Enhanced expression of inducible nitric oxide synthase without vasodilator effect in chronically infected lungs

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Cadogan, Elaine, Natalie Hopkins, Shay Giles, John G. Bannigan, John Moynihan, and Paul McLoughlin. Enhanced expression of inducible nitric oxide synthase without vasodilator effect in chronically infected lungs. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L616–L627, 1999.—We hypothesized that abnormal ventilation-perfusion matching in chronically infected lungs was in part due to excess nitric oxide (NO) production after upregulation of inducible NO synthase (iNOS) expression. Rats were anesthetized and inoculated intratracheally with Pseudomonas aeruginosa incorporated into agar beads (chronically infected) or with sterile agar beads (placebo inoculated) and killed 10–15 days later. Immunohistochemistry demonstrated increased expression of iNOS and reduced expression of endothelial NO synthase (eNOS) in chronically infected compared with placebo-inoculated or noninoculated lungs. In isolated lungs from chronically infected rats, NOS inhibition with Nω-nitro-L-arginine methyl ester increased the mean perfusion pressure (14.4 ± 2.7 mmHg) significantly more than in the placebo-inoculated (4.8 ± 1.0 mmHg) or noninoculated (5.3 ± 0.8 mmHg) lungs (P < 0.01). Although the chronically infected lungs were more sensitive to NOS inhibition, further evidence suggested that the increased iNOS expression was not associated with enhanced iNOS activity. Selective inhibitors of iNOS did not produce an increase in vascular resistance similar to that produced by nonselective inhibitors. Accumulation of nitrate/nitrite in the perfusate of isolated lungs was unchanged by chronic infection. Thus although iNOS expression was increased in chronic pulmonary infection, iNOS activity in the intact lung was not. Nonetheless, endogenous NO production was essential to maintain normal vascular resistance in these lungs.

pulmonary vascular resistance; hypoxia; Pseudomonas aeruginosa; aminoguanidine; nitrate; nitrite

CHRONIC BACTERIAL INFECTION of the airways with Pseudomonas aeruginosa leads to progressive pulmonary damage and impairment of gas exchange. A further consequence of chronic infection is abnormal pulmonary vascular control leading to ventilation-perfusion mismatching, which contributes substantially to the impaired gas exchange observed in such conditions (11). Ultimately, these vascular abnormalities lead to persistent pulmonary hypertension and right ventricular (RV) overload. The exact pathogenic mechanisms underlying these disturbances of pulmonary vascular control are unknown.

Endothelial nitric oxide (NO) synthase (eNOS) is constitutively expressed in the pulmonary vascular endothelium, the airway epithelium (18), alveolar macrophages, and type II pneumocytes (24, 26). Inhibitors of NOS are reported to cause an elevation in the baseline pulmonary vascular resistance in normal lungs (6, 8, 17, 20, 32) and to enhance hypoxic vascular constriction (2, 4, 20), indicating that NO plays an important role in modulating the normal control of pulmonary vascular resistance. Acute sepsis, endotoxin-derived lipopolysaccharide (LPS), and certain cytokines lead to the expression of the high-output, calcium-independent inducible isoform of NOS (iNOS) in a variety of cells. These include vascular smooth muscle cells, endothelial cells, alveolar macrophages, and type II alveolar epithelial cells (1, 30). Isolated pulmonary and systemic arteries from rats treated with LPS demonstrate hyporesponsiveness to vasoconstrictors, and NOS inhibitors restore responses toward normal levels (13, 37). NOS inhibitors also restore vascular responsiveness and increase blood pressure in animal models of sepsis and in humans with septic shock (1, 35). This evidence indicates that, in acute inflammatory conditions, iNOS expression causes important abnormalities in the control of both systemic and pulmonary vascular resistance.

We hypothesized that chronic bacterial infection of the airways leads to persistent increased expression of iNOS in the lungs with a markedly enhanced production of NO, resulting in abnormalities of pulmonary vascular control. We used a previously described model (12) to establish chronic pulmonary P. aeruginosa infection in rats and compared the expression of iNOS and eNOS in the lungs of chronically infected and control animals by immunohistochemical techniques. In isolated, ventilated, blood-perfused lungs, we examined the changes in pulmonary vascular resistance produced by both nonselective inhibitors of NOS and selective inhibitors of iNOS. In addition, the rate of increase in the concentrations of the stable end products of NO metabolism, nitrate and nitrite, in the perfusate of the isolated lungs was determined as an index of NO production by the high-output iNOS.

METHODS

Infection of animals. A mucoid P. aeruginosa strain isolated from a patient with cystic fibrosis was used to prepare the inoculum for all experiments. Chronic infection was produced by incorporating the organism into agarose beads as previously described (12). In brief, a suspension of P. aeruginosa grown overnight in peptone water resulted in a concentration of ~3 × 10⁸ colony-forming units/ml. Agarose was dissolved in water (2.1% wt/vol) and maintained at 45°C. To 19 ml of this agarose solution, 1 ml of the peptone broth was added, and the resultant agarose solution was injected into 20 ml of heated mineral oil (50°C) and mixed vigorously for a further
in air at 37°C. Airway pressure was continuously monitored, and a modified pediatric laryngoscope was used to introduce a polyethylene cannula (1.2 mm outside diameter) into the trachea via the larynx. In the group to be chronically infected, 104 colony-forming units of P. aeruginosa in agar beads suspended in PBS (total volume 200 µl) were inoculated intratracheally through this cannula in each rat, and the animals were then allowed to recover from the anesthesia. A second group of animals (placebo-inoculated group) was anesthetized and inoculated with sterile agarose beads, that is, agar beads prepared in a similar manner except that Pseudomonas organisms were omitted. A third group consisted of animals that were not inoculated (noninoculated group). Isolation of the lungs for hemodynamic, histological, and immunohistochemical analyses was carried out 10–15 days postinoculation.

Lung isolation for hemodynamic studies. The rats were anesthetized (60 mg/kg of pentobarbital sodium) and mechanically ventilated (SAR-830P small-animal ventilator, CWE, Ardmore, PA) at a tidal volume of 1.8 ml and a frequency of 80 breaths/min. The animals were then anticoagulated (300 IU of heparin intravenously) and killed by exsanguination. Plasma was reserved for determination of total nitrate and nitrite concentration. The thoracic contents were exposed through a midline sternotomy, and canulas were inserted into the main pulmonary artery and left atrium and tied in place. The thoracic contents were then removed en bloc and suspended in a chamber maintained at 37°C while ventilation continued with a warmed and humidified mixture of 5% CO2 in air at 37°C. Airway pressure was continuously monitored, and a positive end-expiratory pressure of 2.0 cmH2O was maintained. The lungs were briefly hyperinflated to an airway pressure of 16 cmH2O every 5 min to prevent development of progressive atelectasis.

The vascular perfusion circuit consisted of, in order, the left atrial cannula, a venous outflow pressure transducer (Sensor Nor 840, Horten, Norway), a venous-occlusion pinch valve (PO-NE-MAH, Sibsmty, CT), a warmed, thermostatically controlled perfusate reservoir, a roller pump (Stockert Instruments, Munich, Germany), connecting tubing, bubble trap, arterial occlusion pinch valve (PO-NE-MAH), an arterial pressure transducer (Sensor Nor 840), and the pulmonary artery cannula. The perfusion circuit was primed with a mixture of 20 ml of rat blood obtained by exsanguination under general anesthesia (60 mg/kg of pentobarbital sodium) of two anticoagulated (300 IU of heparin intravenously) donor animals and 10 ml of physiological saline solution (in mM: 119 NaCl, 24 NaHCO3, 4.7 KCl, 0.9 MgSO4, 1.2 KH2PO4, 2.5 CaCl2, and 5.5 glucose) with 4% (wt/vol) bovine serum albumin. Perfusion was maintained at a constant flow (0.04 ml·min⁻¹·g⁻¹) so that changes in arterial perfusion pressure reflected changes in total pulmonary vascular resistance. Venous outflow pressure was maintained at 2.0 mmHg to ensure zone 3 conditions at end expiration. All measurements of arterial perfusion pressure were made at end expiration. Capillary pressure was determined with the double-occlusion technique, i.e., by simultaneous occlusion of arterial and venous cannulas at end expiration (34). Arterial, venous, and airway pressures were continuously recorded with an analog-to-digital system (Biopac MP100 WS, Linton Instrumentation, Norfolk, UK) connected to a desktop computer (Power Macintosh 7100/80), and the data were stored on a hard disk for later analysis.

Hemodynamic studies. After isolation, a period of equilibration was allowed until airway and vascular pressures were stable while the lungs were ventilated with normoxic gas (a mixture of 5% CO2 and 95% air). A hypoxic challenge was presented by switching the ventilating gas to a mixture of 3% O2, 5% CO2, and 92% N2 for 10 min. Hemodynamic measurements were made at the end of this period. Ventilation with normoxic gas was then resumed, and the perfusion pressure was allowed to return to baseline values. After addition of the NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; 10⁻⁴ M) to the perfusate, the perfusion pressure was allowed to stabilize for 20 min, and the hemodynamic measurements were repeated. A second hypoxic challenge was then presented as described above. The perfusate was sampled regularly, and its pH, Pco₂, and Po₂ were measured with an automatically calibrating blood gas analyzer (Ciba Corning model 278, Medfield, MA). pH was maintained in the range of 7.38–7.44 under all conditions by the addition of 0.3 M NaHCO3 as required.

Protocols with specific inhibitors of NOS isoenzymes. The isolated lung protocol as described in Hemodynamic studies was carried out in further separate groups of chronically infected lungs except that L-NAME was replaced by one of the following: 1) NG⁻nitro-L-arginine (L-NNa; 10⁻⁴ M), a nonspecific inhibitor of NOS (1); 2) aminoouanidinie (10⁻⁴ M), an inhibitor of iNOS (14); 3) S-(2-aminoethyl)isothiourea dihydrobromide (AETU; 10⁻⁴ M), a second inhibitor of iNOS (31); or 4) 1-(2-trifluoromethylphenyl)imidazole (TRIM; 10⁻⁴ M), an inhibitor of iNOS and neuronal NOS (15).

Vasodilator response to sodium nitroprusside. In a further series of experiments with the isolated,perfused preparation described in Lung isolation for hemodynamic studies, the response to the NO donor sodium nitroprusside (SNP) was examined in the lungs from each of the three experimental groups. In these lungs, endogenous NOS activity was inhibited by L-NAME (10⁻⁴ M), and pulmonary vascular resistance was then increased by adding PGF2α, incrementally to the perfusate until the pulmonary arterial pressure had risen by 30 mmHg. SNP (10⁻⁸ to 10⁻⁴ M) was added cumulatively to the perfusate so that its concentration increased in half-logarithmic increments, and the resultant changes in pulmonary arterial pressure were recorded.

Nitrate/nitrite accumulation. In isolated, perfused lungs ventilated with normoxic gas, samples of the perfusate were taken on two occasions separated by 20 min for determination of the total concentration of nitrate and nitrite (NO3⁻/NO2⁻) before the addition of NOS inhibitors.

To provide a positive control group, rats were injected intraperitoneally 6 h before death with 20 mg/kg of salmonella enteritidis LPS, a procedure that has previously been shown to increase iNOS expression and activity in the lungs (7, 13, 14, 19). These animals were killed by exsanguination under anesthesia as described in Lung isolation for hemodynamic studies, and the plasma was reserved for determination of the nitrate/nitrite concentration. Samples of the perfusate from the isolated lungs of these animals were also reserved for nitrate/nitrite assay.

Bronchoalveolar lavage. After the hemodynamic studies, bronchoalveolar lavage (BAL) was carried out by intratracheal instillation of 20 ml of saline (PBS) for inoculation.
iNOS EXPRESSION IN CHRONICALLY INFECTED LUNGS

Cheil instillation of 5 ml of normal saline and collection of the returned fluid by free drainage. Total cell number per milliliter in the BAL fluid was counted, and differential cell counts were performed after staining with Diff-Quik (Dade).

Histological and immunohistochemical protocols. After BAL, the lungs were inflated by intratracheal instillation of optimal cutting temperature (OCT) embedding compound. Random blocks of tissue were removed from the lungs and frozen in OCT embedding compound, and cryostat sections (12 µm) were prepared on gelatin-coated slides. For routine histological examination, the sections were stained with hematoxylin and eosin.

Immunolocalization of iNOS was carried out as previously described (18). In brief, the sections were allowed to air-dry and were then fixed for 5 min in 2% (wt/vol) paraformaldehyde. After the endogenous binding sites were blocked by 10 min of preincubation with 10% (vol/vol) normal goat serum, the tissue sections were incubated at room temperature for 1 h with an affinity-purified, polyclonal rabbit anti-iNOS antibody diluted 1:500. This was followed immediately by an antibody incubation with iNOS purified from RAW 264.7 cells and a wash for 20 min in PBS with 0.2% (wt/vol) gelatin added. Anti-iNOS antibody binding was demonstrated by incubation for 1 h with a biotin-conjugated goat anti-rabbit IgG (diluted 1:200) anti-neuronal pentraxin antibody for the anti-iNOS antibody. The sections were counterstained with hematoxylin, dehydrated through graded alcohols, cleared with xylene, and mounted. Negative control slides included 1) the standard staining procedure with omission of the primary anti-iNOS antibody, 2) substitution of preimmune rabbit serum for the anti-iNOS antibody, and 3) the standard procedure with substitution of affinity-purified polyclonal rabbit antineuronal pentraxin antibody for the anti-iNOS antibody. The antibody to neuronal pentraxin was chosen as a negative control because this antigen is not present in the lungs.

Immunostaining was blocked by preincubation of the primary antibody with iNOS purified from RAW 264.7 cells activated by interferon-γ and bacterial LPS, i.e., preincubation with the protein against which the primary antibody was raised. Intensity of the immunostaining was assessed independently by three blinded reviewers who used a semiquantitative scale (0–4) to assess each lung, and the final score assigned to an individual lung was the mean of the three independent determinations.

Immunolocalization of eNOS was carried out by incubating tissue sections overnight at 4°C with an affinity-purified polyclonal rabbit anti-eNOS antibody diluted 1:1,000. Positive staining was visualized with the nickel-enhanced DAB-peroxidase reaction, and the sections were subsequently counterstained with methyl green. Immunostaining for eNOS was blocked by preincubation of the primary antibody with human endothelial cell lysate (Affiniti). Intensity of the immunostaining was assessed independently by three blinded reviewers who used a semiquantitative scale (0–4) to assess separately the intensity of staining in the blood vessel endothelium and alveolar walls in each lung. The final score assigned to an individual parameter was the mean of the three independent determinations.

Measurement of RV weights. Before the lungs were frozen, the rat hearts were excised and placed in fixative (1.5 mM neutral formaldehyde in normal saline). The RV free wall was dissected free from the left ventricle and septum, each ventricle was weighed separately, and the results are expressed as milligrams per 100 g of body weight.

Measurement of nitrate/nitrite concentration. To determine total nitrate and nitrite concentrations in the perfusate and plasma samples, nitrate was first enzymatically converted to nitrite with nitrate reductase (total NO assay kit, R&D Systems, Oxon, UK). Nitrite was further reduced to NO by addition of the sample to a solution of potassium iodide (0.1 M) in hydrochloric acid (0.1 M). The NO produced was detected electrochemically (World Precision Instruments, Herts, UK) as previously described (3).

Reagents. Bovine serum albumin, l-NNAME, amingoguanidine, l-NNA, mineral oil, all salts, DAB, LPS, and SNP were supplied by Sigma (Poole, UK). Heparin (mucous) was obtained from Leo Laboratories (Princes Risborough, UK); pentobarbital sodium was from Rhône Mérieux (Harlow, UK); Hynporm was from Janssen (Wantage, UK), and midazolam was from Roche Products (Welwyn Garden City, UK). AETU and TRIM were obtained from Toscris-Cookson (Bristol, UK).

OCT embedding compound was obtained from Miles laboratory (Elkhart, IN); polyclonal rabbit anti-iNOS was from TCS Biologicals (Bristol, UK); polyclonal rabbit anti-eNOS and anti-neuronal pentraxin were from Affiniti Research Products (Exeter, UK); avidin-biotin-peroxidase complex elite kit was from Vector Laboratories (Burlingame, CA).

Data analysis. Increases in total pulmonary vascular resistance in response to hypoxia are expressed as the change in perfusion pressure at the end of a hypoxic challenge. Significantly, responses to the inhibition of NOS are expressed as the change in perfusion pressure from baseline after the addition of inhibitors to the perfusate and the development of a stable response. After experiments in which capillary pressure was measured, pressure drops across the pre- and postcapillary segments were determined and used to calculate the resistance of each segment. The response after each concentration of SNP is expressed as the cumulative fall in pressure below the initial PFG2-induced plateau.

Statistical analysis. Data are presented as means ± SE. Statistical comparisons of means were made with analysis of variance (ANOVA), and when this indicated significant differences between groups, the Student-Newman-Keuls post hoc test was used to assess the significance of the differences between specific means. Where appropriate (total and differential cell counts), data were log transformed before ANOVA. For clarity, the untransformed values are presented in the text. To determine whether the mean values were significantly different from zero, a one-sample t-test was used. A value of P < 0.05 was accepted as significant.

RESULTS

Mucoid colonies of P. aeruginosa were grown on blood agar plates from the BAL fluid obtained from each chronically infected lung, but none were isolated from either the noninoculated or placebo-inoculated lungs. The mean total cell count in the BAL fluid from Pseudomonas-infected lungs was significantly greater than that in the noninoculated and placebo-inoculated groups (Table 1). Differential cell counts showed that the mean percentage of neutrophils and lymphocytes in the Pseudomonas-infected lungs was significantly elevated above that in the other two groups (Table 1).

The mean (±SE) RV weight in the Pseudomonas-inoculated group was 54.4 ± 4.5 mg/100 g body wt,
which was not significantly different (by ANOVA) from the noninoculated (51.6 ± 3.7 mg/100 g body wt) and placebo-inoculated (56.3 ± 5.7 mg/100 g body wt) groups. The two control groups did not differ significantly from one another (by ANOVA).

Histological examination of chronically infected lungs showed extensive inflammatory cell infiltrate associated with thickening of the alveolar walls and distortion of the bronchioles (Fig. 1A). The inflammatory cells were predominantly mononuclear, with lesser numbers of neutrophils. Medial hypertrophy was observed in the intra-acinar blood vessels (Figs. 1B and 2, C and D). Sections from placebo-inoculated lungs showed normal pulmonary structure, although occasional areas of inflammatory cell infiltrate were observed in some lungs (Fig. 1C).

In noninoculated lungs, immunostaining showed the presence of iNOS in the epithelium of large airways and occasionally in the epithelium of larger bronchioles (Fig. 3C). It was distributed in a patchy manner throughout normal alveolar walls, being virtually absent in some areas and relatively more prominent in others (Fig. 3A). iNOS was not observed in the vascular or airway smooth muscle of the normal lungs. In chronically infected lungs, the alveolar walls stained more intensely and more extensively for iNOS (Fig. 1A), and it was also found in vascular smooth muscle (Fig. 1B). In the placebo-inoculated lungs, the alveolar walls stained in a manner similar to that in the noninoculated lungs (Fig. 1C) and no staining was seen in the vascular or airway smooth muscle. The mean immunostaining score in the chronically infected lungs was 2.8 ± 0.3, which was significantly different from that in the noninoculated (1.9 ± 0.4) and placebo-inoculated (1.5 ± 0.4) lungs (P < 0.01). The intensity of immunostaining in the blood vessel endothelium in the noninoculated and placebo-inoculated lungs did not differ significantly.

Localized staining of eNOS was also seen in the alveolar walls of the noninoculated lungs (Fig. 2E). Macrophages, which were occasionally identified in the alveolar lumen, were also stained in the control lungs. In the chronically infected lungs, there was virtually no staining for eNOS in the alveolar walls (Fig. 2F), whereas in the placebo-inoculated lungs, the alveolar walls stained in a manner similar to that in the noninoculated lungs (data not shown). The mean immunostaining score of alveolar walls in the chronically infected lungs was 0.2 ± 0.1, which was significantly lower than that in both the noninoculated (2.6 ± 0.5) and placebo-inoculated (1.6 ± 0.6) lungs (P < 0.05).

In the noninoculated lungs, staining of eNOS was also seen in the epithelium of large airways and occasionally in the epithelium of larger bronchioles. However, there was no visible staining observed in the airways of the chronically infected group, whereas the placebo-inoculated lungs showed limited staining in the epithelium of the airways. Because large airways were only seen occasionally, it was not possible to score eNOS immunostaining separately in these airways.

Table 1. Cell counts in BAL fluid from noninoculated, Pseudomonas-inoculated, and placebo-inoculated lungs

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<th>Noninoculated</th>
<th>Pseudomonas Inoculated</th>
<th>Placebo Inoculated</th>
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<tbody>
<tr>
<td>n</td>
<td>22</td>
<td>31</td>
<td>26</td>
</tr>
<tr>
<td>Total cell count, ×10^7</td>
<td>3.8 ± 0.9</td>
<td>32.2 ± 2.3*</td>
<td>3.5 ± 0.5</td>
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<td>Neutrophils, %</td>
<td>2.3 ± 0.4</td>
<td>6.9 ± 1.8*</td>
<td>3.9 ± 1.3</td>
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<tr>
<td>Macrophages, %</td>
<td>96.0 ± 0.6</td>
<td>72.1 ± 2.8*</td>
<td>94.7 ± 2.4</td>
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<tr>
<td>Lymphocytes, %</td>
<td>1.5 ± 0.3</td>
<td>10.3 ± 1.7*</td>
<td>2.1 ± 1.2</td>
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Values are means ± SE; n, no. of lungs. *Significantly different from noninoculated and placebo-inoculated groups, P < 0.01.

To allow identification of the vascular segments in which resistance increased after L-NAME, the double-occlusion technique was used to measure capillary pressure in further groups of placebo-inoculated, Pseudomonas-inoculated, and noninoculated lungs. Neither Pseudomonas infection nor placebo inoculation altered the baseline capillary pressure compared with that in...
the noninoculated lungs (data not shown). NOS inhibition did not significantly increase capillary pressure in any of the three groups examined (data not shown). L-NAME caused a significantly greater rise in precapillary resistance in the infected lungs than in either of the other groups (Fig. 4). In contrast, L-NAME did not lead to significant changes in postcapillary resistance. The change in perfusion pressure in response to hypoxia was not altered from the noninoculated values by either Pseudomonas infection or placebo inoculation.

Fig. 1. A: photomicrograph of Pseudomonas aeruginosainoculated, chronically infected lung tissue showing intense staining of inducible nitric oxide synthase (iNOS) in alveolar walls. Note extensive inflammatory cell infiltrate and loss of normal alveolar structure. Original magnification, ×190. B: photomicrograph demonstrating immunoperoxidase staining of iNOS in vascular smooth muscle of a remodeled intra-acinar blood vessel from a chronically infected lung (arrow). Original magnification, ×760. C: photomicrograph of placebo-inoculated lung showing sparse, patchy immunoperoxidase staining of iNOS in alveolar walls similar to that observed in noninoculated lung. Original magnification, ×190.
Addition of L-NAME increased the hypoxic vasoconstriction significantly in the noninoculated lungs ($P < 0.05$). Similarly, L-NAME caused an increase in the hypoxic response in the Pseudomonas-infected lungs, although this was not significant ($P = 0.09$). In the placebo-inoculated lungs, L-NAME reduced the hypoxic vasoconstrictor response significantly, in contrast to the enhanced hypoxic vasoconstrictor response observed in the presence of L-NAME in both the noninoculated and Pseudomonas-inoculated lungs (Fig. 5).

The mean changes in nitrate/nitrite concentration over a 20-min period in the perfusate of isolated lungs from the chronically infected (0.8 ± 0.6 µM), noninoculated (1.3 ± 0.5 µM), or placebo-inoculated (−0.1 ± 0.5 µM) groups were not significantly different (by ANOVA) from one another ($n = 6$ lungs/group). In contrast, the mean increase in lungs ($n = 3$) isolated from LPS-treated rats was 12.2 ± 0.7 mM, significantly greater than in the other three groups ($P < 0.01$ by ANOVA).

Mean plasma nitrate/nitrite concentration in chronically infected rats ($n = 12$) was 14.4 ± 0.9 µM, not significantly different from that in noninoculated (13.7 ± 1.0 µM; $n = 7$) or placebo-inoculated (13.4 ± 1.3 µM) animals ($n = 12$). The LPS-treated rats had a plasma nitrate/nitrite concentration of 181.1 ± 7.4 µM, which was significantly greater than that in the other three groups ($P < 0.01$ by ANOVA).

The sensitivity of the lung vasculature to the vasodilator effects of NO was examined with the NO donor SNP. Lungs from the chronically infected group were significantly more sensitive to SNP than lungs from one another ($n = 6$ lungs/group).
either the placebo-inoculated or noninoculated group (P < 0.05 by ANOVA; Fig. 6).

**DISCUSSION**

In this series of experiments, we have provided immunohistochemical evidence that chronic pulmonary infection with P. aeruginosa leads to an increased expression of iNOS and a reduced expression of eNOS in the rat lung. Nonspecific inhibition of NOS activity leads to an increase in baseline pulmonary vascular resistance in chronically infected lungs, which is significantly greater than that observed in the control lungs. Inhibitors that selectively block the activity of iNOS without inhibiting eNOS did not cause a greater increase in pulmonary vascular resistance in the chronically infected lungs than in either group of noninfected lungs.

**Table 2. Baseline PAP and Δ PAP in response to L-NAME and aminoguanidine**

<table>
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<th>Noninoculated</th>
<th>Pseudomonas Inoculated</th>
<th>Placebo Inoculated</th>
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<tr>
<td><strong>Baseline PAP, mmHg</strong></td>
<td></td>
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<tr>
<td>(n = 16)</td>
<td>(n = 21)</td>
<td>(n = 18)</td>
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<tr>
<td>16.8 ± 1.9</td>
<td>17.7 ± 1.2</td>
<td>16.5 ± 0.7</td>
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<td><strong>ΔPAP, mmHg</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>L-NAME (n = 10)</td>
<td>(n = 13)</td>
<td>(n = 11)</td>
<td></td>
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<tr>
<td>5.3 ± 0.8*</td>
<td>14.4 ± 2.7†</td>
<td>4.8 ± 1.0*</td>
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<tr>
<td>Aminoguanidine (n = 6)</td>
<td>(n = 8)</td>
<td>(n = 7)</td>
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<tr>
<td>4.5 ± 0.7*</td>
<td>6.3 ± 1.3*</td>
<td>5.7 ± 0.7*</td>
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Values are means ± SE; n, no. of lungs. PAP, pulmonary arterial pressure; ΔPAP, change in PAP; L-NAME, N-nitro-L-arginine methyl ester. Significantly different (P < 0.01) from: *zero; †changes in noninoculated and placebo-inoculated groups in presence of L-NAME and changes in all groups in presence of aminoguanidine.
lungs. Hypoxic vasoconstriction was unaltered in either the Pseudomonas-inoculated or placebo-inoculated lungs. After NOS inhibition with L-NAME, hypoxic vasoconstriction was increased in the inoculated and noninoculated groups but was significantly reduced in the placebo-inoculated lungs. The rate of increase in the nitrate/nitrite concentration in the perfusate of isolated lungs from infected animals was not significantly different from that in the lungs from the two control groups, whereas it was markedly elevated in the lungs from animals exposed to LPS. Elevations in in vivo plasma nitrate/nitrite concentration above the control value were not detected in the plasma from infected rats but were observed in the plasma from LPS-exposed rats.

To establish chronic pulmonary Pseudomonas infection, we used a previously described model that leads to pathological changes characteristic of bronchiectasis (12). We confirmed that chronic infection was successfully established by demonstrating that Pseudomonas organisms could be isolated from the BAL fluid of the infected lungs. The elevated total cell counts in this BAL fluid, together with an increased proportion of neutrophils and lymphocytes, are compatible with an airway inflammatory response to infection. Furthermore, the histopathological changes that we observed in chronically infected lungs are similar to those previously reported in this model (12). Occasional areas of inflammatory cell infiltrate were observed on histological examination of placebo-inoculated lungs, suggesting that agar beads may have induced a mild inflammatory reaction in some areas of the lung. There was, however, no elevation in the total cell count in BAL fluid in this group.

Although we have used agar beads as previously described by others (12, 23) to produce chronic airway infection, it is important to note a number of significant differences between the methods used in the present study and those in the previous reports on the effect of chronic Pseudomonas infection on the pulmonary circulation. First, we instilled Pseudomonas organisms into the trachea to produce a widespread, bilateral pulmonary infection. In previous studies (12, 23), agar beads have been instilled into a single lobe, producing localized pneumonia. Second, the effects of this infection on pulmonary hemodynamics have previously been examined in vivo. We used an isolated lung preparation to avoid the direct, reflex-mediated attenuation of hypoxic vasoconstriction that is observed in intact animals (27). This preparation also removes reflex-induced responses such as hyperventilation or changes in cardiac output that lead to secondary effects on pulmonary vascular resistance in vivo, which modulate the direct effects of hypoxia. Such influences are reflected in the observation that changes in pulmonary arterial pressure in vivo frequently do not parallel changes in pulmonary vascular resistance.

Immunostaining of noninoculated lungs showed a pattern of distribution of iNOS similar to that previously reported by others in normal lungs (18), i.e., prominent staining in large airway epithelium, staining in the epithelium of some bronchioles, and patchy staining of the alveolar walls. With 12-μm cryostat sections viewed with light microscopy, it is not possible to resolve the detailed structure of the alveolar wall, and we were unable to identify specifically the cells that were positively stained. In agreement with Kobzik et al. (18), no iNOS was detected in the smooth muscle of the blood vessels or airways of normal lungs. In the lungs chronically infected with Pseudomonas, there was widespread, intense staining throughout the alveolar walls, but we were unable to distinguish the exact cellular locations of the iNOS. Inflammatory, capillary endothelial, and alveolar epithelial cells may all express iNOS when stimulated (1, 30). Pseudomonas inoculation also led to iNOS expression in the vascular and airway smooth muscle. Our immunohistochemical demonstration of increased iNOS expression in chronically infected lungs is supported by the findings of
Lovchik et al. (22), who have reported, using Western blot assays, increased expression of iNOS in the lungs of mice after 14 days of infection with Cryptococcus neoformans. Our observation that lungs inoculated with placebo (agar-alone) beads showed a similar pattern of staining to that of noninoculated lungs demonstrates that the increased iNOS expression observed after Pseudomonas inoculation was a specific effect of infection.

Immunostaining of noninoculated lungs showed a pattern of distribution of eNOS similar to that reported by others in normal lung tissue. In agreement with Kobzik et al. (18), eNOS was detected in the endothelium of blood vessels and airways in normal lungs. The immunostaining that we observed in the alveolar walls was unevenly distributed, with some areas totally devoid of staining. This would suggest that it was not the capillary endothelium that stained for eNOS. It has previously been reported (24, 25) that eNOS is expressed by alveolar macrophages and type II pneumocytes in cell culture. The patchy distribution of eNOS staining in control lungs is compatible with the expression of eNOS in vivo in these cell types and is further supported by our observation that isolated macrophages in the alveolar space stained positively for eNOS. For the reasons outlined above, direct confirmation of this interpretation was not possible in the cryostat sections used in the present series of experiments. In the lungs chronically infected with Pseudomonas, we found that eNOS expression was reduced throughout the alveolar walls and the blood vessels. In this context, it is interesting to note the recent demonstration that in the chronic inflammatory conditions of the atherosclerotic plaque, eNOS protein expression was markedly reduced (28). Furthermore, Liu et al. (19) reported that when acute pulmonary inflammation was produced in rats by the administration of LPS in vivo, there was a reduced expression of eNOS mRNA in the lung accompanied by a simultaneous upregulation of iNOS mRNA. Those results are compatible with our observation that in the chronically infected lung, eNOS expression was reduced, whereas iNOS expression was increased.

In isolated lungs, we found that baseline pulmonary vascular resistance was not significantly increased after chronic infection despite the presence of pulmonary vascular remodeling. This finding is in agreement with previous results reported by Graham et al. (12) in chronically infected rats in vivo at a similar time after inoculation. Furthermore, the absence of RV hypertrophy in chronically infected rats in the present series of experiments suggests that pulmonary vascular resistance was not increased in vivo. It has been previously demonstrated in the systemic circulation that vascular wall remodeling can occur while the lumen size remains unchanged (10). Such a mechanism would account for the simultaneous absence of an increase in pulmonary vascular resistance and the presence of vascular remodeling that we observed, although this hypothesis remains to be confirmed experimentally. In contrast to our findings, McCormack and Paterson (23) found that a small increase in pulmonary vascular resistance occurs in this model of chronic infection in vivo, although this occurred after a shorter interval postinoculation.

We observed a small but significant increase in pulmonary perfusion pressure in response to L-NAME in noninoculated lungs, suggesting that basal NO production tonically reduces pulmonary vascular resistance in normal lungs. Previous evidence regarding the role of NO in modulating baseline pulmonary vascular resistance has been conflicting. NOS inhibition has been reported to increase (6, 20) or have no effect (4, 29) on baseline perfusion pressure in isolated blood-perfused rat lungs. Cremona et al. (9) reported that L-NAME at the same concentration used in the present study caused significant increases in pulmonary vascular resistance in pig, sheep, and human lungs, although not in isolated dog lungs. Sprague et al. (32) found that NOS inhibition caused an increase in baseline pulmonary vascular resistance in blood-perfused, isolated rabbit lungs but not when the lungs were perfused with a physiological saline solution. It is interesting to note that in the case of the most commonly used nonsel ective NOS inhibitor, L-NAME, those studies, including the data presented here, which used higher concentrations (10^-4 M or greater), reported an increased baseline pulmonary vascular resistance (6, 9, 20), whereas when a lower concentration was used, no such increase was found. We chose to use the higher concentration because previous work in our laboratory has demonstrated that in isolated pulmonary arteries, this concentration was required to produce a 90% inhibition in acetylcholine-induced, endothelium-dependent relaxation (M. Sweeney, D. Beddy and P. McLoughlin, unpublished observations).

In chronically infected lungs, addition of the nonspecific NOS inhibitor L-NAME caused a greater increase in pulmonary vascular resistance than in either the placebo-inoculated or noninoculated group, an observation that appeared to support our initial hypothesis. A second, structurally different NOS inhibitor, L-NNA, produced a similar effect in Pseudomonas-infected lungs, whereas D-NAME, the eNOS inhibitor, L-NAME that does not inhibit NOS activity, was without an effect on perfusion pressure. All these results indicate that the increase in pulmonary resistance caused by L-NAME was due to NOS inhibition. In a further group of lungs examined with the double-occlusion technique, we found that the increase in total pulmonary vascular resistance was exclusively due to an elevation in the precapillary component. McCormack and Paterson (23) reported that NOS inhibition caused an increase in pulmonary vascular resistance in vivo in rats infected with Pseudomonas for a shorter period, although the increase observed was not significant. These data suggest that in chronically infected lungs, basal NO production tonically inhibits pulmonary vasoconstriction, thus acting to prevent pulmonary hypertension. This finding parallels the similar observation that NOS inhibitors cause abnormally large increases in pulmonary vascular resistance in lungs adapted to chronic hypoxia (4,
Our observation that placebo-inoculated lungs responded to \( \text{L-NAME} \) in a manner similar to noninoculated lungs demonstrates that the augmented sensitivity to NOS inhibition in infected lungs was specifically due to the presence of \( P. \ aeruginosa \).

To identify the isoenzyme of NOS that was responsible for maintaining normal vascular resistance in chronically infected lungs, we examined the effects of aminoguanidine, a selective inhibitor of iNOS (14), on pulmonary vascular resistance. The observation that aminoguanidine caused a similar increase in vascular resistance in all three groups examined, which was significantly less than that in response to \( \text{L-NAME} \) in the chronically infected lungs, suggests that iNOS activity was not the source of the NO that maintained pulmonary arterial pressure at normal values in the infected lungs. The similar effect of a second selective iNOS inhibitor, AETU, supports this interpretation. It is important to note that the increase in perfusion pressure after the addition of aminoguanidine to the perfusate of the three groups of isolated lungs may not have been as a result of iNOS inhibition. Aminoguanidine has been reported to cause an increase in systemic vascular resistance in normal rats by an action unrelated to inhibition of iNOS (17). Similarly, the rise in pulmonary perfusion pressure produced in infected lungs by AETU may not have been due to iNOS inhibition. Both aminoguanidine and mercaptoethylguanidine, the active metabolite of AETU, partially inhibit cyclooxygenase activity at the concentrations used in the present experiments (38). Because baseline prostacyclin production is an important vasodilator influence in the normal lung, this action may in part account for the observed increase in pulmonary vascular resistance. Despite the fact that the increase in perfusion pressure produced by aminoguanidine may be in part due to activities other than iNOS inhibition, the similarity of its effect in both the chronically infected and control lungs suggests that iNOS activity does not exert an augmented vasodilator effect on pulmonary vascular resistance in the chronically infected group.

To further explore this issue, we examined the effect of TRIM, an inhibitor of iNOS and neuronal NOS that, in contrast to aminoguanidine and AETU, is not a guanidine derivative (15). The absence of an effect of TRIM on pulmonary perfusion pressure supports our interpretation that iNOS was not producing quantities of NO sufficient to exert a vascular effect in the chronically infected lungs and, furthermore, excludes neuronal NOS as a source of vasodilator NO production. These observations suggest that eNOS was the isofrom of the enzyme that produced the NO that maintained normal pulmonary vascular resistance in chronically infected animals (5).

In view of our finding that chronic infection did not lead to an increase in basal pulmonary vascular resistance, we wished to test the possibility that chronic infection led to an enhanced vasoconstrictor response to hypoxia. We found that the increase in pulmonary vascular resistance produced by hypoxia was not significantly altered after either placebo or \( P. \ aeruginosa \) inoculation. Graham et al. (12) reported that the hypoxic vasopressor response was attenuated in chronically infected rats, but McCormack and Paterson (23) found that the increase in pulmonary vascular resistance in response to hypoxia was unchanged, a finding in agreement with the present data.

Inhibition of NOS by \( \text{L-NAME} \) increased hypoxic pulmonary vasoconstriction in the noninoculated lungs, corresponding with previous observations in normal lungs (8, 21). In the chronically infected lungs, \( \text{L-NAME} \) increased the hypoxic vasoconstrictor response, although the change was not significant, a finding in agreement with that previously reported in vivo (23). In contrast, \( \text{L-NAME} \) attenuated the hypoxic response in placebo-inoculated lungs, suggesting that hypoxic vasoconstriction had become partially dependent on a reduction in basal NO production under these circumstances. We are unable to offer an explanation for this unexpected observation. However, it indicates that the specific effect of chronic \( P. \ aeruginosa \) infection, separated from that of agar inoculation, was to produce enhanced hypoxic vasoconstriction that was antagonized by endogenous NO production.

Our observation that iNOS expression was increased in chronically infected lungs, yet that the vascular effect of specific inhibitors of this isoenzyme was not different from that found in control lungs, was unexpected. iNOS expression is associated with a rate of NO production greatly in excess of that of the constitutive isoforms and, furthermore, this production is unregulated (1). In acute inflammatory conditions, activity of iNOS in rats in vivo has been previously shown to cause an increased concentration of plasma nitrate/nitrite (17, 26), the major metabolite of NO under physiological conditions (36, 16). Plasma nitrate/nitrite concentrations in our noninoculated, \( P. \ aeruginosa \)-inoculated, and placebo-inoculated rats were not significantly different from one another and were similar to those previously reported in normal control animals (17, 26). In contrast, in LPS-exposed animals, plasma nitrate/nitrite concentration was markedly elevated, a finding consistent with the induction of highly active iNOS in acute inflammation as previously reported (7, 19).

To indirectly examine NOS activity in the pulmonary tissues, we measured nitrate/nitrite accumulation in the perfusate of isolated lungs and found that there was no significant difference between the control, placebo-inoculated, and chronically infected lungs, suggesting that NO production was relatively similar in the three groups. In contrast, in the lungs of animals exposed to intraperitoneal LPS, an intervention that has been previously shown to lead to increased iNOS expression and activity in the lung (7, 13, 14, 17, 19, 38), a large increase in nitrate/nitrite concentration in the perfusate was detected. Taken together with our data on the effects of selective inhibitors of iNOS on pulmonary vascular resistance, these findings demonstrate that the increased iNOS expression that we detected immunohistochemically in chronically infected lungs was not associated with the high NO production that this enzyme displays when expressed in acute inflamma-
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In the absence of evidence of markedly increased NOS activity, a possible explanation for the enhanced vasconstrictor effect of nonspecific inhibitors of NOS (L-NAME and L-NNA) in the chronically infected lungs is that the pulmonary vasculature was more sensitive to the vasodilator effect of normal, endogenous NO production. The increased vasodilator response of the infected lungs to the NO donor SNP that we observed is compatible with this interpretation.

In summary, we have demonstrated for the first time that in a rat model, chronic pulmonary Pseudomonas infection led to a widespread increase in expression of iNOS in the alveolar walls and pulmonary vascular smooth muscle, whereas eNOS expression was reduced in the vascular endothelium and alveolar walls. The newly expressed iNOS did not demonstrate the high NO production typically seen when this isoform is expressed in acute inflammatory conditions nor did it cause excessive pulmonary vasodilatation. Nonetheless, in chronically infected lungs, endogenous NO production was essential to the maintenance of normal precapillary resistance and the prevention of the development of pulmonary hypertension.

We thank D. Briton (St. Vincents Hospital, Dublin, Ireland) for help and advice in preparing the Pseudomonas aeruginosa inoculum. This work was supported by the Health Research Board of Ireland. Address for reprint requests and other correspondence: P. McLaughlin, Dept. of Human Anatomy and Physiology, Univ. College, Earlsfort Terrace, Dublin 2, Ireland (E-mail: paul.mclaughlin@ucd.ie).

Received 18 May 1998; accepted in final form 22 March 1999.

REFERENCES


