Pharmacological Assessment of the Nitric-Oxide Synthase Isoform Involved in Eosinophilic Inflammation in a Rat Model of Sephadex-Induced Airway Inflammation

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Received September 10, 2002; accepted December 4, 2002

ABSTRACT

Excessive local production of nitric oxide (NO) has been suggested to play a role in rodent models of airway inflammation and in pulmonary diseases such as asthma. However, even given the plethora of data available including gene expression data, pharmacological data, and gene deletion studies in animal models, it is still not clear which nitric-oxide synthase (NOS) isoform is involved in eosinophilic airway inflammation. In this rat study, the nonselective NOS inhibitor L-NAME (N\textsuperscript{ω}-nitro-L-arginine methyl ester), but not a selective inducible NOS (iNOS) inhibitor 1400W (N\textsuperscript{\textomega}-3-(aminomethyl)benzyl)acetamidine), impacted on Sephadex-induced inflammation by significantly inhibiting lung edema, eosinophil infiltration, tumor necrosis factor \( \alpha \), interleukin-13, and eotaxin levels in the lung tissue. Furthermore, iNOS gene expression was not induced following Sephadex administration, which confirms that iNOS does not play a role in this model. To demonstrate that this phenomenon was not restricted to this model of asthma, L-NAME, but not 1400W, was shown to reduce eosinophilia in an antigen-induced model. However, in contrast to the Sephadex model, there was an induction of iNOS gene expression after antigen challenge. In a model of aerosolized lipopolysaccharide-induced inflammation, where iNOS gene expression is increased, 1400W inhibited the increased neutrophilia. These data suggest that the compound has been administered using an appropriate dosing regimen for iNOS inhibition in the rat lung. In conclusion, it appears that constitutive, not inducible, NOS isoforms are important in NO production in models of allergic inflammation, which questions whether there is a role for iNOS inhibitors as therapy for the treatment of asthma.

Nitric oxide (NO) has been shown to be involved in a variety of biological processes including host defense, immune regulation, platelet aggregation, neurotransmission, and inflammation (Moncada et al., 1991; Moncada and Higgs, 1993). It is synthesized from \( \text{L}-\text{arginine} \) by the enzyme nitric oxide synthase (NOS), which exists in three forms. Two are constitutive, nNOS (also known as NOS I) and eNOS (also known as NOS III) and one is inducible, iNOS (also known as NOS II) (Moncada et al., 1997). In the lung, eNOS is found in endothelial cells, bronchial epithelial cells, platelets, neutrophils, and mast cells; nNOS is found in nerve cells, whereas iNOS is also found in bronchial epithelial cells, macrophages, fibroblasts, smooth muscle cells, and endothelial cells (Kobzik et al., 1993; Gaston et al., 1997). There is increasing evidence that endogenous NO plays a key role in physiological regulation of the airways and is implicated in the pathophysiology of airways disease (Barnes, 1995). Exhaled levels of NO are increased in patients with asthma (Kharitonov et al., 1994) and increase even further during the inflammatory late response to allergen (Kharitonov et al., 1995). Interestingly, iNOS is expressed in airway epithelial cells in asthmatics but not in nonasthmatics (Hamid et al., 1993). However, the cellular source of the increased NO seen in the exhaled air from asthmatic patients, the NOS isotype respon-
sible for the NO production, and its physiological role in the asthmatic airway remains unclear.

Although there is a great deal of information in the public domain regarding the role of NO and the NOS isoform involved in animal models of antigen-induced airway inflammation the situation is still not clear. Generally, three approaches have been used to identify the NOS isoform responsible for NO-mediated allergic inflammation. First, several studies have implicated a role for the iNOS isoform in allergic inflammation due to the increased gene expression seen following antigen challenge in animal models (Yeadon and Price, 1995; Liu et al., 1997) and the increased iNOS expression seen in diseased versus normal tissues in biopsy studies in man (Hamid et al., 1993). Second, pharmacological data exist, and there seems to be general agreement that nonselective NOS inhibitors reduce allergen-induced eosinophilia in several animal models (Feder et al., 1997; Ferreira et al., 1998; Iijima et al., 1998). However, the data incorporating the use of iNOS inhibitors are very confusing and often conflicting with many investigators using compounds with minimal selectivity for iNOS (Trifilieff et al., 2000), and several studies not demonstrating a dose-response relationship with the compounds used and not benchmarking their data by using nonselective NOS inhibitors (to rule out effects of compounds not due to NOS inhibition) (Koarai et al., 2000; Muijsers et al., 2001). More recently, molecular approaches have been adopted in the form of gene deletion studies, which have again yielded conflicting results with one study showing reduced eosinophilia in antigen-challenged iNOS gene knockout mice (Xiong et al., 1999) and another using nNOS, iNOS, or eNOS gene knockout mice suggesting that none of the NOS isoforms are individually involved in antigen-induced eosinophilia (De Sanctis et al., 1999).

Instillation of Sephadex into the airways of a rat is a model of acute alveolitis and bronchiolitis, leading to inflammatory cell infiltration and interstitial edema, which appears to parallel many of the pathophysiological features associated with human interstitial lung diseases such as asthma (Cogtgrave et al., 1988). Sephadex-induced airway inflammation is characterized by the development of lung edema and a profound tissue eosinophilia similar to that observed in ovalbumin challenge models (Bjørmer et al., 1994). Therefore, the purpose of this study was to clarify for the first time the role of iNOS in the pathophysiology of Sephadex-induced airway eosinophilia in a rat model by first adopting a comprehensive pharmacological approach and incorporating the use of both a nonselective inhibitor [N(G)-nitro-L-arginine methyl ester (l-NAME)] and a highly selective iNOS inhibitor [N-3-(aminomethyl)benzyl]acetamide (1400W)] (Garvey et al., 1997) in a dose-ranging study. Second, NOS isoform gene expression was measured in the lung tissue after Sephadex instillation to support the pharmacological studies. Finally, the same pharmacological studies were repeated in a model of allergen-induced eosinophilia in the Brown Norway rat to investigate whether the same mechanisms were operative in two distinct models of profound airway eosinophilia.

**Materials and Methods**

**Animals**

Male Sprague-Dawley (SD) rats (330–350 g, n = 172), male Brown Norway (BN) rats (180–200 g, n = 178), and male Wistar rats (180–200 g, n = 80) were purchased from Harlan-Olac (Bicester, UK) and housed for 5 days before initiating experiments. Food and water were supplied ad libitum. United Kingdom Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act 1986 were strictly observed. A total of 430 animals were used for these experiments.

**Sephadex-Induced Airway Eosinophilia**

**Effect of l-NAME and 1400W against Sephadex-Induced Airway Inflammation in the SD Rat.** SD rats were dosed intratracheally (i.t.) with vehicle (saline) or Sephadex beads (5 mg/kg) at a dose volume of 1 ml/kg under halothane anesthesia (4% in oxygen for 4 min). l-NAME or 1400W (3, 10, 30, or 100 mg/kg) or vehicle (saline, 1 ml/kg) was administered i.p. 2 h before and 4 and 12 h after Sephadex administration (Garvey et al., 1997; Laszló and Whittle, 1997). The positive standard, dexamethasone (0.3 mg/kg), was dosed orally 24 and 1 h before Sephadex.

**Measurement of Lung Edema following Sephadex-Induced Airway Inflammation.** Rats were sacrificed 24 h post Sephadex with an overdose of sodium pentobarbitone (200 mg/kg, i.p.) and the heart and lungs were removed en bloc. The lung wet weights were determined and expressed per 100 g of initial body weight. Percentage of inhibition of edema (compared with the Sephadex i.t./vehicle i.p. control group) was then calculated for each treatment group. A dose-response curve was generated, a sigmoidal fit obtained for the data, and an ED50 value (dose that produced 50% of the maximum inhibition of lung edema) calculated.

**Measurement of Cell Recruitment in the Airway Tissue following Sephadex.** After the lungs were weighed, the left lobe was perfused with ice-cold RPMI 1640 to remove any blood and then finally chopped. A 300-μl aliquot of lung was weighed and kept at 4°C; the remaining lung was flash-frozen in liquid nitrogen and stored at −80°C. Cells were isolated from the lung tissue by enzymatic disaggregation using 10 ml of RPMI 1640/10% fetal bovine serum (FBS) containing collagenase (1 mg/ml) and DNase (25 μg/ml) in a shaking water bath at 37°C for 1 h. The resulting mixture was then passed through a 70-μm cell sieve and spun at 800g for 10 min at 4°C. The supernatant was discarded, 10 ml of RPMI 1640/10% FBS added, and the pellet resuspended. The pellet-washing step was carried out twice to remove the collagenase and DNase. After the final wash, the pellet was resuspended in 1 ml of RPMI 1640/10% FBS containing penicillin/streptomycin. Total white blood cells were determined using an automated cell counter (Cyte Counter, Codos, Argos, Roche ABX Hematology, Montpellier, France). Differentiation of white blood cells was performed by examination, under light microscopy, of the slide preparations made from the samples.

**Measurement of Cytokine Protein Levels Present in the Airway Tissue following Sephadex-Induced Airway Inflammation.** The remaining lung was flash frozen in liquid nitrogen and stored at −80°C. Later an accurately weighed piece of rat lung was homogenized with 1 ml of ice-cold saline. The homogenized sample was then spun at 800g for 10 min. The resulting supernatant was taken off and stored at −20°C. IL-13 and IFN-γ levels in the lung tissue supernatant were determined using a rat-specific solid-phase sandwich ELISA kit (Biosource International, Camarillo, CA). The minimum detectable concentration of IL-13 was 1.5 pg/ml, IFN-γ was <13 pg/ml, and there was no detectable cross-reactivity with other rat and mouse cytokines and chemokines. TNFα levels were determined in the lung tissue using a rat-specific sandwich immunoassay kit obtained from R&D Systems (Abingdon, UK). The minimum detectable concentration was found to be <5 pg/ml, and there was no significant cross-reactivity with other cytokines/chemokines. Because of the high degree of similarity maintained in chemokines across species, a mouse ELISA kit containing a polyclonal antibody that recognizes mouse eotaxin was used to detect the rat cognate. Thus, rat eotaxin levels were determined using a mouse ELISA kit (R&D Systems). No significant cross-reactivity was detected with...
other cytokines/chemokines, and the minimum detectable concentration of eotaxin was found to be <3 pg/ml.

**Treatment to Assess the Time Course of NOS Gene Expression following Sephadex Administration.** SD rats were dosed i.t. with vehicle (saline) or Sephadex beads (5 mg/kg) at a dose volume of 1 ml/kg under halothane anesthesia (4% in oxygen for 4 min). At 2, 4, 6, 12, 24, 48, and 72 h after insult, the animals were sacrificed, and samples were taken for gene expression.

**Lung NOS Gene Expression (RT-PCR). RNA extraction.** Lung samples were collected after treatment with Sephadex and antigen. Total cellular RNA was isolated by guanidium thiocyanate-phenol-chloroform extraction. As positive controls for eNOS, nNOS, and iNOS gene expression, RNA was extracted from naive rat lung, naive rat brain, and lung from an LPS-treated rat (20 mg/kg, i.p. sacrificed 4 h later), respectively. Purity and integrity of the RNA samples was assessed by A260/A280 spectrophotometric measurements on the GeneQuant RNA/DNA calculator (Amersham Pharmacia Biotech, Albans, Hertfordshire, UK).

**RT-PCR.** A 1-μg portion of total RNA was subjected to first strand cDNA synthesis in a 25-μl reaction mixture containing avian myeloblastosis virus reverse transcriptase (10 U), deoxyctydine triphosphate mixture (2 mM of each dNTP), oligo(dT)15 primer (10 μM), and reaction buffer as supplied with the enzyme (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl2, 0.5 mM spermidine, and 10 mM dithiothreitol). The samples were incubated in a PerkinElmer 480 thermal cycler (PerkinElmer Life Sciences, Boston, MA) at 42°C for 60 min followed by an enzyme denaturation step at 94°C for 2 min. The reverse transcription mixture was diluted with 25 μl of RNase-free water and stored at −80°C for use in PCR. All the reagents were obtained from Promega (Southampton, UK).

PCR was performed on 4 μl of reverse transcriptase product using Ready-To-Go PCR beads (Amersham Pharmacia Biotech), containing Taq DNA polymerase, dNTP, buffer and gene-specific forward and reverse primers (0.5 μM each) in a total volume of 25 μl. Gene-specific oligonucleotide primers were obtained from Invitrogen (Paisley, UK), and the primer sequences are shown in Table 1. PCR was carried out in a PerkinElmer GeneAmp PCR system 9700. After an initial denaturation at 95°C for 5 min, amplification was carried out through 28 to 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C (GAPDH) or 60°C (all NOS isoforms) for 30 s (1 min for nNOS) and extension at 72°C for 30 s (1 min for nNOS). Final extension was at 72°C for 7 min followed by a final hold at 4°C. Negative controls (PCR mixture without cDNA) and positive controls (PCR mixture with a standard cDNA sample) were included in preliminary PCR runs. Initial experiments were carried out to determine the optimal annealing temperature for each set of gene-specific primers and also the linear phase of the product amplification curve (data not shown).

The PCR products were separated by electrophoresis using 2% agarose gels stained with ethidium bromide to visualize cDNA products. Bands of each target transcript were visualized by ultraviolet transillumination, and the image was photographed (GAPDH at 28 cycles, all NOS genes at 35 cycles) using a UVP BioImaging and Analysis system (Ultra-Violet Products Ltd., Cambridge, UK). Optical densities of each band were calculated by image analysis software (Phoretix; Ultra-Violet Products Ltd.). For each sample, the level of gene expression was normalized to that of the housekeeping gene GAPDH.

**Effect of l-NAME and 1400W on Antigen-Induced Airway Inflammation in the BN Rat.** BN rats were sensitized with ovalbumin (100 μg OA plus 100 mg aluminum hydroxide in 1 ml of sterile saline, i.p.) on days 0, 14, and 21. The rats were challenged with an aerosol of saline or OA (1% w/v for 30 min) on day 28. l-NAME (10, 30, or 100 mg/kg), the inactive isomer d-NAME (100 mg/kg), 1400W (10, 30, or 100 mg/kg), or vehicle (saline, 1 ml/kg) was administered i.p. 2 h before and 4 and 12 h after antigen challenge. The positive standard, dexamethasone (1 mg/kg) was dosed orally 24 and 1 h before antigen challenge.

**Measurement of Cell Recruitment in the Airway Tissue after Antigen.** Rats were sacrificed 24 h postantigen with an overdose of sodium pentobarbital (200 mg/kg, i.p.), and the left lung lobe was removed. The number of cells in the lung tissue was determined by the same method used for the Sephadex model.

**Effect of 1400W on LPS-Induced Airway Inflammation in the Wistar Rat.** Wistar rats were challenged with an aerosol of saline or LPS (0.3 mg/ml for 30 min). Vehicle (saline, 1 ml/kg) or 1400W (10, 30, or 100 mg/kg) was administered i.p. 2 h before and 1 h after challenge. Rats were sacrificed 6 h after challenge with an overdose of sodium pentobarbital (200 mg/kg, i.p.), and the lungs treated the same as in the Sephadex study.

Sodium pentobarbital (200 mg/kg) and halothane were obtained from Rhône Mérieux (Harlow, UK) and RPMI 1640, PBS, and primers for PCR from Invitrogen. Roche Diagnostics (Lewes, UK) supplied the DNAse and collagenase. Sephadex G-200 was purchased from Pharmacia (Uppsala, Sweden). Dexamethasone, l-NAME, and the inactive isomer d-NAME were purchased from Sigma (Poole, UK) along with ovalbumin and LPS. 1400W was synthesized by the Chemistry Department of Aventis Pharma (Vitry, France). TNFα and eotaxin ELISA kits were obtained from R&D Systems and IL-13 from Lifescreen (Watford, UK). Reagents for RNA extraction were purchased from Sigma. All RT-PCR reagents were obtained from Promega, except Ready-To-Go beads (Amersham Pharmacia Biotech).

All values are presented as mean ± S.E.M. from n = 6 to 8 rats per group. The percentage inhibition of edema (compared with the Sephadex-administered, vehicle-treated group) was determined for each treated group. The dose-response curve for inhibition of lung edema by l-NAME was calculated by least-squares, nonlinear iterative regression with the PRISM curve-fitting program (Graphpad Instat software program; GraphPad Software, Inc., San Diego, CA). An ED50 value (dose of l-NAME required to produce 50% maximum inhibition) was subsequently interpolated from a curve of best fit. The data were analyzed using one-way analysis of variance followed by Dunn’s post test. A p value of less than 0.05 was considered statistically significant (*p < 0.05).
crease in lung wet weight. However, d-NAME had no significant effect at the same doses (17.4 ± 23.3, 17.0 ± 16.7, and 47.9 ± 11.2% inhibition).

In the next set of experiments, Sephadex instillation evoked significant lung edema (from 0.437 ± 0.016 to 0.547 ± 0.017 lung wet weight/body weight (in grams), 25%, \( p < 0.05 \)). L-NAME caused a dose-dependent inhibition of lung edema (ED_{50} of 21 mg/kg; maximum inhibition of 102.18 ± 11.5 at 100 mg/kg, Fig. 1) when compared with Sephadex-instilled, vehicle-treated animals. In contrast, the selective iNOS inhibitor, 1400W, only impacted on lung edema at 100 mg/kg (65.2 ± 22.4% inhibition). The positive control, dexamethasone (0.3 mg/kg), also significantly reduced lung wet weight (to 0.382 ± 0.01 lung wet weight/body weight (in grams), \( p < 0.05 \)).

Sephadex instillation caused an increase in lung tissue eosinophils (from 0.209 ± 0.096 to 1.069 ± 0.119 cells/10^9 mg of tissue) but not neutrophil and macrophage numbers. L-NAME treatment reduced eosinophilia in a dose-related manner that reached significance at 100 mg/kg, whereas 1400W had no effect at any dose tested (Fig. 2). The positive standard, dexamethasone, significantly reduced eosinophil number (to 0.188 ± 0.086 cells/10^9 mg of tissue).

**Effect of Compounds on Cytokine Protein Levels in the Lung Tissue.** Sephadex treatment significantly increased lung tissue TNF-\( \alpha \) levels (from 80.3 ± 8.3 to 1868.8 ± 282.0 pg/g of lung tissue, \( p < 0.05 \)), eotaxin (mouse equivalent) levels (from 441.3 ± 104.9 to 2879.2 ± 497.8 pg/g of lung tissue, \( p < 0.05 \)), and IL-13 levels (from 23.5 ± 3.7 to 253.2 ± 71.8 pg/g of lung tissue, \( p < 0.05 \)). L-NAME (100 mg/kg) and dexamethasone significantly reduced TNF-\( \alpha \), eotaxin, and IL-13 levels (Fig. 3), whereas 1400W at the doses tested had no impact on any of the cytokines. None of the compounds tested altered altered lung tissue IFN-\( \gamma \) levels (data not shown).

**Measurement of NOS mRNA Expression after Sephadex.** Expression of iNOS mRNA, as measured by RT-PCR, was not induced in the lung after Sephadex treatment at any time point investigated. In contrast, LPS-treatment (LPS 20 mg/kg, i.p., and lungs harvested 4 h later) exhibited a marked induction of iNOS mRNA. Endothelial NOS was present at all time points and there was no further induction following Sephadex treatment. nNOS was not expressed in any of the samples but the positive control, naive rat brain, was present (Fig. 4A).

**Effect of Compounds on Antigen-Induced Airway Eosinophilia.** Antigen challenge caused a significant increase in eosinophils (from 1.6 ± 0.2 to 3.6 ± 0.5 cells/10^9 mg of tissue, \( p < 0.05 \)). d-NAME (3.0 ± 0.4 cells/10^9 mg of tissue) and 1400W (3.6 ± 0.5, 5.6 ± 1, and 4.5 ± 1 cells/10^9 mg of tissue at 10, 30, and 100 mg/kg, respectively) had no effect on eosinophilia. Alternatively, L-NAME evoked a dose-related decrease in cell number (29, 45, and 65% at 10, 30, and 100 mg/kg, respectively). The positive standard, dexamethasone, completely blocked eosinophilia.

**Measurement of NOS mRNA Expression after Antigen Challenge.** iNOS gene expression was induced only at 4 h; eNOS was present constitutively and there was no further induction following antigen challenge, and nNOS was only very faintly expressed and was not up-regulated with antigen. All positive controls were present, as with the Sephadex-treated animals (Fig. 4B).

The OA solution (1%) used to challenge the rats was assessed for the presence of LPS using an E-TOXATE limulus assay, according to manufacturer’s instructions (Sigma). This semiquantitative assay showed that there was LPS present in the OA but not in the saline solution that was used to challenge the animals.

**Effect of 1400W on LPS-Induced Airway Neutrophilia.** LPS challenge caused a significant increase in lung tissue neutrophils (from 13.2 ± 2.77 to 31.6 ± 2.76 cells/10^9 mg of lung tissue). Treatment with 1400W caused a significant dose-related inhibition of lung tissue neutrophils (to 26.76 ± 1.6 and 23.6 ± 2.98 cells/10^9 mg of lung tissue, \( p < 0.05 \), at 30 and 100 mg/kg, respectively).

**Discussion**

The role of NO in human asthma or in animal models of asthma is unclear, as is the relative contribution of each of the NOS isoforms to NO production in eosinophilic inflammation. We compared the effect of the nonselective NOS inhibitor, L-NAME, with the selective iNOS inhibitor, 1400W in a model of Sephadex-induced inflammation and found that...
L-NAME, and not 1400W, inhibit Sephadex-induced lung edema and lung tissue eosinophilia. This result is consistent with other workers who demonstrated that L-NAME reduced Sephadex-induced lung edema in the rat (Andersson et al., 1995) and inhibited antigen-induced airway microvascular permeability and eosinophilia in the guinea pig (Iijima et al., 1998) and eosinophilia in the sensitized and challenged rat (Ferreira et al., 1998). The studies described in this manuscript have taken these findings further by investigating the effects of a selective iNOS inhibitor 1400W, validating its activity in an LPS model of inflammation and testing its effectiveness in two models of eosinophilic lung inflammation.

In this study, 1400W failed to impact on either Sephadex- or antigen-induced airway eosinophilia, suggesting that activation of cNOS and not iNOS is responsible for the airway inflammatory responses mediated by NO. The inhibitory effect of 1400W on LPS-induced airway neutrophilia suggests that the dosing regime used for 1400W throughout these studies is appropriate.

The lack of impact of 1400W on eosinophilia concurs with other published studies. Feder et al. (1997) showed that L-NAME and not L-N⁶-(1-iminoethyl)lysine (purported to be a selective iNOS inhibitor) blocked antigen-induced airway eosinophilia in the mouse. Muijsers et al. (2001) using a similar antigen-driven mouse model demonstrated 1400W to block airway hyperresponsiveness but not airway eosinophilia. In complete contrast to both the Muijsers study and our data, Koarai et al. (2000) reported that 1400W inhibited antigen-induced airway hyperresponsiveness and airway eosinophilia in the mouse. However, in this study, only one dose of 1400W was used, and this was administered by continuous infusion throughout the study; there is a distinct possibility that at the doses achieved, 1400W could be losing its selectivity for iNOS. In another, more comprehensive study, a role for iNOS was suggested in a murine model of allergic inflammation following data obtained with the "so-called" specific and potent iNOS inhibitors 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine and S-ethylisothiourea and their ability to inhibit eosinophil infiltration into the bronchoalveolar lavage...
fluid (Trifilieff et al., 2000). Again, these data would appear to be in complete contrast to this study. However, in the Trifilieff study the effect of iNOS inhibitors was assessed on cellular infiltration into the bronchoalveolar lavage fluid and not the lung tissue. Second, the iNOS inhibitors used in this study do not have a suitable selectivity profile and should not be considered as acceptable pharmacological tools with which to investigate the role of iNOS in vivo model systems. In contrast, 1400W is one of the most selective inhibitors of purified human iNOS to date (Garvey et al., 1997).

Molecular approaches in the form of gene deletion studies have again yielded conflicting results with one study showing reduced eosinophilia in antigen-challenged iNOS gene knockout mice (Xiong et al., 1999) and another using nNOS, iNOS, or eNOS gene knockout mice, suggesting that none of the NOS isoenzymes are individually involved in antigen-induced eosinophilia (De Sanctis et al., 1999). The reasons for these differences are not clear. Although the use of selective NOS isoform-deficient mice is an interesting approach for attempting to dissect the mechanisms involved in NO-mediated eosinophilia, one cannot rule out the effects of gene deletion on development. For these reasons, we have decided to embrace a pharmacological approach that provides the investigator with a robust method for determining the role of iNOS in these model systems when selective tools such as 1400W are available.

In an attempt to confirm our pharmacological data suggesting that cNOS and not iNOS is involved in these models of asthma, we measured the levels of iNOS gene expression and found that after Sephadex, there was no measurable iNOS expression in the lung. At first, it appeared that the increase in iNOS expression evident at 4 h after antigen challenge was a confounding factor that may cast doubt on our hypothesis, but this observation may be explained by the presence of LPS in the ovalbumin used. However, it would appear that this level of iNOS expression is not of functional significance given the lack of effect of 1400W on the eosinophilic response. Furthermore, confirming our finding, another group has shown the presence of LPS in the ovalbumin used in their study, using a similar antigen challenge model, and stated that the amount found was 10,000 times less than that needed to evoke cell recruitment (Rudmann et al., 2000). It should be stated that in Rudmann et al. (2000) and in our study, grade V antigen was used for sensitization and challenging, which is the same grade used by the majority of laboratories. These results add more weight to the hypothesis that the isoform of NOS involved in airway eosinophilia is cNOS and not iNOS. However, an alternative explanation that should not be overlooked, even though we failed to measure iNOS expression after Sephadex, is the possibility that it is necessary to inhibit all NOS isoforms, including iNOS, to inhibit airway eosinophilia as is achieved when one administers the nonselective iNOS inhibitor L-NAME. To test this hypothesis, it would be necessary to obtain commercially available, selective nNOS and eNOS inhibitors whose use is not cost prohibitive and that have an appropriate pharmacokinetic profile to be used in vivo studies.

The mechanisms involved in NO-mediated eosinophil recruitment into the lung are not clear. NO induces airway microvascular leakage (Kuo et al., 1992; Miura et al., 1996), which may lead to the development of lung edema as measured in this study, and one could speculate that this may augment the migration of eosinophils from the blood into the airways. In fact, from the data obtained in this study there would appear to be an excellent correlation between the dose of L-NAME required to inhibit edema and eosinophilia. NO has also been demonstrated to be chemotactic for a variety of cell types including eosinophils and may, therefore, play a role in the recruitment of eosinophils into the lung (Belenky et al., 1993; Ferreira et al., 1996). Additionally, NO has effects on T cell function in that it inhibits the proliferation of cloned Th-1 cells but not Th-2 cells (Taylor-Robinson et al., 1994). The Th-1-derived cytokine IFN-γ is known to inhibit Th-2 cell proliferation (Gajewski and Fitch, 1988); hence, by reducing the population of Th-1 cells, the Th-1/Th-2 balance is altered enhancing eosinophilic inflammation. The Sephadex model is an acute model, so it is unlikely that T cell switching is responsible for the NO-mediated eosinophilia. Furthermore, L-NAME treatment did not result in a decrease in lung levels of the Th-1 cytokine IFN-γ.

Increased levels of TNFα, eotaxin, and IL-13 in the airways following Sephadex have been suggested to play a causative role in Sephadex-induced lung pathology (Haddad et al., 2002). In theory, L-NAME could be inhibiting eosinophilia by inhibiting the lung tissue levels of the eosinophil chemoattractants IL-13 and eotaxin as shown in this study. However, L-NAME only inhibited lung tissue cytokine levels, following Sephadex, at doses much higher than those required to inhibit Sephadex-induced lung edema and eosinophilia. This may suggest that the reduction of these cytokines is a consequence of the inhibition of eosinophil infiltration rather than the underlying mechanism behind the inhibition.

Another possible mechanism of action of L-NAME could be due to the effect of L-NAME on blood pressure and heart rate. Rees et al. (1990) demonstrated that L-NAME (0.03–300 mg/kg, i.v.) increased mean arterial blood pressure and reduced heart rate when given intravenously to anesthetized rats, whereas in the experiments described here, the rats were dosed i.p. while conscious. Furthermore, NOS inhibitors such as L-NAME increase vascular resistance, and despite any increase in blood pressure, this could in some vascular beds lead to a reduced perfusion pressure, which in turn would mitigate edema formation. In fact, we have previously demonstrated that L-NAME increases plasma leakage under normal conditions (Bernareggi et al., 1997).

In conclusion, these data suggest that activation of the constitutive isoforms of NOS play a role in Sephadex- and antigen-induced airway inflammation. These data question the commonly held belief that the constitutive isoforms of NOS subserve a “physiological” role whereas iNOS is involved in the “pathophysiology” of airway inflammatory diseases. However, to introduce a note of caution, most of the data presented in this article relate to Sephadex-induced eosinophilic inflammation in rats, whereas most of the cited observations on the role of NOS are generated in antigen-induced eosinophilic inflammation in mice. Furthermore, there may be species differences between rats and mice and/or strain differences regarding the induction of iNOS and the role of NOS isoforms in inflammation, indicating that further studies need to be addressed in the clinic.

References


