

Increased expression of inducible nitric oxide synthase in nasal mucosae of guinea pigs with induced allergic rhinitis

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ABSTRACT

Background: Nitric oxide (NO) is produced by the action of NO synthase (NOS) isoforms and is considered an important mediator of inflammatory response including airways. In this study, the changes in the expression levels of NOS isoforms in nasal mucosae were determined in a guinea pig model of allergic rhinitis.

Methods: An allergic rhinitis model was prepared in guinea pigs by repeated challenge with aerosolized dinitrophenylated ovalbumin antigen. Twenty-four hours after the last antigen challenge, the expression levels of NOS isoforms in nasal mucosae were determined by immunoblottings. Changes in the isometrical tension of isolated mucosal tissues of nasal septa induced by histamine were measured also.

Results: Although the expression levels of endothelial NOS (eNOS) and neuronal NOS (nNOS) in nasal mucosae were not affected by the repeated antigen exposure, the inducible NOS (iNOS) level was markedly and significantly increased in the challenged animals. In isolated nasal mucosal tissues, histamine induced a concentration-dependent relaxation, which was sensitive to an H_1 -receptor antagonist, mepyramine, and an NOS inhibitor, L-NMMA. No significant change in the histamine responsiveness was observed between the sensitized control and repeatedly antigen-challenged groups.

Conclusion: The expression of three isoforms of NOS, including eNOS, nNOS, and iNOS, was presented in guinea pig nasal mucosa. A marked increase in iNOS expression in the repeatedly antigen-challenged animals suggests an important role of iNOS in the pathogenesis of allergic rhinitis. However, the pathophysiological role(s) of NO generated by iNOS in nasal allergy is still unclear.

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Allergic rhinitis is characterized by sneezing, rhinorrhea, and nasal obstruction. The nasal obstruction associated with allergic rhinitis probably is caused by the dilatation of plexus cavernosum and mucosal congestion by the increase in nasal microvascular permeability although the exact mechanism(s) is not clear now. The nasal obstruction is thought to decline the quality of life (QOL) in patients with allergic rhinitis. However, there is little effective medication for the nasal obstruction in allergic rhinitis.

High levels of nitric oxide (NO) have been found in the nasal airways and paranasal sinuses as compared with the lower airways in normal subjects,^{1–3} indicating that NO contributes to physiological processes in the upper airways. NO is produced by the action of NO synthase (NOS) isoforms and is considered an important mediator of physiological and pathological functions including airways. One of these functions is the relaxation of vascular smooth muscle cells, regulating blood flow through vessels.^{4,5} Thus, NOS and NO also may play an important role in nasal obstruction in allergic rhinitis. In fact, an increased nasal NO level has been reported in patients with allergic rhinitis,^{6,7} although the different results also have been obtained.^{8,9} Recently, three isoforms of NOS, *i.e.*, the endothelial NOS (eNOS; type III NOS), the neuronal NOS (nNOS; type I NOS), and the inducible NOS (iNOS; type II NOS), have been identified. The eNOS and nNOS are constitutively expressed mainly in endothelial/

epithelial cells and some neurons, respectively, and generate relatively low levels of NO, whereas the iNOS generally is induced transcriptionally by inflammatory stimuli and generates a large amount of NO continuously.^{10,11} Therefore, the high levels of NO in nasal airways of patients with allergic rhinitis may reflect the augmented expression of iNOS isoform. However, the nasal expression levels of each NOS isoform in allergic rhinitis still are unclear. In this study, the changes in the expression levels of NOS isoforms in nasal mucosae were determined in guinea pigs with induced allergic rhinitis.¹² Moreover, the NO-mediated vascular relaxation of nasal mucosa isolated from this animal model, which has an *in vivo* nasal hyperresponsiveness,¹² was compared with that from control guinea pigs.

MATERIALS AND METHODS

Animals

Male Hartley guinea pigs (250–350 g) were purchased from Tokyo Laboratory Animals, Inc., Japan. The animals were housed in a temperature ($23 \pm 1^\circ\text{C}$)- and humidity ($60 \pm 10\%$)-controlled rooms with free access to food and water. All experiments were approved by the Animal Care Committee at the Hoshi University (Tokyo, Japan).

Sensitization and Antigen Challenge

An allergic rhinitis model was prepared as previously reported.¹² In brief, guinea pigs were sensitized by inhalation of dinitrophenylated ovalbumin antigen (DNP-OVA; 10 mg of protein/mL) with an ultrasonic nebulizer (TUR-3000; Nihon Kohden, Tokyo, Japan) for 10 minutes under the unanesthetized condition in a Plexiglas box (300 × 200 mm, 150-mm height; day 0). Seven days after the sensitization (day 7), the

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animals were boosted by the same inhalation procedure described previously. Then, the animals received a total of 21 times challenges with aerosolized antigen (each for 3 minutes) every 24 hours for 3 weeks from days 14 to 34 (challenged group). The antigen concentrations used in the challenge period were 0.5 mg of protein/mL at the first antigen challenge (day 14) and 1 of protein/mL on the other days (days 15–34). Twenty-four hours after the last antigen challenge (day 35), the following experiments were performed. Control animals received the same immunization on days 0 and 7 but were inhaled saline aerosol on days 14–34 instead of DNP-OVA challenge (sensitized control group).

Western Blot Analyses

The protein levels of eNOS, nNOS, and iNOS in nasal mucosae were measured by Western blot analyses as previously reported¹³ with minor modification. In brief, 24 hours after the last antigen challenge, nasal mucosal tissues were isolated from guinea pigs under urethane (1.6 g/kg, i.p.) anesthesia. The mucosal tissue segments were quickly frozen with liquid nitrogen, and the tissue was crushed to pieces by CryopressTM (CP-100Wk, 15 seconds $\times$ 3; Microtec, Co., Ltd., Chiba, Japan). The tissue powder was homogenized in ice-cold tris(hydroxymethyl)aminomethane (Tris, 10 mM; pH 7.5) buffer containing 5 mM of $MgCl_2$, 2 mM of EGTA, 250 mM of sucrose, 1 mM of dithiothreitol, 1 mM of 4-(2-aminoethyl)benzenesulfonyl fluoride, 20 μ g/mL of leupeptin, 20 μ g/mL of aprotinin, 1% Triton X-100, and 1% sodium cholate. The tissue homogenate was then centrifuged (3000 $\times$ g, 4°C for 15 minutes) and the resultant supernatant was stored at –85°C until use. The preparations were dissolved in SDS sample buffer and heated at 100°C for 4 minutes. The samples (20 μ g of protein per lane) were then subjected to 7.5% SDS-PAGE. Proteins were then electrophoretically transferred for 4 hours to PVDF membranes (Amersham, Bucks, UK) in cold transfer buffer (20% methanol containing 25 mM Tris and 192 mM glycine). After five washes with Tris buffer (20 mM of Tris, 500 mM of NaCl, pH 7.5), the nitrocellulose membranes were incubated with blocking buffer (3% gelatin in Tris buffer) for 1 hour at room temperature. The PVDF membranes were then incubated with the primary antibodies in antibody buffer (1% gelatin, 0.1% Tween 20 in Tris buffer) for 2 hours at room temperature. The primary antibodies used were polyclonal rabbit antibodies against nNOS (1:2000 dilution), eNOS (1:2000 dilution), and iNOS (1:1000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The PVDF membranes were then washed four times (each time for 15 minutes) with washing buffer (0.1% Tween 20 in Tris buffer), incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:5000 dilution; Amersham) for 1 hour at room temperature, and then washed four times with washing buffer. The images were developed using an enhanced chemiluminescent method (ECL System; Amersham). The resultant images were analyzed by densitometry system (Atto Densitograph Software for the Macintosh version 4.0; Atto Co., Tokyo, Japan).

In Vitro Measurement of Changes in Tension of Isolated Nasal Septum Mucosae

Changes in the vascular smooth muscle tension of isolated nasal septum mucosae were measured by the method de-

scribed previously¹⁴ with minor modification. In brief, nasal septa with mucosa were isolated carefully from guinea pigs under urethane (1.6 g/kg, i.p.) anesthesia. The nasal mucosa was dissected from the nasal septum carefully and an $\sim 5 \times 5$ mm of tissue strip was suspended in an organ bath at a resting tension of 0.5 g. The organ bath contained modified Krebs-Henseleit solution with the following composition (mM): NaCl, 118.0; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 25.0; KH_2PO_4 , 1.2; and glucose, 10.0 (pH 7.4). The isometrical contraction of the nasal mucosa was measured as described previously.¹⁵ After an equilibration period, smooth muscle responsiveness to cumulatively administered histamine (10^{-7} – 10^{-4} M) or sodium nitroprusside (SNP; 10^{-8} – 10^{-3} M) was determined in norepinephrine (3×10^{-3} M)-precontracted tissues. A higher concentration of histamine or SNP was successively added after attainment of a plateau response to the previous concentration. In some experiments, NG-monomethyl-L-arginine (10^{-4} M; an NOS inhibitor), L-arginine (10^{-3} M; a substrate for NOS), mepyramine (10^{-6} M; a histamine H_1 -receptor antagonist), and/or cimetidine (10^{-6} M; a histamine H_2 -receptor antagonist) was pretreated 30 minutes before application of norepinephrine. The changes in the tension induced by histamine were expressed as percent of maximal contraction induced by norepinephrine.

Statistical Analyses

All data are expressed as the mean with SE. Statistical significance of difference was determined by unpaired Student's *t*-test or two-way ANOVA.

RESULTS

Effects of Repeated Antigen Exposure on the Expression of NOS Isoforms in Guinea Pig Nasal Mucosa

The changes in the expression of NOS isoforms in nasal mucosae were assessed by using Western blot analyses (Figs. 1–3). In the sensitized control guinea pigs, a distinct single 132-kDa band corresponding to eNOS was observed in the homogenate of nasal mucosa (Fig. 1). Distinct nNOS (150 kDa, Fig. 2) and iNOS (130 kDa, Fig. 3) bands also were detected in these tissues. All of these NOS isoforms were expressed also in nasal mucosae of the repeatedly antigen-challenged guinea pigs. Although the expression levels of eNOS and nNOS were not affected by the repeated antigen exposure, the iNOS level was markedly and significantly increased in the challenged animals (Fig. 3).

Involvement of NO in Histamine-Induced Relaxation of Isolated Guinea Pig Nasal Mucosa

To determine an involvement of NO in histamine response of nasal mucosa, the effects of L-NMMA and L-arginine on histamine-induced relaxation of isolated guinea pig septum mucosae were investigated by using standard organ-bath technique. Because histamine had no effect on basal tension of the isolated nasal mucosa (data not shown), the tissue preparations were precontracted by norepinephrine (3×10^{-5} M) before application of histamine. As shown in Figs. 4 and 5, histamine (10^{-7} – 10^{-4} M) induced a concentration-dependent relaxation of nasal mucosae of

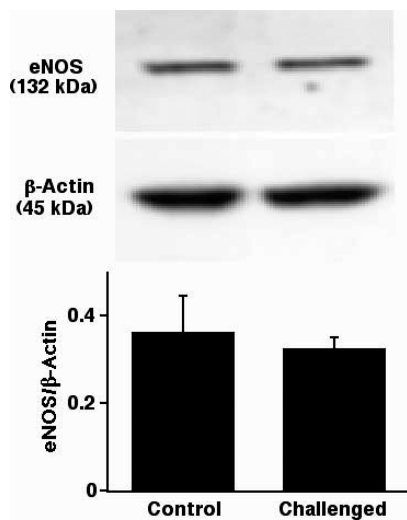


Figure 1. Expression of eNOS in guinea pig nasal mucosae. The upper panel shows typical immunoblots of eNOS and β -actin in nasal mucosae of sensitized control (control; the left lane) and repeatedly antigen-challenged animals (challenged; the right lane). Nasal mucosal homogenates (30 μ g of protein per lane) were subjected to 7.5% SDS-PAGE and immunoblottings were performed by using specific antibodies against eNOS and β -actin. After the band densities were measured by densitometry, relative expression levels of eNOS to β -actin were calculated in each lane and summarized in the lower panel. Data represent the mean $\pm$ SE from four animals, respectively. No significant difference in the eNOS expression was observed between groups.

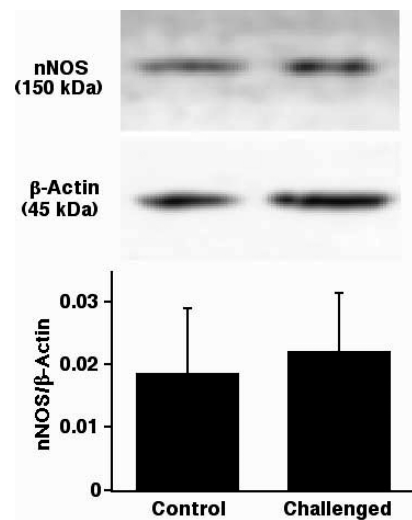


Figure 2. Expression of nNOS in guinea pig nasal mucosae. The upper panel shows typical immunoblots of nNOS and β -actin in nasal mucosae of sensitized control (control; the left lane) and repeatedly antigen-challenged animals (challenged; the right lane). Nasal mucosal homogenates (30 μ g of protein per lane) were subjected to 7.5% SDS-PAGE and immunoblottings were performed by using specific antibodies against nNOS and β -actin. After the band densities were measured by densitometry, relative expression levels of nNOS to β -actin were calculated in each lane and summarized in the lower panel. Data represent the mean $\pm$ SE from four animals, respectively. No significant difference in the nNOS expression was observed between groups.

nonsensitized normal guinea pigs. The histamine-induced relaxation of nasal mucosa was significantly inhibited by treatment with an NOS inhibitor, L-NMMA (10^{-4} M), and the inhibitory effect of L-NMMA was reversed by L-arginine (10^{-3} M; Fig. 4), indicating that NO is involved in the histamine-induced relaxation. Moreover, the histamine-induced relaxation of guinea pig nasal mucosa also was inhibited by pretreatment with an H_1 -receptor antagonist, mepyramine (10^{-6} M), completely (Fig. 5).

Effects of Repeated Antigen Exposure on the Histamine-Induced Relaxation of Guinea Pig-Isolated Nasal Mucosa

The previous study showed an augmented histamine-induced increase in intranasal pressure in the repeatedly antigen-challenged guinea pigs.¹² To clarify the mechanism(s) of the augmented *in vivo* histamine responsiveness, the *in vitro* histamine responsiveness of mucosal tissues of nasal septa isolated from the repeatedly antigen-challenged guinea pigs was compared with that from the sensitized control animals. As shown in Fig. 6, histamine (10^{-7} – 10^{-4} M) induced a concentration-dependent relaxation of isolated septum mucosae both in the sensitized control and the repeatedly antigen-challenged guinea pigs. No significant difference in the histamine responsiveness was observed between groups. Likewise, application of an NO donor, SNP (10^{-8} – 10^{-3} M), also induced a concentration-dependent relaxation in both the groups (Fig. 7). As the case of histamine, no significant dif-

ference in the SNP responsiveness of isolated septum mucosa was observed between the sensitized control and the repeatedly antigen challenged animals.

DISCUSSION

The presence of NO in high concentrations has been found in the upper airways of normal subjects.^{1–3} The higher levels of NO in nasal mucosa also have been reported in patients with untreated allergic rhinitis.^{6,7} Although the exact role(s) of NO in the upper airways in normal and diseased patients still is unclear, its contributions to airway host defense,¹⁶ epithelial cell ciliary activity,¹⁷ and collagen expression in nasal fibroblast cells¹⁸ have been suggested. The current study also revealed an implication of NO in the nasal vascular relaxation in guinea pigs (Fig. 4).

This study clearly showed an increased expression of iNOS in nasal mucosa of a guinea pig allergic rhinitis model.¹² On the other hand, no change in the expression levels of eNOS and nNOS in nasal mucosa was observed in this animal model of allergic rhinitis. The results might be consistent with previous reports in upper and lower airways in allergy,^{13,19,20} suggesting that iNOS may play an important role in the pathogenesis of allergic airway diseases, such as asthma and rhinitis.

The smooth muscles of vasculature in nasal mucosa have been believed to be the only tissue that possesses contractility.²¹ An organ-bath technique has been used for determining the nasal vascular responsiveness in experimental animals.^{14,21} By using this method, it is possible to measure the

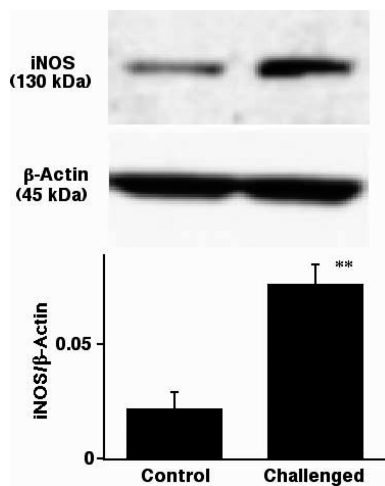


Figure 3. Expression of iNOS in guinea pig nasal mucosae. The upper panel shows typical immunoblots of iNOS and β -actin in nasal mucosae of sensitized control (control; the left lane) and repeatedly antigen-challenged animals (challenged; the right lane). Nasal mucosal homogenates (30 μ g of protein per lane) were subjected to 7.5% SDS-PAGE and immunoblottings were performed by using specific antibodies against iNOS and β -actin. After the band densities were measured by densitometry, relative expression levels of iNOS to β -actin were calculated in each lane and summarized in the lower panel. Data represent the mean $\pm$ SE from four animals, respectively. The iNOS expression in nasal mucosae was significantly increased in the repeatedly antigen-challenged guinea pigs ($p < 0.05$ by unpaired Student's *t*-test).

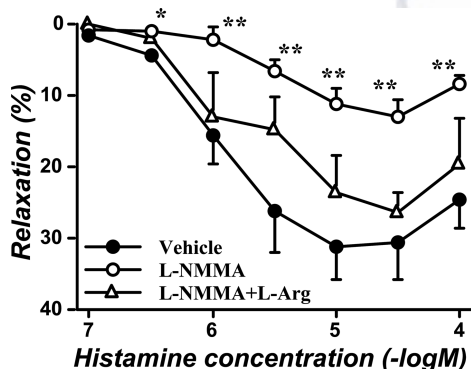


Figure 4. Involvement of NO in histamine-induced relaxation of isolated guinea pig nasal mucosa precontracted with norepinephrine (NE; 3×10^{-5} M). The histamine responsiveness of isolated septum mucosae from nonsensitized normal animals was measured in the absence (vehicle) or presence of N^G-monomethyl-L-arginine (L-NMMA; 10^{-4} M, an NOS inhibitor) or L-NMMA (10^{-4} M) plus L-arginine (L-Arg; 10^{-3} M, a substrate for NO). The relaxation was expressed as percent of maximal contraction induced by 3×10^{-5} M of NE. Each point represents the mean $\pm$ SE from six animals (* $p < 0.05$ and ** $p < 0.01$ versus vehicle group by unpaired Student's *t*-test). The histamine-induced relaxation was significantly inhibited by pretreatment with L-NMMA ($p < 0.01$ versus vehicle group by two-way ANOVA). The inhibitory effect of L-NMMA was reversed by cotreatment with L-arginine ($p < 0.01$ versus L-NMMA group by two-way ANOVA).

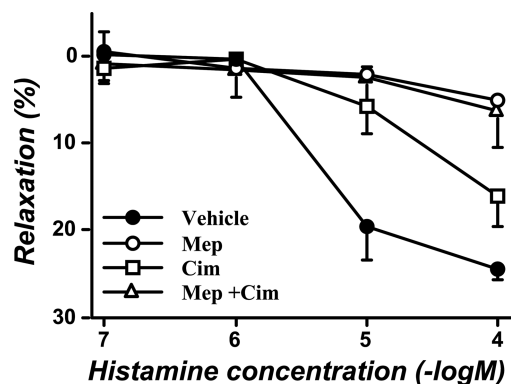


Figure 5. Involvement of H₁-receptors in histamine-induced relaxation of isolated guinea pig nasal mucosa precontracted with norepinephrine (NE; 3×10^{-5} M). The histamine responsiveness of isolated septum mucosae from nonsensitized normal animals was measured in the absence (vehicle) or presence of mepyramine (Mep; 10^{-6} M, an H₁-antagonist) and/or cimetidine (Cim; 10^{-6} M, an H₂-antagonist). The relaxation was expressed as percent of maximal contraction induced by 3×10^{-5} M of NE. Each point represents the mean $\pm$ SE from five animals. The histamine-induced relaxation was inhibited completely by pretreatment with Mep ($p < 0.01$ versus vehicle group by two-way ANOVA).

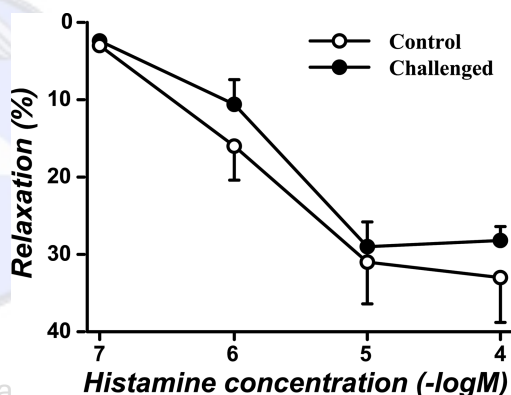


Figure 6. Effect of repeated antigen challenge on histamine responsiveness of isolated guinea pig nasal mucosa. The septum mucosae isolated from sensitized control (control) and repeatedly antigen-challenged guinea pigs (challenged) were precontracted with norepinephrine (NE; 3×10^{-5} M) before application of histamine. The relaxation was expressed as percent of maximal contraction induced by 3×10^{-5} M of NE. Each point represents the mean $\pm$ SE from 8 control and 10 challenged animals. No significant difference was observed between groups.

contraction and/or relaxation of vascular smooth muscle in nasal mucosa directly. In this study, the histamine-induced relaxation of the norepinephrine-precontracted mucosal tissue of the nonsensitized normal guinea pig was almost completely blocked by pretreatment with L-NMMA (an NOS inhibitor; Fig. 4) or mepyramine (an H₁-antagonist; Fig. 5), indicating that NO and H₁-receptors are involved in the histamine-induced relaxation. Distinct expression of NOS isoforms also was shown in guinea pig nasal mucosa (Figs. 1–3).

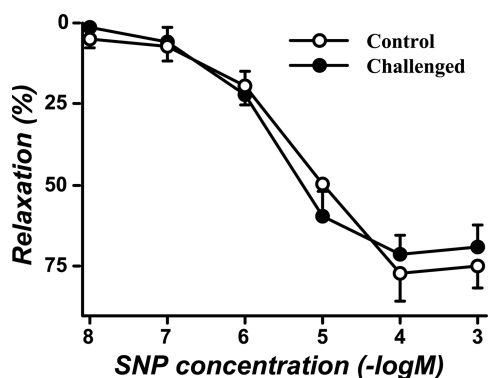


Figure 7. Effect of repeated antigen challenge on SNP responsiveness of isolated guinea pig nasal mucosa. The septum mucosae isolated from sensitized control (control) and repeatedly antigen-challenged guinea pigs (challenged) were precontracted with nor-epinephrine (NE; 3×10^{-5} M) before application of SNP. The relaxation was expressed as percent of maximal contraction induced by 3×10^{-5} M of NE. Each point represents the mean $\pm$ SE from four animals. No significant difference was observed between groups.

The localization of NOS in endothelium and epithelium has been reported in nasal mucosa.^{22,23} Thus, it is possible that stimulation of H_1 -receptors located on endothelial and epithelial cells²⁴ causes NO release from these cells, resulting in the relaxation of nasal vasculature.

An important finding of this study is that no change in the *in vitro* histamine responsiveness of isolated nasal mucosa was observed in this animal model of allergic rhinitis (Fig. 6). However, this result may not be a surprising event because the expression levels of eNOS and nNOS were not changed after the repeated antigen challenge (Figs. 1 and 2). Moreover, no change in mucosal responsiveness to SNP was observed in the repeatedly antigen-challenged group (Fig. 7), suggesting that the smooth muscle responsiveness of mucosal vasculature to NO itself also is normal in this animal model of allergic rhinitis. NO is generated by the action of NOS, using L-arginine as a substrate. eNOS and nNOS are the constitutively expressed isoforms and their activities depend on intracellular Ca^{2+} and calmodulin, whereas iNOS generally is induced transcriptionally by inflammatory stimuli and its activity is independent of intracellular Ca^{2+} concentration.²⁵ In general, stimulation of histamine H_1 -receptor increases intracellular Ca^{2+} concentration to activate calmodulin *via* an activation of a heterotrimeric GTP-binding protein, $G_{q/11}$. In this study, the histamine-induced mucosal tissue relaxation, which is sensitive to both mepyramine and L-NMMA (Figs. 4 and 5), reached to plateau rapidly (data not shown) and was observed in a histamine concentration-dependent manner. Thus, it is possible that the Ca^{2+} -dependent isoform(s), eNOS and/or nNOS, might mainly contribute to the histamine-induced relaxation of isolated nasal mucosa in guinea pigs.

Although the discrepancy between the histamine responsiveness *in vivo* (the augmented increase in intranasal pressure¹²) and *in vitro* (the normal relaxing response of isolated mucosal tissues; Fig. 6) in the guinea pig model of allergic

rhinitis is not explainable now, factors other than the smooth muscle tone of mucosal vasculature might be involved. For example, histamine also has an ability to induce nasal vascular exudation and mucus secretion.^{26,27} It is possible that the histamine-mediated increase in interstitial and intraluminal nasal fluids might mainly affect the intranasal pressure, and the response may be augmented in this animal model of allergic rhinitis. Alternatively, relaxation of the mucosal vasculature other than nasal septum (e.g., lateral turbinate and medial turbinate) may be important for the development of nasal obstruction in guinea pigs.

In this study, a marked increase in the expression of iNOS isoform was established in the repeatedly antigen-challenged animals, suggesting that a large amount of NO might be produced in the nasal mucosa by iNOS after allergic challenge as reported in a model of allergic asthma.¹³ Although the role of NO generated by iNOS in nasal mucosa is not clear now, the increased nasal iNOS expression was reported also in allergic rhinitis models of rats²⁸ and mice²⁰ and in patients with allergic rhinitis.²² It seems that measurement of the NO levels in the nasal airways is useful for monitoring allergic rhinitis.

In conclusion, the expression of three isoforms of NOS, including eNOS, nNOS, and iNOS, was shown in guinea pig nasal mucosa. A marked increase in iNOS expression in the repeatedly antigen-challenged animals suggests an important role of iNOS in the pathogenesis of allergic rhinitis. However, the pathophysiological role(s) of NO generated by iNOS in nasal allergy is still unclear.

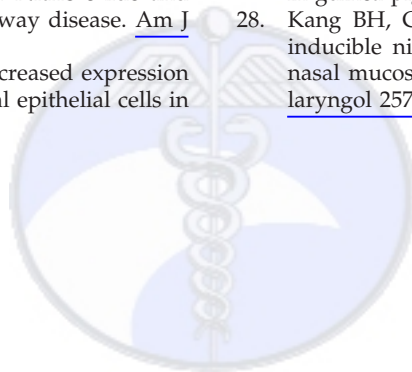
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