Endogenous pulmonary nitric oxide in the regulation of airway microvascular leak

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Mehta, Sanjay, Jacques Boudreau, Craig M. Lilly, and Jeffrey M. Drazen. Endogenous pulmonary nitric oxide in the regulation of airway microvascular leak. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L961–L968, 1998.—Endogenous nitric oxide (NO) is an important modulator of airway function, but its role in the regulation of airway microvascular leak (AMVL) remains unclear. Thus we assessed the effects of NO synthase (NOS) inhibition on expired NO (ENO) levels and on AMVL measured by the Evans blue dye technique in guinea pigs. In control unsensitized animals, systemic N^\text{N}-nitro-\text{L}-arginine methyl ester (L-NAME) reduced ENO by 70 ± 8% (P < 0.01) and reduced AMVL by 92 ± 1 and 44 ± 17% (P < 0.05 for both) in the extrapulmonary and intrapulmonary airways, respectively. In animals sensitized and challenged with intratracheal antigen, markedly increased levels of AMVL and ENO were similarly attenuated by L-NAME. In contrast, aminoguanidine, a relatively selective type II NOS inhibitor, reduced ENO in both antigen-sensitized and control unsensitized animals by 39 ± 3% (P < 0.01) but had no effect on AMVL. These data indicate that endogenous pulmonary NO contributes to both basal and antigen-stimulated levels of AMVL in guinea pigs and that this NO-dependent activity does not appear to be derived from type II NOS.

Evans blue dye; nitric oxide synthase inhibitors; aminoguanidine; expired nitric oxide

ENDOGENOUSLY PRODUCED NITRIC OXIDE (NO) is a recognized regulator of a number of physiological systems (13, 24, 25). NO is synthesized by a family of NO synthase (NOS) isforms that are classified into two categories based on the strength of binding to calmodulin: low-NO output isforms weakly bind calmodulin and thus are sensitive to the local calcium concentration (calcium-dependent NOS; types I and III), whereas high-NO output isforms tightly bind calmodulin, resulting in an apparent independence of local calcium levels (calcium-independent NOS; type II) (25). Both types of NOS isforms have been identified in a variety of cell types in the lungs and airways (2, 8, 15, 17, 30, 31). The endogenous pulmonary production or excretion of NO may be directly measured in the expired gas of animals and humans by a chemiluminescence-based technique (16, 22, 28), although the precise cellular source of expired NO (ENO) remains uncertain.

Among its many roles, NO is an important modulator of airway function in both health and disease (4, 5, 11, 22, 26, 28, 34). For example, airway obstruction induced by pulmonary antigen exposure in sensitized animals is associated with a transient elevation of ENO that reflects an important, endogenous pulmonary NO-related modulatory effect during the acute allergic bronchoconstrictor response (21, 22, 28). It is likely that endogenous NO also has an important role in the regulation of airway microvascular leak (AMVL), although there are conflicting data on the nature of this role and the isoform of NOS that is the source of this NO (6, 9, 18). Thus we investigated the role of endogenously produced NO in the regulation of AMVL in both normal guinea pigs and the setting of airway inflammation induced by repeated pulmonary antigen exposure. We hypothesized that 1) basal endogenous pulmonary production of NO maintains vascular integrity and reduces basal levels of AMVL as described in the systemic circulation and, 2) in contrast, the increased presence of NO in inflamed airways contributes to the increase in AMVL characteristic of airway inflammation.

MATERIALS AND METHODS

Animal Preparation

Exposure protocol. All studies were performed on male Hartley guinea pigs [450- to 650-g body weight; Charles River Laboratories, Kingston, NY (cohort 1) and Raleigh, NC (cohort 2)]. The study protocol had the prior approval of the Institutional Animal Care and Use Committee.

On days 0 and 7, guinea pigs (n = 48) in the antigen exposure group were sensitized to aerosolized antigen (ovalbumin grade II; Sigma, St. Louis, MO) as previously described (22). Unanesthetized animals were placed in a 30-l Plexiglas chamber into which 8 ml of 7% ovalbumin in phosphate-buffered saline (PBS; pH 7.4) were delivered over 6 min as an aerosol generated by two nebulizers driven by compressed air (Marquest Medical Products, Englewood, CO). Thirty minutes before each antigen exposure, animals were pretreated with 10 mg/kg of pyrilamine maleate (Sigma) intraperitoneally. An unsensitized control group of animals (n = 42) was not subjected to the 7-day antigen exposure protocol but remained in the animal facility during this period and received intraperitoneal pyrilamine on days 0 and 7 because this treatment had no effect on AMVL in unsensitized animals (data not shown). AMVL was assessed on day 14 immediately after either antigen, saline, or sham intratracheal instillation.

Invasive monitoring. On day 14, before the assessment of AMVL, the animals were anesthetized by intraperitoneal injection of 100 mg/kg of ketamine and 15 mg/kg of xylazine; additional doses of 50 mg/kg of ketamine were administered at 30-min intervals to maintain anesthesia. The anterior neck was dissected, the internal jugular vein was catheterized with silicone elastomer tubing (0.025-mm OD, 0.012-mm ID; Technical Products, Decatur, GA), and the common carotid artery was catheterized with a 24-gauge Teflon catheter (Quik-Cath, Mehta, Sanjay, Jacques Boudreau, Craig M. Lilly, and Jeffrey M. Drazen. Endogenous pulmonary nitric oxide in the regulation of airway microvascular leak. Am. J. Physiol. 275 (Lung Cell. Mol. Physiol. 19): L961–L968, 1998.—
Baxter Healthcare, Deerfield, IL), and the subglottic trachea was cannulated with PE-240 polyethylene tubing (Intra-
medic, Becton Dickinson, Parsippany, NJ) through an ante-
rior tracheotomy. The animals were mechanically ventilated (ventilator model 683, Harvard Apparatus, South Natick, MA) at the following constant ventilatory settings: a tidal volume of 6 ml/kg, a respiratory frequency of 60 breaths/min, a positive end-expiratory pressure of 2–3 cmH2O, and a fixed ratio of inspiratory to expiratory time of 1:0. The animals were ventilated with inspired air that was essentially NO free (≤1 part/billion [ppb]) and had an oxygen fraction of 0.21. For the measurement of expired NO levels (see Measurement of 
\( E_{NO} \)), mixed expired gas was intermittently collected from the expiratory port of the ventilator into an inert Mylar bag over 3 min. Airway opening pressure (\( P_{ao} \)) and phasic systemic arterial pressure (\( SAP \)) were continuously monitored in the carotid artery by a liquid-filled pressure transducer system (P23D6, Statham, Hato Rey, PR).

After instrumentation and institution of mechanical venti-
lation, baseline measurements of \( P_{ao} \) SAP, and \( E_{NO} \) were made in triplicate over at least 15 min; the coefficients of variation were ≤15, ≤10, and ≤15% for each of these parameters, respectively. A large-volume breath (approximately three times tidal volume) was given 30 s before each baseline measurement to standardize volume history. Antigen-
sensitized animals were treated with 10 mg·kg\(^{-1}\) of pyrilamine maleate intraperitoneally 30 min before intratracheal challenge with either antigen or saline. Control unsensitized animals received either a sham or saline intratracheal instillation.

Assessment of AMVL

AMVL was assessed by the Evans blue (EB) dye (Sigma) technique (32). After instrumentation and establishment of baseline conditions, \( P_{ao} \) and SAP were continuously moni-
tored while the animals received an intravenous bolus of 30 mg·kg\(^{-1}\) of EB dye (20 mg/ml in PBS). At time = 1 min, the animals received an intratracheal instillation (sham, saline, or antigen) in a final volume of 200 µl·kg\(^{-1}\), followed by a large-volume breath to maintain lung volume. At time = 5 min, heparin (1,000 U·kg\(^{-1}\)) and pentobarbital sodium (50 mg·kg\(^{-1}\)) were administered intravenously, an anterior thora-
cotom y was performed, and the right atrium was incised. At time = 6 min, the systemic circulation was perfused through the carotid arterial line at a perfusion pressure of =50 cmH2O with 200 ml of PBS that were then expelled through the right atrioventricular tricuspid valve. The lungs and trachea were removed and blotted dry on filter paper, and the airways were dissected free of connective tissue. The tracheobronchial tree was divided into two samples: the extrapulmonary (EP), comprising the distal 1 cm of trachea and proximal 0.5 cm of both main stem bronchi, and intrapulmonary (IP) airways. Airway tissue samples were placed in tared, stopped tubes, and the tissue EB dye was extracted by incubation for 16 h at 37°C in 2 ml of formamide (Sigma). Tissue content of the EB dye was deter-
mined from the absorbance at 630 nm of the tissue sample extracts and from a standard curve of the absorbance at 630 nm vs. the EB dye in formamide (0.5–10 µg/ml). Tissue content of the EB dye is expressed as nanograms of EB per milligram of tissue wet weight.

The airway contractile response to the intratracheal chal-
lenge was assessed by continuous monitoring of \( P_{ao} \) before and after the intratracheal instillation. From continuous tracings, baseline and peak postantigen \( P_{ao} \) values were identified. After collection of baseline mixed expired gas samples in triplicate, a single sample of mixed expired gas was collected immediately after intratracheal challenge and refer-
cenced to the mean \( E_{NO} \) level of the three baseline samples.

Measurement of \( E_{NO} \)

Mixed expired gas samples were collected from the expira-

tory port of the ventilator over 3 min and were analyzed within 30 min of collection as previously described (21, 22). The expired gas sample was vigorously mixed manually for 5–10 s before determination of the NO concentration in parts per billion by chemiluminescence (425 NO analyzer, Thermo Environmental Instruments, Franklin, MA). The NO ana-
lyzer was calibrated daily against a reference gas of known NO concentration (Matheson Scientific Gas, Houston, TX).

In Vitro NOS Activity Assay

In vitro lung homogenate NOS activity was measured by the conversion of \( L-[^{3}H] \)arginine to \( L-[^{3}H] \)citrulline as previously described (22). In brief, 1 M KCl-extracted, homog-

enized lung tissue samples were incubated with 1.25 mM CaCl\(_2\), 1 µM flavin mononucleotide, 1 µM FAD, 1 µM tetrahy-
drobiopterin, 1 mM NADP, and 100 µM \( L-[2,3,4,5-{^{3}H}] \)arginine (0.3 Ci/mmol) at 37°C for 45 min. \( L-[^{3}H] \)citrulline was eluted from a cation-exchange resin (AG 50W-X8, Na\(^{+}\) form; Bio-

ey, Hercules, CA) and quantified by liquid scintillation spectrophotometry.

Lung homogeneate samples were incubated 1) as above, 2) in the presence of 100 µM N\(^{6}\)-monomethyl- \( L- \)arginine ( \( L-NMMA) \) to assess background non-NOS-dependent conversion of \( L-[^{3}H] \)arginine to \( L-[^{3}H] \)citrulline, and 3) in the pres-


eence of excess EGTA (3 mM) to assess the calcium-indepen-
dent fraction of total NOS activity (type II NOS). The sample protein concentration was determined by the biuret reaction (Micro BCA, Pierce, Rockford, IL) with bovine serum albumin as the standard. Total and calcium-independent non- \( \text{L-NMMA}) \)-inhibitable in vitro maximal pulmonary NOS activities are expressed as picomoles of \( L- \)citrulline produced per milligram of protein per minute.

Pilot Dose-Response Studies of NOS Inhibition

The effect of inhibition of endogenous pulmonary NO production on AMVL was investigated with two NOS inhibi-
tors: \( N^{6}\)-nitro-L-arginine methyl ester ( \( L-NNAME) \); Sigma), a competitive inhibitor that is not selective for any NOS isoform, and aminoquinidine (AG; Sigma), which is relatively selective for type II NOS (7, 23). In a pilot dose-response study, the acute effects of intravenous \( \text{L-NAM} \) \( (1, 3, 10, \text{ and } 30 \text{ mg/kg}; n = 4–7 \text{ animals/dose}, \text{N}^{6}\)-nitro- \( \text{L} \)-arginine methyl ester ( \( \text{L-NAM}) \); Sigma), the biologically inactive enantiomer, and AG (\( 100 \text{ mg/kg}; n = 3–5 \text{ animals/dose}; \text{Sigma}) were assessed. Two of four animals receiving 100 mg·kg\(^{-1}\) of \( L- \)NAME died during the 48-h continuous infusion. Thus an effective, maximally tolerated dose of \( L-NAME \) (30 mg·kg\(^{-1}\)·day\(^{-1}\); \( n = 4 \text{ animals/dose}) \), \( L-NAME \) (30 mg·kg\(^{-1}\)·day\(^{-1}\); \( n = 4 \text{ animals/dose}) \), or AG (3, 10, 30, \text{ and } 100 \text{ mg/kg}; \text{AG} = 6–8 \text{ animals/dose}\), or AG (3, 10, 30, \text{ and } 100 \text{ mg/kg}; \text{AG} = 6–8 \text{ animals/dose}) \) were administered as a continuous subcuta-

neous infusion via an osmotic pump (Alza, Palo Alto, CA). The chronic effects of these agents on \( E_{NO} \), \( SAP \), and in vitro NOS activity were assessed. Two of four animals receiving 100 mg·kg\(^{-1}\)·day\(^{-1}\) of \( L-NAME \) died during the 48-h continuous infusion. Thus an effective, maximally tolerated dose of \( L-NAME \) (30 mg·kg\(^{-1}\)·day\(^{-1}\)) and an effective, type II NOS-selective dose of AG (100 mg·kg\(^{-1}\)·day\(^{-1}\)) identified in the pilot trials were chosen for subsequent experiments on the effects of NOS inhibition on AMVL.

Effect of NOS Inhibition on AMVL

Animals were treated with either \( \text{L-NAM} \) (30 mg·kg\(^{-1}\)·day\(^{-1}\)), \( \text{d-NAM} \) (30 mg·kg\(^{-1}\)·day\(^{-1}\)), or AG (100 mg·kg\(^{-1}\)·day\(^{-1}\)).
day−1) administered as a continuous subcutaneous infusion over 48 h (days 12–14) preceding assessment of AMVL. The effects of L-NAME and AG were assessed in both unsensitized and antigen-sensitized animals, whereas D-NAME was only administered to unsensitized animals. To control for the potential airway effects of changes in systemic vascular hemodynamics induced by systemically administered L-NAME, the effects of local airway NOS inhibition via intratracheal instillation of L-NAME on AMVL, ENO, and SAP were determined in both unsensitized and antigen-sensitized animals. In pilot dose-response studies, a dose of 2 mg/kg of L-NAME (intratracheal instillation of 200 µl/kg) was shown to reduce ENO in the absence of systemic vascular effects as reflected by a lack of change in SAP.

Statistical Analysis

Descriptive group data are expressed as means ± SE. Changes in Pao, SAP, and ENO after intratracheal antigen challenge were assessed with a Student-Newman-Keuls t-test for paired variates. Between-group differences were assessed with an analysis of variance (ANOVA), and multiple comparisons were performed with a Student-Newman-Keuls t-test where appropriate (SigmaStat, Jandel Scientific, San Rafael, CA). The relationships among Pao, ENO levels, and AMVL after antigen exposure were determined by the Pearson product correlation method. Significance was inferred at a two-tailed P < 0.05.

RESULTS

Pilot Dose-Response Studies of NOS Inhibition

The acute intravenous infusion of 1, 3, 10, and 30 mg/kg of L-NAME but not of D-NAME significantly and dose dependently reduced ENO by a maximum of 85 ± 0% (P < 0.01) and markedly increased SAP (Fig. 1) but had no effect on Pao (data not shown). In contrast, acute intravenous infusion of 1, 3, 10, 30, and 100 mg/kg of AG had a lesser maximal effect on ENO than L-NAME, reducing ENO by a maximum of 62 ± 2% (P < 0.01), and had no effect on SAP or on Pao.

The chronic infusion of 3, 10, 30, and 100 mg·kg−1·day−1 of L-NAME dose dependently inhibited both calcium-dependent (types I and III) and calcium-independent (type II) NOS isoforms as assessed by the in vitro conversion of L-arginine methyl ester (L-NAME; n = 4–7 animals/dose) but not N0-nitro-D-arginine methyl ester (D-NAME; n = 3–5 animals/dose) dose dependently reduced ENO and increased SAP. In contrast, aminoguanidine (n = 4–6 animals/dose) had a lesser effect on ENO and did not affect SAP. Significantly different from saline and D-NAME: *P < 0.05; **P < 0.01 (by 2-way repeated-measures ANOVA and post hoc Student-Newman-Keuls t-test).

Effect of L-NAME on Basal AMVL in Unsensitized Animals From Cohort 1

The baseline AMVL in control, unsensitized guinea pigs receiving a sham intratracheal instillation was 5.5 ± 1.0 and 6.3 ± 0.1 ng EB dye/mg tissue in the EP and IP airways, respectively (Table 2). In these unsensitized animals, intratracheal instillation of saline had no effect on the basal level of AMVL. Continuous systemic administration of 30 mg/kg of L-NAME over 48 h before the assessment of AMVL in unsensitized pigs receiving a sham intratracheal instillation on day 14 of L-NAME dose dependently inhibited NOS isoforms as assessed by the in vitro conversion of L-arginine methyl ester (L-NAME; n = 4–7 animals/dose) but not N0-nitro-D-arginine methyl ester (D-NAME; n = 3–5 animals/dose) dose dependently reduced ENO and increased SAP.

Table 1. Effects of chronic in vivo administration of NOS inhibitors on in vitro NOS activity in guinea pig lung homogenate

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>n</th>
<th>Total Cit·protein−1·min−1</th>
<th>Type II Cit·protein−1·min−1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>8</td>
<td>77 ± 8</td>
<td>38 ± 5</td>
</tr>
<tr>
<td>D-NAME (mg·kg−1·day−1)</td>
<td>3</td>
<td>70 ± 10</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>L-NAME (mg·kg−1·day−1)</td>
<td>3</td>
<td>38 ± 4*</td>
<td>17 ± 2†</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>18 ± 4†</td>
<td>7 ± 5†</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>2 ± 1†</td>
<td>1 ± 1†</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>4 ± 1†</td>
<td>2 ± 1†</td>
</tr>
<tr>
<td>Aminoguanidine (mg·kg−1·day−1)</td>
<td>3</td>
<td>70 ± 6</td>
<td>35 ± 4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>67 ± 3</td>
<td>32 ± 7</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>61 ± 7</td>
<td>25 ± 5*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>48 ± 7*</td>
<td>9 ± 3†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals/dose. NOS, nitric oxide (NO) synthase; D-NAME, N0-nitro-D-arginine methyl ester; L-NAME, N0-nitro-L-arginine methyl ester; Cit, citrulline. Significantly different from untreated control groups: *P < 0.05; †P < 0.01.
animals reduced $E_{NO}$ by 70 ± 8% ($P < 0.01$) and reduced basal levels of AMVL by 92 ± 1 and 44 ± 17% in the EP and IP airways, respectively ($P < 0.05$ for both; Fig. 3). In contrast, the administration of d-NAME had no effect. The acute intratracheal administration of 2 mg/kg of L-NAME reduced $E_{NO}$ by 46 ± 4% from baseline ($P < 0.01$) but reduced basal AMVL by 91 ± 3 and 32 ± 9% in the EP and IP airways, respectively ($P < 0.05$ for both), a comparable effect to systemically administered L-NAME (Table 2).

**Effect of Intratracheal Antigen Challenge in Antigen-Sensitized Animals From Cohort 1**

The intratracheal instillation of antigen in antigen-sensitized animals was associated with marked increases in $P_{ao}$ (7.9 ± 0.2 to 46.9 ± 4.3 cmH2O; $n = 12$ animals; $P < 0.01$) and $E_{NO}$ levels (15.5 ± 0.8 to 37.5 ± 3.8 ppb; $P < 0.01$). There was a significant correlation between peak $P_{ao}$ and the increase in $E_{NO}$ levels after antigen challenge ($r = 0.88; P < 0.001$). In contrast, intratracheal saline instillation had a minimal effect on

**Table 2. Effects of NOS inhibition on expired NO levels and airway microvascular leak in unsensitized and antigen-sensitized guinea pigs**

<table>
<thead>
<tr>
<th>Group</th>
<th>Intratracheal Challenge</th>
<th>n</th>
<th>Expired NO, parts/billion</th>
<th>Airway Microvascular Leak, mg Evans blue dye/mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Baseline</td>
<td>Postchallenge</td>
</tr>
<tr>
<td>Unsensitized</td>
<td>Sham</td>
<td>5</td>
<td>18.1 ± 2.6</td>
<td>19.7 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>6</td>
<td>16.0 ± 1.9</td>
<td>15.0 ± 1.9</td>
</tr>
<tr>
<td>Unsensitized + sc L-NAME</td>
<td>Sham</td>
<td>6</td>
<td>5.5 ± 0.6</td>
<td>6.0 ± 0.7</td>
</tr>
<tr>
<td>Unsensitized + it L-NAME</td>
<td>Sham</td>
<td>6</td>
<td>11.2 ± 0.9</td>
<td>10.4 ± 0.9</td>
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<tr>
<td>Unsensitized + sc d-NAME</td>
<td>Sham</td>
<td>6</td>
<td>17.5 ± 1.5</td>
<td>17.9 ± 1.8</td>
</tr>
<tr>
<td>Antigen sensitized</td>
<td>Saline</td>
<td>4</td>
<td>17.7 ± 1.0</td>
<td>16.9 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Antigen</td>
<td>12</td>
<td>15.5 ± 0.8</td>
<td>37.5 ± 3.8a</td>
</tr>
<tr>
<td>Antigen sensitized + sc L-NAME</td>
<td>Antigen</td>
<td>12</td>
<td>6.3 ± 0.7b</td>
<td>6.1 ± 0.7</td>
</tr>
<tr>
<td>Antigen sensitized + it L-NAME</td>
<td>Antigen</td>
<td>5</td>
<td>9.7 ± 1.3b</td>
<td>16.5 ± 3.7a</td>
</tr>
<tr>
<td>Unsensitized</td>
<td>Sham</td>
<td>6</td>
<td>25.8 ± 2.2</td>
<td>26.9 ± 1.7</td>
</tr>
<tr>
<td>Unsensitized + it L-NAME</td>
<td>Sham</td>
<td>3</td>
<td>17.3 ± 1.4b</td>
<td>19.0 ± 3.5</td>
</tr>
<tr>
<td>Unsensitized + sc aminoguanidine</td>
<td>Sham</td>
<td>6</td>
<td>15.5 ± 1.5b</td>
<td>14.3 ± 1.5</td>
</tr>
<tr>
<td>Antigen sensitized</td>
<td>Antigen</td>
<td>6</td>
<td>28.1 ± 1.8</td>
<td>45.1 ± 6.2b</td>
</tr>
<tr>
<td>Antigen sensitized + sc aminoguanidine</td>
<td>Antigen</td>
<td>9</td>
<td>16.0 ± 0.9b</td>
<td>45.1 ± 6.6b</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals/group. sc, Subcutaneous; it, intratracheal. Significantly different from respective within-cohort unsensitized sham-treated group: *P < 0.05; **P < 0.01. Significantly different from respective within-cohort antigen-sensitized group: †P < 0.05. Significantly different from respective baseline value: ‡P < 0.05; ‡‡P < 0.01.
P\(_\infty\) (7.9 ± 0.2 to 13.0 ± 0.6 cmH\(_2\)O; n = 4 animals; P < 0.05) and no effect on E\(_N\)\(_O\) (17.7 ± 1.0 to 16.9 ± 0.8 ppb; P = not significant (NS)) in antigen-sensitized animals. The 7-day antigen sensitization protocol had no effect on AMVL because antigen-sensitized animals challenged with intratracheal saline had levels of AMVL similar to those of unsensitized control animals challenged with either intratracheal saline or sham instillation (Table 2). However, intratracheal antigen instillation in antigen-sensitized animals markedly increased AMVL in both EP and IP airways (Fig. 4). In antigen-sensitized animals, the antigen-stimulated increase in AMVL was significantly related to peak P\(_\infty\) (sensitized animals, the antigen-stimulated increase in ENO over antigen challenge. *Significantly different from antigen-challenged animals; r = 0.96, P < 0.0001 and r = 0.75, P < 0.01 for EP and IP airways, respectively) after intratracheal antigen instillation.

Effect of L-NAME on AMVL in Antigen-Sensitized Animals From Cohort 1

The chronic subcutaneous administration of 30 mg·kg\(^{-1}\)·day\(^{-1}\) of L-NAME to antigen-sensitized guinea pigs reduced basal E\(_N\)\(_O\) levels before antigen challenge by 64 ± 5% (P < 0.01) and eliminated the increase in E\(_N\)\(_O\) that followed intratracheal antigen instillation in non-L-NAME-treated, antigen-sensitized animals (Table 2). Although the peak P\(_\infty\) response to antigen challenge was not significantly different in animals receiving continuous systemic L-NAME (531 ± 84 vs. 590 ± 51% of baseline in non-L-NAME-treated animals; P = NS), the antigen-stimulated increase in AMVL was significantly attenuated by 67 ± 9 and 51 ± 12% in the EP and IP airways, respectively (P < 0.05 for both).

In contrast to systemic L-NAME, the intratracheal administration of 2 mg/kg of L-NAME in antigen-sensitized animals had a smaller effect on E\(_N\)\(_O\), reducing basal E\(_N\)\(_O\) by 39 ± 5% (P < 0.01) compared with non-L-NAME-treated antigen-sensitized animals. Moreover, intratracheal L-NAME did not eliminate the antigen-induced increase in E\(_N\)\(_O\), although it was significantly attenuated in comparison with non-L-NAME-treated antigen-sensitized animals (Table 2). However, the antigen-stimulated increase in AMVL was blunted by the intratracheal administration of L-NAME to a comparable extent (by 75 ± 7 and 43 ± 10% in EP and IP airways, respectively; P < 0.05 for both) as that by systemically administered L-NAME.

Effect of AG on AMVL in Unsensitized and Antigen-Sensitized Animals From Cohort 2

Although the basal levels of AMVL and E\(_N\)\(_O\) were quite consistent between animals within a given cohort, the basal levels of both AMVL and E\(_N\)\(_O\) were significantly greater in animals from cohort 2 obtained from a different source and studied at a different time than animals from cohort 1 (Table 2). There was a significant correlation between basal levels of AMVL and E\(_N\)\(_O\) in control, unsensitized animals from each of the cohorts, as well as when animals from the two cohorts were combined (r = 0.76, P < 0.0001 and r = 0.52, P < 0.05 for EP and IP airways, respectively). Yet, antigen-sensitized animals from both cohorts responded similarly with regard to changes in P\(_\infty\), E\(_N\)\(_O\), and AMVL after antigen challenge. To facilitate comparison of the effects of NOS inhibition in cohorts 1 and 2, the effects of intratracheal L-NAME on AMVL and E\(_N\)\(_O\) in animals from cohort 1 (see Effect of L-NAME on Basal AMVL in Unsensitized Animals From Cohort 1) were successfully reproduced in animals from cohort 2 (Table 2).

The chronic systemic administration of 100 mg·kg\(^{-1}\)·day\(^{-1}\) of AG significantly reduced basal E\(_N\)\(_O\) levels in both unsensitized and antigen-sensitized guinea pigs by 40 ± 4 and 38 ± 2%, respectively (P < 0.01 for both). In antigen-sensitized animals, the acute increase in E\(_N\)\(_O\) induced by antigen challenge was unaffected by administration of AG (peak E\(_N\)\(_O\) 45.1 ± 6.6 vs. 45.1 ± 6.2 in non-AG-treated animals, P = NS). Despite the reduction in basal E\(_N\)\(_O\), systemic AG had no effect on AMVL in both unsensitized and antigen-sensitized animals in comparison with non-AG-treated animals (Table 2, Figs. 3 and 4).

**DISCUSSION**

In the guinea pig, endogenously produced pulmonary NO is an important modulator of AMVL of plasma protein as assessed by the EB dye technique. The low basal level of AMVL in control animals was significantly reduced by both the local intratracheal and systemic administration of L-NAME, an L-arginine competitive NOS inhibitor that does not show any NOS isoform selectivity. Pulmonary antigen challenge in sensitized animals resulted in an acute marked increase in AMVL and a concurrent elevation in mixed E\(_N\)\(_O\) levels. Inhibition of NOS with L-NAME both dramatically decreased E\(_N\)\(_O\) levels and attenuated the increase in AMVL after antigen exposure. In contrast to the effect of L-NAME, AG, an NOS inhibitor that is relatively selective for the type II, cytokine-inducible

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**Fig. 4. Effects of chronic administration of inhibitors of NOS on airway microvascular leak assessed by Evans blue dye technique in antigen-sensitized guinea pigs intratracheally challenged with antigen. L-NAME (n = 12 animals/dose) attenuated increase in microvascular leak in both extrapulmonary and intrapulmonary airways after antigen challenge. *Significantly different from antigen-challenged animals not treated with NOS inhibitors (P < 0.05), whereas aminoguanidine (n = 9 animals/dose) had no effect.**
NOS isoform, had no effect on either basal or antigen-stimulated AMVL despite significantly reducing $E_{\text{NO}}$ levels. These data indicate that endogenously produced NO, derived primarily from the calcium-dependent (types I and III) NOS isoforms, contributes significantly to both the basal level of AMVL and stimulated AMVL in the setting of allergic airway inflammation in the guinea pig.

Among its many roles, endogenously produced NO is thought to be an important homeostatic mediator in the airways of both humans and animals (3, 4, 13, 21, 22, 26). For example, NO mediates neural bronchodiatory responses (5, 34) and modulates bronchoconstrictor responses to both endogenous and exogenous contractile agonists (4, 21, 22, 26). As Mehta and colleagues (21, 22) previously showed, the measurement of $E_{\text{NO}}$ levels in the guinea pig serves as an index of the endogenous pulmonary production of NO. Furthermore, the antigen-induced increase in $E_{\text{NO}}$ levels and the significant relationship between the increased $E_{\text{NO}}$ level and the magnitude of the antigen-induced pulmonary constrictor response observed in the present study confirm our previous finding that antigen-induced increases in $E_{\text{NO}}$ reflect an endogenous pulmonary NO-dependent modulatory mechanism in the setting of acute allergic bronchoconstriction in the guinea pig (22).

It has been suggested that endogenously produced pulmonary NO may also have a role in the regulation of AMVL, although there are conflicting data over the exact nature of this role (6, 9, 18). In the present study, the EB dye technique was used to assess AMVL because it has been extensively used for the measurement of microvascular leak into airways, skin, and other tissues (10, 32, 33). The assessment of AMVL in the present study was unaffected by the actual instillation procedure because saline instillation, in comparison with sham instillation, had no effect on the measured AMVL in control, unsensitized animals. Furthermore, although acute intratracheal antigen instillation markedly increased AMVL in antigen-sensitized animals, the 7-day antigen sensitization protocol itself, in the absence of intratracheal antigen challenge, had no significant effect on AMVL; saline instillation in antigen-sensitized animals yielded values for measured AMVL similar to those in control animals not sensitized to antigen.

The attenuation in antigen-induced AMVL with NOS inhibition observed in the present study supports the proposed role for NO, i.e., as a mediator of AMVL, and is consistent with the findings of others (6, 18). Kuo et al. (18) reported that L-NAME blunted the increased leak of intravascularly administered $^{125}$I-labeled albumin into tracheal tissue after vaginal stimulation in guinea pig airways but that L-NAME had no effect on the basal level of leak in the absence of neurogenic stimulation. In contrast, using the EB dye technique, we found that NOS inhibition with L-NAME reduced AMVL both in the basal state and in the setting of increased leak after antigen challenge in antigen-sensitized animals. Given that the assessment of microvascular leak by these two methods, the EB dye technique and the radioiodinated albumin method, have been shown to correlate strongly (33, 35), the conflicting nature of these observations is most likely due to the different methods used to stimulate increased leak, the different routes of NOS inhibition (acute intravenous vs. chronic subcutaneous infusions or intratracheal instillation), or animal strain differences. Recently, this role for endogenous NO in mediating AMVL has been confirmed in another species: NOS inhibition attenuated endotoxin-stimulated, type II NOS-dependent AMVL in rat airways (6).

The findings described above by us and by Kuo et al. (18) are also inconsistent with those of Erjefalt et al. (9), who assessed airway plasma protein leak in guinea pig airways by two techniques, tissue and luminal recovery of $^{125}$I-labeled albumin and tissue colloidal gold deposition. Tracheal mucosal application of L-NAME increased $^{125}$I recovery in tracheal washes and increased gold deposition, although there was no change in tracheal tissue $^{125}$I counts (9). The described discrepancy between tissue $^{125}$I and gold deposition highlights the difficulty in comparing different techniques for assessment of microvascular leak. For example, although it has previously been shown that there is a strong linear correlation between measurements of plasma exudation by the EB dye technique and by the radioiodinated albumin method, this is not so for luminal transudation (33).

Although the precise cellular sources of both $E_{\text{NO}}$ and the endogenous pulmonary NO-related activity modulating AMVL remain undetermined, the use of NOS inhibitors with different potencies against the different NOS isoforms permits some insight into this question. In the present report, pilot studies demonstrated that L-NAME, an NOS inhibitor without isoform selectivity, significantly reduced $E_{\text{NO}}$ levels. These changes in $E_{\text{NO}}$ were closely associated with dose-dependent increases in SAP, a well-recognized manifestation of inhibition of type III endothelial NOS (1, 14, 24). AG has been shown to be relatively selective for the type II isoform of NOS, with an inhibitory potency ~100-fold greater than that for the calcium-dependent NOS isoforms such as the endothelial type III NOS (7, 23). In both the acute and chronic pilot dose-response studies, AG dose dependently reduced basal $E_{\text{NO}}$ levels, with a maximum inhibitory effect of 40–45%, without any observable effect on SAP, in marked contrast to the hypertensive effect of the systemic administration of L-NAME. These studies are consistent with a difference in potency of at least 100-fold for the inhibition of type III NOS by L-NAME and AG as reflected by their respective effects on in vitro NO activity and on SAP measured in vivo. Moreover, the observed reduction in basal $E_{\text{NO}}$ levels by AG, presumably due to the inhibition of type II NOS, is consistent with the previous findings by Mehta et al. (22) of basal or "constitutive" expression of both calcium-dependent (comprising type I, neuronal NOS, and type III, endothelial NOS) and calcium-independent (type II) NOS isoforms in the guinea pig lung and with similar findings in human airway epithelial cells (15).
Despite the significant inhibition of type II NOS and the associated reduction of  \( E_{NO} \) levels after AG administration, the lack of effect on AMVL strongly suggests that calcium-dependent NOS isoforms (types I and III; i.e., those not inhibited by the calcium-independent NOS-selective doses of AG employed in this study) are the likely sources of the endogenous pulmonary NO-related activity that supports AMVL in both the basal state and the setting of antigen-induced increases in AMVL. The findings of the present study are consistent with the previous demonstration by Mehta et al. (22) of a lack of induction of type II NOS expression after repeated pulmonary antigen challenge in the guinea pig. In contrast, systemic endotoxin challenge is associated with enhanced microvascular leak in rat airways, as well as in other tissues, which is mediated by enhanced expression of type II NOS (6, 20).

The mechanism through which endogenously produced pulmonary NO contributes to AMVL remains unclear. It is recognized that systemic nonselective inhibition of type III NOS with L-arginine analogs such as L-NAME, as in our model, has a number of physiological effects, including changes in systemic vascular tone and blood pressure (1, 14, 29). Guinea pigs that received a continuous systemic L-NAME infusion at 30 \( \text{mg}\cdot\text{kg}^{-1}\cdot\text{day}^{-1} \), which was associated with a reduction in \( E_{NO} \) by 70%, had a significant elevation in SAP. The latter observation indicates a general increase in systemic arterial tone due to endothelial type III NOS inhibition. Thus it is possible that the disturbed regulation of systemic vascular tone due to inhibition of endothelial type III NOS altered hemodynamics in the bronchial circulation and resulted in a reduction in airway mucosal blood flow and the observed reduction in AMVL. However, the local intratracheal administration of a much smaller dose of L-NAME reduced AMVL to a similar degree as systemic administration, without any effect on SAP; thus the attenuation of AMVL after NOS inhibition cannot be attributed simply to an alteration of systemic hemodynamics. However, our experiments cannot exclude the possibility that the reduction in AMVL may be due to an alteration of local bronchial microcirculatory hemodynamics resulting from administration of intratracheal L-NAME. Alternatively, NO is known to preserve vascular integrity and downregulate microvascular leak in the systemic circulation via inhibition of endothelial cell-neutrophil adherence in postcapillary venules, possibly via scavenging of oxygen free radicals (12, 19, 27). The exact mechanism of the effect of NO on microvascular leak at the level of the airway microcirculation remains to be elucidated.

In summary, on the basis of the differential inhibition of \( E_{NO} \) by L-NAME and AG, 40% of the NO present in the mixed expired gas of guinea pigs is derived from type II NOS isoforms. Endogenously produced pulmonary NO, predominantly from calcium-dependent NOS isoforms, contributes significantly to the low basal level of AMVL in normal airways and to the markedly increased AMVL in the setting of airway inflammation induced by repeated pulmonary antigen exposure in guinea pigs. It remains to be determined whether the role of NO in promoting AMVL, which is apparently different from its modulatory role in the systemic circulation, is mediated through an alteration of bronchial microcirculatory hemodynamics, cellular interactions, or some other mechanism.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-19170 and HL-03293.

S. Mehta is the recipient of a fellowship from the Medical Research Council of Canada.

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Received 2 March 1998; accepted in final form 3 August 1998.

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