

Increased Expression of Arginase I and II in Allergic Nasal Mucosa

Woo Sung Cho, MD; Tae Hoon Kim, MD; Ki Hyoung Kim, MD; Heung Man Lee, MD;
Seung Hoon Lee, MD; Young Ho Ju, MD; Euy Hyun Park, MD; Kang Woo Kim, MD; Sang Hag Lee, MD

Objectives/Hypothesis: It is known that arginase may be a regulator of diverse pathways, including production of nitric oxide (NO). Increased expression of arginase has been reported in several inflammatory lung diseases, including allergic asthma, suggesting that this may be a common feature underlying the pathophysiology of airway hyperreactivity. Thus, arginase I and II may play a role in the pathogenesis of allergic rhinitis. The distribution pattern and level of expression of arginase I and II were therefore determined in normal and allergic nasal mucosa.

Study Design: Controlled, prospective study.

Methods: The distribution pattern and level of expression of arginase I and II in normal and allergic nasal mucosa were evaluated using RT-PCR, immunohistochemistry, and Western blotting.

Results: The level of expression of arginase I and II mRNA was increased in allergic nasal mucosa in comparison with normal nasal mucosa. In normal nasal mucosa, arginase I and II were expressed in the surface epithelium, submucosal glands, vascular endothelium, and fibroblasts. In allergic nasal mucosa, both enzymes were also localized to similar sites, in addition to inflammatory cells, and the level of expression were greatly increased compared with normal nasal mucosa. These findings were verified by Western blotting.

Conclusions: These results indicate that arginase I and II may play a role in the pathophysiology of allergic rhinitis, and suggest the possible role of the L-arginine metabolic pathway through modulation of L-arginine availability as a substrate for nitric oxide synthase (NOS) and arginase in the pathogenesis of allergic rhinitis.

Key Words: Arginase I, arginase II, allergic nasal mucosa.

Level of Evidence: 5

Laryngoscope, 121:236–240, 2011

INTRODUCTION

Allergic rhinitis is a chronic inflammatory disorder of the nasal airways, characterized by allergen-induced, IgE-mediated early and late nasal obstructive reactions, increased nasal hyperresponsiveness, and infiltration of inflammatory cells into the nasal mucosa, particularly eosinophils and Th2 lymphocytes.¹ All of these changes can be induced by a cascade of inflammatory reactions involving various mediators, including nitric oxide (NO). High levels of NO have been found in the nasal airways and paranasal sinuses compared with the lower airways in normal subjects, indicating that NO contributes to physiological processes in the upper airways.² An increased nasal NO level has been reported in patients

with allergic rhinitis, although the different results have also been obtained.³

NO is synthesized from L-arginine and oxygen as a result of the process catalyzed by the action of several forms of nitric oxide synthesis. In this process, arginase can regulate NOS by controlling the bioavailability of L-arginine to NOS.^{4–6} Arginase exists as two distinct isoenzymes: arginase I and II. Arginase I is a cytosolic enzyme and is the prominent isoform in the liver, where it is highly expressed. Arginase II is mainly expressed in extrahepatic tissues.^{4–6} Upregulation of arginase expression has also been shown to contribute to the pathophysiology of various diseases, which exhibit dysregulated NO signaling. Further, increased arginase induction has been observed in a variety of animal models of asthma and human asthma, suggesting that increased arginase activity in allergic asthma could also increase airway inflammation and airway hyperresponsiveness by increasing the production of inflammatory cytokines.^{4–6}

Thus, an altered level of expression of arginase may play an important role in the pathogenesis of allergic rhinitis. Nevertheless, the expression of arginase I and II in normal nasal mucosa and the role in the pathogenesis of allergic rhinitis have not been elucidated. The present study was undertaken to determine the levels of expression and distribution pattern of arginase I and II in normal and allergic nasal mucosa in order to evaluate the possible effects in the pathogenesis of allergic rhinitis.

From the Department of Otorhinolaryngology—Head and Neck Surgery (w.s.c.), Bundang Jeseang General Hospital, Daejin Medical Center, Seongnam, Korea; and Department of Otorhinolaryngology—Head and Neck Surgery (T.H.K., K.H.K., H.M.L., S.H.L., Y.H.J., E.H.P., K.W.K., S.H.L.), College of Medicine, Korea University, Seoul, South Korea.

Editor's Note: This Manuscript was accepted for publication August 10, 2010.

This work was supported by a Grant-in-Aid for Scientific Research from the Communication Disorders Center of Korea University, Seoul, Korea.

The authors have no financial disclosures for this article.
The authors have no conflicts of interest to disclose.

Send correspondence to Dr. Sang Hag Lee, Department of Otorhinolaryngology—Head and Neck Surgery, College of Medicine, Korea University, 126-1, 5Ka, Anam-Dong, SungBuk-Ku, Seoul, 136-705, South Korea. E-mail: sanghag@kumc.or.kr

DOI: 10.1002/lary.21288

TABLE I.
Sequences of PCR Primers Specific for Arginase I and II.

Gene	Size (bp)	Sequence
Arginase I	382	Sense: 5'-CTTGTTCGGACTTGCTCGG-3' Antisense: 5'-CACTCTATGTATGAGTTA-3'
Arginase II	229	Sense: 5'-GTTAGCAGAGCTGTGTCAGA-3' Antisense: 5'-GGAGTTGTGGTACCTTATCC-3'
GAPDH	502	Sense: 5'-ATCTTCCAGGAGCGA GATCC-3' Antisense: 5'-ACCACTGACACGTTGGCAGT-3'

Annealing temperature = 57°C.

PCR = polymerase chain reaction; GAPDH = glyceraldehydes-3-phosphate dehydrogenase.

MATERIALS AND METHODS

Tissue Samples and Preparation

Thirty-six normal nasal mucosa specimens were obtained from the inferior turbinates of 36 patients (17 males, 19 females: age range, 22–35 years) who had a normal nasal mucosa and were admitted for augmentation rhinoplasty. The patients had no history of nasal infections or allergy, smoking, or ongoing drug treatment. Allergic nasal mucosa was obtained from 36 patients (19 males, 17 females: age range, 20–39 years) with perennial allergic rhinitis during septoturbinate surgery who had typical symptoms of perennial nasal allergy, nasal eosinophilia, and a positive skin test or positive radioallergen sorbent test to house dust mites. The turbinate mucosa had an edematous and congested appearance. Before obtaining the tissue specimens, the institutional review boards for human beings at our institution approved the protocols and informed consent form.

Tissues (normal nasal mucosa [$n = 18$] and allergic nasal mucosa [$n = 18$]) were cut into two portions; one portion was dissected, frozen in liquid nitrogen, and stored at -70°C for subsequent RNA isolation, and the other portion was used for protein isolation. For immunohistochemical analysis, samples (normal mucosa [$n = 18$] and allergic mucosa [$n = 18$]) were immersed overnight in a freshly prepared fixative containing 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). All samples were then dehydrated in a graded series of ethanol to xylene and embedded in paraffin wax.

Total RNA Isolation, Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), and Semiquantitative RT-PCR

Total RNA (1 μg) from each sample was reverse-transcribed in 20 μL of a reaction mixture containing 2.5 U of Moloney murine leukemia virus reverse transcriptase (RT; Gibco BRL, Grand Island, NY, USA) and 50 pmol of random hexanucleotides at 42°C for 60 minutes. RNA integrity and the success of the reverse-transcription reaction were monitored by PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts. Negative controls consisted of omission of the RT enzyme from the cDNA synthesis for each specimen. The primer sequences of each gene used in the study are listed in Table I. Amplified PCR products were resolved in 2% agarose gels, stained with ethidium bromide, and photographed under ultraviolet light. The identity of each PCR product was confirmed by sequencing and found to be identical to the mRNA sequence of each gene as deposited in the National Center for Biotechnology Information database.

Semiquantitative RT-PCR was performed on the samples to assess the differential expression of each gene mRNA in nor-

mal and allergic nasal mucosa. The optimum number of PCR cycles for each cDNA species was determined by plotting the PCR product yield of different cycles on a semilogarithmic graph and the cycle number representing exponential amplification was chosen for the final amplification. For quantification of the PCR products of all samples, the expected bands were analyzed by densitometry and the data are expressed as the ratio of each gene to the corresponding GAPDH cDNA. The data are presented as the mean $\pm$ SD. The statistical significance of differences between groups was evaluated using the Mann-Whitney U test, and the level of significance was set at $P < .05$.

Immunohistochemical and Western Blot Analysis

Immunohistochemical staining was performed using a peroxidase-labeled streptavidin-biotin technique. Briefly, paraffin sections (4 μm) were cut and placed on poly-L-lysine-coated glass slides. The sections were treated with 0.03% H_2O_2 in methanol for 15 minutes to quench endogenous peroxidase activity. After washing in PBS, the sections were then incubated overnight at 4°C with a 1:100 dilution of antiarginase I antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or a 1:100 dilution of antiarginase II antibody (Santa Cruz Biotechnology, Inc.). The color was developed using 3,3'-diaminobenzidine. The negative immunohistochemical control procedure included omission of the primary antibodies and replacement of the primary antibodies by normal rabbit IgG in appropriate concentrations. Five sections and four random fields per section were analyzed for each subject. In addition, to assess the staining intensity of arginase I and II, all tissue sections were independently examined by two different investigators. The intensity of arginase I and II expression was classified into 4 degrees as follows: (–), negative; (+), weakly positive; (++) , moderately positive; and (+++) , strongly positive. Quantitative analyses of the intensities for each patient was calculated as the mean of the total score in the tissue specimen.

For Western blot analysis, frozen tissue was crushed and vigorously vortexed in a buffer solution containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and a proteinase inhibitor cocktail. The extracted protein (50 μg) was resuspended in sodium dodecyl sulfate sample buffer and boiled for 5 minutes. Equal amounts of total protein were separated on 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to Immobilon (Millipore, Bedford, MA, USA). The blots were blocked for 1 hour at room temperature with PBS-T containing 1% skim milk, then incubated with anti-arginase I or II antibody in PBS-T overnight at room temperature. Antibody reactions were detected using the ECL detection kit (Amersham Bioscience, Piscataway, NJ, USA), followed by detection of chemiluminescence on X-ray film. As an internal control, β -actin expression was analyzed in parallel blots using β -actin antibody (Sigma, St. Louis, MO, USA). The intensity of the detected bands was quantified using Scion Image Beta 4.0.2 (Scion Corporation, Frederick, MD, USA). The relative intensities of each protein signals were obtained by dividing the intensities of each protein signal by β -actin signals. The statistical significance of differences was evaluated by the Mann-Whitney U test, and the level of significance was set at $P < .05$.

RESULTS

The expression of arginase I and II, and GAPDH mRNA was determined by RT-PCR in total RNA preparations isolated from normal and allergic nasal mucosa. As shown in Figure 1A, arginase I and II mRNAs were

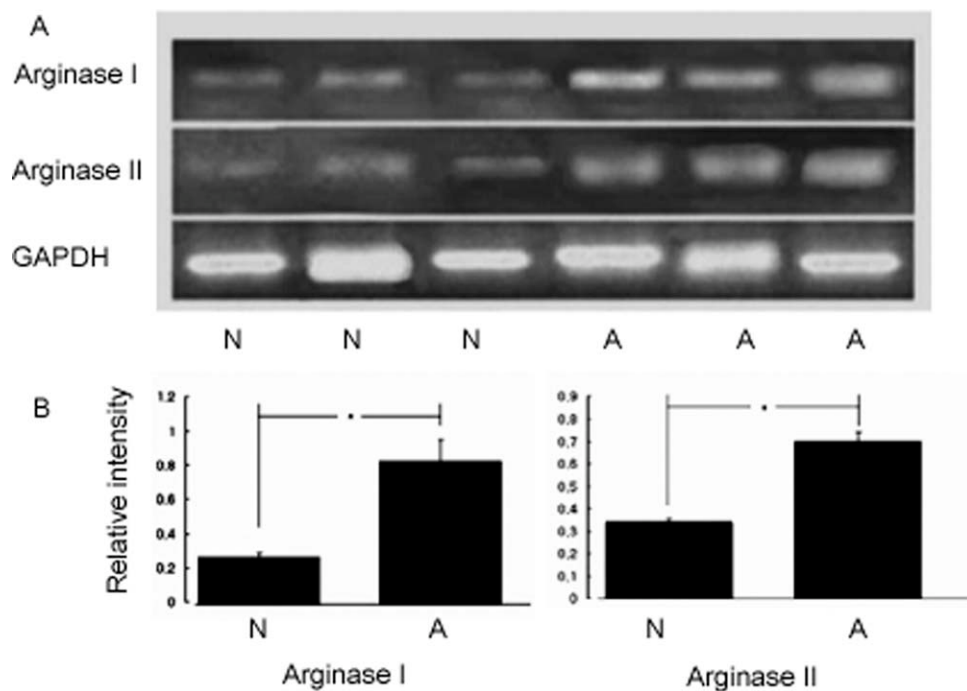


Fig. 1. (A) Semiquantitative RT-PCR analysis of arginase I and II mRNA level in normal and allergic turbinates mucosa. (B) Bands were quantified by densitometric scanning and the relative amount of each gene was calculated by dividing by the internal control (GAPDH). The bar indicates the mean $\pm$ SD. N = normal turbinates mucosa; A = allergic turbinates mucosa. *Indicates $P < .05$.

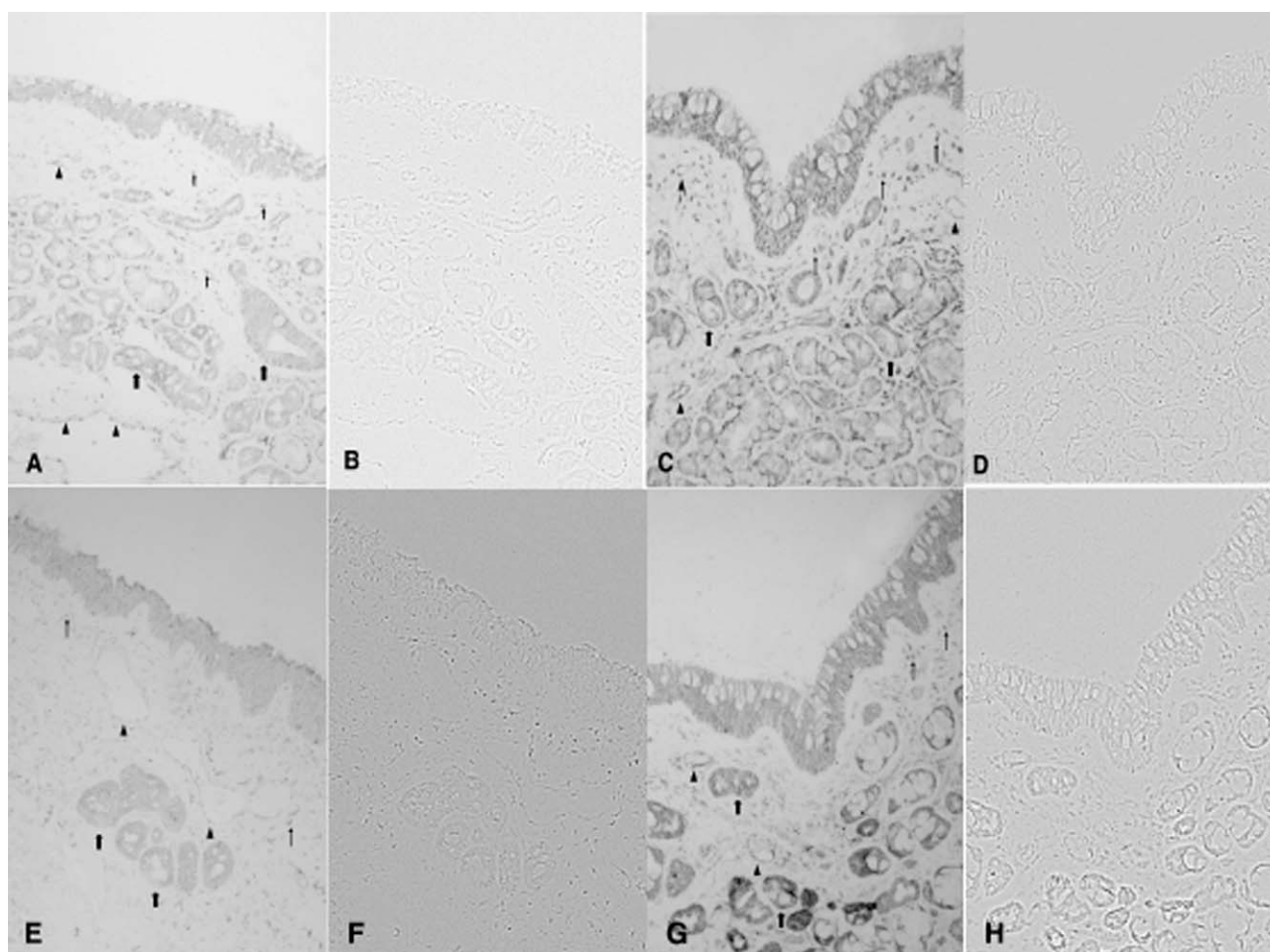


Fig. 2. Immunohistochemical localization of arginase I (A, C) and arginase II (E, G) in normal (A, B, E, and F) and allergic turbinates mucosa (C, D, G, and H). In both normal and allergic nasal mucosa, arginase I (A, C) and arginase II (E, G) were detected in surface epithelium, submucosal glands (thick arrow), vascular endothelium (arrow head), fibroblasts, and inflammatory cells (thin arrow), where intense staining was found in allergic rhinitis. Negative isotype control for arginase I (B, F) and arginase II (D, H) do not show any staining. Original magnification $\times 100$.

TABLE II.
Distribution Pattern of Arginase I and II in Normal and Allergic Nasal Mucosa.

	Normal Nasal Mucosa		Allergic Nasal Mucosa	
	Arginase I	Arginase II	Arginase I	Arginase II
Epithelium	+	+	+++	++
Submucosal gland	+	+	+++	++
Endothelium	+	+	+++	++
Fibroblast	+	+	+++	++
Inflammatory cells	+	+	+++	++

expressed in normal and allergic nasal mucosa. The PCR products of the expected size corresponding to 382 bp for arginase I, 229 bp for arginase II, and 502 bp for GAPDH were detected in normal and allergic nasal mucosa and showed 100% sequence homology with the published sequences. Negative control reactions without RT in the cDNA synthesis process showed no bands, indicating that there was no contamination by genomic DNA in the RNA samples (data not shown). The relative abundance of arginase I and II with respect to GAPDH were significantly increased in allergic nasal mucosa compared with normal nasal mucosa (Fig. 1B; $P < .05$).

Immunohistochemical staining showed a homologous pattern in all samples of nasal mucosa from normal and allergic turbinate mucosa (Fig. 2). In normal nasal mucosa, arginase I and II were localized to the superficial epithelium, submucosal glands, vascular endothelium, and fibroblasts (Fig. 2A and E). In allergic nasal mucosa, arginase I and II were also expressed in the superficial epithelium, submucosal glands, vascular endothelium, and fibroblasts (Fig. 2C and G). The staining intensity was largely strong in allergic nasal mucosa compared with normal nasal mucosa (Fig. 2 and Table II). In addition, a large number of inflammatory cells showing arginase I and II immunoreactivity were identified in allergic nasal mucosa (Fig. 2 and Table II). The

nonimmune isotype control did not show any staining (Figs. 2B, D, F, and G).

Western blot analysis with the arginase I and II antisera detected both proteins in normal and allergic turbinate mucosae (Fig. 3A). The relative abundance of arginase I and II with respect to β -actin was significantly increased in allergic turbinate mucosa compared with normal turbinate mucosa, demonstrating that the levels of expression of arginase I and II were increased in allergic turbinate mucosa in comparison with normal turbinate mucosa (Fig. 3B; $P < .05$).

DISCUSSION

In the present study, we have characterized the expression and distribution pattern of arginase I and II in normal and allergic nasal mucosa. At the level of mRNA and protein, arginase I and II was expressed in the normal and allergic nasal mucosae tested in the present study. Furthermore, our results showed that the level of expression of arginase I and II mRNA and protein in allergic nasal mucosa is increased compared with normal nasal mucosa. Findings from the immunohistochemical examination demonstrated that arginase I and II are localized to the superficial epithelium, submucosal glands, and vascular endothelium in both normal and allergic turbinate mucosa. Strongly positive staining was also observed in allergic nasal mucosa compared with normal nasal mucosa, verifying the results of Western blotting. Therefore, our data demonstrate that arginase I and II are constitutively expressed in normal human nasal mucosa and upregulated in allergic nasal mucosa, and suggest that these enzymes may play a role in the pathophysiology of allergic rhinitis. This is the first detailed analysis of the expression and distributional patterns of arginase I and II mRNA and protein in normal and allergic nasal mucosa.

The existence of arginase in various extrahepatic tissues that do not have complete urea cycle indicates that this enzyme may be functional in various

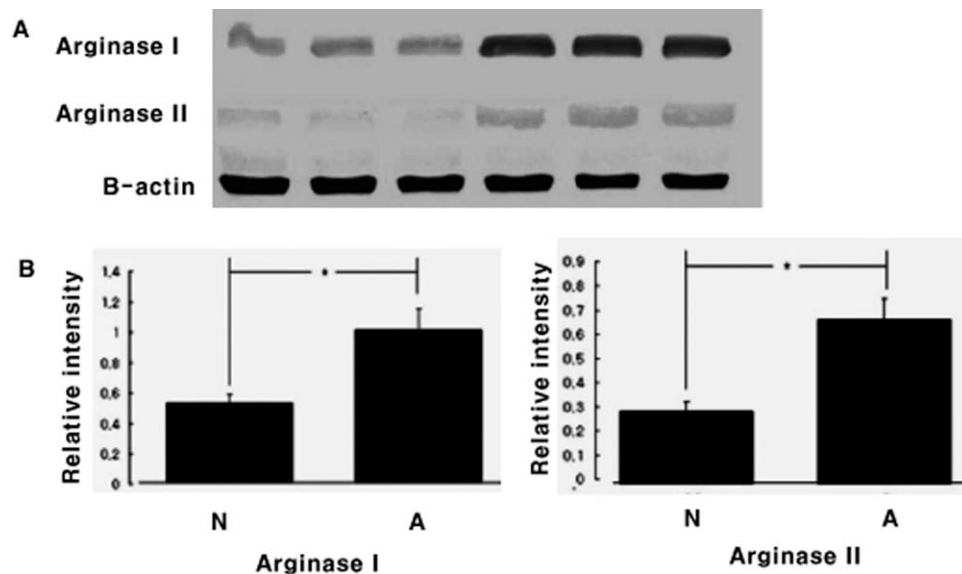


Fig. 3. (A) Western blot analysis of arginase I and II in normal and allergic nasal mucosa. (B) Bands were quantified by densitometric scanning and the relative amount of each gene was calculated by dividing by the internal control (β -actin). The bar indicates the mean $\pm$ SD. N = normal turbinate mucosa; A = allergic turbinate mucosa. *Indicates $P < .05$.

physiological processes in many organs.⁵ However, the physiologic role of the arginase in normal human nasal mucosa has not been elucidated. Based upon existing literature, arginine is utilized by NOS for NO synthesis, forming citrulline as a coproduct. Arginine is also metabolized to urea and ornithine by arginase.⁴ Because both arginase and NOS utilize L-arginine as a common substrate, elevated levels of arginase activity can inhibit NO production via substrate limitation.⁴ A similar effect of arginase in the inhibition of normal function of NOS has been shown in many other diseases.⁷ Various studies have explored the complex interrelationships between the components of the L-arginine-NO pathway in the pathogenesis of allergic rhinitis and chronic rhinosinusitis.⁸ Nasal NO has been reported to be decreased in acute and chronic rhinosinusitis, nasal polyposis, and primary ciliary dyskinesia, and normal or increased in allergic rhinitis.² Korn et al.⁸ reported that following a nasal allergen challenge, the level of nasal NO decreases more than that of exhaled NO in the early phase response, but in the late phase response the expired NO level decreases. Based on these results, it is reasonable to speculate that decreased NO in various sinunasal diseases may be related to data showing that arginase may compete with NOS for arginine and downregulate NO production. Therefore, NOS/arginase balance may contribute to the maintenance of homeostasis in normal human nasal mucosa, including the regulation of blood flow and nasal secretion, and the imbalance can contribute to the development of human sinunasal diseases. The functional role for constitutively expressed arginase in the airways has been established in guinea pig tracheal preparations using the specific arginase inhibitor N^ω-hydroxy-nor-L-arginine, providing experimental evidence that arginase is involved in the control of airway responsiveness through attenuation of substrate availability to NOS.^{9,10} Further studies using specific inhibitors are required to better understand the role of the arginases in normal human nasal mucosa.

A high level of NO has been found in nasal airways and paranasal sinuses compared with the lower airways in normal subjects, indicating that NO contributes to pathophysiologic processes in the upper airways.³ As previously reported, nasal NO may be produced from L-arginine by any of the three NOS isoforms.^{4,5} Thus, production of nasal NO may be regulated by the amounts of NOS enzyme available, and by the availability of the substrate for NOS, L-arginine.^{4,5} Therefore, it has been suggested that any biochemical perturbation in the L-arginine:NO pathway may affect the pathogenesis of allergic rhinitis.³ Several studies have attempted to assess nasal NO levels in allergic rhinitis; however, the results have been conflicting.³ Several studies have demonstrated differences in the expression of NOS and NO metabolites in human nasal epithelial cells between normal subjects and patients with allergic rhinitis.³ However, the L-arginine–L-ornithine pathway regulated

by arginase has not been evaluated in allergic rhinitis. Because arginase and NOS have L-arginine as a common substrate as mentioned above, the two enzymes compete for the same substrate, and reciprocal regulation of both L-arginine metabolic pathway has been shown.^{4,5} Due to the potential of arginase isoforms for affecting the L-arginine availability for NO synthesis, there has been increased interest in arginase expression in various diseases, including asthma.⁵ In the present study, we found that the expression of both arginase isoforms is increased in allergic rhinitis compared with normal human nasal mucosa. Based on our results and the published literature, it is hypothesized that increased expression of arginase in allergic human nasal mucosa suggests a possible role of the L-arginine–L-ornithine pathway in the pathogenesis of allergic rhinitis through competition for the common L-arginine substrate. This suggestion is supported by the results that the depletion of NO caused by arginase contributes to airway hyperreactivity, and direct administration of an arginase inhibitor to the airway may be beneficial and useful in treating asthma.¹¹

CONCLUSION

The present study showed that two functional arginase isoforms, arginase I and II are constitutively expressed in normal human nasal mucosa, and the level of expression is increased in allergic nasal mucosa. Based on our results and on published data, the pathophysiology of allergic rhinitis may be associated with the L-arginine metabolic pathway through modulation of L-arginine availability as a substrate for NOS and arginase, and elevated arginase activity.

BIBLIOGRAPHY

1. Wang Y, Bai C, Li K, Adler KB, Wang X. Role of airway epithelial cells in development of asthma and allergic rhinitis. *Respir Med* 2008;102:949–955.
2. Scadding G. Nitric oxide in the airways. *Curr Opin Otolaryngol Head Neck Surg* 2007;15:258–263.
3. Maniscalco M, Sofia M, Pelaia G. Nitric oxide in upper airways inflammation diseases. *Inflamm Res* 2007;56:58–69.
4. Maarsingh H, Zaagsma J, Meurs H. Arginine homeostasis in allergic asthma. *Eur J Pharmacol* 2008;585:375–384.
5. Zimmermann N, Rothenberg ME. The arginine-arginase balance in asthma and lung inflammation. *Eur J Pharmacol* 2006;533:253–262.
6. Munder M. Arginase: an emerging key player in the mammalian immune system. *Br J Pharmacol* 2009;158:638–651.
7. Durante W, Johnson FK, Johnson RA. Arginase: a critical regulator of nitric oxide synthesis and vascular function. *Clin Exp Pharmacol Physiol* 2007;34:906–911.
8. Korn S, Beier J, Heilmann C, Kornmann O, Buhl R, Michael Beeh K. Discrepant nasal and bronchial nitric oxide kinetics during early and late phase allergic reactions. *Respir Med* 2005;99:1595–1599.
9. Meurs H, Hamer MA, Pethe S, Vandon-Le Goff S, Boucher JL, Zaagsma J. Modulation of cholinergic airway reactivity and nitric oxide production by endogenous arginase activity. *Br J Pharmacol* 2000;130:1793–1798.
10. De Boer J, Meurs H, Flendrig L, Koopal M, Zaagsma J. Role of nitric oxide and superoxide in allergen-induced airway hyperactivity after the late asthmatic reaction in guinea pigs. *Br J Pharmacol* 2001;133:1235–1242.
11. Takahashi N, Ogino K, Takemoto K, et al. Direct inhibition of arginase attenuated airway allergic reactions and inflammation in a Dermatophagoides farinae-induced NC/Nga mouse model. *Am J Physiol Lung Cell Mol Physiol* 2010; in press.