

EXPRESSION OF NITRIC OXIDE SYNTHASES IN NASAL MUCOSA FROM A MOUSE MODEL OF ALLERGIC RHINITIS

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Nitric oxide (NO), which is produced by nitric oxide synthase (NOS), has been recently identified as a multifunctional mediator. As for nasal tissue, however, the distribution and expression patterns of 3 isoforms of NOS, including neuronal NOS (nNOS, type I NOS), inducible NOS (iNOS, type II NOS), and endothelial NOS (eNOS, type III NOS), are still unclear. To evaluate the function of NO in the pathophysiology of nasal allergy, we investigated the distribution of NOSs in the nasal mucosa of C57BL/6 mice with allergic rhinitis to the house dust mite, *Dermatophagoides farinae*. Immunoreactivity to each isoform of NOS was immunohistochemically observed. In the allergic nasal mucosa, many eosinophils had infiltrated. Immunoreactivity to NOS types I and III was localized to the surface epithelial and vascular endothelial cells in both allergic and control groups without a statistically significant difference. In contrast, the type II NOS immunoreactivity was weak in normal mice and increased after allergic sensitization. The type II NOS expression of the surface epithelial and vascular endothelial cells was significantly elevated in the allergic group as compared with the control group. These findings suggest that a large amount of NO may be produced in the nasal mucosa of mice by type II NOS after allergic sensitization and that type II NOS may play an important role in the pathogenesis of allergic rhinitis.

KEY WORDS — allergic rhinitis, nasal epithelial cell, nitric oxide, nitric oxide synthase.

INTRODUCTION

Nitric oxide (NO) is known as a defensive molecule against parasites, tumor cells, intracellular microorganisms, and viruses. This defensive function of NO is mediated by cytotoxic metabolites of NO such as peroxynitrite and nitrosonium.¹ The nitrous metabolites with biological activities can inhibit iron sulfate-containing enzymes such as hemoglobin, inhibit electron transfer in mitochondria, and suppress cellular replication by activating ribonucleotide reductase.² Nitric oxide can also function as an immunomodulator. It can inhibit leukocyte adhesion on the vascular endothelial cells in inflammatory processes and can increase activities of natural killer cells or lymphokine-activated killer cells.

Many studies on the biological function of NO have been recently performed in the rhinologic field. The expiratory air in humans contains a large amount of NO, which is mostly produced by the paranasal sinuses or nasal cavities.^{3,4} In particular, NO is produced more by the nasal cavities of patients with allergic rhinitis than by those of normal subjects.^{5,6}

However, it is not definite which cells in the nasal cavity mucosa produce NO and what the role of NO is in the pathogenesis of allergic rhinitis. There have been several studies identifying the presence of 3 isoforms of nitric oxide synthase (NOS), but the reports

show different results according to the subjects and analytical methods. Therefore, this immunohistochemical study was performed in a murine model of experimentally induced allergic rhinitis in order to standardize the experimental conditions. We observed the distribution of NOS isoforms in the nasal cavity mucosa of both normal mice and mice with allergic rhinitis in order to identify the role of NO in the pathogenesis of allergic rhinitis.

MATERIALS AND METHODS

Twenty C57BL/6 male mice were used. All of them weighed between 20 and 30 g and were specific pathogen-free. All procedures followed the Principles of Laboratory Animal Care of Seoul National University. A lyophilized crude body extract of *Dermatophagoides farinae* (Allergopharma, Reinbek, Germany) was used as an allergen. The allergen was stored at 4°C and solubilized in phosphate-buffered saline solution (PBS).

Allergic rhinitis was induced in the mice by systemic sensitization. Ten mice were injected at their tail base with a mixture of 100 µg of *D. farinae* in 100 µL of PBS and 100 µg of complete Freund's adjuvant (Pierce, Rockford, Illinois). At 24 and 72 hours, respectively, 300 mg of pertussis toxin in PBS was intraperitoneally injected. After 7 days, the mice were injected at their tail base with a mixture of 100

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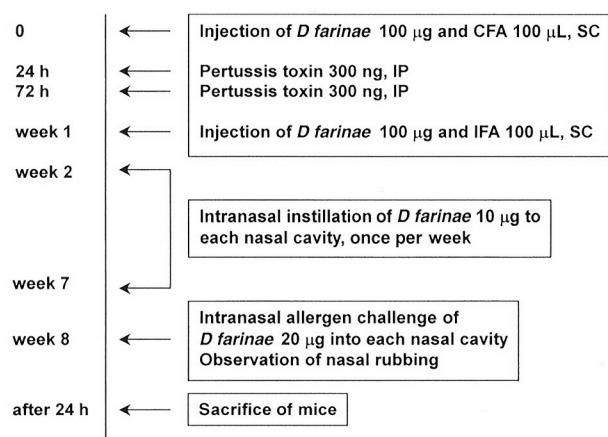


Fig 1. Protocol for induction of nasal allergy in mice. CFA — complete Freund's adjuvant; SC — subcutaneously; IP — intraperitoneally; IFA — incomplete Freund's adjuvant.

µg of *D farinae* in 100 µL of PBS and 100 µg of incomplete Freund's adjuvant (Pierce). After the induction of general sensitization, each of the *D farinae* allergens was instilled into the nasal cavities at a concentration of 1 µg/µL once every week for 6 weeks (Fig 1). The mice included in the control group were given PBS instead of *D farinae* allergen during intranasal instillation.⁷ After the final intranasal challenge of *D farinae* allergen, an immediate allergic response was observed. The mice were given 20 µg of *D farinae* allergen (1 µg/µL) intranasally into each of the nasal cavities with a micropipette. After 15 minutes, symptom scores were calculated from the frequency of nasal rubbing over 15 minutes.

The mice were painlessly sacrificed at 24 hours, and the nasal cavity mucosa was obtained. The mucosa was immediately fixed in 10% formalin in PBS and embedded in paraffin for routine histologic examination and immunohistochemical analysis. Paraffin sections (4 µm thick) were first stained with hematoxylin-eosin so we could count the number of infiltrated eosinophils. The number of eosinophils was measured at 5 different areas under a magnification of 200 by 2 pathologists independently, and the average was calculated.

The sections were immunostained with the avidin-biotinylated horseradish peroxidase complex method using rabbit polyclonal antiserum against NOS types I (nNOS, 1/100), II (iNOS, 1/100), and III (eNOS, 1/100; all from Transduction Laboratories, Lexington, Kentucky). After 3 washes in PBS, the sections were incubated with biotinylated goat anti-rabbit IgG antiserum, followed by treatment with the avidin-biotin-peroxidase complex (Dako, Copenhagen, Denmark) according to the manufacturer's instructions. The sections were visualized with 3,3'-diaminobenzidine and hydrogen peroxide. Normal

IMMUNOREACTIVITY TO NOS IN NASAL MUCOSAE OF CONTROL AND ALLERGIC GROUPS

Group	NOS	Epithelium	Endothelium	Gland
Control	eNOS (type III)	2.1 ± 0.6	2.2 ± 0.6	0
	nNOS (type I)	2.3 ± 0.6	2.1 ± 0.5	0
	iNOS (type II)	0.4 ± 0.5	0.3 ± 0.5	0.3 ± 0.5
Allergy	eNOS (type III)	2.1 ± 0.5	2.3 ± 0.6	0
	nNOS (type I)	2.3 ± 0.6	2.1 ± 0.6	0
	iNOS (type II)	2.6 ± 0.6*	2.5 ± 0.6*	0.4 ± 0.5

NOS — nitric oxide synthase; eNOS — endothelial NOS; nNOS — neuronal NOS; iNOS — inducible NOS.

*p < .05 compared with control group.

rabbit IgG was used as a negative control.^{8,9}

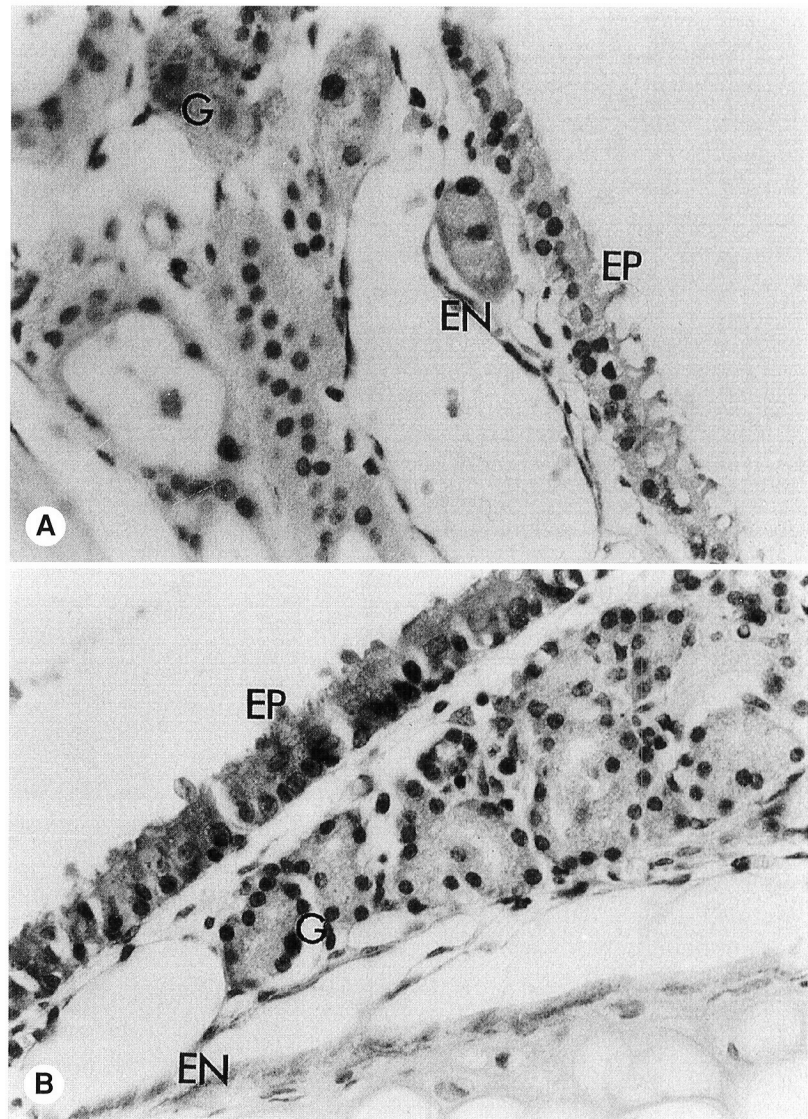
The intensity of immunoreactivity was graded semiquantitatively from 0 to 3, with 0 representing no immunoreactivity and 3 representing strong immunoreactivity in comparison with the negative control. A nonparametric Mann-Whitney U test was used to compare the allergic rhinitis and control groups. Statistical significance was taken to be p < .05.

RESULTS

The allergic symptom scores were significantly higher in the allergic rhinitis group than in the negative control group. The average symptom scores were 21.8 ± 6.6 in the allergic rhinitis group and 4.6 ± 2.5 in the control group (p < .05). Light microscopic sections showed severe infiltration of chronic inflammatory cells, especially eosinophils. The average number of eosinophils was 41.4 ± 9.7 in the allergic rhinitis mucosa and 4.6 ± 1.5 in the control mucosa (p < .05).

The Table shows the immunoreactivity to NOS in the nasal mucosae of the control and allergic groups. Type III NOS was mainly localized to the surface epithelium (2.1 ± 0.6) and vascular endothelium (2.2 ± 0.6) in the control mucosa. There was no type III NOS immunoreactivity in the glandular tissue of control and allergic rhinitis mucosae. Type III NOS was also strongly expressed in the surface epithelium (2.1 ± 0.5) and vascular endothelium (2.3 ± 0.6) of allergic rhinitis mucosa. Type I NOS showed the same pattern as type III NOS. Immunoreactivity to type I NOS was strong in the surface epithelium (2.3 ± 0.6) of allergic rhinitis mucosa and vascular endothelium (2.1 ± 0.5), whereas it was absent in the glandular tissue. In the mucosa of mice with induced allergic rhinitis, immunoreactivity to type I NOS was also strong in the surface epithelium (2.3 ± 0.6) and

Fig 2. Immunoreactivity to inducible nitric oxide synthase in nasal mucosa (original $\times 400$). EP — epithelium; EN — endothelium; G — glands. **A)** Control group. In contrast to endothelial nitric oxide synthase or neuronal nitric oxide synthase, inducible nitric oxide synthase is expressed weakly in epithelial cells, endothelial cells, and submucosal glands. **B)** Allergy group. Epithelial and endothelial cells show strong immunoreactivity.



vascular endothelium (2.1 ± 0.6), whereas it was absent in the glandular tissue.

The control mucosa showed weak immunoreactivity to type II NOS in the surface epithelium (0.4 ± 0.5), vascular endothelium (0.3 ± 0.5), and glandular tissue (0.3 ± 0.5 ; Fig 2A). Type II NOS caused significantly stronger immunoreactivity in the surface epithelium (2.6 ± 0.6) and vascular endothelium (2.5 ± 0.6) of allergic rhinitis mucosa than in those of normal control mucosa ($p < .05$). Glandular tissue showed the same extent of immunoreactivity (0.4 ± 0.5) in the allergic rhinitis mucosa as in the normal mucosa (Fig 2B).

DISCUSSION

Immunohistochemical studies have shown that isoforms of NOS are present in the nasal cavity mucosa.¹⁰ Since the surface area of the nasal cavity mucosa is wider than that of the paranasal sinus mucosa, na-

sal cavity mucosa may mainly contribute to the production of NO of the expiratory air.^{4,11} The presence of NOS isoforms in human and animal nasal cavity mucosae has been found in many studies. However, the distribution of each isoform of NOS is different according to the reports.

Lundberg et al³ showed no expression of type I NOS in the human nasal mucosa, but other researchers demonstrated that type I NOS was consistently expressed in the vascular endothelium, surface epithelium, and submucosal gland tissue of normal rat and mouse nasal mucosae.^{8,12,13} Type I NOS was also present in the nasal cavity mucosa of rats with allergic rhinitis.¹³

Type III NOS was reported to be equally present in the vascular endothelium, surface epithelium, and submucosal glandular tissue.^{10,14} However, Sawada et al⁸ showed that type III NOS was localized to the surface epithelium, and Lundberg et al³ reported that

there was no expression of type III NOS in the normal human nasal mucosa. In animals, type III NOS was expressed in the vascular endothelium and submucosal glandular tissue of rats¹³ and in the vascular endothelium of guinea pigs.¹⁵ As for allergic rhinitis, type III NOS was present in the vascular endothelium, surface epithelium, and submucosal glandular tissue of patients with allergic rhinitis,¹⁴ and rats with induced allergic rhinitis also showed the same distribution as did humans.

Our study demonstrated that NOS types I and III are present in the surface epithelium and vascular endothelium of both normal and allergic rhinitis mucosae without a significant difference. These results of our study and other researchers suggest that NOS types I and III may participate in normal physiologic function of nasal cavity mucosa through maintaining homeostasis by regulating nasal blood flow and possibly through mucociliary clearance. In addition, the coexistence of constitutive and inducible NOS in epithelial cells may reflect a complex mechanism promoted by surface epithelial cells to protect the host from noxious assault, as well as to prevent the induction of airway hyperreactivity.¹⁶ Type II NOS shows various expression patterns. Yuasa et al¹³ showed that type II NOS was present in the surface epithelium of normal rat nasal mucosa, and Sawada et al⁸ demonstrated that it was not present in the surface epithelium, vascular endothelium, or submucosal glandular tissue. Other researchers reported very weak expression of type II NOS in normal human nasal mucosa.^{3,14,15} In patients with allergic rhinitis, the expression of type II NOS increased only in the surface epithelium.¹⁴ The presence of macrophages with type II NOS in the submucosa of rats with allergic rhinitis may reveal the participation of this NOS in the pathogenesis of allergic rhinitis.¹³

In our study, the expression of type II NOS was

very weak in the surface epithelium, vascular endothelium, and submucosal glandular tissue of the normal mucosa and in the submucosal glandular tissue of the allergic rhinitis mucosa. The surface epithelium showed increased immunoreactivity to type II NOS after allergic rhinitis was induced. Our study showed the peculiar finding that the expression of type II NOS increased also in the vascular endothelium after induction of allergic rhinitis. This suggests that NO produced by type II NOS in the vascular endothelium may be directly involved in the pathogenesis of the allergic response. This NO may increase vascular permeability in allergic inflammation. The concentration of NO measured in the air from the nasal cavity increased in allergic rhinitis⁵ and decreased after topical use of steroid spray in the nasal cavity.⁶ Therefore, this finding suggests that NO may be produced in the nasal cavity mucosa during allergic inflammation. Type II NOS seems to mainly account for the increased NO production, because steroids can suppress the activity of type II NOS, and not that of types I and III.^{6,17} In regard to the activity of type II NOS in the submucosal tissue, the expression of type II NOS was very weak in our animal study. However, Kang et al¹⁸ demonstrated in a human study that type II NOS increased during allergic inflammation and that it may be involved in the secretory function of the submucosal gland. There is a possibility that NO may act on secretion by the submucosal gland during allergic inflammation.

In conclusion, NOS types I and III are present in the nasal cavity irrespective of the presence of allergic inflammation, and this finding suggests that they may play homeostatic roles in the normal physiology of the nasal cavity. Type II NOS is expressed in the surface epithelium and vascular endothelium during allergic inflammation, and this finding suggests that it may play a crucial role in mediating allergic inflammation.

ACKNOWLEDGMENT — The authors thank Sarah B. Jung for excellent word processing.

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