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

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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-β1, 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;125:626-35.)

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

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

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

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(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 asthma[10] 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 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 μg 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-μL 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 previously[16] by using a standard clinical ENO analyzer (CLD88sp; Ecomedics, Durnten, 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 account 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, IgG_1, 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 μg cytosolic fraction protein in 40 μL volume was mixed with 10 μL substrate buffer containing arginine, and $MnCl_2$, 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 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.