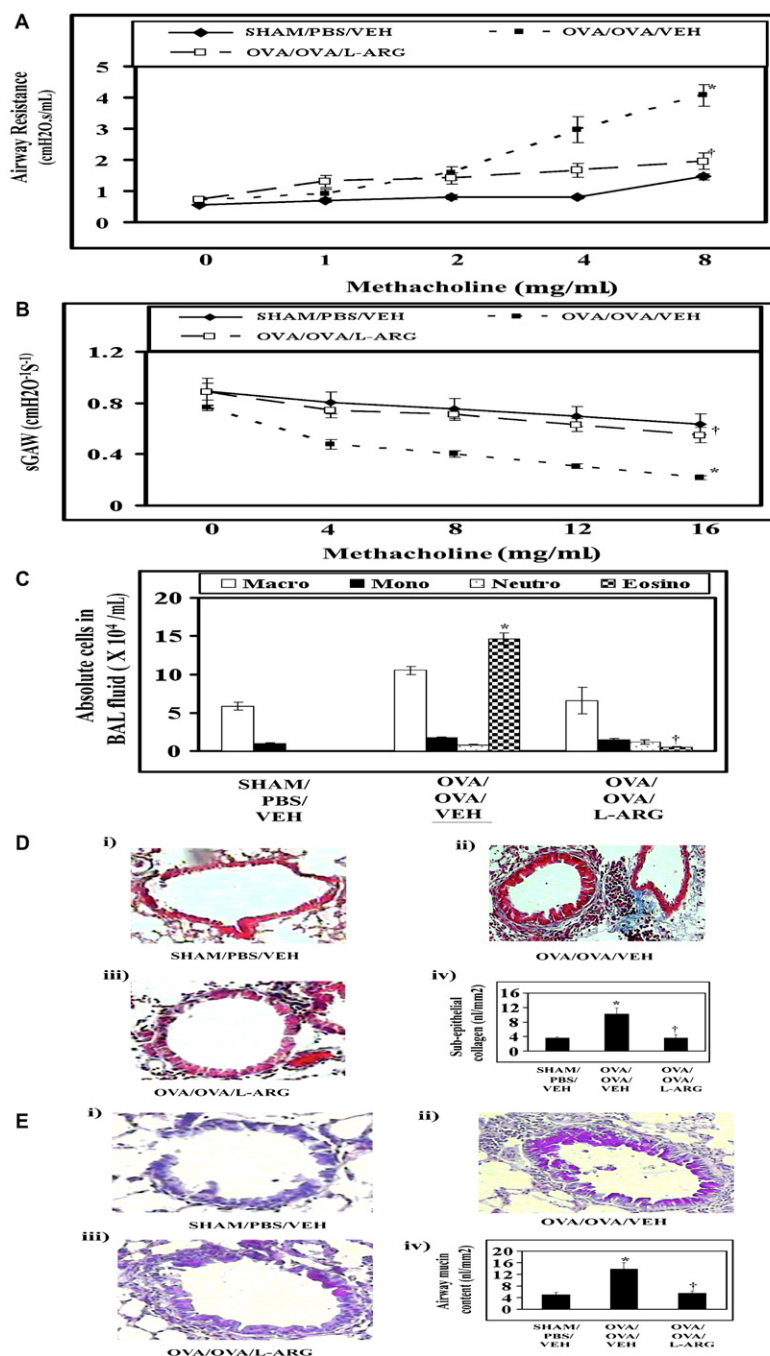


FIG 3. Effect of L-ARG on airway resistance, sGAW, BAL fluid eosinophilia, goblet cell metaplasia, and subepithelial fibrosis. Data were means $\pm$ SEMs of 3 independent experiments. * $P < .05$ vs SHAM/PBS/VEH and $\dagger P < .05$ vs OVA/OVA/VEH. **A**, Airway resistance. **B**, sGAW. **C**, Absolute cell types in BAL. *Eosino*, Eosinophils; *Macro*, macrophages; *Mono*, monocytes and lymphocytes; *Neutro*, neutrophils. Masson Trichrome (**D**) and periodic acid (**E**) stainings (i, ii, and iii) were performed and analyzed by quantitative morphometry (iv). All representative microphotographs were at $\times 10$ magnification.

was prominently seen in allergic controls, and was significantly attenuated by L-ARG treatment (Fig 3, E).

Arginase expression is increased in AAI and is reduced by L-ARG treatment

To determine the effect of L-ARG on various arginine metabolizing enzymes, we measured arginase activity in lung cytosols.

As shown in Fig 4, A, arginase activity was significantly increased in the lung cytosols of allergic controls compared with lung cytosols of sham controls, in which arginase activity was not measurable. Western blot analysis (Fig 4, B and C) also confirmed that Arginase I, detected as dimeric forms (36 and 41 kD), was significantly increased in the lung cytosols of allergic control mice compared with sham controls. Immunohistochemistry revealed that arginase was predominantly expressed in inflammatory cells

TABLE I. Effects of L-ARG on IL-4, IL-5, IL-13, OVA-specific IgE, IgG_{2a}, eotaxin, and TGF- β ₁ levels

Groups	IL-4 (pg/100 μ g protein)	IL-5 (pg/100 μ g protein)	IL-13 (pg/100 μ g protein)	OVA Specific IgE (A.U.)	OVA Specific IgG _{2a} (A.U.)	Eotaxin (pg/25 μ g protein)	TGF- β ₁ (pg/50 μ g protein)
SHAM/PBS/VEH	63.3 $\pm$ 7.1	27.9 $\pm$ 10.4	5.2 $\pm$ 1.1	0.8 $\pm$ 0.1	1.3 $\pm$ 0.1	13.7 $\pm$ 0.7	318.5 $\pm$ 57.0
OVA/OVA/VEH	97.0 $\pm$ 3.3*	63.2 $\pm$ 7.0*	35.5 $\pm$ 12.8*	1.9 $\pm$ 0.1*	2.6 $\pm$ 0.7*	38.9 $\pm$ 2.4*	521.8 $\pm$ 63.4*
OVA/OVA/L-ARG	75.2 $\pm$ 7.9†	30.7 $\pm$ 10.8†	5.3 $\pm$ 4.0†	1.3 $\pm$ 0.1†	2.4 $\pm$ 0.4	24.0 $\pm$ 3.7†	356.4 $\pm$ 33.9†

A.U.; Arbitrary Units.

**P* < .05 vs SHAM/PBS/VEH.†*P* < .05 vs OVA/OVA/VEH.

around the bronchial and vascular regions of allergically inflamed lungs but was undetectable in normal lungs (Fig 4, D). L-ARG treatment significantly reduced the activity and expression of Arginase I compared with allergic control mice (Fig 4, A-D). As mentioned, L-ARG treated mice had significantly less inflammation (Fig 2).

L-ARG enhances ENO and cGMP but reduces peroxynitrite

To determine the effect of L-ARG treatment on NO metabolism, we measured ENO as a marker of NO synthesis, cGMP as a marker of guanylyl cyclase activation by NO, and nitrotyrosine as a marker of peroxynitrite generation. Mice with AAI had increased ENO but lower cGMP than sham controls (Fig 5, A and B). This was further associated with increased nitro-oxidative stress as shown by significantly increased 8-isoprostane and nitrotyrosine, which represent lipid peroxidation and peroxynitrite, respectively (Fig 5, C and D). L-ARG treatment was associated with significantly greater ENO than either sham control mice or allergically inflamed mice (Fig 5, A). As shown in Fig 5, B, cGMP levels were significantly increased in L-ARG-treated mice compared with mice with allergic inflammation but similar to sham controls. Importantly, despite even greater levels of ENO in L-ARG-treated mice, oxidative stress markers were lower than allergic control mice, and similar (8-isoprostane) or reduced (nitrotyrosine) compared with the sham controls.

L-ARG enhances eNOS expression and reduces iNOS

To investigate whether different sources of NO could be responsible for the differences in cellular reactive nitrogen species, we studied the NOS expression patterns by immunohistochemistry in lung tissue sections. Sham control mice showed mild expression of eNOS in bronchial epithelial cells (Fig 6, A) and no expression of iNOS (Fig 6, B). Semiquantitative analysis scores correlating to stain intensity for sham control mice were 4.1 ± 0.85 for eNOS and 0.23 ± 0.07 for iNOS (means $\pm$ SEMs, arbitrary units). In contrast, mice with AAI showed a significant high expression of iNOS in inflammatory cells such as macrophages and eosinophils and bronchial epithelia (Fig 6, B). Semiquantitative analysis scores were 4.4 ± 0.4 for eNOS and 3.1 ± 0.5 for iNOS. However, L-ARG-treated allergic mice showed a significantly greater expression of eNOS in bronchial epithelia compared with either sham controls or mice with AAI and reduced expression of iNOS compared with mice with AAI. Semiquantitative analysis scores were 7.2 ± 0.3 for eNOS and 1.5 ± 0.3 for iNOS (*P* < .05 compared with allergic controls

for both eNOS and iNOS). Thus, the major source for NO synthesis was iNOS in allergically inflamed lungs and eNOS in normal and L-ARG-treated lungs.

DISCUSSION

L-arginine is a conditionally essential basic amino acid, and human beings normally consume 3.5 to 5 g daily.²¹ It has multiple pharmacologic properties (antioxidant, anti-inflammatory, antihypercholesterolemic, muscle relaxant, immunomodulatory, and so forth).¹² These widespread activities could be a result of its effect on various metabolizing enzymes and their final metabolites.²² The use of L-ARG has been reported for various disease conditions (angina pectoris, congestive heart failure, hypertension, intermittent claudication, pre-eclampsia, AIDS, burns, cancer, diabetes, gastritis, male and female infertility, senile dementia, and so forth).¹² Because most of the L-ARG-metabolizing enzymes are also present in lung, various studies have been performed to understand pulmonary arginine metabolism.²³ A recent study reported that L-ARG bioavailability is strongly associated with airflow obstructions in human beings.²⁴ Because dietary intake is the primary determinant of plasma arginine levels, dietary supplementation or oral administration has been tried as a potential therapy for asthma at doses up to 50 mg/kg. Although these studies have found L-ARG to be ineffective in the treatment of asthma, there have been no studies at higher doses that are shown to be safe and effective in other diseases.²⁵ In this study, we report for the first time the beneficial effects of a 250 mg/kg dose of L-ARG in a murine model of allergic airway inflammation and AHR. Consistent with previous studies, these effects were not seen at a lower dose (25 mg/kg). At this dose (25 mg/kg), there were no significant effects on the expressions of eNOS, iNOS, and arginase and ENO levels (data not shown). These results indicate that exogenous arginine at low doses may not be sufficient to increase the substrate availability of eNOS.

Importantly, we found that L-ARG administration profoundly enhances eNOS expression in bronchial epithelia (Fig 6, A) and increases the levels of ENO (Fig 5, A). It is known that L-ARG supplementation increases the transcription of eNOS.²⁶ A simultaneous downregulation of iNOS and arginase noted in our study indicates that the administered L-ARG was predominantly used by eNOS, as evidenced by the increased ENO levels, increased guanylyl cyclase activation, and low reactive nitrogen species. In contrast, mice with allergic airway inflammation that also had high levels of ENO probably had iNOS as the source, because eNOS expression was low. Further, the high arginase levels in such mice may lead to generation of L-ornithine, which is a precursor for polyamines and proline that may induce structural changes in the airway such as subepithelial fibrosis.²⁷ Taken

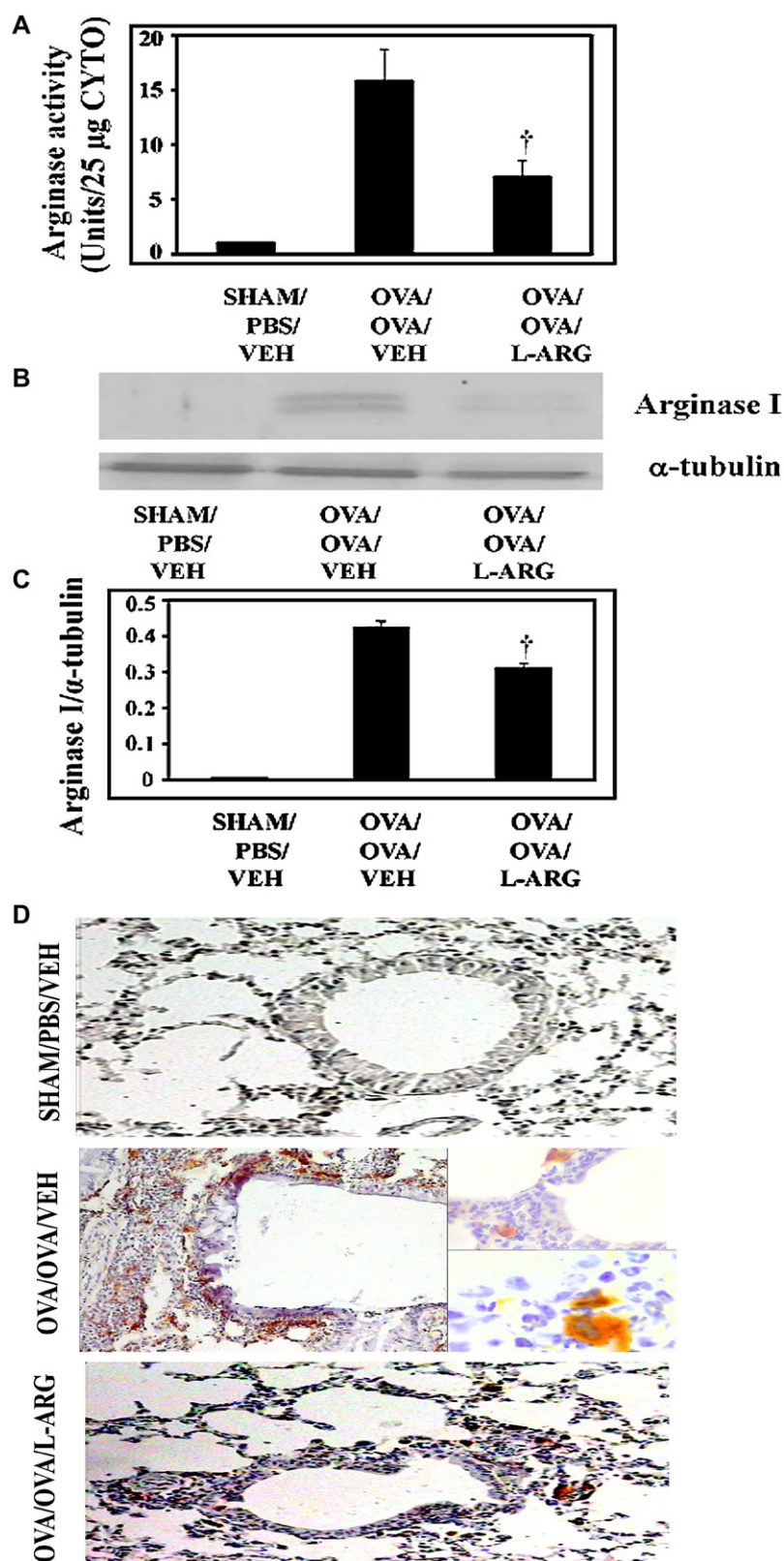


FIG 4. L-ARG reduces arginase activity, expression in lung. Data were means $\pm$ SEMs of 3 independent experiments. * $P < .05$ vs OVA/OVA/VEH. **A**, Arginase activities in lung cytosols. **B and C**, Western blots for Arginase I and α -tubulin and spot densitometry signals. **D**, Immunohistochemistry for Arginase I expression. Brown indicates the positive expressions. In arginase activity and densitometry graphs, the sham group was given values of 1 and 0.01, respectively, to make the graphs. All representative photographs were at $\times 10$ except insets of OVA/OVA/VEH (upper, $\times 40$; and lower, $\times 100$). CYTO, Cytosolic fraction.

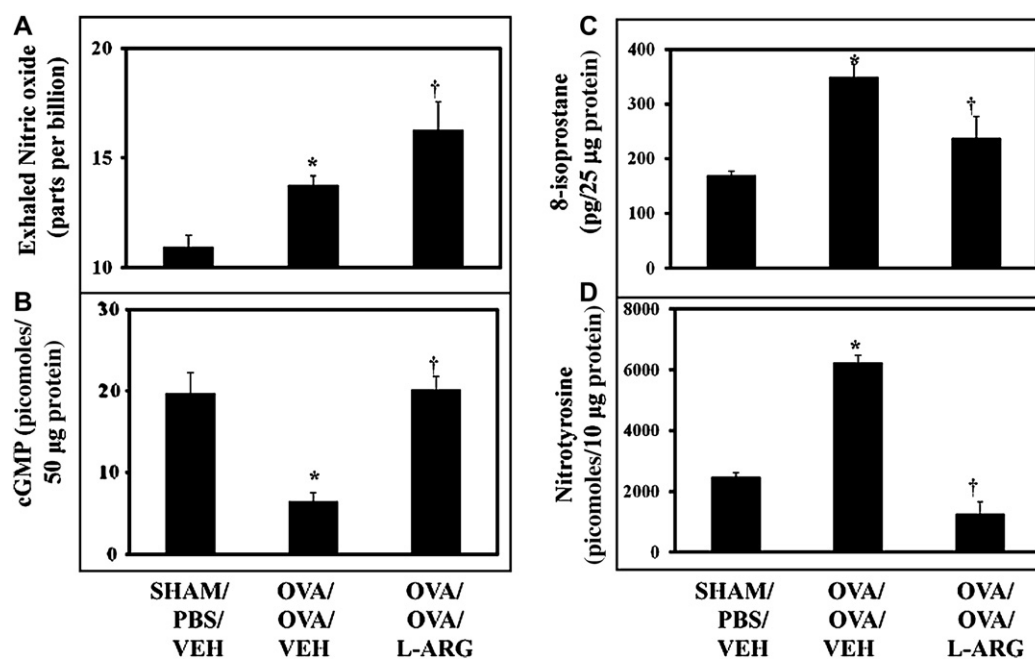


FIG 5. L-ARG enhances the levels of ENO and cGMP and reduces nitro-oxidative stress in the lung. Data were means $\pm$ SEMs of 3 independent experiments. * $P < .05$ vs SHAM/PBS/VEH; † $P < .05$ vs OVA/OVA/VEH. ENO levels (A), cGMP (B), 8-isoprostane (C), and nitrotyrosine, a marker of peroxynitrite (D), were determined.

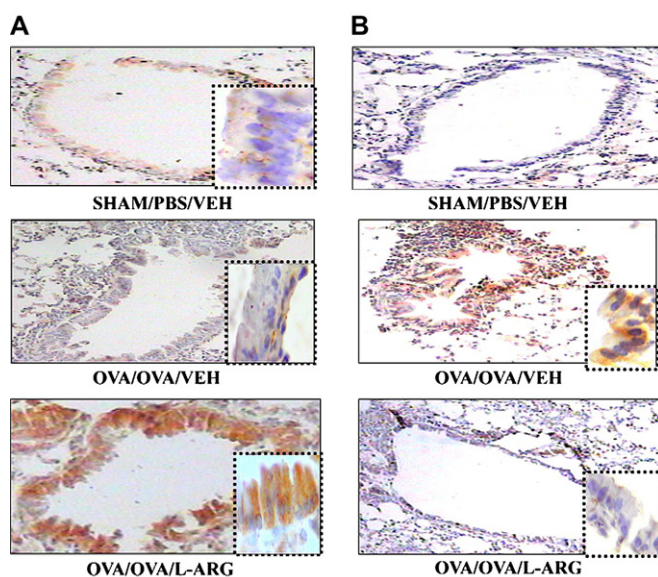


FIG 6. L-ARG enhances eNOS expression and reduces iNOS. Immunohistochemistry was determined to determine the expression of eNOS (A) and iNOS (B). Brown indicates the positive expressions. Representative photomicrographs from 3 independent experiments are shown. All photographs were at $\times 10$ magnification except insets ($\times 100$).

together, downregulations of both iNOS and arginase and upregulation of eNOS by L-ARG treatment could be responsible for the reduction of airway inflammation and AHR (Figs 2 and 3). To support this view, eNOS overexpression or arginase inhibition alleviated the features of asthma in similar models.^{28,29} Most of the biological effects of NO are determined by its cellular source and

by-products after combination with other biomolecules. NO originating from constitutive NOS such as eNOS leads to increased production of cGMP by soluble guanylyl cyclase, which leads to bronchodilation. In this study, L-ARG administration increased cGMP and attenuated AHR. On the other hand, NO originating from iNOS may lead to recruitment of inflammatory cells in the airway, including eosinophils.⁶ L-ARG treatment reduced eosinophils in BAL fluid along with the reduction in the expression of iNOS.

It is interesting to note that increasing the availability of the substrate decreased the expressions of iNOS and arginase, although it increased the expression of eNOS and its activity, which is evidenced by increased ENO and cGMP levels. This can be explained in the context of the interactions between L-ARG and asymmetric dimethyl arginine (ADMA). ADMA, an L-ARG analog and an endogenous inhibitor of different isoforms of NOS, has been shown to be increased in allergic airway inflammation.³⁰ It increases reactive oxygen, nitrogen species such as peroxynitrite in murine lung epithelial cells,³¹ and the collagen deposition in allergically inflamed lungs.³² In normal airways, where extracellular L-ARG and ADMA are at physiological concentrations, eNOS can produce physiological concentration of NO, which is required for maintenance of airway tone.⁶ In asthmatic airways, the increased levels of ADMA could inhibit eNOS in the presence of limited amounts of endogenous L-ARG (Fig 7). It also should be noted that although ADMA is known to inhibit constitutive and inducible forms in various cell types, it does not inhibit iNOS protein and its induced nitrite levels significantly in murine epithelial cells.³¹ We have recently shown that L-ARG administration reduced the levels of both ADMA and nitrotyrosine in the lungs of OVA-sensitized and challenged mice.³⁰ In pathological conditions such as airway inflammation, where ADMA may be

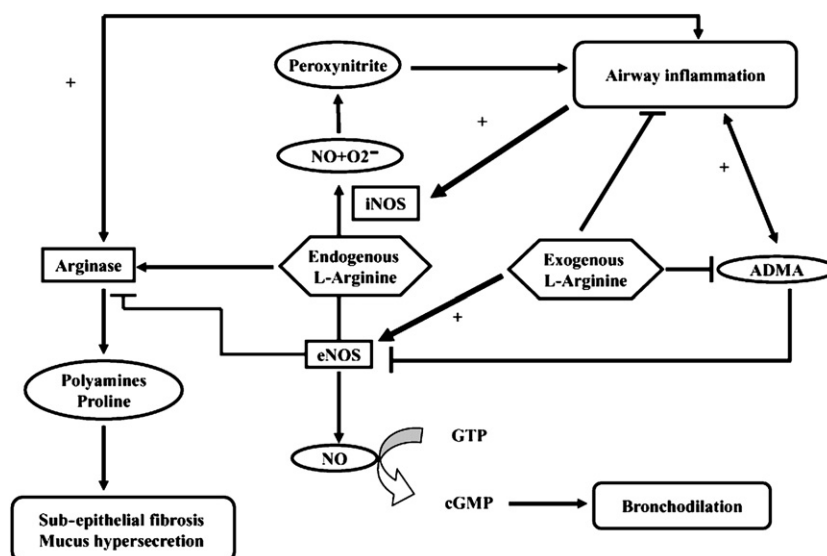


FIG 7. Schematic diagram to illustrate the effects of L-ARG availability and exogenous administration of L-ARG on NO metabolism and asthma features. Arginine is a sole substrate for various arginine-metabolizing enzymes including NOS (constitutive NOS isoforms such as eNOS and neuronal NOS, and iNOS) and arginase. In normal airways, constitutive NOS isoforms consume endogenous arginine to produce NO to maintain the airway tone through cGMP activation. In allergic airways, proinflammatory cytokines induce the expressions of arginase and iNOS; the former leads to airway remodeling through the polyamines, and the latter releases NO, which has detrimental effects such as infiltration of inflammatory cells. Arginase activation also limits the availability of endogenous arginine as a substrate for iNOS, which leads to release both NO and superoxide anion (O₂⁻) from iNOS to produce peroxynitrite, which has dangerous effects on the airway by causing airway inflammation and bronchoconstriction. Exogenous administration of L-ARG leads to displacement of ADMA, an L-ARG analog, to increase eNOS activity, and has also been shown to increase the expression of eNOS to enhance its beneficial effects through the NO-cGMP pathway and to reduce the inflammation on the one hand, and on the other hand eNOS also produces N^G-hydroxy-L-ARG, which inhibits arginase. +, Positive modulation. Lines with buffers at the end indicate the inhibitory pathways.

inhibiting eNOS activity, supplementation with L-ARG is likely to displace ADMA competitively, as shown in Fig 7, and the increased L-ARG/ADMA ratio would restore the eNOS activity.^{30,33} It is known that eNOS overexpression reduces airway inflammation, and it is to be noted that arginase and iNOS are predominantly expressed in inflammatory cells. Further, eNOS also produces N^G-hydroxy-L-ARG, which inhibits the arginase pathway, compounding the effect. However, these mechanisms are speculative, and alleviation of asthma features by L-ARG might additionally or entirely be a result of many other mechanisms that are not studied here because L-ARG is the substrate not only to NOS but also to various enzymes, as detailed.²²

In conclusion, our findings reveal that L-ARG could reduce the AHR and airway inflammation by correcting the balance between L-ARG-metabolizing enzymes and reducing nitro-oxidative stress in the lung. Thus, further studies are desirable to examine the role of L-ARG, especially at higher doses, in the development of better therapeutics for diseases such as asthma in which AAI is the main feature.

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Clinical implications: These results indicate a possible role for dietary arginine supplementation in the treatment of allergic airway inflammatory diseases such as bronchial asthma.

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