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Decreased Bronchial Eosinophilic Inflammation and Mucus Hypersecretion in Asthmatic Mice Lacking All Nitric Oxide Synthase Isoforms

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Abstract
Background Asthma is characterized by airflow limitation with chronic airway inflammation, hyperresponsiveness and mucus hypersecretion. NO is generated by three nitric oxide synthase (i/n/eNOSs) isoforms, but conflicting results have been reported using asthmatic mice treated with NOSs inhibitors and NOS-knockout mice. To elucidate the authentic role of NO/NOSs in asthma, we used asthmatic mice lacking all NOSs (n/i/eNOS$^{-/-}$).
Methods Wild-type and n/i/eNOS$^{-/-}$ mice were sensitized and challenged with ovalbumin. Pathological findings and expressions of interferon (IFN)-γ, interleukin (IL)-4, -5, -10, -13 and chemokines in the lung were evaluated.
Results Decreased eosinophilic inflammation, bronchial thickening and mucus secretion, IL-4, -5 and -13, monocyte chemoattractant protein-1, eotaxin-1 and thymus and activation-regulated chemokine expressions were observed in n/i/eNOS$^{-/-}$ mice compared to wild-type, but expressions of IFN-γ and IL-10 were similar.
Conclusion Using asthmatic n/i/eNOS$^{-/-}$ mice, NO plays important roles in accelerating bronchial eosinophilic inflammation and mucus hypersecretion in the pathophysiology of asthma.

Keywords Nitric oxide · Nitric oxide synthase · Eosinophilic inflammation · Bronchial asthma

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Introduction

Asthma is characterized by airflow limitation with airway hyperresponsiveness, bronchial inflammation, mucus hypersecretion and remodeling. A fraction of exhaled nitric oxide (FENO) is clinically used as a marker of eosinophilic bronchial inflammation [1], and the role of nitric oxide (NO) in asthma is of particular interest. NO is generated by three isoforms of nitric oxide synthase (NOS): neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) NOSs, and all three NOS isoforms are found in the bronchial epithelium [2]. NO is involved in the Th2-cell-mediated (mainly eosinophils) asthmatic inflammatory responses and has multiple roles from proinflammatory to anti-inflammatory actions, regulating bronchomotor tone. Accelerated NO production with oxidative stress may be harmful in asthma, and iNOS-induced epithelial NO production by inflammatory stimuli such as proinflammatory cytokines is observed during asthma exacerbation [3]. In addition to iNOS, eNOS and nNOS are also involved in the pathophysiology of allergic asthma [4, 5].

The roles of NO and NOSs in asthma have been examined using selective or non-selective pharmacological inhibitors of NOSs, and also using NOS-knockout mice, however, conflicting results have been observed. Reduced eosinophilic bronchial inflammation was reported in

asthmatic mice treated with inhibitors of nonselective NOSs [6, 7] and selective iNOS inhibitors [8, 9], but no effect on bronchoalveolar lavage fluid (BALF) eosinophis were observed in mice treated with selsctive iNOS inhibitor [6]. Similar eosinophilic inflammation among nNOS$^{-/-}$, iNOS$^{-/-}$, eNOS$^{-/-}$ and n/eNOS$^{-/-}$ asthmatic mice was observed, and nNOS was considered to be important in the airway hyperresponsiveness [10]. Whereas, reduced eosinophilic bronchial inflammation was also reported in iNOS$^{-/-}$ [11] and eNOS transgenic mice [12]. The limitations of these animal studies were due to the specificity and pharmacokinetic concerns of NOS inhibitors, compensatory reactions between NOS isoforms to provide NO by the other NOS isoforms in NOS-knockout mice, differences between asthmatic murine models and mouse strains, that might be responsible for these conflicting findings. Because of heterogeneous and complicated activities of NO and NOSs, the comprehensive roles of NO and NOSs in the pathophysiology of allergic asthma is not fully understood so far.

A murine model lacking all three NOSs (n/i/eNOS$^{-/-}$) was developed to evaluate the essential role of NO and the authentic role of NOSs [13, 14] to overcome these issues described above. Using this triply-n/i/eNOSs$^{-/-}$ mice, we evaluated whether complete deletion of all NOS isoforms has an impact on allergic asthma.

Materials and Method

Male wild-type (WT; C57/BL6) and n/i/eNOS$^{-/-}$ mice (6 to 8-weeks-old) were sensitized with an intraperitoneal (i.p.) injection of 20 μg ovalbumin (OVA) (GradeV, Sigma-Aldrich, St. Louis, MO) and 2.25 mg of $Al(OH)_3$ (Sigma-Aldrich) as an adjuvant on days 1 and 14. The mice were exposed to aerosolized 1 % OVA for 20 min on days 26–28, and were sacrificed under deep anesthesia on day 30 [15]. Total RNA was extracted from homogenized right lung and reverse-transcribed. The expression of each mRNA (interferon (IFN)-γ, interleukin (IL)-4, IL-5, IL-10, IL-13, C–C chemokines (monocyte chemoattractant protein (MCP)-1, eotaxin-1 and thymus and activation-regulated chemokine (TARC) and GAPDH) was evaluated using real-time quantitative PCR (ABI Prism 7000, Applied Biosystems, Foster City, CA). The left lung was fixed with formalin and embedded in paraffin, sectioned and stained with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS). This study was approved by the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health, Japan, and was carried out according to the related guidelines.

Results

Comparing with WT, HE-stained and PAS-stained lung sections of n/i/eNOS$^{-/-}$ mice showed a decrease in eosinophilic inflammation and bronchial thickening (Fig. 1a, c) and decreased airway mucus hypersecretion (Fig. 1b, d), respectively. Compared to WT, IL-4, IL-5, MCP-1, eotaxin-1, TARC and especially IL-13 mRNA expressions in the lung were significantly reduced in n/i/eNOS$^{-/-}$ mice (Fig. 2b, c, f, g, h, and e, respectively), but the expressions of IFN-γ and IL-10 were similar (Fig. 2a, d).

Discussion

Pathological findings of allergic asthmatic n/i/eNOS$^{-/-}$ mice revealed a significant decrease in bronchial eosinophilic inflammation, bronchial thickening, mucus secretion and mRNA levels of IL-4, IL-5, IL-13, MCP-1, eotaxin-1 and TARC. These findings indicate that NO itself may have promotive effects on airway eosinophilic inflammation and airway mucus hypersecretion in allergic asthma.

Conflicting findings of pulmonary eosinophilic inflammation have been reported in asthma model using pharmacological suppressors of NOSs and NOS-knockout or NOS-transgenic mice as described above [3–12]. In addition to pulmonary eosinophilic inflammation, conflicting results of cytokines such as increased IL-4 and IL-5 production from activated lung T cells in mice treated with pharmacological selective iNOS inhibitor [8], decreased production of IL-5 and IL-10 from isolated thoracic lymph node cells from eNOS-transgenic asthmatic mice [12], and

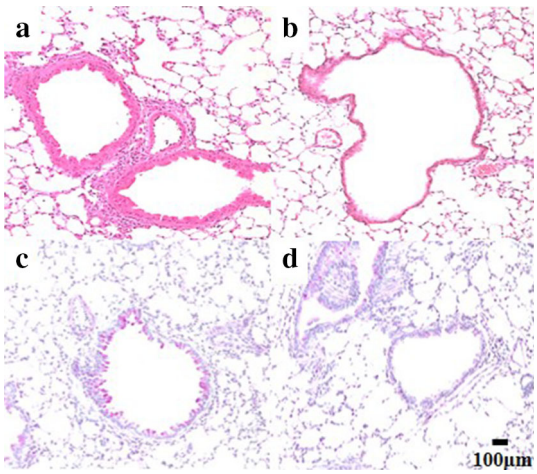

Fig. 1 Hematoxylin and eosin staining of lung sections from wild-type (WT) (**a**) and n/i/eNOS$^{-/-}$ mice (**b**) and periodic acid-Schiff staining of lung sections from WT (**c**) and n/i/eNOS$^{-/-}$ mice (**d**). Mice were sensitized and challenged with ovalbumin

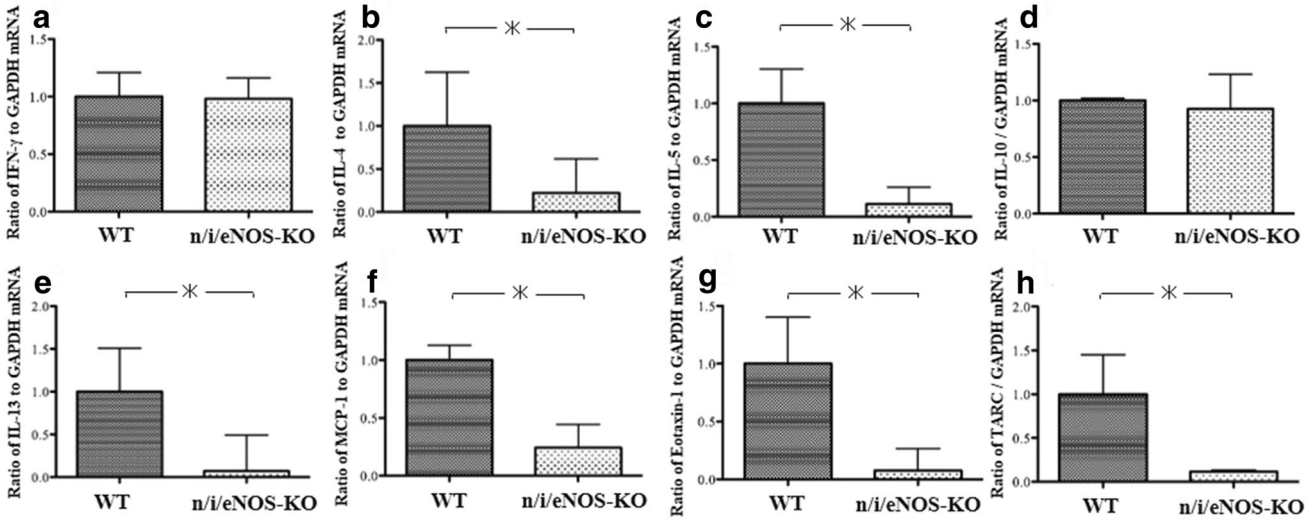

Fig. 2 The mRNA expressions of interferon (IFN)-γ (**a**), interleukin (IL)-4 (**b**), IL-5 (**c**), IL-10 (**d**), IL-13 (**e**), monocyte chemoattractant protein (MCP)-1 (**f**), eotaxin-1 (**g**), and thymus and activation-regulated chemokine (TARC) (**h**) in the lungs of WT ($n = 4$) and n/i/eNOS$^{-/-}$ mice ($n = 4$). *Data* are represented as the ratio to GAPDH. $^{*}P < 0.05$ by *t* test. *WT* wild-type, *NOS* nitric oxide synthase, *IFN* Interferon, *IL* interleukin, *MCP-1* monocyte chemoattractant protein-1, *TARC* thymus and activation-regulated chemokine, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase

unchanged production of IL-4 and IL-5 isolated from lung cells in the iNOS$^{-/-}$ than in WT instead of decreased eosinophilic inflammation [11] were reported. In addition, there have been only limited data of chemokine expressions in the lung using asthmatic murine model. Decreased expressions of MCP-1, eotaxin, C10 were observed in asthmatic mice treated with NOS inhibitor with a decrease of eosinophilic bronchial inflammation compared to wild type mice [7]. Decreased eosinophilic inflammation, expressions of macrophage inflammatory protein (MIP)-2 and MCP-1 were reported in asthmatic mice treated with iNOS inhibitor with unchanged expressions of regulated on activation, normal T cell expressed and secreted (RANTES), eotaxin and T cell activation gene 3 (TCA-3) [8]. By using n/i/eNOS$^{-/-}$ mice, our results indicated that NO plays an important role in promoting eosinophilic inflammation, mucus hypersecretion that might be related to an increase of Th2 cytokines such as IL-4, IL-5, IL-13, and C–C chemokines such as MCP-1, eotaxin-1 and TARC in the pathophysiology of asthma.

Comparing to WT, expressions of pulmonary IFN-γ and IL-10 in n/i/eNOS$^{-/-}$ mice were similar (Fig. 2a, d). With a decrease of eosinophilic inflammation, decreased expression of IFN-γ in activated lung T cells in asthmatic mice treated with iNOS inhibitor [8], and also a decrease of IFN-γ expression in isolated thoracic lymph node cells in asthmatic eNOS-transgenic mice [12] were observed, whereas an increase of IFN-γ expression was shown in asthmatic mice lacking iNOS [10], compared to WT mice. IL-10 is known as anti-inflammatory cytokine and suppresses iNOS and production of NO [16], and decreased levels of IL-10 in the BALF of asthmatic patients compared with those in normal subjects have been reported [17]. IL-4 was significantly increased in asthmatic mice lacking IL-10 [18], but only limited data regarding the relationship between IL-10 and NOSs in murine asthma models was reported. According to our results, decreased levels of Th2 cell-mediated IL-4, IL-5 and IL-13 were not due to IFN-γ and IL-10 production, and NO and its metabolic products or mediators might be related to the decrease of Th2 cytokines.

In contrast to decreased inflammation in asthmatic model using triply-NOS$^{-/-}$ mice, our previous report of a bleomycin-induced pulmonary fibrosis using this n/i/eNOS$^{-/-}$ mouse showed an exacerbation of pulmonary fibrosis [19]. Based on these contrary pulmonary findings of n/i/eNOS$^{-/-}$ mice, further studies are necessary to elucidate complicated multiple roles of NO and NOSs in the lung.

Phase 2 clinical trial of iNOS inhibitor in patients with asthma, GW274150 showed a decrease of FENO but no remarkable effects on asthma symptoms [20]. In this study using n/i/eNOS$^{-/-}$ mice, our results provided the evidence that NO undeniably plays important promotive roles in bronchial eosinophilic inflammation and mucus hypersecretion in asthmatic airway, partially through an increase in Th2 cytokines. We believe that these results of this study might provide a clue to find some targets for drug development to treat bronchial asthma.

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Conflict with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

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