

NOS 1 Is Required for Allergen-Induced Expression of NOS 2 in Mice

Hiroaki Iijima Meri K. Tulic Alexandre Duguet Jichuan Shan
Paolo Carbonara Outayba Hamid David H. Eidelman

Meakins-Christie Laboratories, Respiratory Division, Department of Medicine, McGill University Health Centre,
Montréal, Canada

Key Words

Neuronal nitric oxide synthase · Nitric oxide synthase ·
Animal models · Eosinophils · Lung · Asthma · Mice

Abstract

Background: Although increased nitric oxide (NO) production in asthma is mediated largely by upregulation of the inducible form of nitric oxide synthase (iNOS, or NOS 2), some studies have suggested an important role for the usually constitutive neural NOS isoform (nNOS, or NOS 1). **Aim:** To investigate how NOS 1 may influence allergic inflammation, we used NOS 1 knockout mice and their wild-type (WT) controls. **Methods:** Mice were sensitized and challenged with ovalbumin (OVA) using a protocol known to upregulate NOS 2 in the airways. **Results:** In addition to expected increases in NOS 2 activity, OVA challenge led to increases in calcium-dependent NOS activity, which was accounted for by increased expression of NOS 1 at both mRNA ($n = 5$, $p < 0.001$) and protein levels ($n = 5$, $p < 0.01$). In NOS-1-deficient mice, OVA challenge induced less eosinophilia ($n = 7$, $p < 0.05$) and much less NO production ($n = 10$, $p < 0.01$) than in WT controls, reflecting not only the expected absence of NOS 1, but also lack of upregulation of NOS 2. This interaction appeared to be stimulus specific as NOS-1-deficient mice did upregulate NOS 2 following exposure to lipopolysaccharide ($n = 5$, $p < 0.001$). **Conclusions:** These findings underscore the importance of NOS 1 in allergic

airway inflammation and suggest a mechanism by which NOS 1 may influence overall NO production in the airways.

Copyright © 2005 S. Karger AG, Basel

Introduction

Since the first clinical studies demonstrating nitric oxide (NO) in exhaled air of healthy volunteers [1, 2] and increased exhaled NO concentrations in asthma [3], there has been growing interest in the role of NO in asthma and other inflammatory diseases of the airways. The bronchial epithelium of asthmatics exhibits evidence of increased expression of the inducible isoform of nitric oxide synthase (NOS 2) [4], which may be suppressed by inhaled corticosteroids [5–7]. In asthma, as in other inflammatory states, NOS 2 is thought to be the most important source of NO. NOS 2, which produces NO in a calcium-independent fashion, is capable of producing NO very efficiently compared to other NOS isoforms. NOS 2 is expressed both in inflammatory cells such as macrophages and in the airway epithelium and is upregulated under the influence of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α), interferon-gamma (IFN- γ) and interleukin-1 beta (IL- β) through mechanisms dependent on several transcription factors, most notably nuclear factor kappa beta (NF- κ B).

Despite the importance of NOS 2, however, recent research has demonstrated that the neuronal NOS isoform (NOS 1, also termed nNOS) may also be important. NOS 1 may be responsible for the modulation of airway hyperresponsiveness in rodents [8–10], although these reports are conflicting. While NO produced by NOS 1 in rats has been shown to limit OVA-induced airway hyperresponsiveness in vivo using preferential NOS 1 inhibitors [8], in NOS 1 knockout (KO) mice NOS-1-produced NO promotes OVA-induced airway hyperresponsiveness [10] and contributed about 40% of the NO in expired air [9]. In man, genetic studies have found an association between a polymorphism in the NOS 1 gene and asthma [11–13]. Furthermore, while NOS 1 was originally thought to be constitutively expressed, increasing evidence indicates that its expression can be dynamically regulated by a large array of physiological or pathological conditions [14].

In the present study, we set out to examine the role of NOS-1-derived NO in an animal model of allergic airway inflammation using mice with a targeted NOS 1 gene deletion. In view of studies suggesting a role for NOS 1 in the pathogenesis of asthma, we wished to determine the influence of NOS 1 on the inflammatory response to specific antigen challenge in the airways. We hypothesized that like the inducible isoform of NOS (iNOS, also termed NOS 2), NOS 1 protein and mRNA may be upregulated following ovalbumin (OVA) challenge in the lungs of sensitized animals. In addition to demonstrating that NOS 1 is indeed upregulated, our findings suggest an important role for NOS 1 in the inflammatory response to acute allergen challenge mediated in large part through an interaction with NOS 2.

Material and Methods

Animals

Male C57BL/6J wild-type (WT) mice were obtained from a commercial supplier (The Jackson Laboratory, Bar Harbor, Me., USA) and were 10 weeks old at the time of data collection. A homozygote breeder pair of NOS KO mice (C57BL/6J background) [15] was kindly provided by Dr. S. Hussain (Royal Victoria Hospital, Montréal, Canada). These mice are homozygous for the nNOS^{tm1Plh}-targeted mutation, in which a neomycin cassette has replaced exon 1 (encoding the initiation site and amino acids 1–159 of the protein), leading to absence of detectable mRNA. All animals were housed and bred in a conventional animal facility at our laboratory. All methods used in these experiments were evaluated and approved by the Animal Ethics Committee at McGill University.

Sensitization and Allergen Challenge

Animals were sensitized twice at 7-day intervals with 100 µg OVA, given by subcutaneous injection in 0.4 ml of 4 mg/ml suspension of Al(OH)₃. Seven days after the second injection, mice were lightly anesthetized with halothane and challenged intranasally with 10 µg OVA suspended in 50 µl sterile saline. This type of protocol is known to elicit an eosinophilic inflammatory response in C57BL/6 mice that, in our hands, is dependent on IL-5 [16] and has been shown to increase serum levels of IgE and IgG1 in this strain.

Experimental Protocols

To examine the effect of OVA challenge on NOS induction and NO production, sensitized animals were challenged with OVA or saline on 3 consecutive days and cells harvested 24 h after the last OVA challenge. Bronchoalveolar lavage (BAL) was performed to determine the total and differential cell count and to measure the NO metabolites (NO_x; NO₂ plus NO₃). After BAL was performed, the lungs were removed for ex vivo measurements of NOS 2 and constitutive NOS (cNOS) isoenzyme activities. cNOS reflects the activities of NOS 1 and endothelial cell NOS (NOS 3) [10]. We used immunohistochemistry, Western blot analysis and a reverse transcriptase polymerase chain reaction (RT-PCR) to study the regulation of these enzymes at both protein and gene transcription levels in WT and NOS 1 KO mice. Details of these protocols are described below.

Bronchoalveolar Lavage

Animals were deeply anesthetized with an intraperitoneal injection of 50 mg/kg of sodium pentobarbital. The upper trachea was cannulated and the lungs lavaged with cold PBS containing 0.5 mM ethylenediaminetetraacetic acid (EDTA). Routinely, greater than 80% of the BAL fluid (BALF) was recovered from the lungs. Samples were centrifuged (250 g for 10 min at 4°C), and the supernatant removed and stored at –80°C for later analysis. After resuspension, total cell number was counted under a light microscope using a Neubauer hemocytometer (Hausser Scientific Co., Horsham, Pa., USA). Cytospin preparations were stained with May-Grünwald-Giemsa (BDH Laboratory, Poole Dorset, UK) for differential cell count. A minimum of 200 cells was counted at random and cells were identified by standard morphology.

Tissue Preparation

Following BAL, the chest was opened and the lungs including the trachea were removed and rinsed with PBS. All connective tissue, vasculature and parenchyma were gently removed and the airway trees frozen in liquid nitrogen and stored at –80°C until use. In some animals, the left lung was removed and embedded in ornithyl carbamyltransferase (OCT), then slowly frozen by immersion in isopentane cooled liquid nitrogen. Cryostat sections (7 µm) were cut onto poly-L-lysine-coated glass slides (Microm HM500, Microm International GmbH, Walldorf, Germany). Slides were fixed in acetone/methanol (solution ratio of 6:4) at room temperature (RT) for 7 min and stored at –80°C after 1 h of air-drying.

NO_x Assay

We measured NO_x levels in BALF as an index of NO production using the colorimetric Griess reaction using a commercial assay kit (Catalog 78001, Cayman Chemical Co., Ann Arbor, Mich., USA) and following the manufacturer's instructions. BAL samples

were filtered through a 10-kDa molecular weight cut-off filter (Millipore, Bedford, Mass., USA) to avoid artifact of hemoglobin. In order to convert NO_3^- into NO_2^- , 80 μl of BALF was mixed with 10 μl of NO_3^- reductase and 10 μl of its enzyme co-factor and incubated at RT for 3 h. 100 μl of Griess reagent was then added and the mixture incubated for 10 min before reading. Absorbance was measured with a plate reader (SLT 400 ATC, SLT Lab Instruments, Salzburg, Austria) at 540 nm. The concentration of NO_2^- was determined from standard curves constructed using serial concentrations of NaNO_2 . The detection limit of this assay is 2.5 μM .

Measurements of NOS 2, cNOS and Total NOS Activity

Frozen lung and tracheal samples were homogenized using a Polytron homogenizer (Brinkmann Instruments, Westbury, N.Y., USA) in 5 vol (wt/vol) of homogenization buffer [pH 7.4; 25 mM Tris; 1 mM ethylene glycol-bis β -aminoethyl ether-N,N',N'-tetraacetic acid (EGTA); 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride (PMSF); 1 $\mu\text{g}/\text{ml}$ aprotinin; 1 $\mu\text{g}/\text{ml}$ leupeptin; 1 $\mu\text{g}/\text{ml}$ pepstatin A]. The crude homogenates were centrifuged at 4°C for 15 min at 10,000 g and NOS activity was measured using an NOS assay kit. The supernatant (10 μl) was added to 40 μl of pre-warmed (37°C) reaction buffer for total NOS activity [pH 7.4; final concentration; 25 mM Tris, 3 μM tetrahydrobiopterin (BH_4), 1 μM flavin adenine dinucleotide (FAD), 1 μM flavin adenine mononucleotide (FMN), 1 mM β -nicotinamide adenine dinucleotide phosphate, tetrasodium salt (NADPH), 0.6 mM CaCl_2 , 0.1 μM calmodulin, 1 mM magnesium acetate and 1 μCi L -[2,3,4,5- ^3H]arginine monohydrochloride], calmodulin, magnesium acetate, with or without CaCl_2 . All samples were incubated for 30 min at RT and the reaction terminated by addition of stop buffer [pH 5.5: 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 5 mM EDTA]. To obtain free L -[^3H]citrulline, equilibrated resin was added to the sample to eliminate excess L -[^3H]arginine and samples were then passed through the separation column. The supernatant was added to 4.5 ml of liquid scintillation fluid, and radioactivity was measured by liquid scintillation counter (6100IC, Beckman Coulter, Inc., Fullerton, Calif., USA). Enzyme activity was expressed in picomoles of L -citrulline produced/min/mg total protein. Protein concentration was measured by the Bradford technique with bovine serum albumin (BSA) as standard. The Bradford reagent (Sigma) was prepared according to the manufacturer's instructions. NOS activity was also measured in presence of 1 mM of L -NAME. NOS activity in presence of CaCl_2 was referred to as total NOS, in absence of CaCl_2 as NOS 2 activity and the difference between total NOS and NOS 2 activity was taken as the cNOS activity.

Immunohistochemistry

NOS 2 and NOS 1 protein in frozen cryostat sections was identified using APAAP immunocytochemistry previously described [5]. Briefly, sections were incubated with universal blocking solution for 15 min to block nonspecific binding of antibodies then incubated with the primary antibody overnight at 4°C in a humidified chamber. For NOS 2, the primary antibody used was anti-mouse macrophage NOS 2 rabbit polyclonal IgG (1:200) and for NOS 1 we used an anti-rat NOS 1 rabbit polyclonal IgG (1:20). The next day, slides were washed in 0.05 M Tris-buffered saline and then incubated with the secondary antibody (biotinylated anti-rabbit swine immunoglobulin at 1:300) at RT for 50 min. After washing, slides were incubated with Streptavidin-AP (1:200) for 50 min.

All slides were developed with Fast red in substrate solution (0.1 M Tris base, 0.5 mM naphthol AS-MX phosphate, 2% dimethylformamide and 1 mM levamisole, pH 8.2). Negative controls included use of buffer alone or dilution of nonspecific purified rabbit IgG in the primary layer. As an additional control to confirm the specificity of NOS 1 staining, some slides were incubated with blocking peptides to block NOS 1.

Western Blot Analysis

Proteins from each test group (40 $\mu\text{g}/\text{lane}$ for NOS 2 and 100 $\mu\text{g}/\text{lane}$ for NOS 1 detection) were mixed with NuPage LDS sample buffer and reducing agent before separation on 3–8% Tris-acetate gels at 150 V for 1 h. Proteins were then electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes with 25 mM of bicine, 25 mM of bis-tris 1 mM EDTA and 20% of methanol at 20 V for 1 h. Transferred membranes were stained with Ponceau red for 1 min to confirm uniform protein loading. Membranes were then blocked with washing buffer (pH 7.5; 0.02 M Tris base, 0.5 M sodium chloride and 0.1% of Tween 20) containing 7% non-fat milk and 1% fetal bovine serum and were probed with anti-NOS 2 antibody (1:2,000) or anti-NOS 1 antibody (1:50) at 4°C overnight. The membranes were subsequently rinsed in 200 ml of washing buffer for 30 min and exposed to the secondary horseradish peroxidase-conjugated antibody (anti-rabbit Ig from donkey) for 1 h at RT. A chemiluminescent detection system (ECL Plus; Amersham Pharmacia Biotech, Bucks., UK), Hyperfilm (Amersham Pharmacia Biotech) and Fluorchem 8000 (Alpha Innotech Corporation, San Leandro, Calif., USA) were used to detect the binding of this antibody. As a positive control for NOS 1 and NOS 2 isoenzymes, protein extracted from a lysate of murine macrophages (RAW 264.7 cells) stimulated with IFN- γ (10 ng/ml) and LPS (1 $\mu\text{g}/\text{ml}$) for 12 h or protein extracts from a rat pituitary were used. Density measurements and molecular weight calculations were performed with commercial software (FluorChem FC, Alpha Innotech Corporation, San Leandro, Calif., USA). Each density was calculated as the sum of the pixel values after background correction.

RNA Extraction and RT-PCR

Total RNA was extracted by RNeasy total RNA kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized from 1 μg of total RNA using Advantage system (Clontech Laboratories, Inc., Palo Alto, Calif., USA). The volume of RNA was adjusted to 12.5 μl by DEPC-treated H_2O . RNA was then heated at 70°C for 2 min and quenched on ice. 50 mM Tris-HCl (pH 8.3), 75 mM KCl and 3 mM MgCl_2 , 200 units of Moloney murine leukemia virus (MMLV) recombinant reverse transcriptase (Invitrogen, Burlington, Ontario, Canada), 0.5 mM of dNTP mix and 1 μM of random hexamer primer were mixed and incubated at 42°C for 1 h. The reaction mixture was heated at 94°C for 5 min to stop the cDNA synthesis and destroy any DNase activity. This mixture was used for specific amplifications of cDNA using AdvanTaq Plus system (Clontech Laboratories Inc., Palo Alto, Calif., USA). For PCR, AdvanTaq PCR buffer (final concentration; 40 mM Tricine-KOH pH 8.0, KCl 16 mM, MgCl_2 3 mM, BSA 3.75 $\mu\text{g}/\text{ml}$), AdvanTaq Plus DNA polymerase (final concentration; 1.0% glycerol, 0.4 mM Tris-HCl pH 8.0, 2 mM KCl, 2 μM EDTA pH 8.0, 0.005% Tween 20, 0.005% Nonidet P-40 and 0.2 mM dNTP) and 0.2 μM of sense and anti-sense primers were added to the reaction mixture (50 μl total volume). The oligonucleotide primers were synthesized in the Mc-

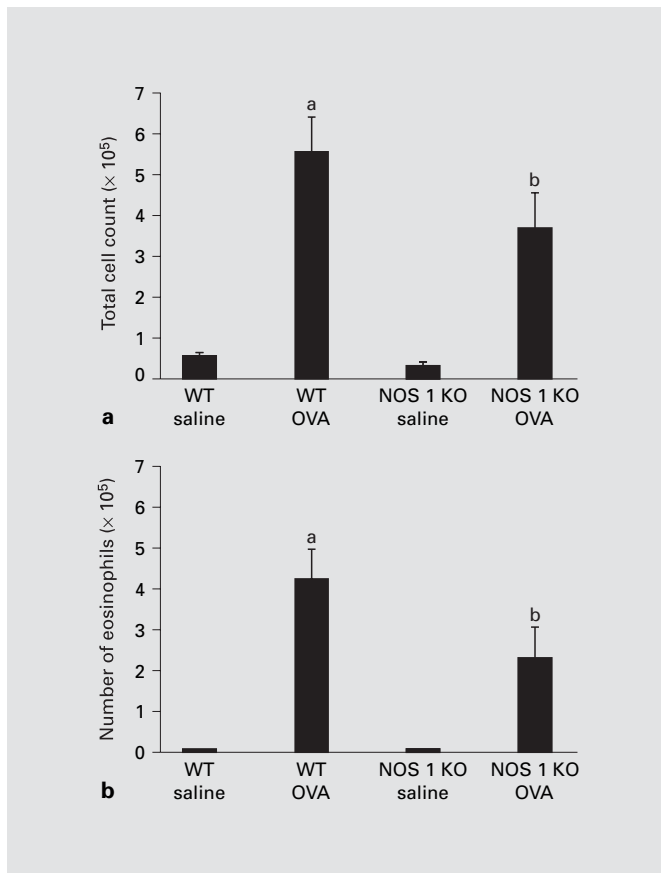


Fig. 1. Effect of OVA on cellular inflammation in BALF. Total cell count (**a**) and the number of eosinophils (**b**) in the BAL fluid from sensitized WT mice ($n = 5$) and NOS 1 KO mice ($n = 7$) 24 h after OVA challenge. Results are expressed as means $\pm$ SEM. ^a $p < 0.001$ vs. WT-sensitized mice challenged with saline ($n = 6$) and ^b $p < 0.01$ vs. NOS 1 KO-sensitized mice challenged with saline ($n = 7$).

Gill University DNA synthesis facility (Sheldon Biotechnology, Montreal, Canada). The PCR products were electrophoresed on a 1.8% agarose gel containing 0.5 $\mu\text{g/ml}$ of ethidium bromide in 0.5 $\times$ Tris-Borate-EDTA (TBE) buffer. $\Phi\text{X174/Hae III}$ digest was used as a size marker. The amplified DNA bands were visualized and quantified with FluorchemTM 8000 (Alpha Innotech Corporation, San Leandro, Calif., USA).

Materials

OVA (grade V), $\text{Al}(\text{OH})_3$, lipopolysaccharide (LPS: *Escherichia coli* 0111:B4), Tween 20, aprotinin, ethylene glycol-bis β -aminoethyl ether-EGTA, Fast red, leupeptin, $\text{N}\omega$ -nitro-*L*-arginine methyl ester (*L*-NAME), NADPH, pepstatin A, Ponceau red, bicine, sodium pentobarbital, PMSF, conventional LPS from *E. coli* 0111:B4 and poly-*L*-lysine were purchased from Sigma Chemical Company (St. Louis, Mo., USA). *L*-[2,3,4,5- ^3H]arginine monohydrochloride and PVDF membranes were purchased from Amersham Pharmacia Biotech, Inc. (Baie d'Urfé, Québec, Canada). Tris

base was from Fisher Scientific (Nepean, Ontario, Canada), while BH_4 , FAD, FMN and HEPES were purchased from Cayman Chemical Co. (Ann Arbor, Mich., USA). The NOS assay kit which contained the homogenization, stop and reaction buffers, equilibrated resin, CaCl_2 , rat cerebellum extract and NADPH was purchased from Calbiochem-Novabiochem (San Diego, Calif., USA). OCT was purchased from Sakura Finetechnical Co. (Tokyo, Japan). Antibody dilution buffer (ADB), universal blocking solution, biotinylated anti-rabbit swine immunoglobulin, Streptavidin-AP, FITC-conjugated anti-rabbit swine immunoglobulin and horseradish peroxidase-conjugated anti-rabbit donkey immunoglobulin were all from DAKO Diagnostics Canada Inc. (Mississauga, Ontario, Canada). Anti-mouse macrophage NOS 2 rabbit polyclonal IgG, anti-rat and anti-human NOS 1 rabbit polyclonal IgG and NOS 1 blocking peptides were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, Calif., USA). NuPage LDS sample buffer (4 $\times$) and Tris-acetate gels were from Invitrogen (Burlington, Ontario, Canada).

Statistical Analysis

A one-way analysis of variance (ANOVA) with Fisher's PLSD analysis was used to assess differences between control and treated groups of animals. Results are expressed as means $\pm$ standard error of mean (SEM) of number (n) of experiments, and p values less than 0.05 were considered statistically significant. All statistics were performed with the commercial software package InStat Mac (GraphPad Software, San Diego, Calif., USA).

Results

Cellular Inflammation

OVA challenge in sensitized mice significantly increased the total number of inflammatory cells in the BAL fluid of both WT ($n = 13$, $p < 0.001$) and NOS 1 KO mice ($n = 7$, $p < 0.01$) when compared to sensitized animals challenged with saline (fig. 1a). In WT mice, OVA challenge caused a large increase in the number of eosinophils ($n = 13$, $p < 0.001$) (fig. 1b), macrophages ($p < 0.01$), neutrophils ($p < 0.001$) and lymphocytes ($p < 0.001$). In NOS 1 KO mice, OVA challenge also caused a similar increase in the number of neutrophils ($p < 0.001$) and lymphocytes ($p < 0.05$); however the increase in eosinophils was half that of the increase seen in WT mice following OVA challenge ($p < 0.05$).

NO_x Metabolites in BALF

OVA challenge caused an increase in the total amount of NO_x measured in the BAL fluid of sensitized WT mice. NO_x was increased from $1.5 \pm 0.2 \mu\text{M}$ in saline-challenged animals to $3.3 \pm 0.6 \mu\text{M}$ after OVA challenge (or from a 4.3% increase above baseline to a 130% increase above baseline) ($n = 10$, $p < 0.01$) (fig. 2). NO_x in NOS 1 KO mice did not change after OVA challenge. Baseline NO_x was similar in WT and NOS 1 KO mice (fig. 2).

Measurements of NOS Activity in Tracheobronchial Tree

Endogenous NOS 2 and cNOS activity was detected in tracheobronchial homogenates of WT sensitized mice challenged with saline ($n = 5$) (fig. 3). In these animals, ~70% of the total NOS activity was due to NOS 2 (fig. 3). Exposure of WT mice to OVA caused an increase in total

NOS activity (fig. 3a) ($n = 6$, $p < 0.01$) as a result of increased NOS 2 activity ($p < 0.01$) (fig. 3b) and cNOS activity ($p < 0.05$) (fig. 3c). In NOS 1 KO mice, the total NOS (fig. 3a), NOS 2 (fig. 3b) and cNOS (fig. 3c) activity remained unchanged after OVA challenge ($n = 6$) compared to saline-challenged animals ($n = 5$) and this activity was significantly reduced when compared to WT mice challenged with OVA (fig. 3).

NOS 2 and NOS 1 Protein Expression Inducible NOS (NOS 2)

Baseline NOS 2 protein expression was detected in both WT and NOS 1 KO mice challenged with saline (fig. 4a). Expression of NOS 2 was increased following OVA challenge in sensitized WT mice (fig. 4a, lanes 4–5) compared to WT mice challenged with saline (fig. 4a, lanes 2–3). OVA challenge in NOS 1 KO mice had no effect on NOS 2 protein expression (fig. 4a, lanes 7–9) compared to saline exposure (fig. 4a, lane 6). NOS 1 KO mice exposed to OVA had significantly reduced expression of NOS 2 when compared to WT mice challenged with OVA (fig. 4a, lanes 7–9). Relative intensity of NOS 2 expression was increased after OVA challenge in WT mice ($n = 5$, $p < 0.001$) but not in NOS 1 KO animals (fig. 4a).

Neuronal NOS (NOS 1)

Baseline NOS 1 protein was detected in WT but not in NOS 1 KO mice challenged with saline (fig. 4b). Expression of NOS 1 was significantly increased following OVA challenge in sensitized WT mice (fig. 4b, lane 5) compared to WT mice challenged with saline (fig. 4b, lane

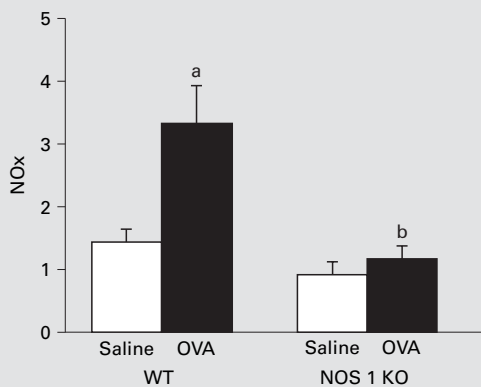
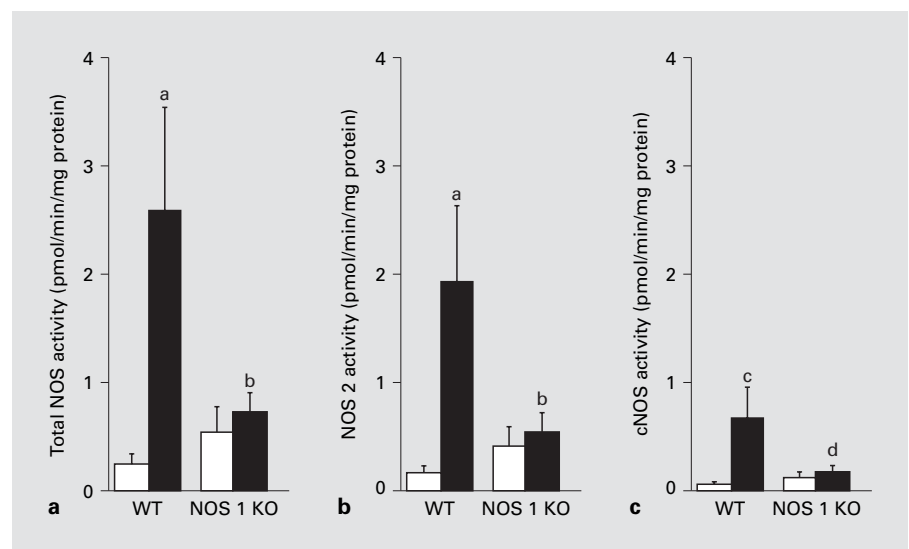


Fig. 2. NO_x metabolites in BAL fluid. Total amount of NO_x (NO₂ + NO₃) measured in BAL fluid in sensitized WT mice 24 h after saline ($n = 6$: open bars) or OVA ($n = 10$: closed bars) challenge or in NOS 1 KO mice 24 h after saline ($n = 6$: open bars) or OVA ($n = 10$: closed bars) challenge. Results are represented as means ± SEM. ^a $p < 0.05$ vs. WT-sensitized mice challenged with saline and ^b $p < 0.05$ vs. WT-sensitized mice challenged with OVA.

Fig. 3. Total NOS, NOS 2 and cNOS activity in tracheobronchial homogenates. Measurement of total NOS (a), NOS 2 (b) and cNOS (c) activity in tracheobronchial homogenates in sensitized WT mice 24 h after saline ($n = 5$: open bars) or OVA ($n = 6$: closed bars) challenge or in NOS 1 KO mice 24 h after saline ($n = 5$: open bars) or OVA ($n = 6$: closed bars) challenge. Results are expressed as mean NOS activity ± SEM. ^a $p < 0.01$, ^c $p < 0.05$, vs. WT-sensitized mice challenged with saline and ^b $p < 0.01$, ^d $p < 0.05$, vs. WT-sensitized mice challenged with OVA.



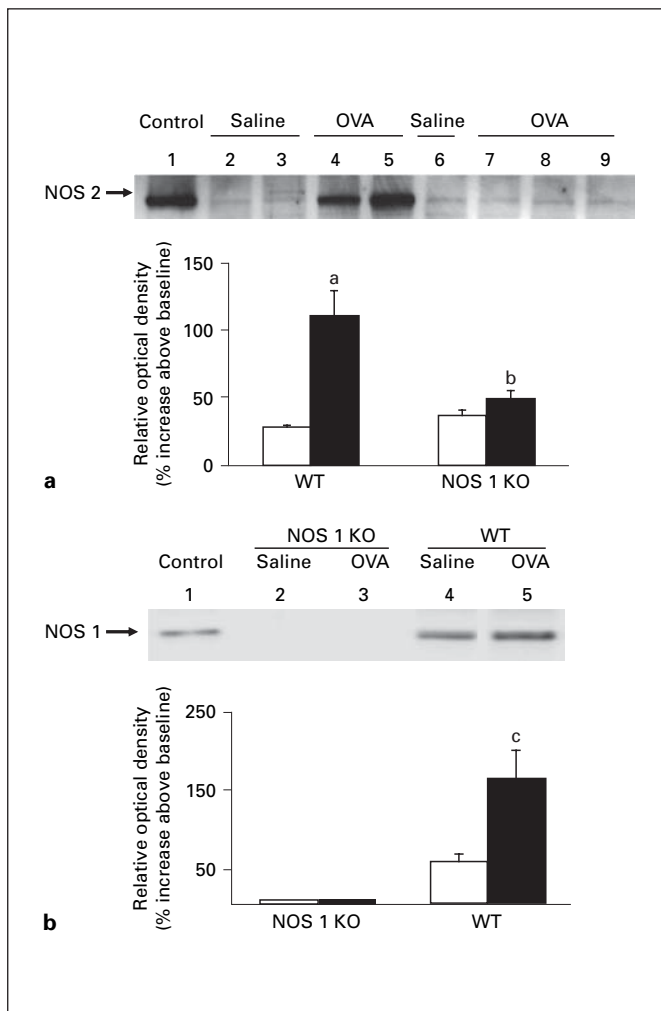


Fig. 4. Expression of NOS 2 and NOS 1 proteins in tracheobronchial homogenates. NOS 2 (**a**) and NOS 1 (**b**) protein expression and their relative optical densities in sensitized WT mice 24 h after saline (n = 5) or OVA (n = 6) challenge or in NOS 1 KO mice 24 h after saline (n = 5) or OVA challenge (n = 6). Lane 1 is a positive control (murine macrophages stimulated with IFN- γ and LPS for NOS 2 and mouse brain for NOS 1). Relative optical density is given as a percentage increase above baseline and is expressed as means $\pm$ SEM. ^a p < 0.001 vs. WT-sensitized mice challenged with saline and ^b p < 0.01 vs. WT-sensitized mice challenged with OVA. ^c p < 0.05 saline vs. WT-sensitized mice challenged with OVA.

4). OVA in NOS 1 KO mice had no effect on NOS 1 protein expression (fig. 4b, lane 3).

NOS 2 mRNA and NOS 1 mRNA Expression

NOS 2 mRNA is constitutively expressed in both WT (fig. 5a, lane 1) and in NOS 1 KO mice (fig. 5a, lanes 2–3).

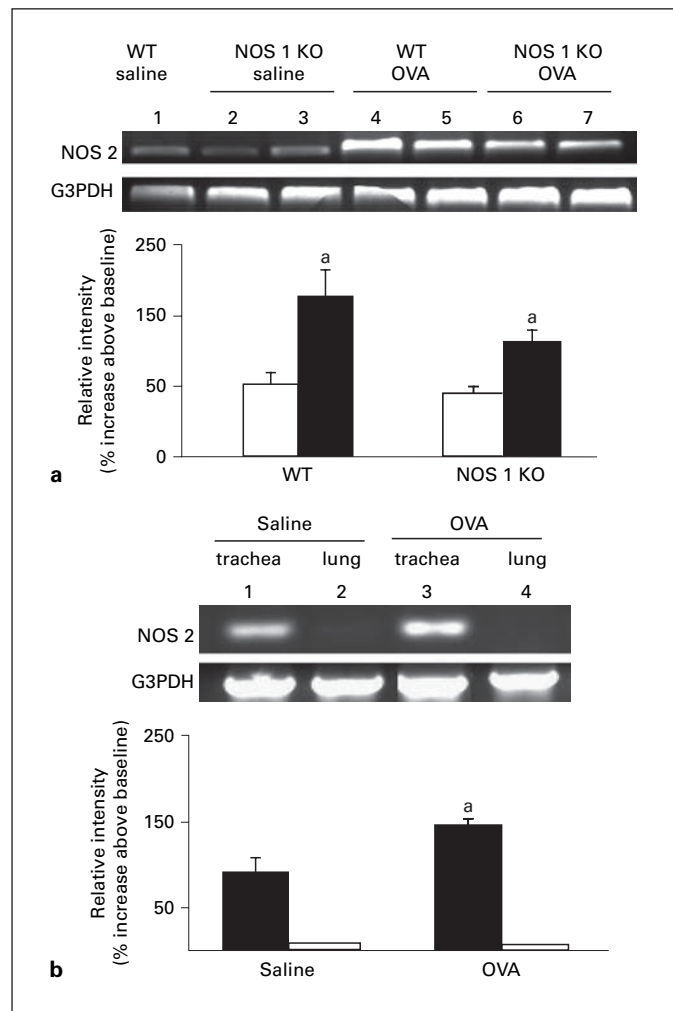


Fig. 5. Expression of NOS 2 mRNA and NOS 1 mRNA in tracheobronchial homogenates. NOS 2 mRNA (**a**) and NOS 1 mRNA (**b**) expression and their relative optical densities in sensitized WT mice 24 h after saline (n = 5) or OVA (n = 6) challenge or in NOS 1 KO mice 24 h after saline (n = 5) or OVA challenge (n = 6). NOS 1 mRNA expression was studied in both the trachea (black bars) and the lungs (white bars) of WT mice challenged with saline (**b**, lanes 1–2) or OVA (**b**, lanes 3–4). Lane 1 is a positive control (murine macrophages stimulated with IFN- γ and LPS for NOS 2 and mouse brain for NOS 1). G3PDH was used as a house-keeping gene. Relative optical density is given as a percentage increase above baseline and is expressed as means $\pm$ SEM. ^a p < 0.05 vs. saline-challenged mice.

Exposure to OVA increased NOS 2 mRNA expression in WT (fig. 5a, lanes 4–5) but not in NOS 1 KO mice (fig. 5a, lanes 6–7). The relative intensity of NOS 2 mRNA expression was increased after OVA challenge in WT mice (n = 5, p < 0.05) but not in NOS 1 KO animals (fig. 5a). NOS 1 mRNA was detected only in the trachea (fig. 5b,

lane 1) but not the lungs (fig. 5b, lane 2) of WT mice. OVA caused a significant increase in tracheal NOS 1 mRNA expression (fig. 5b, lane 3). OVA had no effect on NOS 1 mRNA in the lungs (fig. 5b, lane 4).

Immunolocalization of NOS 2 and NOS 1 following OVA Exposure

OVA challenge caused an increase in NOS-2-positive cells in both WT (fig. 6b) and NOS 1 KO mice (fig. 6d) when compared to saline-challenged WT (fig. 6a) and NOS 1 KO (fig. 6c) animals. NOS 2 immunoreactivity was present in the airway epithelium and the smooth muscle layer of the lungs in both groups of mice; however, the NOS 2-immunoreactivity was less intense in NOS 1 KO (fig. 6d) compared to WT controls (fig. 6b). OVA caused an increase in NOS 1 immunoreactivity in the airway epithelial and sub-epithelial layers of the trachea in sensitized WT mice (fig. 6f) when compared to WT and saline-challenged animals (fig. 6e).

NOS 2 Protein Expression following LPS Exposure

To exclude the possibility that mutant mice deficient in NOS 1 may have a defect in NOS 2 expression, we exposed mice to 50 µg LPS in 50 µl of sterile saline by intranasal administration and harvested lung and airways 18 h later. As shown in figure 7, NOS-1-deficient mice were as capable of responding to LPS by NOS 2 protein expression as WT mice.

Discussion

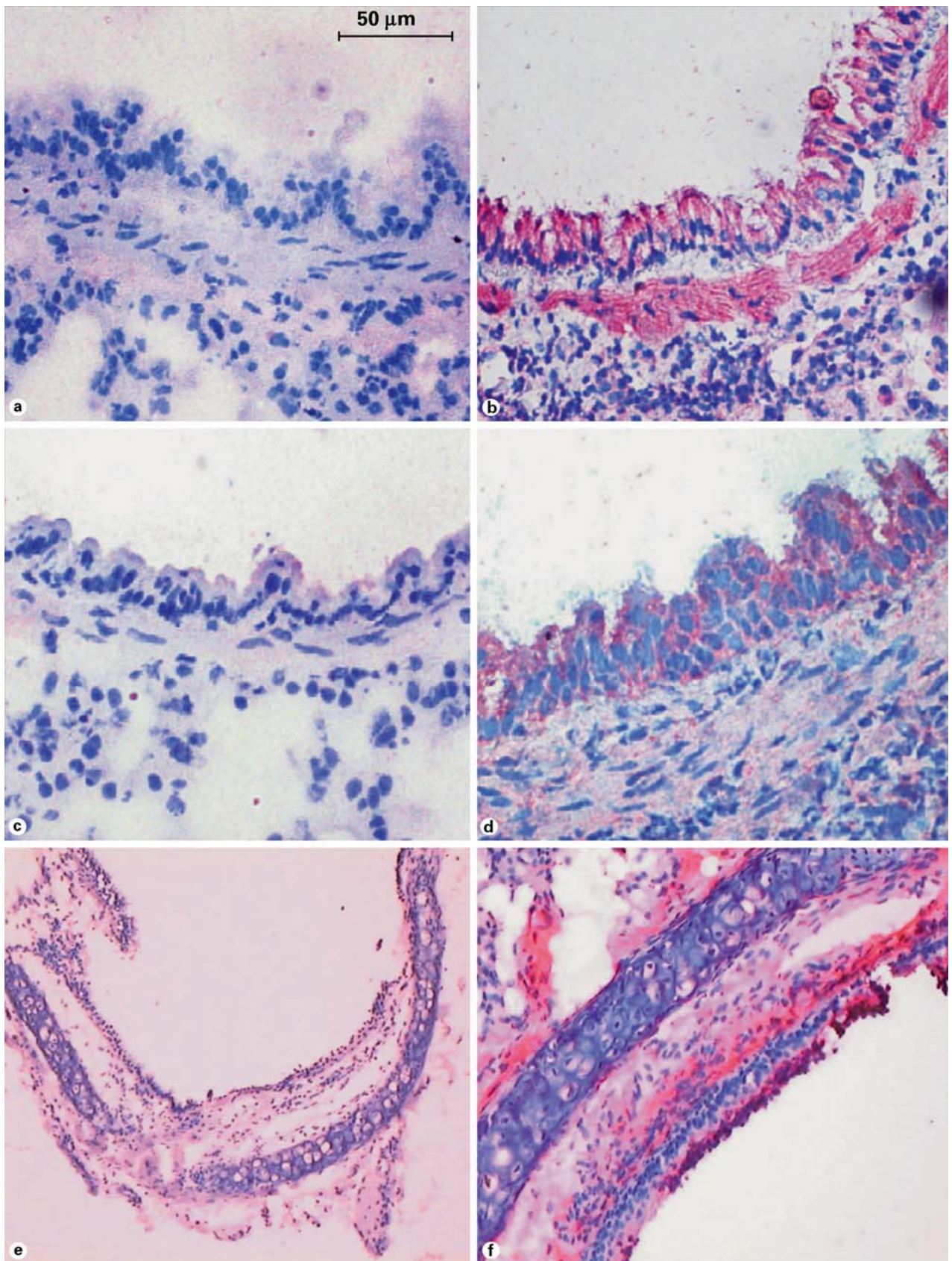
Our results demonstrate that NOS 1 is associated with the development of allergic airway inflammation following specific antigen challenge in mice. Following OVA challenge, NOS 1, like NOS 2, is inducible in murine airways. Mice deficient in NOS 1 exhibited a more modest inflammatory cell influx in bronchoalveolar lavage with fewer eosinophils and less evidence of NO production. Moreover, the influence of NOS 1 is mediated at least in part through an interaction with NOS 2. These findings indicate a novel and potentially important role for NOS 1 in the modulation of allergic airway responses.

Specific airway challenge with OVA led to eosinophilic inflammation in WT mice as previously described [17]. We have previously demonstrated that suppression of NOS 2 activity leads to a reduction in BAL eosinophilia [17], in agreement with other reports [18, 19] and underscoring the importance of inducible NOS activity and NO production in this type of inflammation. In the present

study, OVA challenge led to increased NO production as demonstrated by increased concentrations of NO_x in BAL (fig. 2), and a marked increase in overall pulmonary NOS activity (fig. 3a). To our surprise, however, in NOS-1-deficient mice, the eosinophilic response to OVA challenge was reduced by about half compared with OVA-exposed WT mice (fig. 1). Although NOS 2 activity had previously been associated with eosinophilic inflammation, we were surprised to find that deficiency of NOS 1 also led to a reduction in eosinophilic inflammation following specific OVA challenge (fig. 1). In the present study we found several lines of evidence that NOS 1 plays an important role following OVA challenge. Firstly, NOS-1-deficient mice failed to increase the concentration of NO metabolites in BAL (fig. 2). Indeed, although not statistically significant, there was a tendency for baseline NO_x levels to be lower in NOS-1-deficient mice than in WT controls (fig. 2). This finding is consistent with the previous observation that exhaled NO levels are much reduced in NOS-1-deficient mice [8]. Furthermore, the contribution of NOS 1 to overall NO production increased after OVA challenge as demonstrated by increased constitutive NOS activity (fig. 3c), which was paralleled by increases in NOS 1 protein (fig. 4b), NOS 1 mRNA (fig. 5b) and NOS 2 mRNA (fig. 5a). The increase in constitutive NOS activity is likely to be all accounted for by NOS 1 since NOS-1-deficient mice failed to increase constitutive NOS 1 activity (fig. 3c) and no evidence of increased endothelial cell nitric oxide synthase (NOS 3, also termed eNOS) protein levels was detected after OVA exposure (data not shown).

To our knowledge, this is the first evidence that NOS 1 can be induced in the respiratory tract, although up-regulation of NOS 1 has been described in the nervous system [20] and in the gastrointestinal tract [21]. In this case, the increase in NOS 1 likely occurs at least in part, at a transcriptional level given the parallel increases in NOS 1 mRNA and protein. However, the regulation of

Fig. 6. Immunodetection of NOS 2 and NOS 1 protein in the lungs and the trachea. Immunodetection of NOS 2 protein in the lungs of sensitized WT mice 24 h after saline (**a**) or OVA challenge (**b**) or in NOS 1 KO mice 24 h after saline (**c**) or OVA (**d**) challenge. NOS 2 is detected in the airway epithelium and airway smooth muscle after OVA challenge (**b**, **d**). Less intense NOS 2 staining was observed in sensitized NOS 1 KO mice after OVA (**d**) when compared to WT mice challenged with OVA (**b**). NOS 1 protein expression was significantly increased in the trachea of WT mice after OVA challenge (**f**) compared with tracheal expression of NOS 1 in saline-challenged animals (**e**). Original magnification × 200.



NOS 1 expression is extremely complex occurring at multiple levels including transcription, pre-mRNA splicing and translation [22]. NOS 1 exhibits striking promoter diversity [22, 23] and complex patterns of alternative splicing that may be associated with tissue and promoter specificity [22, 24, 25]. Regulation of NOS 1 expression may be influenced by a variety of mediators that are pertinent to allergic airway inflammation such as IFN- γ and vasoactive inhibitory peptide (VIP) [21] and may be dependent on intracellular calcium levels [26].

In previous studies, NOS 1 expression has been seen in several structures within the lung including nerves and epithelium. Although we were able to confirm epithelial staining, confirmation of NOS 1 expression in nerves was beyond the scope of this study as it requires advanced stereological techniques and careful dissection of neuronal pathways. In this regard, a recent elegant paper by Dinh et al. [27] demonstrated NOS 1 in sensory nerves of murine airways, often in association with substance P. We therefore cannot exclude that some upregulation of NOS 1 is occurring in these nerves. Nevertheless, our findings suggest that in the mouse, the tracheal epithelium is a prominent site of NOS 1 expression.

In addition to augmented NOS 1 expression, we detected a marked increase in NOS 2 activity in WT mice challenged with OVA (fig. 3b) consistent with previous reports [17] and with observations in asthma [4, 5]. This expected increase was absent in NOS 1 KO mice (fig. 3b) in which little evidence of increased NOS 2 was detected. The failure to upregulate NOS 2 appeared to occur at a transcriptional level, in that increases in both NOS 2 mRNA (fig. 6) and protein (fig. 4) were inhibited after OVA challenge in the KO mice. Administration of lipopolysaccharide did lead to increased NOS 2 expression, confirming that NOS-1-deficient mice can increase NOS 2 expression. Therefore, the absence of an increase in NOS 2 in the NOS-1-deficient mice is not the result of a defect in the synthetic mechanisms required for NOS 2 expression such as an unexpected removal of promoter regions for NOS 2 along with the coding region for NOS 1. Rather, there must be some sort of interaction between the two enzyme systems. Interactions between NOS 1 and NOS 2 have been previously described in the central nervous system [20] and in the gut [21] where it has been suggested that low-level production of NO by NOS 1 acts to inhibit NOS 2 production by interfering with the transcription factor NF- κ B that is known to be important in the regulation of NOS 2. In those systems, NOS 1 appears to be acting as part of a feedback mechanism that minimizes NOS 2 production under basal conditions [14, 21].

This is the opposite of the interaction in the present system, where NOS 1 functions as a pro-inflammatory gene, priming the expression of NOS 2 rather than inhibiting it.

Although elucidation of the precise molecular mechanism of the interaction between NOS 1 and NOS 2 is beyond the scope of this study, several hypotheses present themselves to explain the dependence of NOS 2 upregulation on the presence of functional NOS 1 in this model. Firstly, both NOS 1 and NOS 2 (fig. 6) are expressed in the epithelial cell in this model, raising the possibility of an interaction between these genes within the same cell. NOS isoforms are known to be subject to the influence of protein-protein interactions [28]. Although numerous interactions have been described for both NOS 1 and NOS 3, less is known about molecular regulation of NOS 2 expression. Kunciewicz et al. [29] have described an interaction between NOS 2 and the Rho family GTPase Rac2 in which over-expression of Rac2 led to increased NOS 2 activity. Rac-2 did not influence NOS 2 mRNA or protein levels, however; so this mechanism would not explain our data. Perhaps production of NO by NOS 1 regulates the redox state of the epithelial cell by influencing nitrosothiol levels [30]. Changes in redox state could influence the activity of the transcription factor NF- κ B and thereby influence NOS 2 activity. Our data suggest the possibility that the interaction may be specific to the stimulus. Inhibition of NOS 2 expression was restricted to allergic challenge and did not affect the response to LPS (fig. 7). It is also possible that the NOS 2-NOS 1 interaction between these NOS enzymes is indirect. Lack of NOS 1 could potentially influence the function of other cells reducing the level of pro-inflammatory signals reaching the epithelium. NOS 1 is known to be expressed in airway nerves where NO functions as a neurotransmitter [31]. Deficiency of NOS 1 in the nerves could dampen some of the signaling reaching the epithelium or, alternatively, could lead to the production of anti-inflammatory signals that normally would be suppressed.

The interaction between NOS 1 and NOS 2 in this study of allergic airway inflammation contrasts to what has been reported by De Sanctis et al. [10], who studied mutant mice deficient in all three NOS isoforms. Their principal finding was that while NOS 1 expression was essential to the development of bronchial hyperresponsiveness after allergic challenge, neither of the other NOS isoforms was involved. In their system, none of the NOS isoforms influenced airway eosinophilia. The differences between the present results and those of De Sanctis are

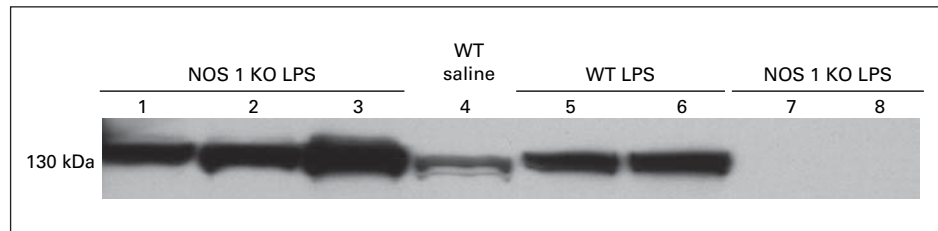


Fig. 7. Expression of NOS 2 protein after exposure to LPS. Expression of NOS 2 protein in WT mice 18 h after exposure to saline (lane 4) or LPS (lanes 5, 6) or in NOS 1 KO mice (lanes 1–3). Lanes 7 and 8 demonstrate the absence of NOS 2 expression after LPS exposure in mutant mice deficient in NOS 2. All tissues were harvested 18 h after LPS or saline exposure. Lanes represent pooled protein from 5 or 6 animals. NOS 2 expression after LPS is intact in NOS-1-deficient mice after LPS, despite being diminished after OVA.

unexplained, but may relate to the different type of protocol used in the two studies. Whereas we measured airway inflammation following a single challenge, De Sanctis et al. [10] used 5 daily repeated allergic challenges before studying their mice, suggesting that the interaction between NOS 1 and NOS 2 is dependent on the intensity of allergen exposure. Supporting the notion that intensity of allergen exposure is important, studies demonstrating a role for NOS 2 following allergic airway challenge in mice [17], guinea pigs [32] and rats [33] also used less intense exposure than did De Sanctis et al., who failed to find any association role for NOS 2 in allergic challenge. Since none of the rodent models is a realistic recapitulation of antigen exposure in man, it is impossible to state which is more relevant to human disease. Instead, the discrepancies between these less intense and more intense allergen exposures underscore the complexity of the mechanisms of allergic airway inflammation.

Regardless of the mechanism, the influence of NOS 1 on NOS 2 production is potentially important in understanding the role of NOS 1 in allergic airway inflammation and asthma. Suggestions of the importance of NOS 1 in bronchial responsiveness in the mouse [9, 10] have led to population-based studies in humans, in whom polymorphisms of the NOS 1 gene have been linked to altered levels of NO production in both asthmatics [12, 13] and cystic fibrosis patients [34]. This is puzzling since NOS-1-like endothelial cell NOS, NOS 1 is relatively inefficient in producing NO compared to NOS 2 [35]. An interaction between NOS 1 and NOS 2 could help explain this paradox. In individuals with variants of NOS 1 that were less efficient in producing NO, not only would NO production be decreased because of NOS 1 itself, but potentially because of a consequent decrease in NOS 2 production.

In summary, in the mouse, specific antigen challenge of the airways leads to the upregulation of NOS 1 as well as NOS 2 in the bronchial epithelium. Deficiency of NOS 1 not only blocks increases in constitutive NOS activity but also interferes with the upregulation of NOS 2, suggesting a possible mechanism by which NOS 1 may influence the production of NO in asthma and associated allergic inflammatory diseases.

Acknowledgements

The authors acknowledge the financial support of the Montreal Chest Institute Research Center, Canadian Thoracic Society and Medical Research Council of Canada. H.I. was a recipient of a Glaxo Wellcome Fellowship from the Canadian Thoracic Society. M.K.T. is a GSK/CLA/CIHR Post-Doctoral Fellow and A.D. was the recipient of a Merck Frosst Fellowship from the Canadian Thoracic Society. Q.H. and D.H.E. were recipients of Chercheur-Boursier awards from the Fonds pour la Recherche en Santé du Québec. We thank Dr. S. Hussain from the Royal Victoria Hospital (Montréal, Québec, Canada) for helpful discussions.

References

- ▶ 1 Leone AM, Gustafsson LE, Francis PL, Persson MG, Wiklund NP, Moncada S: Nitric oxide in exhaled breath in humans: Direct GC-MS confirmation. *Biochem Biophys Res Commun* 1994;201:883–887.
- ▶ 2 Gustafsson LE, Leone AM, Persson MG, Wiklund NP, Moncada S: Endogenous nitric oxide is present in the exhaled air of rabbits, guinea pigs and humans. *Biochem Biophys Res Commun* 1991;181:852–857.
- ▶ 3 Kharitonov SA, Yates DH, Robbins RA, Logan-Sinclair R, Shinebourne EA, Barnes PJ: Increased nitric oxide in exhaled air of asthmatic patients. *Lancet* 1994;343:133–135.
- ▶ 4 Hamid Q, Springall DR, Riveros-Moreno V, Chanez P, Howarth P, Redington A, Bousquet J, Godard P, Holgate S, Polak JM: Induction of nitric oxide synthase in asthma. *Lancet* 1993;342:1510–1513.
- ▶ 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 Kharitonov SA, Yates DH, Barnes PJ: Inhaled glucocorticoids decrease nitric oxide in exhaled air of asthmatic patients. *Am J Respir Crit Care Med* 1996;153:454–457.
- ▶ 7 Barnes PJ: NO or no NO in asthma? *Thorax* 1996;51:218–220.
- ▶ 8 Tulic MK, Wale JL, Holt PG, Sly PD: Differential effects of nitric oxide synthase inhibitors in an in vivo allergic rat model. *Eur Respir J* 2000;15:870–877.
- 9 De Sanctis GT, Mehta S, Kobzik L, Yandava C, Jiao A, Huang PL, Drazen JM: Contribution of type I NOS to expired gas NO and bronchial responsiveness in mice. *Am J Physiol* 1997;273:L883–L888.
- ▶ 10 De Sanctis GT, MacLean JA, Hamada K, Mehta S, Scott JA, Jiao A, Yandava CN, Kobzik L, Wolynec WW, Fabian AJ, Venugopal CS, Grasemann H, Huang PL, Drazen JM: 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.
- ▶ 11 Grasemann H, Yandava CN, Storm van's Gravesande K, Deykin A, Pillari A, Ma J, Sonna LA, Lilly C, Stampfer MJ, Israel E, Silverman EK, Drazen JM: A neuronal NO synthase (NOS1) gene polymorphism is associated with asthma. *Biochem Biophys Res Commun* 2000;272:391–394.
- ▶ 12 Wechsler ME, Grasemann H, Deykin A, Silverman EK, Yandava CN, Israel E, Wand M, Drazen JM: Exhaled nitric oxide in patients with asthma. Association with nos1 genotype. *Am J Respir Crit Care Med* 2000;162:2043–2047.
- ▶ 13 Grasemann H, Yandava CN, Drazen JM: Neuronal NO synthase (NOS1) is a major candidate gene for asthma. *Clin Exp Allergy* 1999;29:39–41.
- ▶ 14 Forstermann U, Boissel JP, Kleinert H: Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III). *FASEB J* 1998;12:773–790.
- ▶ 15 Huang PL, Dawson TM, Bredt DS, Snyder SH, Fishman MC: Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* 1993;75:1273–1286.
- ▶ 16 Duguet A, Iijima H, Eum SY, Hamid Q, Eidelman DH: Eosinophil peroxidase mediates protein nitration in allergic airway inflammation in mice. *Am J Respir Crit Care Med* 2001;164:1119–1126.
- ▶ 17 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.
- ▶ 18 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.
- ▶ 19 Feder LS, Stelts D, Chapman RW, Manfra D, Crawley Y, Jones H, Minnicozzi M, Fernandez X, Paster T, Egan RW, Kreutner W, Kung TT: Role of nitric oxide on eosinophilic lung inflammation in allergic mice. *Am J Respir Cell Mol Biol* 1997;17:436–442.
- ▶ 20 Togashi H, Sasaki M, Frohman E, Taira E, Ratan RR, Dawson TM, Dawson VL: Neuronal (type I) nitric oxide synthase regulates nuclear factor kappaB activity and immunologic (type II) nitric oxide synthase expression. *Proc Natl Acad Sci USA* 1997;94:2676–2680.
- ▶ 21 Qu XW, Wang H, De Plaen IG, Rozenfeld RA, Hsueh W: Neuronal nitric oxide synthase (NOS) regulates the expression of inducible NOS in rat small intestine via modulation of nuclear factor kappa B. *FASEB J* 2001;15:439–446.
- ▶ 22 Wang Y, Newton DC, Robb GB, Kau CL, Miller TL, Cheung AH, Hall AV, VanDamme S, Wilcox JN, Marsden PA: RNA diversity has profound effects on the translation of neuronal nitric oxide synthase. *Proc Natl Acad Sci USA* 1999;96:12150–12155.
- ▶ 23 Xie J, Roddy P, Rife TK, Murad F, Young AP: Two closely linked but separate promoters for human neuronal nitric oxide synthase gene transcription. *Proc Natl Acad Sci USA* 1995;92:1242–1246.
- ▶ 24 Lee MA, Cai L, Hubner N, Lee YA, Lindpaintner K: Tissue- and development-specific expression of multiple alternatively spliced transcripts of rat neuronal nitric oxide synthase. *J Clin Invest* 1997;100:1507–1512.
- ▶ 25 Silvagno F, Xia H, Bredt DS: Neuronal nitric oxide synthase-mu, an alternatively spliced isoform expressed in differentiated skeletal muscle. *J Biol Chem* 1996;271:11204–11208.
- ▶ 26 Lee SJ, Beckingham K, Stull JT: Ca²⁺-independent activity of nitric oxide synthase. *Biochem Biophys Res Commun* 2001;284:526–530.
- ▶ 27 Dinh QT, Groneberg DA, Peiser C, Joachim RA, Frossard N, Arck PC, Klapp BF, Fischer A: Expression of substance P and nitric oxide synthase in vagal sensory neurons innervating the mouse airways. *Regul Pept* 2005;126:189–194.
- ▶ 28 Kone BC: Protein-protein interactions controlling nitric oxide synthases (review). *Acta Physiol Scand* 2000;168:27–31.
- 29 Kunczewicz T, Balakrishnan P, Snuggs MB, Kone BC: Specific association of nitric oxide synthase-2 with Rac isoforms in activated murine macrophages. *Am J Physiol* 2001;281:326–336.
- ▶ 30 Stamler JS: Redox signaling: Nitrosylation and related target interactions of nitric oxide. *Cell* 1994;78:931–936.
- ▶ 31 Belvisi MG, Ward JK, Mitchell JA, Barnes BJ: Nitric oxide as a neurotransmitter in human airways. *Arch Int Pharmacodyn Ther* 1995;329:97–110.
- ▶ 32 Iijima H, Uchida Y, Endo T, Xiang A, Shirato M, Nomura A, Hasegawa S: Role of endogenous nitric oxide in allergen-induced airway responses in guinea-pigs. *Br J Pharmacol* 1998;124:1019–1028.
- ▶ 33 Eynott PR, Groneberg DA, Caramori G, Adcock IM, Donnelly LE, Kharitonov S, Barnes PJ, Chung KF: Role of nitric oxide in allergic inflammation and bronchial hyperresponsiveness. *Eur J Pharmacol* 2002;452:123–133.
- ▶ 34 Grasemann H, Knauer N, Buscher R, Hubner K, Drazen JM, Ratjen F: Airway nitric oxide levels in cystic fibrosis patients are related to a polymorphism in the neuronal nitric oxide synthase gene. *Am J Respir Crit Care Med* 2000;162:2172–2176.
- ▶ 35 Al-Ali MK, Howarth PH: Nitric oxide and the respiratory system in health and disease. *Respir Med* 1998;92:701–715.