Transiently, paralleled upregulation of arginase and nitric oxide synthase and the effect of both enzymes on the pathology of asthma

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We established an experimental asthmatic model using NC/Nga mice administered intranasally with mite extract (44). The model has several features such as increases in airway hyperresponsiveness, eosinophilia, and IgE. The present study investigated the involvement of arginase, NOS, and NO using Dermatophagoides farinae (Df) extract-treated NC/Nga mice.

IN ASTHMA, NITRIC OXIDE (NO) is capable of relaxing the airway via cGMP-dependent pathways (34) and contributes to remodeling. NO has various roles in the asthmatic airway from bronchoconstriction to proinflammation. In an ex vivo study, hyperreactivity of the airway was observed on the inhibition of NOS by arginine-analog (6). However, NO can have a deleterious or harmful effect on the airway when converted to a more reactive species such as peroxynitrite (ONOO−) via a reaction of NO with superoxide anion radicals and nitrogen dioxide (NO2), oxidized from nitrite (NO−2) by the catalytic anion of peroxides (34) and contributes to remodeling. NO has various roles in the asthmatic airway from bronchoconstriction to proinflammation.
MATERIALS AND METHODS

Animals. NC/Nga mice (7-wk-old male) were obtained from Charles River Laboratories Japan (Yokohama, Japan). All mice were housed in a specific pathogen-free environment with a 12-h light and 12-h dark cycle. The mice were allowed to take water and food ad libitum. The care and handling of the animals were in accordance with the Guidelines for the Care and Use of Laboratory Animals at Kanazawa University and Okayama University, and approved by these committees.

Chemicals. Df crude extracts were purchased from COSMO BIO (Tokyo, Japan). The antibody against nitrotyrosine was purchased from Upstate (Charlottesville, VA). Antibodies against iNOS, endothelial NOS (eNOS), nNOS, arginase I, and arginase II were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Oligonucleotide primers for RT-PCR were synthesized by TAKARA BIO (Otsu, Shiga, Japan). All other chemicals were of the highest quality and commercially available.

Administration to mice. Mite crude extract from Df was administered to NC/Nga mice as previously described (44). Briefly, 100 µg of Df extract (in 50 µl of saline) was administered by intranasal instillation on 5 consecutive days (experimental day 0–4) and on day 11 (single challenge) under anesthesia with pentobarbital sodium, as shown in Fig. 1. For the control group, simply 50 µl of saline was administered. For the “nonchallenged” control group, 50 µl of saline was administered on day 11 instead of Df extract. Mice were killed on experimental days 5 and 12 to evaluate the effect of Df.

In several experiments, mice were killed on days 8, 12, and 25 to investigate the asthmatic state with regard to remodeling (Fig. 1). Serum and whole lung tissue were collected for further analysis. In some mice, bronchoalveolar lavage fluid (BALF) was collected with 1.0 ml of saline after death (44). Cells in BALF were collected by centrifugation and lysed in ISOGEN (Nippongene, Tokyo, Japan) to retrieve RNA.

RT-PCR for cytokines and NOS isoforms. Total RNA from lung tissue was isolated using ISOGEN with modifications based on the manufacturer’s instructions to strictly collect RNA. Briefly, the resultant RNA pellet was dissolved in ISOGEN again and washed with chloroform twice to remove contaminating DNA and proteins thoroughly. The RNA samples were stored at −80°C until the analyses. Reverse transcription and PCR were performed using TaKaRa RNA PCR kit AMV ver. 3.0 (TAKARA BIO) with oligo(dT) primers according to the manufacturer’s instructions. The sequences for the primers used in this study are listed in Supplemental Table S1.
respectively. Protein samples (40 mg with 1% Triton X-100 for the detection of arginase I and arginase II, NaCl, and 1 mM EDTA) containing protease inhibitor without and for 10 min, the amount of urea generated was determined by measuring the mixture was heated at 100°C for 45 min. After storage in a dark place 1,2-propanedione-2-oxime in 100% ethanol was then added, and the product was visualized by UV transilluminator. The expression of each mRNA was analyzed using the Scion Image program (Scion, Frederick, MD). The results were compared as relative values using GAPDH as an internal reference.

**Immunohistochemical and histological experiments.** Immunostaining was performed as reported previously (27). Briefly, deparaffinized lung tissue sections were incubated for 30 min in methanol containing 1% H2O2 to inactivate endogenous peroxidase. After three washes in Tris-buffered saline for 5 min, sections were incubated in 5% normal goat serum. The specimens were incubated overnight at 4°C with polyclonal antibodies against arginase (1:10), eNOS (1:500), iNOS (1:500), nNOS (1:100), or nitrotyrosine (1:800). Then specimens were treated with goat anti-rabbit immunoglobulin conjugated with peroxidase-labeled dextran polymer (Dako) for 1 h at room temperature. Visualization was performed with 3,3-diaminobenzidine tetrahydrochloride (Dako) as a substrate, and counterstaining was carried out with hematoxylin. As a negative control, rabbit nonimmune immunoglobulin (Dako) was used. The specificity of staining for nitrotyrosine was verified by preincubation of the anti-nitrotyrosine antibody with 10 mM nitrotyrosine or by incubation of tissue sections with 100 mM sodium dithionite to convert nitrotyrosine to aminotyrosine. To observe the deposition of collagen in the interstitial space and goblet cell hyperplasia in the epithelium of bronchioles, lung sections were stained with Azan and the periodic acid-Schiff (PAS) method. The proportions of collagen fibers in the peribronchiolar region and goblet cells in the bronchial epithelium were digitized by ImagePro software (Media Cybernetics, Bethesda, MD).

**Determination of arginase activity.** Arginase activity in lung homogenate was determined by methods described previously (5). Briefly, lung tissue homogenate (25 µg of protein) was adjusted to a volume of 50 µl with 20 mM Tris·HCl buffer (pH 7.5). After the addition of 50 mM MnCl2 in 50 mM Tris·HCl (pH 7.5), arginase was activated by heating at 55°C for 10 min. Then, 25 µl of 0.5 M L-arginine (pH 9.7) was added to 25 µl of activated solution, and the conversion of arginine to urea was carried out at 37°C for 60 min. The reaction was stopped by adding 400 µl of acidic solution (H2SO4: H2O2: H2O = 1:3:7 vol). Twenty-five microliters of 9% 1-phenyl-2,3-diaminobenzidine tetrahydrochloride (Dako) was used. The specificity of staining for nitrotyrosine was verified by preincubation of the anti-nitrotyrosine antibody with 10 mM nitrotyrosine or by incubation of tissue sections with 100 mM sodium dithionite to convert nitrotyrosine to aminotyrosine. To observe the deposition of collagen in the interstitial space and goblet cell hyperplasia in the epithelium of bronchioles, lung sections were stained with Azan and the periodic acid-Schiff (PAS) method. The proportions of collagen fibers in the peribronchiolar region and goblet cells in the bronchial epithelium were digitized by ImagePro software (Media Cybernetics, Bethesda, MD).

**Measurement of nitrotyrosine.** The amount of nitrotyrosine in the proteins was measured as previously described (18). Briefly, acetonitrile was added to sedimented BALF. After washing for the removal of residual NO2-, NO3-, and other contaminants, digestion of protein by Pronase (Roche) was performed at 50°C for 16 h. A 10-kDa molecular cut off filter (Ultrafree-MC, Millipore) was washed with ultrapure water to remove any contaminants and artifacts. The product of the digestion was passed through the filter to remove the precipitate. Then, the amount of nitrotyrosine was quantified by HPLC with electrochemical detection (HPLC-ECD; Eicom, Kyoto, Japan) and a SC-50DS column (3 × 150 mm, 5 µm, Eicom). Fifty microliters of the filtrate was injected into the HPLC system for detection. The mobile phase contained 2% acetonitrile and 0.5 mg/l EDTA in 0.2 M sodium dihydrogenphosphate (pH 3.0). The flow rate was 0.5 ml/min. To correct the nitrotyrosine content, the amount of tyrosine was also measured by HPLC using an Spherisorb ODS2 column (4.6 × 150 mm, 5 µm; Waters, Milford, MA). The mobile phase contained 93% 50 mM potassium phosphate (pH 3.0) and 7% methanol. The flow rate was 1.0 ml/min. Results are expressed as the molar ratio of nitrotyrosine to tyrosine.

**Measurement of nitrite + nitrate.** A model 280i NO analyzer (Sievers, Boulder, CO) was used to measure the amount of NO generated as nitrate and nitrite. Lung tissue was homogenized in buffer containing 150 mM NaCl, 20 mM Tris·HCl (pH 7.2), and 1 mM EDTA. The homogenate was divided into two portions. Nitrate reductase (0.33 U/ml) from Aspergillus niger (Sigma-Aldrich, St. Louis, MO) and NADPH (6.6 µM) were added to one portion to convert nitrate to nitrite. After incubation at room temperature for 30 min, protein was removed by adding acetonitrile and subsequent centrifugation. Nitrite was quantified in a mixture of acetic acid, iodine, and potassium iodide (50) under argon gas. To validate the increase of nitrite + nitrate (NOx), lung homogenate was obtained from mice administered LPS intranasally (300 µg/kg in saline), as previously described (28).

**Statistical analysis.** The results are presented as means ± SD. Data were analyzed by one-way ANOVA to examine whether there was significant difference among groups. Statistical analysis was performed with optimized conditions. A representative image is shown in A. Relative expression was calculated with GAPDH as an internal standard (B). Data are expressed as the means ± SD from 6 mice. **P < 0.01 from the saline-exposed control group.
any statistical difference among groups. If the difference evaluated with the ANOVA was significant, a Scheffe multiple comparisons test was used for paired comparisons. A two-tailed unpaired t-test was used to compare with control values, using the SPSS 11.0 windows program. A P value less than 0.05 was considered statistically significant.

RESULTS

Immunolocalization of NOS isoforms, nitrotyrosine, and arginase. Immunohistochemical staining for NOS, nitrotyrosine, and arginase was detected on day 12 in the Df-exposed group. Increased immunostaining for nitrotyrosine was observed in type II alveolar epithelial cells, infiltrating granulocytes, alveolar macrophages, and epithelial cells of bronchioles in the Df-exposed lung (Fig. 2A), but not in the saline-exposed control lung (Fig. 2B). Immunostaining for nitrotyrosine was abolished by treatment with dithionite (not shown), which is a reductant forming aminotyrosine from nitrotyrosine, meaning that immunostaining signal was derived from nitrotyrosine. Immunostaining for iNOS was detected in infiltrating granulocytes and in epithelial cells of bronchioles (Fig. 2D) and that for eNOS was observed in alveolar macrophages (Fig. 2E). The intensity of nNOS immunostaining, observed in airway nerves of the lung, was unchanged in the Df-exposed lung compared with the saline-exposed control lung (not shown). Arginase I was located particularly in infiltrating cells around the bronchioles (Fig. 2F) and alveolar macrophages. Immunostaining for arginase was not detected in the saline-exposed control group (Fig. 2G).

Increased formation of nitrotyrosine. The formation of reactive nitrogen species was evaluated as the amount of nitrotyrosine generated. Significantly large amounts of nitrotyrosine were detected in BALF cells on day 12 (Fig. 2C).

Fig. 4. Paralleled expression of arginase and iNOS. Enzymatic activity of arginase in lung and serum was measured at the critical time shown in A. Lung arginase activity for the Df-exposed experimental group is shown (■) and compared with that in the saline-exposed control group on day 12 (□) or nonchallenged control group on day 12 (●). Serum arginase activity for the Df-exposed experimental group is shown (□) and compared with that in the saline-exposed control group on day 12 (●). Representative PCR bands for arginase I mRNA on day 12 are shown in B. Each band was quantified by densitometric analysis for the control (open bar) and Df-exposed group (closed bar) in C. The amounts of arginase I and II were also evaluated by immunoblotting (D). Total lung homogenate proteins (40 μg, without detergents for arginase I; 100 μg, with 1% of Triton X-100 for arginase II) were separated by SDS-PAGE and transferred onto PVDF membranes. Anti-arginase I or anti-arginase II antibodies from rabbits were used for each isozyme. Tissue homogenates from liver and kidney were used as a positive control for arginase I and II, respectively. Equality of protein loading was confirmed by the expression of β-actin. Expression of mRNA for iNOS in BALF cells on day 12 (E) and total lung tissue on days 0, 5, 12, and 25 (F) was compared with that in the saline-exposed control group on the same day. Data are expressed as the means ± SD from 3 (immunoblotting) or at least 5 (RT-PCR and arginase activity) mice. **P < 0.01 from the nonchallenged control group. #P < 0.05, ##P < 0.01 from the saline-exposed control group.
mRNA expression of cytokines. To confirm the expression of arginase I and to investigate Th1/Th2 balances, levels of several cytokines in lung tissue were analyzed by RT-PCR. After a single challenge (day 12), the mRNA expression of IL-4, IL-5, and IL-13, all Th2-type cytokines, was significantly induced under asthmatic conditions. In contrast, the expression of IL-12 p40, a proinflammatory component of IL-12 (1), and of IFN-γ, both Th1-type cytokines, was significantly suppressed (Fig. 3B).

Enzymatic activity and mRNA level of arginase. A significant increase in arginase activity was observed on days 5 and 12 after exposure to Df extract for sensitization and challenge compared with the activity in the saline-exposed control group on the same day and that in the nonchallenged control group on day 12 (Fig. 4A). Arginase activity on day 25 was maintained at half the maximal level achieved on day 12. Arginase activity in serum was also investigated to clarify the relationship in terms of enzymatic activity between the lung and blood. The level of arginase mRNA on day 12 was significantly elevated compared with that in the saline-exposed control group (Fig. 4, B and C). The changes of arginase I and II expression were also investigated with immunoblotting. Levels of both isozymes were significantly increased on day 12 compared with day 0 (P = 0.006 and 0.029 for arginase I and arginase II from control group, respectively; Fig. 4D).

mRNA expression of NOS isoforms. In BALF cells, significantly induced expression of iNOS mRNA was observed on day 12 (Fig. 4E) compared with day 0. Upregulation of iNOS mRNA expression was also observed in lung tissue on day 5 and 12 (Fig. 4F). Additionally, on day 25, the expression of iNOS mRNA was reduced to the basal level (Fig. 4F). Thus, on days 5, 12, and 25, the course of mRNA expression of iNOS paralleled that of arginase. No change was found in the eNOS and nNOS mRNA levels (data not shown).

Collagen deposition and goblet cell hyperplasia. Thickening of bronchial smooth muscle was detected by hematoxylin-eosin staining in the Df-exposed group (Fig. 5B) compared with the saline-exposed control group (Fig. 5A). An accumulation of collagen, as revealed by Azan blue staining, was observed around bronchioles and arteries on day 25 (Fig. 5D) compared with day 12 (Fig. 5C). Evidence of significant fibrosis was also obtained through the quantification of collagen fibers in the peribronchial regions (Fig. 5E). On day 12, overlapping PAS-positive goblet cells had formed thick layers in the epithelium of bronchioles (Fig. 5G). Few cells were stained on day 0 (Fig. 5F). On day 25, most PAS-stained cells had disappeared in bronchioles (data not shown), despite that the bronchial smooth muscle remained thickened. The proportion of goblet cells in the bronchial epithelium was increased significantly on day 12 compared with day 0 (Fig. 5H).

Changes of NOx. NO production was expressed as NOx. No significant change in the concentration of NOx was observed in the experimental lung throughout the experiment (day 0: 2.73 ± 0.82, day 12: 3.35 ± 0.92, day 25: 2.22 ± 1.06 pmol/mg tissue), despite a higher expression level of iNOS on day 12. There was no significant difference in the concentration of NOx in BALF supernatants on day 12 between the Df-exposed group and saline-exposed control group (data not shown). However, a very high concentration of NOx was observed in the LPS-exposed mice (31.77 ± 2.99 pmol/mg tissue, P < 0.01 compared with both the control and Df-exposed groups).

DISCUSSION

Arginase, an enzyme of the urea cycle, has two isozymes, types I and II. The isozymes are encoded by different genes (16). Arginase I is a cytoplasmic protein that is primarily expressed in the liver, whereas arginase II is a mitochondrial protein expressed in a variety of tissues, particularly in the kidney and prostate (16). The function of arginase is to convert L-arginine to L-ornithine, a precursor in the production of

Fig. 5. Distribution of collagen and goblet cells. Paraffin-embedded sections were stained with hematoxylin-eosin (A, day 12, saline-exposed; B, day 12, Df-exposed), Azan (C, day 12, Df-exposed; D, day 25, Df-exposed), and the periodic acid-Schiff (PAS) technique (F, day 0, saline-exposed; G, day 12, Df-exposed). Bars indicate 50 µm. We also quantified the proportions of collagen fibers in peribronchial regions (E) and goblet cells in the bronchial epithelium (H) with ImagePro. Data are expressed as means ± SD. Proportions were calculated from at least 50 and 17 bronchioles from 5 mice for Azan and PAS stain, respectively. **P < 0.01 from day 12 (E) or day 0 (H).
polyamines and proline, which regulate cell proliferation and collagen synthesis. Therefore, the expression of arginase in the lung has been a focus in experimental asthma regarding remodeling and airway hyperresponsiveness through competition between L-arginine and NOS (52).

An increase in arginase I activity was observed not only in the lung but also in serum. Immunohistochemical staining for arginase I was detected in inflammatory cells and fibroblasts. Moreover, increased levels of both arginase I and II proteins were detected in the Df-challenged lung. Although the expression of arginase was not determined in the cellular fraction of the blood, the rise in serum arginase activity after the challenge implies the release of arginase enzymes from the lung. Two previous studies have shown an upregulation of arginase I gene expression in the lung in an experimental model of asthma involving exposure to OVA and A. fumigatus using microarrays (13, 52). In a human study, the arginase activity of BALF increased in asthma patients. However, the serum arginase activity in asthma patients varied between two reports (4, 23).

One showed an increase and the other a decrease in serum arginase activity in asthmatic patients. Although the precise reasons for this discrepancy are unknown, and there was a methodological difference between these reports, our study supported an increase in arginase activity in asthmatic patients. Recent reports suggest that arginase is expressed in many types of white blood cells, including polymorphonuclear leukocytes (PMN) (25), mononuclear cells (24, 26), and eosinophils (25). In the PMNs, arginase is not only a cytoplasmic protein but also found in the granules, colocalized with myeloperoxidase (25). Moreover, the induction mechanisms for arginase differ between humans and rodents (25). Arginase is constitutively expressed in human PMNs, whereas its expression in murine PMNs is stimulated by Th2-type cytokines (25). Although these factors may contribute to the discrepancy between humans and rodents, more data are needed to evaluate the change of serum arginase activity in human asthma.

Airway remodeling is a pathological feature of asthma involving goblet cell hyperplasia (2), airway smooth muscle hypertrophy (9), and subepithelial fibrosis (41). Overproduction of a downstream product of arginase (ornithine, a precursor of proline and polyamines) in the asthmatic lung might provoke the proliferation of mesenchymal cells and deposition of collagen, thus promoting airway remodeling (38, 52). Therefore, although the amounts of polyamines and proline in the lung were not determined in this study, the upregulation of arginase activity and concomitant goblet cell hyperplasia and collagen deposition suggested the involvement of arginase and remodeling in the pathophysiology of this experimental model of asthma.

An increase in the iNOS mRNA level was observed. However, the concentration of NOx in lung homogenate and BALF was unchanged. The expression of iNOS is usually observed in experimental asthma models and human asthmatic lung (14, 15, 35). Moreover, several studies have revealed high NOx levels in BALF (8, 32). An elevation of the NOx level has been demonstrated, and our data are inconsistent with previous investigations. On the other hand, recent studies have focused on the contribution of nNOS to airway hyperresponsiveness and increased NO levels in expiratory air. Our study showed no changes in the expression of nNOS, and we did not have any data regarding nNOS. Therefore, it is not clear whether the NOx levels in this study are meaningful or not. However, it is possible that increased levels of arginase isozymes inhibit the consumption of arginine by NOS, which contributes to sustained asthmatic conditions.

Moreover, increased formation of nitrotyrosine was observed in asthmatic lung despite that the NOx concentration remained almost unchanged. It has been suggested that nitrotyrosine can be produced in different ways: 1) through an attack on tyrosine residues by peroxynitrite (ONOO–), a powerful oxidant generated by the reaction of NO with superoxide (O2−) (3, 33). 2) Peroxidases such as myeloperoxidase and eosinophil peroxidase can also catalyze the nitration of tyrosine by the oxidation of nitrite (3). This peroxidase-dependent nitration occurs at physiological levels of nitrite. 3) NOS can produce NO and superoxide simultaneously under conditions where the amount of arginine is decreased and contribute to the formation of nitrotyrosine, or the bioavailability of tetrahydrobiopterin (BH4), which is an important cofactor for coupling for NOS, is limiting (31, 43, 46, 47). It is not clear which mechanism is involved in the present findings because precise immunohistochemical data for the colocalization of arginase, iNOS, and nitrotyrosine were not obtained, and the state of BH4 is unclear. However, if arginase is involved in the depletion of arginine and consequential NOS uncoupling, the formation of nitrotyrosine in arginase-expressing cells cannot be ruled out.

An upregulation of the production of Th2-type cytokines and a downregulation of that of Th1-type cytokines, frequently seen in allergic reactions, were confirmed to have occurred in this study. Increased mRNA levels of IL-4 and IL-13, involved in the inducing expression of arginase via the JAK1/JAK3 and STAT6 pathway (29), were also observed. However, it is known that the expression of iNOS is induced via the STAT1 pathway, and IFN-γ is involved in this process (7). The discrepancy in the regulation of iNOS and IFN-γ needs to be investigated further. This study presented only one measurement (day 12). Therefore, the gene expression of arginase and NOS should be investigated with more time points, and one should consider unknown factors.

In conclusion, transiently elevated levels of arginase activity (both I and II isozymes) may play a role in the pathology of asthma and airway remodeling. The expression of iNOS was also upregulated in parallel with the increase in arginase; nevertheless, the concentration of NOx was unchanged. Arginine depletion may be involved in this phenomenon. Theoretically, artificial control of arginase activity in the lung may be a useful approach to the prevention and treatment of asthma.

**REFERENCES**

PARALLELED UPREGULATION OF NOS AND ARGINASE IN ASTHMA

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