Effect of inhibitors of nitric oxide release and action on vascular tone in isolated lungs of pig, sheep, dog and man

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1. The actions of inhibitors of the release or action of nitric oxide (NO) on pulmonary vascular resistance (PVR) were investigated in lungs isolated from pig, sheep, dog and man.

2. In pig, sheep and human lungs perfused with Krebs–dextran solution, both N^{ω}-nitro-L-arginine methyl ester (L-NAME; 10^{-5} M) and Methylene Blue (10^{-4} M) increased basal PVR. This increase was reversed by sodium nitroprusside (10^{-5} M). In pig lungs N^{ω}-monomethyl-L-arginine (10^{-4} M) increased PVR by 154%. This increase was partially reversed by L-arginine (10^{-3} M). L-NAME had no effect in dog lungs.

3. Pulmonary artery pressure–flow ($P_{PA}/\dot{Q}$) relationships were studied over a wide range of flows. In pig, sheep and human lungs perfused with Krebs–dextran solution, L-NAME increased the $P_{PA}/\dot{Q}$ slope. This increase was reversed by sodium nitroprusside. In dog lungs L-NAME had no effect.

4. In blood-perfused lungs, the respective responses to L-NAME were similar to those observed with saline. Acute hypoxia in pig and dog lungs increased intercept pressure. Addition of L-NAME during hypoxia increased the $P_{PA}/\dot{Q}$ slope in both species.

5. In the human, there was no difference in the absolute increase of PVR or $P_{PA}/\dot{Q}$ slope elicited by L-NAME between hypertensive and control lungs.

6. We conclude that NO is continuously released in the pulmonary vascular bed of pig, sheep and humans under normoxic conditions. In dog lungs inhibition of NO synthesis increases PVR only under hypoxic conditions. In human lungs with pulmonary hypertension, NO is still released under basal conditions.

It is now established that systemic vascular endothelial cells *in vitro* and *in vivo* continually produce nitric oxide (NO) derived from L-arginine by a constitutive synthase (Moncada, 1992). NO exerts a potent vasodilator action, and inhibition of nitric oxide synthase by substituted analogues of L-arginine such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) or N^{ω}-monomethyl-L-arginine (L-NMMA) markedly increase systemic vascular resistance (Griffith, Edwards, Davies & Henderson, 1989; Rees, Palmer & Moncada, 1989; Vallance, Collier & Moncada, 1989).

The production and role of NO in the pulmonary circulation is uncertain. Pharmacological stimulation releases NO from conduit pulmonary arteries in man (Dinh-Xuan, Higenbottam, Clelland, Pepke-Zaba, Wells & Wallwork, 1990*b*) and resistance pulmonary arteries in the rat (Adnot, Raffestin, Eddahibi, Braquet & Chabrier, 1991). In the intact lungs of rabbits, guinea-pigs (Persson, Gustafsson, Wiklund, Moncada & Hedqvist, 1990) and cats (Hyman, Kadowitz & Lippton, 1989; McMahon, Hood, Bellan & Kadowitz, 1991) investigators have reported that NO regulates basal pulmonary vascular tone. Conversely, studies in isolated lungs of rats (Mazmanian, Baudet, Brink, Cerrina, Kirkiacharian & Weiss, 1989; Archer, Rist, Nelson, DeMaster, Cowan & Weir, 1990; Hasunuma, Yamaguchi, Rodman, O'Brien & McMurtry, 1991; Barer, Emery, Stewart, Bee & Howard, 1993) and in conscious dogs (Nishiwaki *et al.* 1992) have found little evidence of NO release in the absence of stimuli. These discrepancies may relate to species differences in the regulation of pulmonary vascular tone or to differences in experimental procedure.

In order to investigate the production and role of pulmonary NO in a range of larger mammals including humans, experiments were conducted to examine the

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effect of inhibitors of the release or action of NO on pulmonary vascular resistance. The lungs of these species are of comparable size but differ considerably in the structure and responsiveness of their pulmonary arteries (Peake, Harabin, Brennan & Sylvester, 1981; Hakim & Malik, 1988). Lungs from humans with pulmonary hypertension were also studied to discover whether deficiency of NO contributes to the elevated resistance. In all four species, the lungs were perfused and ventilated in isolation to avoid the influence of neural or humoral factors and the passive mechanical effects of varying cardiac performance.

METHODS

Surgical procedures

Animal lungs. The animal experiments in this study were carried out under a project licence granted by The Home Office under the Animals (Scientific Procedures) 1986 Act. Pigs (40–60 kg; $n = 28$), sheep (20–40 kg; $n = 18$) and dogs (20–25 kg; $n = 12$) weighing between 20 and 25 kg were anaesthetized with intravenous sodium pentobarbitone (up to 30 mg kg^{-1}; Sagatal; Rhône-Poulenc Rorer Ltd, Eastbourne, Sussex, UK) and then animals were intubated, paralysed with 0·2 mg kg^{-1} alcuronium (Alloferin; Roche, Welwyn, Herts, UK) and ventilated by a Manley ventilator (Blease Medical, Bucks, UK) with 40% O_2–60% N_2 at a rate of 15 breaths min^{-1}. The pigs were sedated prior to anaesthesia with 0·5 mg kg^{-1} droperidol (Droleptan; Janssen Pharmaceutical Ltd, Wantage, Oxon, UK) and 0·3 mg kg^{-1} midazolam (Hypnovel; Roche). The adequacy of anaesthesia was assessed by monitoring the responses of heart rate and systemic blood pressure to noxious stimuli. A mid-line sternotomy was performed. Heparin (1000 U kg^{-1}) was administered intravenously and the aorta, pulmonary artery (PA) and venae cavae were mobilized and isolated. Cannulae were placed in the aorta and PA. The animals were then killed by exsanguination and the thorax was filled with cold saline.

The procedure for preservation of the lung was adapted from the technique described for human donor lungs for transplant surgery (Wallwork, Jones, Cavarocchi, Hakim & Higenbottam, 1987). The lungs were pretreated with an infusion of 20 ng prostacyclin (Epoprostenol; Wellcome Foundation, Beckenham, Kent, UK) in 10 ml of saline as a bolus in order to vasodilate the pulmonary arteries and assist uniform penetration of the preservation solution. Under gravity (30 mmHg), 2 l of cold (10 °C) extracellular preservation solution was infused into the PA. This contained (mmol l^{-1}): Na^+, 130; K^+, 5; Ca^{2+}, 2; Cl^-, 111; lactate, 29; glucose, 12; mannitol, 66; citrate, 10; together with bovine serum albumin 5 g l^{-1} and heparin 1000 U l^{-1}. The pH was adjusted to 7·3–7·4 by addition of small quantities of sodium bicarbonate solution (1 M). The lungs were continuously ventilated throughout the procedure.

The left atrium was incised to prevent pulmonary venous overload. When the venous effluent was completely clear of blood, the organs were excised and the heart was dissected free. The lungs were inflated to an airway pressure of 10 mmHg and the trachea and pulmonary artery were clamped.

Human lungs. Lungs were obtained at the time of excision of heart and lungs from heart–lung transplant (HLT) recipients ($n = 13$) and from single lung or cardiac donors ($n = 9$). The HLT recipients were premedicated with intramuscular morphine sulphate (0·2 mg kg^{-1}; Martindale Pharmaceuticals, Romford, Essex, UK) and midazolam (80 μg kg^{-1}). Anaesthesia was induced with intravenous fentanyl (1 mg kg^{-1}; Sublimaze; Janssen) and sodium methohexitone (1·5 mg kg^{-1}; Brietal Sodium; Lilly, Basingstoke, Hants, UK) and the patients were paralysed with intravenous vecuronium (10 mg; Organon-Teknika, Cambridge, UK). The surgical procedure for HLT has been described in detail (Reitz *et al.* 1982). After median sternotomy and intravenous administration of heparin, cardiopulmonary bypass was instituted. The recipient's heart was removed followed by sequential removal of the lungs. In the course of lung resection, the bronchial arteries were ligated, and the main bronchi were divided just distal to the carina. A few centimetres of extraparenchymal pulmonary artery were retained but only a few millimetres of pulmonary veins remained outside the lung surface.

Diseased lungs were obtained from patients undergoing transplantation for primary or secondary pulmonary hypertension. All the patients preoperatively had clinical signs of arterial hypoxaemia, pulmonary hypertension and right heart failure. Lungs obtained from cardiac or single lung transplant donors served as a control group. Haemodynamic measurements, arterial blood gas analysis and chest radiographs were taken on these patients as part of the routine assessment for organ donation (Wallwork *et al.* 1987). All the lungs had normal gas exchange (arterial oxygen tension to fractional inspired oxygen concentration ratio >300; mean, 408; range, 366–564) and haemodynamics (mean PA pressure, 16·4 mmHg; range, 12–21 mmHg) as well as clear chest radiographs. In these patients the heart–lung block was excised with the same donor procedure as for HLT and the lungs were dissected out immediately on excision. The recipient human lungs were preserved by the same technique as was used in the animals except that perfusion of the lungs with preservation fluid was carried out immediately after excision rather than before.

Perfusion of the lungs

The lungs were suspended by the hilar structures from a gravimetric balance (Model 235; Salter, London, UK) and enclosed in a Perspex chamber to conserve humidity. Temperature was maintained by heat lamps (Philips, Eindhoven, Holland) placed around the chamber. The lungs were ventilated with a gas mixture containing 20% O_2, 5% CO_2 and 75% N_2 by means of a Manley ventilator set at a rate of 15 breaths min^{-1}. The tidal volume was set at 10–12 ml (kg body weight)$^{-1}$ (half for single lung experiments) to give a maximum airway pressure of 10 mmHg. The inflation pressure was measured through a side-arm in the endobronchial tube connected to a pressure transducer (Model P50; Spectramed, Coventry, UK). A deep breath was simulated periodically to prevent atelectasis of the lung.

A recirculating circuit provided a controlled flow perfusion (Fig. 1). A roller pump (Model 16670; American Optical

Corporation, MA, USA) delivered the perfusate to the lungs through a cannula in the left main pulmonary artery. The venous drainage was free and the effluent ran into a constant-temperature reservoir through a filter. Flow rate was varied by altering the speed of the pump. In constant flow experiments only the left lung was used whereas both lungs were used for experiments at varying flow rates. The perfusate consisted of 3 l of buffered Krebs–Henseleit solution (containing in mmol l^{-1}: Na^+, 137; Cl^-, 125; K^+, 4·6; HCO_3^-, 15; HPO_4^-, 0·7; $H_2PO_4^-$, 1·5; Mg^{2+}, 0·5; Ca^{2+}, 1·2; glucose, 10) to which 35 g l^{-1} dextran was added to maintain colloid osmotic pressure. Bovine serum albumin (5 g l^{-1}) was also added to maintain normal endothelial function (Michel, 1988). In order to investigate the effects of blood on the responses to inhibition of NO release, autologous heparinized blood was used in a number of animal experiments. The perfusate was warmed to 37 °C in human experiments and 38 °C in animal experiments.

Pulmonary artery pressure (P_{PA}), referred to the level of the hilum, was recorded with a transducer (Model P50, Spectramed) through a catheter, of outer diameter 2 mm, placed in the main pulmonary artery. As venous drainage was free, pulmonary outflow pressure, equivalent to left atrial pressure, was therefore atmospheric pressure. Pulmonary flow ($\dot{Q}$) was recorded by a doppler ultrasound probe (Model T101D; Transonic Systems Inc., New York, USA) placed on the inflow cannula. Calibration curves were supplied by the manufacturers for saline and blood at different temperatures. A calibration curve was constructed for Krebs–dextran perfusate by measuring the time taken for collection of fixed volumes of perfusate at temperatures of 37 and 38 °C. Flow and pressure signals were displayed on a chart recorder (Model 404; W&W Scientific Instruments, Basel, Switzerland). The analog pressure and flow signals were digitized (sample rate 500 Hz; MP100; Biopac Systems Inc, Goleta, CA, USA) and data was stored on a microcomputer (Macintosh SE30; Apple Computer Inc, Cupertino, CA, USA).

Samples of the perfusate were collected anaerobically from a side-arm in the pulmonary artery cannula and the gas tensions and pH measured by means of a blood gas analyser (Model 1312; Instrumentation Laboratory, Milano, Italy). Samples were taken at the beginning of the experiment and subsequently before the addition of each of the pharmacological agents. Oxygen tension was never allowed to fall below 90 mmHg and pH was maintained between 7·3 and 7·4 by the addition of small volumes of sodium bicarbonate solution (1 M).

After the perfusion of the lung had been established, indomethacin (10^{-5} M) was added to the perfusate to inhibit formation of products of cyclo-oxygenase (Ferreira, Moncada & Vane, 1971).

A period of 20–30 min equilibration was allowed before the baseline pulmonary arterial pressure and flow were recorded. Measurements were made when the ventilator was switched off at end-expiration, so that airway pressure was atmospheric.

To detect development of pulmonary oedema sufficient to affect pulmonary vascular responses, the weight of the lung was monitored continuously. In preliminary experiments, P_{PA} and weight had been measured in lungs perfused at different flow rates. Pressure–flow relationships remained stable over time until the weight of the lungs had increased by more than 120% and oedema fluid appeared in the airways. Similar results have been reported in which hydrostatic oedema was induced in isolated lungs (Bhattacharya, Nakahara & Staub, 1980; Wang, Hakim, Michel & Chang, 1985). For the purposes of our study, a weight gain greater than 50% of the initial weight was taken to indicate that the lungs were damaged and the experiment was terminated.

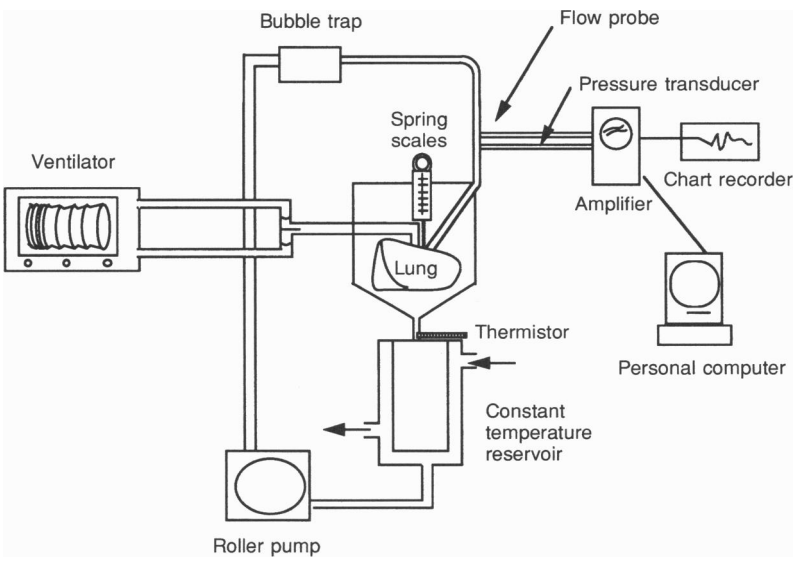

Figure 1. The isolated perfused lung system
Pulmonary artery pressure was measured from the system perfused at constant flow. The perfusate consisted of buffered Krebs–Henseleit solution with 35 g l^{-1} dextran and 5 g l^{-1} albumin, or autologous blood.

Drugs and chemicals

Methylene Blue, N^{ω}-nitro-L-arginine methyl ester (L-NAME), N^{G}-monomethyl-L-arginine (L-NMMA), indomethacin, sodium nitroprusside, bovine serum albumin and dextran were purchased from Sigma Chemical Company, Poole, Dorset, UK. Indomethacin was dissolved in 3% sodium carbonate. All other drugs were dissolved in physiological saline solution. Drugs were added as a bolus in a fixed volume of 2 ml of vehicle to the reservoir.

Experimental protocols

Constant flow experiments. The actions of inhibitors of NO synthesis or action were examined in single lungs from pigs, sheep, dogs and humans perfused with Krebs–dextran solution at a constant flow of 40 ml min^{-1} kg^{-1}. When perfusion conditions were stable, L-NAME was tested in human, pig, sheep and dog lungs. In the animal lungs, cumulative doses of L-NAME were added (from 0·1 to 100 mg in 2 ml vehicle; giving a final concentration in the perfusate of 10^{-7} to 10^{-4} M) whereas in human lungs only a single dose was given (10 mg in 2 ml vehicle; final concentration, 10^{-5} M). L-NMMA was tested in pig lungs in cumulative doses (0·1–100 mg in 2 ml vehicle; final concentration 10^{-7} to 10^{-4} M) and after the maximum response was achieved, L-arginine hydrochloride (600 mg in 2 ml vehicle; final concentration approximately 10^{-3} M) was added to attempt to restore NO production. The effect of Methylene Blue (100 mg in 2 ml vehicle; final concentration approximately 10^{-4} M) was studied in pig, sheep and human lungs. Subsequently, sodium nitroprusside (10 mg in 2 ml; giving a final concentration in the perfusate of approximately 10^{-5} M) was added to assess the responsiveness of the pulmonary smooth muscle to endothelium-independent nitrovasodilatation.

Experiments at varying flow. Measurements of P_{PA} were obtained at 6–8 different levels of flow. After each flow change a stable pressure trace was obtained before measurements were taken. The measurements were repeated both at increasing as well as decreasing flow rates. Pressure–flow ($P_{PA}/\dot{Q}$) relationships were determined for pig, sheep, dog and human lungs perfused with Krebs–dextran solution before and after addition of L-NAME (10 mg in 2 ml vehicle; final concentration approximately 10^{-5} M). Subsequently sodium nitroprusside (10 mg in 2 ml vehicle; final concentration approximately 10^{-5} M) was added and measurements of P_{PA} at different rates of $\dot{Q}$ were repeated to assess the responsiveness of the vessels.

The relationships of $P_{PA}/\dot{Q}$ were also investigated in pig, sheep and dog lungs perfused with autologous blood; in pig and dog lungs approximately 500 ml of Krebs–dextran solution was added to the perfusate to make up the required volume. In pig and dog lungs the effects of acute hypoxia on the response to L-NAME was also tested by generating $P_{PA}/\dot{Q}$ plots during ventilation with 8% O_2–5% CO_2–87% N_2 before and after addition of L-NAME. The responsiveness of the preparation to nitrovasodilatation was subsequently tested by generating a further $P_{PA}/\dot{Q}$ plot after the addition of sodium nitroprusside (10^{-5} M) in normoxic conditions.

Oedematous lungs. A total of four human and three sheep but no pig studies were stopped because of excessive weight gain and oedema formation. All the human lungs rejected were obtained from patients with a diagnosis of emphysema; the oedema may have been a result of the extensive alveolar structural abnormalities in these lungs.

Analysis. Maximum pressure changes were calculated as the difference between P_{PA} reached after treatment and the baseline P_{PA} (expressed in mmHg). Pulmonary vascular resistance was calculated by the formula:

$$\text{Pulmonary vascular resistance} = P_{Pa}\,\dot{Q}^{-1}\ \text{body wt}^{-1}.$$

Mean venous outflow pressure is taken as zero. Adjustment for the size of the different animal species was made using body weight (Milnor, 1982). Results are presented as means ± S.E.M. Mean values were compared by one-way analysis of variance and Scheffe's F test was used for multiple comparisons, or Wilcoxon signed-rank test for data which was not normally distributed. A linear portion of the $P_{PA}/\dot{Q}$ relationships was chosen for analysis (flow rate > 10 ml kg^{-1}). The data were analysed by analysis of covariance with P_{PA} as the dependent variable, $\dot{Q}$ as covariate and treatment as factor. Differences between treatments were assessed by Scheffe's F test; P values < 0·05 were considered significant. Using the parameter estimates obtained from the analysis, fitted lines and 95% confidence intervals were calculated for the range of flow explored.

RESULTS

Constant flow experiments

Effects of L-NAME and L-NMMA on pulmonary vascular resistance

In pig and sheep lungs, L-NAME caused a dose-dependent increase in pulmonary vascular resistance. In pigs resistance increased from 0·25 ± 0·04 to 0·69 ± 0·1 mmHg ml^{-1} min^{-1} kg^{-1}, (Fig. 2*A*; $n = 5$, $P < 0·0001$; F test) and in sheep from 0·16 ± 0·01 to 0·35 ± 0·03 mmHg ml^{-1} min^{-1} kg^{-1} (Fig. 2*C*; $n = 4$, $P = 0·0002$; F test). Similarly, L-NAME (10^{-4} M) increased pulmonary vascular resistance in human donor lungs from 0·13 ± 0·02 to 0·51 ± 0·19 mmHg ml^{-1} min^{-1} kg^{-1} (Table 1; $n = 5$, $P = 0·04$; Wilcoxon test). No effect of L-NAME on pulmonary vascular resistance was observed in the dog lungs ($n = 3$, Fig. 2*D*).

L-NMMA (10^{-7} to 10^{-4} M) increased pulmonary vascular resistance in three pig lungs from 0·26 ± 0·02 to 0·66 ± 0·1 mmHg ml^{-1} min^{-1} kg^{-1} (Fig. 2*B*; $P = 0·04$; F test). L-Arginine hydrochloride (10^{-3} M) then reduced pulmonary vascular resistance to 0·27 ± 0·01 mmHg ml^{-1} min^{-1} kg^{-1} which was not significantly different from initial levels.

Effects of Methylene Blue on pulmonary vascular resistance

Methylene Blue increased pulmonary vascular resistance in pigs from 0·23 ± 0·03 to 0·75 ± 0·1 mmHg ml^{-1} min^{-1} kg^{-1},

Table 1. Constant flow experiments, characteristics of patients undergoing HLT

Diagnosis	Age (years)	Sex	FiO_2	P_{a,O_2} (mmHg)	PVR_B (mmHg ml^{-1} min^{-1} kg^{-1})	ΔPVR (mmHg ml^{-1} min^{-1} kg^{-1})	Inhibitor
Donor	41	M	0·35	146·5	0·11	+0·09	L-NAME
Donor	17	M	0·45	171	0·13	+0·15	L-NAME
Donor	47	F	0·45	169	0·10	+1·14	L-NAME
Donor	23	M	0·45	174	0·14	+0·27	L-NAME
Donor	30	M	0·35	133	0·19	+0·24	L-NAME
CF	19	M	0·21	52	0·27	+0·82	MB
CF	31	M	0·21	40	0·34	+0·40	MB
PPH	40	M	0·21	54	1·40	+1·61	MB
PPH	47	F	0·21	48	1·58	+1·32	MB
ES	33	F	0·21	61	1·48	+0·34	L-NAME
ES	35	F	0·21	55	1·15	+0·33	L-NAME
ES	44	F	0·21	65	0·88	+0·19	L-NAME
CF	29	F	0·21	58	0·41	+0·27	L-NAME
CF	24	F	0·21	48	0·39	+0·23	L-NAME
COAD	47	M	0·21	63	0·63	+0·66	L-NAME

Abbreviations: P_{a,O_2}, preoperative arterial oxygen tension; PVR_B, initial pulmonary vascular resistance; ΔPVR, change in pulmonary vascular resistance with inhibitor; CF, cystic fibrosis; PPH, primary pulmonary hypertension; ES, Eisenmenger's syndrome; COAD, chronic obstructive airways disease; MB, Methylene Blue (10^{-4} M); L-NAME, N^{ω}-nitro-L-arginine methyl ester (10^{-5} M).

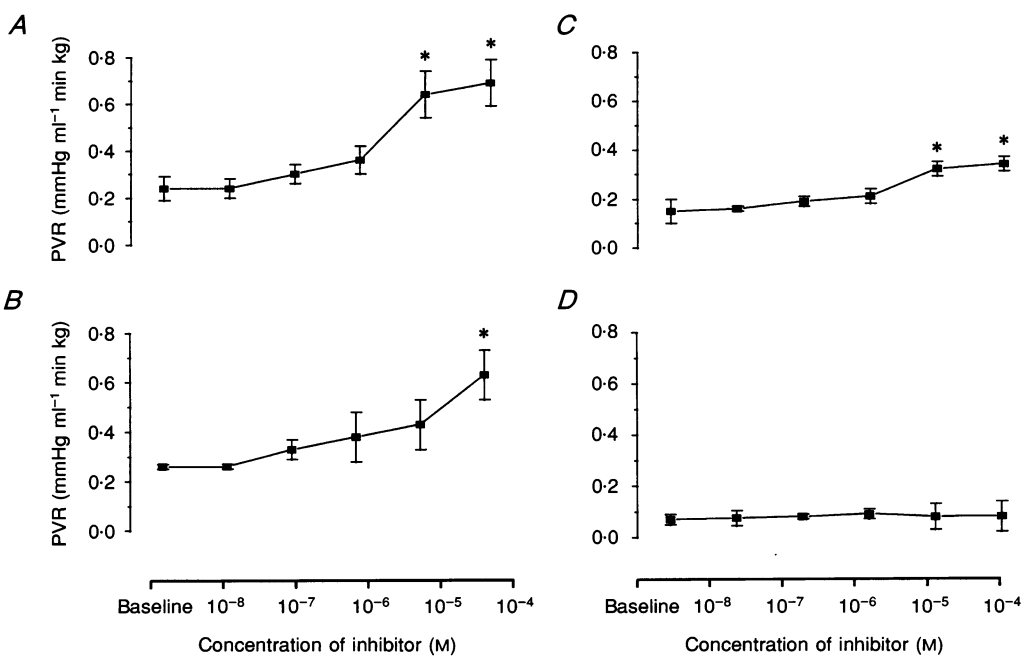

Figure 2. Effects of inhibitors of NO synthesis on pulmonary vascular resistance
Dose–response relationships of pulmonary vascular resistance to inhibitors of NO synthase. *A*, L-NAME in pig lungs ($n = 5$); *B*, L-NMMA in pig lungs ($n = 3$); *C*, L-NAME in sheep lungs ($n = 4$); *D*, L-NAME in dog lungs ($n = 3$). Values are means ± S.E.M.; asterisks denote values significantly different from baseline ($P < 0·05$).

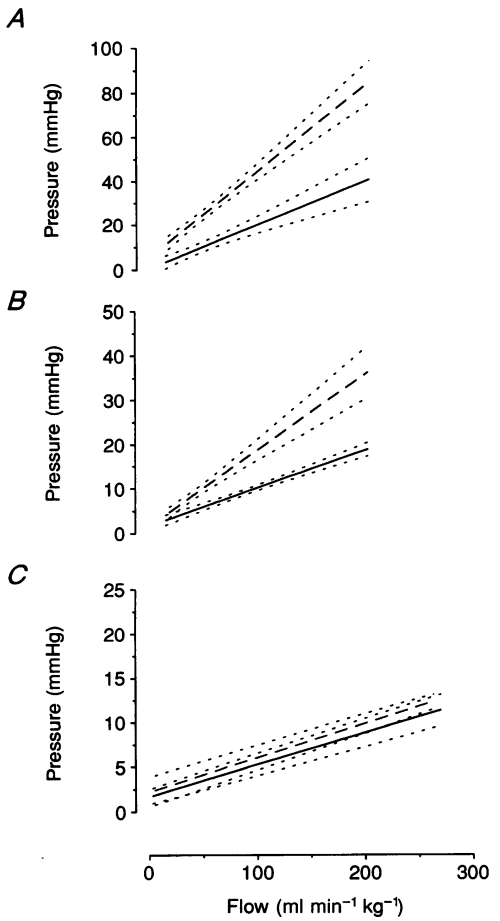

Figure 3. Changes in pressure–flow relationships after L-NAME in isolated lungs perfused with Krebs–dextran solution
Mean pressure–flow lines in isolated lungs of pig (*A*; $n = 6$), sheep (*B*; $n = 4$), and dog (*C*; $n = 3$), perfused with Krebs–dextran solution before (continuous line) and after (dashed line) L-NAME (10^{-5} M). Dotted lines show 95% confidence limits.

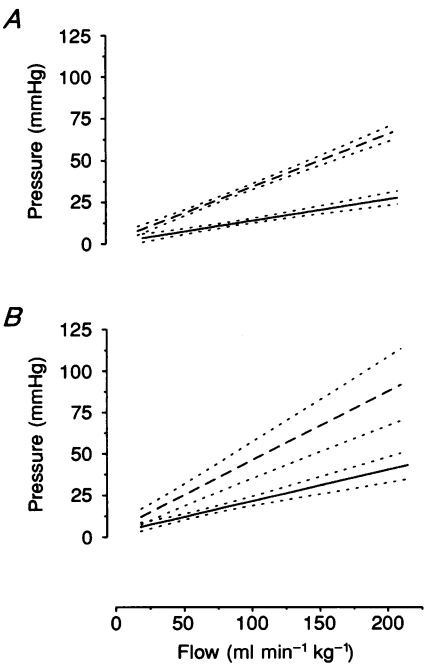

Figure 4. Changes in pressure–flow relationships after L-NAME in human isolated lungs perfused with Krebs–dextran solution
Mean pressure–flow lines in isolated human lungs perfused with Krebs–dextran solution before (continuous line) and after (dashed line) L-NAME (10^{-5} M). *A*, lungs from cardiac donors ($n = 4$); *B*, lungs from patients with cystic fibrosis ($n = 3$). Dotted lines show 95% confidence limits.

($n = 5$, $P = 0{\cdot}0002$; F test). Similarly, in sheep pulmonary vascular resistance rose from $0{\cdot}15 \pm 0{\cdot}01$ to $0{\cdot}5 \pm 0{\cdot}13$ mmHg ml^{-1} min^{-1} kg^{-1} ($n = 4$, $P = 0{\cdot}04$; F test) after addition of Methylene Blue. In both species sodium nitroprusside (10^{-5} M) reduced pulmonary vascular resistance to initial levels (pig, $0{\cdot}30 \pm 0{\cdot}02$ and sheep, $0{\cdot}34 \pm 0{\cdot}1$ mmHg ml^{-1} min^{-1} kg^{-1}; P = not significant; F test).

Experiments at varying flow

The difference between pressures obtained at increasing and decreasing flow rates was small. Both sets of data with equal weighting were used to generate $P_{PA}/\dot{Q}$ plots in all experiments.

Effect of L-NAME on $P_{PA}/\dot{Q}$ relationships

$P_{PA}/\dot{Q}$ plots were obtained for isolated pig (Fig. 3*A*), sheep (Fig. 3*B*), dog (Fig. 3*C*) and human donor lungs (Fig. 4*A*) perfused with Krebs–dextran solution. In the range of flow rates studied the $P_{PA}/\dot{Q}$ relationship was linear with a correlation coefficient always greater than 0·97. L-NAME (10^{-5} M) increased the slope of $P_{PA}/\dot{Q}$ lines by 0·19 mmHg ml^{-1} min^{-1} kg^{-1} in pig lungs ($n = 6$, $P < 0{\cdot}001$; F test), by 0·09 mmHg ml^{-1} min^{-1} kg^{-1} in sheep ($n = 4$, $P = 0{\cdot}02$; F test) and by 0·19 mmHg ml^{-1} min^{-1} kg^{-1} in human donor lungs ($n = 4$, $P = 0{\cdot}001$; F test). An increase in the intercept pressure was observed only in pig lungs. Addition of sodium nitroprusside (10^{-5} M) reversed the

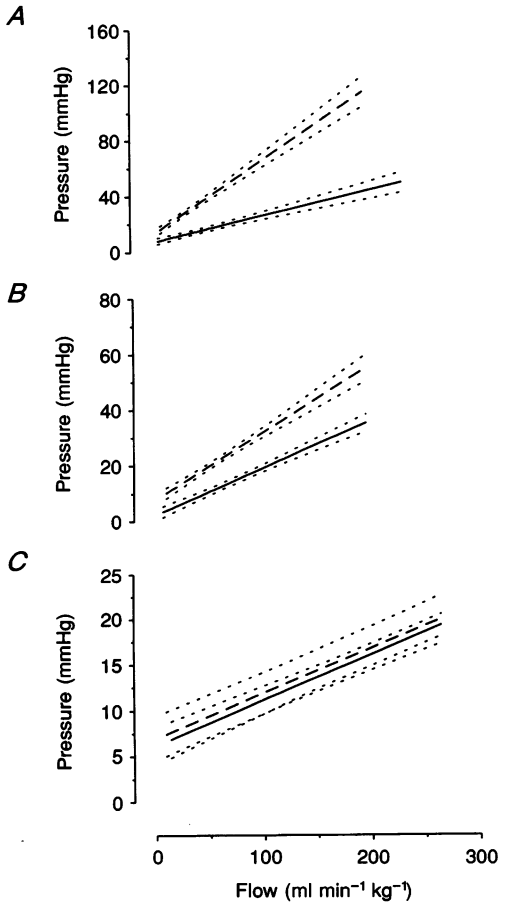

Figure 5. Changes in pressure–flow relationships after L-NAME in isolated lungs perfused with autologous blood

Mean pressure flow lines in isolated pig (*A*; $n = 5$), sheep (*B*; $n = 5$), and dog (*C*; $n = 3$) lungs perfused with autologous blood before (continuous line) and after (dashed line) L-NAME (10^{-5} M). Dotted lines show 95% confidence limits.

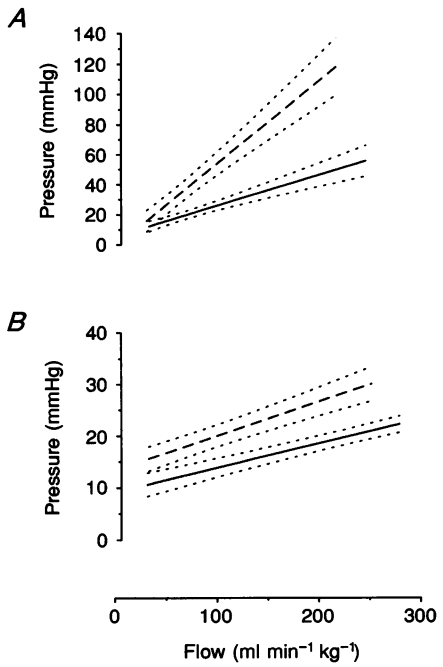

Figure 6. Effects of hypoxic ventilation and L-NAME on pressure–flow relationships of isolated lungs blood perfused with autologous blood

Mean pressure–flow lines in isolated lungs of pig (*A*; $n = 4$), and dog (*B*; $n = 3$), perfused with autologous blood and ventilated with 8% O_2 before (continuous line) and after (dashed line) L-NAME (10^{-5} M). Dotted lines show 95% confidence limits.

changes caused by L-NAME in the three species ($P > 0{\cdot}1$; *F* test, compared with initial values of slope and intercept in pig, sheep and human lungs). No effect on slope or intercept was seen with L-NAME in dog lungs perfused with Krebs–dextran solution (Fig. 3*C*; $n = 3$, $P = 0{\cdot}48$; *F* test).

Effect of blood on the response to L-NAME

The mean haematocrit was lower in pig and dog lungs ($21 \pm 1{\cdot}5$ and 23 ± 2%, respectively) than in sheep lungs (38 ± 4%) due to the addition of Krebs–dextran solution. Initial pulmonary vascular resistance was higher in the blood-perfused lungs than those perfused with Krebs–dextran solution in all the three species. In the sheep lungs the increase was reflected in a steeper slope of the $P_{PA}/\dot{Q}$ lines when perfused with blood whereas in the pig and dog lungs the $P_{PA}/\dot{Q}$ intercept pressures were higher but the slopes were unchanged. Addition of L-NAME (10^{-5} M) increased the slope of $P_{PA}/\dot{Q}$ lines in blood-perfused pig lungs by 0·34 mmHg ml^{-1} min^{-1} kg^{-1} (Fig. 5*A*; $n = 5$, $P < 0{\cdot}001$; *F* test). In sheep the slope of the $P_{PA}/\dot{Q}$ lines increased by 0·07 mmHg ml^{-1} min^{-1} kg^{-1} (Fig. 5*B*; $n = 5$, $P = 0{\cdot}001$, *F* test). The inhibitor increased the intercept pressure significantly in sheep lungs from 1·4 to 5·3 mmHg ($P = 0{\cdot}002$; *F* test) but not in pig lungs. Sodium nitroprusside (10^{-5} M) restored $P_{PA}/\dot{Q}$ lines to initial levels. No change in the slope or intercept pressure was observed in dog lungs after addition of L-NAME (Fig. 5*C*; $n = 3$, $P = 0{\cdot}9$; *F* test).

Effect of acute hypoxia on the response to L-NAME

Acute hypoxia increased the mean $P_{PA}/\dot{Q}$ intercept pressure by 3·7 mmHg in pig lungs ($n = 4$, $P < 0·05$; F test) and by 4·2 mmHg in dog lungs ($n = 3$, $P < 0·05$; F test). No increase in the slope of the $P_{PA}/\dot{Q}$ lines was observed in either of the species. L-NAME added during hypoxic ventilation increased the slope of $P_{PA}/\dot{Q}$ lines in pig lungs by 0·34 mmHg ml^{-1} min^{-1} kg^{-1} (Fig. 6*A*; $P < 0·05$; F test) while in dog lungs the slope increased by 0·02 mmHg ml^{-1} min^{-1} kg^{-1} and the intercept increased by 4·7 mmHg (Fig. 6*B*; $P < 0·05$; F test).

Effects of inhibition of NO synthesis and action in diseased human lungs

In lungs obtained from patients with secondary pulmonary hypertension (Table 1), L-NAME increased pulmonary vascular resistance from 0·66 ± 0·2 to 0·99 ± 0·2 mmHg ml^{-1} min^{-1} kg^{-1} (Table 1; $n = 6$, $P = 0·03$; Wilcoxon test). Similarly, Methylene Blue increased pulmonary vascular resistance in diseased lungs from 0·9 ± 0·3 to 1·94 ± 0·6 mmHg ml^{-1} min^{-1} kg^{-1} ($n = 4$; $P = 0·04$; Wilcoxon test). Sodium nitroprusside (10^{-5} M) reduced pulmonary vascular resistance to initial levels in both L-NAME and Methylene Blue-treated lungs. The rise in pulmonary vascular resistance induced by L-NAME was similar in both diseased and donor lungs (0·34 ± 0·07 and 0·38 ± 0·2 mmHg ml^{-1} min^{-1} kg^{-1}, respectively; $P = 0·8$; Wilcoxon test for unpaired data).

$P_{PA}/\dot{Q}$ plots were also generated for lungs from three patients in respiratory failure from cystic fibrosis (Fig. 4*B*). The mean slope and intercept pressure of the $P_{PA}/\dot{Q}$ lines in these lungs were higher than those in human donor lungs (Fig. 4*A* and *B*; $P < 0·05$, F test). Addition of L-NAME increased the slope of the $P_{PA}/\dot{Q}$ lines by 0·23 mmHg ml^{-1} min^{-1} kg^{-1} ($P < 0·01$, F test) in the diseased lungs. There was no difference between the increases of the $P_{PA}/\dot{Q}$ slope in the diseased and the donor lungs (0·23 and 0·19 mmHg ml^{-1} min^{-1} kg^{-1}, respectively, $P = 0·18$, F test).

DISCUSSION

Effects of inhibitors of NO release and action on normotensive lungs

In normoxic conditions, L-NAME, a competitive inhibitor of NO synthase, increased pulmonary vascular resistance in the isolated lungs of man, pig and sheep, but had no effect in dog lungs. Another analogue of L-arginine, L-NMMA, increased pulmonary vascular resistance in pig lungs in a similar fashion although effective inhibition occurred only at higher concentrations than with L-NAME. In the latter experiments, the reduction of pulmonary vascular resistance by excess L-arginine (10^{-3} M) implies that the action of L-NMMA was specific to the L-arginine–NO pathway (Rees, Palmer, Hodson & Moncada, 1989). A similar increase in pulmonary vascular resistance was observed in pig and sheep lungs when Methylene Blue, an inhibitor of NO production and action was used. Methylene Blue has also been shown to inhibit prostacyclin synthesis (Martin, Drazen & Newby, 1989). However, all the experiments were carried out in the presence of indomethacin, an inhibitor of cyclo-oxygenase, and it is therefore unlikely that the increase in pulmonary vascular resistance was caused by inhibition of prostacyclin. Sodium nitroprusside (10^{-5} M) fully restored initial pulmonary vascular resistance after inhibition with Methylene Blue or L-NAME, indicating that neither agent caused an irreversible inhibition of smooth muscle cytosolic guanylate cyclase (Feelisch & Noack, 1987).

Effects of L-NAME on $P_{Pa}/\dot{Q}$ relationships

In contrast to single point calculations, the use of $P_{PA}/\dot{Q}$ lines permits the assessment of flow-dependent effects of inhibitors of NO release in the pulmonary circulation. Although the $P_{PA}/\dot{Q}$ relationship of the pulmonary circulation is linear at the physiological range of flow, it is generally curvilinear at low flow rates. It has been suggested that in the linear range of $P_{PA}/\dot{Q}$, the pulmonary vascular bed is fully recruited and the dynamic resistance remains fairly constant (Mitzner, 1983). Extrapolation of the rectilinear portion of the $P_{PA}/\dot{Q}$ curve to zero flow has been considered to provide a weighted estimate of collapse pressure of the entire pulmonary vascular system, allowing the separation of the overall resistance into Starling and Ohmic components (Hakim, Chang & Michel, 1985). This study was not designed to elucidate the nature and distribution of pulmonary vascular resistance and although the Starling–Ohmic resistor model may be a simplification, changes in the slope and intercept of $P_{PA}/\dot{Q}$ lines remain valuable descriptors of active vascular responses in the lung.

In pig, sheep and human lungs, a similar increase of the $P_{PA}/\dot{Q}$ slopes was observed with L-NAME, indicating that inhibition of NO synthesis leads to an increase in the ohmic resistance of the pulmonary vascular bed of these three species. The effects on intercept pressure, however, were less consistent. In contrast, no effect on either slope or intercept was observed in the dog lungs treated with L-NAME.

Increasing haematocrit has been shown to increase pulmonary vascular resistance (Julien, Hakim, Vahi & Chang, 1985), and free haemoglobin (Martin, Villani, Jothianandan & Furchgott, 1985), as well as red blood cells (Evans, Ryley, Hallett & Lewis, 1989), reduces the vascular responses to NO. The affinity of haemoglobin for NO is about 280 times that for oxygen (Doyle & Hoekstra, 1981) and recent evidence indicates that the oxygen saturation of haemoglobin influences the binding of NO (Wennmalm, Benthin & Petersson, 1992). In our study, the slopes of the $P_{PA}/\dot{Q}$ lines were higher when the lungs were perfused with blood than with Krebs–dextran solution. However,

the response to L-NAME was not dependent on the type of perfusate used, suggesting that blood did not affect the NO acting on the vascular smooth muscle. The increase in the initial pulmonary vascular resistance may therefore have been due to the difference in the rheological properties of the perfusates, although a direct effect of red blood cells on NO cannot be ruled out. Studies on rabbit arteries (Bassenge, Busse & Pohl, 1987) have shown a greater abluminal secretion of NO by endothelial cells which may explain in part the lack of effect of blood on the action of L-NAME.

Shear stress has been shown to increase basal NO release in the systemic resistance arteries (Rubanyi, Romero & Vanhoutte, 1986; Griffith *et al.* 1989). The effects of L-NAME were similar at all flow rates in the lungs of each of the species studied in spite of the wide range of flow rates studied (10–200 ml min^{-1} kg^{-1}). This would suggest that either shear stress does not affect NO release in pulmonary vessels or that the increase in NO induced by shear stress in the pulmonary circulation is not sufficient to cause overall changes in pulmonary vascular resistance.

Effects of hypoxia on $P_{Pa}/\dot{Q}$ relationships

Acute hypoxia has been shown to decrease NO release in cultured pulmonary endothelial cells (Warren, Maltby, MacCormack & Barnes, 1989) and isolated pulmonary arteries (Johns, Linden & Peach, 1989), suggesting that failure of NO release may play a role in hypoxic vascoconstriction. On the other hand, hypoxic pulmonary vasoconstriction is generally enhanced after block of NO release (Mazmanian *et al.* 1989; Archer, Tolins, Raij & Weir, 1989; Persson *et al.* 1990). The discrepancy may be due to the different degrees of hypoxia achieved in cultured cells and isolated arteries as opposed to intact or *in vivo* preparations. The effects of acute hypoxia have been shown to be localized mainly on distal precapillary vessels (Hakim, Michel, Minami & Chang, 1983), and increase the intercept pressure of $P_{PA}/\dot{Q}$ lines. In our study, hypoxia, unlike L-NAME, increased the intercept of the $P_{PA}/\dot{Q}$ lines but did not affect the slope suggesting that hypoxia acts at a different site to L-NAME. Furthermore in both pig and dog lungs exposed to hypoxia, pulmonary vascular resistance fell after return to normoxic ventilation in spite of inhibition of NO synthesis. This would suggest that the mechanisms underlying hypoxic vasoconstriction are independent of changes in basal NO release.

Species differences in the response to L-NAME

In the dog, L-NAME increased the slope of the $P_{PA}/\dot{Q}$ lines only during hypoxic ventilation. Initial pulmonary vascular resistance was lowest in the dog and it is possible that the response to L-NAME may have been masked by the low vascular tone in normoxic conditions. In conscious dogs (Nishiwaki *et al.* 1992), inhibition of NO synthesis also had no effect on initial pulmonary vascular resistance except when the initial tone was raised by other agents. Similarly, in rats Barer *et al.* (1993) found no effect of L-NAME or L-NMMA on the pulmonary vascular resistance of isolated lungs except when the initial tone was raised by acute or chronic hypoxia or indeed by many other vasoconstrictors. The authors suggested that NO is not released in normoxic conditions unless vascular tone is raised and that the narrowing of the pulmonary vessels increases shear stress in turn stimulating NO release. In all the species used in our study the initial vascular tone was minimal and could not be significantly reduced by nitroprusside. Except in the dog, there was no correlation between the magnitude of the response to inhibitors and initial pulmonary vascular resistance. Likewise, although significant differences in the structure of the muscular pulmonary arteries exist in these species (Kay, 1983), they cannot fully account for the difference in response to NO inhibition. A similar lack of correlation between structure and response has been described for acute hypoxic vasoconstriction and may reflect an important difference between mammalian species in the regulation of basal pulmonary vascular tone.

The present observations therefore imply that continuous basal production of NO from L-arginine contributes to the regulation of basal vascular tone in the isolated lungs of pigs, sheep and humans but not in dogs.

Studies in several other species also suggest an important role for basal release of NO in modulating pulmonary vascular tone. Lobar infusion of Methylene Blue (Hyman *et al.* 1989) or L-NAME (McMahon *et al.* 1991) increased pulmonary vascular resistance in anaesthetized cats. Similar results were observed in spontaneously breathing newborn lambs with both Methylene Blue (Fineman, Crowley, Heymann & Soifer, 1991) or L-NAME (Fineman, Heymann & Soifer, 1991) and in anaesthetized but spontaneously breathing rabbits (Persson *et al.* 1990) with L-NAME. Recent work has shown that neural stimulation of intact lungs may cause NO release either directly from non-adrenergic, non-cholinergic nerve stimulation (Liu, Crawley, Evans & Barnes, 1992) or as a secondary consequence of the release of acetylcholine (McMahon, Hood & Kadowitz, 1992); such alternate sources of NO could account for the changes observed *in vivo*. Use of isolated lungs avoids conflicting influences from neural mechanisms and precludes such a source of NO. Furthermore, the isolated lung preparation avoids the passive mechanical effects of varying cardiac output and airway pressure.

Effects of Methylene Blue and L-NAME in diseased human lungs

In this study both Methylene Blue and L-NAME increased pulmonary vascular resistance in lungs from patients with various forms of pulmonary hypertension, indicating that NO is released from resistance vessels in these conditions. We were able to compare the action of L-NAME in human lungs without pulmonary vascular disease and in lungs

from patients with severe secondary pulmonary hypertension (Table 1). The absolute rise in mean pulmonary vascular resistance with L-NAME was similar in donor and pulmonary hypertensive lungs despite the higher level of pulmonary vascular resistance in the latter. Similarly, the increase in the $P_{PA}/\dot{Q}$ slope was comparable in both groups. Studies on isolated conduit pulmonary arteries from patients with secondary pulmonary hypertension have revealed impaired endothelium-dependent relaxation (Dinh-Xuan, Higenbottam, Clelland, Pepke-Zaba, Cremona & Wallwork, 1990*a*; Dinh-Xuan *et al.* 1991). This discrepancy may reflect differences in behaviour between conduit and resistance vessels (Orton, Reeves & Stenmark, 1988) or perhaps between basal and stimulated release of NO (Griffith, Edwards & Henderson, 1987). In our study, sodium nitroprusside, a nitrovasodilator which acts independently of the endothelium, reversed the rise in pulmonary vascular resistance caused by L-NAME in both groups of lungs, indicating that the vascular smooth muscle is capable of responding normally to NO. However, in the lungs from pulmonary hypertensive patients, sodium nitroprusside did not reduce pulmonary vascular resistance below initial values even at higher concentrations (10^{-3} M), suggesting that the elevated pulmonary vascular resistance in these lungs is more likely to be due to the extensive structural changes rather than a deficiency of basal release of NO.

In conclusion, inhibition of NO production and its effects in the pulmonary vasculature increased pulmonary vascular resistance in the isolated lungs of three mammalian species including man but had no effect in the dog. Continuous production of NO in the pulmonary vascular bed could therefore contribute to the low pulmonary vascular tone in some, but not all, species. Moreover, basal NO production appears to be conserved in human lungs with secondary pulmonary hypertension. The lack of effect of inhibition of NO synthesis in dog lungs may reflect a species difference in the regulation of pulmonary vascular tone of particular importance when interpreting results used as a model for disease in man.

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