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Investigation of the contributions of nitric oxide and prostaglandins to the actions of endothelins and sarafotoxin 6c in rat isolated perfused lungs

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1 The aims of the study were to assess the contribution of prostaglandins and nitric oxide (NO) to the effects of endothelin (ETs) and sarafotoxin 6c (SX6c) in perfused rat lungs. This was carried out by using indomethacin, a cyclo-oxygenase inhibitor and N^G-nitro-L-arginine (L-NOARG), a NO synthase inhibitor. Responses were studied under basal perfusion conditions and in other experiments after the elevation of vascular tone with the thromboxane-mimetic, U46619. The sub-types of ET receptors involved were characterized by use of ET receptor antagonists and cross-tachyphylaxis.

2 Pulmonary perfusion pressure (PPP), lung weight and pulmonary inflation pressure (PIP), were continuously recorded. Although L-NOARG (100 μM) did not alter basal parameters it markedly augmented the vasoconstriction and lung weight increases induced by ET-1 (50-400 pmol) or SX6C (25-200 pmol) while vasoconstrictor responses to phenylephrine were not affected by L-NOARG.

3 L-NOARG markedly potentiated the bronchoconstriction induced by ET-1 or SX6C whereas it had no effect on responses to carbachol.

4 When vascular tone was elevated, low doses (1.25-40 pmol) of ET-1, ET-3 and SX6C produced falls in PPP. The vasodilator potencies were SX6C > ET-1 = ET-3. The ET_A receptor antagonist, BQ123, did not affect these depressor responses, whereas the mixed ET_A/ET_B antagonist, bosentan, blocked them.

5 Indomethacin (10 μM) partially inhibited vasodilator response to ET-1, whereas it had no effect on SX6C-induced vasodilatation.

6 L-NOARG plus indomethacin completely blocked ET-1 induced vasodilatation, whereas responses to SX6C were blocked by L-NOARG alone.

7 Repeated injections of submaximal doses of ET-1 or SX6C caused tachyphylaxis to vasodilator responses. Subsequent injections of SX6C or ET-1 did not elicit depressor responses showing cross tachyphylaxis had occurred.

8 These findings indicate that under basal conditions the pulmonary vasoconstrictor, lung weight and bronchoconstrictor responses to ET-1 and SX6C are attenuated by evoked release of nitric oxide (NO). When vascular tone was elevated, lower doses of ETs and SX6C produced vasodilatation. These vasodilator responses are indirect, those to SX6C being mediated via NO production, whereas those to ET-1 involve both NO and prostanoid(s). Tachyphylaxis and ET antagonist experiments indicate that the same receptor subtype is involved in mediating the vasodilatation and that this is of the ET_B type located on the endothelium. However the post-receptor vasodilator events triggered by ET-1 or SX6C appear to be different.

Keywords: Rat lung, pulmonary vasoconstriction/vasodilatation; bronchoconstriction; ETs and SX6C; nitric oxide; prostanoids

Introduction

There is increasing evidence that endothelins may have a role to play in pulmonary disease (see Barnes, 1994; Hay & Goldie, 1995). We (Lal *et al.*, 1995a) and others (Rodman *et al.*, 1992; Bonvallet *et al.*, 1993; MacLean *et al.*, 1994) have provided pharmacological evidence that the pulmonary vasoconstrictor actions of the endothelins in the rat lung are mediated by ET_A and ET_B receptor subtypes and that ET_A receptors are located primarily on the venous side of the pulmonary circulation while the ET_B receptors mediating vasoconstriction are on the arterial side. Stimulation of venous ET_A receptors leads to a rise in capillary perfusion pressure and the development of hydrostatic oedema (Rodman *et al.*, 1992; Ercan *et al.*, 1993; Lal; *et al.*, 1995a).

ET_B receptors were originally considered as receptors mediating vasodilatation linked to nitric oxide production (Warner *et al.*, 1989). However, subsequently it was shown that ET_B receptors were also involved in smooth muscle contraction (Moriland *et al.*, 1992). Thus ET_B receptors have been subdivided into ET_{B1} (located on the endothelium and mediating vasodilatation) and ET_{B2} (located on the vascular smooth muscle and inducing contraction) (Douglas *et al.*, 1994). Sokolovsky *et al.* (1992) also reported that there appeared to be two subtypes of ET_B receptors i.e. the 'super high affinity', ET_{B1} receptors and the 'high affinity', ET_{B2} receptors. It was suggested that the super high sites showing binding affinity in the picomolar range are related to the vasodilatation property of endothelins whereas the high affinity sites with binding affinity in the nanomolar range participate in the vasoconstrictor actions.

In view of these observations it would be expected that if there were any ET_{B1} receptors in the pulmonary vasculature they should induce relaxation of the pulmonary circulation. However, in our previous experiments (Lal *et al.*, 1995b) examining the effects of ETs on the rat isolated lung we were unable to demonstrate any vasodilator actions, possibly because the pulmonary vessels were already maximally dilated. The present experiments were carried out to examine the effects of endothelins and the ET_B agonist, sarafotoxin 6c (SX6C), in more detail using preparations in which the perfusion pressure was elevated by the infusion of the thromboxane analogue, U46619 (Coleman *et al.*, 1981). For comparison the effects of phenylephrine and the endothelium-

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dependent and endothelium-independent vasodilator agents, carbachol and sodium nitroprusside, were used. Preliminary findings of these studies have been presented to the British Pharmacological Society (Lal *et al.*, 1995c).

Methods

Isolated ventilated perfused lung preparation

Lungs were isolated and perfused as described previously (Lal *et al.*, 1994). Male Wistar rats were anaesthetized with an intraperitoneal injection of Sagatal, 60 mg kg^{-1} body weight. Heparin (500 i.u.) was injected via the tail vein, 5 min later the chest wall was opened. A small cut was made in the right ventricular free wall and the pulmonary artery cannulated with a stainless steel cannula (external diameter = 1.5 mm, internal diameter = 1 mm) via the right ventricle. The left atrium was cut and the major part of the ventricles removed to allow free efflux of perfusate. The trachea was cannulated and thereafter the lungs were isolated and perfused via the pulmonary artery at a constant flow of 5 ml min^{-1} with Krebs solution of following composition (mM): KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 1.25, $MgSO_4$ 1.2, NaCl 118, $NaHCO_3$ 25, glucose 11.1 (gassed with 95% O_2 and 5% CO_2). Pulmonary arterial perfusion pressure (PPP) was recorded via a side arm on the arterial cannula. Lungs were ventilated via the trachea with room air (stroke volume 1 ml, 28 strokes min^{-1}, no positive end expiratory pressure). Pulmonary inflation pressure (PIP) was measured via the tracheal cannula. Lungs were suspended from an isometric transducer to record changes in lung weight. All parameters were recorded on a multichannel recorder (Grass model 7D polygraph). Lungs were allowed to stabilize for 30 min before drug administration. Drugs were administered as bolus doses (10–100 μl) or infusions (100 μl min^{-1}) via the pulmonary artery.

Elevation of basal PPP with U46619

In a series of experiments basal PPP was elevated by infusion of the thromboxane-mimetic, U46619 (40–80 nM), commencing 20 min after the initial stabilisation period. Ten minutes later bolus injections of agonists were administered at 7–10 min intervals.

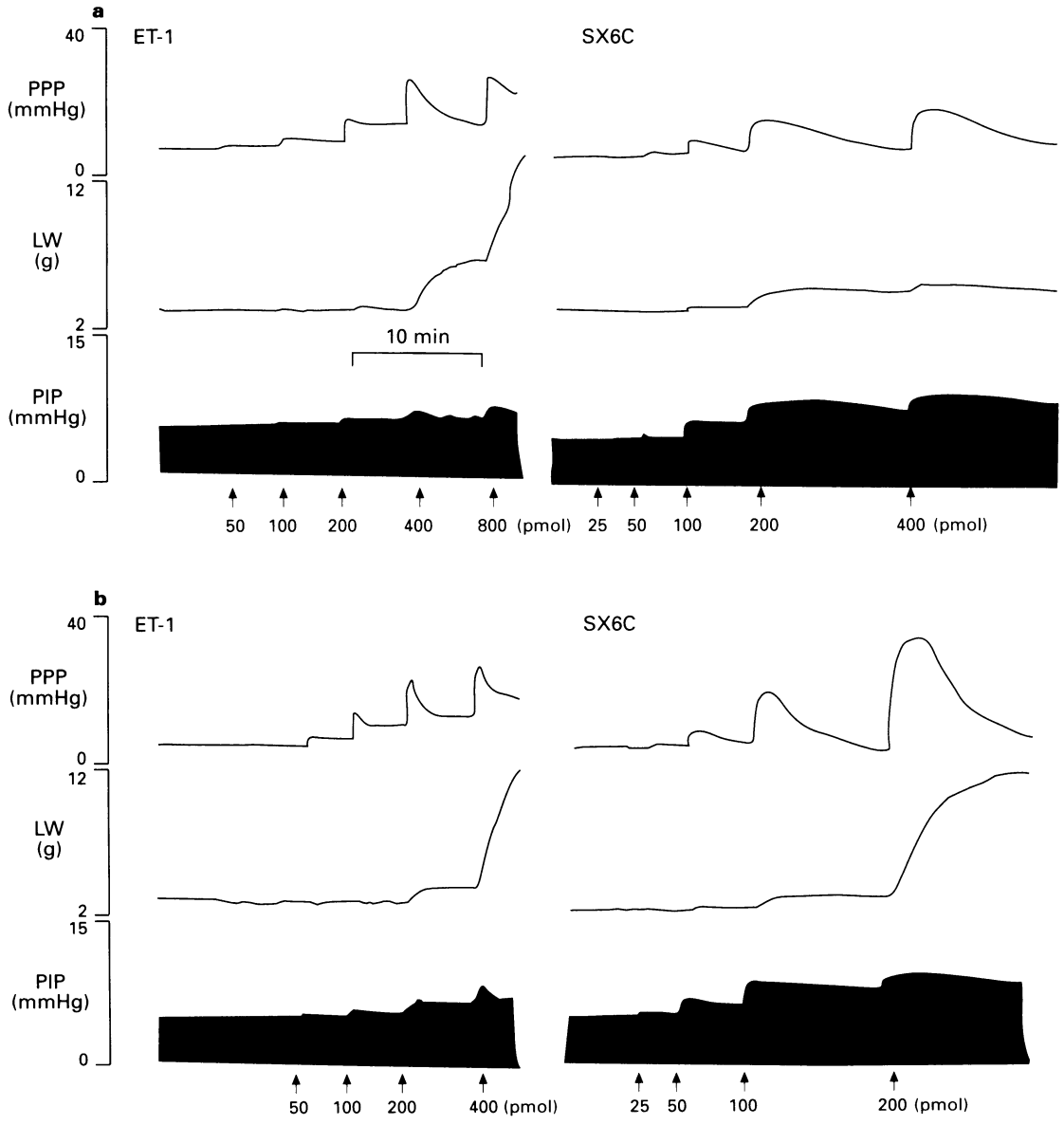

Figure 1 The effects of ET-1 and SX6C on pulmonary perfusion pressure (PPP), lung weight (LW), and pulmonary inflation pressure (PIP) in the absence of L-NOARG (a) and in the presence of L-NOARG (100 μM) (b). This trace is one of 4 such experiments.

Effects of inhibitors and receptor antagonists

Indomethacin (10 μM) and/or N^G-nitro-L-arginine (100 μM) were included in the perfusate for 30 min before agonist injection and were present for the rest of the experiment. Receptor antagonists were included in the perfusate for 20 min before bolus injections of the appropriate agonist were given. The selectivity of inhibitors and receptor antagonists was studied by use of phenylephrine, carbachol and sodium nitroprusside.

Drugs and chemicals

BQ123, (cyclo[D-Asp-L-Pro-D-Val-L-Leu-D-Trp]) was supplied by Dr A.G. Roach, Rhone Poulenc Rorer, Dagenham. Bosentan (4-*tert*-butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxyphenoxy)-2,2'-bipyrimidin-4-yl]-benzenesulphonamide sodium salt) was provided by Hoffmann-La Roche Ltd (Basel, Switzerland). Endothelin-1 and endothelin-3 were obtained from Novabiochem (UK) Ltd. Sarafotoxin 6c was from Peninsula Laboratories. Indomethacin, L-phenylephrine hydro-chloride,9,11-dideoxy-11$_\alpha$,9$_\alpha$-epoxy-methanoprostaglandin $F_{2\alpha}$ (U46619), sodium nitroprusside, N^G-nitro-L-arginine and N^G-nitro-D-arginine were from Sigma, Poole, Dorset. U46619 was dissolved in 95% ethanol and stored at −80°C; working dilutions were made in isotonic saline. Stock solutions of all other drugs were prepared in isotonic saline and stored at −20°C.

Statistical analyses

Increases in PPP, PIP and lung weight induced by bolus injections of phenylephrine, carbachol, endothelin-1 and sarafotoxin 6c are expressed relative to the value prior to the addition of each dose. Pulmonary vasodilator responses are expressed as % decreases of the U46619-induced increase in pressure. When lung weight measurement was needed for comparative purposes, weight gain was calculated 7 min after giving the maximum dose of agonist (Lal *et al.*, 1995a). Data are expressed as mean ± standard error of the mean (s.e.mean), in many cases the error bars fall within the symbols and are therefore not shown. ANOVA followed by Dunnett's test for multiple comparisons or Student's (unpaired) *t* test for comparison of individual means. Probability values of $P<0.05$ were considered significant.

Results

(a) Effects of nitro-L-arginine (L-NOARG) on responses to ET-1 and sarafotoxin 6c (SX6C)

Inclusion of L-NOARG (100 μM) in the perfusate for 30 min did not affect basal PPP, measured immediately prior to L-NOARG addition (5.4 ± 0.6 mmHg, $n=7$ in control vs. 5.4 ± 0.5, $n=9$). In addition there were no effects on bronchial tone (basal PIP 4.15 ± 0.2 mmHg, $n=7$ in control vs. 5.24 ± 0.3 mmHg, $n=9$) or lung weight (2.85 ± 0.14 g, $n=7$ in control vs. 2.84 ± 0.12 *g*, $n=9$).

The effects of L-NOARG (100 μM) on responses to ET-1 and SX6C under basal conditions are shown in Figures 1 and 2. Figure 1 shows that PPP, lung weight and PIP responses to ET-1 50–400 pmol (b left panel) or, SX6C 25–200 pmol (b right panel) in the presence of L-NOARG were markedly potentiated when compared to control ET-1 50–400 pmol (a left panel) or SX6C 25–200 pmol (a right panel) respectively in the absence of L-NOARG. Furthermore, L-NOARG produced more marked potentiations in the responses to SX6C as compared to ET-1 (b). The quantitative data is expressed in Figure 2. From Figure 2a it can be seen that L-NOARG potentiated PPP increases in responses to ET-1 100–400 pmol ($P<0.001$, $n=4$), similarly, SX6C (50–200 pmol)-mediated increases in PPP were also significantly augmented ($P<0.001$, $n=5$). The maximum constrictor response to SX6C was also increased in the presence of L-NOARG. Figure 2b illustrates that in the presence of L-NOARG lung weight increases caused by ET-1 and SX6C were also significantly potentiated.

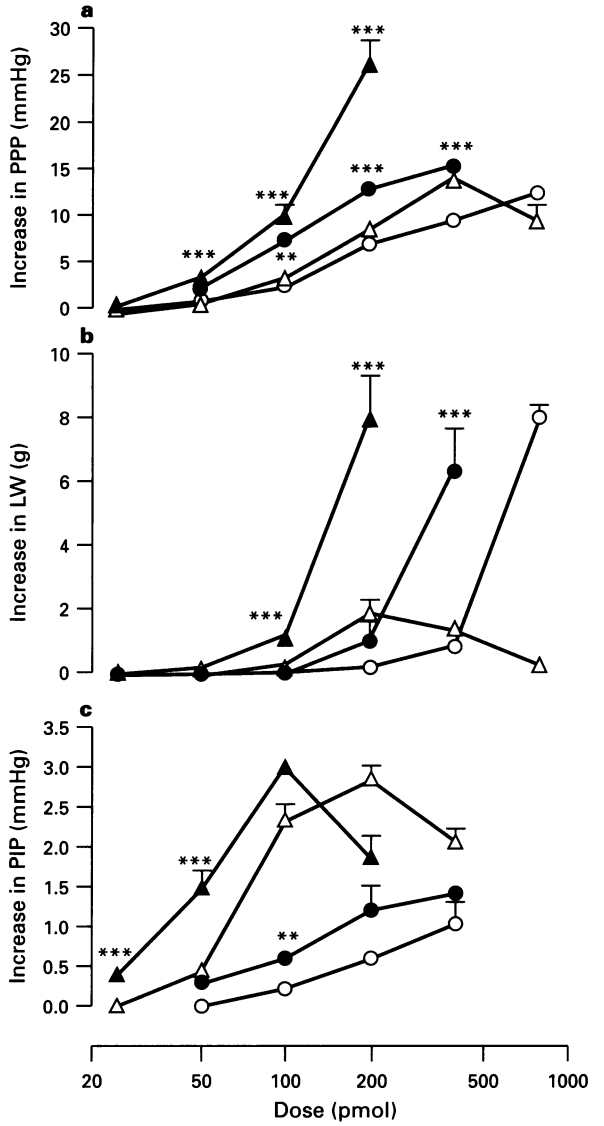

Figure 2 The effects on (a) pulmonary perfusion pressure (PPP), (b) lung weight and (c) pulmonary inflation pressure (PIP) of ET-1 (○, ●) and SX6C (△, ▲) in the absence (open symbols) and presence (solid symbols) of L-NOARG (100 μM) compared with control ET-1 or SX6C. Each point represents mean ± s.e.mean, $n=4-13$ experiments. $^{**}P<0.01$; $^{***}P<0.001$ ANOVA followed by Dunnett's test.

In contrast to the potentiation of responses to ET-1 and SX6C, it was of interest to note that L-NOARG did not affect the increases in PPP induced by the α-adrenoceptor agonist, phenylephrine (3.12–50 nmol) (ED_{50} 4 ± 0.7, $n=5$ in controls vs. 5.4 ± 1.7, $n=3$) at any dose.

The increases in PIP in response to ET-1 were significantly augmented in the presence of L-NOARG (ED_{50} 242 ± 33 pmol, $n=7$ in control vs. 132 ± 5 pmol, $n=4$; $P<0.05$). Similarly, the SX6C-induced increases in PIP were also significantly potentiated (ED_{50} 85 ± 6 pmol, $n=13$ in control vs. 48 ± 5 pmol, $n=5$; $P<0.01$) (Figure 2c). The apparent reduction in response to the highest doses of SX6C on each curve is a consequence of expressing each increase relative to the PIP measured immediately prior to injection. It was necessary to include these points to establish clearly that maximal responses had been achieved.

The presence of L-NOARG did not alter the bronchoconstrictor responses to carbachol (ED_{50} 4.25 ± 0.4 nmol, $n=7$ in controls vs. 4 ± 0.3 nmol, $n=4$) at any dose.

(b) Effects of ETs and SX6C in lungs where vascular tone is increased by U46619

Infusion of U46619 (80 nM) into the pulmonary artery produced an increase in PPP of 9±0.5 mmHg over the basal PPP of 5±0.5 mmHg ($n=16$). Under these conditions bolus injections of ET-1 (2.5–40 pmol), ET-3 (2.5–40 pmol) and SX6C (1.25–40 pmol) all produced dose-dependent falls in PPP (Figure 3). The vasodilator potencies of ET-1 (ED_{50} 3.6±0.6 pmol, $n=5$) and ET-3 (ED_{50} 3.9±1.05 pmol, $n=4$) were similar. However, SX6C (ED_{50} 2.2±0.14 pmol, $n=7$; $P<0.05$) was significantly more potent than either of the ETs (Figure 3), although the difference was small.

(c) Effect of inhibitors and ET receptor antagonists on vasodilator responses to ET-1 and SX6C

L-NOARG, D-NOARG and indomethacin Figure 4 shows ET-1 and SX6C responses in the absence (Figure 4a) and presence of the nitric oxide synthase inhibitor, L-NOARG (Figure 4b). L-NOARG (100 μM) had no effect on basal PPP. However, it augmented the vasoconstrictor action of U46619. Therefore a lower concentration of U46619 was used in order to elevate the PPP by a similar amount to that seen in the earlier experiments (approximately 9 mmHg). In this series of experiments (40 nM) U46619 produced an increase in PPP of 8.5±0.8 mmHg, $n=8$. Figure 4b (left panel) illustrates that in the presence of L-NOARG a normally sub-threshold dose of ET-1 (1.25 pmol) produced vasodilatation, whereas, at higher doses (5–20 pmol) vasoconstrictor responses were seen. In contrast to the ET-1-induced vasodilatation, the vasodilator responses to SX6C (1.25–20 pmol) were completely abolished in the presence of L-NOARG (100 μM). The highest dose of SX6C (20 pmol) now produced an increase in PPP (Figure 4b right). Accumulated results from a series of experiments are shown in Figure 5. D-NOARG did not affect the response to any of the agonists ET-1 (ED_{50} 3.6±0.6 pmol, $n=5$ in control vs. 3.2±1.2 pmol, $n=4$) or SX6C (ED_{50} 2.2±0.14 pmol, $n=7$ in control vs. 2.3±0.6 pmol, $n=4$). Perfusion of L-NOARG (100 μM) had no effect on vasodilatation produced by the NO donor, sodium nitroprusside (SNP), 10–1000 pmol (data not shown).

In U46619-preconstricted preparations the inclusion of the cyclo-oxygenase inhibitor, indomethacin (10 μM), tended to reduce the vasodilator response to ET-1 (2.5–20 pmol) but this was only significant at the 5 pmol dose (Figure 5a). However, the ED_{50} vasodilator value for ET-1 was significantly increased from 3.6±0.6 pmol, $n=5$ in controls to 9±3.4 pmol, $n=3$ ($P<0.05$). In a different set of experiments, perfusion of indomethacin (10 μM) and L-NOARG (100 μM) in combination completely abolished the vasodilator response to ET-1. ET-1 (5–20 pmol) now produced marked rises in PPP (Figure 5a).

The combination of inhibitors was not tried in the case of SX6C as L-NOARG completely abolished the vasodilator responses to SX6C (see above), whereas indomethacin (10 μM) had no effect (ED_{50} 2.2±0.14 pmol, $n=7$ in control vs. 1.7±0.3 pmol, $n=4$) (Figure 5b).

BQ123 Inclusion of BQ123 (10 μM) in the perfusate did not alter basal PPP or the increase in PPP in response to U46619 (80 nM), $n=3$. In U46619 pre-constricted preparations BQ123

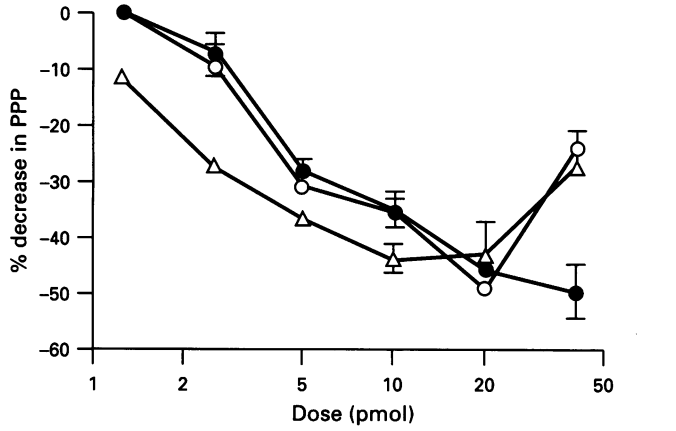

Figure 3 Graph showing the pulmonary vasodilator responses to ET-1 (○), ET-3 (●) and SX6C (△) in U46619 pretreated preparations. Each point represents mean±s.e.mean, $n=4-7$ experiments.

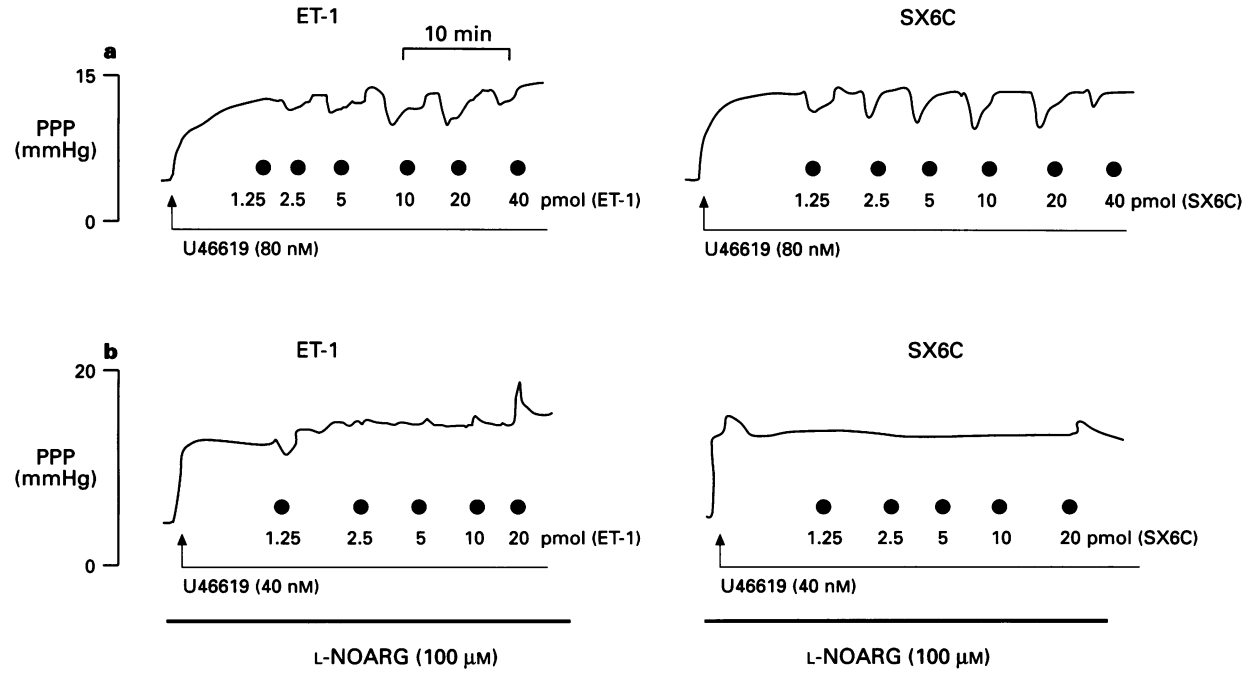

Figure 4 Experimental traces showing the elevation of basal pulmonary perfusion pressure (PPP) with U46619 (40–80 nM) and subsequent changes in PPP in response to ET-1 and SX6C (a) in control and (b) in the presence of L-NOARG (100 μM). Each trace is one of 4–7 similar experiments.

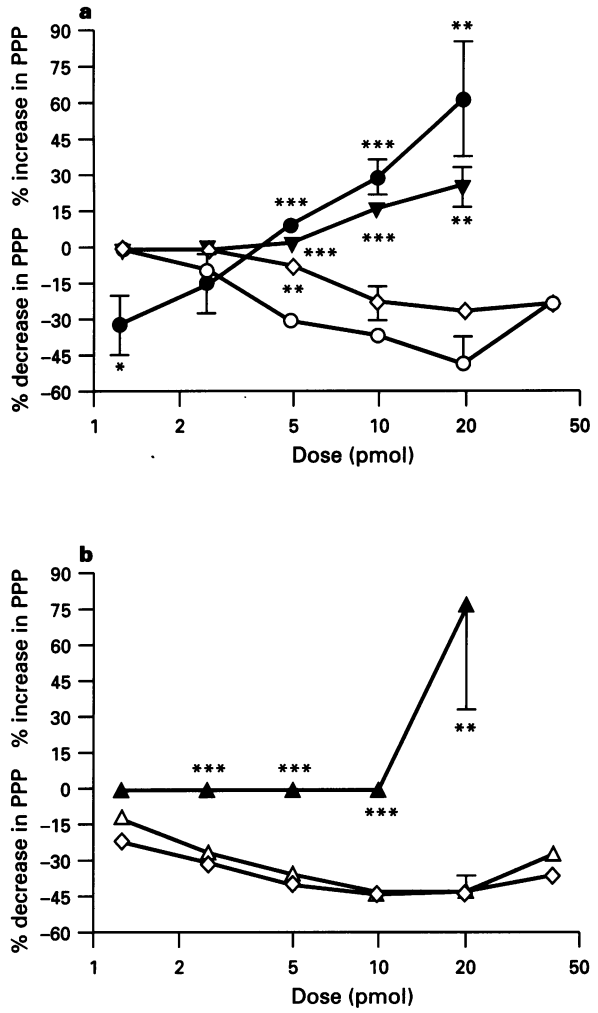

Figure 5 (a) Graph showing the pulmonary vascular responses to ET-1 in U46619 (40–80 nM) pretreated preparations, control (○), plus L-NOARG (100 μM) (●), plus indomethacin (10 μM) (◇), and L-NOARG (100 μM) plus indomethacin (10 μM) (▼), $n=3-5$. (b) Graph showing the pulmonary vascular responses to SX6C in U46619 (40–80 nM) pretreated preparations, control (△), plus L-NOARG (100 μM) (▲), and in the presence of indomethacin (10 μM) (◇), $n=4-7$. Each point represents mean ± s.e.mean. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$, ANOVA, followed by Dunnett's test.

was without any marked effect on the vasodilator responses to ET-1 (2.5–40 pmol) control ED_{50} 3.6 ± 0.6 ($n=5$) vs. 5.6 ± 1 pmol ($n=3$).

Bosentan The effects of the mixed ET_A/ET_B receptor antagonist, bosentan, were studied on pulmonary vasodilator responses to ET-1 and SX6C. Perfusion of bosentan (5 μM) had no effect on basal PPP (5.1 ± 0.5 mmHg, $n=8$). The infusion of U46619 (80 nM) produced an increase in PPP of 9.25 ± 0.6 mmHg, $n=8$. Bosentan abolished ($P<0.001$) the decreases in PPP produced in response to ET-1 (1.25–40 pmol) or SX6C (1.25–40 pmol). Bosentan had no effect on SNP-induced decreases in PPP (100 pmol of SNP in the presence of bosentan produced a fall in PPP of 27% ± 2 in control lungs vs. 28% ± 4, $n=3$ in test preparations).

(d) Tachyphylaxis to ET-1 and SX6C

Although, dose-response curves to the vasodilator effects of ET-1 and SX6C could be constructed, it was noted that repeated injections of the dose which produced a maximum vasodilatation, caused rapid loss of response. Therefore, a series of experiments were carried out to study this phenomenon in more detail. In these experiments the infusion of U46619 (80 nM) produced an increase in PPP of 9 ± 0.6 mmHg, $n=6$. The effects of repeated injection of low doses of ET-1 or SX6C (20 pmol each) are shown in Figure 6. As shown, the dilator responses to ET-1 given at 5–7 min intervals showed a marked tachyphylaxis, the fifth dose of ET-1 actually produced vasoconstriction rather than vasodilatation. When SX6C was given after ET-1 it also produced dose-dependent increases in PPP (Figure 6a). Similarly, serial doses of SX6C caused marked depressor responses which decreased in magnitude with repeated injections; as can be seen, the sixth injection actually produced a vasoconstriction. Subsequent injection of ET-1 then caused dose-dependent increases in PPP (Figure 6b).

Discussion

In this lung model the pulmonary perfusion pressure was low. To determine if constant release of NO is responsible for this, the NO synthase inhibitor L-NOARG was studied at a concentration reported to block constitutive NOS (Moore *et al.*, 1990). L-NOARG had no effect on basal PPP suggesting that NO is not a major factor contributing to the low vascular tone. This was confirmed as L-NOARG did not potentiate the vasoconstrictor responses to phenylephrine, whereas in other vascular tissues where basal production of NO occurs, phenylephrine responses are potentiated after NO synthase inhibition (Frew *et al.*, 1993).

ET-1 and SX6C-induced pulmonary vascular and bronchial actions were studied under basal conditions and in the presence of L-NOARG. L-NOARG markedly augmented the vasoconstrictor responses to ET-1 and SX6C. A possible explanation of this potentiation is that ET-1 and SX6C release NO via activation of endothelial ET_B receptors (Douglas *et al.*, 1994). Inhibition by L-NOARG would then potentiate vasoconstriction mediated via ET_A and ET_B receptors on the vascular smooth muscle. This is supported by the observation that in rat isolated pulmonary arteries, potentiation of ET-1-induced contractions in the presence of a NOS inhibitor were reversed after removal of the endothelium (Maclean *et al.*, 1994).

In the presence of L-NOARG the increases in PPP induced by SX6C were far greater than those induced by ET-1. This indicates that SX6C is more potent than ET-1 in releasing NO. Alternatively, this could be explained if ET-1 acts on different subtypes of ET_A receptors on smooth muscle cells one of which elevates cyclic AMP thus offsetting the constriction mediated via the other subtype (Eguchi *et al.*, 1993). Our previous finding that a low dose of BQ123 potentiated pulmonary vasoconstrictor responses to ET-1 whilst higher doses blocked them would support this hypothesis (Lal *et al.*, 1995b).

L-NOARG also potentiated lung weight increases in response to ET-1 or SX6C. These increases are probably secondary to the enhanced vasoconstrictor responses. The relationship between pulmonary vasoconstriction and pulmonary oedema formation (lung weight increase) has been explored (Lal *et al.*, 1995a). The primary conclusion was that the venoconstrictor action of these substances increases lung weight by producing a hydrostatic oedema. In this earlier study it was also shown that SX6C acted primarily on the arterial side of the circulation whereas the action of ET-1 was predominantly venous. However, in the present experiments L-NOARG potentiated lung weight increases to SX6C more than to ET-1. This further supports the suggestion that SX6C is more potent in releasing NO than ET-1 and indicates that activation of ET_B receptors on the venous endothelium by SX6C in normal lungs must be sufficient to mask the venoconstrictor responses (and hence increases in lung weight) which are only revealed after inhibition of NO synthesis. This concept is supported by the work of Zellers *et al.* (1994) and Gao *et al.* (1995) who found that ET- and SX6C-induced release of NO and prostacyclin in pulmonary veins is greater

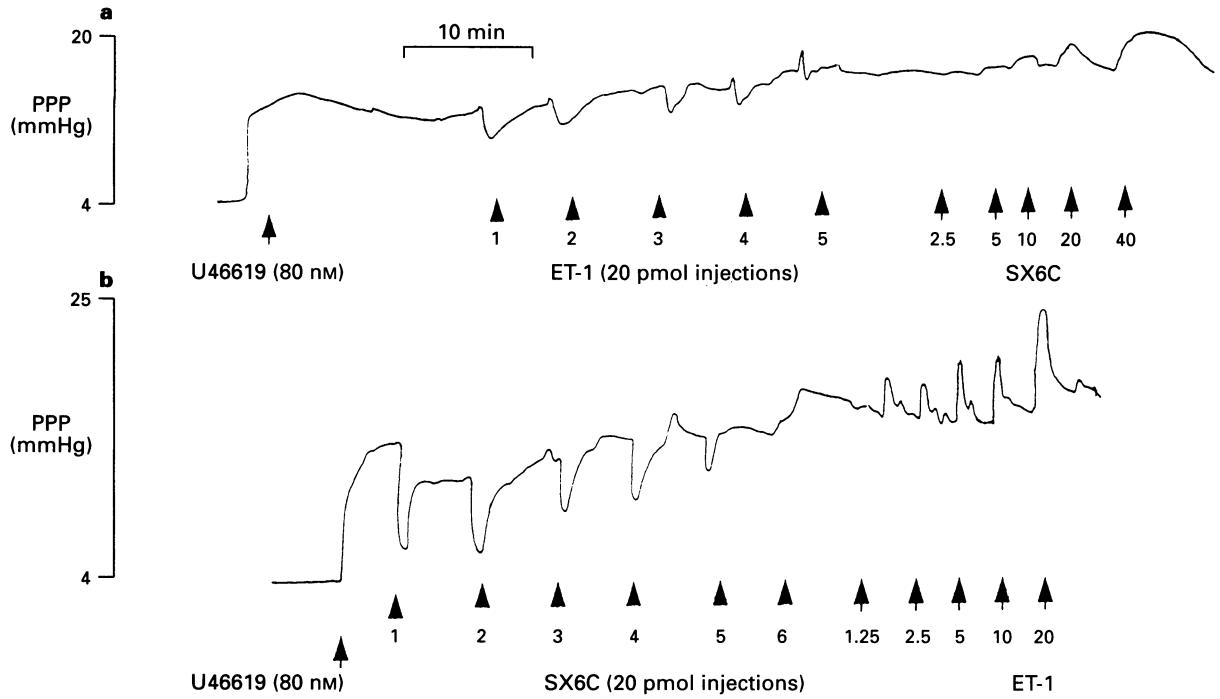

Figure 6 Experimental traces showing the tachyphylaxis/cross tachyphylaxis to pulmonary vasodilator responses to ET-1 and SX6C in U46619 pretreated preparations. Each trace is one of 3 similar experiments.

than that from pulmonary arteries. The second possibility is that NO causes preservation of endothelial barrier function to prevent extravasation (Zi-Qiang *et al.*, 1994).

ET-1 and SX6C-induced bronchoconstriction were also potentiated by L-NOARG suggesting that evoked release of NO was inhibiting their bronchial constrictor actions. Both lung endothelial and epithelial cells can release NO (Xue *et al.*, 1994) and although both sources would be inhibited by L-NOARG, the source of NO in these responses is probably the airway epithelium rather than vascular endothelium. Indeed (Filep *et al.*, 1993) have implicated epithelium-derived NO in ET-1-induced relaxation of guinea-pig tracheal smooth muscle. The bronchoconstrictor effects of SX6C in the presence of L-NOARG were potentiated to a greater extent than the effects of ET-1, again indicating that SX6C is more potent in releasing NO. L-NOARG had no effect on bronchoconstrictor responses to carbachol suggesting that it did not stimulate NO production.

In U46619 pre-constricted preparations, very low doses of ET-1, ET-3 and SX6C produced dose-dependent falls in PPP. ET-1 was equipotent with ET-3 whereas, the highly selective ET_B receptor agonist, SX6C (Williams *et al.*, 1991) was significantly more potent. Furthermore, a ET_A receptor antagonist, BQ123, did not block the vasodilator response to ET-1, whereas, a mixed ET_A/ET_B receptor antagonist, bosentan, completely abolished the dilator responses to ET-1 and SX6C, indicating a role for ET_B receptors in these responses. Bosentan did not affect vasodilatation caused by sodium nitroprusside, suggesting that it is having a selective action. These observations are in agreement with those of Eddahibi *et al.* (1995).

In U46619 pre-contracted preparations, indomethacin did not affect SX6C-induced vasodilatation but it did reduce the vasodilator effects of ET-1 as shown by the increase in the ED_{50}. This suggests that ET-1-induced pulmonary vasodilatation is partially mediated by the release of vasodilator prostanoids. This is further supported by the fact that in the presence of L-NOARG there was an indomethacin-sensitive ET-1-mediated vasodilator response at the lowest dose used. Vasodilator actions of ETs have also been shown to be partially inhibited by indomethacin *in vivo* in cats (Ekelund *et al.*, 1994), while DeNucci *et al.* (1988) have shown ET-1 stimulated prostacyclin synthesis in rat lungs.

The possible role of NO in ET-1- and SX6C-induced vasodilatation was evaluated by use of L-NOARG. L-NOARG potentiated the pressor responses to U46619, suggesting that U46619 also evoked endogenous production of NO in the rat pulmonary circulation. Similar findings have been reported in lungs from neonatal pigs (Pinheiro & Malik, 1993). Therefore, in lungs treated with L-NOARG, a lower concentration of U46619 had to be used in order to elevate the PPP to a similar level seen in the absence of L-NOARG.

In the present experiments L-NOARG blocked the vasodilatation induced by the higher doses of ET-1. However, at the lowest dose of ET-1, L-NOARG revealed a significant vasodilator response which was inhibited by indomethacin. This could be explained by the suggestion of Keen *et al.* (1990) who showed the feed-back control of vasodilator prostanoid by nitric oxide. D-NOARG was without effect. Vasodilator responses to ET-1 were abolished by the combination of indomethacin and L-NOARG, indicating that the vasodilator actions of low doses of ET-1 in the rat pulmonary circulation are mediated by prostanoid release while higher doses cause release of both prostanoids and NO. Similar findings have been reported in porcine pulmonary blood vessels (Zellers *et al.*, 1994), and perfused lungs (Pinheiro & Malik, 1993). As L-NOARG alone completely abolished the vasodilatation caused by SX6C, whereas D-NOARG did not, this indicates mediation via NO production. However, these findings are at variance with the observations of Eddahibi *et al.* (1993). This may be due to their use of a different NOS inhibitor, N^G-monomethyl-L- arginine (L-NMMA) which has been reported to inhibit selectively basal release of NO without affecting the agonist-evoked release (Frew *et al.*, 1993). In support of this suggestion Namiki *et al.* (1992) have shown that L-NOARG blocked the ET-1-induced relaxation of porcine isolated pulmonary arteries. The selectivity of action of L-NOARG in our work was demonstrated by its lack of any effect on the vasodilator actions of sodium nitroprusside.

Repeated injections of a single dose of ET-1 or SX6C (20 pmol) caused a rapid loss of pulmonary vasodilator responses. Other investigators have reported a similar phenomenon when measuring vasodepressor responses in cats and rats (LeMonnier DeGouville *et al.*, 1990; 1991; Lippton *et al.*, 1993; Ekelund *et al.*, 1994) and dilatations of rat basilar arteries

(Kitazono *et al.*, 1995) which are mediated by ET_B receptors. In ET-1-desensitized lungs, further injections of very low doses of SX6C also failed to cause vasodilatation, instead they produced dose-dependent vasoconstriction. Similarly in lungs desensitized with SX6C, injections of low doses of ET-1 produced dose-dependent vasoconstriction. This suggests the development of cross-tachyphylaxis to the vasodilator actions of ET-1 and SX6C. Several possibilities could explain this phenomenon. First, ET-1 and SX6C may stimulate a single type of receptor that becomes rapidly refractory to activation by these peptides. Alternatively, ET-1 and SX6C could bind to distinct receptor sites which share at least one common point in the second messenger system. This shared portion of the reaction chain may then become impaired or refractory, after repeated stimulation of the receptors.

In summary, the present study has shown that under basal conditions evoked release of NO attenuates the pulmonary vasoconstrictor and bronchoconstrictor actions of ET-1 and SX6C. When vascular tone was elevated, lower doses of ET-1, ET-3 and SX6C produced vasodilatation probably via ET_B receptors located on the endothelial cells. In addition we have also shown that in this model, repeated injections of ET-1 or SX6C produced tachyphylaxis to the vasodilator responses. The rapid development of tachyphylaxis to the vasodilator response may have important implications in the pathogenesis of pulmonary hypertension where pulmonary ET-1 production is enhanced (Stewart *et al.*, 1991) as the constrictor effects of ET-1 would not be opposed by its dilator actions. We have also provided evidence that the vasodilator responses to ET-1 are mediated by the release of both NO and prostanoids, whereas the vasodilator effects of SX6C are mediated solely by NO production.

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