ELSEVIER

European Journal of Pharmacology 452 (2002) 123–133

www.elsevier.com/locate/ejphar

Role of nitric oxide in allergic inflammation and bronchial hyperresponsiveness

Paul R. Eynott, David A. Groneberg, Gaetano Caramori, Ian M. Adcock, Louise E. Donnelly, Sergei Kharitonov, Peter L. Barnes, K. Fan Chung *

National Heart and Lung Institute, Imperial College School of Medicine, Dovehouse Street, London SW3 6LY, UK

Received 7 March 2002; received in revised form 5 August 2002; accepted 9 August 2002

Abstract

The role of nitric oxide (NO) in allergic inflammation and bronchial hyperresponsiveness is unclear. We studied a selective prodrug nitric oxide synthase (NOS)-2 inhibitor, L-N^6-(1-iminoethyl)lysine 5-tetrazole amide (SC-51). In ovalbumin-sensitized and challenged rats, exhaled NO levels increased by 3 h following challenge (3.73 ± 0.74 ppb; $P<0.05$), peaking at 9 h (11.0 ± 2.75; $P<0.01$) compared to saline controls (1.87 ± 0.26; $P<0.05$ and 2.81 ± 0.18; $P<0.01$). Immunoreactive lung NOS2 expression was increased in ovalbumin-challenged rats compared with ovalbumin-sensitized, saline-challenged rats at 8 h post-challenge. SC-51 (10 mg/kg; p.o.) inhibited allergen-induced increase in exhaled NO levels to 1.3 ± 0.17 ppb. SC-51 inhibited bronchial hyperresponsiveness in ovalbumin-sensitized and challenged rats ($P<0.05$). In sensitized non-exposed rats, SC-51 increased bronchial responsiveness ($P<0.05$). SC-51 reduced the allergen-induced increase in bronchoalveolar lavage neutrophils, but caused a nonsignificant reduction in bronchial mucosal eosinophil numbers. NO generated through NOS2 contributes to allergen-induced bronchial hyperresponsiveness but not to bronchial eosinophilia, indicating that these are independently expressed.

Keywords: Nitric oxide (NO); Nitric oxide (NO) synthase 2; Bronchial hyperresponsiveness; Airway inflammation; SC-51

1. Introduction

Endogenous nitric oxide may play an essential role in the physiological regulation of airway function and has been implicated in the pathogenesis of airway diseases, such as bronchial asthma (Barnes, 1995). Asthma is a chronic disease of the airways characterized by airway inflammation and bronchial hyperresponsiveness, and is associated with increased concentrations of nitric oxide (NO) in the expired air (Alving et al., 1993; Kharitonov et al., 1995b). Nitric oxide is synthesized by a variety of cell types from the amino acid, L-arginine, by the action of enzymes, nitric oxide synthases (NOS) (Moncada et al., 1991). Three main isoforms of NOS have been characterized, each the product of genes located on different chromosomes, with each isoform differing in structural and biochemical properties. Neuronal NOS or NOS1 and endothelial NOS or NOS3 are constitutively expressed isoforms activated by depolarization- or agonist-induced intracellular calcium changes, resulting in the generation of small (picomolar) amounts of NO that serves as a diffusible signaling molecule mediating intracellular processes. NOS1-derived NO may be involved in neurotransmission (Synder and Fleisch, 1989), and NOS3-derived NO may be important for the relaxation of vascular smooth muscle and in the regulation of systemic and pulmonary blood pressure. In contrast, the inducible isoform of NOS or NOS2 is expressed via prolonged calcium-independent mechanisms, leading to the production of relatively large (nanomolar) amounts of NO, which may not only activate soluble guanylyl cyclase, but may additionally have cytostatic and cytotoxic effects (Nathan, 1992; Liew, 1994).

NO is detected in the exhaled air of humans and various experimental animals and is increased in the exhaled air of asthmatic patients (Kharitonov et al., 1995b; Alving et al., 1993). Increased expression of NOS2 has been detected in

* Corresponding author. Tel.: +44-207-3528121x3052/8; fax: +44-207-3518126.

E-mail address: f.chung@ic.ac.uk (K.F. Chung).

0014-2999/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0014-2999(02)02237-9

the bronchial epithelium of asthmatic patients (Hamid et al., 1993), and following allergen challenge in sensitized rats, NOS2 is expressed mainly by alveolar macrophages and to a lesser extent in the airway epithelium (Liu et al., 1997). After bronchial provocation with specific allergen, atopic asthmatic patients demonstrate an increase of exhaled NO (Kharitonov et al., 1995a). In sensitized rats (Yeadon and Price, 1995) and guinea pigs (Yan et al., 1995), allergen exposure results in enhanced endogenous NO production during the late phase, together with an induction of NOS2 in the lungs (Liu et al., 1997).

The precise role of NOS2-derived NO in allergic inflammation and in bronchial hyperresponsiveness remain unclear. In in vivo studies of mice rendered deficient of NOS2, there was a reduction in pulmonary eosinophilia following allergen exposure of sensitized mice (Xiong et al., 1999), while another study showed no effects on pulmonary eosinophilia (De Sanctis et al., 1999). However, in both studies, allergen-induced bronchial hyperresponsiveness was fully expressed despite the absence of NOS2. Because unconditional gene disruption may not fully reveal the role played by NOS enzymes in view of potential effects to the development of innate immunity, pharmacological isoenzyme inhibition using highly selective inhibitors may be a better way of investigating the role of NOS enzymes.

We examined the role of endogenously produced NO in a rat model of allergic asthma. We examined the levels of exhaled NO and NOS isoform protein expression, and determined the effect of NOS inhibitor, L-N^6-(1-iminoethyl)-lysine 5-tetrazole amide (SC-51), which is a prodrug of L-NIL, L-N^6-(1-iminoethyl lysine 5-tetrazole amide) (Hallinan et al., 2002), in allergic inflammation and bronchial hyperresponsiveness following allergen challenge in sensitized Brown–Norway rats. This inhibitor has some selectivity against NOS2.

2. Materials and methods

2.1. Animals, sensitization procedures and allergen-exposure

Pathogen-free male Brown–Norway rats (weighing 200–280 g, 9–13 weeks old; Harlan Olac, Bicester, UK) were sensitized on days 1, 2 and 3 using 1 mg/kg intraperitoneal injections of ovalbumin (grade V, salt-free, Sigma, Dorset, UK) in 0.9% (w/v) sterile sodium chloride solution containing 100 mg aluminium hydroxide as adjuvant. Three weeks later, rats were exposed to ovalbumin aerosol (1% w/v, 20 min) or saline (0.9% w/v, 20 min) with the use of a 6.5-l Plexiglas acrylic plastic connected to an ultrasonic nebulizer (model no. 2512; DeVilbiss Health Care UK, Feltham, UK) which generated the aerosol mist pumped into the exposure chamber by the airflow supplied by a small animal ventilator (Harvard Apparatus, Peterborough, UK), set at 60 strokes/min, with a pumping volume of 10 ml (as previously described by Elwood et al., 1991; Haczku et al., 1996; Liu et al., 1997). At all other times, rats were housed in a caging system receiving clean filtered air (Maximiser, Thorens Caging System, Hazleton, PA, USA).

The study was performed in three parts. In the first part (study 1), a time-course of possible changes in exhaled nitric oxide and NOS2 lung protein levels following allergen challenge were examined in sensitized rats. Exhaled NO was measured at the following time-periods of 1–4, 5–8, 9–12 and 24 h following allergen challenge in separate groups of rats. A naive group was included as control to compare sensitized rats with nonsensitized rats since the effect of sensitization alone can increase gene expression of NOS2 in the lungs (Liu et al., 1997).

Three groups were studied:

(i) Nonsensitized, saline-challenged animals (group NS, $n=2$): animals were injected with 1 ml 0.9% (w/v) saline for three consecutive days and exposed to saline on day 21.
(ii) Sensitized and saline-challenged animals (group SS, $n=7$ for each time-point group of 1–4, 5–8, 9–12 and 24 h): animals were injected with 1 ml of 1 mg ovalbumin/100 mg aluminium hydroxide in 0.9% (w/v) saline suspension for three consecutive days and exposed to saline on day 21.
(iii) Sensitized and ovalbumin-challenged animals (group SO, $n=7$ for each of the above time-points): animals were injected with 1 ml of 1 mg ovalbumin/100 mg aluminium hydroxide in 0.9% (w/v) saline suspension for three consecutive days and exposed to ovalbumin on day 21.

In study 2, our aim was to determine the effects of the NOS 2 inhibitor, SC-51, on exhaled NO following allergen challenge in sensitized rats. Three groups were studied:

(i) Sensitized and saline-challenged (group SS, $n=7$): animals were injected with 1 ml of 1 mg ovalbumin/100 mg aluminium hydroxide in 0.9% (w/v) saline suspension for three consecutive days and exposed to saline on day 21.
(ii) Sensitized and ovalbumin-challenged (group SO, $n=7$): animals were injected with 1 ml of 1 mg ovalbumin/100 mg aluminium hydroxide in 0.9% (w/v) saline suspension for three consecutive days and exposed to ovalbumin on day 21.
(iii) Sensitized, SC-51-treated and ovalbumin-challenged (group SOSC, $n=5$): animals were injected with 1 ml of 1 mg ovalbumin/100 mg aluminium hydroxide in 0.9% (w/v) saline suspension for three consecutive days and exposed to ovalbumin on day 21. Animals received 10 mg/kg SC-51 by oral gavage (volume 0.5 ml; in sterile water) following allergen challenge.

In study 3, we investigated the effects of inhibition of NOS2 by SC-51 on the effects of allergen challenge. We studied four groups.

(i) Sensitized, vehicle-treated and saline-exposed animals (group SS, $n=6$): animals were injected with 1 ml of 1 mg ovalbumin/100 mg aluminium hydroxide in 0.9% (w/v) saline suspension for three consecutive days, 2 h prior to saline aerosol exposure, 0.5 ml (vehicle for SC-51) by oral gavage. Subsequently, rats were exposed to saline aerosol for 20 min followed by three further 0.5-ml doses of sterile water vehicle at 4, 10 and 18 h post-challenge and then studied 2–4 h thereafter (18–24 h post-challenge).

(ii) Sensitized, SC-51-treated and saline-exposed (SSSC, $n=7$): the procedures were the same as for group SS, except the four oral doses of sterile water vehicle was replaced with four oral doses of 10 mg/kg SC-51 dissolved in 0.5 ml sterile water.

(iii) Sensitized, vehicle-treated and ovalbumin-exposed animals (SO, $n=6$): the procedures were the same as for group SS except that the challenge was with 1% (w/v) ovalbumin aerosol for 20 min.

(iv) Sensitized, SC-51-treated and ovalbumin-exposed animals (SOSC, $n=7$): the procedures were the same as for group SSSC except that the challenge was 1% (w/v) ovalbumin aerosol for 20 min and that four oral doses of 10 mg/kg SC-51 in 0.5-ml sterile water were administered.

2.2. L-N^6-(1-iminoethyl)lysine 5-tetrazole amide (SC-51)

The NOS2 selective inhibitor, SC-51 (L-N^6-(1-iminoethyl)lysine tetrazole-amide), is a prodrug of L-NIL, L-N^6-(1-iminoethyl lysine 5-tetrazole amide), which is an inhibitor of NOS2. SC-51 itself has little activity as an inhibitor of NOS2. The IC_{50} values for human NOS2 of L-NIL is 5.9 μM, with lesser activities for NOS3 ($IC_{50}=138$ μM) and NOS1 (35 μM); SC-51 itself has an IC_{50} of >1850 μM for these three NOS (Hallinan et al., 2002). SC-51 is rapidly metabolised to L-NIL in the systemic circulation after oral administration in the rat.

2.3. Measurement of exhaled NO levels

The on-line NO analyser (Model 280 NOA™: Sievers Instruments, USA) was calibrated at the beginning and again at the end of each experiment in order to assess the stability of the zero and signal levels. Fifty parts per million (ppm, by volume) nitric oxide/nitrogen (290422-AV-C; purity 99.99%; BOC Gases, Worsley, Manchester) was used to calibrate the machine. The NO analyser was set to sample air at 100 ml/min. The detection limit of the analyser was <1 ppb with a resolution of 0.3 ppb and was linear over the range 1 ppb–50 ppm. The response time was <0.5 s. Rats were anaesthetised at various time-points after either ovalbumin or saline challenge such that measurements of exhaled NO levels were obtained at time-points 1–4, 5–8, 9–12 and 24 h. Anaesthetised rats breathed air with exactly defined concentrations of constituent gases (330 ppm CO_2/argon/oxygen/nitrogen) and free of any oxides of nitrogen (Zero air, Cat No: 10043442, BOC, Surrey, UK). Exhaled NO levels were measured directly from within the trachea via a catheter connected directly to the exhaled port of the chemiluminescence analyser. This is a direct and highly specific method of NO detection, based on a photochemical reaction between NO and ozone generated within the chemiluminescence analyser and detected by a photomultiplier (Archer, 1993). Exhaled NO was sampled for a 10-min period at 30-min intervals directly from the trachea using a small catheter connected to a filter (Acrodisk, 0.2 μl; Gelman Sciences, MI) to the NO analyser.

2.4. Measurement of airway resistance

For the measurement of airways resistance, all animals were anaesthetised with 0.3 ml/kg Hypnorm (i.m) consisting of fentanyl (0.315 mg/ml) and fluanisone (10 mg/ml) (Roche Chemicals, UK) and 1.5 mg/kg Hypnovel (i.p) consisting of midazolam (Janssen Animal Health, UK) and ventilated (10 ml/kg tidal volume; 60/min rate). Tracheostomised and ventilated rats were monitored for airflow with a pneumotachograph (model F1L, Mercury Electronics, Glasgow, Scotland) connected to a transducer (model FC040; 200 mm H_2O, Furness Controls, Sussex, UK) and transpulmonary pressure via a transpleural catheter connected to a transducer (model FCO40; 1000 mm H_2O, Furness Controls). Lung resistance was simultaneously calculated, according to the method of Neergard and Wirz (1927), using software (LabView, National Instruments, Austin, TX, USA) on a Macintosh 11 computer (Apple Computer, California, USA). Briefly, one side of the transducer was attached to an air-filled catheter inserted into the right pleural cavity, while the other was attached to a catheter connected to a side-port of the intratracheal cannula. Airflow was measured with a pneumatograph connected to a transducer. The signals from the transducers were digitized with a 12-bit analogue-digital board connected to a Macintosh II computer and analyzed with the LabView software, which is programmed to instantaneously calculate pulmonary resistance.

2.5. Measurement of airway responsiveness

Eighteen to twenty-four hours following saline or ovalbumin challenge, animals were injected with propranolol (1 mg/kg i.v.) prior to measurements in order to eliminate the adrenergic effects of surgical anaesthesia. Saline was given by inhalation (45 breaths), and the subsequent lung resistance value was the baseline. Aerosol generated from increasing half $\log_{10}$ concentrations of acetylcholine (Sigma) was administered by inhalation (45 breaths of 10 ml/kg

stroke volume), with the initial concentration of $10^{-3.5}$ mol/l and the maximal concentration of 10^{-1} mol/l starting 3 min after the initial saline exposure. Each concentration of acetylcholine was separated by 10-min intervals with hyperinflation of the airways to twice the tidal volume, performed by blocking the outflow of the ventilator manually. Hyperinflation was used in order to reverse any residual atelectasis and ensure a constant volume history prior to challenge. Between the acetylcholine challenges, the tracheal cannula was cleared of secretions by suction with a 20-ml syringe through a polyethylene catheter when necessary. The concentration of acetylcholine needed to increase lung resistance 200% above baseline (provocative concentration: PC_{200}) was calculated by interpolation of the log concentration-lung resistance curves from individual animals, and $-\log PC_{200}$ was used as a measure of bronchial responsiveness.

2.6. Bronchoalveolar lavage and cell counting

After measurement of lung function parameters, rats were administered an overdose of sodium pentobarbitone (100 mg/kg intravenously), and the lungs lavaged with 10×2 ml aliquots of 0.9% w/v saline through a polyethylene syringe introduced through the tracheostomy. Lavage fluid was then centrifuged ($500 \times g$ for 10 min at 4 °C) and the cell pellet resuspended in 1 ml of Hanks balanced salt solution. Differential cell counts were made from cytospin preparations and then stained with May–Grunwald stain. Slides were then washed and air-dried prior to mounting in DPX™ mountant with coverslips. Cells were identified as macrophages, eosinophils, neutrophils and lymphocytes by standard morphology and at least 500 cells counted under $\times 400$ magnification. The percentage and absolute numbers of each cell type were calculated.

2.7. Collection of lung tissues

In study 1, lung tissue was collected from animals 8 h following either saline or ovalbumin. Briefly, lungs were perfused with saline (0.9% NaCl) via the right ventricle. Subsequently, the lungs were rapidly removed and insufflated with OCT Tissue Tek™ mounting medium (Raymond A Lamb, London, UK) diluted 1:1 with phosphate-buffered saline. Regions of the right lung lobes were mounted on cork blocks with the main bronchi uppermost, snap-frozen in melting isopentane and stored at −25 °C for later immunohistochemistry studies. The left lung was snap-frozen and ground for later protein studies.

2.8. Immunohistochemistry

Immunostaining was carried out on transverse 6-μm-thick frozen lung sections cut at the level of the tracheobronchial tree using a cryostat. Sections were mounted on gelatin-coated glass slides and air-dried for 1 h.

2.8.1. NOS isoforms

Three saline and three ovalbumin-challenged rats receiving either vehicle or SC-51 were stained for NOS -1, -2 and -3 with previously characterized antibodies (Steudel et al., 1999). All sections were fixed with cold acetone, rinsed several times in phosphate-buffered saline and coated with 0.1 M phosphate buffer containing 1% bovine serum albumin and 10% normal swine serum for 1 h at room temperature. After washing in phosphate-buffered saline, the tissues were then incubated with either NOS1-antiserum (monoclonal mouse, diluted 1:400, N-2280, Sigma, St. Louis), NOS2-antisera (polyclonal rabbit, diluted 1:250,

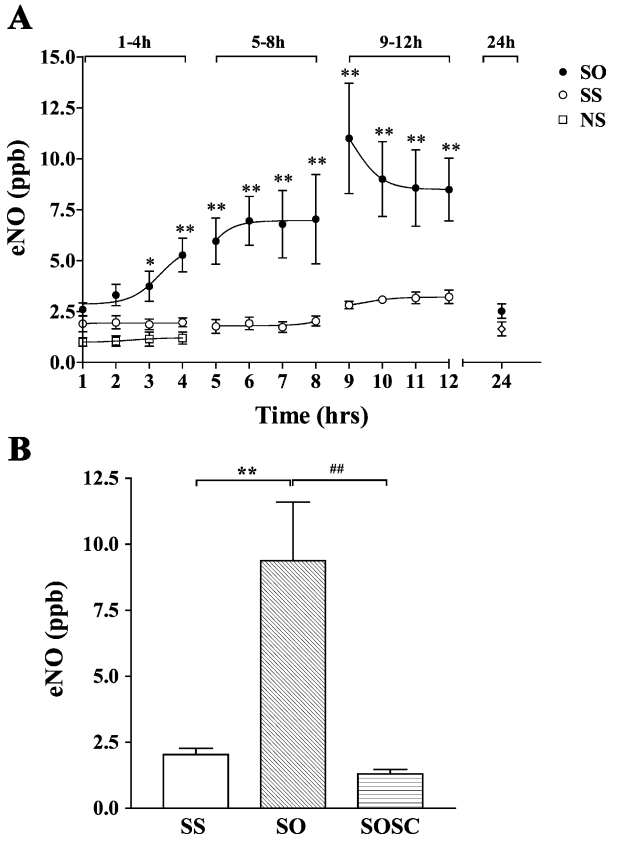

Fig. 1. (Panel A) Time-course of exhaled nitric oxide following allergen challenge. Separate groups of rats are represented for the time-points 1–4, 5–8, 9–12 and 24 h. In rats sensitized and exposed to either saline (SS) or ovalbumin aerosol (SO), there was a gradual increase in exhaled NO in the SO group, maximal at the 9-h time-point, returning to baseline by 24 h. There was no difference between SS rats and naïve rats (NS) at 1–4 h. Significant differences between sensitized, saline-exposed and sensitized, ovalbumin-exposed are expressed as *: $P<0.05$; **: $P<0.01$. Data expressed as mean ± S.E.M. (Panel B) SC-51 inhibits allergen-induced increase in exhaled nitric oxide (exhaled NO). Exhaled nitric oxide was measured at 8 h after allergen exposure of sensitized rats (SO; $n=7$) or after saline exposure of sensitized rats (SS; $n=7$) or after treatment with SF-51 (10 mg/kg) of allergen exposed sensitized rats (SOSC; $n=5$). There was a significant inhibition of the increased exhaled NO by SC-51 in SO rats. Significant differences between sensitized, ovalbumin-exposed and sensitized, SC-51 treated and ovalbumin-exposed groups are expressed as ##: $P<0.01$, whereas significant differences between sensitized ovalbumin-exposed and sensitized saline-exposed are expressed as **: $P<0.01$. Data shown as mean ± S.E.M.

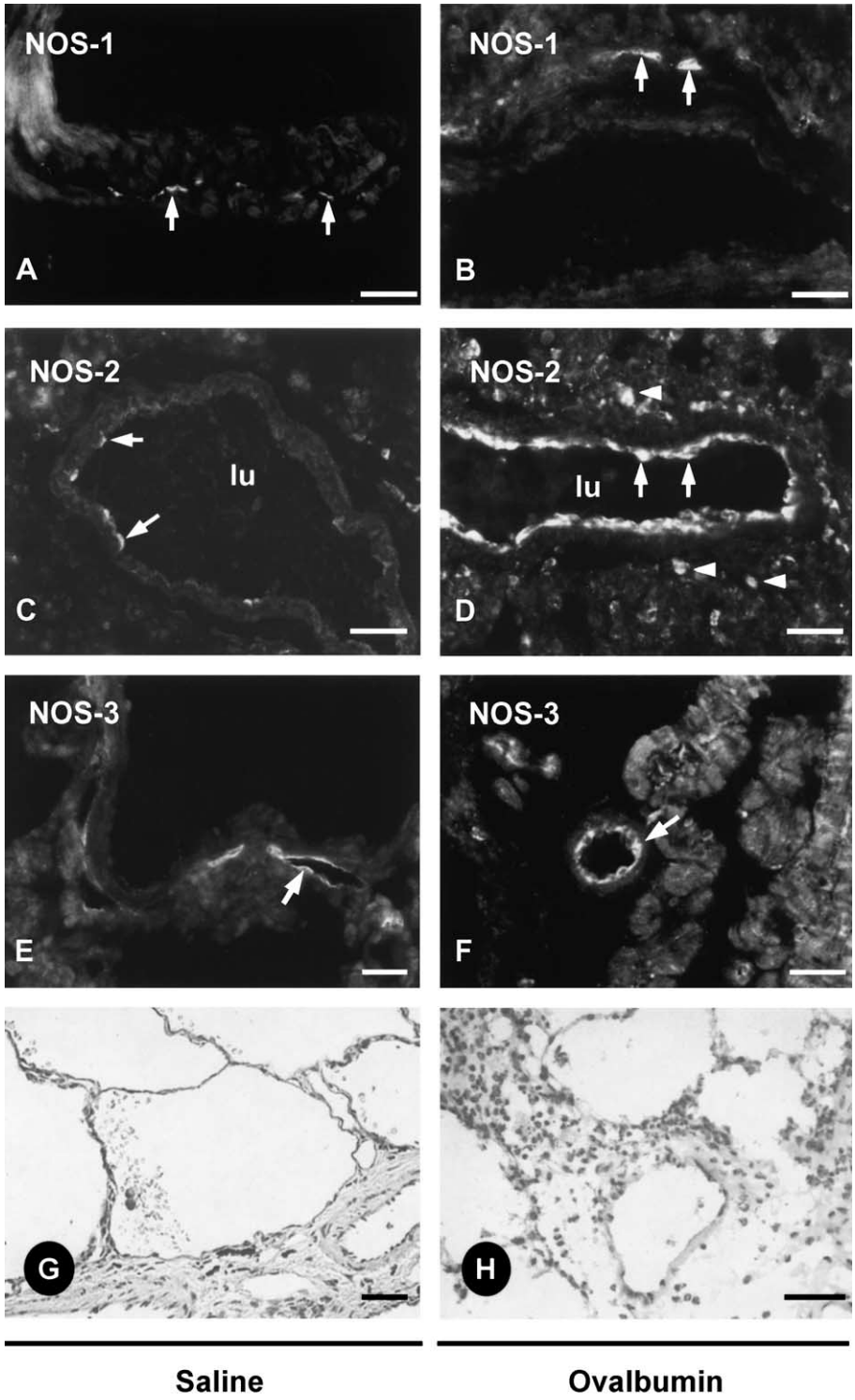

Fig. 2. Immunohistochemistry for NOS1, NOS2 and NOS3 in rat lung. NOS1-immunoreactivity is present in nerve fibres (arrows) innervating the airway smooth muscle of allergen challenged (A) and saline-challenged rats (B). In ovalbumin-sensitized, saline-challenged animals, NOS2 is present in epithelial cells (C). In contrast, significant stronger labelling in ovalbumin-sensitized and ovalbumin-challenged rats in epithelial, endothelial and subepithelial inflammatory cells (arrowheads in D). NOS3-immunoreactivity is restricted to endothelial cells (arrows) and the epithelial cell layer of ovalbumin- (F) and saline- (E) challenged animals. Haematoxylin–eosin staining of the different groups shows inflammatory cell influx in the ovalbumin-challenged animals (H) as compared to the saline-challenged rats (G). Scale bars represent 50 μm (A–F), 80 μm (G, H).

SA-200, Biomol, Hamburg, Germany) or NOS3-antisera (polyclonal rabbit, diluted 1:500, SA-201, Biomol) in the preincubation solution at room temperature. After overnight incubation and rinsing in phosphate-buffered saline, the sections were incubated with anti-rabbit or mouse fluorescein isothiocyanate secondary antibody (FITC, Dianova, Hamburg, Germany, dilution 1:500) for 1 h at room temperature. Specificity of the antibody reaction was verified in parallel sections, which were incubated with preabsorbed NOS2 antiserum or only secondary antibodies. Slides were mounted in carbonate-buffered glycerol (pH 8.6) and viewed using epifluorescence microscopy. The sections were scored independently by two blinded observers as strong (+++), moderate (++), weak (+) or negative immunoreactivity (0) as described elsewhere (Wislez et al., 2001).

2.8.2. Eosinophil major basic protein (MBP) and $CD2^+$, $CD4^+$, $CD8^+$ T-cells

Cryostat sections, 6 μm thick, were fixed in cold acetone and then incubated with either a monoclonal antibody against human major basic protein (clone BMK-13; Monosan, Uden, The Netherlands) at a concentration of 1:80 for 1 h at room temperature for detection of rat eosinophils or with either mouse anti-rat CD2, CD4 or CD8 monoclonal antibody (pan T-cell markers, Pharmingen, Cambridge Bioscience, Cambridge, UK) at a dilution of 1:500 for 1 h at room temperature. After labeling with a biotinylated, horse anti-mouse monoclonal antibody, positive cells were visualised by using an avidin–biotin complex reagent conjugated to alkaline phosphatase (Vector Laboratories, Peterborough, UK). All cells were visualised using Sigma FAST in Tris buffer with positive cells appearing red. Sections were counterstained with haematoxylin (BDH, Lutterworth, UK) and mounted under glass coverslips. Eosinophil (MBP^+) and $CD2^+$, $CD4^+$ and $CD8^+$ T-cell influx around the five largest airways in each lung section were assessed as the number of positively stained cells in the epithelium, submucosa, smooth muscle and lamina propria, and expressed per millimeter of basement membrane.

2.9. Data analysis

Data are presented as the mean ± S.E.M. For multiple comparisons of different groups, Kruskall–Wallis test for analysis of variance was used. If the Kruskall–Wallis test for analysis of variance was significant, we applied Mann–Whitney *U*-test for comparison between two individual groups. The data was analyzed using Graphpad™ for Windows statistical package. A P value of less than 0.05 was considered significant.

3. Results

3.1. Exhaled NO levels

Exhaled NO following ovalbumin challenge of ovalbumin-sensitized rats was significantly increased by 3 h (3.73 ± 0.74 ppb) when compared to ovalbumin-sensitized and saline-exposed rats (1.87 ± 0.26; $P<0.05$). This allergen-induced increase in exhaled NO remained significantly elevated for 12 h, reaching a maximum at around 9 h (11.0 ± 2.75), when compared to ovalbumin-sensitized and saline-exposed rats (2.81 ± 0.18; $P<0.01$), returning to baseline at 24 h (2.51 ± 0.35). There was no significant difference in exhaled NO when naive rats were compared to ovalbumin-sensitized, saline-exposed rats (Fig. 1A). In a separate experiment, at 8 h, there was a significant increase in the levels of exhaled NO in the ovalbumin-sensitized and

Table 1
NOS immunoreactivity in lungs challenged with ovalbumin

Structure	SS ($n=3$)			SO ($n=3$)			SOSC ($n=3$)		
	NOS1	NOS2	NOS3	NOS1	NOS2	NOS3	NOS1	NOS2	NOS3
Epithelial cells									
Bronchial	+	+	+	+	+++	+	+	+++	+
Bronchiolar	+	+	+	+	+++	+	+	+++	+
Alveol. type I	0	+	+	0	+	+	0	+	+
Alveol. type II	0	+	+	0	+	+	0	+	+
Smooth muscle									
Airway	+	+	0	+	+	0	+	+	0
Vascular	+	0	0	+	0	0	+	0	0
Endothelial cells									
Arteries	0	+	+++	0	+	+++	0	+	+++
Veins	0	0	+++	0	0	+++	0	+	+++
Capillary	0	0	+	0	0	+	0	0	+
Macrophages	0	+	0	0	+++	+	0	+++	+
Nerve fibres	+++	0	0	+++	0	0	+++	0	0
Chondrocytes	0	0	0	0	0	0	0	0	0

Scoring of intensity of staining from 0 to +++ (see Materials and methods). Key: SS: ovalbumin-sensitized, vehicle treated, saline-challenged; SO: ovalbumin-sensitized, vehicle treated, ovalbumin-challenged; SOSC: ovalbumin-sensitized, SC-51-treated, ovalbumin-challenged.

ovalbumin-challenged group (9.38 ± 2.22 ppb) when compared to the ovalbumin-sensitized and saline-challenged group (2.03 ± 0.24; $P < 0.001$). Treatment with 10 mg/kg (p.o.) SC-51 of the ovalbumin-sensitized and ovalbumin-challenged group inhibited levels of exhaled NO back to baseline levels as found in ovalbumin-sensitized and saline-challenged animals (1.3 ± 0.17 versus 2.03 ± 0.24; Fig. 1B).

3.2. NOS isoenzyme immunohistochemistry

Immunohistochemistry for NOS1 revealed abundant staining of nerve fibres innervating the airway smooth muscle (Fig. 2). NOS1-like immunoreactivity was also displayed in extraneuronal structures such as smooth muscle cells and airway epithelial cells. There was no difference in the pattern of distribution of NOS1 between the different experimental groups (Table 1). NOS3 immunoreactivity was restricted to endothelial cells of submucosal vessels and to the epithelial cell layer (Fig. 2; Table 1). In ovalbumin-sensitized, saline-challenged and naive, saline-challenged animals, sections incubated with NOS2 antiserum showed staining restricted to epithelial cells. In tissues of ovalbumin-sensitized and ovalbumin-challenged rats, the intensity of NOS2-immunoreactivity was significantly stronger in the epithelium and present in subepithelial inflammatory cells, such as macrophages (Fig. 2; Table 1). SC-51 did not affect the distribution or intensity of NOS1, NOS2 or NOS3 immunoreactivity (data not shown).

3.3. Bronchial responsiveness to acetylcholine

At 24 h, there were no significant differences in the baseline lung resistance values following saline challenge in the five experimental groups (data not shown). There was a significant increase in bronchial responsiveness to increasing concentrations of acetylcholine between the sensitized, allergen-exposed and vehicle-treated rats ($-\log PC_{200}$: 2.60 ± 0.08) compared to sensitized, saline-exposed and vehicle-treated rats (1.93 ± 0.09; $P < 0.01$). Treatment of allergen-exposed rats with SC-51 significantly inhibited the allergen-induced increase in bronchial responsiveness to a level similar to that of saline-exposed control rats (2.16 ± 0.06; $P < 0.05$). There was a significant increase in bronchial responsiveness when SC-51 was administered to sensitized, saline-exposed animals (2.24 ± 0.13) when compared to sensitized, vehicle treated and saline-exposed (Fig. 3).

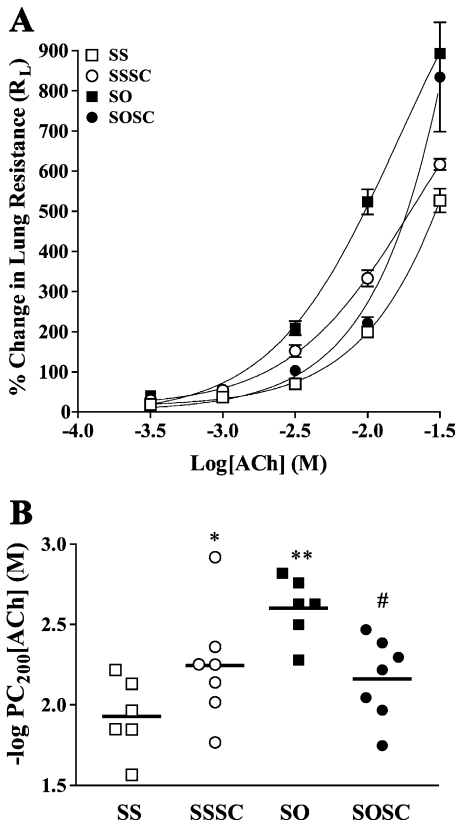

Fig. 3. Effect of SC-51 on bronchial responsiveness. (Panel A) Mean percentage increase in lung resistance to increasing concentrations of acetylcholine in four groups of sensitized rats: SS: ovalbumin-sensitized and saline exposed, $n = 6$; SO: ovalbumin-sensitized and ovalbumin-exposed, $n = 7$; SSSC: ovalbumin-sensitized, SC-51 (10 mg/kg p.o.)-treated and saline-exposed, $n = 6$; SOSC: ovalbumin-sensitized, SC-51 (10 mg/kg p.o.)-treated and ovalbumin-exposed, $n = 7$. The concentration–response curves are significantly shifted leftward for the groups SO ($P < 0.01$) and SSSC ($P < 0.05$) when compared to group SS. Data expressed as mean ± S.E.M. (Panel B) Individual and mean $-\log PC_{200}$, the negative logarithm of the provocative concentration of acetylcholine needed to increase baseline lung resistance by 200% at 18–24 following allergen challenge. Following allergen challenge (group SO), there was a significant increase in $-\log PC_{200}$ compared to saline-challenged group (group SS; $P < 0.01$). SC-51 in sensitized rats (group SSSC) increased $-\log PC_{200}$ ($P < 0.05$), while SC-51 in sensitized and allergen-exposed rats (SOSC) inhibited the increase in $-\log PC_{200}$ after allergen challenge ($P < 0.05$). Significant differences between sensitized, ovalbumin-exposed and sensitized, SC-51-treated and ovalbumin-exposed groups are expressed as #: $P < 0.05$, whereas significant differences between sensitized, ovalbumin-exposed and sensitized, saline-exposed are expressed as *: $P < 0.05$; **: $P < 0.01$.

3.4. Inflammatory cell response

3.4.1. Bronchoalveolar lavage

In the ovalbumin-sensitized and ovalbumin-exposed group, there was a significant increase in total cell count when compared to ovalbumin-sensitized and saline-exposed group ($P < 0.01$), eosinophils ($P < 0.01$), lymphocytes ($P < 0.01$) and neutrophils ($P < 0.01$). SC-51 significantly reduced the allergen-induced increase in neutrophils in sensitized rats when compared to sensitized and allergen-exposed rats ($P < 0.05$) (Table 2). SC-51 had no significant effect on the total number of cells recovered from BAL, and had no significant effect on the other differential cell counts when compared to both the sensitized, saline-challenged group and the sensitized, allergen challenged group.

Table 2
Effect of SC-51 on total and differential cell counts in bronchoalveolar lavage fluid from sensitized rats challenged with either saline or allergen

	SS ($n=6$)	SSSC ($n=7$)	SO ($n=6$)	SOSC ($n=7$)
Total[a]	180.0 ± 38.2	312.9 ± 49.6	621.7 ± 48.13 * *	740.0 ± 149.6
Macs[b]	91.69 ± 5.35	88.51 ± 1.04	65.15 ± 3.40	79.55 ± 1.71
Eos[b]	1.92 ± 0.65	2.91 ± 0.42	10.12 ± 1.04 * *	5.98 ± 0.60
Lym[b]	5.26 ± 4.31	5.66 ± 0.99	7.78 ± 3.06 * *	7.03 ± 1.28
Neu[b]	1.13 ± 0.46	2.91 ± 0.28	16.94 ± 2.79 * *	7.56 ± 1.41[#]

Macs = macrophages; Eos = eosinophils; Lym = lymphocytes; Neu = neutrophils. SS: ovalbumin-sensitized, vehicle-treated, saline-challenged; SSSC: ovalbumin-sensitized, SC-51-treated, saline-challenged; SO: ovalbumin-sensitized, vehicle-treated, ovalbumin-challenged; SOSC: ovalbumin-sensitized, SC-51-treated, ovalbumin-challenged. All statistical changes have been made on the absolute counts.

[a] Expressed as $\times 10^4$ per ml of recovered bronchoalveolar lavage fluid ± S.E.M.

[b] Expressed as percentages ± S.E.M.

** $P<0.01$ versus saline pretreatment.

[#] $P<0.05$ versus sensitized animals challenged with ovalbumin.

3.4.2. Airways

Allergen exposure caused a significant increase in MBP^+ cells (21.6 ± 5.4) and $CD2^+$ (18.2 ± 3.0), but had no effect on $CD4^+$ and $CD8^+$ T-cells. SC-51 did not inhibit allergen-induced increase in MBP^+ cells (16.7 ± 3.9), $CD2^+$ (15.5 ± 1.9), $CD4^+$ (9.9 ± 0.9) and $CD8^+$ T-cells (5.4 ± 1.2) (Fig. 4).

4. Discussion

We have used a relatively selective inhibitor of inducible nitric oxide synthase, SC-51, in order to examine the role played by endogenous nitric oxide production under the stimulation of NOS2 in allergic inflammation and allergen-induced bronchial hyperresponsiveness. We showed that nitric oxide in exhaled air of allergen-provoked rats is increased over the ensuing 9 h, and that these levels are suppressed by the NOS2 inhibitor, indicating that this increase in exhaled NO is derived from NOS2. In addition, the finding of increased NOS2 expression, but not of NOS1 or NOS3, in the lungs of allergen-exposed rats indicate that NOS2 activity is increased in the lungs of allergen-exposed rats. Of interest, SC-51-treated rats did not inhibit eosinophilic lung inflammation, while allergen-induced bronchial hyperresponsiveness was suppressed. Therefore, NOS2-derived NO is important in modulating bronchial hyperresponsiveness without an effect on eosinophilic inflammation.

While there is a large body of evidence on the importance of endogenous NO in inflammatory diseases, a precise role for NOS2-generated NO in human asthma and in animal models of allergen-induced bronchial hyperresponsiveness and airway inflammation has not yet been fully determined. Because the maturation and development of the innate immune system may be important for the expression

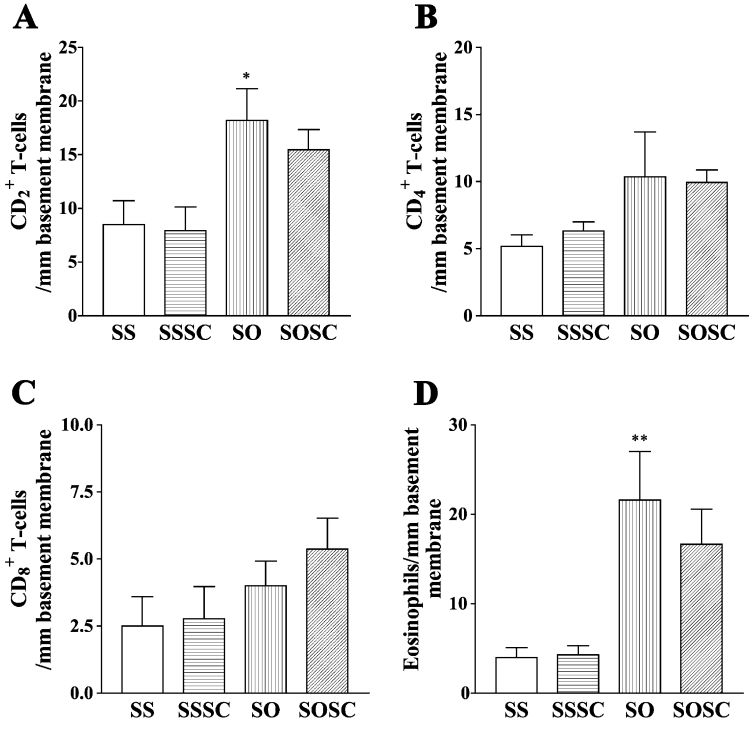

Fig. 4. Mean number of $CD2^+$ (A), $CD4^+$ (B) and $CD8^+$ (C) T-cells and MBP^+ eosinophils (D) in the airway submucosa. Allergen exposure of sensitized rats increased MBP^+ eosinophils and $CD2^+$ T-cells following allergen challenge, but this was not affected by pretreatment with SC-51. Significant differences between sensitized saline and sensitized ovalbumin-exposed groups are expressed as *: $P<0.05$ and **: $P<0.01$. Data shown as mean ± S.E.M.

of the allergic response, it may not be advantageous to use genetically NOS-deficient mice to examine the role of NO in the induction of allergic inflammation. Indeed, experiments in NOS knockout mice have produced conflicting results (Xiong et al., 1999; De Sanctis et al., 1999), perhaps due to the variable effect of NOS deficiency on the development of T-helper cell responses underlying the allergic response.

The ovalbumin-sensitized and exposed Brown–Norway rat model of allergic asthma was chosen because it has many characteristics that mimic human asthma (Elwood et al., 1991; Haczku et al., 1996). Following sensitization, these rats generate high levels of immunoglobulin E by 14–21 days (Pauwels et al., 1979). After allergen challenge, they develop both early and late phase responses and display bronchial hyperresponsiveness (Waserman et al., 1992; Elwood et al., 1991). In addition, there is an increased gene expression for Th2 'type' cytokines in the lung, namely interleukin-4 and interleukin-5, another prominent feature of human asthma (Haczku et al., 1996). In this model, we established the measurement of exhaled NO. Exhaled NO levels were increased following allergen challenge of sensitized rats, but not after allergen challenge of nonsensitized rats. The maximal increase in exhaled NO was observed at 8 h after allergen exposure, accompanied by an increase in the expression of the inducible isoform of nitric oxide synthase, NOS2, but not of the constitutively expressed isoforms, NOS1 and NOS3. NOS2 expression was localised to inflammatory cells and the airway epithelium. Inhibition of NOS2 activity with a selective NOS2 inhibitor, SC-51, led to a reduction in exhaled NO, implicating NOS2 as the major NO-generating isoenzyme during allergen-induced airway inflammation.

Selective NOS 2 inhibition by SC-51 resulted in suppression of the increase in exhaled NO following allergen challenge in sensitized rat, but did not inhibit the allergen-induced increase in NOS 2 protein levels, as would be expected. SC-51 itself has little activity against NOS2, but it is rapidly converted in vivo to L-NIL which has selective inhibitory activity on NOS2 (Hallinan et al., 2002). SC-51 reduced paw swelling in a rat model of carrageenan-induced acute paw inflammation with an ED_{50} of 10 mg/kg. We used this inhibitory dose of SC-51.

Bronchial hyperresponsiveness is one of the major characteristics of asthma and in our study, the inhibitor of NOS2 inhibited the allergen-induced bronchial hyperresponsiveness. Interestingly, SC-51 in sensitized, non-exposed rats induced bronchial hyperresponsiveness itself, indicating a dual effect of NO in modulating bronchial responsiveness. The induction of bronchial hyperresponsiveness by SC-51 has also been observed with higher doses of aminoguanidine in conscious guinea pigs (Schuiling et al., 1998). Thus, NOS1/NOS3-derived NO at low levels may be acting as a functional antagonist of methacholine-induced bronchoconstriction. On the other hand, high levels induced by NOS2 activation during allergic inflammation may be deleterious. Using aminoguanidine, Schuiling showed dual effects of NOS2-derived NO on ovalbumin-induced airway hyperresponsiveness, with low doses, given acutely, potentiating airway hyperresponsiveness, and high doses, given prophylactically, inhibiting airway hyperresponsiveness. Our data, using SC-51, are similar to the effects of the high-dose aminoguanidine observed by Schuiling et al. The changes in bronchial responsiveness may reflect a direct action of NO on airways smooth muscle since direct exposure of animals to inhaled NO protected against agonist-induced bronchoconstriction (Dupuy et al., 1992). On the other hand, the deleterious effect of high endogenous production of NO during allergic inflammation may be related to other mechanisms such as the production of toxic metabolites such as peroxynitrite and 3-nitrotyrosine formed by the rapid reaction between nitric oxide and superoxide (Beckman et al., 1990), which are both released during allergic inflammation (Calhoun et al., 1992). Peroxynitrite can oxidise sulfhydryl groups and initiate lipid peroxidation, and may be involved in the induction of bronchial hyperresponsiveness (Muijsers et al., 2001; Sadeghi-Hashjin et al., 1996; Tsukagoshi et al., 1994). This concept of low versus high production of NO is also supported by studies on airway microvascular leakage (Bernareggi et al., 1997).

Our data on the effect of SC-51 on bronchial hyperresponsiveness are in sharp contrast to the reports of studies obtained in the NOS2 knockout mouse where there was no effect observed on allergen-induced bronchial hyperresponsiveness (Xiong et al., 1999; De Sanctis et al., 1999). However, in NOS1 knockout mice, allergen-induced bronchial hyperresponsiveness was reduced (De Sanctis et al., 1999). These conflicting data may be due to the other factors controlling airways responsiveness induced by long-term depletion of NOS2 and NO.

Airway inflammation particularly characterized by activation of eosinophils has been suggested as a mechanism responsible for airway hyperresponsiveness in asthma (Bentley et al., 1992; Chung, 1986). However, in our study, NOS2 inhibition with our selective and potent inhibitor did reduce the accumulation of eosinophils into the airways, but this did not achieve statistical significance. This observation is in agreement with a study of allergen effects in a NOS2 knockout mouse, but not in another study with the same genetically deficient mouse (Xiong et al., 1999; De Sanctis et al., 1999). In a pharmacological study using a selective inhibitor of NOS2 in the sensitized mouse, L-N^6-(1-iminoethyl)lysine (L-NIL), which is the active inhibitor of NOS2 derived from SC-51 used in the present study, similar results as in our study were obtained in that this compound did not inhibit allergen-induced eosinophilia, while *N*-nitro-L-arginine methyl ester (L-NAME), an inhibitor of endothelial NOS (NOS3), had some inhibitory effect (Feder et al., 1997). A less selective inhibitor of NOS2, aminoguanidine, which is only twice more selective for NOS2 than for NOS3, has been reported to reduce allergen-induced lymphocytic, eosinophilic and neutrophilic increases in bron-

choalveolar lavage fluid in the PVG rat (Tulic et al., 2000). These effects could have been the result of combined NOS2 and NOS3 inhibition. Studies in mice showed that another selective NOS2 inhibitor, 1400W, inhibited ovalbumin-induced airway hyperresponsiveness but, to a lesser extent, eosinophil accumulation in the airways (Koarai et al., 2000; Muijsers et al., 2001). In our study, inhibition of allergen-induced bronchial hyperresponsiveness was coincident with inhibition of allergen-induced neutrophilia. This observation is consistent with the observation that inhibitors of nitric oxide synthase attenuate human neutrophil chemotaxis in vitro (Belenky et al., 1993), and that nitric oxide may be involved in the chemotaxis of neutrophils (Beauvais et al., 1995). It is also possible that neutrophils may be important for the expression of allergen-induced bronchial hyperresponsiveness, but the role of eosinophils is still not excluded.

In summary, we have demonstrated that a highly selective inhibitor of NOS2 can prevent allergen-induced bronchial hyperresponsiveness, with inhibition of allergen-induced neutrophilia, but not eosinophilia. These observations indicate that these selective inhibitors may be beneficial in patients with hyperreactive airways and allergic inflammation such as in asthma.

Acknowledgements

We thank Mark Currie and Pamela Manning of Pharmacia for their support of this study and for the provision of SC-51.

References

Alving, K., Weitzberg, E., Lundberg, J.M., 1993. Increased amount of nitric oxide in exhaled air of asthmatics. Eur. Respir. J. 6, 1368–1370.

Archer, S., 1993. Measurement of nitric oxide in biological models. FASEB J. 7, 349–360.

Barnes, P.J., 1995. Nitric oxide and asthma. Res. Immunol. 146, 698–710.

Beauvais, F., Mitchel, L., Dubertret, L., 1995. Exogenous nitric oxide elicits chemotaxis of neutrophils in vitro. J. Cell. Physiol. 165, 610–614.

Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A., Freeman, B.A., 1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc. Natl. Acad. Sci. U. S. A. 87, 1620–1624.

Belenky, S.N., Robbins, R.A., Rennard, S.I., Gossman, G.L., Nelson, K.J., Rubinstein, I., 1993. Inhibitors of nitric oxide synthase attenuate human neutrophil chemotaxis in vitro. J. Lab. Clin. Med. 122, 388–394.

Bentley, A.M., Menz, G., Storz, C., Robinson, D.S., Bradley, B., Jeffery, P.K., Durham, S.R., Kay, A.B., 1992. Identification of T-lymphocytes, macrophages and activated eosinophils in the bronchial mucosa of intrinsic asthma: relationship to symptoms and bronchial hyperresponsiveness. Am. Rev. Respir. Dis. 146, 500–506.

Bernareggi, M., Mitchell, J.A., Barnes, P.J., Belvisi, M.G., 1997. Dual action of nitric oxide on airway plasma leakage. Am. J. Respir. Crit. Care Med. 155, 869–874.

Calhoun, W.J., Reed, H.E., Moest, D.R., Stevens, C.A., 1992. Enhanced superoxide production by alveolar macrophages and air-space cells, airway inflammation, and alveolar macrophage density changes after segmental antigen bronchoprovocation in allergic subjects. Am. Rev. Respir. Dis. 145, 317–325.

Chung, K.F., 1986. Role played by inflammation in the hyperreactivity of the airways in asthma. Thorax 41, 657–662.

De Sanctis, G., Maclean, J.A., Hamada, K., Mehta, S., Scott, J.A., Jiao, A., Yandava, C.N., Kobzik, L., Wolyniec, W.W., Fabian, A.J., Venugopal, C.S., Grasemann, H., Huang, P.L., Drazen, J.M., 1999. Contribution of nitric oxide synthases 1, 2, and 3 to airway hyperresponsiveness and inflammation in a murine model of asthma. J. Exp. Med. 189, 1621–1630.

Dupuy, P.M., Shore, S.A., Drazen, J.M., Frostell, C., Hill, W.A., Zapol, W.M., 1992. Bronchodilator action of inhaled nitric oxide in guinea pigs. J. Clin. Invest. 90, 421–428.

Elwood, W., Lotvall, J.O., Barnes, P.J., Chung, K.F., 1991. Characterisation of allergen-induced inflammation and bronchial hyperresponsiveness in sensitised Brown–Norway rats. J. Allergy Clin. Immunol. 88, 951–960.

Feder, L.S., Stelts, D., Chapman, R.W., Manfra, D., Crawley, Y., Jones, H., Minnicozzi, M., Fernandez, X., Paster, T., Egan, R.W., Kreutner, W., Kung, T.T., 1997. Role of nitric oxide on eosinophilic lung inflammation in allergic mice. Am. J. Respir. Cell Mol. Biol. 17, 436–442.

Haczku, A., Macary, P., Haddad, E.-B., Huang, T.J., Kemeny, M., Moqbel, R., Chung, K.F., 1996. Expression of Th-2 cytokines IL-4 and IL-5 and of Th-1 cytokine IFN-γ in ovalbumin-exposed sensitised Brown–Norway rats. Immunology 88, 247–251.

Hallinan, E.A., Tsymbalov, S., Dorn, C.R., Moore, W.M., Jerome, G.M., Connor, J.R., Branson, L.F., Widomski, D.L., Zhang, Y., Currie, M.G., Manning, P.T., Pitzele, B.S., Hansen, D.W., 2002. Synthesis and biological chracterisation of L-N^6-(1-Iminoethyl)lysine tetrazole-amide, a prodrug of a selective iNOS inhibitor. J. Med. Chem. 45 (8), 1686–1689.

Hamid, Q., Springall, D.R., Riveros-Moreno, V., Chanez, P., Howarth, P., Redington, A., Bousquet, J., Godard, P., Holgate, S., Polak, J.M., 1993. Induction of nitric oxide synthase in asthma. Lancet 342, 1510–1514.

Koarai, A., Ichinose, M., Sugiura, H., Yamagata, S., Hattori, T., Shirato, K., 2000. Allergic airway hyperresponsiveness and eosinophil infiltration is reduced by a selective iNOS inhibitor, 1400W, in mice. Pulm. Pharmacol. Ther. 13 (6), 267–275.

Kharitonov, S.A., O'Connor, B.J., Evans, D.J., Barnes, P.J., 1995a. Allergen-induced late asthmatic reactions are associated with elevation of exhaled nitric oxide. Am. J. Respir. Crit. Care Med. 151, 1894–1899.

Kharitonov, S.A., Yates, D., Barnes, P.J., 1995b. Increased nitric oxide in exhaled air of normal human subjects with upper respiratory tract infections. Eur. Respir. J. 8, 295–297.

Liew, F.Y., 1994. Regulation of nitric oxide synthesis in infectious and autoimmune diseases. [Review]. Immunol. Lett. 43, 95–98.

Liu, S.F., Haddad, E.B., Adcock, I., Salmon, M., Koto, H., Gilbey, T., Barnes, P.J., Chung, K.F., 1997. Inducible nitric oxide synthase after sensitization and allergen challenge of Brown Norway rat lung. Br. J. Pharmacol. 121, 1241–1246.

Muijsers, R.B.R., van Ark, I., Folkerts, G., Koster, A.S., van Oosterhout, A.J.M., Postma, D.S., Nijkamp, F.P., 2001. Apocynin and 1400W prevents airway hyperresponsiveness during allergic reactions in mice. Br. J. Pharmacol. 134, 434–440.

Moncada, S., Palmer, R.M.J., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol. Rev. 43, 109–142.

Nathan, C., 1992. Nitric oxide as a secretory product of mammalian cells. FASEB J. 6, 3051–3064.

Neergard, K.V., Wirz, K., 1927. Die messung der stromuns-winderstande in den Atemwegen des menschen, inbe sondere bei asthma und emphysema. Z. Klin. Med. 105, 51–82.

Pauwels, R., Bazin, H., Platteau, B., Van Der Straeten, M., 1979. Relation between total serum IgE levels and IgE antibody production in rats. Int. Arch. Allergy Appl. Immunol. 58, 351–357.

Sadeghi-Hashjin, G., Folkerts, G., Henricks, P.A., Verheyen, A.K., Van Der Linde, H.J., Van Ark, I., Coene, A., Nijkamp, F.P., 1996. Peroxynitrite induces airway hyperresponsiveness in guinea pigs in vitro and in vivo. Am. J. Respir. Crit. Care Med. 153, 1697–1701.

Schuiling, M., Meurs, H., Zuidhof, A.B., Venema, Zaagsma, N., 1998. Dual action of iNOS-derived nitric oxide in allergen-induced airway hyper-

reactivity in conscious, unrestrained guinea-pigs. Am. J. Respir. Crit. Care Med. 158, 1442–1449.

Steudel, W., Watanabe, M., Dikranian, K., Jacobson, M., Jones, R.C., 1999. Expression of nitric oxide synthase isoforms (NOS II and NOS III) in adult rat lung in hyperoxic pulmonary hypertension. Cell Tissue Res. 295, 317–329.

Synder, D.W., Fleisch, J.H., 1989. Leukotriene receptor antagonists as potential therapeutic agents. Annu. Rev. Pharmacol. Toxicol. 29, 123–143.

Tsukagoshi, H., Robbins, R.A., Barnes, P.J., Chung, K.F., 1994. Role of nitric oxide and superoxide anions in interleukin-1 beta- induced airway hyperresponsiveness to bradykinin. Am. J. Respir. Crit. Care Med. 150, 1019–1025.

Tulic, M.K., Wale, J.L., Holt, P.G., Sly, P.D., 2000. Differential effects of nitric oxide synthase inhibitors in an in vivo allergic rat model. Eur. Respir. J. 15, 870–877.

Waserman, S., Olivenstein, R., Renzi, P., Xu, L.-J., Martin, J.G., 1992. The relationship between late asthmatic responses and antigen-specific immunoglobulin. J. Allergy Clin. Immunol. 90, 661–669.

Wislez, M., Fleury-Feith, J., Rabbe, N., Moreau, J., Cesari, D., Milleron, B., Mayaud, C., Antoine, M., Soler, P., Cadranel, J., 2001. Tumor-derived granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor prolong the survival of neutrophils infiltrating bronchoalveolar subtype pulmonary adenocarcinoma. Am. J. Pathol. 159, 1423–1433.

Xiong, Y., Karupiah, G., Hogan, S.P., Foster, P.S., Ramsay, A.J., 1999. Inhibition of allergic airway inflammation in mice lacking nitric oxide synthase 2. J. Immunol. 162, 445–452.

Yan, Z.Q., Hansson, G.K., Skoogh, B.E., Lotvall, J.O., 1995. Induction of nitric oxide synthase in a model of allergic occupational asthma. Allergy 50, 760–764.

Yeadon, M., Price, R., 1995. Induction of calcium-independent nitric oxide synthase by allergen challenge in sensitized rat lung in vivo. Br. J. Pharmacol. 116, 2545–2546.