Eur Respir J 2002; 20: 609–616
DOI: 10.1183/09031936.02.00274902
Printed in UK – all rights reserved

Copyright ©ERS Journals Ltd 2002
European Respiratory Journal
ISSN 0903-1936

iNOS depletion completely diminishes reactive nitrogen-species formation after an allergic response

A. Koarai*, M. Ichinose*, H. Sugiura*, M. Tomaki*, M. Watanabe#, S. Yamagata*, Y. Komaki*, K. Shirato*, T. Hattori*

iNOS depletion completely diminishes reactive nitrogen-species formation after an allergic response. A. Koarai, M. Ichinose, H. Sugiura, M. Tomaki, M. Watanabe, S. Yamagata, Y. Komaki, K. Shirato, T. Hattori. ©ERS Journals Ltd 2002.

ABSTRACT: Nitric oxide (NO) shows proinflammatory actions mainly *via* reactive nitrogen species (RNS) formation through superoxide- and peroxidase-dependent mechanisms. The purpose of this study was to examine the role of inducible NO synthase (iNOS) in RNS production, airway hyperresponsiveness, and inflammation after allergen challenge.

Ovalbumin (OVA)-sensitised, iNOS-deficient and wild-type mice were used. RNS production was assessed by nitrotyrosine (NT) immunoreactivity in the airways. Airway inflammation and responsiveness were evaluated by eosinophil accumulation and methacholine (*i.v.*) challenge, respectively.

In wild-type mice, OVA-inhalation challenge increased iNOS immunoreactivity in airway epithelial cells as well as iNOS protein measured by Western blotting. The total amounts of nitrite and nitrate in bronchoalveolar lavage (BAL) fluid were increased, and NT immunoreactivity was also observed abundantly in airway inflammatory cells. In iNOS-deficient mice, both iNOS expression and NT formation were completely abolished, and the total amounts of nitrite and nitrate in BAL fluid were significantly decreased. In contrast, OVA-induced airway eosinophil recruitment and hyperresponsiveness were observed almost equally in wild-type and iNOS-deficient mice.

These data suggest that reactive nitrogen species production after allergic reaction occurs totally *via* inducible nitric oxide synthase-dependent pathways. Allergen-mediated airway eosinophil recruitment and hyperresponsiveness appear to be independent of reactive nitrogen species production.
Eur Respir J 2002; 20: 609–616.

*Division of Respiratory and Infectious Diseases, Tohoku University Graduate School of Medicine and #Dept of Pathology, Tohoku University Hospital, Sendai, Japan.

Correspondence: M. Ichinose
Division of Respiratory and Infectious Diseases
Tohoku University Graduate School of Medicine
1-1, Seiryo-machi, Aoba-ku
Sendai 980-8574
Japan
Fax: 81 227177156
E-mail: ichinose@int1.med.tohoku.ac.jp

Keywords: Airway inflammation
airway responsiveness
asthma
nitric oxide
nitrotyrosine

Received: August 21 2001
Accepted after revision: April 3 2002

Nitric oxide (NO) is a gas molecule synthesised by NO synthase (NOS) that has many physiological and detrimental functions [1]. NOS has at least three isoforms which are composed from two types of constitutive NOS (cNOS) and an inducible NOS (iNOS). cNOS consists of neural NOS (nNOS) and endothelial NOS (eNOS). cNOS-derived NO may be important for physiological regulations, such as vasodilation, neurotransmission, and inhibition of platelet aggregation [1].

In contrast, iNOS, presumably induced by inflammatory cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and interferon (IFN)-γ, produces much larger amounts of NO than cNOS. There is increasing evidence that these large amounts of NO derived from iNOS may participate in the pathophysiology of asthmatic-airway inflammation. iNOS has been reported to be expressed on airway epithelial cells and some inflammatory cells in asthmatic airways [2]. It has also been reported that exhaled NO levels are elevated in asthmatics compared with those in healthy subjects [3], and that the elevation of exhaled NO is correlated with eosinophilic inflammation [4], which is a prominent feature of bronchial asthma.

NO-mediated inflammatory actions are mainly due to conversion to reactive nitrogen species (RNS) [5]. NO reacts with the superoxide anion released from inflammatory cells, yielding the potent oxidant peroxynitrite [6, 7]. NO also reacts directly with oxygen (O_2) to form nitrite which yields nitrogen dioxide by eosinophil peroxidase [8] and myeloperoxidase [9]. These mechanisms seem to be involved in the pathophysiology of the inflammatory process in asthmatic airways [10, 11]. Recently, the authors reported that hyperproduction of RNS occurs during the late-allergic phase in guinea pigs and that the inhibition of RNS production suppresses airway inflammation [12]. However, the precise pathway of RNS production and the role of this molecule in the pathogenesis of asthma are still unclear.

The aim of this study was to examine the iNOS-dependent pathway for RNS production after allergen challenge by means of iNOS-deficient and wild-type mice. The effect of iNOS deficiency on allergen-induced airway hyperresponsiveness and eosinophil accumulation in the airways, an area in which controversial results have been reported, were examined further [13, 14]. The authors demonstrated that RNS hyperproduction occurs after allergen challenge *via*

iNOS-dependent pathways, and that the depletion of iNOS does not affect airway hyperresponsiveness and eosinophil infiltration into the airways in mice.

Materials and methods

Mice

Homozygous iNOS-deficient mice were purchased from Jackson Laboratory (ME, USA) [15], and C57BL/6 mice, which are strain-specific controls for ZZ mice, were purchased from the Institute for Experimental Animals (Tohoku University School of Medicine, Sendai, Japan). Mice were maintained in conventional animal housing under specific pathogen-free conditions, at a constant temperature and humidity, with regular 12-h cycles of light and darkness. Only male mice were used for these studies and were examined between 10–13 weeks of age. All of the experiments performed in this study were conducted with the consent of the Ethics Committee for the Use of Experimental Animals of the Tohoku University School of Medicine.

Sensitisation and allergen challenge

As in a previous study, mice were sensitised and challenged with modification. Briefly, mice were sensitised by an intraperitoneal injection of 0.5 mL solution containing 50 µg of ovalbumin (OVA) (Sigma Chemical Co., St Louis, MO, USA) and 4 mg of aluminum hydroxide in saline, twice, 5 days apart. Twelve days after the second sensitisation, mice were placed in a plexiglass chamber (10×15×25 cm) and challenged with aerosolised saline or 0.5% OVA in saline for 1 h on two occasions 4 h apart. The aerosolised OVA was produced by an ultrasonic nebuliser (NE-12; Omuron, Tokyo, Japan (output 0.8 $mL \cdot min^{-1}$)). One or 3 days after the challenge, mice were anaesthetised by intraperitoneal injection of pentobarbital sodium (70 mg·kg body $weight^{-1}$) and used for the following experiments.

Bronchoalveolar lavage. One or 3 days after the challenge, mice were sacrificed, and bronchoalveolar lavage (BAL) was performed. After dissection of the lungs, the trachea was cannulated with a polyethylene tube (0.6 mm outer diameter). The lungs were lavaged twice with phosphate-buffered saline (PBS) (0.25, 0.20 mL each time) and ~0.4 mL of instilled fluid was consistently recovered. Total cell numbers were counted with a haemocytometer. After centrifugation at 3,000×*g* for 5 min, cell pellets were resuspended in 100 µL of PBS and cytospin preparations were prepared using a Shandon III cytocentrifuge (Shandon Southern Instruments, Seuekley, PA, USA) at 300×*g* for 5 min. All preparations were stained with Diff-Quik stain (Baxter, McGraw Park, IL, USA). Differential cell counts in BAL fluid were carried out with ⩾300 leucocytes. BAL supernatant was stored at -80°C until further examination.

Western-blot analysis for inducible nitric-oxide synthase. One or 3 days after the challenge, mice were sacrificed. After opening the thorax, the systemic circulation was perfused with 50 mM ice cold PBS containing 1 mM ethylenediamine tetraacetic acid (EDTA) (Sigma Chemical Co.), for 5 min, at 100 mmHg, using an 18G-needle passed through a left ventriculoctomy into the ascending aorta. The pulmonary circulation was also perfused with 5 mL of the same solution from the right ventricle. The lungs were then isolated and connective tissue, vasculature and parenchyma were gently scraped off. The airways were homogenised in 0.8 mL of 50 mM Tris-HCl buffer (pH 7.8) containing 150 mM NaCl, 1 mM EDTA, 1 mM sodium vanadate (Sigma Chemical Co.), 1% NP-40 (Nacalai Tesque, Inc., Kyoto, Japan) and 1 mM para-amidino-phenylmethylsulphonyl fluoride (para-amidino-PMSF). The samples were centrifuged at 10,000×*g* for 15 min at 4°C and the supernatants were concentrated by a microcon 10 (Amicon, Inc., Beverly, USA) at 14,000×*g* for 50 min at 4°C. The protein content was determined using the method described by LOWRY *et al.* [16], with bovine serum albumin as the standard. Sample protein was added at one-quarter of the volume of a 250 mM Tris buffer containing 20% β-mercaptoethanol (Bio-Rad Laboratories, Hercules, CA, USA), 0.01% bromophenol blue, 8% sodium dodecyl sulphate (SDS) (Bio-Rad Laboratories), and 40% glycerol and boiled for 5 min. Protein (90 $\mu g \cdot lane^{-1}$) was separated on a 7.5% SDS polyacrylamide gel, transferred to a hydrophobic polyvinylidene difluoride membrane (Amersham Life Science, Little Chalfont Buckinghamshire, UK) and probed with an 1:2,000 diluted antimouse iNOS rabbit antisera (Wako Pure Chemical Industries, Osaka, Japan). The secondary antibody used was an antirabbit goat immunoglobulin (Ig)G conjugated horseradish peroxidase (DAKO Japan Ltd, Kyoto, Japan). Antibody binding was detected using enhanced chemiluminescence (ECL)-plus (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer′s recommendation. The membrane was photographed by a midnight sun camera (C-1; Fuji Photo Film Co., Tokyo, Japan) and the intensity of the bands was quantified by densitometry (MCID image analyzer; Imaging Inc., Research, St. Catherines, ON, Canada).

Inducible nitric-oxide synthase immunostaining. One day after the challenge, mice were sacrificed and the systemic and pulmonary circulation were perfused with 1% paraformaldehyde (PFA) (Sigma Chemical Co.). The lungs were isolated and immersed in 4% PFA fixative solution for 12 h at 4°C, and further immersed for 24 h at 4°C in 0.1 M phosphate buffer containing 15% sucrose. The tissues were then sectioned at a thickness of 6 µm with a cryostat. All sections were mounted on chrom-alum gelatin-coated glass slides. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in 100% methanol for 5 min at room temperature. After washing in PBS, sections were incubated with a primary antibody (polyclonal iNOS rabbit antisera, 1:250 dilution). In order to reduce nonspecific binding, tissues were

preincubated with 4% skimmed milk in PBS containing 0.3% Triton-X (Sigma Chemical Co.) for 30 min and then incubated with 10% heat inactivated normal goat serum for 30 min at room temperature. The immunoreactions were visualised by the indirect immunoperoxidase method using ENVISION polymer reagent, which is an antirabbit goat IgG conjugated with peroxidase-labelled dextran (DAKO Japan Ltd.), for 1 h at room temperature [12]. The diaminobenzidine (Sigma Chemical Co.) reaction was performed, followed by counterstaining with haematoxylin. Stained sections were viewed on a microscope (BX-40; Olympus, Tokyo, Japan) and captured with a 3-charged coupled device (CCD) colour video camera (KY-F55MD; Olympus) using image analysis software (Mac SCOPE; Mitani Co., Fukui, Japan).

Nitrotyrosine immunostaining. One day after the challenge, the mice were sacrificed, and the lung tissues were treated using the same protocol as in the iNOS-immunostaining method. An antinitrotyrosine rabbit IgG was used (1:100 dilution; Upstate Biotechnology, Lake Placid, NY, USA) as a primary antibody and ENVISION polymer reagent for the immunoreactions [12].

Measurement of nitrite and nitrate in bronchoalveolar lavage fluid. The total amount of nitrite and nitrate in BAL fluid, after the reduction of nitrate to nitrite with nitrate reductase, was determined by a commercial nitrite/nitrate colourimetric assay kit (BioDynamics Laboratory, Inc., OH, USA). Briefly, 70 µL of each sample was incubated with the cofactor reduced nicotinamide adenine dinucleotide phosphate (NADPH) for 30 min in the presence of nitrate reductase, followed by the addition of Griess reagent and a subsequent incubation for 20 min. Absorbance was measured by a spectrophotometer at 540 nm, and the nitrite concentration was determined using $NaNO_2$ and $NaNO_3$ standards.

Measurement of airway responsiveness. Airway responsiveness was measured *in vivo* 1 or 3 days after the challenge, using a modified oscillation method as described by SCHUESSLER and BATES [17]. Briefly, mice were anaesthetised with an intraperitoneal injection of pentobarbital sodium (70 $mg \cdot kg^{-1}$) (Tokyo Kasei Kogyo Co., Tokyo, Japan) and the trachea was cannulated with a tracheal tube (0.6 mm outer diameter). Spontaneous breathing of animals was then suppressed with an intraperitoneal injection of succinylcholine chloride (10 $mg \cdot kg^{-1}$) (Sigma Chemical Co.). When the breathing stopped, the tracheal cannula was connected to a computer-controlled small-animal ventilator (flexiVent® Scientific Respiratory Equipment Inc., Montreal, Canada) [17]. Mice were ventilated quasisinusoidally at 150 $strokes \cdot min^{-1}$ with a tidal volume of 6 $mL \cdot kg^{-1}$. Small-amplitude volume oscillations contained discrete frequency components at 0.3–19.6 Hz. A total of 19 points were then applied at constant lung volume to the tracheal opening for 16 s, and pulmonary resistance (RL) was estimated at each frequency. Measurements were made at baseline and following increasing doses of methacholine (MCh), cumulatively administered (33–330 $\mu g \cdot kg^{-1}$) intrajugularly at the start of the 16-s oscillation.

Measurement of immunoglobulin E content in serum. Three days after the challenge, blood was drawn from the right ventricule, and serum was obtained by centrifugation at 1,500×*g* for 10 min. Serum IgE levels were determined with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Yamasa, Chiba, Japan).

Statistical analysis

Data are expressed as mean±SEM. Multiple comparisons of mean data of cell counts in BAL, iNOS Western blotting, total nitrite and nitrate, airway hyperreactivity, and total IgE among the groups were performed by one-way analysis of variance (ANOVA) followed by Bonferroni/Dunn test as a *post hoc* test. A $p<0.05$ was considered statistically significant.

Results

Cell counts in bronchoalveolar lavage after antigen challenge

OVA-inhalation challenge caused eosinophil infiltration into the airways in wild-type sensitised mice 1 and 3 days after challenge. In the BAL cell counts, total cell numbers did not increased 1 day after OVA inhalation, but significantly increased 3 days after the challenge as compared with those after saline inhalation ($p<0.05$) (table 1). Eosinophil numbers in BAL were significantly increased 1 day after, and more greatly increased 3 days after OVA inhalation ($p<0.05$). As seen in wild-type mice, in iNOS-deficient mice, the same OVA-inhalation challenge caused eosinophil infiltration into the airways. When estimated by the BAL cell counts, the degree of allergen-induced increases in total cells and eosinophils were no different in the iNOS-deficient mice and the wild-type mice.

Immunostaining of inducible nitric-oxide synthase expression and nitrotyrosine formation

Immunostaining studies were performed in the lung tissues obtained 1 day after OVA-inhalation challenge. iNOS expression was scanty within the tissues from saline-challenged wild-type mice (fig. 1a). In contrast, obvious iNOS expression was observed in epithelial cells and infiltrated inflammatory cells (figs. 1b and c) within the submucosa in OVA-challenged wild-type mice. In iNOS-deficient mice, iNOS expression was not detected in any type of cell after OVA challenge (fig. 1d).

Nitrotyrosine formation was not observed within the tissues from saline-challenged wild-type mice (fig. 1e). In contrast, nitrotyrosine formation was clearly seen in infiltrated polynuclear cells, within the tissues in OVA-challenged wild-type mice (fig. 1f).

Table 1. – Differential cell counts on bronchoalveolar lavage (BAL) cells from ovalbumin (OVA)-sensitised and challenged mice

Genotype	Treatment	Time after challenge	Total number of BAL cells 1×10^5	Macrophages %	Eosinophils %	Lymphocytes %	Neutrophils %
(+/+)	Saline	1 day	1.67±0.20	1.59±0.21 (95.33)	0.00±0.00 (0.18)	0.08±0.02 (4.49)	0.00±0.00 (0.12)
(-/-)	Saline	1 day	1.60±0.24	1.52±0.23 (95.18)	0.01±0.00 (0.19)	0.07±0.02 (4.20)	0.01±0.00 (0.31)
(+/+)	OVA	1 day	1.68±0.18	1.15±0.01 (68.28)	0.45±0.10* (26.95)	0.06±0.02 (3.70)	0.02±0.01 (1.31)
(-/-)	OVA	1 day	1.98±0.19	1.30±0.08 (65.60)	0.51±0.12* (25.95)	0.10±0.02 (4.96)	0.06±0.02*,(*) (3.19)
(+/+)	OVA	3 day	3.07±0.30*,(*)	1.91±0.32(*) (62.15)	0.97±0.24*,(*) (31.63)	0.16±0.03*,(*) (5.11)	0.04±0.01* (1.20)
(-/-)	OVA	3 day	2.94±0.38*,(*)	1.77±0.45 (60.37)	1.04±0.23*,(*) (35.48)	0.12±0.20 (3.98)	0.01±0.00 (0.24)

Data are presented as mean±SEM of six animals. *: $p<0.05$ compared with wild type mice at 1 day after saline challenge; (*): $p<0.05$ compared with wild type mice at 1 day after OVA challenge.

In iNOS-deficient mice, nitrotyrosine formation was not detected in those cells after OVA challenge (fig. 1g).

Effects of antigen sensitisation and challenge on inducible nitric-oxide synthase protein levels

Basal iNOS protein levels in the airways were not significantly different between control (nonsensitised) and sensitised wild-type mice (data not shown). One and 3 days after OVA-inhalation challenge, the iNOS protein levels were significantly increased in wild-type mice compared with those of saline-challenged mice ($p<0.0001$ and $p<0.05$, respectively) (fig. 2). The increase in iNOS protein levels in the airways was larger, 1 day after OVA challenge when compared to 3 days after the challenge in wild-type mice ($p<0.01$). In iNOS-deficient mice, iNOS protein levels in the airways after OVA challenge were almost the same as background levels.

Nitrite and nitrate in bronchoalveolar lavage fluid after antigen challenge

OVA-inhalation challenge significantly increased the total amount of nitrite and nitrate in BAL fluid 3 days after OVA challenge in the wild-type mice ($p<0.05$) (fig. 3). In iNOS-deficient mice, the amount of nitrite and nitrate in BAL fluid was not increased after OVA challenge.

Airway responsiveness after antigen challenge

OVA-inhalation challenge significantly increased airway responsiveness to MCh 1 and 3 days after OVA challenge in wild-type mice ($p<0.05$) (fig. 4). The degree of the airway responsiveness to MCh after OVA challenge in iNOS-deficient mice was not significantly different from that in the wild-type mice.

Serum total immunoglobulin E levels

The serum total IgE levels after OVA sensitisation and challenge were not significantly different between iNOS-deficient and wild-type mice (fig. 5).

Discussion

In this study, it has been demonstrated that RNS production in mice airways after allergen challenge is completely dependent upon the NO derived from iNOS. In the present study, the RNS immunoreactivity after allergen challenge in the airway tissues and the total increases in the nitrite and nitrate levels in BAL fluid after allergen challenge were completely abolished in iNOS-deficient mice, while airway eosinophil infiltration and hyperresponsiveness were still observed.

With regard to the role of endogenous NO derived

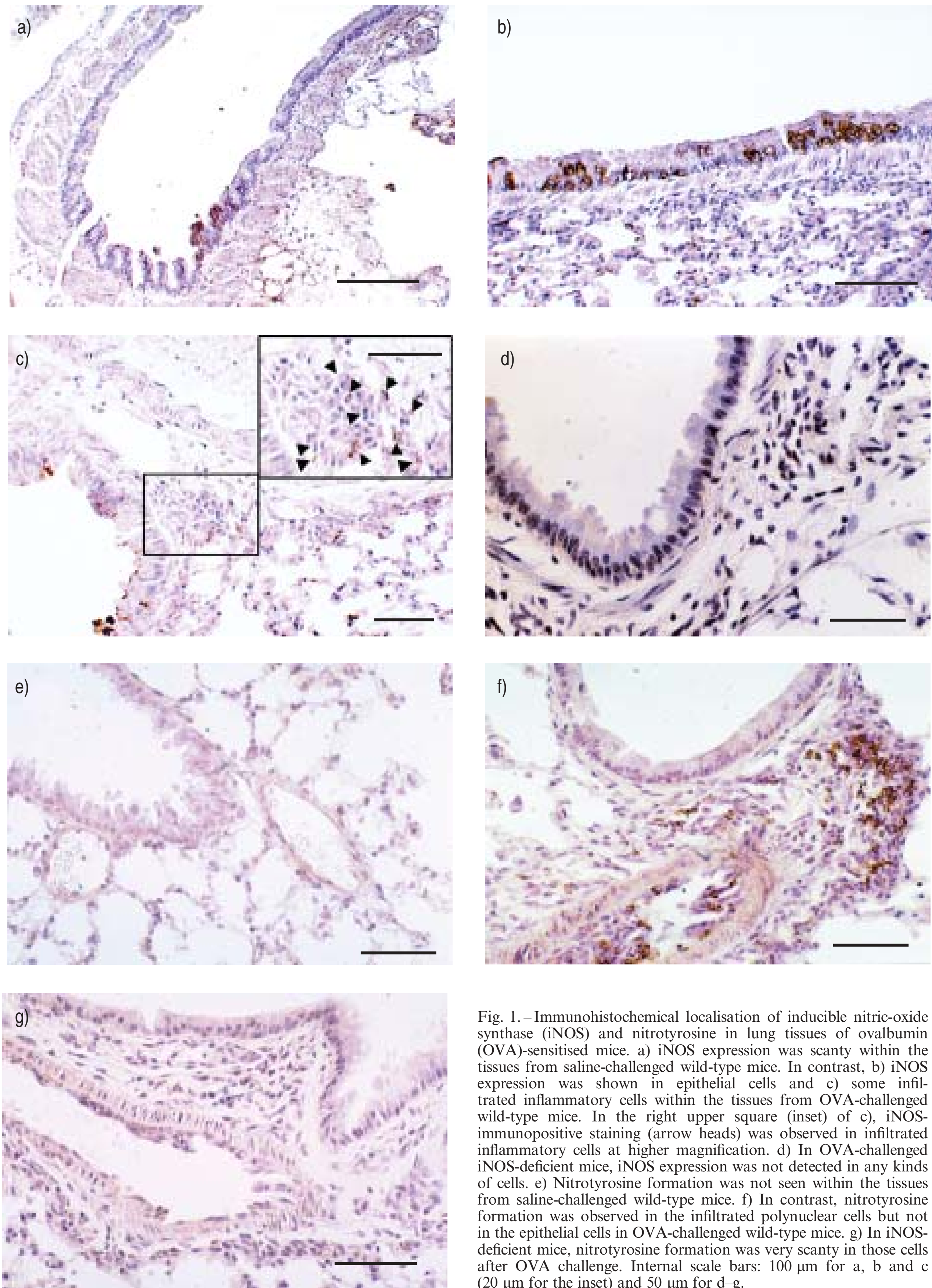

Fig. 1. – Immunohistochemical localisation of inducible nitric-oxide synthase (iNOS) and nitrotyrosine in lung tissues of ovalbumin (OVA)-sensitised mice. a) iNOS expression was scanty within the tissues from saline-challenged wild-type mice. In contrast, b) iNOS expression was shown in epithelial cells and c) some infiltrated inflammatory cells within the tissues from OVA-challenged wild-type mice. In the right upper square (inset) of c), iNOS-immunopositive staining (arrow heads) was observed in infiltrated inflammatory cells at higher magnification. d) In OVA-challenged iNOS-deficient mice, iNOS expression was not detected in any kinds of cells. e) Nitrotyrosine formation was not seen within the tissues from saline-challenged wild-type mice. f) In contrast, nitrotyrosine formation was observed in the infiltrated polynuclear cells but not in the epithelial cells in OVA-challenged wild-type mice. g) In iNOS-deficient mice, nitrotyrosine formation was very scanty in those cells after OVA challenge. Internal scale bars: 100 μm for a, b and c (20 μm for the inset) and 50 μm for d–g.

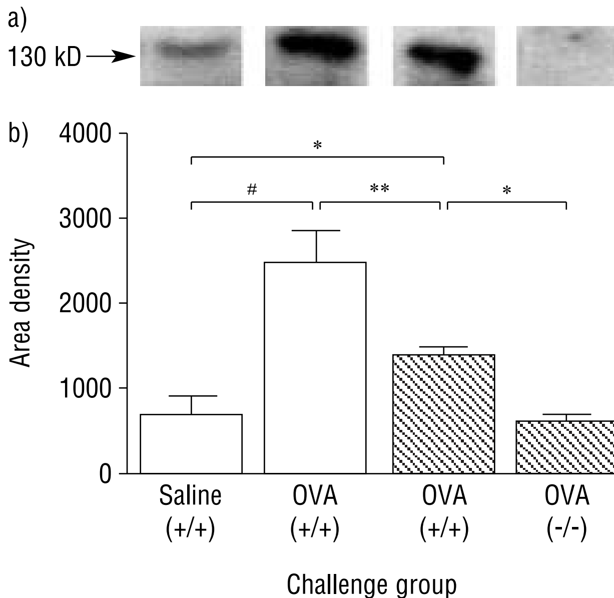

Fig. 2. – a) Immunoblot analysis of the expression of inducible nitric-oxide synthase (iNOS) in airway tissues isolated from ovalbumin (OVA)-sensitised and -challenged wild (+/+) and iNOS-deficient (-/-) mice. b) iNOS protein levels in the airways were quantified with densitometry. Each value indicates mean±SEM of four animals. □: day 1; ▧: day 3. *: $p<0.05$; **: $p<0.01$; #: $p<0.0001$.

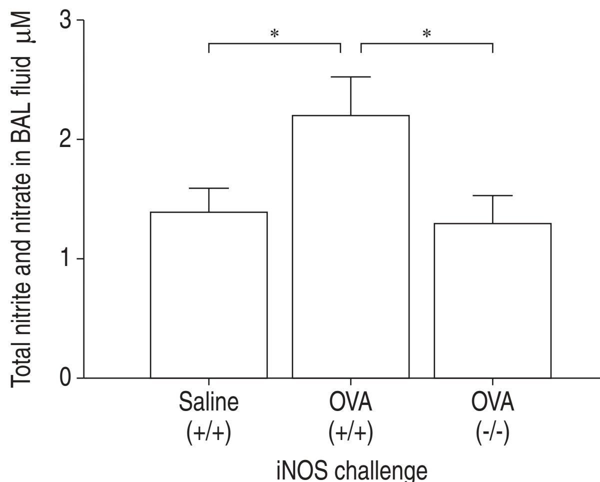

Fig. 3. – Total nitrite and nitrate in bronchoalveolar lavage (BAL) fluid from ovalbumin (OVA)-sensitised and -challenged wild (+/+) and inducible nitric-oxide synthase (iNOS)-deficient (-/-) mice. Each value indicates mean±SEM of six animals.

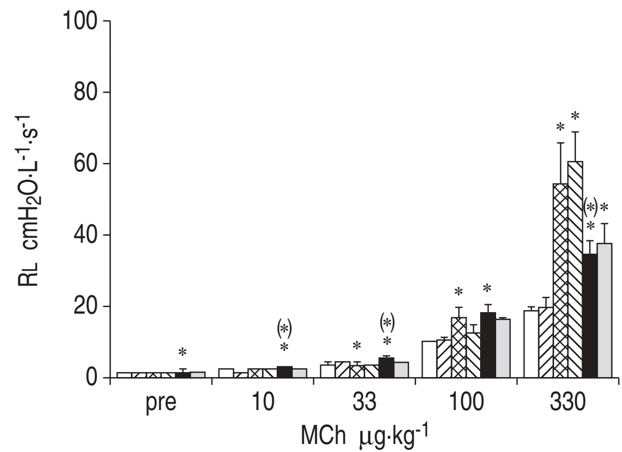

Fig. 4. – Airway responsiveness to intravenous methacholine (MCh) in wild (+/+) and inducible nitric-oxide synthase (iNOS)-deficient (-/-) mice. Each value indicates mean±SEM of 6–14 animals. RL: lung resistance. *: $p<0.05$ compared with wild-type mice 1 day after saline challenge; (*): $p<0.05$ compared with wild-type mice 1 day after ovalbumin (OVA) challenge. □: saline, day 1, +/+; ▨: saline, day 1, -/-; ▩: OVA, day 1, +/+; ▧: OVA, day 1 -/-; ■: OVA, day 3, +/+; ▒: OVA, day 3, -/-.

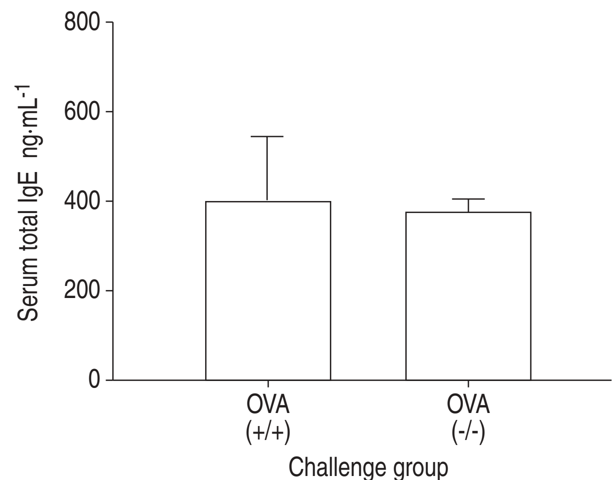

Fig. 5. – Serum total immunoglobulin (Ig)E levels in wild (+/+) and iNOS-deficient (-/-) mice. Each value indicates mean±SEM of six animals.

from iNOS in allergic-airway inflammation, two studies utilising iNOS-deficient mice have been reported. XIONG *et al.* [13] showed that allergen-induced eosinophil recruitment into the airway was significantly reduced in iNOS-deficient mice compared with wild-type mice. However, they have also reported that airway hyperresponsiveness after allergen challenge was not different between iNOS-deficient and wild-type mice [13]. In contrast, DE SANCTIS *et al.* [14] reported that the degrees of both allergen-induced airway eosinophilia and hyperresponsiveness were similar in iNOS-deficient mice and wild-type mice. The present results are in agreement with the latter study.

NO reacts with superoxide anion released from inflammatory cells, yielding the potent oxidant peroxynitrite [6]. NO also reacts directly with O_2 to form nitrite that yields nitrogen dioxide by myeloperoxidase [9] and eosinophil peroxidase [8]. These RNS have been thought to cause detrimental effects in various inflammatory diseases, including bronchial asthma. Recently, using an allergic guinea-pig model, the authors showed that the production of RNS is increased in the airways after allergen challenge [12].

In that study, a nonspecific NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME), xanthine oxidase inhibitor (4-amino-6-hydroxypy-razolo (3, 4-d) pyrimidine (AHPP)), and peroxynitrite scavenger (ebselen) inhibited airway microvascular hyperpermeability during the late-allergic phase in guinea pigs, suggesting an important role for RNS in allergic airway inflammation [12]. However, in the present study, the RNS elimination by iNOS deficiency inhibited neither airway eosinophil accumulation nor airway hyperresponsiveness after allergen challenge.

The airway microvasculature is involved in inflammatory cell infiltration and airway wall oedema, which are important factors for airway hyperresponsiveness [18]. Thus, between the previous guinea-pig study and the present study with iNOS-deficient mice there is a discrepancy in the effect of RNS on the airway hyperreactivity *via* microvascular hyperpermeability. One possible explanation for this discrepancy may be differences in the airway structures between the two species. Since the microvasculature in airway submucosa of mice is not as well developed as that of guinea pigs, the degree of oedema formation in the murine-airway submucosa of mice, caused by microvascular hyperpermeability during the late-allergic phase, might be much smaller than that of guinea pigs [19]. There is also another possible explanation. In a previous guinea-pig study by the authors, allergen-induced nitrotyrosine immunoreactivity was observed in airway microvascular endothelium as well as in infiltrated polynuclear cells [12]. In contrast, in the present murine model, nitrotyrosine immunoreactivity after allergen challenge was seen in infiltrated cells but not in microvascular endothelium. If RNS production in microvascular endothelium were essential for causing the leakage from airway microvasculature and for the airway oedema formation, this would explain why iNOS deficiency did not affect the leakage from airway microvasculature and lead to airway hyperresponsiveness in mice.

With regard to the allergen-induced eosinophilia, in the guinea-pig study, L-NAME inhibited eosinophil accumulation in the airways, but AHPP and ebselen had no effect during the late-allergic phase [12]. The elimination of RNS had no effect on airway eosinophilia in guinea pigs or mice. There have been several reports that nonspecific NOS inhibitors inhibited eosinophil accumulation in the airways and eosinophil activities [20, 21]. In other studies, iNOS-specific inhibitor inhibited allergen-induced airway eosinophilia [22, 23]. Until now, it was uncertain whether the cNOS- or iNOS-derived NO participate in eosinophil accumulation in the airways during the allergic response. In this iNOS-deficient study, the elimination of iNOS-derived NO had no effect on airway eosinophilia after allergen challenge. Further, it has been previously reported that the inhibition of cNOS, but not iNOS, suppressed the airway eosinophilia in the late-allergic response in mice [24]. These results suggest that the inhibition of eosinophilia by L-NAME might be due to the inhibition of cNOS, but not iNOS.

Recently, nitrotyrosine, which is a marker of protein nitration by RNS, has been detected in airway epithelial cells and infiltrated inflammatory cells in asthmatic airways by immunohistochemistry [5]. MACPHERSON *et al.* [25] also demonstrated nitrotyrosine formation in eosinophils in asthma using a double immunostaining for nitrotyrosine and eosinophil peroxidase. In mice, IIJIMA *et al.* [26] reported nitrotyrosine immunoreactivity in airway epithelial cells and infiltrated cells including mainly eosinophils, macrophages, and neutrophils after antigen challenge. In the present study, nitrotyrosine immunoreactivity was observed mainly in infiltrated polynuclear cells, which could be assumed to be eosinophils. Because eosinophils can express iNOS protein to produce NO after allergen challenge [27] and eosinophils also have eosinophil peroxidase [8], it seems likely that eosinophils may produce RNS through these pathways. DUGUET *et al.* [28] also reported that nitrotyrosine formation was reduced in eosinophil peroxidase-deficient mice after antigen challenge, suggesting that eosinophil peroxidase plays a role in RNS production.

Recent studies have suggested the functional consequences of protein nitration by RNS. Exposure of surfactant protein A (SP-A) to peroxynitrite or tetranitromethane leads to nitration of protein, and decreases the ability of SP-A to aggregate lipids [29, 30]. Nitrated SP-A protein has been detected in pulmonary oedema fluid from patients with acute lung injury [31]. The enzyme inactivation caused by nitration of a tyrosine residue by peroxynitrite has been shown in human mitochondrial manganese-superoxide dismutase [32]. In the present study, the involvement of protein nitration in the pathogenesis of allergic airway diseases is suggested. Further studies are required to reveal the precise mechanisms of nitrotyrosine formation and to determine the functional significance of protein nitration in allergic airways.

In the present study, there was no difference in the serum total IgE level between wild-type and iNOS-deficient mice after allergen challenge, which is in accordance with two recent reports [13, 14]. These results, suggested that iNOS-derived NO did not influence the capacity of B-type lymphocytes to secret antibodies.

To conclude, it has been shown that reactive nitrogen species hyperproduction after allergen challenge occurs totally *via* inducible nitric-oxide synthase-dependent pathways. Since allergen-induced airway eosinophil recruitment and hyperresponsiveness were observed to almost the same degree in wild-type and inducible nitric-oxide synthase-deficient mice, these two phenotypes appear to be independent of reactive nitrogen-species production.

Acknowledgements. The authors would like to thank. H. Sakurada for technical assistance and B. Bell for reading the manuscript.

References

1. Schmidt HH, Walter U. NO at work. *Cell* 1994; 78: 919–925.
2. Hamid Q, Springall DR, Riveros-Moreno V, *et al.*

Induction of nitric oxide synthase in asthma. *Lancet* 1993; 342: 1510–1513.
3. Kharitonov SA, Yates D, Robbins RA, Logan-Sinclair R, Shinebourne EA, Barnes PJ. Increased nitric oxide in exhaled air of asthmatic patients. *Lancet* 1994; 343: 133–135.
4. Jatakanon A, Lim S, Kharitonov SA, Chung KF, Barnes PJ. Correlation between exhaled nitric oxide, sputum eosinophils, and methacholine responsiveness in patients with mild asthma. *Thorax* 1998; 53: 91–95.
5. Saleh D, Ernst P, Lim S, Barnes PJ, Giaid A. Increased formation of the potent oxidant peroxynitrite in the airways of asthmatic patients is associated with induction of nitric oxide synthase: effect of inhaled glucocorticoid. *FASEB J* 1998; 12: 929–937.
6. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 1990; 87: 1620–1624.
7. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* 1996; 271: C1424–C1437.
8. Wu W, Chen Y, Hazen SL. Eosinophil peroxidase nitrates protein tyrosyl residues. Implications for oxidative damage by nitrating intermediates in eosinophilic inflammatory disorders. *J Biol Chem* 1999; 274: 25933–25944.
9. Eiserich JP, Hristova M, Cross CE, *et al.* Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature* 1998; 391: 393–397.
10. Barnes PJ, Chung KF, Page CP. Inflammatory mediators of asthma: an update. *Pharmacol Rev* 1998; 50: 515–596.
11. Sadeghi-Hashjin G, Folkerts G, Henricks PA, Muijsers RB, Nijkamp FP. Peroxynitrite in airway diseases. *Clin Exp Allergy* 1998; 28: 1464–1473.
12. Sugiura H, Ichinose M, Oyake T, *et al.* Role of peroxynitrite in airway microvascular hyperpermeability during late allergic phase in guinea pigs. *Am J Respir Crit Care Med* 1999; 160: 663–671.
13. Xiong Y, Karupiah G, Hogan SP, Foster PS, Ramsay AJ. Inhibition of allergic airway inflammation in mice lacking nitric oxide synthase 2. *J Immunol* 1999; 162: 445–452.
14. De Sanctis GT, MacLean JA, Hamada K, *et al.* Contribution of nitric oxide synthases 1, 2, and 3 to airway hyperresponsiveness and inflammation in a murine model of asthma. *J Exp Med* 1999; 189: 1621–1630.
15. Laubach VE, Shesely EG, Smithies O, Sherman PA. Mice lacking inducible nitric oxide synthase are not resistant to lipopolysaccharide-induced death. *Proc Natl Acad Sci USA* 1995; 92: 10688–10692.
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951; 193: 265–275.
17. Schuessler TF, Bates JH. A computer-controlled research ventilator for small animals: design and evaluation. *IEEE Trans Biomed Eng* 1995; 42: 860–866.
18. Kimura K, Inoue H, Ichinose M, *et al.* Bradykinin causes airway hyperresponsiveness and enhances maximal airway narrowing. Role of microvascular leakage and airway edema. *Am Rev Respir Dis* 1992; 146: 1301–1305.
19. Baluk P, Thurston G, Murphy TJ, Bunnett NW, McDonald DM. Neurogenic plasma leakage in mouse airways. *Br J Pharmacol* 1999; 126: 522–528.
20. Ferreira HH, Medeiros MV, Lima CS, *et al.* Inhibition of eosinophil chemotaxis by chronic blockade of nitric oxide biosynthesis. *Eur J Pharmacol* 1996; 310: 201–207.
21. Zanardo RC, Costa E, Ferreira HH, *et al.* Pharmacological and immunohistochemical evidence for a functional nitric oxide synthase system in rat peritoneal eosinophils. *Proc Natl Acad Sci USA* 1997; 94: 14111–14114.
22. Schuiling M, Meurs H, Zuidhof AB, Venema N, Zaagsma J. Dual action of iNOS-derived nitric oxide in allergen-induced airway hyperreactivity in conscious, unrestrained guinea pigs. *Am J Respir Crit Care Med* 1998; 158: 1442–1449.
23. Koarai A, Ichinose M, Sugiura H, Yamagata S, Hattori T, Shirato K. Allergic airway hyperresponsiveness and eosinophil infiltration is reduced by a selective iNOS inhibitor, 1400W, in mice. *Pulm Pharmacol Ther* 2000; 13: 267–275.
24. Feder LS, Stelts D, Chapman RW, *et al.* Role of nitric oxide on eosinophilic lung inflammation in allergic mice. *Am J Respir Cell Mol Biol* 1997; 17: 436–442.
25. MacPherson JC, Comhair SA, Erzurum SC, *et al.* Eosinophils are a major source of nitric oxide-derived oxidants in severe asthma: characterization of pathways available to eosinophils for generating reactive nitrogen species. *J Immunol* 2001; 166: 5763–5772.
26. Iijima H, Duguet A, Eum SY, Hamid Q, Eidelman DH. Nitric oxide and protein nitration are eosinophil dependent in allergen-challenged mice. *Am J Respir Crit Care Med* 2001; 163: 1233–1240.
27. del Pozo V, de Arruda-Chaves E, de Andres B, *et al.* Eosinophils transcribe and translate messenger RNA for inducible nitric oxide synthase. *J Immunol* 1997; 158: 859–864.
28. Duguet A, Iijima H, Eidelman DH. Nitrotyrosine formation is reduced in eosinophil peroxidase deficient mice after antigen challenge. *Am J Respir Crit Care Med* 2000; 161: A920.
29. Greis KD, Zhu S, Matalon S. Identification of nitration sites on surfactant protein A by tandem electrospray mass spectrometry. *Arch Biochem Biophys* 1996; 335: 396–402.
30. Haddad IY, Zhu S, Ischiropoulos H, Matalon S. Nitration of surfactant protein A results in decreased ability to aggregate lipids. *Am J Physiol* 1996; 270: L281–L288.
31. Zhu S, Ware LB, Geiser T, Matthay MA, Matalon S. Increased levels of nitrate and surfactant protein a nitration in the pulmonary edema fluid of patients with acute lung injury. *Am J Respir Crit Care Med* 2001; 163: 166–172.
32. Yamakura F, Taka H, Fujimura T, Murayama K. Inactivation of human manganese-superoxide dismutase by peroxynitrite is caused by exclusive nitration of tyrosine 34 to 3-nitrotyrosine. *J Biol Chem* 1998; 273: 14085–14089.