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Functionally important role for arginase 1 in the airway hyperresponsiveness of asthma

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¹Institutes of Medical Sciences and ⁶Occupational and Environmental Health Program, Dalla Lana School of Public Health, Faculty of Medicine, ²Divisions of Occupational and Respiratory Medicine and ⁴Nephrology, Department and Faculty of Medicine, and ⁵Physiology and Experimental Medicine and Division of Respiratory Medicine, The Hospital for Sick Children, University of Toronto; and ³Keenan Research Centre, Li Ka Shing Knowledge Institute and Gage Occupational and Environmental Health Unit, St. Michael's Hospital Research Centre, Toronto, Ontario, Canada

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North ML, Khanna N, Marsden PA, Grasemann H, Scott JA. Functionally important role for arginase 1 in the airway hyperresponsiveness of asthma. *Am J Physiol Lung Cell Mol Physiol* 296: L911–L920, 2009. First published March 13, 2009; doi:10.1152/ajplung.00025.2009.—L-Arginine metabolism by the arginase and nitric oxide (NO) synthase (NOS) families of enzymes is important in NO production, and imbalances between these pathways contribute to airway hyperresponsiveness (AHR) in asthma. To investigate the role of arginase isozymes (ARG1 and ARG2) in AHR, we determined the protein expression of ARG1, ARG2, the NOS isozymes, and other proteins involved in L-arginine metabolism in lung tissues from asthma patients and in acute (3-wk) and chronic (12-wk) murine models of ovalbumin-induced airway inflammation. Expression of ARG1 was increased in human asthma, whereas ARG2, NOS isoforms, and the other L-arginine-related proteins (i.e., cationic amino acid transporters 1 and 2, agmatinase, and ornithine decarboxylase) were unchanged. In the acute murine model of allergic airway inflammation, augmentation of ARG1 expression was similarly the most dramatic change in protein expression. However, ARG2, NOS1, NOS2, and agmatinase were also increased, whereas NOS3 expression was decreased. Arginase inhibition in vivo with nebulized S-(2-boronoethyl)-L-cysteine attenuated the methacholine responsiveness of the central airways in mice from the acute model. Further investigations in the chronic murine model revealed an expression profile that more closely paralleled the human asthma samples: only ARG1 expression was significantly increased. Interestingly, in the chronic mouse model, which generates a remodeling phenotype, arginase inhibition attenuated methacholine responsiveness of the central and peripheral airways. The similarity in arginase expression between human asthma and the chronic model and the attenuation of AHR after in vivo treatment with an arginase inhibitor suggest the potential for therapeutic modification of arginase activity in asthma.

S-(2-boronoethyl)-L-cysteine; agmatinase; nitric oxide

THE SEMI-ESSENTIAL AMINO ACID L-arginine is substrate for the nitric oxide (NO) synthase (NOS) and arginase enzyme families (28). Studies in affected patients and animal models have indicated that L-arginine metabolism is altered in asthma through increased expression and activity of arginase (3, 23, 32, 42). Previous studies have shown that arginase contributes to the regulation of airway smooth muscle tone, likely by reducing the bioavailability of L-arginine for NOS and, subse-

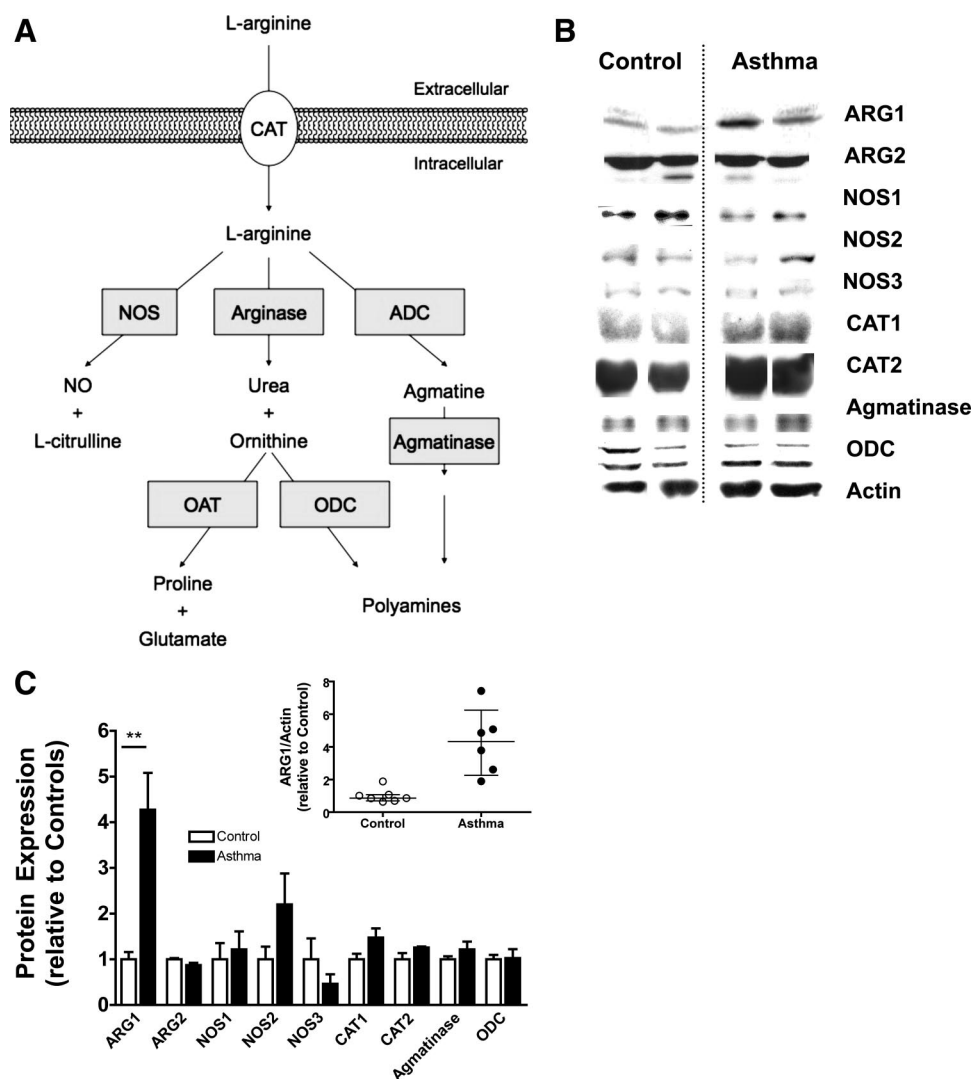
quently, NO production (17, 27, 30). Genetic association studies have suggested functionally important contributions from haplotypes/alleles of the arginase genes *ARG1* and *ARG2* in asthma and atopy, as well as a role for *ARG1* in the responsiveness to bronchodilators (13, 25, 26). Thus there is a need to develop a thorough understanding of the consequences of biochemical imbalances of the L-arginine pathways in asthma, the suitability of common model systems in terms of mimicking these alterations, and the potential for arginase inhibition to attenuate airway hyperresponsiveness (AHR) in multiple animal models to predict the effectiveness of these pharmacological agents for future human asthma therapies.

A comprehensive examination of the protein expression profile for L-arginine-related enzyme pathways and transporters has not been performed. L-Arginine bioavailability within the cell is directly related to its uptake by the cationic amino acid transporters (CATs) and catabolism via the arginase, NOS, and arginine decarboxylase pathways (Fig. 1A) (28). Increases in CAT2 expression have been detected in murine models of allergic inflammation using microarray analysis, and mediators released from eosinophils can inhibit the function of CAT (15, 42, 43). However, the expression levels of CAT2 and CAT1 have not previously been described in lung tissue from asthma patients. The NOS isozymes NOS1, NOS2, and NOS3 are immunologically distinct proteins that are expressed from independent genes and have been of significant interest in asthma because of the observation that exhaled NO levels are increased in asthma (1, 22). Immunohistochemical staining has demonstrated expression of the NOS isoforms in bronchial biopsies from asthma patients (14, 24). Studies in animal models have also highlighted arginase as a novel therapeutic target in asthma through the use of specific inhibitors. Meurs et al. (31) demonstrated that the increase in contractile responsiveness of isolated tracheal preparations from ovalbumin (OVA)-sensitized and -challenged guinea pigs to methacholine was attenuated by treatment with an arginase inhibitor (31). The same group recently investigated the acute and protective effects of arginase inhibition in vivo in a guinea pig model of asthma and demonstrated reversal of allergen-induced AHR when the inhibitor was administered before allergen challenge (29). They further noted a reduction in sensitivity to allergen with chronic arginase inhibition (29). In murine models of allergic airway inflammation, systemic administration of arginase inhibitor caused increases in NO metabolite concentrations (5, 20). However, the expression profiles of L-arginine-related proteins have not been investi-

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Fig. 1. L-arginine-related pathways and expression of proteins in lungs from control subjects and asthma patients. **A**: L-arginine-related pathways include cationic amino acid transporters (CATs), nitric oxide (NO) synthase (NOS) isozymes, arginase isozymes (ARG1 and ARG2), and agmatine decarboxylase (ADC). Ornithine, a by-product of arginase activity, is metabolized downstream by ornithine amino transferase (OAT) and ornithine decarboxylase (ODC). Agmatine, the decarboxylation product of L-arginine, is metabolized by agmatinase. **B** and **C**: Western blot determination of protein expression of ARG1, ARG2, NOS2, NOS3, CAT1, CAT2, and ODC in lung homogenates of control subjects and asthma patients, with actin as a loading control. Immunoprecipitation was used to assess expression of NOS1 and agmatinase. Fold change in human asthma is expressed relative to control lung samples. ARG1 expression was significantly increased in asthma patients compared with control subjects (*C* and *inset*). Values are means $\pm$ SE ($n = 6$ –7/group) in *C* and median and interquartile range in *inset*. Expression of ARG2, NOS1, NOS2, NOS3, CAT1, CAT2, agmatinase, and ODC was not significantly different between groups. $**P < 0.01$.



gated in common murine models of allergic airway inflammation and compared with human asthma. Additionally, the ability of arginase inhibition to attenuate AHR in acute and chronic murine models (i.e., in the absence and presence of airway remodeling, respectively) has not been evaluated. Furthermore, agmatinase, an enzyme that catabolizes the decarboxylation product of L-arginine, has not been investigated in human asthma. Such investigations may be important to fully understand the causes and implications of dysregulation of L-arginine metabolism in asthma.

On the basis of these important insights derived from animal models and observations in asthma patients and to test the hypothesis that augmented expression of arginase contributes to the AHR of asthma, we investigated the expression of proteins related to L-arginine metabolism and compared the biochemical profiles of two commonly used acute and chronic murine models of allergic inflammation with those of lung tissue from asthma patients. Using a treatment-based protocol, we also investigated and compared the immediate effects of nebulized arginase inhibitors on methacholine responsiveness in these murine models.

MATERIALS AND METHODS

Lung homogenization and Western blotting. Lung specimens from control and physician-diagnosed asthma patients ($n = 7$ and 6 specimens, respectively) were obtained from the National Disease Research Interchange (Philadelphia, PA) and frozen at -80°C until homogenization and determination of protein expression (Table 1). Frozen tissues were thawed and macroscopically dissected, and airway tissues were isolated. Human and mouse specimens were homogenized 1:1 (wt/vol) in 0.1% Triton X-100 containing 0.7 $\mu\text{g}/\text{ml}$ pepstatin A (Bioshop, Burlington, ON, Canada), 0.5 $\mu\text{g}/\text{ml}$ antipain (Bioshop), and 2 mM EDTA (pH 7.3). Insoluble debris was cleared by

Table 1. Age and sex of control subjects and asthma patients from whom lung specimens were obtained

	Control ($n = 7$)	Asthma ($n = 6$)
Age, yr	19–70	29–68
Male/female	6/1	4/2

All specimens were obtained from the National Disease Research Interchange. One asthma patient had a prior history of smoking. There was no indication of steroid use among any of the subjects.

centrifugation at 20,000 *g* for 15 min at 4°C. Protein concentrations were determined using the QuantiPro bicinchoninic acid assay (Sigma, St. Louis, MO). Western blotting of 30–40 µg of tissue homogenate was performed to assess ARG1, ARG2, NOS2, NOS3, CAT1 (SLC7A1), and CAT2 (SLC7A2) expression. NOS1 and agmatinase expression was assessed by immunoprecipitation (300 and 250 µg, respectively, for mouse and human samples) followed by Western blotting (ExactaCruz, Santa Cruz Biotechnologies, Santa Cruz, CA). All antibodies were purchased from Santa Cruz Biotechnologies, except SLC7A2, which was obtained from Orbigen (San Diego, CA). (Further details of each antibody are provided in Table 2.) Western blots were stripped of the primary antibodies and reprobed for actin as a loading control. Proteins were visualized using Western Lightning reagent (PerkinElmer Life Sciences, Boston, MA), and the polyvinylidene difluoride membranes were exposed to X-Omat Blue XB-1 film (Kodak, Rochester, NY). Densitometry was performed using GelEval 1.22 (Frogdance Software, University of Dundee).

Arginase activity measurements. Arginase activity was measured in lung tissue homogenates by methods that have been described previously (9).

Immunohistochemistry assessment. Immunohistochemistry for ARG1, ARG2, and NOS1 was performed using the antibodies described above for Western blotting, as described previously (Pathology Core Facility, Centre for Modeling Human Disease, Toronto Centre for Phenogenomics, Toronto, ON, Canada) (2). Slides were visualized on a Leica inverted microscope, and images were captured digitally using a micropublisher RTV 5.0 camera with QCapture image capture software (Quorum Technologies, Guelph, ON, Canada).

Acute and chronic murine OVA-sensitization and -challenge models of allergic airway inflammation. To investigate the role of arginase in AHR, we used acute (3-wk) and chronic (12-wk) OVA-sensitization and -challenge murine models. These protocols were approved by the University of Toronto Faculty Advisory Committee on Animal Services and conducted in accordance with the guidelines of the Canadian Council on Animal Care.

The acute model utilized a 3-wk OVA-sensitization and -challenge protocol, as described previously (6). Briefly, 8–14 female Balb/C mice per group (6–8 wk old; Charles River Laboratories, Saint-Constant, PQ, Canada) were sensitized to chicken OVA on *days 0* and *7* [25 µg ip OVA, endotoxin-free, grade V (Sigma Chemical, Mississauga, ON, Canada), with 1 mg of aluminum hydroxide gel in 0.2 ml of PBS, pH 7.4 (Invitrogen, Grand Island, NY)]. Beginning on *day 14*, the animals were randomized to repeated exposure to nebulized 6% OVA in PBS or PBS alone (AeroNebLab nebulizer, SciReq, Montréal, PQ, Canada) for 25 min/day up to *day 20*. On *day 21*, pulmonary function testing and methacholine challenge were performed before tissue harvesting and euthanasia.

The chronic model consisted of a sensitization regimen identical to that described for the acute model, with a prolonged, but less severe,

challenge period. On *day 14*, mice were randomized to repeated exposure to nebulized 2.5% OVA in PBS or PBS alone for 2 consecutive days (25 min/day) every 2 wk up to *week 12* (i.e., 2 consecutive days of exposure followed by 12 days of rest between exposures) (18).

Pulmonary function testing and methacholine challenge. At 24 h after the final OVA or PBS challenge, mice were anesthetized with ketamine (50 mg/kg ip; Bioniche, Belleville, ON, Canada)-xylazine (10 mg/kg ip; Bayer, Toronto, ON, Canada) and then intubated with an 18-gauge stainless steel cannula (BD Biosciences Canada, Mississauga, ON, Canada) for in vivo, ventilator-based assessment of methacholine responsiveness with use of the FlexiVent system (Scireq). After baseline determination of airway resistance, mice were challenged with methacholine (0–100 mg/ml in sterile PBS; Sigma) nebulized directly into the ventilatory circuit (AeroNebLab nebulizer). Two models of respiratory mechanics were used to assess lung resistance: the linear first-order single-compartment model, which provides resistance of the total respiratory system, and the constant-phase model, which utilizes forced oscillation to differentiate between central airway Newtonian resistance (R_N) and peripheral tissue damping (G) and elastance (H) (16, 38). Anesthesia was maintained throughout the experiment by repeated administration of ~25% of the initial dose of ketamine-xylazine every 25 min until completion. All data points were collected using the FlexiVent software and analyzed offline using Excel (Microsoft, Redmond, WA).

Arginase inhibition in vivo. After determination of baseline pulmonary mechanics parameters in the OVA-sensitized and OVA- or PBS-challenged mice, *S*-(2-boronoethyl)-L-cysteine (BEC, 40 µg/g body wt; Alexis Biochemicals, San Diego, CA) was delivered via nebulization directly into the ventilatory circuit 15 min before reassessment of pulmonary mechanics and initiation of the methacholine dose-response curve.

Statistical analyses. Statistical analyses were performed using the statistical software package in Prism 4.0b (GraphPad Software, San Diego, CA). Values are means ± SE, except where noted. Mann-Whitney *t*-tests or one-way ANOVA with Kruskal-Wallis test and Dunn's post hoc test were used for between-group comparison. Differences were considered significant when $P < 0.05$. All Western blot and immunoprecipitation findings were derived from at least three independent experiments, based on samples from 6–14 mice per group. Immunohistochemical staining was conducted on at least four mice per treatment group. Pulmonary function and methacholine responsiveness were evaluated in 8–14 mice per group.

RESULTS

Lung ARG1 expression and activity is increased in human asthma. We examined the expression of several proteins related to L-arginine metabolism in the lung samples from asthma patients and control subjects. Expression of ARG1 was in-

Table 2. Antibodies used for Western blotting, immunoprecipitation, and immunohistochemistry

Target	Epitope	Type	Catalog No.	Source
Actin	COOH terminus (human)	Goat polyclonal IgG	sc-1616 (I-19)	SCBT
Agmatinase	Internal (human)	Goat polyclonal IgG	sc-46713 (K-14)	SCBT
ARG1	NH ₂ terminus (human)	Goat polyclonal IgG	sc-18351 (N-20)	SCBT
ARG2	COOH terminus (human)	Goat polyclonal IgG	sc-18357 (L-20)	SCBT
CAT1	COOH terminus (human)	Goat polyclonal IgG	sc-33087 (C-12)	SCBT
CAT2	Internal (human)	Rabbit polyclonal IgG	Pab-11689	Orbigen
NOS1	COOH terminus (rat)	Rabbit polyclonal IgG	sc-648 (R-20)	SCBT
NOS2	2-175 (human)	Rabbit polyclonal IgG	sc-8310 (H-174)	SCBT
NOS3	COOH terminus (human)	Rabbit polyclonal IgG	sc-654 (C-20)	SCBT
ODC	Internal (human)	Goat polyclonal IgG	sc-21516 (F-14)	SCBT

ARG1 and ARG2, arginase isozymes 1 and 2; CAT1 and CAT2, cationic amino acid transporter types 1 and 2; NOS1, NOS2, and NOS3, nitric oxide synthase isozymes 1, 2, and 3; ODC, ornithine decarboxylase; SCBT, Santa Cruz Biotechnology.

creased 4.4 ± 1.0 -fold in lung tissue homogenates from asthma patients compared with controls ($P < 0.001$; Fig. 1). A significant elevation in arginase activity was also detected in samples from the asthma patients (35.7 ± 4.6 and 62.2 ± 6.0 pmol urea $\cdot$ min $^{-1} \cdot \mu$ g protein $^{-1}$ for control and asthma, respectively, $P = 0.0087$). However, no significant differences in ARG2, NOS1, NOS2, or NOS3 protein expression were detected between asthma patients and controls by Western blotting or immunoprecipitation (Fig. 1). Additionally, no alterations in the expression of CAT1 (SLC7A1), CAT2 (SLC7A2), or ornithine decarboxylase were observed in asthma patients (Fig. 1). Thus, augmented ARG1 expression was solely responsible for the increased arginase activity in human asthma.

Protein expression, localization, and arginase inhibition in the acute murine model of allergic airway inflammation. After examining the protein expression profile in human asthma, we used an acute murine model of allergic airway inflammation to determine the functional relevance of the augmentation of ARG1 protein expression. This is one of the most common animal models employed in the study of allergic asthma, and we employed this system to determine the effects of arginase inhibition on AHR *in vivo*. First, we confirmed that the augmentation of ARG1 was modeled in this system. Indeed, in OVA/OVA lung homogenates, ARG1 was significantly upregulated (11.3-fold) compared with the OVA/PBS controls ($P < 0.001$; Fig. 2), as was total arginase activity (35.4 ± 3.8 and 584.5 ± 91.4 pmol urea $\cdot$ min $^{-1} \cdot \mu$ g protein $^{-1}$ for OVA/PBS and OVA/OVA, respectively, $P < 0.001$). ARG2 was also increased (2.6-fold) in the acute model ($P < 0.001$; Fig. 2). NOS1 and NOS2 expression were upregulated 3.4- and 3.0-fold, respectively, in the OVA/OVA lung homogenates ($P < 0.001$ and $P < 0.01$, respectively; Fig. 2), whereas expression of NOS3 was decreased 2.1-fold ($P < 0.01$; Fig. 2). Expression of agmatinase was increased 1.65-fold in OVA/OVA compared with OVA/PBS lung homogenates ($P < 0.05$; Fig. 2). Although the expression of many proteins involved in L-arginine metabolism was altered in this model, the increase in ARG1 expression was the largest in magnitude, similar to the samples from asthma patients.

Immunohistochemistry was performed to assess the potential for increased expression of arginase to lead to substrate limitation for NOS by coexpression in the same cells or tissues within the lungs. ARG1 was visibly increased throughout the lungs, and strong-positive staining was observed in infiltrating inflammatory cells, bronchiolar and alveolar macrophages, and the peribronchiolar regions in the OVA/OVA mice (Fig. 3A). Increased ARG1 expression was also detected at the apical surface of ciliated airway epithelial cells compared with the respective OVA/PBS controls (Fig. 3A). Diffuse ARG2 staining was detected in lung tissues from OVA/OVA and OVA/PBS mice in the acute model, with more prominent staining in areas of inflammation in the lungs from the OVA/OVA mice (Fig. 3B). Immunohistochemical staining for NOS1 revealed expression in airway epithelial cells (Fig. 3C). The results of immunohistochemical staining in the acute model revealed coexpression of ARG1 and NOS1 in airway epithelial cells in this model, indicating the potential for increased ARG1 expression to result in substrate deficiency for NOS in the same cell populations within the lung.

Finally, we assessed the effect of the nebulized arginase inhibitor BEC on airway responsiveness to methacholine in

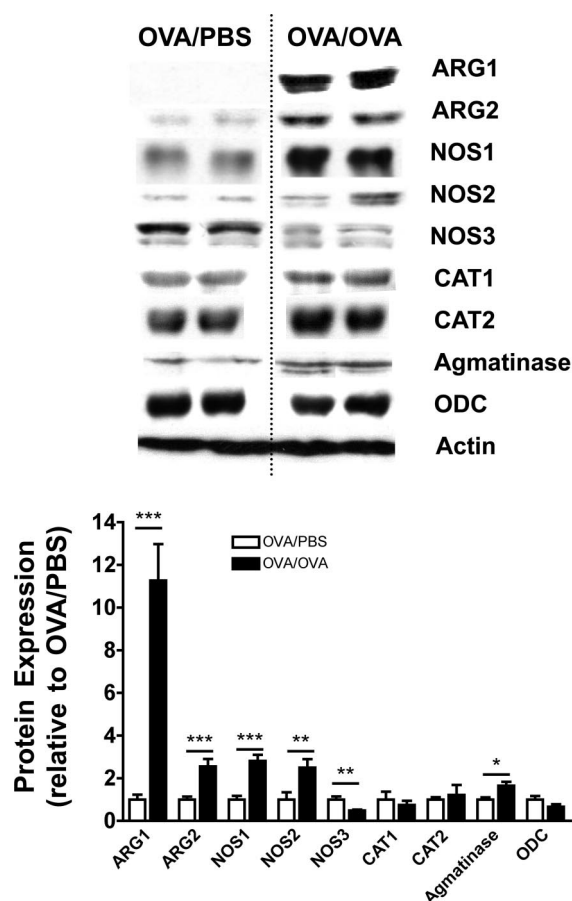


Fig. 2. Expression of arginine-related proteins in the acute murine model of allergic airway inflammation. Western blotting was used to determine ARG1, ARG2, NOS2, NOS3, CAT1, CAT2, agmatinase, and ODC expression in murine lung homogenates from the acute model, which consisted of a 3-wk ovalbumin (OVA)-sensitization and -challenge protocol. Immunoprecipitation was performed to assess expression of NOS1 and agmatinase. Fold change in OVA/OVA mice was expressed relative to the OVA/PBS group. Western blot for ARG1 and ARG2 demonstrated significant upregulation in OVA/OVA compared with OVA/PBS mice. NOS1, NOS2, and agmatinase also exhibited significantly increased expression in OVA/OVA mice. NOS3 expression was significantly attenuated in OVA/OVA relative to OVA/PBS mice. Values are means $\pm$ SE ($n = 6$ –9/group). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

this model to predict the functional consequences of the increased ARG1 expression observed in the samples from asthma patients. Interestingly, responsiveness of the central airways to methacholine (R_{Nmax}) was significantly attenuated after treatment with nebulized BEC ($P < 0.05$; Fig. 4A). The maximum peripheral tissue damping/resistance (G_{max}) and peripheral tissue elastance (H_{max}) were unaffected by BEC in the acute OVA model (Fig. 4, B and C). The decreased AHR in the central airways in this model supports the notion that increased arginase expression and activity in asthma are directly relevant to airway function.

Protein expression, localization, and arginase inhibition in the chronic murine model of allergic airway inflammation. Although the acute murine model was similar to the protein expression profile of human asthma, in that the protein with the largest observed alteration in expression was ARG1, the acute model also exhibited some changes in protein expression that were not detected in the human specimens. Specifically, the

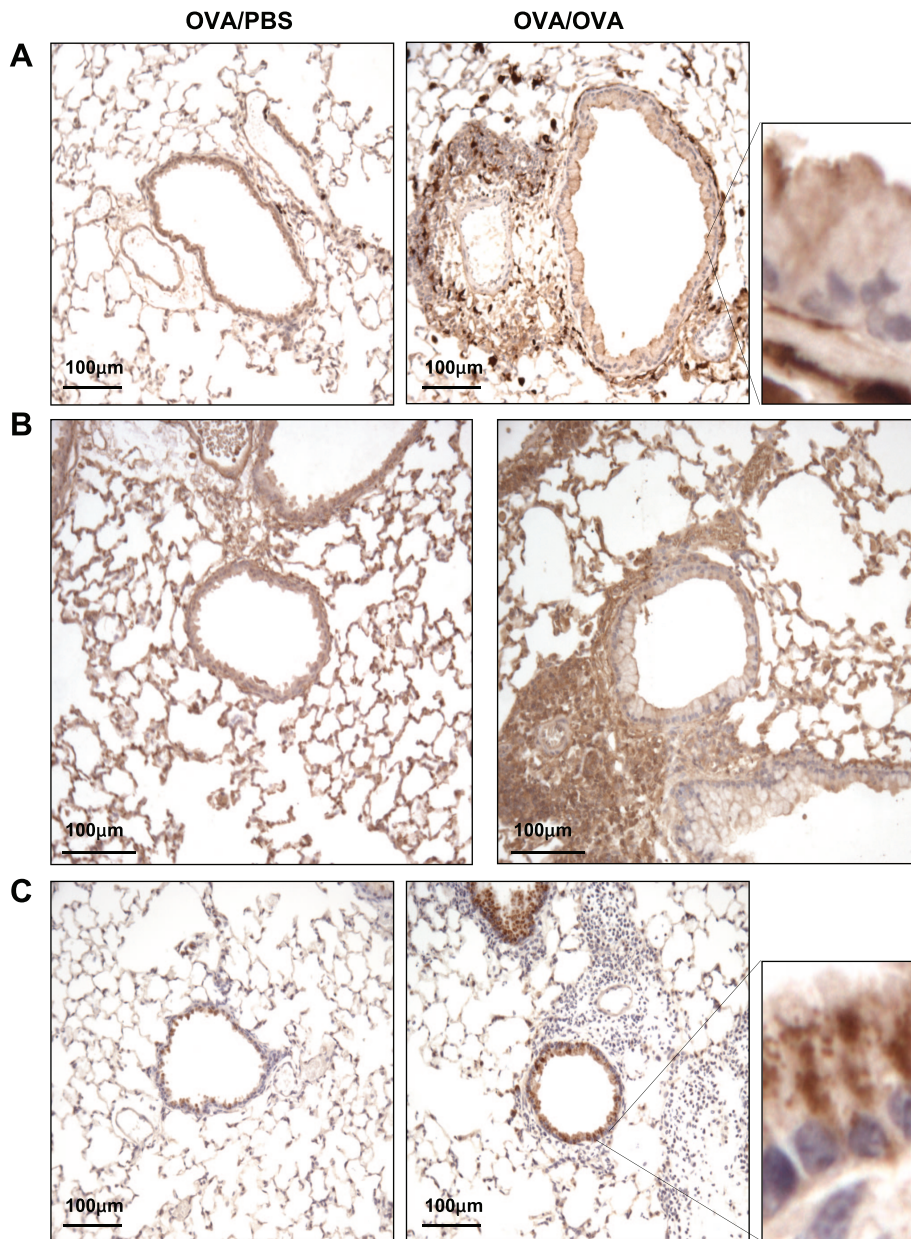


Fig. 3. Tissue localization of altered protein expression determined by immunohistochemistry in the acute model of allergic airway inflammation. *A*: increased ARG1 staining in inflammatory cells, epithelial cells, and peribronchiolar region in OVA/OVA and OVA/PBS (control) mice. *B* and *C*: increased ARG2 staining in areas of inflammation (*B*) and increased epithelial NOS1 staining (*C*) in OVA/OVA and OVA/PBS mice. Photomicrographs are representative of sections from 4 mice per group. Magnification $\times 200$.

increased ARG2, NOS1, and NOS2 expression and decreased NOS3 expression might accentuate the functional relevance of increased ARG1 expression in human asthma. Thus we employed a chronic murine model of allergic airway inflammation to determine whether a less severe, but prolonged, challenge phase would result in an expression profile of L-arginine-related proteins that was more similar to that of human asthma. This chronic model system was then used to test the functional relevance of the increased ARG1 expression to AHR.

Similar to the specimens from asthma patients, ARG1 expression was increased 26-fold in the chronic model ($P < 0.001$; Fig. 5). Total arginase activity was increased significantly (5.3 ± 1.1 and 405.1 ± 63.3 pmol urea $\cdot$ min $^{-1} \cdot$ μ g protein $^{-1}$ for OVA/PBS and OVA/OVA, respectively, $P < 0.0001$). However, no significant alterations of ARG2, NOS2, or NOS3 expression were detected by Western blotting (Fig. 5). No differences in NOS1 or arginase expression were

detected by immunoprecipitation. Furthermore, there were no significant changes in the expression of CAT1, CAT2, or ornithine decarboxylase between OVA/OVA and OVA/PBS mice in the chronic model. The sole augmentation of ARG1 in the expression profile of L-arginine-related proteins in the chronic murine model was very similar to that observed in human asthma, suggesting that this model would be suitable for examination of the functional relevance of increased ARG1 expression.

Immunohistochemical staining demonstrated increased ARG1 expression in OVA/OVA mice in the chronic model at the apical surface of ciliated airway epithelial cells compared with their respective OVA/PBS controls, similar to the acute model (Fig. 6A). Immunohistochemical staining for NOS1 also revealed expression in airway epithelial cells (Fig. 6B) that was not modified by OVA challenge. Thus ARG1 augmentation was the only significant alteration in protein expression detected in the chronic model.

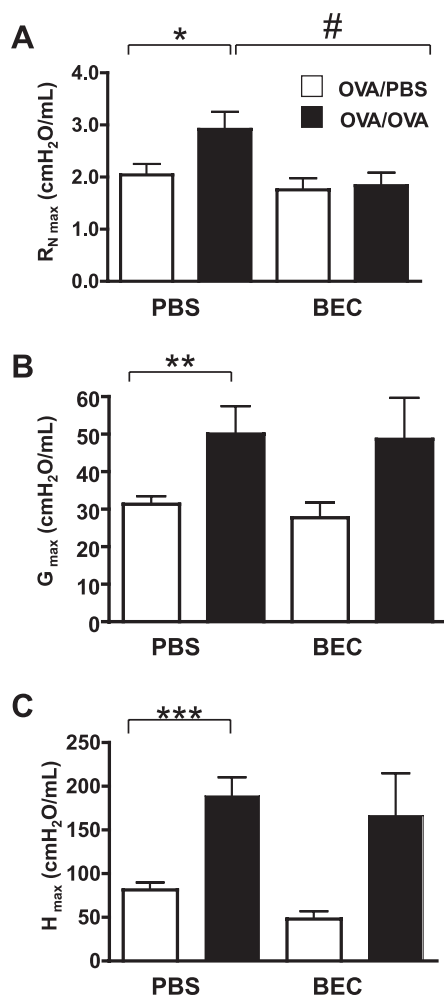


Fig. 4. Functional improvement in central airway responsiveness to methacholine with S-(2-boronoethyl)-L-cysteine (BEC) treatment in OVA/OVA mice. **A**: maximum response of central airway resistance (R_{Nmax}) was attenuated in OVA/OVA mice treated with nebulized BEC compared with PBS-treated controls. **B** and **C**: no significant changes in peripheral tissue resistance (G_{max}) or peripheral tissue elastance (H_{max}). Values are means $\pm$ SE ($n = 8-14$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, OVA/PBS vs. OVA/OVA, with the same drug treatment/control. # $P < 0.05$, PBS vs. BEC, with the same challenge group.

After determining that the chronic murine model of allergic airway inflammation closely mimicked the protein expression profile of human asthma, we investigated the functional relevance of the augmented ARG1 expression by administering an arginase inhibitor in vivo. Responsiveness to methacholine was significantly attenuated after treatment with nebulized BEC, as determined by decreased peak response of the central airway R_{Nmax} , G_{max} , and H_{max} ($P < 0.01$, 0.05 , and 0.001 , respectively) and shown in Fig. 7. Thus, in this chronic model, a model system that effectively mimics the expression of L-arginine-related proteins in lung samples from asthma patients, inhibition of arginase resulted in reduction of the central and peripheral airway responsiveness to methacholine.

DISCUSSION

Summary. In this study, we examined the expression of the arginase and NOS isoforms and other proteins related to

L-arginine uptake and metabolism in lung specimens from asthma patients and nonasthmatic control subjects. We found that ARG1 alone was significantly upregulated in lungs from asthma patients. To determine the functional relevance of dysregulation of L-arginine metabolism due to increased expression of ARG1 to AHR, we then investigated the effects of arginase inhibition in two murine models of OVA-induced allergic airway inflammation. In the acute murine model, in which expression of all arginase and NOS isozymes, as well as agmatinase, was significantly altered, arginase inhibition resulted in reduced AHR to methacholine in the central, but not peripheral, airways. In the chronic murine model, the expression profile of L-arginine-related proteins more closely mimicked those observed in human asthma, with only ARG1 being significantly upregulated. Nebulized arginase inhibitor in this model resulted in attenuation of central and peripheral AHR to methacholine. These findings support the potential for pharmacological inhibition of arginase, specifically inhibition of ARG1, to reduce AHR in asthma.

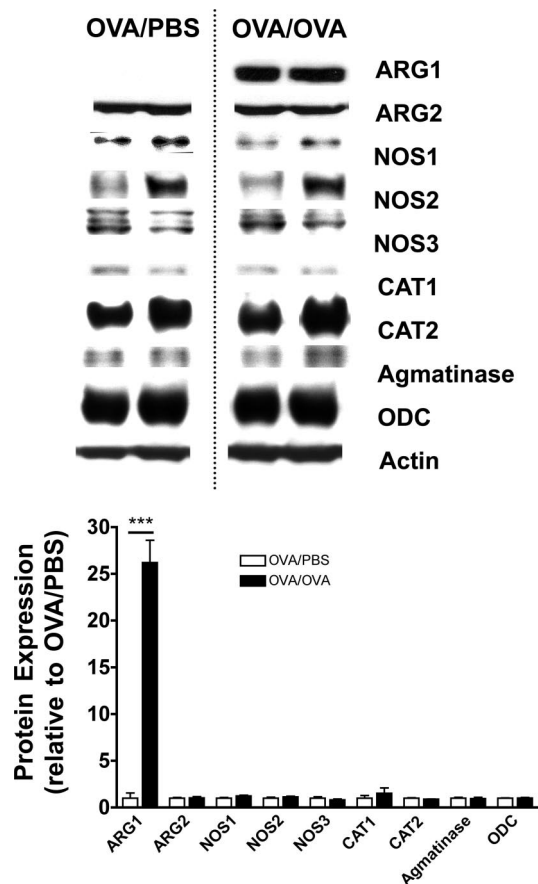


Fig. 5. Expression of arginine-related proteins in the chronic murine model of allergic airway inflammation. Western blotting was used to determine expression of ARG1, ARG2, NOS2, NOS3, CAT1, CAT2, and ODC in murine lung homogenates from the chronic model, and expression was normalized to actin as a loading control. Immunoprecipitation was performed to assess expression of NOS1 and agmatinase. Fold change in OVA/OVA mice is expressed relative to OVA/PBS group. Western blotting for ARG1 demonstrated significantly increased expression in lung homogenates from OVA/OVA mice compared with OVA/PBS controls in the chronic model. ARG2, NOS1, NOS2, NOS3, CAT1, CAT2, agmatinase, and ODC expression was not significantly different between groups. Values are means $\pm$ SE ($n = 6-14$ /group). *** $P < 0.001$.

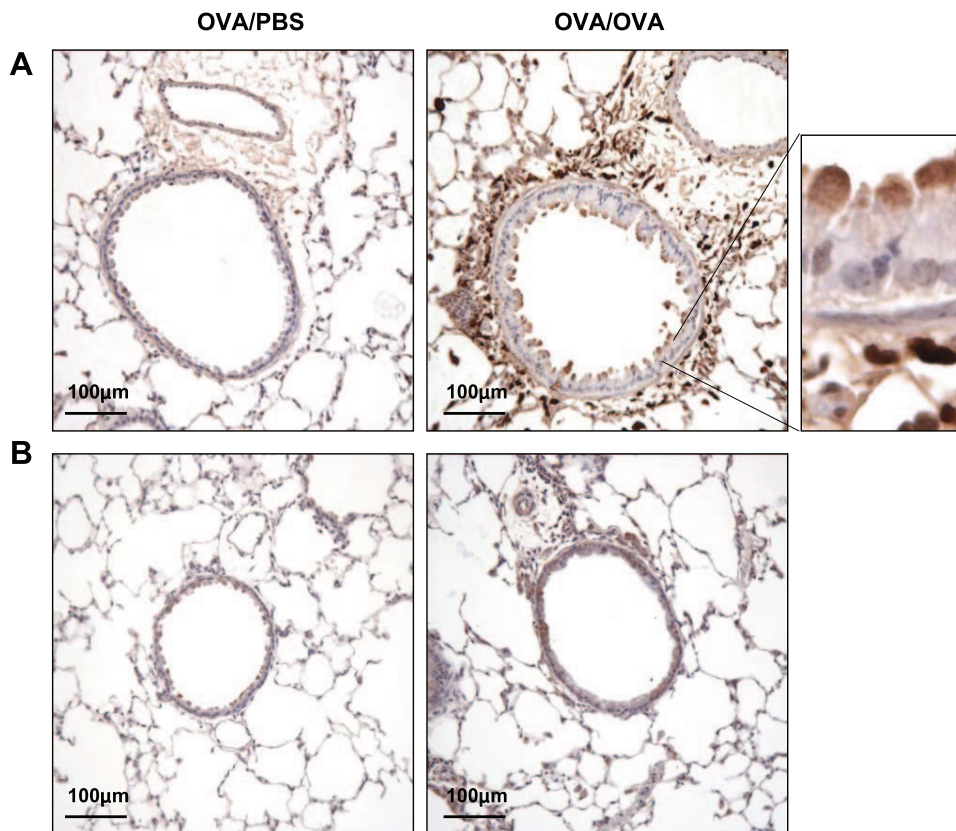


Fig. 6. Tissue localization of altered protein expression determined by immunohistochemistry in the chronic model of allergic airway inflammation. A: increased ARG1 positivity in inflammatory cells, epithelial cells, and throughout the peribronchiolar region in chronic OVA/OVA mice. B: NOS1 staining in airway epithelia in chronic model mice. Photomicrographs are representative of sections from 4 mice in each group. Magnification $\times 200$.

Expression of proteins relevant to L-arginine metabolism in asthma. Although genetic linkage and association studies have provided compelling evidence suggesting role(s) for the NOS and arginase isozymes in asthma (10, 11, 13, 25, 40), the specific contributions of imbalances in L-arginine metabolism have only recently become appreciated (23, 26, 32, 39). Morris et al. (32) reported decreased L-arginine bioavailability and increased serum arginase activity in pediatric asthma patients. In a subgroup of patients who were admitted to hospital with respiratory distress and status asthmaticus, these investigators also reported increased arginine bioavailability and reduced serum arginase activity at discharge (32). Lara et al. (23) recently described increased bioavailability in plasma, increased L-arginine catabolism, and a direct relationship between L-arginine bioavailability and airflow obstruction in severe asthma. These reports support the delicate balance in L-arginine metabolism in asthma. The data reported here build on these previous findings and provide new information regarding the expression of a panel of proteins related to L-arginine metabolism in lung tissue from asthma patients.

The absence of a significant increase in ARG2 in human tissues in this study also demonstrated, for the first time, that the increased arginase activity in human asthma was solely attributable to changes in ARG1 expression. Consistent with previous studies (14), we observed a trend toward increased NOS2 expression in our asthma samples; however, this increase was not significant ($P = 0.1$). One caveat in the interpretation of the protein expression relates to the fact that the severity of asthma in these patients was unknown, since detailed clinical information regarding the asthma patients was not available and the sample size was limited. This may

explain the lack of a significant increase in NOS2. These findings reinforce the need for further investigation of the expression of proteins related to the arginine metabolome in a properly characterized patient population.

Although CAT2 is induced in certain murine models of asthma and mediators released from eosinophils can inhibit the function of CAT (15, 42, 43), the expression of CAT2 and CAT1 proteins has not been examined in human asthma. Uptake of L-arginine by CAT is critical to L-arginine bioavailability within the cell. Inasmuch as expression of ARG1 was significantly upregulated in the lung specimens from asthma patients compared with nonasthmatic controls, in the absence of augmented expression of CAT1 or CAT2, our results support the potential for decreased intracellular bioavailability of L-arginine for NOS in asthmatic airways.

L-Arginine metabolism and arginase inhibition in an acute murine model of allergic airway inflammation. Mouse models can be useful for investigation of specific mechanisms related to disease pathology (34). However, one of the limitations in their utility is that mice do not develop asthma (35, 41). Thus allergen-induced airway inflammation to otherwise innocuous agents (i.e., OVA) has been used to elicit augmented AHR, increased IgE levels, goblet cell metaplasia, mucus production, and airway remodeling as surrogates of the asthma phenotype (8, 35, 37). Furthermore, these models can be subacute, acute, or chronic, to elicit different specific asthma phenotypes (i.e., greater AHR, inflammatory cell infiltration/activation, or remodeling) (18). To investigate the functional importance of the increase in ARG1 protein expression detected in the human samples, we employed a commonly used acute murine model of OVA-induced allergic airway inflammation as a first step.

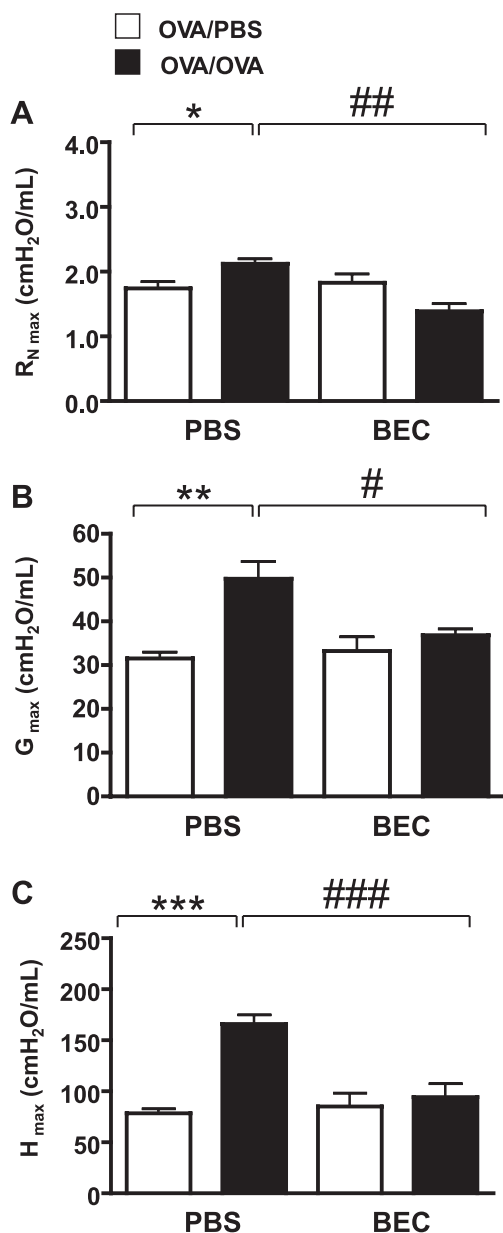


Fig. 7. Functional improvement in airway responsiveness to methacholine with BEC treatment in the chronic model of allergic airway inflammation. Maximum central airway resistance (R_{Nmax} ; A), peripheral tissue resistance (G_{max} ; B), and peripheral tissue elastance (H_{max} ; C) in OVA/OVA mice were attenuated with nebulized BEC treatment relative to control. Values are means $\pm$ SE ($n = 8-14$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, OVA/OVA vs. OVA/PBS, with the same drug treatment/control. # $P < 0.05$; ### $P < 0.01$; #### $P < 0.001$, PBS vs. BEC, with the same challenge group.

In the acute (3-wk) OVA model, ARG1 protein expression was increased, consistent with previous studies in mice (12, 20, 36, 42). The concomitant expression of NOS1 and arginase in airway epithelial cells supports the hypothesis that augmented arginase activity has the potential to decrease the local bioavailability of L-arginine for NOS1 in these cell types (5, 36). Indeed, previous studies in guinea pigs have suggested that arginase activity can affect airway tone through increased competition with NOS1 in homeostasis and under conditions of allergic inflammation (27, 29, 30). Expression of ARG1 in the

airway epithelium is also consistent with studies in humans, inasmuch as increased ARG1 mRNA expression has been described in these cell types and in areas of inflammation in asthma patients (42). ARG1 protein has also been demonstrated in the airway epithelia in human asthma by immunohistochemistry (3). However, the *in vivo* effects of arginase inhibitors on pulmonary function have only recently been described in guinea pigs by Maarsingh et al. (29), who demonstrated that the arginase inhibitor 2(*S*)-amino-6-boronohexanoic acid reduced AHR after the early and late allergic response. Decreased responsiveness to methacholine has also been observed after arginase inhibition in a murine model (4, 5). In this study, using a treatment-based administration protocol and the mouse as a model system, we inhibited arginase to examine the functional relevance of increased ARG1 protein expression in asthma. We found that acute arginase inhibition reduced central airway responsiveness to methacholine in the acute murine model, supporting the notion that increased ARG1 expression and arginase activity likely play a functional role in AHR.

Our observation of augmented ARG2 expression in the acute murine model is consistent with previous findings obtained in similarly acute models using gene expression microarray (42) and Western blotting (20). However, ARG2 protein expression was not upregulated in the chronic murine model or the human asthma specimens. Although genetic studies have demonstrated association of polymorphisms of ARG2 with the diagnosis and severity of asthma in children (25) and have identified chromosome 14q24 as a major susceptibility gene for asthma (13), the present findings do not support a functional role for ARG2 in the AHR that develops in the chronic model of asthma, nor do they support a role for ARG2 in human asthma. Although Zimmermann et al. (42) reported upregulation of ARG1 in human asthma, they did not report ARG2 expression in their patient population. Thus we speculate that the augmented ARG2 expression is related to the acute inflammatory response in the acute murine model and does not play a role in chronic allergic airway inflammation.

Many of the proteins were up- or downregulated in the acute model, likely indicative of the severity of the inflammation induced by repeated exposure to OVA for 7 consecutive days. One interesting alteration was the increase in agmatinase in the OVA/OVA lungs from this model. Agmatinase is synthesized from L-arginine by arginine decarboxylase and is subsequently catabolized by agmatinase, which is part of the arginase superfamily (19). Agmatinase has been implicated in the inflammatory response and may be involved as a temporal switch between the generation of NO and the induction of arginase (33). Thus the alterations in protein expression that were observed only in the acute model might be related to the time course of the acute inflammatory response.

L-Arginine metabolism and arginase inhibition in a chronic murine model of allergic airway inflammation. Interestingly, in the chronic murine model of experimental asthma, ARG1 was the sole significantly upregulated isozyme; this expression profile was more similar to that of samples from asthma patients. The chronic model is typically used for investigations of airway remodeling (18, 21). However, we also found it useful for modeling the more subtle functional changes in L-arginine metabolism associated with chronic disease.

Localization of ARG1 in the peribronchiolar regions was consistent with the features of remodeling, such as collagen deposition, that are typical of this model (7, 18, 21). Additionally, coexpression of ARG1 and NOS1 in the airway epithelia supports the hypothesis that competition for L-arginine substrate is increased in allergic asthma.

The finding that the responsiveness of the peripheral lung and airways to methacholine was attenuated in the chronic, but not the acute, model of allergic airway inflammation is intriguing. This difference may be due to the severity of the acute model and the altered expression of L-arginine-related proteins described above. Chronic murine models of allergic airway inflammation similar to that described here have been shown to exhibit features of remodeling and sustained airway dysfunction (7, 18, 21). Despite the presence of airway remodeling, arginase inhibition was effective in significantly reducing central and peripheral AHR to methacholine in the chronic model. These findings from the chronic model provide strong support for the importance of ARG1 upregulation in asthma. Inasmuch as ARG2 was not increased in this model, the effects of arginase inhibition on central and peripheral airway responsiveness in this study were due solely to inhibition of ARG1. The reduction in methacholine responsiveness after in vivo treatment with BEC in both murine models supports the importance of L-arginine bioavailability in asthma. Furthermore, the concordance between the findings of augmented ARG1 expression in asthma patients and in the murine and guinea pig models of asthma supports the potential for arginase as a therapeutic target in human asthma.

Conclusions. Arginine metabolism contributes to the maintenance of homeostasis in the respiratory system, and imbalances can contribute to disease. This study demonstrated altered expression of ARG1 in lung samples from asthma patients, and upregulation of ARG1 was consistently observed in the acute and chronic murine models of allergic airway inflammation. The expression of other proteins related to L-arginine metabolism (e.g., NOS isozymes and transporters) was also determined. Colocalization of ARG1 and NOS1 was demonstrated in the airway epithelia in both murine models and supports the direct competition for substrate in these cell types. Finally, arginase inhibition in vivo attenuated airway responsiveness to methacholine in acute and chronic murine models of allergen-induced airway inflammation. These results support the therapeutic potential of specific arginase inhibitors as a novel treatment for asthma and suggest that ARG1 should be targeted specifically.

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