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Direct inhibition of arginase attenuated airway allergic reactions and inflammation in a *Dermatophagoides farinae*-induced NC/Nga mouse model

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Takahashi N, Ogino K, Takemoto K, Hamanishi S, Wang D-H, Takigawa T, Shibamori M, Ishiyama H, Fujikura Y. 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* 299: L17–L24, 2010. First published April 9, 2010; doi:10.1152/ajplung.00216.2009.—The expression of arginase I has been a focus of research into the pathogenesis of experimental asthma, because arginase deprives nitric oxide synthase (NOS) of arginine and therefore participates in the attenuation of bronchodilators such as nitric oxide (NO). The present study used an intranasal mite-induced NC/Nga mouse model of asthma to investigate the contribution of arginase to the asthma pathogenesis, using an arginase inhibitor, *N*^ω-hydroxy-nor-L-arginine (nor-NOHA). The treatment with nor-NOHA inhibited the increase in airway hyperresponsiveness (AHR) and the number of eosinophils in bronchoalveolar lavage fluid. NOx levels in the lung were elevated despite suppressed NOS2 mRNA expression. Accompanied by the attenuated activity of arginase, the expression of arginase I at both the mRNA and protein level was downregulated. The levels of mRNA for T helper 2 cytokines such as IL-4, IL-5, and IL-13, and for chemotactants such as eotaxin-1 and eotaxin-2, were reduced. Moreover, the accumulation of inflammatory cells and the ratio of goblet cells in the bronchiole were decreased. The study concluded that the depletion of NO caused by arginase contributes to AHR and inflammation, and direct administration of an arginase inhibitor to the airway may be beneficial and could be of use in treating asthma due to its anti-inflammatory and airway-relaxing effects, although it is not clear whether the anti-inflammatory effect is direct or indirect.

experimental asthma; arginase inhibitor; nor-NOHA

ACCORDING TO THE World Health Organization, asthma is a chronic inflammatory disorder of the airways affecting 300 million people worldwide (40). The major characteristics of asthma include airway inflammation, airway hyperresponsiveness (AHR), and variable airflow obstructions (2). Common triggers of asthmatic symptoms include cold air, tobacco smoke, strong odors or fumes, respiratory infections, as well as allergens such as dust mites, pollens, molds, and pet dander (40).

Nitric oxide (NO) is produced by a variety of cells in the respiratory tract such as airway epithelial cells, airway nerve cells, vascular epithelial cells, and inflammatory cells (32). NO is capable of relaxing the airway via cGMP-dependent path-

ways (32) and contributes to a remission of oxidative stress in asthma. NO is also involved in the control of airway tone via a cGMP-independent pathway, namely *S*-nitrosylation. There are a number of mechanisms for *S*-nitrosylation (8). *S*-nitroso-glutathione (GSNO) is generated by transnitrosylation between protein *S*-nitrosothiol (SNO) and glutathione, and eliminated by GSNO reductase (GSNOR). GSNO and GSNOR play a key role in asthma. In a previous study, a lack of GSNOR resulted in protection from AHR in OVA-exposed mice (28). In human asthma, depletion of *S*-nitrosothiol and increased expression of GSNOR were observed (10, 29). Although several types of nitric oxide synthase (NOS) are involved in the pathophysiology of experimental asthma (31) and human asthma (12), NOS2-derived NO is thought to be important in the pathophysiology of allergic airway disease (32). Recent studies have demonstrated that alterations in L-arginine homeostasis play a major role in allergen-induced NO deficiency and AHR in experimental asthma models and asthmatic patients (19, 26). Although high concentrations of exhaled NO were observed in asthmatic patients (14), NO depletion from constitutive NOS has been confirmed for AHR in animal models (1, 17) and in patients (33). Moreover, it was demonstrated that expression of arginase was closely associated with airway NO depletion (23). Expression of arginase and subsequent NO depletion have been suggested in several animal models of asthma induced by different allergens and T helper 2 (Th2) cytokines (17, 47).

The importance of arginase in the development of AHR in allergic asthma was demonstrated by recent findings in mice and guinea pigs showing inhibition of arginase activity by RNA interference or arginase inhibitors (19, 26, 47). The inhibition of experimental asthma using arginase inhibitors has been investigated in only three studies (5, 19, 26). Two of the studies revealed inhibition of OVA-induced AHR or inflammation in guinea pigs (19) and mice (26). However, the third one showed augmentation of inflammation after treatment with an arginase inhibitor in OVA-exposed mice (5).

Instead of OVA-induced animal model of asthma, the present study used a mite-challenged mouse model that is considered bearing a closer resemblance to human asthma (34). The aim of this study was to investigate the role of arginase in asthma using an arginase inhibitor, *N*^ω-hydroxy-nor-L-arginine (nor-NOHA), in a mite-challenged NC/Nga mouse model that develops allergic asthma-like responses by intranasal exposure to *Dermatophagoides farinae* (Df) extract as common asthma allergens without adjuvant (34).

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MATERIALS AND METHODS

Chemicals and reagents. All chemicals and reagents without annotation were of analytical reagent grade and purchased from Wako Pure Chemical Industries (Osaka, Japan).

Animals. Male NC/Nga mice, 7 wk old, were obtained from Charles River Laboratories Japan (Yokohama, Japan). The mice were maintained under specific pathogen-free conditions with a 12-h light/12-h dark cycle and had free access to standard laboratory food and tap water. They were acclimatized for at least 1 wk before the experiments. The care and handling of the animals were in accordance with the Guidelines for the Care and Use of Laboratory Animals at Shikata Campus of Okayama University and approved by the Okayama University Institutional Animal Care and Use Committee.

Intranasal administrations. NC/Nga mice were sensitized to mite crude extract (Cosmo Bio, Tokyo, Japan) based on a previously described protocol (34) as in Fig. 1. Briefly, the anesthetized mice were intranasally instilled with Df crude extract (50 μ g/25 μ l saline) for 5 consecutive days (days 0–4), and, from day 11, given intranasal administrations of nor-NOHA (100 μ g/10 μ l saline) or saline for 3 consecutive days (days 11–13). On day 14, Df crude extract and nor-NOHA were administered simultaneously.

Measurement of AHR. On day 14, the degree of bronchoconstriction was measured according to the overflow method (34). Briefly, mice were anesthetized with pentobarbital (80 mg/kg) and connected to an artificial ventilator following surgical incision of the trachea. A pulmotor system was constructed with a rodent ventilator (model 132; New England Medical Instrument, Medway, MA), a bronchospasm transducer (model 7020; Ugo Basile, Comerio-Barese, Italy), and a DATA recorder (Omniace II data acquisition system, model RA1300; NEC San-ei, Tokyo, Japan). Gallamine triethiodide (350 μ g/mouse) was intravenously administered immediately to eliminate spontaneous respiration and followed by administrations of acetylcholine with stepwise increases in the concentration from 62.5 to 2,000 μ g/kg. Dose-response curves for acetylcholine in anesthetized, mechanically ventilated mice were obtained. Bronchoconstriction was expressed as the respiratory overflow volume provoked by acetylcholine as a percentage of the maximal overflow volume (100%) obtained by totally occluding the tracheal cannula (25). Severity of AHR and sensitivity towards acetylcholine were evaluated with maximum airway constriction (Emax) and pEC₅₀ ($-\log_{10}$ EC₅₀) values, respectively, according to the method reported by Meurs et al. (22).

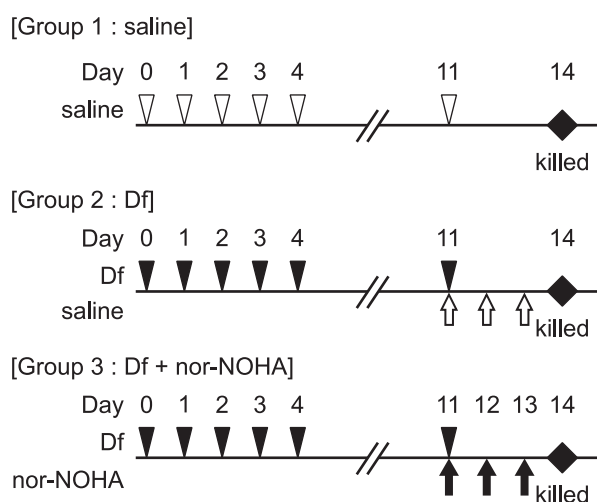


Fig. 1. Schematic representation of the experiment. One-hundred micrograms of *Dermatophagoides farinae* (Df) extract dissolved in 50 μ l of saline was administered intranasally on day 0–4 and day 11 to NC/Nga mice in group 2. The treatment group (group 3) received 100 μ g of nor-NOHA in 50 μ l of saline on days 11, 12, and 13. The control group (group 1) received an equal volume of saline.

Bronchoalveolar lavage fluid. Immediately after the assessment of acetylcholine-induced AHR, the lungs were lavaged with 1-ml aliquots of cold Hanks' balanced salt solution without calcium and magnesium, containing 0.05 mM EDTA (HBSS). The collected bronchoalveolar lavage fluid (BALF) was then centrifuged at 150 g for 10 min at 4°C. The supernatant was stored at -80°C for further analysis, and the cell pellet was resuspended in HBSS. An aliquot was stained with Türk's solution, and a total cell count was performed in a Bürker-Türk chamber. Another aliquot for a differential cell count was applied to cytopspin preparations at 800 rpm for 3 min (Cytospin 3; Thermo Fisher Scientific, Waltham, MA) and stained with Diff-Quik (Sysmex, Kobe, Japan).

Lung specimens. The lung tissue was removed to extract total RNA and fixed in 10% buffered formalin for morphological examination. The remaining lung tissue was homogenized in a homogenizing buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM EDTA containing a protease inhibitor cocktail) with or without 1% Triton X-100 for Western blotting and measurements of arginase activity and NOx concentrations.

RT-PCR. After the total RNA of each sample was extracted with ISOGEN (Nippon Gene, Tokyo, Japan), RT-PCR was performed using TakaRa RNA PCR kit AMV, ver. 3.0, and a Takara PCR thermal cycler MP (Takara Bio, Ohtsu, Japan) with oligo-dT primers according to the manufacturer's instructions. Primer sets and PCR conditions are detailed in Table 1. PCR products were detected by agarose gel electrophoresis and ethidium bromide staining. PCR bands were quantified by ImageJ software (National Institutes of Health, Bethesda, MD) and normalized against GAPDH.

Western blotting. The protein expression of arginase and nitrotyrosine was evaluated by Western blotting (15). Aliquots of lung tissue homogenate with or without 1% Triton X-100 were subjected to SDS-PAGE, and proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). After blocking with 5% dried skimmed milk in Tris-buffered saline containing 0.5% Tween 20 (TBS-T), blots were incubated with appropriate primary antibodies (polyclonal rabbit, 1:100 to 1:500 dilutions; Santa Cruz Biotechnology, Santa Cruz, CA) and a horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000 to 1:4,000; Sigma-Aldrich, St. Louis, MO). Antibody-specific bands were detected using an enhanced chemiluminescence Western blot detection system (Perkin-Elmer, Boston, MA) and quantified by Scion Image software (Scion, Frederick, MD). For nitrotyrosine immunoblotting, a rabbit polyclonal antibody developed against octapeptide containing nitrotyrosine (9, 13) was also employed (1:4,000 dilution). The specificity of the antibody was confirmed by immunoblotting using halogenated or nitrated bovine serum albumin.

Measurement of arginase activity. An aliquot of the lung tissue homogenate with 1% Triton X-100 was ultracentrifuged at 105,000 g for 60 min at 4°C (Beckman TL-100; Beckman Coulter, Fullerton, CA). Arginase activity was determined as described previously (6). Briefly, arginase was activated for 10 min at 55°C in the presence of MnCl₂, followed by the addition of arginine and incubation for 60 min at 37°C. The hydrolysis of arginine was stopped with acid, and the reaction product, urea, was measured by a colorimetric assay at 560 nm after incubation with α -isonitrosopropiophenone for 45 min at 100°C.

Measurement of NOx concentrations. To estimate NO production, the NOx concentration in the lung tissue homogenate was determined with a NO analyzer (model 280i NOA with the Purge Vessel; Sievers, Boulder, CO) (46). An aliquot of the homogenate was treated with nitrate reductase (Sigma-Aldrich) to convert nitrate to nitrite for 30 min at room temperature. Nitrite was further reduced to NO in a Purge Vessel containing the reducing agent potassium iodide in acetic acid, and NO was subsequently detected by the ozone-chemiluminescence method.

Histopathological observations. The fixed lung tissues were dehydrated, embedded in paraffin, and sectioned. Hematoxylin and eosin

Table 1. List of primers and RT-PCR conditions

Target Gene	Sense	Antisense	Denaturation	Amplification	Cycles	Elongation
Arginase I	CAGAAAGATGGAAGATCAG	CAGATATGCGAGGAGTGACC	95°C, 5min	56°C, 20 s	35	72°C, 5 min
Arginase II	TGATTGCGAAAGGACAGG	CTAGAGTAGGAAGGTGGTC	95°C, 5min	56°C, 20 s	35	72°C, 5 min
IL-4	CTAGTTGCTATCTCTCTCTTT	CTTTAGGCTTCCAGGAAGCTTTT	94°C, 3min	55°C, 75 s	40	72°C, 7 min
IL-5	GTGAAAGAGACCTTGACACAGCTG	CAGACCAAGGAACCTCTTGAGGTA	94°C, 3min	55°C, 75 s	35	72°C, 7 min
IL-13	CTCCCTCTGACCTTAAAGGAG	GAAAGGGCCGTGGGAAACAG	94°C, 3min	55°C, 75 s	35	72°C, 7 min
NOS1	CCTTAGAGAGTAAGGAAGGGGGGGG	GGCGGATCATGAGCGCGAGATCATG	94°C, 3min	60°C, 45 s	35	72°C, 5 min
NOS3	GGGCTCCCTCCCTCCGCTGCGACC	GGATCCCTGGAAAAGGGGTGAGG	94°C, 3min	64°C, 45 s	33	72°C, 5 min
NOS2	ATGGCTTGGCCCTGGAAGTTTCTC	CCTCTGATGGTGCCATCGGGCATC	94°C, 3min	60°C, 45 s	31	72°C, 5 min
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCCACCTGTTGCTGTA	94°C, 3min	60°C, 45 s	30	72°C, 5 min
Eotaxin-1	GCTCCACAGCGCTTCTATTG	TTGTGGCATCTCTGGAGC	94°C, 3min	56°C, 60 s	30	72°C, 5 min
Eotaxin-2	GCTCTGCTACGATCGTTG	AGCAAACTTGGTTCTCACTG	94°C, 3min	56°C, 60 s	30	72°C, 5 min
MIP-2	TGCCTGAAGACCCCTGCCAAGG	GTTAGCCCTTGGCTTTGTTGAG	94°C, 3min	60°C, 30 s	34	72°C, 5 min

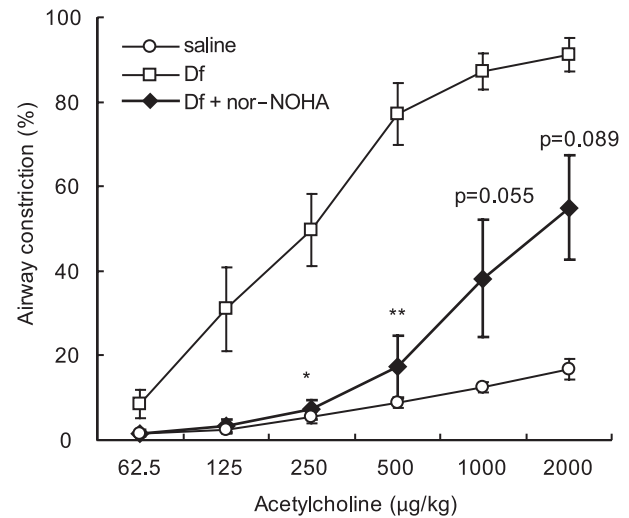


Fig. 2. Airway hyperresponsiveness (AHR) to acetylcholine. Effect of nor-NOHA on the Df-induced increase in AHR in NC/Nga mice. Data were obtained from 5 to 7 animals/group. Bronchoconstriction (%) is expressed as means $\pm$ SE. * P < 0.05, ** P < 0.01 vs. the Df-exposed group.

(H&E) staining was used to assess the degree of inflammation (16). Azan and periodic acid-Schiff (PAS) stainings were also done to observe interstitial collagen deposition and goblet cell hyperplasia, respectively (35). The levels of inflammation in peribronchial and perivascular spaces of the lung were determined by an ordinal scale ranging from 0 to 3, as described elsewhere (36). A value of 0 meant no inflammation was detectable; a value of 1, occasional cuffing with inflammatory cells; a value of 2, most bronchi or vessels were surrounded by a thin layer (1–5 cells thick) of inflammatory cells; and a value of 3, most bronchi or vessels were surrounded by a thick layer (more than 5 cells thick) of inflammatory cells.

Statistical analysis. Data, unless otherwise noted, are expressed as means $\pm$ SD and were analyzed using Student's unpaired t -test or ANOVA with a post hoc test. A P value less than 0.05 was considered statistically significant. Statistical analyses were performed using the SPSS 11.0 windows program.

RESULTS

Effect of nor-NOHA on AHR. Mice treated with Df and saline showed an increasing AHR. However, treatments with Df and nor-NOHA caused a significant reduction in AHR (Fig. 2). $E_{\max}$ and pEC_{50} values were calculated with the entire set of data. Both were significantly increased in the Df-treated group compared with the control, whereas similar $E_{\max}$ and significantly high pEC_{50} values were observed in the Df+nor-NOHA-treated group compared with the Df-treated group (Table 2).

Effect of nor-NOHA on BALF cells and inflammatory scores. The increased cell numbers in BALF and inflammatory scores for the peribronchial and perivascular space observed in Df-

Table 2. Maximum airway constriction ($E_{\max}$) and concentration of half-maximal response (pEC_{50}) in the acetylcholine-induced AHR

	$E_{\max}$ (% Constriction)	P	pEC_{50} (μ g/kg)	P
Saline	30.0 $\pm$ 7.5	<0.01	1,282 $\pm$ 301	<0.01
Df	95.4 $\pm$ 6.9		206 $\pm$ 114	
Df+nor-NOHA	93.3 $\pm$ 6.4	N.S.	1,346 $\pm$ 547	<0.01

P value: compared with Df group; N.S., not significant.

exposed mice were significantly reduced by treatment with nor-NOHA (Fig. 3, A and B). No significant difference in the inflammation score was found between peribronchial and perivascular sites (data not shown).

Effect of nor-NOHA on the activity and mRNA and protein levels of arginase. The effect of nor-NOHA on arginase expression is shown in Fig. 4. By Df challenge, protein (Fig. 4B) and mRNA (Fig. 4C) levels of arginase I and II determined by Western blotting were significantly augmented compared with levels in the control group given saline. Intranasal inoculation of Df-treated mice with nor-NOHA significantly attenuated the activity (Fig. 4A) and protein (Fig. 4B) and mRNA (Fig. 4C) expression of arginase I. However, levels of mRNA and protein were unchanged for arginase II by treatment of nor-NOHA (Fig. 4, B and C).

Effect of nor-NOHA on mRNA expression of Th2 cytokines. Messenger RNA levels of the Th2 cytokines IL-4, IL-5, and IL-13 increased after the treatment of Df (Fig. 5A). The increases in mRNA levels of IL-4, IL-5, and IL-13 were attenuated by inoculation with nor-NOHA. The expression of chemotactants was also investigated. Levels of eotaxin-1, eotaxin-2, and macrophage inflammatory protein 2 (MIP-2) increased significantly with exposure to Df. The expression of eotaxin was markedly downregulated on additional treatment with nor-NOHA, although the level of MIP-2 was unchanged by nor-NOHA (Fig. 5B).

Effect of nor-NOHA on mRNA expression of NOS isoforms and NOx concentrations. Effects of nor-NOHA on NOS isoforms in Df-exposed mice are shown in Fig. 6A. The mRNA expression for NOS2 was upregulated, whereas NOS1 and

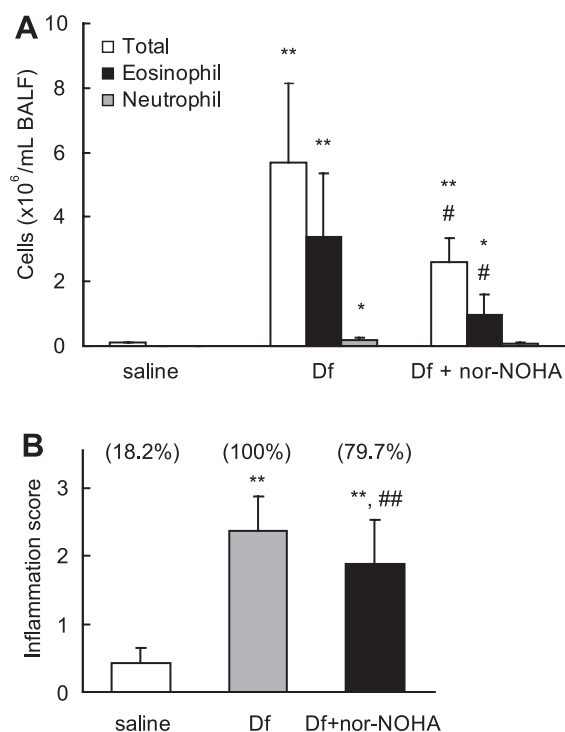


Fig. 3. Effect of nor-NOHA on BALF cell numbers (A) and inflammatory scores in H&E-stained sections of the lung (B). Data obtained from 5 to 7 animals/group are shown. The total cell, eosinophil, and neutrophil counts are expressed as means $\pm$ SD. * P < 0.05 and ** P < 0.01 vs. the control group. # P < 0.05 and ## P < 0.01 vs. Df-exposed group.

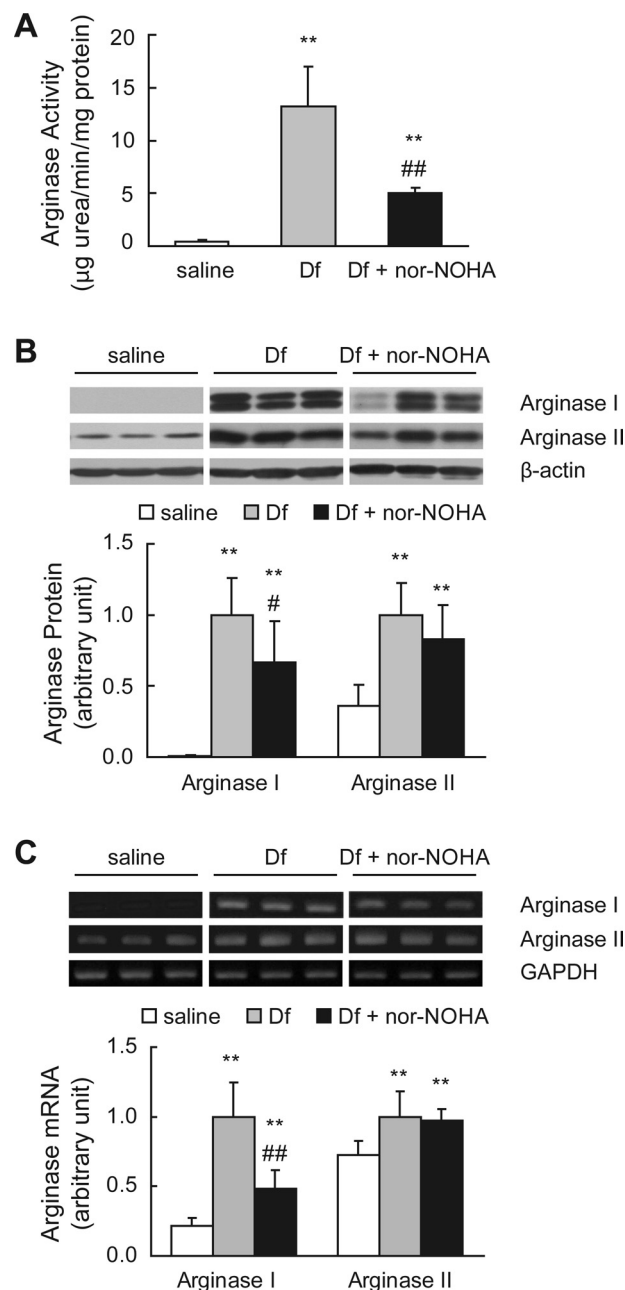


Fig. 4. Effect of nor-NOHA on Df-induced arginase expression. Total arginase activity (A), protein expression (B), and mRNA expression (C) for arginase I and II are shown. Values are expressed as means $\pm$ SD for 5–7 mice. ** P < 0.01 vs. the control group. # P < 0.05 and ## P < 0.01 vs. the Df-exposed group.

NOS3 mRNA levels were unchanged. Inoculation of the Df-exposed mice with nor-NOHA reduced the NOS2 levels. The concentration of NOx was not changed in the lungs of Df-treated mice compared with the control mice. However, treatment with nor-NOHA increased NOx levels significantly (Fig. 6B).

Effect of nor-NOHA on histopathological findings. The histopathological changes in the lung on treatment with Df and nor-NOHA were investigated. With intranasal exposure to Df, a severe accumulation of inflammatory cells around the peribronchial and perivascular space (Fig. 7A) was observed. However, additional treatment with nor-NOHA eliminated this

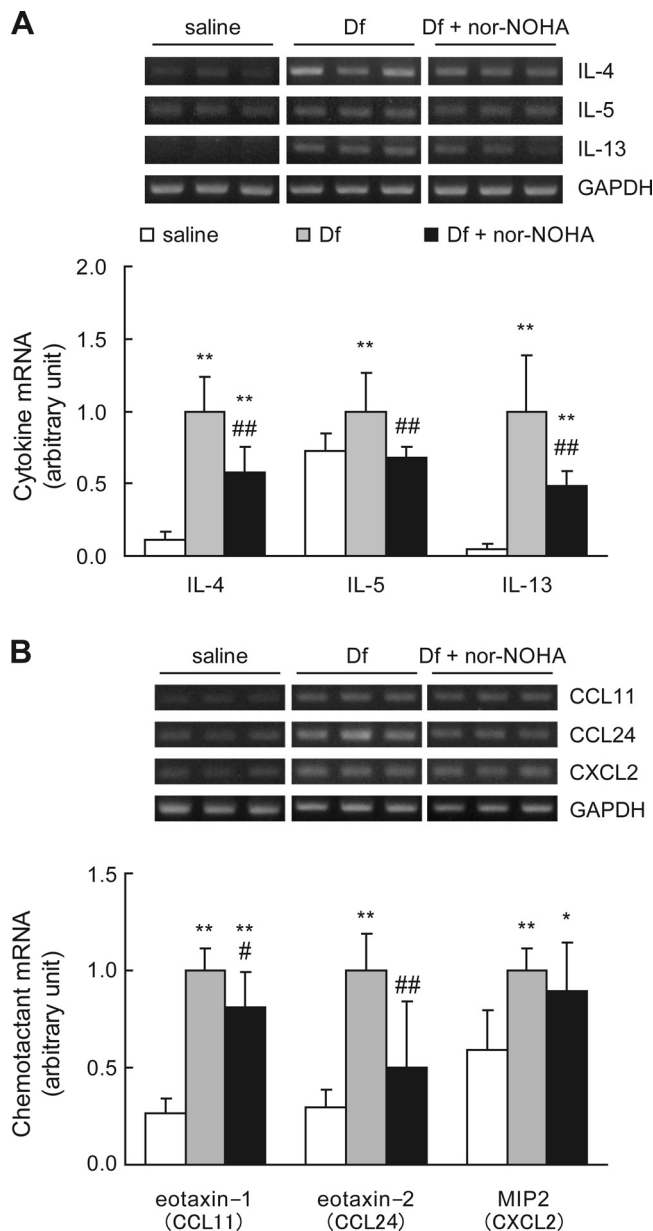


Fig. 5. *A* and *B*: effect of nor-NOHA on Df-induced mRNA expression of Th2 cytokines. Each value is the mean $\pm$ SD for 5–7 mice. * P < 0.05, ** P < 0.01 vs. the control group. ## P < 0.01 vs. Df-exposed group.

layer of inflammatory cells (Fig. 7*B*, Fig. 3*B*). We next investigated the hypersecretion of mucus in the epithelium. The distribution of goblet cells in the lung was analyzed with a PAS staining technique. With exposure to Df intranasally, goblet cells were extensively distributed in the bronchiole epithelium (Fig. 7*C*). In contrast, additional treatment with nor-NOHA dramatically reduced the number of goblet cells in the bronchiole (Fig. 7, *D* and *E*). Azan staining showed no significant increase in the deposition of collagen (data not shown).

Effect of nor-NOHA on nitrotyrosine production. Changes in nitrotyrosine levels in the lung were evaluated by Western blotting. Nitrotyrosine was detected in a wide range of proteins (Fig. 8), and most of the bands showed similar intensity among control, Df-, and Df+nor-NOHA-treated mice. In Df exposure, some minor bands in the low-molecular-weight range pre-

sented higher nitrotyrosine content than those in saline control; however, there was no difference when compared with those in nor-NOHA treatment.

DISCUSSION

The present study demonstrated a reduction in arginase activity caused by an inhibitor of arginase, nor-NOHA, which attenuated pathophysiological findings of asthma such as AHR, goblet cell hyperplasia, and airway inflammation in a Df-exposed animal model via the airways. There is some evidence from studies *in vivo* that arginase inhibitors attenuate allergic symptoms (3, 19). In an OVA-induced asthmatic model in guinea pigs, pretreatment with an arginase inhibitor, 2(s)-amino-6-boronohexanoic acid, inhibited early- and late-phase asthmatic reactions, AHR, and inflammatory cell infiltration (19). Another arginase inhibitor, S-(2-boronoethyl)-L-cysteine (BEC), attenuated AHR in the central airway in an acute mouse model and in the central airway and peripheral airways in a chronic mouse model (26). In our study, although the experimental animal model and allergens were different from previ-

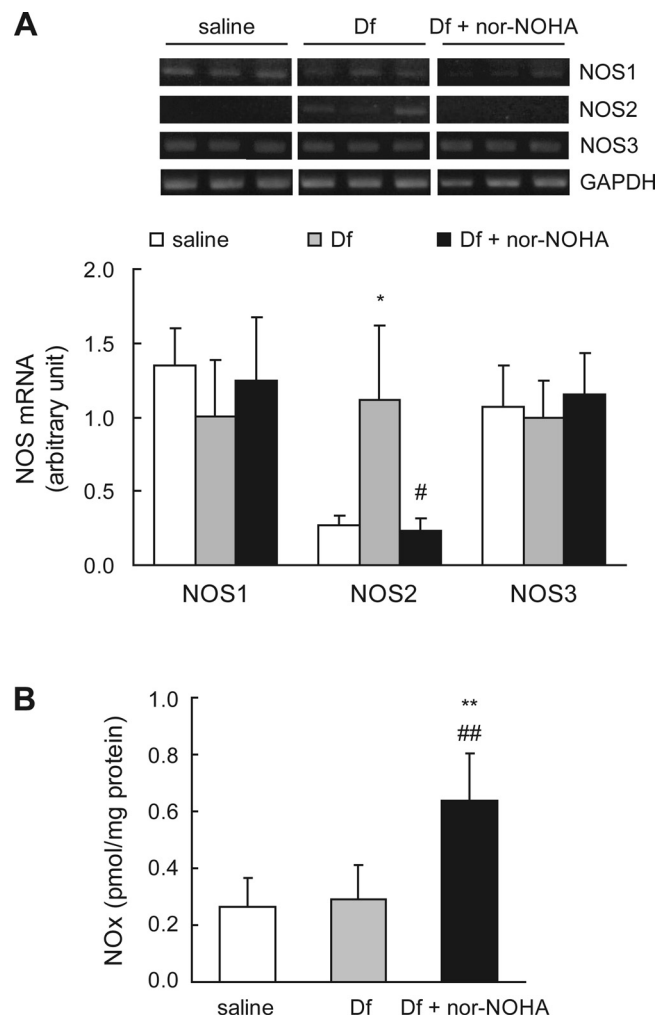


Fig. 6. Effect of nor-NOHA on Df-induced mRNA expression of nitric oxide synthase (NOS) isoforms and NOx concentrations in lung tissue. NOS (1, 2, 3) are shown in *A*. NOx levels are in *B*. Each value is the mean $\pm$ SD for 5–7 mice. * P < 0.05, ** P < 0.01 vs. the control group. # P < 0.05, ## P < 0.05 vs. the Df-exposed group.

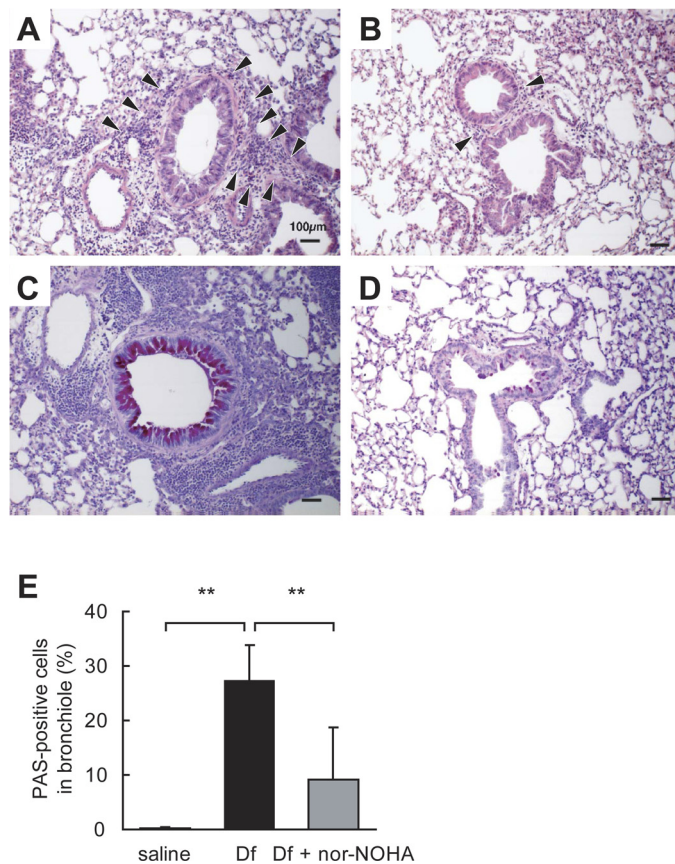


Fig. 7. Histopathology of lungs from Df-exposed and nor-NOHA-treated Df-exposed NC/Nga mice. H&E-stained lung sections from the Df-exposed mice (A) and nor-NOHA-treated Df-exposed mice (B). Arrowheads indicate the accumulation of inflammatory cells. Periodic acid-Schiff (PAS)-stained lung sections from the Df-exposed mice (C) and nor-NOHA-treated Df-exposed mice (D) are shown. The red-violet color in the bronchiolar epithelium indicates the existence of goblet cells. The ratio of PAS-positive cells was determined with ImageJ software (E). H&E-stained peribronchial and perivascular inflammatory cell layers were counted to define the inflammatory score shown in Fig. 3B.

ous studies, treatment with nor-NOHA, an arginase inhibitor used by two other reports (20, 23), had the same inhibitory effect on AHR. However, BEC did not inhibit AHR, but augmented inflammation in another OVA-induced model of allergic asthma in mice, although BEC inhibited IgE and IL-4 (5). In this report, the dose of BEC inhaled was 2.7 $\mu\text{g}/\text{mouse}$, lower than that of 800 $\mu\text{g}/\text{mouse}$ in the study mentioned above (26). An inadequate dose of arginase inhibitor was suggested from data indicating that the inhibition rate for arginase activity by BEC worsened from 24 to 48 h (5). However, to clarify this discrepancy, comparative studies evaluating these inhibitors in the same experimental model are needed.

In this study, inflammatory findings such as BALF cell numbers, tissue levels of Th2 cytokines and proinflammatory cytokines, and histopathological inflammatory scores decreased after treatment with nor-NOHA. Since nor-NOHA is an inhibitor of arginase, a reduction in arginase activity was expected. In support of our findings, an attenuation of AHR and cell numbers in BALF was observed on nor-NOHA treatment in OVA-exposed C57BL/6 mice (3).

However, a reduction was observed not only in arginase activity, but also in protein and mRNA levels for arginase I.

Changes in arginase expression have been a central topic in the study of asthma. Upregulation of arginase expression or enhancement of arginase activity has been observed in various species, including humans, mice, guinea pigs, and rats (18). In previous studies, the expression of arginase I was regulated by Th2 cytokines. IL-4 and IL-13 activate a nuclear transcription factor, STAT6, through common receptors (24), and the activated STAT6 is involved in the expression of arginase I (11). In this study, upregulation or downregulation of IL-4, IL-13, and arginase I expression was paralleled by the treatment. Therefore, although the mechanism for the reduction in levels of Th2 cytokines is not clear, the reduction in arginase I on treatment with nor-NOHA may be due to attenuated expression of IL-4 and IL-13.

Concerning the production of NO, we observed an increase of NOx levels in the lung homogenate on treatment with Df and nor-NOHA, compared with levels in Df-treated mice. The inhibitor nor-NOHA binds to the active site of arginase competitively (7), and this may lead to an increase in the bioavailability of L-arginine to NOS. Finally, elevated levels of NO resulted in the augmentation of NOx expression. In addition, an increase in NOS2 mRNA levels was observed in Df-treated mice, and the levels dropped following treatment with nor-NOHA. The transcription factor NF- κ B is involved in the regulation of NOS2 (45). NO modulates NF- κ B activity, including S-nitrosylation of the p50 subunit. This modification is related to suppressed phosphorylation and subsequent degradation of I κ B (21). Therefore, the significant reduction in NOS2 levels caused by treatment with nor-NOHA in our study suggests the involvement of S-nitrosylation and inactivation of NF- κ B by NO.

An internal NOS inhibitor, named asymmetric dimethylarginine (ADMA), has been identified. ADMA is a naturally occurring analog of L-arginine and a competitive inhibitor of

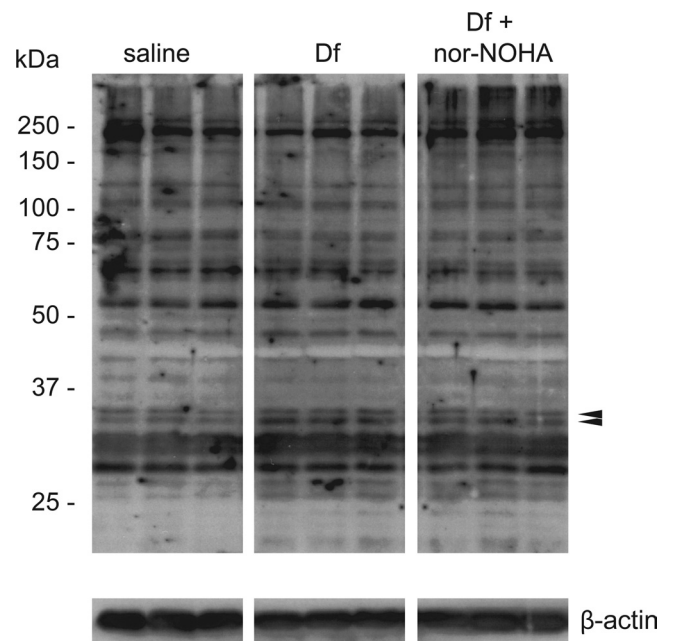


Fig. 8. Effect of nor-NOHA on Df-induced nitrotyrosine production in the lung. Arrowheads indicate the protein bands that intensified significantly on exposure to Df. Equal protein loading was confirmed based on the expression of β -actin as an internal standard.

all isoforms of NOS. A study of ADMA found augmentation of AHR and collagen deposits in the absence of inflammation (38). The increase in AHR seems to be caused by three mechanisms: 1) direct inhibition of NOS and following insufficiency of NO (39), 2) the enhanced availability of L-arginine to arginase, and 3) a reduction in NOHA production, with these mechanisms contributing to the increased activity of arginase. NOHA, the intermediate in the NOS-catalyzed reaction leading from L-arginine to NO, is a potent competitive inhibitor of arginase (4, 7). This suggests ADMA to be involved in asthma through NOS inhibition and enhancement of arginase activity. Since the level of ADMA in the lungs was not determined in mice in the present study, the contribution of ADMA is unknown.

Tyrosine residues in protein are nitrated by peroxynitrite (ONOO^-), and nitrotyrosine is formed. ONOO^- , a reactive nitrogen species formed via the reaction of superoxide (O_2^-) with NO (30), is thought to be the main reason for the detection of nitrotyrosine. Therefore, nitrotyrosine is considered to be a biomarker of the formation of ONOO^- in tissue. However, an alternative pathway for nitrotyrosine production is peroxidase-dependent oxidation of nitrite in the presence of hydrogen peroxide (H_2O_2) (37). Myeloperoxidase in neutrophils or macrophages and eosinophil peroxidase in eosinophils are known to be involved in this pathway (37, 41). Previously, we demonstrated increased levels of nitrotyrosine in lung tissue using an HPLC-electrochemical detection method (35). In the present study, Western blotting was performed to determine whether certain proteins had a higher nitrotyrosine content in the Df-exposed group than control group. Increased nitrotyrosine levels were detected as proteins with low-molecular-weights. Therefore, ONOO^- and a peroxidase-dependent system in infiltrated macrophages or granulocytes may have contributed. However, given the upregulation of arginase expression, some other mechanism should be considered. Uncoupled NOS is formed under conditions such as ischemia, loss of cofactor BH₄, and low levels of L-arginine (27, 42, 43, 44). Superoxide generated from the uncoupled oxidase domain of NOS and NO from coupled NOS can easily react with each other to form ONOO^- . Recently, production of ONOO^- from uncoupled NOS was shown in a guinea pig model (20). Inhibition of L-arginine uptake and Th2-induced upregulation of arginase expression were involved in this process and led to the formation of NO and superoxide from NOS2. Although L-arginine levels in lung tissue were not determined here, the levels in inflamed lung tissue may not have changed with a shift in substrate from arginase to NOS to repair uncoupled NOS because the intensity of immunoreactive protein bands for nitrotyrosine was not changed by treatment with an arginase inhibitor, nor-NOHA. Additionally, augmentation of nitrosative stress was not observed after treatment with nor-NOHA, contrary to a previous study (5). As mentioned, upregulation of arginase expression is related to the depletion of L-arginine for NOS and subsequent formation of ONOO^- from uncoupled NOS. However, the contribution of arginase to the formation of nitrotyrosine may be slight.

We demonstrated improvements in AHR and an anti-inflammatory effect on treatment with an arginase inhibitor, nor-NOHA, in Df-exposed mice. We also observed the expression of NOx, a metabolite of NO, on supplemental treatment with nor-NOHA, indicating the restoration of arginine's availability

to NOS. The expression of Th2 cytokines (IL-4, IL-5, and IL-13) was reduced by nor-NOHA. These extensive effects may be beneficial and contribute to the relief of asthmatic symptoms. However, elucidation of the precise mechanisms behind the anti-inflammatory effect of nor-NOHA and comparative studies of several arginase inhibitors in animal models are needed to evaluate candidate drugs for treating asthma.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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