

The effect of overexpression of endothelial nitric oxide synthase on eosinophilic lung inflammation in a murine model

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Abstract

The effects of nitric oxide (NO) on allergic inflammation are controversial. In particular, the role of endothelial nitric oxide synthase (eNOS) in asthma remains uncertain. In the present study, we examined the effects of overexpression of eNOS on allergic inflammation using eNOS transgenic (eNOS-Tg) mice, in which eNOS protein is overexpressed in the vascular endothelium and airway epithelium. We found that eNOS-Tg mice showed a reduction of the asthmatic response to allergen challenge. Eosinophilic accumulation in the airspaces, eosinophilic activity, and bronchial responsiveness to acetylcholine were significantly attenuated in eNOS-Tg mice, as compared with wild-type mice following ovalbumin sensitization/challenge, even though the levels of circulating eosinophils were comparable in the wild-type and eNOS-Tg mice. The concentrations of eotaxin in the bronchoalveolar lavage fluid were significantly less in eNOS-Tg mice than in the wild-type mice. In addition, immunohistochemical analysis showed that the expressions of both intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 on the pulmonary endothelium of eNOS-Tg mice was decreased compared with the controls. These results suggest that chronic eNOS overexpression contributes to the suppression of allergic inflammation by reducing the production of eotaxin in the airspaces and/or the expression of adhesion molecules in the vascular endothelium.

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1. Introduction

Bronchial asthma is an airway disease characterized by airway hyperresponsiveness, reversible airway obstruction and chronic inflammation [1–3]. Airway inflammation plays a critical role in the pathogenesis of bronchial asthma and nitric oxide (NO) has a significant influence on this process. Nitric oxide is

a free radical molecule, an important mediator of lung inflammation and regulates airway smooth muscle contractility.

Nitric oxide is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS), which has three isoforms, including two constitutive forms, the neuronal (nNOS) and endothelial (eNOS) isoforms, and one inducible (iNOS) form. All of these isoforms are observed in the lung and airways of every animal species. The nNOS isoform is mainly identified in nonadrenergic noncholinergic nerves and plays a role in the regulation of airway tone [4].

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On the other hand, the iNOS isoform is induced in a variety of cell types, such as alveolar macrophages, epithelial cells, endothelial cells, T cells and eosinophils, by several cytokines or microbial products. Elevated iNOS expression was found in the airway epithelium of patients with asthma, whose NO in the exhaled air is increased compared with those of normal subjects. The iNOS knock out mice showed a reduction of both circulatory and pulmonary eosinophilic numbers following aeroallergen treatment, while the levels of airway hyperresponsiveness in these mice were markedly higher than those found in wild mice after OVA challenge [5]. However, another study showed that iNOS-derived NO has no effect on the pulmonary eosinophil infiltration [4]. Thus, iNOS plays a pivotal role in asthma and there have been many studies about it.

However, there have been few experimental studies into the relationship between the endothelial NOS isoform and allergic inflammation. The expression of eNOS protein in the airway epithelium is decreased and there is a significant correlation between eNOS protein expression and airway hyperresponsiveness [6]. In a mouse model, it was reported that mice lacking the eNOS gene show airway hyperresponsiveness compared with wild-type mice [7]. However, the role of eNOS in the pathogenesis of asthma is poorly understood. In the present study, we examined the effect of eNOS overexpression on asthmatic phenotypes by using eNOS transgenic mice.

2. Materials and methods

2.1. Animal preparation

We used 8- to 10-week-old heterozygous transgenic (eNOS-Tg) mice overexpressing the bovine eNOS gene using the preproendothelin-1 promoter and C57BL/6J mice as their littermate control (WT) mice [8]. All animal experiments were conducted in accordance with the "Guidelines for Animal Experiments at Kobe University Graduate School of Medicine".

2.2. Antigen sensitization and challenge protocol

The sensitization and challenge protocols used the same methods as described previously, with some modifications [9]. To induce an immune response mediated by IgE [10], mice were sensitized with an intraperitoneal injection of 10 μ g of OVA (Grade V; Sigma Chemical Co., St. Louis, MO, USA) absorbed in 2 mg of aluminum hydroxide (Sigma Chemical Co.) in 0.5 mL sterile Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline (PBS). The mice were subsequently boosted intraperitoneally with the same mixture 7 days later.

On day 14 after the initial sensitization, mice were placed in an acrylic chamber (10 cm $\times$ 10 cm $\times$ 20 cm) and exposed to aerosolized 0.5% OVA in sterile PBS for 25 min, and the same protocol was repeated on days 17 and 21. These groups were termed Tg-OVA and WT-OVA. The aerosolized OVA was generated with an ultrasonic nebulizer (NE-U12; Omron, Tokyo, Japan), repeating the same protocol. Negative controls were injected and exposed to PBS. They were designated Tg-PBS and WT-PBS. In addition, some mice were given the non-specific NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME; Sigma Chemical Co.) in their drinking water (1 mg/mL) from day -7 and treated with OVA. In addition, some mice were given iNOS specific inhibitor, AMT (2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine), intraperitoneally from day 0 to day 14. These mice were termed WT-OVA-L-NAME, Tg-OVA-L-NAME, WT-OVA-AMT and Tg-OVA-AMT. Eight to ten mice in every group were used in every procedure.

2.3. Measurement of airway hyperresponsiveness

On day 22, the mice were anesthetized by the intraperitoneal injection of 80 mg/kg sodium pentobarbital (Abbott Laboratories, Chicago, IL, USA). The mice were injected intravenously with pancuronium bromide (0.1 mg/kg) and ventilated with the aid of a rodent ventilator (Model 683; Harvard Apparatus Co., South Natick, MA, USA) at a rate of 70 strokes/min and a stroke volume of 0.6 mL. The airway responsiveness was assessed according to the Konzett-Rössler overflow method [11] with the use of a Ugo Basile 7020 bronchospasm transducer connected to the tracheal cannula. Increasing doses of acetylcholine were injected into the femoral vein, and the changes in overflow volume were determined. The increase in respiratory overflow volume induced by acetylcholine was expressed as a percentage of the maximal overflow volume obtained by clamping the tracheal cannula.

2.4. Bronchoalveolar lavage

On day 22 (24 h after the final exposure), bronchoalveolar lavage (BAL) was performed on the mice in each group under anesthesia with sodium pentobarbital. BAL fluid was collected using a 22-gauge balltipped needle inserted through a tracheal incision. The lungs were lavaged with 0.8 mL PBS containing 0.6 mM ethylenediaminetetraacetic acid (EDTA) (Sigma Chemical Co.) three times. The total cell counts were determined using standard hematological procedures. After the lavage was centrifuged at 2000 $\times$ g for 10 min, the supernatant was separated from the cell pellet, and aliquots were frozen at -80 °C for cytokines analysis and eosinophil peroxidase (EPO) activity assay. The cell pellets were resuspended in PBS and slides were prepared by spinning the samples at 400 rpm for 3 min (Cytospin 2; Shandon Inc., Pittsburgh, PA, USA). BAL specimens were stained with the Diff Quick Staining kit (Kokusaishiyaku Co., Kobe, Japan), and differential cell counts were determined from at least 200 leukocytes.

2.5. Cytokine and chemokine analysis

The cytokines and chemokines in BAL supernatants were measured by enzyme-linked immunosorbent assays (ELISAs), as previously described [12]. Interleukin (IL)-5 and eotaxin were also determined by ELISAs using purified and biotinylated antibodies purchased from R&D Systems (Minneapolis, MN, USA). The paired antibodies were purchased from BioSource International (Camarillo, CA, USA) for the analysis of IL-4 and Interferon (IFN)- γ .

2.6. Eosinophil peroxidase (EPO) activity assay

This method has been shown to be specific for the peroxidase activity of eosinophils, but not of neutrophils [13]. The samples (100 μ L) were pipetted into the wells of a 96-well plate followed by 100 μ L of the reaction solution (0.1 mM *o*-phenylene-diamine-dihydrochloride, 0.1% Triton X-100, 1 mM hydrogen peroxide in 0.05 M Tris-HCl; all reagents were from Sigma Chemical Co.). The plate was incubated in the dark for 30 min and the reaction was stopped by adding 50 μ L of 4 M sulfuric acid per well. The optical densities were read on a plate reader at 490 nm.

2.7. Nitrite assay

NO_x (defined as the sum of nitrate and nitrite) in the BAL fluid was measured using a spectrophotometric method based on the Griess reaction. Nitrate in the BAL fluid was reduced to nitrite by using the enzyme nitrate reductase and its enzyme cofactors. The samples were mixed with Griess' reagent and incubated at room temperature. Nitrite concentrations in the samples were determined using different concentrations of sodium nitrite as a standard curve. The reaction was read on a plate reader at a wavelength of 570 nm.

2.8. Histopathological analysis

On day 22, after the mice of each group were anesthetized by the intraperitoneal injection of 80 mg/kg sodium pentobarbital, the tracheas were cannulated in each mouse and the lungs were removed with the trachea after sacrifice. These mice were only used for histopathological analysis, not for the BAL procedure. The lungs were gravity-inflated in situ to 20 cm H₂O with 10% buffered formalin. The whole lung was embedded in paraffin and sectioned at 4 μ m thickness. The sections were analyzed with hematoxylin and eosin (H.E.) stain.

Immunohistological examinations were performed by the labeled streptavidin biotin (LSAB) method with an LSAB kit (DAKOPATTS, Copenhagen, Denmark), according to a previous study [8]. 0.8 mL of 50% OCT compound (Miles, Elkhart, IN, USA) in PBS was infused into the lungs to expand the tissue, and the tissues were embedded in OCT at -80°C . Cryostat sections were fixed in acetone for 10 min, blocked with 5% bovine serum albumin (BSA) and incubated overnight with polyclonal antibodies for eNOS, intercellular

adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (dilution, 1:50 in PBS; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C . These sections were then incubated with a second antibody and subsequently with horseradish peroxidase-labeled streptavidin. Endogenous peroxidase was quenched with H₂O₂ and the bound primary antibody was detected with the substrate, diaminobenzidine, and counterstained with hematoxylin.

2.9. Western blot analysis

Western blot experiments were performed as described previously [14]. Whole lungs were homogenized with a buffer (50 mM Tris/HCl, 300 mM NaCl, 1% Triton X-100, 2.5% glycerol, 5 mM CHAPS, 1 mM EGTA, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin), centrifuged and the supernatants were collected. Equal amounts of protein extract were electrophoresed through 10% SDS-PAGE gel. The proteins were transferred to nitrocellulose membranes, blocked with 5% non-fat milk in Tris-buffered saline/Tween20 at room temperature for one hour and incubated with primary antibody at 4°C overnight. These membranes were incubated with horseradish peroxidase-labeled second antibodies at room temperature for 1 h. The signals were detected by detection reagents (Amersham Life Science, Buckinghamshire, UK). The relative intensities of bands were determined by densitometry analysis with NIH Image software, version 1.62.

2.10. Statistical analysis

Data were expressed as means $\pm$ SE. Analysis of variance (ANOVA) was employed to analyze the differences of mean values among different groups. When ANOVA showed a significant difference, the Fisher LSD post-hoc test was used to identify which group differences accounted for the significant *P* value. Significance was accepted at the 0.05 level of probability.

3. Results

3.1. Expression of eNOS protein in the lung of eNOS transgenic mice

A previous study showed the regions of eNOS overexpression in eNOS-Tg mice [11]. We again examined the immunohistochemical staining of eNOS to clarify the eNOS overexpression regions of the lung in greater detail (Fig. 1). A marked increase in eNOS expression was detected in the endothelial cells in pulmonary arteries and veins. Endogenous mouse eNOS in endothelial cells in wild-type mice was slightly stained (Fig. 1A). Similarly, pulmonary airway cells and alveolar cells were more strongly stained for eNOS protein in eNOS-Tg mice (Fig. 1D) than in the wild-type mice (Fig. 1C).

According to Western blots for the eNOS from total lung extracts, the expression of eNOS in this transgenic mouse was about 6-fold that in the wild mouse (Fig. 2A, B). On the other

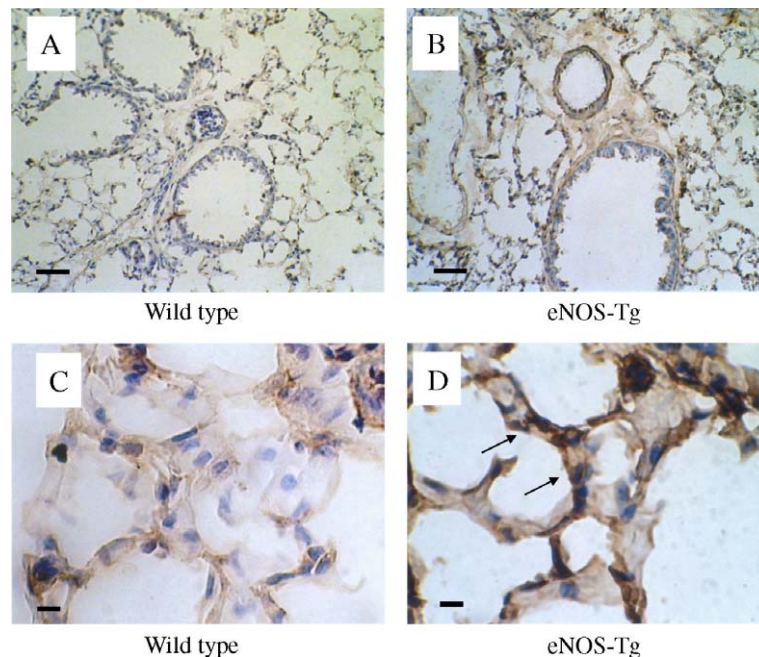


Fig. 1. Immunohistochemical staining for eNOS in the lung. Frozen sections of the lungs from wild-type (A, C) and eNOS-Tg mice (B, D) were immunohistochemically stained using a rabbit polyclonal anti-eNOS Ab. Intense signals were mainly detected in the endothelial cells in pulmonary vessels of eNOS-Tg mice. Pulmonary airway cells and alveolar cells of eNOS-Tg mice were also slightly stained (D). Endogenous mouse eNOS in the endothelial cells and airway epithelium of wild-type mouse were slightly stained compared with eNOS-Tg mice (C). Arrows show eNOS expression in alveolar cells. Original magnification: A, B $\times 200$; C, D $\times 400$. Calibration bars = 150 μm (A, B), 50 μm (C, D).

hand, the eNOS activities (NO production) in the airways of these transgenic mice were slightly higher than those in the wild type mice. However, there was no significant difference between the two mouse groups ($p=0.07$) (Fig. 2C).

3.2. Expression of each NOS isoform in total lung extracts

In these model mice, there was no difference in the eNOS expression between those mice treated and those not treated with OVA (Fig. 2A), while the expression of iNOS increased after OVA challenge (Fig. 2D). The elevation of NO production in the airway, which was probably derived from iNOS, in Tg-OVA mice was slight compared to WT-OVA (Fig. 2C), although there were no significant differences in the iNOS expression and NO production between WT-OVA and Tg-OVA mice. L-NAME could not suppress either eNOS or iNOS expression completely, but reduced their expressions (Fig. 2A,D).

3.3. eNOS transgenic mice show decreased airway eosinophilia and eosinophilic activity following OVA challenge

The BAL fluid revealed that the number of inflammatory cells, including eosinophils, macrophages and lymphocytes migrating into the airspaces, significantly increased following OVA challenge in both mouse genotypes (Fig. 3A). However, the cellular components in the BAL fluid were

markedly different between the Tg-OVA and WT-OVA mice. The absolute number of eosinophils in the BAL fluid of Tg-OVA mice was about 6-fold less than that of WT-OVA mice. WT-OVA mice showed predominant eosinophil infiltration, while Tg-OVA showed similar numbers of monocyte and eosinophil influx. The EPO activities in the BAL fluid of the Tg-OVA mice at 24 h after the last OVA challenge were significantly lower than those of WT-OVA mice (Fig. 3B). To clarify whether the NOS inhibitor abolishes the difference between WT-OVA and Tg-OVA, the BAL fluid of Tg-OVA-L-NAME was examined. The eosinophil infiltration of the Tg-OVA-L-NAME group was more suppressed than Tg-OVA (number of total cells: $13.1 \times 10^4/\text{mL}$ and eosinophil: $6.2 \times 10^4/\text{mL}$). This was probably caused by the effects of L-NAME (NOS non-specific inhibitor), which suppressed not only eNOS expression, but also iNOS expression, because AMT (iNOS specific inhibitor) reduced the eosinophil infiltration in the BAL fluid (Tg-OVA-AMT; number of total cells: $10.0 \times 10^4/\text{mL}$ and eosinophil: $4.1 \times 10^4/\text{mL}$) more than L-NAME.

Histological examination (H&E staining) of the lungs confirmed the results using BAL fluid. WT-OVA mice showed peribronchial and perivascular infiltrates containing abundant eosinophils, and higher airway epithelial cells. In contrast, Tg-OVA mice showed a slight change in the amount of infiltrating leukocytes and in the height of the airway epithelium after antigen challenge (Fig. 4).

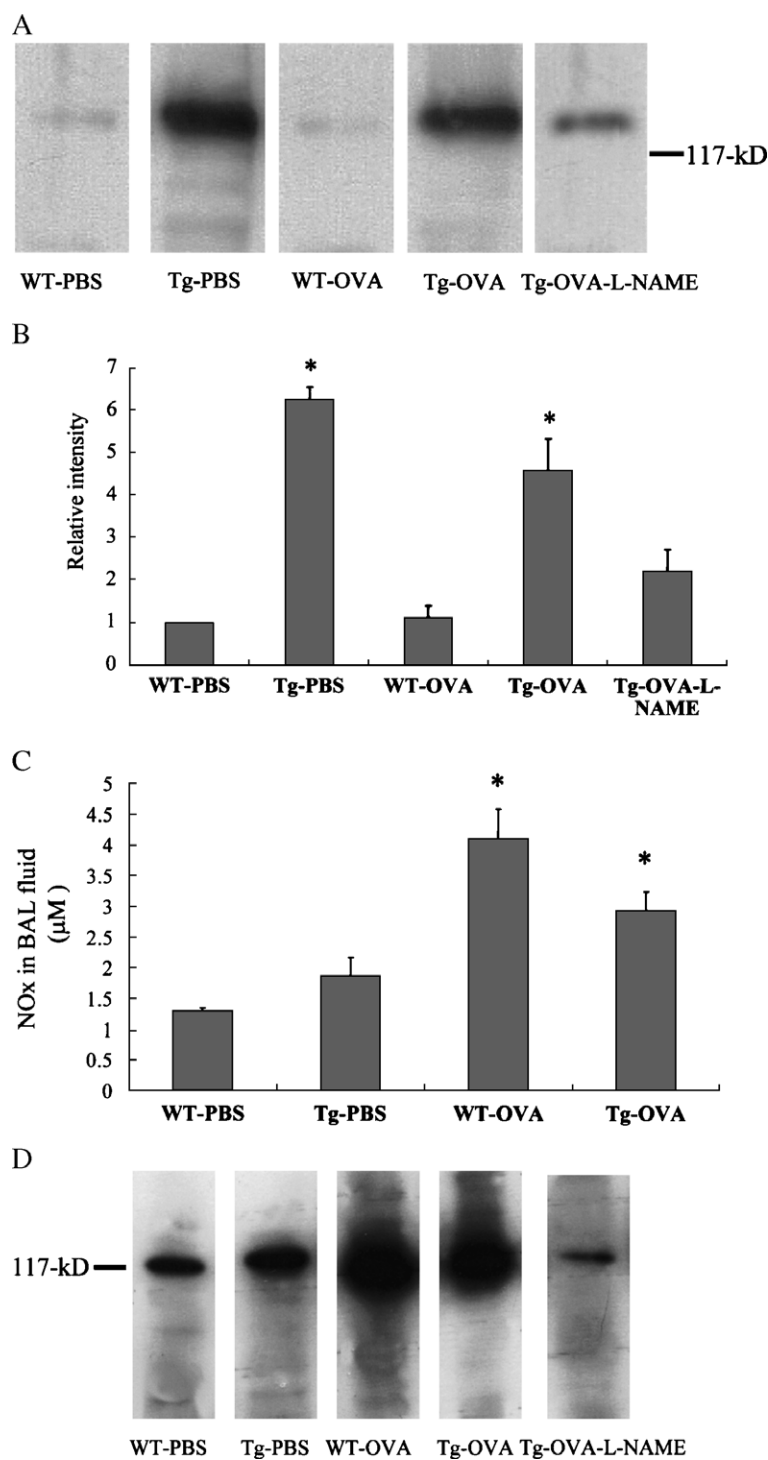


Fig. 2. The expressions of NOS isoforms. Representative Western blots of eNOS (A), iNOS (D) and quantization of eNOS expression by densitometry. The Y-axis represents the relative intensities of eNOS expression compared to WT-PBS (B). Cell-free BAL fluid was analyzed for NOx concentrations (sum of nitrite and nitrate) (C). Results are expressed as means $\pm$ SE. * p < 0.05 versus WT-PBS.

One of the possibilities for the reduction of eosinophilic influx of eNOS-Tg mice is that these mice may be unable to generate eosinophils from bone marrow after antigen chal-

lenge. We counted the numbers of circulating leukocytes and the differential cell counts of both genotypes. There were no significant differences in the circulating eosinophil levels

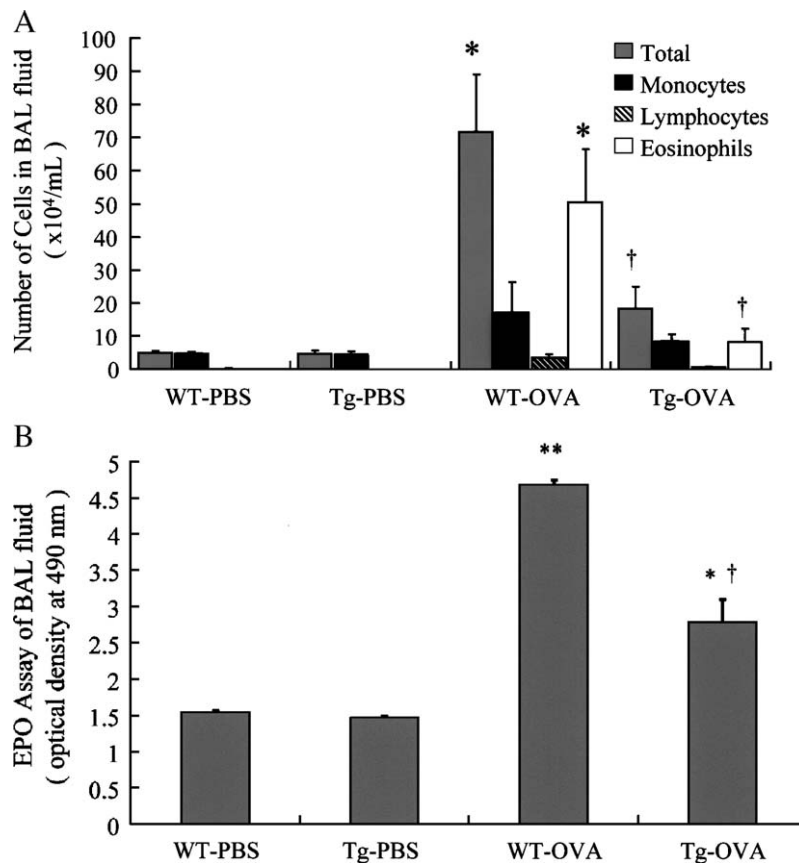


Fig. 3. Effect of eNOS overexpression on eosinophil accumulation and activity in BAL fluid in sensitized and challenged mice. The number of inflammatory cells in the BAL fluid was determined 24 h after the final allergen challenge. The figure shows the absolute number of each infiltrating cellular population (A). Cell-free BAL fluid was analyzed for EPO activity (B). Results are expressed as means $\pm$ SE. * $p < 0.05$, ** $p < 0.01$ versus WT-PBS. † $p < 0.05$ versus WT-OVA.

between Tg-OVA and WT-OVA mice (data not shown). Therefore, this reduction of airway eosinophilia in eNOS-Tg mice may be due to the failure of eosinophilic infiltration from blood vessels into airways.

3.4. Inhibition of airway hyperresponsiveness in eNOS-Tg mice after challenge

In this study model, antigen challenge caused a significant increase in the airway responsiveness to acetylcholine in WT-OVA mice compared with WT-PBS mice. The eNOS-Tg mice also showed an increase in the airway responsiveness after antigen challenge, but not as much as wild-type mice (Fig. 5). The administration of L-NAME diminished this reduction of airway responsiveness of eNOS-Tg mice after OVA treatment.

3.5. Th1 and Th2 cytokines in BAL fluid after antigen challenge

To examine the cause of the inhibition of the allergic response in eNOS-Tg mice, we measured the concentrations of the Th1 and Th2 cytokines in the BAL fluid (Fig. 6). IFN- γ , a

representative Th1 cytokine in BAL fluid, changed little after antigen challenge in both genotypes. Similarly, the production of IL-4, one of the Th2 cytokines, did not differ significantly between the two groups, although this Th2 cytokine production was increased by antigen challenge. The concentration of IL-5 in the BAL fluid of both genotypes treated with OVA was below the detectable level. These results disagree with the suggestion that the reduction of airway eosinophilia in Tg-OVA mice is caused by the shift of Th1/Th2 cytokines production balance by NO derived from eNOS.

3.6. Eotaxin production in airspaces

We measured the concentration of eotaxin (C-C chemokine), which plays a pivotal role in the movement of allergic inflammatory cells, such as eosinophils, basophils and mast cells, and is produced mainly by airway epithelial cells [15]. The level of eotaxin in the serum showed no significant difference among the groups (WT-PBS, Tg-PBS, WT-OVA and Tg-OVA; 1620 ± 290 , 1190 ± 114 , 1320 ± 92 and 1020 ± 184 pg/mL, respectively). On the other hand, the level of eotaxin in the BAL fluid in OVA-treated mice in-

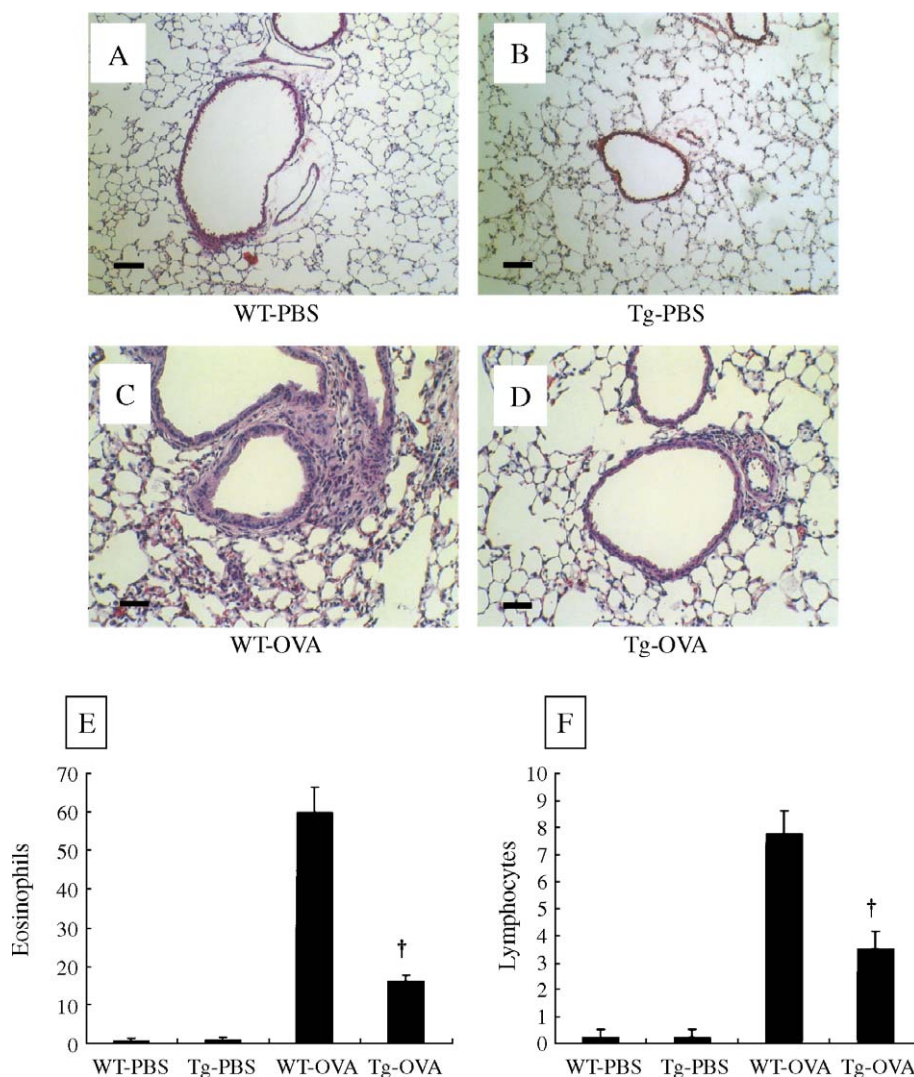


Fig. 4. Reduction of the peribronchial eosinophilic accumulation and bronchial epithelium thickening in Tg-OVA mice compared with that in WT-OVA mice. Lung tissues were routinely fixed, sectioned and stained with hematoxylin and eosin. WT-PBS (A), Tg-PBS (B), WT-OVA (C), and Tg-OVA (D) were prepared as described in Materials and methods. A total of 5 mice in each group were examined, and representative findings are shown. Original magnification $\times 200$. Calibration bars = 150 μm . Arrows show the eosinophils accumulations. Total numbers of eosinophils (E) and lymphocytes (F) obtained from high power fields ($\times 400$ magnification) in histological sections from each lung of mice from all groups. $\dagger p < 0.05$ versus WT-OVA.

creased significantly more than in the PBS-treated mice group, but the degree of eotaxin production in eNOS-Tg mice was significantly suppressed compared with the wild-type mice after exposure (Fig. 7). This result means that NO derived from the overexpression of eNOS protein might modulate the production of eotaxin in the airway epithelium. The levels of eotaxin in the BAL fluid of Tg-OVA-L-NAME (13.5 pg/mL) and Tg-OVA-AMT (13.9 pg/mL) were more suppressed than Tg-OVA, but there were no significant differences among the three groups.

3.7. ICAM-1 and VCAM-1 expression in pulmonary vascular endothelial cells after antigen challenge

In light of the finding that the regions where eNOS protein was mainly overexpressed were pulmonary vascular endothelial cells, we examined the effect of eNOS on adhesion molecules expressions on the endothelium of pulmonary vessels after exposure. ICAM-1 is related to the survival and activation of eosinophils [16], and VCAM-1 is an extremely important adhesion molecule for regulating

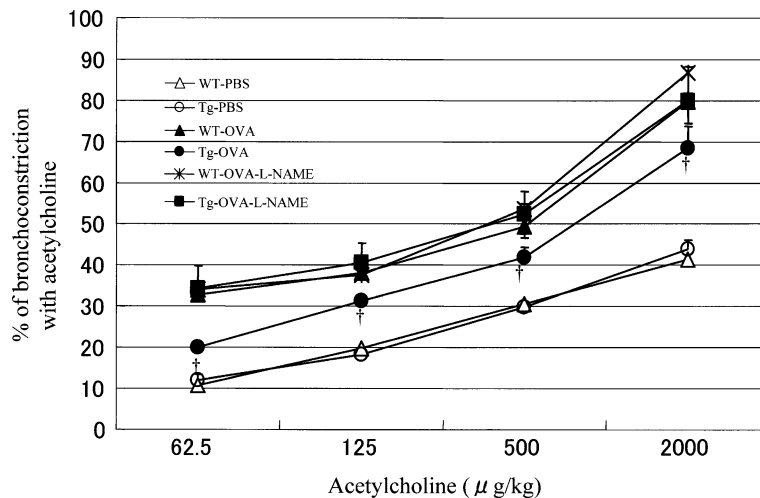


Fig. 5. The dose-response curves of acetylcholine-induced bronchoconstriction in WT-PBS (Δ), Tg-PBS ($\circ$), WT-OVA ($\blacktriangle$), Tg-OVA ($\bullet$), WT-OVA-L-NAME ($\times$), and Tg-OVA-L-NAME ($\blacksquare$) are shown as a percentage of the maximal overflow volume obtained by clamping the trachea. The OVA challenge significantly increased the sensitivity to acetylcholine in the wild-type mice, whereas little increase was detected in the eNOS-Tg mice. The results for each group are means $\pm$ SE ($n=5$). $\dagger p < 0.05$ versus WT-OVA.

eosinophil migration from peripheral vessels into the air-space [17]. By immunohistochemical staining, significant expressions of both adhesion molecules ICAM-1 and VCAM-1 were observed in the vascular endothelial cells of wild-type mice after OVA challenge, compared with WT-PBS mice. The same challenge to eNOS-Tg mice showed the faint expression of these adhesion molecules in the endothelium (Fig. 8). Western blots for ICAM-1 and VCAM-1 confirmed the results of immunohistochemistry. These results suggest that nitric oxide derived from over-expressed eNOS reduces the expressions of both ICAM-1 and VCAM-1, and this mechanism may help prevent allergic inflammation in eNOS-Tg mice.

4. Discussion

The present study demonstrated that eNOS overexpressing mice exhibited a reduction in the response to aerosol OVA challenge. It has been reported that the eNOS isoform is found in vascular endothelial cells, bronchial epithelial cells, mast cells, neutrophils and platelets [18,19]. In the present eNOS-Tg mice, eNOS was chronically overexpressed in both bronchial epithelial cells and pulmonary vascular endothelial cells. Therefore, it was suggested that the reduction of allergic response in eNOS-Tg mice was caused by over-

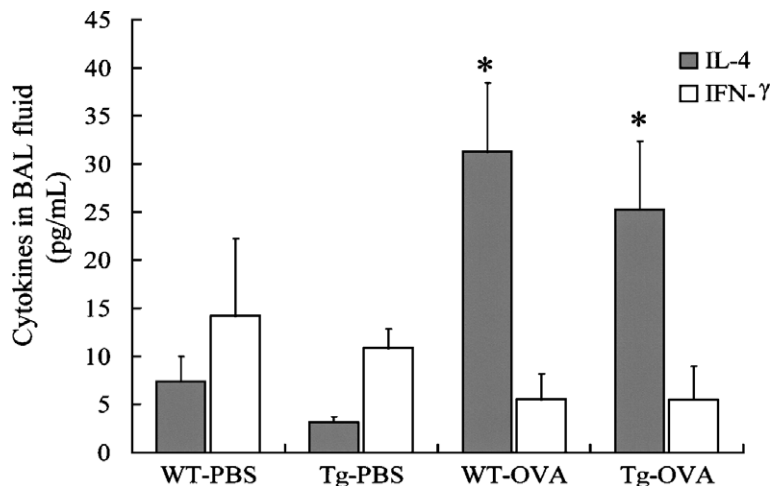


Fig. 6. Effects of eNOS overexpression on the release of Th1/Th2 cytokines in BAL fluid. IL-4 and IFN- γ cytokine concentrations in BAL fluid were assessed 24 h after the last allergen challenge. Values represent the means $\pm$ SE. There were no significant differences of the Th1/Th2 cytokines between WT-OVA and Tg-OVA. * $p < 0.05$ versus WT-PBS.

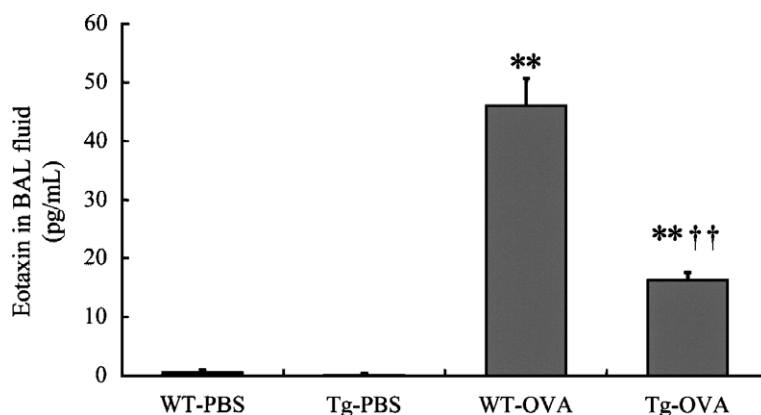


Fig. 7. Effects of eNOS overexpression on the concentration of eotaxin in s BAL fluid. Concentration of eotaxin in BAL fluid were determined using ELISA. The results for each group are the means $\pm$ SE ($n=5$). ** $p<0.01$ versus WT-PBS. †† $p<0.01$ versus WT-OVA.

produced NO derived from overexpressed eNOS from bronchial epithelial cells and/or pulmonary vascular endothelial cells.

The present study showed that Tg-OVA mice generated a lower concentration of eotaxin in the airspaces than WT-OVA. The kinetics of leukocyte accumulation around airways of patients with bronchial asthma correlate with the increased pulmonary expression of C-C chemokines, which are produced by alveolar macrophages, airway epithelial cells, lung fibroblasts and smooth muscle cells [20]. Much evidence exists demonstrating that NO suppresses C-C chemokines in a number of cells during various airway inflammations [21,22]. Blease et al. reported that acute inhibition of NO exacerbated C-C chemokine eotaxin, MCP-1, and C10 generation [23]. The present eNOS-Tg mice showed the overexpression of eNOS protein in airway epithelial cells and the NOx concentrations in the BAL fluids of eNOS-Tg mice were higher than those of wild type mice, although there was no significant difference in the steady state. The difference of the activity of eNOS between the two groups stood out in the inflammatory process. It cannot be denied that the reduction of eotaxin production is only the result of the reduced degree of airway allergic inflammation in eNOS-Tg mice, but it also cannot be also denied that NO derived from overexpressed eNOS may modulate the generation of eotaxin in airway epithelial cells.

A previous study reported that NO derived from pulmonary vascular endothelial cells is involved in the extravasation of eosinophils from the circulation into the lung tissue [14]. However, our eNOS Tg-mice had no difference in the number of eosinophils in BAL fluid before challenge compared with the wild type. Rather, Tg-OVA mice showed the reduction of eosinophil infiltration after challenge. Their conclusion

was led by the result that L-NIL, an iNOS specific inhibitor, could not prevent eosinophil infiltration. In two different studies, iNOS deficiency [4] or iNOS inhibitor treatment [14] had no effect on the lung inflammation. While Trifilieff et al. reported that other iNOS specific inhibitors, EIT and AMT, have the effect of preventing eosinophil infiltration [24] and in the other study, iNOS deficient mice showed the reduction of airway inflammation [5]. These discrepancies were attributed to significant differences in the immunization and challenge protocols [5]. Our additional data revealed that mice treated by AMT showed the reduction of eosinophil influx. Furthermore, in our model, iNOS protein increased after OVA challenge, but Fender's model did not show iNOS expression. In these regards, it is speculated that the degree of inflammation in our model was greater than that of Fender's model and, that in our model, iNOS played a role concerning eosinophil migration to some degree by modulation of the chemotactic process [24]. Tg-OVA mice did not show the reduction of iNOS expression compared to WT-OVA mice (Fig. 2D). Therefore, chronic eNOS overexpression suppressed allergic inflammation via modulation of iNOS down-stream cytokine expression or via another process not concerning iNOS, such as adhesion molecule expression.

The purpose of most previous asthma therapies has been the suppression of airway inflammation and contraction. However, the present study highlights the importance of vascular endothelial cells and also adhesion molecule expression in the pathogenesis of airway hyperresponsiveness and inflammation in a murine model of allergic asthma and in asthma therapy. The signaling by ICAM-1 and the $\alpha 2$ integrin family contributes to the prolongation of survival and activation of eosinophils by augmenting the oxidative metabolism

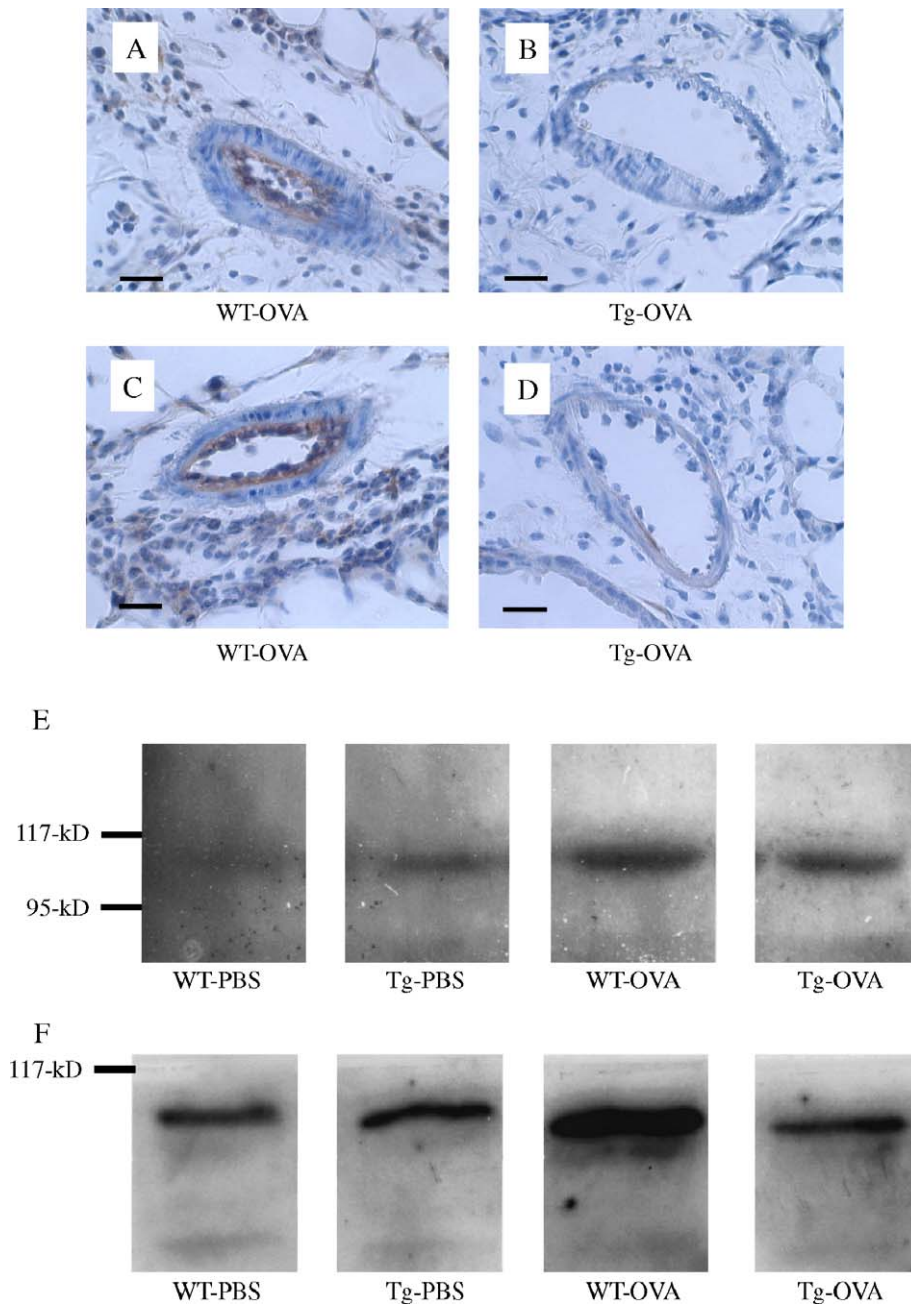


Fig. 8. Marked reduction of adhesion molecule (ICAM-1 and VCAM-1) expression on pulmonary vascular endothelium in Tg-OVA. The figure shows immunohistochemical staining for ICAM-1 of WT-OVA (A) and Tg-OVA (B), and VCAM-1 of WT-OVA (C) and Tg-OVA (D). Less staining for both ICAM-1 and VCAM-1 is seen in the lung sections from Tg-OVA, compared with WT-OVA. A total of 5 mice in each group were examined, and representative findings are shown. Original magnification $\times 400$. Calibration bars = 75 μm . Representative Western blots for ICAM-1 (E) and VCAM-1 (F) from total lung extracts.

[16]. On the other hand, much evidence has demonstrated the extreme importance of the interaction between VCAM-1 and very late activation Ag-4 (VLA-4), a ligand of VCAM-1 in the adhesion of eosinophils to vascular endothelial cells and their transendothelial migration [25,26]. IL-4 is required for the induction

of expression of VCAM-1 in endothelial cells [27]. In the present study, we showed that the extent of VCAM-1 and ICAM-1 expression in vascular endothelial cells was less in Tg-OVA mice, compared with WT-OVA mice, even though there was no significant difference in the production of Th2 cytokines between the two

groups treated with OVA. There is overwhelming evidence that NO inhibits leukocyte adherence to activated endothelium by reducing the cytokine induced adhesion molecules on endothelial cells [28–31]. It was noticed in the present study that eNOS was overexpressed in pulmonary vessels in eNOS-Tg mice, and lung eNOS activity in eNOS-Tg mice was about 20-fold that of wild-type mice [8]. Therefore, it is likely that overexpressed eNOS-derived NO directly suppressed the adhesion molecule expression and reduced the allergic inflammatory changes in Tg-OVA mice compared with WT-OVA mice.

We demonstrated not only that both eosinophil and lymphocyte infiltration into airspaces was less in Tg-OVA mice than in WT-OVA mice, but also that EPO activity in BAL fluid was significantly lower in Tg-OVA mice. Production of cytokines such as IL-4, IL-5, IL-9, IL-10 and IL-13 derived from Th2 cells is recognized to be implicated in the development of asthmatic features, including lung eosinophilia, mucus hypersecretion, and bronchial hyperresponsiveness [32]. IL-4 and IL-5 are known to regulate growth, differentiation, activation, and survival of eosinophils and appear to be essential in the development of allergic airway eosinophilia and airway hyperresponsiveness [33]. On the other hand, IFN- γ , a Th1 cytokine, inhibits the proliferation of mast cells and eosinophils by reducing production of Th2 cytokines [34]. We measured the concentrations of Th1 and Th2 cytokines in BAL fluid from wild-type and eNOS-Tg mice in order to elucidate the mechanism of different asthmatic responses between wild-type and eNOS-Tg mice. OVA challenge induced no significant difference in the concentration of the Th1 and Th2 cytokines in the BAL fluid between wild-type and eNOS-Tg mice. Taken together, it is unlikely that eNOS-derived NO affects the balance of Th1/Th2 cytokine production.

It was reported that NO released from the epithelium limits bronchoconstriction to spasmogens [35]. Neurally mediated bronchoconstriction by bradykinin is limited by several epithelium-derived factors, including peptidases [36], prostaglandin E2 [37], and an especially important factor, NO [38,39]. In addition, eNOS-deficient mice are markedly hyperresponsive to inhaled methacholine [7]. NO derived from eNOS has been recognized to play a physiological role in controlling airway responsiveness. At first, it was suggested that Tg-PBS mice have less sensitivity to acetylcholine compared with wild-type mice. However, there were no significant differences in airway responsiveness between the two PBS groups. This was probably caused by the eNOS-Tg mice having no significant elevation

of NO in the airways compared with wild type mice. In addition, Tg-OVA mice showed a reduced responsiveness to acetylcholine compared with WT-OVA. Fabio et al. reported that allergen exposure in asthma induced increased airway hyperresponsiveness through the impaired release of bronchoprotective NO associated with down-regulation of eNOS in airway epithelial cells [6]. According to this result, eNOS-Tg mice may be resistant to bronchial responsiveness to allergen due to the chronic expression of eNOS in the epithelium, but this mechanism fails to easily explain the reduction of leukocyte infiltration in Tg-OVA mice.

Administration of L-NAME abolished the difference of phenotype between eNOS-Tg and wild type mice. A previous study [8] showed that this dose of L-NAME was sufficient to suppress both the activities of endothelial and inducible NOS. It was through the inhibition of NO production that L-NAME increased the airway responsiveness compared to OVA treated mice [5,7], even though the difference was not significant, while the inhibition of eNOS activity diminished the difference of phenotypes between eNOS-Tg and wild type mice. The reason for the reduction of bronchial responsiveness of Tg-OVA mice may be that NO derived from overexpressed eNOS acted as a bronchodilator, or that overexpression of eNOS systemically suppressed the allergic response, because there were no significant differences in the iNOS expression between WT-OVA and Tg-OVA. That is to say, in our models, the overexpression of eNOS did not influence the expression of iNOS, and the difference of phenotype between WT-OVA and Tg-OVA was not caused by iNOS, but by eNOS.

The present study demonstrated that overexpressed eNOS-derived NO might protect the host from allergic inflammation by reducing the eotaxin production in airway epithelial cells and/or adhesion molecule expression in vascular endothelial cells. In addition, we could demonstrate that a NOS inhibitor (L-NAME) abolishes the difference between eNOS-Tg and wild type mice. It is no wonder that iNOS is an important factor in allergic pulmonary inflammation, but the role of eNOS in the allergic response cannot be neglected. In the present study it could not be clearly determined whether NO derived from eNOS overexpression in vascular endothelial cells or airway epithelium mainly contributed to the reduction of allergic response in the eNOS-Tg mice, because our generated mice showed eNOS overexpression in either the vascular endothelium or airway epithelium. Even though the adenovirus transfection method was used, eNOS gene overexpression was reported in both airway epithelial cells and vascular endothelial cells [40]. It is necessary to generate

eNOS transgenic mice using vascular endothelial cells or airway epithelial cells specific promoter, to solve this problem.

In conclusion, eNOS overexpression inhibits OVA induced eosinophil inflammation by protecting the vascular infiltration of eosinophils through the reduction of adhesion molecules. The present study also warns us not to use NOS inhibitors as asthma therapy, because they may diminish the protective effect of eNOS-derived NO. Adequate modulators of eNOS expression may represent a novel therapeutic approach for bronchial asthma.

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