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Endothelium-dependent relaxations in sheep pulmonary arteries and veins: resistance to block by N^G-nitro-L-arginine in pulmonary hypertension

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1 The effect of the nitric oxide synthase inhibitor, N^G-nitro-L-arginine (L-NOARG), on endothelium-dependent relaxation to a receptor-independent agent, ionomycin, was examined in isolated pulmonary arteries and veins from control, short-term and chronic pulmonary hypertensive sheep. All vessel segments were contracted to optimal levels of active force with endothelin-1 to record endothelium-dependent relaxation.

2 Pulmonary hypertension was induced by continuous pulmonary artery air embolization for 1 day (short-term) and 14 days (chronic) and was associated with a 2 and 3 fold increase in pulmonary vascular resistance respectively.

3 L-NOARG (0.1 mM) reduced the maximum relaxation (R_{max}) to ionomycin in large and medium-sized pulmonary arteries from control sheep by approximately 70%. By contrast, L-NOARG (0.1 mM) did not inhibit the R_{max} to ionomycin in matched vessels from short-term and chronic pulmonary hypertensive sheep.

4 Resistance of ionomycin-induced relaxations to inhibition by L-NOARG, was confined to the arterial vasculature in chronic pulmonary hypertensive animals, as relaxations to ionomycin in large and medium-sized chronic pulmonary hypertensive veins were, like those in control veins, abolished by L-NOARG. Both large and medium-sized pulmonary veins from short-term pulmonary hypertensive sheep, however, were resistant to block by L-NOARG.

5 Neither sensitivity (pEC_{50}) nor R_{max} to ionomycin in large, short-term pulmonary hypertensive arteries was affected when the extracellular concentration of K^+ was increased isotonically to 30 mM. Nifedipine (0.3 μM) was present throughout to prevent high K^+-induced smooth muscle contraction. In the presence of this high extracellular K^+, however, L-NOARG (0.1 mM) caused complete inhibition of the relaxation to ionomycin, whereas in normal extracellular K^+ (4.7 mM), L-NOARG only weakly inhibited ionomycin relaxations.

6 In conclusion, the onset of pulmonary hypertension in sheep following air embolization, is associated with the development of resistance of endothelium-dependent relaxations to block by L-NOARG. The mechanism of L-NOARG resistance appears to be due to the up-regulation of a K^+ channel-mediated backup vasodilator mechanism which can compensate for the loss of nitric oxide (NO)-mediated relaxation. Although this mechanism remains functionally 'silent' in the presence of NO it is able to maintain adequate endothelium-dependent vasodilatation during pulmonary hypertension if NO synthesis is compromised.

Keywords: EDRF; endothelium; nitric oxide; hyperpolarization; K^+ channels; pulmonary hypertension

Introduction

Most endothelium-dependent vasodilators which release nitric oxide (NO, Moncada *et al.*, 1991), also cause hyperpolarization of both endothelial and smooth muscle cells via opening of K^+ channels (Beny *et al.*, 1986; Nagao & Vanhoutte, 1992; Pacicca *et al.*, 1992; Garland & McPherson, 1992; Eckman *et al.*, 1994; for review, see Taylor & Weston, 1988). Studies by Cowan *et al.* (1993) and Kilpatrick & Cocks (1994) indicate that although NO is the principal mediator of endothelial-dependent relaxation in large systemic arteries, a K^+ channel-dependent mechanism acts as an important 'backup' for NO when its synthesis is blocked. Differential mediation of endothelium-dependent relaxation by multiple factors may have a role in the pathophysiology of diseases thought to be associated with endothelial cell dysfunction, such as pulmonary hypertension (Dinh-Xuan *et al.*, 1989; Cremona *et al.*, 1992). Thus, if a K^+ channel-dependent backup mechanism were present in the pulmonary circulation, then its contribution to endothelium-dependent relaxation might become more apparent following the development of pulmonary hypertension.

To examine this hypothesis, we have evaluated the effect of the potent NO synthase inhibitor, N^G-nitro-L-arginine (L-NOARG, Mulsch & Busse, 1990) on endothelium-dependent relaxations produced by the calcium ionophore, ionomycin, in isolated pulmonary arteries and veins obtained from normotensive sheep and those with short-term and chronic pulmonary hypertension induced by continuous pulmonary air embolization (Perkett *et al.*, 1988). In a sub-group of animals with short-term pulmonary hypertension, we also examined the contribution of K^+ channels to ionomycin-mediated relaxation in the absence and presence of L-NOARG. Our results indicate that the onset of pulmonary hypertension is associated with up-regulation of a K^+ channel-dependent relaxation mechanism in pulmonary arteries and veins. This mechanism remains silent until NO synthesis is blocked, whereupon it mediates endothelial-dependent relaxation.

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Methods

The study was performed in 17 chronically instrumented adult Border-Leicester cross ewes, aged 2–3 years. The animals comprised a sham-operated, normotensive, control group ($n=5$), a short-term, pulmonary hypertensive group ($n=5$) and a chronic, pulmonary hypertensive group ($n=7$). Hypertension was induced in the latter two groups with continuous pulmonary arterial air embolization (Perkett *et al.*, 1988; 1991) for 24 h and 14 days respectively. All experiments were conducted in accordance with guidelines established by the National Health and Medical Research Council of Australia and were approved by the Monash Medical Centre and Monash University Animal Experimentation Committees.

Surgical preparation

After an overnight fast and premedication with atropine sulphate (300 mg, s.c.), animals were anaesthetized with Diprivan (propofol 4–5 mg kg^{-1}, i.v., ICI, Australia), intubated and then ventilated with a mixture of 1–2% halothane, 33% O_2 and 66% N_2O. A thoracotomy was performed in the third left intercostal space under sterile conditions and the fourth rib sectioned near its sternal and vertebral ends to increase exposure of the heart and major vessels. The pericardium was incised over the right and left atrial appendages and pulmonary trunk, taking care not to damage the adjacent vagal and phrenic nerves. Polyvinyl catheters (1 mm i.d., 2 mm o.d.) were inserted into the left and right atrial cavities via the appendages for pressure measurement. Two polyvinyl catheters with smaller polyvinyl tubing (0.05 mm i.d., 0.08 mm o.d.) telescoped into their tips were inserted into the right atrial cavity for infusion of air emboli. Teflon cannulae connected to polyvinyl catheters were introduced into the aortic arch and the pulmonary trunk for pressure measurement, as previously described (Woods & Smolich, 1991). An ultrasonic flow probe (model 24S, Transonic Systems, Ithaca, NY, U.S.A.) was placed around the pulmonary trunk, proximal to the pulmonary artery catheter. After ensuring that haemostasis was adequate, the left hemithorax was lavaged with warm, isotonic saline containing antibiotics, and the ribs then re-apposed. Vascular catheters were filled with concentrated heparin (500 iu ml^{-1}) and, together with the flow probe cable, were tunnelled subcutaneously and exteriorized above the left scapula. Following repair of muscle layers and closure of the skin, air was evacuated from the chest cavity via a temporary, wide-bore intercostal tube connected to an underwater drainage system. Antibiotics (procaine penicillin 500 mg and dihydrostreptomycin 500 mg, i.m.) were administered at the start and end of the surgical procedure. At the end of surgery, animals were sedated with acepromazine (2 mg, i.m.) and given the anti-inflammatory agent, Finadyne (flunixin meglumine, 50 mg, i.m.) as required.

Animals were allowed 10 days to recover from surgery. During this time, antibiotics (procaine penicillin 500 mg and dihydrostreptomycin 500 mg, i.m.) and aspirin sulphate (150 mg orally) were administered daily. Postoperatively, vascular catheters were flushed regularly and the dead space filled with sodium heparin (500 iu ml^{-1}). On the 7th day after surgery, sheep were transferred to a mobile cage, where they were fitted with a restraining collar which permitted free access to food and water, but prevented excessive circular movement.

Induction of pulmonary hypertension

Animals were brought into the laboratory in their mobile cage on at least two consecutive days prior to recording of baseline haemodynamic measurements, to familiarize them with the laboratory surroundings. To induce pulmonary hypertension, sterile air microemboli (approximately 300 μm o.d.) were infused continuously into the pulmonary circulation using a technique similar to that described by Perkett *et al.* (1988). Briefly, room air was passed through large (PFF-100, Bentley laboratories) and micropore (Millex-GS 0.22 μm Filter Unit, Millipore) air filters connected via an intervening roller pump (MS 4-Reglo, Ismatec) to extension tubing which was, in turn, connected to a telescoped right atrial catheter.

After measuring baseline haemodynamics and blood gases, air infusion was started at a rate of 0.02–0.04 ml kg^{-1} min^{-1}, in order to increase mean pulmonary artery pressure by approximately 50% and pulmonary vascular resistance 2–3 fold (Perkett *et al.*, 1988; 1991). Haemodynamics and blood gases were monitored at 30 min intervals for at least 2 h after the start of air embolization to ensure stability of pulmonary variables. Pulmonary pressures and blood gases were checked daily and the rate of air infusion adjusted as necessary to maintain a stable level of pulmonary hypertension. During air embolization, animals received 150 mg of aspirin orally every second day, to limit the extent of arterial plugging associated with this model.

Air was continuously infused for 24 h in short-term pulmonary hypertensive sheep and 14 days in chronic pulmonary hypertensive sheep. The air infusion was stopped at the end of these periods and haemodynamics and blood gases were measured when they had stabilized 1 h later (Perkett *et al.*, 1988). The control sheep were treated in an identical manner to the chronic pulmonary hypertensive animals, except that they did not undergo air embolization.

Physiological measurements

Pulmonary arterial, aortic, left atrial and right atrial blood pressures were measured with silicon chip transducers (model CDX-111, COBE Laboratories Inc, Lakewood, CO, U.S.A.) calibrated against a water manometer. Vascular pressures were referenced to atmospheric pressure at the mid-chest position of the animal. Pulmonary blood flow was measured with an ultrasonic flowmeter (model T208, Transonic Systems Inc, Ithaca, NY, U.S.A.). Flow and pressure signals were amplified with a programmable signal conditioner (CyberAmp 380, Axon Instruments, Foster City, CA, U.S.A.) and displayed continuously on a 8-channel chart recorder (Neotrace 800ZF, Neomedix Systems, Sydney, Australia). Pressure and flow data were also collected onto a computer hard disc at a sampling rate of 200 Hz using an analogue-to digital converter (DAS16, MetraByte). Data stored on computer were later analyzed with interactive software to obtain mean pressures, cardiac output and pulmonary vascular resistance, calculated as (mean pulmonary artery pressure − mean left atrial pressure) / cardiac output. Right ventricular output could not be measured in one short-term pulmonary hypertensive sheep because of a flow probe malfunction. In this animal, cardiac output was measured by thermodilution using a Swan-Ganz catheter inserted into the pulmonary arterial tree via the left external jugular vein and a cardiac output computer (model 9520, Edwards Laboratory, Santa Anna, CA, U.S.A.).

Aortic and pulmonary arterial blood pH, P_{O_2}, P_{CO_2} and base excess were measured in 0.5 ml samples at 40°C with a blood analyzer (model ABL 500, Radiometer, Copenhagen, Denmark). Blood haemoglobin content and oxygen saturation were measured in duplicate with a hemoximeter (model OSM2, Radiometer, Copenhagen, Denmark). Oxygen content of aortic or pulmonary arterial blood (ml O_2 per dl blood) was calculated as $(1.36 \times HbS \times Hb/100) + (0.003 \times P_{O_2})$ where HbS = haemoglobin oxygen saturation (%), Hb = haemoglobin level (g dl^{-1}) and P_{O_2} = oxygen tension (mmHg).

Dissection and mounting of pulmonary vessels in organ baths

Control and pulmonary hypertensive sheep were killed with an i.v. overdose of sodium pentobarbitone, after administration of sodium heparin (5000 iu, i.v.) to prevent postmortem coagulation. The right and left lungs were rapidly removed through a median sternotomy and, after flushing the main pulmonary arteries and veins with cold oxygenated Krebs so-

lution, were immersed in Krebs solution prior to dissection. After careful exposure of the intrapulmonary vascular network, the main (large) and a first-order (medium-sized) branch of the pulmonary artery and main (large), as well as a first-order (medium-sized) tributary of the pulmonary vein, were excised and placed in oxygenated Krebs solution.

The vessels were cut into 3 mm ring segments and suspended on 350 μm diameter stainless steel wire hooks, in 25 ml glass organ baths containing Krebs solution. The organ baths were bubbled continuously with carbogen (95% O_2, 5% CO_2) and maintained at 37°C with a heated water jacket. The upper hook was suspended from a force transducer (model FTO3C, Grass, Quincy, MA, U.S.A.) to measure changes in isometric tension, which were recorded on dual channel flat-bed recorders (W & W Scientific Instruments, Basel, Switzerland). The lower hook was fixed to a support leg attached to a micrometer which was used to measure the distance between the two wires. The Krebs solution was composed of (in mM): Na^+ 144, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128.7, HCO_3^- 25, SO_4^{2-} 1.2 and glucose 11, pH 7.4.

Normalization procedure of vessels

A two step procedure was used to determine the passive tension which was optimal for contraction of isolated pulmonary arteries and veins from control and chronic pulmonary hypertensive sheep. First, vessel rings were set to passive tensions equivalent to that required to produce 90% of their internal circumference when exposed to transmural pressures ranging between 5–50 mmHg for arteries and 5–30 mmHg for veins (refer to Mulvany & Halpern, 1977; Angus *et al.*, 1986). Second, at each distending pressure, concentration-contraction responses to KCl (10–100 mM) were constructed to determine the distending pressure at which the contraction to KCl was maximal. Over the range of distending pressures examined, the maximal contraction to KCl did not differ between control and chronic pulmonary hypertensive large arteries (Figure 1a) or large veins (Figure 1b). Similar results were obtained in medium-sized arteries and veins from control and chronic pulmonary hypertensive sheep (data not shown). Therefore 30 mmHg was used as the optimal equivalent distending pressure for large and medium-sized arteries and 20 mmHg for large and medium-sized veins from both control and chronic pulmonary hypertensive sheep. Also, as K^+ contractions did not differ between control and chronic pulmonary hypertensive vessels, the same equivalent distending pressures were employed in both arteries and veins from short-term pulmonary hypertensive sheep.

Evaluation of endothelial-dependent relaxation

Isolated vessel segments in which endothelium-dependent relaxation was to be examined were stretched to the predetermined level of passive tension, left to equilibrate for 1–2 h and then precontracted with endothelin-1. The concentration of endothelin-1 used for this precontraction had been previously determined in a separate vessel ring by generation of a cumulative (0.5 log unit steps) endothelin-1 concentration-contraction curve. Upon completion of the endothelin-1 contraction curve in this vessel segment, K^+ (75 mM) was added to the organ bath to determine the maximal contraction (F_{max}). A submaximal concentration of endothelin-1 (0.3–1 nM), which contracted vessels to <50% F_{max}, was then chosen to precontract vessels used to study endothelium-dependent relaxation. Prior to the addition of endothelin-1, indomethacin (3 μM) was added to inhibit the release of prostanoids (PGI_2). Once the endothelin-1 contraction had reached a plateau, cumulative concentration-relaxation responses to the endothelium-dependent, receptor-independent vasodilator, ionomycin (a calcium ionophore), were constructed in the absence and presence of N^G-nitro-L-arginine (L-NOARG, 0.1 mM). L-NOARG was added after the endothelin-1 contraction had reached a plateau, and ionomycin was then added after a further 20 min equilibration period. The relaxation response to 0.3 μM ionomycin was taken as maximum in all cases since concentrations above 0.3 μM caused only contractions. The maximal relaxation to the endothelium-independent vasodilator, sodium nitroprusside (SNP, 10 μM) was obtained in each vessel segment and all ionomycin relaxation responses (R_{max}) were normalized as percentages of the relaxation to SNP. To assess endothelial-independent relaxation, cumulative-concentration relaxation responses to SNP were also constructed and the results expressed as a percentage reversal of the endothelin-1 contraction. Only one concentration-relaxation curve to ionomycin or SNP was obtained for any one ring of tissue.

In large and medium-sized pulmonary arteries and veins from control sheep, relaxation responses to ionomycin were repeated in rings denuded of endothelium. The endothelium was removed by gently rotating vessels around the tip of fine forceps.

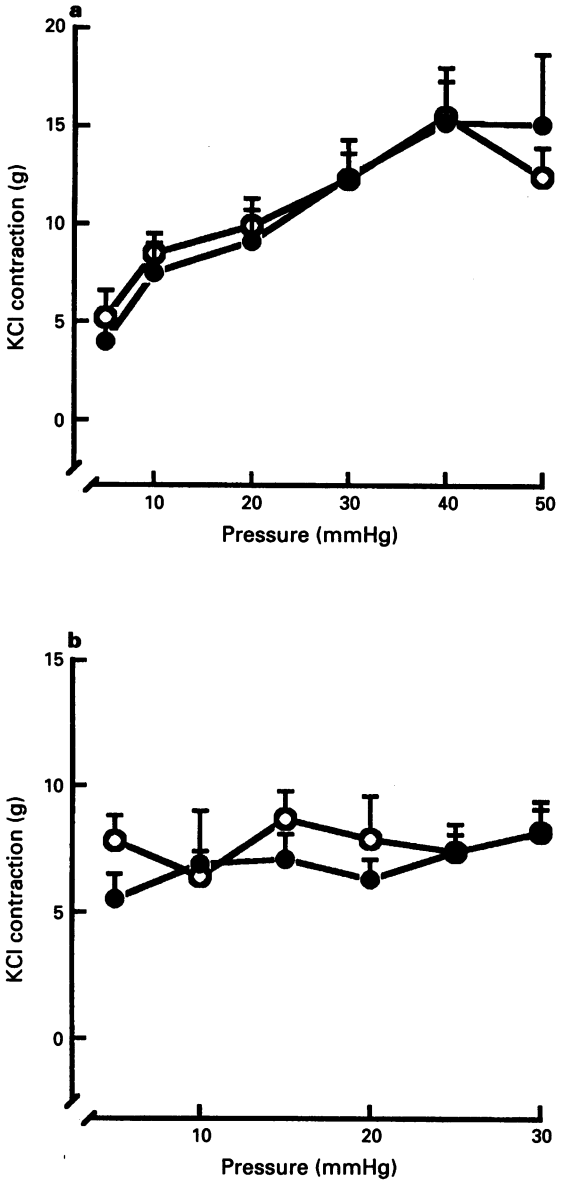

Figure 1 Maximum contraction to KCl (100 mM) over a range of distension pressures in isolated large pulmonary arteries (a) and large pulmonary veins (b) from control (○, $n=5$) and chronic pulmonary hypertensive sheep (●, $n=7$). Values are mean ± s.e.mean. n = number of rings. NB. For arteries from control and chronic pulmonary hypertensive sheep only the contraction to KCl at 5 mmHg was significantly different from any other value ($P<0.01$, ANOVA). Contractions to KCl at distension pressures of 10–50 mmHg did not differ significantly.

Effect of high extracellular K^+ upon endothelium-dependent relaxation

To ascertain the contribution of K^+ channels to ionomycin-mediated relaxation, the effect of high K^+ isotonic Krebs solution upon ionomycin relaxations was examined in a separate group of large pulmonary arteries obtained from short-term pulmonary hypertensive sheep. All arteries were treated with 0.3 μM nifedipine for 30 min following stretch to an optimal level of passive tension, to avoid any functional antagonism resulting from K^+ -induced smooth muscle contraction. The arteries were divided into 2 groups, one which was bathed in normal Krebs solution and another in Krebs solution in which 25 mM NaCl had been isotonically substituted with 25 mM KCl to produce a final K^+ concentration of 30 mM. L-NOARG (0.1 mM) was added to half of the arteries in normal Krebs and half of the arteries in high K^+ Krebs (30 mM). Following a further 20 min equilibration period, all arteries were contracted to a steady state level with a submaximal concentration of endothelin-1 (0.3–1 nM). Cumulative concentration-relaxation response curves to ionomycin were then constructed, and the maximal relaxation to SNP (10 μM) was obtained at the end of each experiment, as described above.

Drugs

Drugs used in the isolated vessel studies and their sources were: endothelin-1 (Auspep, Victoria, Australia); indomethacin, ionomycin, N^G-nitro-L-arginine (L-NOARG) (Sigma, U.S.A.); sodium nitroprusside dihydrate (Roche, Dee Why, NSW, Australia); (-)-nifedipine (Bayer A.G., Wuppertal, Germany). Stock solutions of nifedipine (10 mM) and ionomycin (1 mM) were made up in absolute ethanol, L-NOARG (100 mM) in 1 M $NaHCO_3$ and indomethacin (100 mM) in 1 M Na_2CO_3. All dilutions of stock solutions were in distilled water and all other drugs were made up in distilled water.

Statistical analysis

The individual contraction and relaxation curves were fitted to the sigmoidal logistic equation, $Y = P_1 + P_2/[1 + eP_3(\log X - P_4)]$, where X = agonist concentration, P_1 = lower plateau response, P_2 = range between the lower and the maximal plateau of the concentration-response curve, P_3 = a negative curvature index indicating the slope independent of the range and P_4 = log concentration required to produce a half maximal response (pEC_{50}). From this relationship, computer estimates of the concentrations required to give 50% of the maximum response (pEC_{50}) were determined (see Elghozi & Head, 1990) and expressed as $-\log$ M. pEC_{50} values could not be determined for ionomycin relaxation curves which were non-sigmoidal in nature or where the maximum relaxation to ionomycin was < 20% of the relaxation to SNP. Note, however, that when a plateau response to ionomycin was not obtained at 0.3 μM then the response at this concentration was assumed to be maximal for pEC_{50} determination.

The significance of differences in mean pEC_{50} and R_{max} values within experimental groups (e.g. ionomycin relaxation in the absence and presence of L-NOARG) were tested using Student's two-tailed unpaired t test. Comparisons of the pEC_{50}, R_{max}, F_{max} and vessel parameters between experimental groups were performed by one way analysis of variance (ANOVA). If the F statistic exceeded the critical value, then post ANOVA tests were applied. Specifically, Dunnett's modified t statistic was used to make comparisons between the control and pulmonary hypertensive groups and the Tukey-Kramer modified t statistic was used to make comparisons between all experimental groups. Changes in haemodynamic and blood gas parameters within control, short-term and chronic pulmonary hypertensive animals were analysed by the Student's paired two-tailed t test. Results are expressed as mean ± s.e.mean and statistical significance was accepted at the $P < 0.05$ level.

Results

Physiological measurements

In control animals, pulmonary arterial, aortic and atrial pressures, cardiac output and pulmonary vascular resistance were similar at baseline and 14 days later (Table 1). Short-term air embolization increased pulmonary artery pressure by 50% ($P < 0.05$) and pulmonary vascular resistance by 120% ($P < 0.05$) (Table 1). Chronic air embolization increased pulmonary artery pressure by 70% ($P < 0.05$) and pulmonary vascular resistance by 190% ($P < 0.05$). Air embolization reduced left and right atrial pressures and lowered cardiac output in chronic pulmonary hypertensive animals, but had no effect on aortic pressures (Table 1).

Apart from a small increase in aortic Po_2, blood gas variables at baseline and 14 days later were similar in control animals (Table 2). Short-term air embolization was associated with minor changes in aortic and pulmonary arterial Po_2 and haemoglobin levels, but these had no significant effect on aortic and pulmonary O_2 content. By contrast, chronic air embolization was accompanied by reductions in aortic and pulmonary arterial haemoglobin concentration, haemoglobin saturation, Po_2 and oxygen content (Table 2).

Resting vessel parameters

A summary of normalized resting vessel diameters and F_{max} values from control, short-term and chronic pulmonary hypertensive sheep is given in Table 3. Large and medium-sized pulmonary arteries and medium-sized pulmonary veins from short-term pulmonary hypertensive sheep were significantly

Table 1 Haemodynamic parameters for control, short-term and chronic pulmonary hypertensive sheep

	Control sheep ($n=5$)		*Short-term pulmonary hypertensive sheep* ($n=5$)		*Chronic pulmonary hypertensive sheep* ($n=7$)	
Haemodynamics	*Baseline*	*Day 14*	*Baseline*	*Day 1*	*Baseline*	*Day 14*
PABP (mmHg)	10.3 ± 0.4	10.0 ± 1.7	9.0 ± 0.7	13.5 ± 1.5*	10.9 ± 1.0	18.5 ± 2.4*
SABP (mmHg)	78.8 ± 2.3	78.8 ± 1.8	72.2 ± 2.7	75.2 ± 2.9	79.1 ± 2.9	76.0 ± 2.7
LABP (mmHg)	−0.9 ± 0.6	0.3 ± 0.8	0.1 ± 0.9	−1.7 ± 1.2*	2.6 ± 1.4	−1.5 ± 0.8*
RABP (mmHg)	−2.5 ± 1.2	−2.7 ± 0.8	−2.3 ± 0.7	−5.7 ± 1.4**	−2.2 ± 0.9	−5.0 ± 0.4*
CO ($l\ min^{-1}$)	4.2 ± 0.6	4.3 ± 0.2	3.4 ± 0.4	3.0 ± 0.6	4.3 ± 0.6	3.5 ± 0.5**
PVR ($mmHg\ l^{-1}\ min^{-1}$)	2.7 ± 0.2	2.0 ± 0.2	2.6 ± 0.3	5.7 ± 0.9*	2.2 ± 0.4	6.3 ± 1.2**

Values quoted on Day 1 and Day 14 for short-term and chronic pulmonary hypertensive sheep respectively were obtained 1 h after air embolization was stopped.
PABP, mean pulmonary arterial blood pressure; SABP, mean systemic arterial blood pressure; LABP, mean left atrial blood pressure; RABP, mean right atrial blood pressure; CO, cardiac output; PVR, pulmonary vascular resistance. Values are mean ± s.e.mean. n = number of sheep.
*$P < 0.05$; **$P < 0.01$ compared with baseline value, Student's paired t test.

Table 2 Aortic and pulmonary artery blood gas values

	Control sheep ($n=5$)		*Short-term pulmonary hypertensive sheep* ($n=5$)		*Chronic pulmonary hypertensive sheep* ($n=7$)	
	Baseline	*Day 14*	*Baseline*	*Day 1*	*Baseline*	*Day 14*
Aortic						
Haemoglobin (g dl^{-1})	8.0±0.6	8.0±0.5	9.0±0.2	9.9±0.3**	8.2±0.3	7.3±0.3**
pH	7.47±0.03	7.47±0.01	7.46±0.02	7.47±0.01	7.49±0.01	7.50±0.01
O_2 saturation (%)	93.7±1.0	94.8±1.2	98.2±1.6	94.1±3.0	95.9±0.2	90.8±1.2**
P_{O_2} (mmHg)	100.3±2.0	110.6±4.8*	100.5±3.0	87.9±7.4*	109.6±3.5	86.0±4.5**
P_{CO_2} (mmHg)	39.4±3.1	35.5±0.9	36.8±1.2	37.3±3.4	36.9±1.1	37.4±1.4
Base excess (mmol l^{-1})	5.3±1.1	3.2±0.9	2.1±1.2	3.5±1.9	5.1±0.5	4.6±1.0
O_2 content (ml dl^{-1})	10.5±0.7	10.7±0.7	12.4±0.3	13.0±0.7	11.0±0.4	9.3±0.4**
Pulmonary artery						
Haemoglobin (g dl^{-1})	8.3±0.6	8.5±0.6	9.6±0.3	10.6±0.4*	8.6±0.3	7.8±0.4**
pH	7.44±0.02	7.43±0.01	7.42±0.02	7.43±0.01	7.44±0.01	7.44±0.01
O_2 saturation (%)	53.1±4.4	54.5±2.7	61.9±2.3	56.3±3.8	56.0±1.2	44.3±1.9***
P_{O_2} (mmHg)	45.5±3.1	45.4±1.3	45.3±1.7	40.8±0.6	46.8±1.7	40.5±1.3*
P_{CO_2} (mmHg)	42.8±1.3	42.2±0.3	41.7±1.5	42.4±3.3	43.9±1.2	43.9±1.4
Base excess (mmol l^{-1})	4.8±0.9	4.3±1.0	2.1±1.2	3.1±1.7	5.5±0.4	5.2±0.8
O_2 content (ml dl^{-1})	6.3±0.8	6.5±0.7	8.2±0.4	8.2±0.6	6.7±0.3	4.9±0.3***

Values quoted on Day 1 and Day 14 for short-term and chronic pulmonary hypertensive sheep respectively were obtained 1 h after air emobolization was stopped. Values are mean±s.e.mean. n=number of sheep.
*$P<0.05$; **$P<0.01$; ***$P<0.001$ compared with baseline value, Student's paired t-test

Table 3 Sheep pulmonary artery and vein–summary of vessel diameters at normalization

	Number of sheep	*Number of rings*	*Diameter* (mm)	F_{max} (g)		$F_{max}/Diam$ ($g\,mm^{-1}$)	
Large pulmonary artery							
Control	5	17	7.74±0.26	13.5±0.9	(9)	1.84±0.20	(9)
Short-term pulmonary hypertension	5	23	5.52±0.28**	10.6±1.5	(5)	2.00±0.28	(5)
Chronic pulmonary hypertension	7	7	7.18±0.34	12.4±1.9	(7)	1.70±0.24	(7)
Medium-sized pulmonary artery							
Control	5	21	4.38±0.30	8.2±0.9	(10)	1.81±0.22	(10)
Short-term pulmonary hypertension	5	24	2.97±0.10**	5.5±0.5	(5)	1.70±0.20	(5)
Chronic pulmonary hypertension	7	7	2.93±0.21**	4.4±1.1*	(7)	1.52±0.35	(7)
Large pulmonary vein							
Control	5	13	6.44±0.41	4.9±0.7	(7)	1.26±0.13	(7)
Short-term pulmonary hypertension	5	17	5.51±0.27	3.3±0.7	(3)	1.10±0.10	(3)
Chronic pulmonary hypertension	7	23	5.30±0.30	7.1±1.6	(7)	2.57±0.52*	(7)
Medium-sized pulmonary vein							
Control	5	24	4.23±0.27	9.9±0.9	(9)	1.41±0.18	(9)
Short-term pulmonary hypertension	5	24	2.27±0.17**	12.4±0.6	(4)	2.11±0.17	(4)
Chronic pulmonary hypertension	7	29	2.51±0.20**	11.1±1.9	(8)	2.36±0.37	(8)

Values in parentheses refer to the number of vessels in which F_{max} was obtained. Values are mean±s.e.mean.
*$P<0.05$; **$P<0.01$ compared with control value, Dunnett's modified t statistic.

($P<0.01$) smaller in diameter than the corresponding control vessels (Table 3). With chronic pulmonary hypertension this decrease in diameter was sustained in medium-sized pulmonary arteries and veins but not in large pulmonary arteries (Table 3).

Endothelin-1 contraction

Endothelin-1 (0.01–30 nM) caused concentration-dependent contractions in large and medium-sized control pulmonary arteries with pEC_{50}s of 9.07±0.08 and 8.79±0.17, and maximum contractions of 12.6±1.2g (89.8±2.8% F_{max}) and 8.9±0.9g (93.4±3.8% F_{max}) respectively. In control large and medium sized pulmonary veins, endothelin-1 generated a maximum force of 10.9±0.5g (96.6±2.2% F_{max}) and 4.8±1.4g (93.4±5.1% F_{max}) with pEC_{50} values of 8.86±0.15 and 8.83±0.18 respectively. The pEC_{50} and $\%F_{max}$ to endothelin-1 of large and medium-sized pulmonary arteries and veins from short-term and chronic pulmonary hypertensive sheep did not differ significantly from the control values.

SNP relaxation

The level of precontraction with endothelin-1 in arteries and veins prior to the addition of SNP did not differ between experimental groups. SNP (0.001–30 μM) caused a concentration-dependent relaxation of large ($pEC_{50}=7.46\pm0.14$) and medium-sized control pulmonary arteries ($pEC_{50}=6.84\pm0.17$), reversing the endothelin-1 contraction by 98.8±1.2% and 93.5±4.9% respectively. The pEC_{50} and maximum response to SNP in large and medium-sized arteries from short-term and chronic pulmonary hypertensive sheep did not differ significantly from control. SNP relaxed large ($pEC_{50}=6.86\pm0.33$) and medium-sized control pulmonary veins ($pEC_{50}=6.90\pm0.07$) to 100±0% and 95.0±5.0% respectively. The maximum relaxation to SNP in large and medium-sized short-term and chronic pulmonary hypertensive veins did not differ significantly from control. Large ($pEC_{50}=7.82\pm0.24$) and medium-sized short-term pulmonary hypertensive veins ($pEC_{50}=8.06\pm0.30$) and large chronic pulmonary hypertensive veins ($pEC_{50}=7.83\pm0.13$), however,

were significantly more sensitive to SNP compared with control vessels ($P<0.05$).

Ionomycin relaxations

Relaxation responses to ionomycin in large and medium-sized pulmonary arteries and veins from control sheep were abolished upon removal of the endothelium (data not shown).

Large pulmonary arteries

In control pulmonary arteries, ionomycin (0.01–300 nM) caused concentration-dependent relaxation with a pEC_{50} value of 8.03 ± 0.11 and maximum relaxation (R_{max}) of $90.8\pm4.4\%$ maximum relaxation to SNP (Figure 2a). The pEC_{50} for ionomycin in corresponding arteries from short-term and chronic pulmonary hypertensive sheep did not differ significantly from that in control sheep (Figure 2b, c). Compared to control, the maximal relaxation to ionomycin tended to be reduced in short-term ($62.4\pm10.8\%$ SNP) and chronic pulmonary hypertensive arteries ($67.0\pm8.3\%$ SNP) ($P=0.075$, ANOVA) (Figure 2b, c).

L-NOARG (0.1 mM), significantly attenuated the relaxation curve to ionomycin in control arteries, reducing R_{max} to $26.4\pm10.6\%$ SNP ($P<0.001$) (Figure 2a). By contrast, L-NOARG (0.1 mM) had no significant effect on R_{max} to ionomycin in short-term and chronic pulmonary hypertensive arteries (Figure 2b, c). The pEC_{50} of ionomycin, however, was significantly reduced in both short-term (7.15 ± 0.08, $P<0.01$) and chronic pulmonary hypertensive arteries (7.35 ± 0.09, $P<0.01$) in the presence of L-NOARG.

Medium-sized pulmonary arteries

In control pulmonary arteries, ionomycin caused concentration-dependent relaxation with a pEC_{50} value of 8.40 ± 0.16 and R_{max} of $72.0\pm9.3\%$ SNP (Figure 3a). The relaxations to ionomycin in matched arteries from both short-term and chronic pulmonary hypertensive sheep were not different from control ($P=0.72$). Short-term (pEC_{50} = 7.67 ± 0.12; $P<0.01$), but not chronic pulmonary hypertensive arteries were significantly less sensitive to ionomycin than control arteries (Figure 3b, c).

L-NOARG (0.1 mM) significantly attenuated R_{max} to ionomycin in control ($23.2\pm9.9\%$ SNP, $P<0.05$) and short-term ($50.1\pm10.9\%$ SNP, $P<0.05$), but not chronic pulmonary hypertensive arteries ($61.9\pm7.0\%$ SNP, $P=0.15$) (Figure 3). L-NOARG also caused a small but significant decrease in sensitivity to ionomycin in short-term (pEC_{50}= 7.11 ± 0.12, $P<0.05$) and chronic pulmonary hypertensive arteries ($pEC_{50}=7.31\pm0.12$, $P<0.01$).

Large pulmonary veins

In pulmonary veins from control sheep, the pEC_{50} (8.84 ± 0.29) and R_{max} ($87.2\pm4.6\%$ SNP) for ionomycin did not differ significantly from those values in veins from short-term and chronic pulmonary hypertensive sheep (Figure 4a, b, c). In veins from control and chronic pulmonary hypertensive sheep, L-NOARG (0.1 mM) significantly reduced R_{max} to $16.4\pm4.9\%$ SNP ($P<0.001$) and $16.8\pm4.3\%$ SNP ($P<0.001$) respectively (Figure 4a, c). In veins from short-term pulmonary hypertensive animals, however, the relaxation to ionomycin was not significantly impaired in the presence of L-NOARG (Figure 4b). In each case, a pEC_{50} could not be calculated in the presence of L-NOARG.

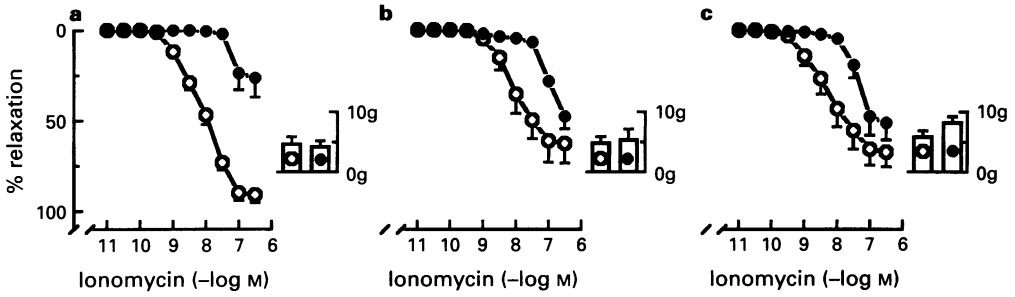

Figure 2 Ionomycin relaxation responses in large isolated pulmonary arteries from control (a, $n=5$), short-term (b, $n=4$) and chronic pulmonary hypertensive sheep (c, $n=7$) in the absence (○) and presence (●) of L-NOARG (0.1 mM). The level of precontraction with endothelin-1 is shown in histograms on the right of the graphs. Note that there is no significant difference between the endothelin-1 contractions within any of the experimental groups. Responses are expressed as percentages of the maximal relaxation to SNP (10 μM) from initial active force. Indomethacin (3 μM) was present throughout. Values are mean ± s.e.mean. n = number of rings.

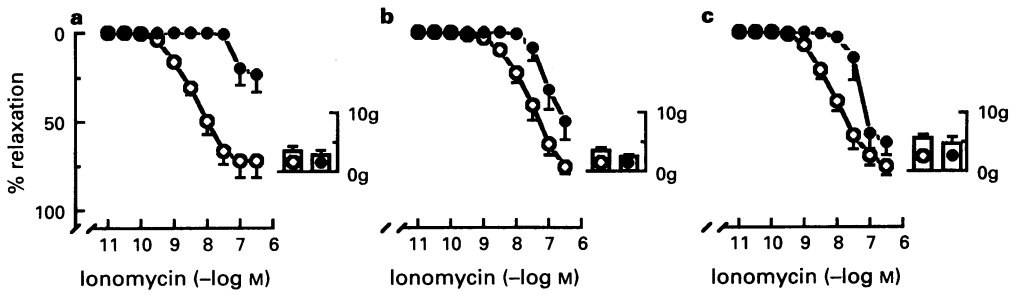

Figure 3 Ionomycin relaxation responses in medium-sized isolated pulmonary arteries from control (a, $n=4$), short-term (b, $n=5$) and chronic pulmonary hypertensive sheep (c, $n=7$) in the absence (○) and presence (●) of L-NOARG (0.1 mM). The level of precontraction with endothelin-1 is shown in histograms on the right of the graphs. Note that there is no significant difference between the endothelin-1 contractions within any of the experimental groups. Responses are expressed as percentages of the maximal relaxation to SNP (10 μM) from initial active force. Indomethacin (3 μM) was present throughout. Values are mean ± s.e.mean. n = number of rings.

Medium-sized pulmonary veins

Ionomycin relaxed control veins 59.9±8.0% SNP (Figure 5a) with a pEC_{50} of 8.41±0.40. The sensitivity and R_{max} to ionomycin in short-term (pEC_{50}= 7.74±0.17, $P<0.05$; R_{max} = 35.9±6.2% SNP, $P=0.09$) and chronic pulmonary hypertensive veins did not differ significantly from control values (Figure 5b, c). L-NOARG (0.1 mM) significantly attenuated the R_{max} to ionomycin in control veins to 22.9±5.7% SNP ($P<0.01$) but had no effect on pEC_{50} (Figure 5a). L-NOARG virtually abolished the relaxation curve to ionomycin in chronic pulmonary hypertensive vessels (R_{max}=9.8±1.9% SNP, $P<0.001$) (Figure 5c). The maximum relaxation to ionomycin in short-term pulmonary hypertensive veins was unaffected by L-NOARG (Figure 5b). Although visual inspection suggested that the sensitivity to ionomycin was unchanged in these vessels, an accurate pEC_{50} could not be calculated because concentration-response curves were non-sigmoidal.

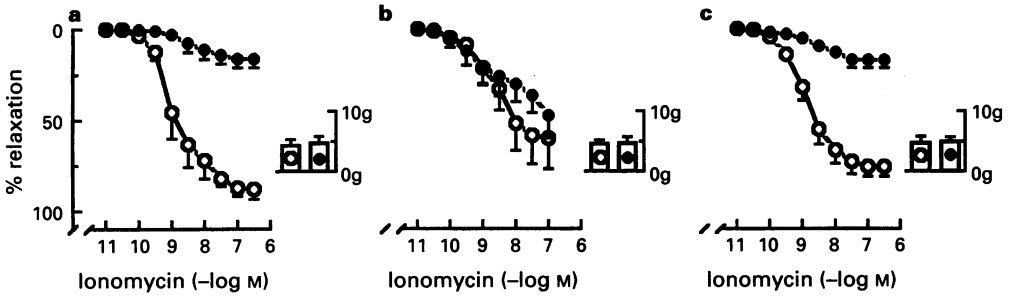

Figure 4 Ionomycin relaxation responses in large isolated pulmonary veins from control (a, $n=4-5$), short-term (b, $n=4$) and chronic pulmonary hypertensive sheep (c, $n=6-7$) in the absence (○) and presence (●) of L-NOARG (0.1 mM). The level of precontraction with endothelin-1 is shown in histograms on the right of the graphs. Note that there is no significant difference between the endothelin-1 contractions within any of the experimental groups. Responses are expressed as percentages of the maximal relaxation to SNP (10 μM) from initial active force. Indomethacin (3 μM) was present throughout. Values are mean±s.e.mean. n = number of rings.

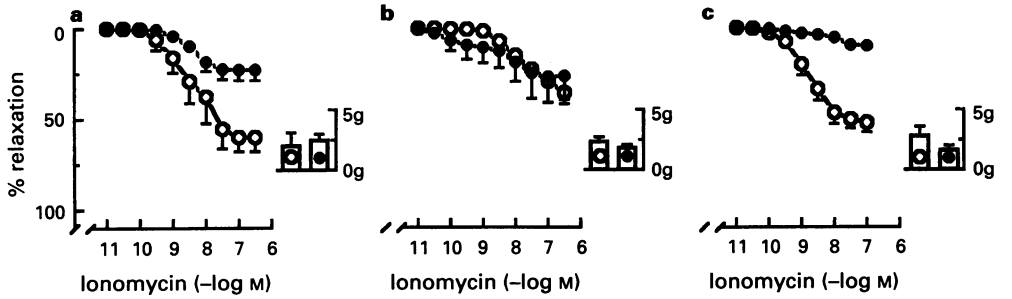

Figure 5 Ionomycin relaxation responses in medium-sized isolated pulmonary veins from control (a, $n=3-5$), short-term (b, $n=4-5$) and chronic pulmonary hypertensive sheep (c, $n=7$) in the absence (○) and presence (●) of L-NOARG (0.1 mM). The level of precontraction with endothelin-1 is shown in histograms on the right of the graphs. Note that there is no significant difference between the endothelin-1 contractions within any of the experimental groups. Responses are expressed as percentages of the maximal relaxation to SNP (10 μM) from initial active force. Indomethacin (3 μM) was present throughout. Values are mean±s.e.mean. n = number of rings.

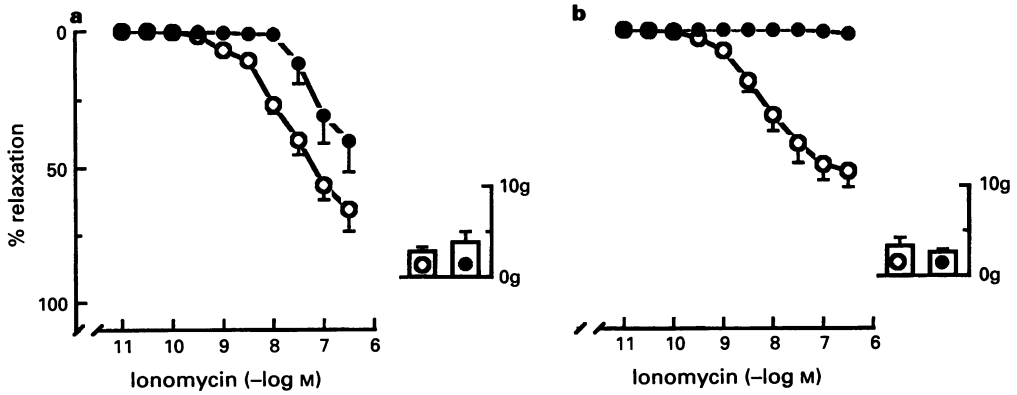

Figure 6 Ionomycin relaxation responses in large isolated pulmonary arteries from sheep with short-term pulmonary hypertension ($n=4$) in the absence (○) and presence (●) of L-NOARG (0.1 mM). (a) Ionomycin responses in normal Krebs solution in the presence of nifedipine (0.3 μM). (b) Ionomycin responses in Krebs solution containing 30 mM K^+ in the presence of nifedipine (0.3 μM). The level of precontraction with endothelin-1 is shown in histograms on the right of the graphs. Note that there is no significant difference between the endothelin-1 contractions within any of the experimental groups. Responses are expressed as percentages of the maximal relaxation to SNP (10 μM) from initial active force. Indomethacin (3 μM) was present throughout. Values are mean±s.e.mean. n = number of rings.

Effect of high extracellular K^+

High K^+ (30 mM) alone, did not significantly alter the pEC_{50} (8.02 ± 0.24) or R_{max} ($51.5 \pm 6.0\%$ SNP) to ionomycin in large short-term pulmonary hypertensive arteries from values observed in matched arteries incubated in normal K^+ (pEC_{50} = 7.65 ± 0.19; R_{max} = $65.2 \pm 8.4\%$ SNP) (Figure 6a, b). In the presence of high K^+, however, L-NOARG (0.1 mM) completely inhibited the relaxation response to ionomycin (Figure 6b).

Discussion

This study has produced two main new findings on the effects of pulmonary hypertension on receptor-independent, endothelial-dependent relaxation in the pulmonary vasculature. First, pulmonary hypertension was accompanied by the emergence of a marked attenuation of the effect of the potent NO synthase inhibitor, L-NOARG, on ionomycin-induced endothelial-dependent relaxation in arteries exposed to short-term and chronic pulmonary hypertension, and in veins from short-term pulmonary hypertensive animals. Second, in arterial segments demonstrating an attenuated response to L-NOARG, relaxation to ionomycin was unaffected by high extracellular K^+ in the absence of L-NOARG, but was abolished completely in the presence of L-NOARG. These findings suggest that pulmonary hypertension is associated with up-regulation of a K^+ channel-dependent mechanism, which maintains receptor-independent, endothelial-dependent relaxation when NO synthesis is blocked.

Four aspects of our experimental design are worthy of comment. First, pulmonary hypertension was induced with continuous air embolization into the pulmonary circulation. The advantages of this experimental model of pulmonary hypertension include a rapid and sustained development of hypertension, minimal effects in the systemic circulation and structural remodelling of the pulmonary vasculature which are similar to those observed in human pulmonary hypertension (Perkett *et al.*, 1988; 1991). Moreover, vessels from both short-term and chronic pulmonary hypertensive animals were examined to separate the effects of elevated pulmonary pressures *per se* from the combined effect of elevated pulmonary pressures and hypertension-induced structural changes within the pulmonary vasculature. In addition, because vasodilator responses vary regionally along the pulmonary vascular tree (Kemp *et al.*, unpublished observations), *in vitro* reactivity studies were performed in both arterial and venous segments.

The second important experimental feature of this study was that the bioassay conditions were optimal for assessment of endothelium-dependent relaxation. Thus, vessels were distended to transmural pressures which produced optimal contraction to K^+ (see Cocks *et al.*, 1993), and any problems with interpretation of relaxation data due to functional antagonism were avoided by pre-contracting vessel segments with endothelin-1 to a value $\leqslant 50\%$ of the maximal K^+ contraction. Moreover, when K^+ channel activity was blocked with a high extracellular K^+ concentration in short-term pulmonary hypertensive arteries, smooth muscle contraction was blocked with nifedipine (Kilpatrick & Cocks, 1994). While the use of nifedipine did not affect endothelium-dependent relaxation (Angus & Cocks, 1989), it permitted arterial segments to be precontracted with endothelin-1 to a level of active force which was similar to that of segments not exposed to high K^+.

The third experimental consideration was the use of L-NOARG to block completely NO synthase activity in normotensive and hypertensive vessels. L-NOARG has been shown to be a potent, irreversible (Furfine *et al.*, 1993) and complete inhibitor of constitutive NO synthase in isolated endothelial cells (Mulsch & Busse, 1990). In addition, Cowan *et al.* (1993) found that N^G-nitro-L-arginine methyl ester (L-NAME), which blocks NO synthase in a similar manner to L-NOARG (Bogle *et al.*, 1992), abolished the accumulation of the second messenger for NO, cyclic cGMP (Ignarro, 1991), in vascular smooth muscle preparations. Upon the basis of these previous observations it was assumed that L-NOARG completely inhibited NO synthase in this study. It is possible, however, that the L-NOARG resistance observed in pulmonary hypertensive vessels may be attributed to a decrease in the potency of L-NOARG as a NO synthase inhibitor in pulmonary hypertension.

The fourth consideration in this study was the confirmation that relaxation responses to ionomycin were endothelium-dependent. This was verified in pulmonary arteries and veins from control sheep in which removal of the endothelium abolished the relaxation response to ionomycin. Although not specifically tested, it was assumed that the relaxation responses to ionomycin in vessels from pulmonary hypertensive animals were also endothelium-dependent. It is unlikely that the development of pulmonary hypertension up-regulated a smooth muscle relaxation response to ionomycin as a previous study in isolated pulmonary arteries from patients with secondary pulmonary hypertension demonstrated that another calcium ionophore, A23187, retained its dependence on the endothelium in pulmonary hypertension (Dinh-Xuan *et al.*, 1993).

Passive vessel properties

The decreased diameter of pulmonary vessels from short-term pulmonary hypertensive sheep was unlikely to be caused by structural alterations in the vessel wall since no change in medial or intimal thickness of large and medium-sized arteries and veins from short-term pulmonary hypertensive sheep was observed (data not shown). Thus, the decreased diameter may indicate that the vessels were in a partially constricted state. Vasoconstrictors such as thromboxane A_2 (Fukushima & Kobayashi, 1986) and endothelin-1 (Wang *et al.*, 1992) are released during air embolization and their effects may be maintained after the vessels are removed from the lung.

The decrease in pulmonary blood flow associated with air embolization may also influence vascular diameter since in the systemic circulation, Langille & O'Donnell (1986) demonstrated that following a 70% reduction in blood flow through the rabbit common carotid artery, the vessel initially constricted and then chronically structurally remodelled. Consequently the diameter of the carotid artery was reduced by approximately 20% after flow was reduced chronically for 2 weeks. In the present study it is tempting to speculate that a similar mechanism may have occurred such that with a maintained decrease in flow, the vessel structurally remodels to the new diameter. Both the reason why large arteries did not maintain the same initial decrease in diameter as medium-sized arteries and why the diameter of large veins were unaffected by short-term and chronic pulmonary hypertension is unclear.

Vasodilator responses

The development of short-term and chronic pulmonary hypertension in response to air embolization in sheep was associated with maintained non-receptor mediated, endothelium-dependent relaxation in pulmonary arteries and veins. In addition, the ability of the smooth muscle to relax via activation of guanylate cyclase was unchanged with the development and maintenance of pulmonary hypertension as the relaxation response to the NO donor, SNP, was preserved in arteries and veins from pulmonary hypertensive animals. The finding of a sustained relaxation response to SNP during pulmonary hypertension is in agreement with previous observations in isolated pulmonary arteries from the spontaneously pulmonary hypertensive rat (Ashmore *et al.*, 1991), chronic hypoxic calves (Orton *et al.*,1988) and patients with chronic obstructive airways diseasc (Dinh-Xuan *et al.*, 1993). By contrast, smooth muscle cell dysfunction in the form of a decreased sensitivity and/or maximum relaxation to SNP has been reported in isolated pulmonary arteries from rats following chronic hy-

poxia (Wanstall *et al.*, 1992; Rodman, 1992; Crawley *et al.*, 1992; Maruyama & Maruyama, 1994) and monocrotaline administration (Wanstall & O'Donnell, 1992). Thus, it is clear that species and model variation exists in the effect pulmonary hypertension has upon pulmonary arterial smooth muscle responsiveness to nitrovasodilators.

Compared to normotensive vessels, pulmonary arteries from short-term and chronic, and pulmonary veins from short-term pulmonary hypertensive sheep demonstrated an attenuated blockade of ionomycin-induced vasodilation by L-NOARG, suggesting that relaxation to ionomycin in these vessels was maintained by a non-NO dependent mechanism. The L-NOARG resistant relaxation was unlikely to be mediated by prostacyclin, an endothelium-derived relaxing factor that hyperpolarizes vascular smooth muscle via opening of ATP-sensitive K^+ channels (Jackson *et al.*, 1993), because all vessels were pretreated with the cyclo-oxygenase inhibitor, indomethacin. A more plausible explanation, supported by the abolition of L-NOARG resistant ionomycin relaxations in vessels from pulmonary hypertensive animals by a high extracellular K^+ concentration, is that the L-NOARG-resistant ionomycin relaxation involves activation of K^+ channels to produce hyperpolarization and subsequent relaxation of the smooth muscle. When NO is available, however, the K^+ channel mechanism does not contribute to endothelium-dependent relaxation in pulmonary hypertensive vessels, as high extracellular K^+ alone did not alter relaxation responses to ionomycin in vessels from pulmonary hypertensive sheep. Thus, NO can mediate all of the relaxation response to ionomycin regardless of the presence of the K^+ channel mechanism, whilst the K^+ channel mechanism acts as a 'backup' system to maintain endothelium-dependent relaxation when NO synthase is blocked.

The features of the K^+ channel-mediated mechanism observed here in pulmonary arteries after the development of pulmonary hypertension are remarkably similar to those described in systemic arteries (Cowan *et al.*, 1993; Kilpatrick & Cocks, 1994). Thus, in the rabbit abdominal aorta and carotid artery, Cowan *et al.* (1993) found that the NO synthase inhibitor, L-NAME, abolished ACh-stimulated cyclic GMP accumulation but only partially inhibited ACh-mediated relaxations. Furthermore, high extracellular K^+ (25–30 mM) Krebs solution and a range of K^+ channel antagonists, did not alter the response to ACh but abolished it in the presence of L-NAME (Cowan *et al.*, 1993). Independently, Kilpatrick & Cocks, (1994) found that the relaxation to the endothelium-dependent dilators, substance P, bradykinin and A23187 in pig isolated coronary artery, were inhibited by only about 40% in the presence of L-NOARG. When the extracellular concentration of K^+ was raised to 30 mM, the relaxation responses to each agent was unaffected, yet high K^+ completely abolished these endothelium-dependent relaxations in the presence of L-NOARG.

Two crucial questions are raised by our study. First, what is the stimulus for up-regulation of a K^+ channel-mediated relaxation mechanism in pulmonary hypertension? Second, why does the up-regulated K^+ channel-mediated relaxation mechanism remain 'silent' in hypertensive arteries in the presence of NO?

At present we can only speculate on the precise stimulus responsible for development of L-NOARG resistance and upregulation of the K^+ channel-mediated backup system in pulmonary hypertension. This phenomenon was not specifically related to a reduction in NO synthesis, because endothelium-dependent relaxation was not impaired in the air embolization model of pulmonary hypertension, but it could have been related to the elevation of pulmonary pressures, hypoxaemia or the inflammatory response. A putative role for elevated pressure in the appearance of L-NOARG resistance and upregulation of K^+ channels in short-term and chronic pulmonary hypertensive arteries is consistent with the observation that L-NOARG resistance exists constitutively in the high pressure systemic vasculature (Cowan *et al.*, 1993; Kilpatrick & Cocks, 1994) including the sheep isolated carotid artery (Kemp *et al.*, unpublished observations). A possible mechanism is suggested by the finding that, in systemic vessels, increases in intraluminal pressure result in the opening of voltage operated or stretch-activated Ca^{2+} channels in the smooth muscle (Laher *et al.*, 1988) which, via a subsequent rise in intracellular Ca^{2+} concentration, may activate Ca^{2+}-dependent K^+ channels in the smooth muscle membrane (Berezi *et al.*, 1992; Brayden & Nelson, 1992). Elevated pressure, however, is unlikely to be the sole factor involved in the upregulation of the K^+ channel-mediated system, because L-NOARG resistance was observed in veins from short-term pulmonary hypertensive sheep in which left atrial pressure (and thus pulmonary venous pressure) was decreased.

Hypoxaemia may be responsible for the upregulation of the K^+ channel-mediated backup system since other studies in chronically hypoxic rats have demonstrated an increase in the maximal relaxation to ATP-sensitive K^+ channel openers (Wanstall & O'Donnell, 1992; Rodman, 1992). Whether these ATP-sensitive K^+ channels contribute to endothelium-dependent relaxation in pulmonary vessels in unknown, although Cowan *et al.* (1993) reported that L-NAME-resistant relaxations of rabbit abdominal aorta were inhibited by the ATP-K^+ channel blockers, glibenclamide and tolbutamide. In the same study, however, the L-NAME-resistant response in the aorta was also blocked by the Ca^{2+}-sensitive K^+ channel inhibitor, charybdotoxin. Furthermore, Cowan *et al.* (1993) also found that glibenclamide did not block similar L-NAME resistant relaxations in the rabbit carotid artery, yet they were blocked by TEA and charybdotoxin.

Hypoxaemia, however, cannot be the sole stimulus of the upregulation of a K^+ channel-dependent dilator mechanism because while the oxygen content of systemic and pulmonary arterial blood was reduced by chronic pulmonary hypertension, it was normal in short-term pulmonary hypertension. In addition, acute hypoxia decreased the amplitude of K^+ currents mediated via Ca^{2+}-sensitive K^+ channels in dog isolated pulmonary arterial smooth muscle (Post *et al.*, 1992) and the activity of voltage gated K^+ channels in cultured rat pulmonary arterial cells (Yuan *et al.*, 1993), while chronic hypoxia inhibited delayed rectifier K^+ channels in rat cultured pulmonary arterial cells (Smirnov *et al.*, 1994). Furthermore, in isolated perfused lungs (Russ & Walker, 1993) and pulmonary arteries (Oka *et al.*, 1993) from chronically hypoxic rats, L-NOARG has been found to inhibit endothelium-dependent relaxation, suggesting that hypoxia alone is unlikely to upregulate K^+ -channel mediated relaxation.

Vascular inflammation may play a role in the appearance of L-NOARG resistance and the upregulation of a K^+ channel-dependent dilator mechanism particularly in pulmonary veins. This proposal is based upon the observation that the resolution of inflammation during the development of chronic pulmonary hypertension (Perkett *et al.*, 1991) was associated with resolution of L-NOARG resistance in pulmonary veins. Inflammation during air embolization involves the accumulation of granulocytes around the air emboli. Although air emboli are unlikely to pass through the arterial circulation, granulocytes not attached to air emboli, have been observed in small pulmonary veins (Staub *et al.*, 1982) and thus may influence the reactivity of pulmonary veins. The precise mechanism via which an inflammatory response would up regulate a K^+ channel-mediated backup relaxation mechanism is unclear. Activated granulocytes, however, may cause endothelial cell damage due to the release of superoxide radicals (Sacks *et al.*, 1978; Weiss *et al.*, 1981) which are known to inactivate NO (Gryglewski *et al.*, 1986; Chen & Gillis, 1991). Consequently a decrease in NO-mediated relaxation, as observed in pulmonary veins from short-term pulmonary hypertensive sheep where ionomycin-mediated relaxations tended to be attenuated, may result in a greater contribution of K^+ channel-mediated mechanisms to endothelium-dependent relaxation.

At present we have no satisfactory explanation for the functional silence of the K^+ channel-mediated relaxation in

the presence of NO. If K^+ channels are involved in this mechanism, then cyclic GMP, the transducer for NO (See Ignarro, 1991), may inactivate the same mechanism via which hyperpolarization transduces its smooth muscle relaxing effect i.e. either a K^+ or Ca^{2+} channel. Thus cyclic GMP has been found to suppress the activity of L-type Ca^{2+} channels in single vascular smooth muscle cells from rabbit portal vein (Ishikawa *et al.*, 1993) and SNP which donates NO, has also been shown to decrease Ca^{2+} influx through voltage-gated Ca^{2+} channels in rabbit isolated pulmonary arteries (Clapp & Gurney, 1991). Therefore, cyclic GMP may prevent the relaxation via smooth muscle hyperpolarization by inhibiting the same Ca^{2+} channels which would be blocked by hyperpolarization. If this mechanism also functions in pulmonary hypertensive arteries then it is unlikely that cyclic GMP inhibits a L-type Ca^{2+} channel as relaxation responses were not affected by the Ca^{2+} channel antagonist, nifedipine. Another possibility is that NO could directly inhibit smooth muscle cell K^+ channels since SNP has been shown to inhibit directly Ca^{2+}-activated K^+ channels independently of its actions upon inward Ca^{2+} currents in avian cultured ciliary ganglia (Cetiner & Bennett, 1993). This is unlikely, however, since hyperpolarization is unaffected even when endothelium-dependent relaxation is abolished by inhibitors of NO (Chen *et al.*, 1988; Chen & Suzuki, 1989).

In conclusion, the findings of this study indicate that a non-NO-mediated relaxation mechanism is upregulated during the development of pulmonary hypertension in sheep and that in short-term pulmonary hypertension at least, it appears to depend on the activity of K^+ channels. An important question with substantial clinical implications is whether a similar phenomenon occurs in human pulmonary hypertension. Studies are currently in progress to address this issue. Therefore, K^+ channel-dependent hyperpolarization and vasorelaxation may play an important role in maintaining endothelium-dependent vasodilation in pulmonary vascular disease where NO synthesis is compromised.

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