Role of Nitric Oxide in Chronic Allergen-Induced Airway Cell Proliferation and Inflammation

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ABSTRACT

Chronic cellular inflammation and airway wall remodeling with subepithelial fibrosis and airway smooth muscle thickening are features of chronic asthma. We determined the role of nitric oxide in the pathogenesis of allergen-induced airway cell proliferation and inflammation by studying the effects of a relatively selective prodrug inhibitor of nitric-oxide synthase type 2 (NOS2), L-NAME-[1-iminoethyl]-lysine-5-tetrazole amide (SC-51). Brown-Norway rats were sensitized to ovalbumin and were exposed to ovalbumin aerosol every 3rd day on six occasions and were treated orally with either vehicle or SC-51 (10 mg/kg/day on six occasions and were treated orally with either vehicle or SC-51 (10 mg · kg⁻¹ · day⁻¹). We measured inflammatory cell accumulation in the airways and proliferation of cells by incorporation of bromodeoxyuridine. There was an increase in the total number of airway smooth muscle cells expressing bromodeoxyuridine from 1.3% of airway smooth muscle cells in saline exposed to 5.4% after allergen-exposure (P < 0.001) and airway epithelial cells from 3.3 cells/mm basement membrane to 9.6 after allergen-exposure (P < 0.001). SC-51 had no effect on airway smooth muscle or epithelial cell proliferation. SC-51 attenuated the allergen-induced increase in major basic protein (MBP+) eosinophil (P < 0.05) and CD4+ T-cell (P < 0.05) accumulation. We conclude that nitric oxide derived during allergic inflammation is involved in the expression of eosinophilic inflammation and not in epithelial or airway smooth muscle cell DNA synthesis induced by chronic allergen exposure.

Asthma is a chronic inflammatory disease of the airways characterized by the presence of activated inflammatory cells, such as eosinophils, mast cells, macrophages, and T-lymphocytes within the bronchial mucosa (Bousquet et al., 1999; Djukanovic et al., 1999). This inflammatory process has an allergic basis in many patients. There are also changes to structural cells of the airways, including increases in airway smooth muscle mass (Dunnill, 1960), goblet cell hyperplasia (Aikawa, 2001), epithelial cell disruption (Laitinen et al., 1985), and subepithelial fibrosis (Roche et al., 1989). The mechanisms underlying the changes in airway wall structure are unclear.

Studies derived from experimental models in mice indicate that certain cytokines of the interleukin-6 type such as interleukin-6 and -11 and of the T helper 2 type such as interleukin-4, -5, and -13 can induce changes of airway wall remodeling (Zhu et al., 1999; Zheng et al., 2001). For example, in interleukin-11 overexpressing transgenic mice, goblet cell hyperplasia, subepithelial fibrosis, and an increased accumulation of airway smooth muscle cells have been observed (Kuhn et al., 2000). In vitro, many mediators cause an increase in airway smooth muscle cell proliferation, including cysteinyl-leukotrienes, endothelin, growth factors such as epidermal growth factor, platelet-derived growth factor, and endothelin, and interactions between these mediators may be important (Panettieri, 1998a). Cysteinyl-leukotrienes can potentiate the proliferation of airway smooth muscle induced by epidermal growth factor (Panettieri et al., 1998b). These indicate that the mediators released in asthma may contribute to the airway wall remodeling characteristics of this disease, particularly the airway smooth muscle hyperplasia. The role of these mediators in airway smooth muscle proliferation in vivo is less clear. In a model of chronic allergic airway inflammation, endothelin and cysteinyl-leukotrienes have been shown to be important mediators (Salmon et al., 1998, 1999a).

In the present study, we have examined whether any inhibitory mediator of airway smooth muscle proliferation released during allergic inflammation could be acting as a brake to the proliferative response in vivo. In this regard, we studied the potential role played by nitric oxide. Increased

ABBREVIATIONS. NOS, nitric-oxide synthase; L-NAME, N^6-nitro-L-arginine methyl ester; SC-51, L-N^6-[1-iminoethyl]lysine-5-tetrazole amide; L-NIL, L-N^6-[1-iminoethyl]-lysine-5-tetrazole amide; MBP, major basic protein.
expression of the inducible form of nitric-oxide synthase, NOS2, has been observed in the bronchial epithelium of patients with asthma (Hamid et al., 1992), together with an increased level of exhaled nitric oxide. A nitric-oxide synthase inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME), reduced exhaled nitric oxide levels in patients with asthma (Yates et al., 1996), indicating that NOS enzyme activates the release of exhaled nitric oxide. In animal models of allergic inflammation in the rat or guinea pig, similar increases in NOS2 expression and activity have been observed in airway epithelium and alveolar macrophages (Eynott et al., 2000). In vitro studies, nitric oxide donors inhibit the proliferation of airway smooth muscle cells (Hamad et al., 1999; Patel et al., 1999), and this antiproliferative effect of nitric oxide has also been demonstrated in vascular smooth muscle proliferation occurring at sites of vascular injury (Marks et al., 1995). Thus, the increased levels of nitric oxide could inhibit the airway smooth muscle proliferation induced by other mediators/cytokines released during allergic inflammation. This protective effect of nitric oxide is also accompanied by a potential effect of nitric oxide as a bronchodilator in the airways (Kackmarek et al., 1996). To test the hypothesis that nitric oxide synthesized during allergic inflammation may modulate airway smooth muscle proliferation, we studied the effect of a NOS inhibitor, SC-51, Nω-(3-iminoethyl)lysine-5-tetrazole amide, which is a produg of L-NIL (L-N(L–l-arginyl)-lysine-5-tetrazole amide), in repeatedly allergen-exposed Brown-Norway rats actively sensitized to ovalbumin. In this model, we have previously shown significant increases in airway smooth muscle and airway epithelial cell proliferation, as shown by incorporation of bromodeoxyuridine with chronic eosinophilic and lymphocytic proliferation, as shown by incorporation of bromodeoxyuridine (4-chloro-2-methylbenzenediazonium/3-hydroxy-2-naphthoic acid 2,4-diamidino-2-phenylindole [4-chloro-2-methylbenzenediazonium/3-hydroxy-2-naphthoic acid 2,4-diamidino-2-phenylindole], Sigma-Aldrich, Dorset, UK) in 0.9% (w/v) sterile saline. Regions of the left and right lung lobes were mounted on cork blocks with the main bronchi uppermost, snap-frozen in melting isopentane, and stored at −80°C.

Materials and Methods

Animals, Sensitization Procedures, and Allergen-Exposure. Pathogen-free, male Brown-Norway rats (weighing 220–280 g, 9–13 weeks old; Harlan Olac, Ltd., Bicester, UK) were housed in a caging system receiving clean filtered air (Maximizer; Thoren Caging System, Inc., Hazleton, PA). Rats were sensitized on days 1, 2, and 3 using 1 mg · kg⁻¹ · intraperitoneal injections of ovalbumin (grade V, salt free; Sigma-Aldrich, Dorset, UK) in 0.9% (w/v) sterile saline containing 100 mg of aluminum hydroxide as adjuvant. On days 6, 9, 12, 15, and 21, animals were exposed to ovalbumin aerosol (1% w/v, 20 min) or 0.9% (w/v) saline. Challenges took place in a 0.8-m³ chamber, with free-breathing animals exposed to either 0.9% (w/v) saline or ovalbumin (1% w/v, 20 min) aerosol mist produced by a DeVilbiss PulmoSonic nebulizer (DeVilbiss Health Care, Ltd., Feltham, UK). The aerosol mist was pumped at a rate of 0.6 l · min⁻¹ by a small animal ventilator.

Experimental Design. Four groups were studied:

1. Sensitized, vehicle-treated, and repeatedly saline-exposed (group SS; n = 7); animals received vehicle for SC-51 (1 ml/dose of sterile water, orally) 2 h before and 8 h after exposure to saline. Rats were exposed to aerosolized saline for 20 min on days 6, 9, 12, 15, 18, and 21 and then studied 18 to 24 h later.

2. Sensitized, SC-51-treated (10 mg · kg⁻¹, orally), and repeatedly saline-exposed (group SSSC; n = 7): the procedures were the same as for group SS.

3. Sensitized, vehicle-treated, and repeatedly ovalbumin-exposed (SO; n = 8): the procedures were the same as for group SS, except that the aerosol was with 1% ovalbumin aerosol.

4. Sensitized, SC-51-treated (10 mg · kg⁻¹), and repeatedly ovalbumin-exposed (SOSC; n = 7): the procedures were the same as for group SO.

All rats were studied 18 to 24 h after exposure to either 1% ovalbumin or 0.9% saline aerosol.

SC-51. The NOS2-selective inhibitor, SC-51, is a produg of L-NIL, which is an inhibitor of NOS2. SC-51 itself has little activity as an inhibitor of NOS2 but is rapidly metabolized to L-NIL in the systemic circulation after oral administration in the rat. The IC₅₀ values for human NOS2 of L-NIL is 5.9 µM, with lesser activities for NOS3 (IC₅₀ = 138 µM) and NOS1 (35 µM); SC-51 itself has an IC₅₀ of >1850 µM for these three NOS (Hallinan et al., 2002). In the rat, oral doses of up to 10 mg/kg inhibited the elevated plasma nitrite levels of rats treated with endotoxin with an ED₅₀ of 0.6 mg/kg (Hallinan et al., 2002). A single dose of SC-51 (10 mg · kg⁻¹) inhibited the increase in exhaled nitric oxide induced by allergen challenge in sensitized rats for a least 24 h (Eynott et al., 2000).

Bromodeoxyuridine Dosing. 5-Bromo-2′-deoxyuridine (Sigma-Aldrich, Poole, UK) was dissolved in dimethyl sulfoxide and diluted with sterile water, giving a final concentration of dimethyl sulfoxide less than 7%. Rats were injected intraperitoneally with 50 mg · kg⁻¹ bromodeoxyuridine in 1 ml of solution immediately following the allergen challenges on days 3, 6, 9, 12, 15, 18, and 21 and received a second dose 8 h later (total of eight injections).

Tissue Collection. Rats were killed using an overdose of sodium pentobarbitone (500 mg · kg⁻¹, i.p.). The lungs were rapidly removed and inflated with optimal cutting temperature Tissue Tek mounting medium (Raymond A Lamb, London, UK) diluted 1:1 with phosphate-buffered saline. Regions of the left and right lung lobes were mounted on cork blocks with the main bronchi uppermost, snap-frozen in melting isopentane, and stored at −25°C.

Bromodeoxyuridine and α-Smooth Muscle Actin Immunohistochemistry. Cryostat sections 6 µm thick were cut and thaw-mounted on glass slides treated with Vectabond (Vector Laboratories, Peterborough, UK). Left lung blocks were cut between the first and second division of the main bronchi at an angle perpendicular and lateral to the conducting airways. Tissue sections were fixed in a 1:1 mixture of acetone and methanol for 10 min at 12°C, and endogenous peroxidase was blocked by immersing sections in methanol containing 0.3% (v/v) hydrogen peroxide for 20 min. A primary anti-bromodeoxyuridine monoclonal antibody (clone BU-1; Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) was applied at 37°C for 75 min. A secondary biotinated rat adsorbed antiserum to mouse IgG (Vector Laboratories) was then applied followed by a 45-min incubation with a peroxidase-linked avidin-biotin complex solution (ABC-Elite kit; Vector Laboratories). Bromodeoxyuridine positive cells were visualized using 3,3-diaminobenzidine tetrachloride solution (Sigma-Aldrich) with glucose oxidase-nickel enhancement to give a black endproduct (Shu et al., 1998). Sections were then rinsed, and a primary anti-α-smooth-muscle actin monoclonal antibody (clone 1A4; Sigma-Aldrich) was applied for 1 h at room temperature. A secondary biotinylated rat adsorbed antiserum to mouse IgG (Vector Laboratories) was then applied to the sections, followed by an avidin-biotin complex reagent conjugated to alkaline phosphatase (Vector Laboratories). The α-smooth-muscle-actin staining was visualized using Sigma FAST [4-chloro-2-methylbenzenediazonium/3-hydroxy-2-naphthoic acid 2,4-dimethylanilide phosphate (naphthol AS-MX) and Fast Red TR] in Tris buffer (Sigma-Aldrich) to give a red endproduct. Nuclei that were not immunoreactive for bromodeoxyuridine were counterstained by application of the fluorescent DNA ligand 4,6-diamidino-2-phenylindole hydrochloride (Sigma-Aldrich) at a concentration of 0.0001% and mounted under glass coverslips. Tissue sections of spleen, colon, and thymus were used to confirm positive bromodeoxyuridine immunoreactivity. Negative controls were performed on sections of lung for bro-
modeoxyuridine and \(\alpha\)-smooth-muscle actin with antibodies of the same immunoglobulin class or in the absence of primary antibody.

**Bronchoalveolar Lavage and Cell Counting.** Lungs were perfused with 10 ml (9% w/v saline) via the right ventricle. The intact lungs were subsequently lavaged with 10 \(\times\) 2-ml aliquots of 0.9% w/v saline through a polyethylene syringe introduced through the tracheostomy. Lavage fluid was then centrifuged (500 g for 10 min at 4\(^\circ\)C), and the cell pellet was resuspended in 0.5 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 before mounting in DPX mountant with coverslips. Cells were identified as macrophages, eosinophils, neutrophils, and lymphocytes by standard morphology, and at least 500 cells were counted under 400\(\times\) magnification. The percentage and absolute numbers of each cell type were calculated.

**Immunohistochemistry of NOS Isoforms.** For immunohistochemistry, 6-\(\mu\)m thick lung cryostat sections from seven rats in each group were used. Sections were mounted on gelatin precoated glass slides and subjected to a standard immunostaining protocol (Lim et al., 2000). The sections were fixed with acetone, rinsed in phosphate-buffered saline, and then preincubated with 10% normal swine serum and 1% bovine serum albumin in 0.1 M phosphate buffer for 1 h. After several washes in phosphate-buffered saline, the sections were incubated with either monoclonal mouse NOS1-antiserum (Sigma-Aldrich) diluted 1:400, polyclonal rabbit NOS 2-antiserum (BIOMOL Research Laboratories, Hamburg, Germany) diluted 1:250, or polyclonal rabbit NOS 3-antiserum (BIOMOL Research Laboratories) diluted 1:500 in the preincubation solution. Overnight incubation at 4\(^\circ\)C was followed by 3 \(\times\) 5-min washes in phosphate-buffered saline and detection of the primary antisera by incubation with either anti-mouse or anti-rabbit fluorescein isothiocyanate antibodies (Dianova, Hamburg, Germany) or a biotin-streptavidin Texas Red detection system (Amersham Biosciences, Braunschweig, Germany) for 1.5 h at room temperature. Slides were then mounted in carbonate-buffered glycerol (pH 8.6) and viewed using epifluorescence microscopy. Blinded observers scored the immunoreactivity for NOS1, -2, and -3 as strongly positive (+ + +), moderate positive (+ +), weakly positive (+), or negative (−) immunoreactivity, as described elsewhere (Wislez et al., 2001).

**Eosinophil Major Basic Protein and T-Cell Immunohistochemistry.** For the detection of eosinophils, we used a IgG, monoclonal antibody against human major basic protein (MBP), clone BMK-13 (Monosan, Uden, The Netherlands), which is both sensitive and specific for staining rat eosinophils through sections. The cryostat sections were incubated with BMK-13 at a concentration of 1:80 for 1 h at room temperature. After labeling with a biotinylated horse anti-mouse monoclonal secondary antibody, positively stained cells were visualized using the alkaline phosphatase-anti-alkaline phosphatase method. For staining of CD2+, CD4+, and CD8+ T-lymphocytes in tissue sections, sections were incubated with either mouse anti-rat CD2, CD4 or CD8 monoclonal antibody (pan T-cell markers; BD PharMingen, San Diego, CA) at a dilution of 1:400 for 1 h at room temperature. A secondary, biotinylated goat anti-mouse monoclonal antibody (Vector Laboratories) against the primary antibody was applied to lung tissue sections for 30 min. Tissue sections were rinsed in buffer and then applied with an avidin-biotin complex reagent conjugated to alkaline phosphatase for 45 min. For all tissue sections, alkaline phosphatase was developed as a red stain after incubation with napthol AS-MX phosphate in 0.1 M trimethylamine-HCl buffer (pH 8.2) containing levamisole to inhibit endogenous alkaline phosphatase and 1 mg/ml Fast Red-TR salt (Sigma-Aldrich). Then, sections were counterstained with Harris hematoxylin (BDH, Manchester, UK) and mounted in Glycergel (DAKO, Carpinteria, CA). System and specificity controls were carried out for all staining.

**Quantification of DNA Synthesis and Airway Smooth Muscle Area.** The quantification procedure has been adapted from that previously described by Salmon et al. (1999b). Briefly, image processing was performed using an Axioskop microscope (Zeiss GmbH, Munich, Germany) fitted for both transmitted light and fluorescence imaging. Images were captured using an Axiocam digital camera (Zeiss GmbH) at maximum sensitivity and analyzed using an image analysis system (Image Associates, Thame, UK). Each selected airway was quantified through a 5\(\times\) objective lens to measure lumen area. Images captured through the 5\(\times\) objective lens were then used to count Bromodeoxyuridine- and 4,6-diamidino-2-phenylindole hydrochloride-labeled cells and measure smooth muscle area and epithelial basement membrane length. For measurement of bromeoxyuridine indices, the airway of interest was selected, and the transmitted light image containing bromodeoxyuridine-positive cells was captured. A blue fluorescence image for 4,6-diamidino-2-phenylindole hydrochloride-positive nuclei was then captured, and both images were converted to stored monochrome images. The transmitted light image was used to create a mask that excluded all regions that were not immunoreactive for \(\alpha\)-actin, and the number of bromodeoxyuridine positively stained nuclei was counted. The mask was then overlaid onto the fluorescence image of the 4,6-diamidino-2-phenylindole hydrochloride-positive nuclei and the number of nuclei counted. Measurement of epithelial bromodeoxyuridine indices was performed in a similar way by creating an epithelial mask using thresholding of the epithelium on 4,6-diamidino-2-phenylindole hydrochloride fluorescence images and counting the number of bromodeoxyuridine-positive nuclei within the mask. Bromodeoxyuridine indices in airway smooth muscle were measured as the number of bromodeoxyuridine immunoreactive nuclei divided by total nuclei (bromodeoxyuridine plus 4,6-diamidino-2-phenylindole hydrochloride nuclei) within the \(\alpha\)-smooth-muscle actin-stained area. Epithelial bromodeoxyuridine index was measured as the number of bromodeoxyuridine-positve cells within the 4,6-diamidino-2-phenylindole hydrochloride-defined epithelial mask divided by the basement membrane length. Statistical power calculations have previously been reported (Salmon et al., 1999b). The five largest conducting airways cut perpendicular and lateral to the plane of the airways between the first and second division of the main bronchi from a single lung section was used to calculate the DNA synthesis and airway smooth muscle thickness indices for rats from each treatment group. As previously described, when using these parameters, a standard error of less than \(\pm\) 15% of the mean index is achieved for airway smooth muscle indices when comparing one against five consecutive sections of lung (Salmon et al., 1999b). All counts in this study were performed with the investigator blinded to treatment group.

**MBP + Eosinophil and T-Cell Counts.** Eosinophil (MBP+) and T-cell (CD2+, CD4+, and CD8+) counts around the five largest airways in each lung section was assessed as the number of positively stained cells in the bronchial submucosa and expressed per millimeter of basement membrane.

**Measurement of Serum Ovalbumin-Specific IgE.** Blood (2–3 ml) was left to clot for 1 to 2 h at 20–24\(^\circ\)C and centrifuged for 15 min at 1000g. Serum was aspirated, aliquoted into Eppendorf tubes, and stored frozen at \(-20^\circ\)C. Ovalbumin-specific IgE titers were estimated by enzyme-linked immunosorbent assay using a method adapted from that of Nonaka et al. (2000). Briefly, microtiter plates (Nunc-Immuno Plate MaxiSorp Surface; Naige-Nunc International, Copenhagen, Denmark) were coated overnight at 4\(^\circ\)C with MARE-1 mouse anti-rat IgE (mouse anti-rat IgE heavy chain; 1 mg/ml, 1/2000 dilution) at 0.5 \(\mu\)g/ml in carbonate bicarbonate buffer (coated buffer). A standard curve was constructed using doubling dilutions of standards (rat serum identified to be of high IgE titer), and samples were added and incubated for 2 h. Biotinylated-ovalbumin (prepared using EZ-Link Sulfo-NHS-LC-Biotinylation Kit; Pierce and Warriner, Chester, UK) was added at an optimized concentration (~10 \(\mu\)g/ml) and incubated for 90 min, followed by incubated for 45 min with 1 \(\mu\)g/ml streptavidin-alkaline phosphatase. The p-nitro-phenyl phosphate enzyme substrate was used for color development, and the plates were read spectrophotometrically at 405 nm with an auto-
mated enzyme-linked immunosorbent assay plate reader (Automated Microplate Reader EL311; Bio-Tek Instruments, Inc., Winooski, VT). Titers were expressed as arbitrary units (AU) per milliliter. A naive control group of animals (n = 5) was also studied.

Analysis of Data. All data are expressed as the arithmetic mean index derived from the five largest airways in one section of lung from each rat. Mean indices were statistically analyzed after logarithmic transformation by two-way analysis of variance, followed by t tests with Bonferroni correction used to evaluate significant differences between groups. Values are expressed as means (95% confidence intervals), with P values of less than 0.05 considered significant.

Results

Airway Smooth Muscle and Epithelial Bromodeoxyuridine Indices

Following repeated allergen challenge, there was a 4-fold increase in the airway smooth muscle cell bromodeoxyuridine index in the sensitized, allergen-exposed, and vehicle-treated group (5.4%; 3.5 to 8.0) compared with the sensitized, saline-exposed, and vehicle-treated group (1.3%; 0.6 to 1.9; P < 0.001). Treatment with SC-51 did not alter airway smooth muscle DNA synthesis following repeated allergen-exposure (Fig. 1A). Repeated allergen exposure also caused a significant increase in the number of bromodeoxyuridine-positive epithelial cells per millimeter of basement membrane in the sensitized and vehicle-treated group of rats (9.6; 6.6 to 15.2) compared with the sensitized, saline-exposed, and vehicle-treated controls (3.3; 2.0 to 5.4; P < 0.001). Treatment with SC-51 did not alter the epithelial cell DNA synthesis following repeated allergen-exposure (Fig. 1B).

Airway Smooth Muscle Thickness

Repeated allergen exposure did not result in significant increase in the airway smooth muscle thickness. The sensitized, allergen-exposed, and vehicle-treated group had a greater mean thickness of 18.8 μm (13.5 to 24.4) compared with 15.2 μm (14.0 to 16.2) in the sensitized, saline-exposed, and vehicle-treated rats, but this did not reach statistical significance (Fig. 2). Treatment with SC-51 had no effect in the airway smooth muscle thickness (Fig. 2).

Inflammatory Cell Response

Bronchoalveolar Lavage Cells. The ovalbumin-sensitized and ovalbumin-exposed group, when compared with ovalbumin-sensitized and saline-exposed group, showed a significant increase in total cell count (531.9 × 10^4 cells/ml versus 62.4; P < 0.01), eosinophils (71.5 versus 3.5; P < 0.01), lymphocytes (136.9 versus 1.8; P < 0.01), and neutrophils (139.9 versus 2.1; P < 0.01). SC-51 had no significant effect on the total number of cells recovered in bronchoalveolar lavage and on the differential cell counts when compared with both the sensitized, saline-challenged group and the sensitized, allergen-challenged group (Fig. 3).

Airways. Repeated allergen-exposure caused a significant increase in MBP+ cells (16.4 versus 3.8; P < 0.01), CD2+ (11.2 versus 7.1; P < 0.01), CD4+ (10.6 versus 5.4; P < 0.01), and CD8+ (2.8 versus 1.3; P < 0.05); T-cells are compared with control (Fig. 4). Treatment of allergen-exposed rats with SC-51 resulted in inhibition of MBP+ cells (8.0; P < 0.05; Fig. 4, A) and CD4+ T-cells (7.0; P < 0.05; Fig. 4, C) in the airways. SC-51 had no effect on the allergen induced increase in CD2+ or CD8+ T-cells in the airways.
Serum Ovalbumin-Specific IgE

Serum levels of ovalbumin-specific IgE were significantly increased in all sensitized rats compared with those of control naive animals (Table 1). SC-51 had no effect on ovalbumin-specific IgE levels in sensitized and ovalbumin-challenged rats.

NOS Isoenzyme Lung Protein Expression

The distributions of NOS isoenzymes were examined by immunohistochemistry (Fig. 5; Table 2). NOS1-like immunoreactivity was abundantly present in nerve fibers innervating the airways and was also found in extraneuronal structures such as airway epithelial cells or smooth muscle myocytes. There was no difference in NOS1 staining between the four different experimental groups (Fig. 5, A–D; Table 2). NOS3-like immunoreactivity was present in endothelial cells of submucosal vessels and in epithelial cells, and no difference was found between the different groups (Fig. 5, I–L; Table 2). NOS2-like immunoreactivity was present in myocytes, epithelial and endothelial cells. In ovalbumin-sensitized, saline-challenged, treated or nontreated animals, NOS2-like immunoreactivity was localized to myocytes, epithelial and endothelial cells. In ovalbumin-sensitized, ovalbumin-challenged rats, the intensity of NOS2-like immunoreactivity was stronger in the epithelial cells, and also large populations of subepithelial NOS2-positive inflammatory cells were present (Fig. 5, E–H; Table 2). SC-51 had no effect on the distribution or intensity of staining of the anti-NOS isoenzyme antibodies.

Discussion

We used a model of repeated allergen exposure of sensitized Brown-Norway rats to investigate the role played by endogenous nitric oxide production during chronic allergen-induced eosinophilic inflammation and airway smooth muscle and epithelial cell mitogenesis. We found increases in bromodeoxyuridine incorporation into airway smooth muscle cells and airway epithelial cells after repeated allergen-exposure, as previously described (Salmon et al., 1999b). Concomitantly, we found increased NOS2 protein expression in the lungs of repeatedly allergen-exposed rats indicating that NOS2 activity is increased in the lungs, but the expression of NOS1 and NOS3 was not altered. NOS2 expression was localized to inflammatory cells and the airway epithelium. There was an increase in eosinophil and CD2+ , CD4+ , and CD8+ T-cell accumulation within the airways together with increased serum levels of antigen-specific IgE. Using the relatively selective inhibitor of NOS2 SC-51 to suppress the generation of nitric oxide, we found that SC-51 had no effect on airway smooth muscle cell or epithelial cell DNA synthesis after repeated allergen exposure, despite attenuating the numbers of eosinophils and CD4+ T-cells in the airway submucosa. SC-51 also had no effect on elevated serum levels of ovalbumin-specific IgE indicating that it did not influence IgE synthesis. Therefore, nitric oxide does not appear to be important in modulating the proliferation of airway smooth muscle and epithelial cells during chronic allergic inflammation and may not provide a significant brake on this proliferative process.

We have used a prodrug, SC-51, which is converted rapidly in the blood to its active moiety, L-NIL, which in turn has selective inhibitory activity on NOS2 (Hallinan et al., 2002). SC-51 has been demonstrated to have anti-inflammatory properties. It reduced paw swelling in a rat model of carrageenan-induced acute paw inflammation with an ED_{50} of 10 mg/kg (Hallinan et al., 2002). In our previous study, we examined the effect of a single dose of SC-51 (10 mg · kg⁻¹) on the acute effects of single allergen exposure in sensitized Brown-Norway rats. SC-51 suppressed allergen-induced in-
crease in exhaled nitric oxide, indicating that an effective dose SC-51 had been administered but did not significantly inhibit the number of eosinophils in tissue and bronchoalveolar lavage fluid in a single allergen inhalation model (Eynott et al., 2000). In the current study, we used a similar dose of SC-51 but administered at 2 h before each allergen exposure and 8 h later so that inhibition of NOS activity was achieved for at least 12 h after each of the six allergen exposures.

In our chronic allergen exposure model, we found an increase in airway smooth muscle cell and epithelial cell DNA incorporation indicating cell proliferation, but the NOS inhibitor we used had no effect on this process. Nitric oxide has been shown to inhibit proliferative responses of airway smooth muscle induced by serum (Hamad et al., 1999; Patel et al., 1999), and therefore inhibiting nitric oxide production could have resulted in an enhanced proliferative response. The contribution of nitric oxide in this process in vivo is likely to be more complex because of other potential effects of nitric oxide. Nitric oxide released may interact with superoxide to form peroxynitrite, which is a very reactive molecule, and this process may occur in the lungs following allergen challenge (Saleh et al., 1998, de Boer et al., 2001). It is also possible that the conversion to peroxynitrite is very rapid such that nitric oxide may not have time to exert its protective effects. Nitric oxide may inhibit proliferation by inhibiting epidermal growth factor receptor tyrosine kinase activity by nitrosylation of the receptor (Estradra et al., 1997) and by inhibiting platelet-derived growth factor-induced protein kinase B cascade (Sandirasegarane et al., 2000). This effect is not shared by peroxynitrite, although peroxynitrite can inhibit proliferation of lymphocytes (Brito et al., 1999). In previous studies in our chronic allergen exposure model, we found that the airway smooth proliferation was inhibited by both a cysteinyl-leukotriene receptor antagonist and by an endothelin receptor antagonist, indicating the involvement of cysteinyl-leukotrienes and of endothelin in this process (Salmon et al., 1998, 1999b). Nitric oxide can reduce endothelin-induced airway smooth muscle proliferation in vitro (Kizawa et al., 2001), and interestingly, endothelin-1 may inhibit the synthesis of nitric oxide in smooth muscle (Ikeda et al., 1997). Thus, these in vitro experiments indicate that these mediators, which are released during allergic inflammation, may antagonize each other in the control of airway smooth muscle proliferation state. The present study indi-

**TABLE 1**

Effect of ovalbumin sensitization and exposure on ovalbumin specific IgE levels in the serum

Data are presented as mean ± S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>Naive (n = 3)</th>
<th>SS (n = 7)</th>
<th>SSSC (n = 7)</th>
<th>SO (n = 8)</th>
<th>SOSC (n = 7)</th>
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<tr>
<td>0.13 ± 0.01</td>
<td>(0.08 to 0.17)</td>
<td>0.27 ± 0.01*</td>
<td>0.29 ± 0.01*</td>
<td>0.42 ± 0.12*</td>
<td>0.28 ± 0.01*</td>
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<tr>
<td>0.10 ± 0.01</td>
<td>(0.06 to 0.17)</td>
<td>0.26 ± 0.01*</td>
<td>0.28 ± 0.01*</td>
<td>0.41 ± 0.11*</td>
<td>0.26 ± 0.01*</td>
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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.

* P < 0.05 versus naive.

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**Fig. 4.** Mean number of MBP⁺ eosinophils (A), CD2⁺ (B), CD4⁺ (C), and CD8⁺ (D) T-cells in the airway submucosa. Allergen exposure of sensitized rats increased MBP⁺ eosinophils, CD2⁺, and CD4⁺ T-cells following allergen-challenge (*, P < 0.05), 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 and ***, P < 0.001, whereas those between sensitized, ovalbumin-exposed and sensitized, SC-51 treated and ovalbumin-exposed groups are expressed as #, P < 0.05. Data shown as mean ± S.E.M.
cates that nitric oxide does not contribute to inhibition of airway smooth muscle proliferation in this in vivo model of chronic allergic inflammation.

Eosinophils may be an important source of growth factors and, therefore, may be responsible for airway smooth muscle proliferation. The level of eosinophils was inhibited by NOS inhibitor indicating that nitric oxide may be involved in the recruitment of eosinophils to the airways. This is the first study to demonstrate the role of nitric oxide in eosinophil recruitment in a chronic allergen model. In acute models of allergic inflammation, there have been conflicting results. For example, in studies describing the allergen-effects in a NOS2 knockout mouse, there have been conflicting data regarding the effects on airway eosinophils (De Sanctis et al., 1999; Xiong et al., 1999). A selective inhibitor of NOS2 in the sensitized mouse, L-NIL, which is the active inhibitor of NOS2 derived from SC-51 used in the present study, did not inhibit allergen-induced eosinophilia, whereas 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, reduced allergen-induced lymphocytic, eosinophilic, and neutrophilic increases in bronchoalveolar lavage fluid in the PVG rat (Tulic et al., 2000). These effects could have been the result of concomitant NOS3 inhibition. In another study by Koarai et al. (2000), a reduction in allergen-induced pulmonary eosinophilia was observed following treatment with 1400W, a selective inhibitor of NOS2. Similarly, Trifilieff et al. (2000) reported a reduction in allergen-induced pulmonary eosinophilia following treatment and L-NAME. The discrepancies between our data and those of others may be attributed to species differences and/or sensitization and challenge protocols.

In summary, we found that a selective NOS2 inhibitor inhibited partially the eosinophil inflammation induced by chronic allergen exposure but had no effect on airway smooth muscle and airway epithelial cell proliferation. These results indicate that nitric oxide produced during allergic inflammation is unlikely to alter proliferative indices in the airway.

Acknowledgments

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<table>
<thead>
<tr>
<th>Structure</th>
<th>Saline-Challenged</th>
<th>SC-51-Treated</th>
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<tbody>
<tr>
<td></td>
<td>Vehicle-Treated</td>
<td>SC-51-Treated</td>
</tr>
<tr>
<td></td>
<td>(n = 7)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td></td>
<td>NOS1</td>
<td>NOS2</td>
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<tr>
<td>Epithelial cells</td>
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<tr>
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</tr>
<tr>
<td>Inflammatory cells</td>
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<td>+</td>
</tr>
<tr>
<td>Smooth muscle</td>
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<tr>
<td>Endothelial cells</td>
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<tr>
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</tr>
<tr>
<td>Fibroblasts</td>
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</tr>
</tbody>
</table>

SS, ovalbumin-sensitized, vehicle-treated, saline-challenged; SO, ovalbumin-sensitized, vehicle-treated, ovalbumin-challenged; SOSC, ovalbumin-sensitized, SC-51-treated, ovalbumin-challenged.


