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Development of endothelin receptors in perinatal rabbit pulmonary resistance arteries

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1 Contractile responses to endothelin-1 (ET-1) and sarafotoxin S6c (S6c) were studied in pulmonary resistance arteries (~320 μm i.d.) from fetal, 0–24 h, 4 day and 7 day rabbits. The effects of the ET_A-selective antagonist FR139317, the selective ET_B receptor antagonist BQ-788 and the non-selective ET_A/ET_B receptor antagonist SB 209670, on these responses, were determined. Acetylcholine-induced vasodilation and noradrenaline-evoked contractions were also examined.

2 ET-1 potency was in the following order (pEC_{50} values): fetal (8.7) = 0–24 h (8.8) = 4 day (8.6) > 7 day (8.0). The order of potency for S6c was 7 days (11.1) = 4 days (10.8) >0–24 h (9.7) > fetal (8.6). Hence, S6c and ET-1 were equipotent in the fetus but S6c was increasingly more potent than ET-1 with increasing age, being some 1000 times more potent by 7 days. By 7 days, responses to ET-1 were also resistant to both FR139317 and BQ-788. FR139317 inhibited responses to ET-1 in vessels from 0–24 h and 4 day, but not fetal, rabbits (pK_b: 6.4 in 4 day rabbits). BQ-788 inhibited responses to ET-1 at all age points except for 7 days (pK_b: 6.7 at 0–24 h; 6.2 at 4 days). BQ-788 inhibited responses to S6c at all age points (pK_b: 8.5 at 4 days). SB 209670 inhibited responses to ET-1 and S6c at 0–24 h and 4 days (pK_b for ET-1: 8.3 and 8.0 respectively; pK_b for S6c: 9.2 and 10.2 respectively).

3 Acetylcholine (1 μM) induced vasodilation at all age points (inhibited by 100 μM L-N^{ω}-nitroarginine methylester) although the degree of vasodilation was significantly reduced (~75%) at 0–24 h. Noradrenaline induced contraction at all age points except 7 days and its response was significantly enhanced at 0–24 h.

4 Over the first week of life, the potency of S6c increases whilst that to ET-1 decreases suggesting differential development of responses to ET-1 and S6c and heterogeneity of ET_A- or 'ET_B-like' receptor-mediated responses. There is no synergism between ET_A and ET_B receptors at birth but this is established by 7 days. Immediately after birth rabbit Pulmonary Resistance Arteries are hyperresponsive to ET-1 and noradrenaline but exhibit impaired nitric-oxide dependent vasodilation.

Keywords: Perinatal rabbit pulmonary resistance arteries; endothelin-1; sarafotoxin S6c; BQ-788; FR139317; SB 209670; acetylcholine; noradrenaline

Introduction

At birth, the ventilation and exposure of the lungs to the high O_2 content of atmospheric air results in loss of hypoxic pulmonary vasoconstriction and hence a marked reduction in the pulmonary vascular resistance (PVR). This change is vital for the marked reduction in pulmonary pressure required to deal with the 10 fold increase in pulmonary blood flow which occurs in the normal transition of the role of gas exchange from the placenta to the lungs (Heymann & Soifer, 1988). However in some newborns, this normal decrease in PVR and increase in pulmonary blood flow does not occur and results in persistent pulmonary hypertension of the newborn (PPHN). This condition results in substantial morbidity and mortality in more than 1 in 1000 newborn infants (Reece *et al*., 1987; Hageman *et al*., 1984).

The endothelins (ETs) and sarafotoxins are a family of potent vasoconstrictor peptides (Inoue *et al*., 1989; Kloog & Sokolovsky, 1989). Two subtypes of mammalian endothelin (ET) receptor have been cloned and sequenced. The first was denoted ET_A and demonstrates selectivity for endothelin-1 (ET-1) over ET-3 (Arai *et al*., 1990). The other receptor, ET_B, is non-isopeptide selective (Sakurai *et al*., 1990). Whilst both receptors have been shown to mediate contraction, the ET_B receptor may also mediate vasorelaxation via endothelial release of nitric oxide (Masaki *et al*., 1991).

ETs have been implicated in many pathophysiologic conditions including pulmonary hypertension (PHT). Elevated circulating ET-1 levels have been reported in patients with both primary and secondary PHT and in infants with PPHN (Stewart *et al*., 1991; Rosenberg *et al*., 1993). Kumar *et al*. (1996) have also shown a significant elevation in PPHN and a positive correlation with disease severity, suggesting that ET-1 may serve as a marker of the disease severity in these infants. ET-1 is one of many endothelial-derived products that play an important role in the transition from *in utero* to postnatal pulmonary circulation (Zielger *et al*., 1995). Loesch and Burnstock (1995) showed that the endothelial cells of the main pulmonary artery in the newborn rat are rich in ET, suggesting a substantial involvement in the vasomotor control of the pulmonary circulation during the early stages of postnatal development.

ET receptor subtype distribution varies with their localization and between species, especially in the pulmonary circulation. Vasoconstriction is evoked via ET_A receptor activation in human, rat, dog and pig large pulmonary arteries (Nakamiche *et al*., 1992; Douglas *et al*., 1993; Fukuroda *et al*., 1994b; MacLean *et al*., 1994). However, in the rabbit large and small pulmonary artery and in the rat pulmonary resistance artery (PRA), 'atypical' ET_B-like receptors mediate vasoconstriction (MacLean *et al*., 1994; Hay *et al*., 1996; Docherty & MacLean, 1998; McCulloch *et al*., 1998). In human PRAs ET-1-mediated vasoconstriction is also mediated via stimulation of

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ET_B receptors at low concentrations of ET-1 whilst ET_A receptors may mediate contraction at higher concentrations (McCulloch *et al*., 1996; 1998).

It is the PRAs which are the important determinants of pulmonary vascular resistance, hypoxic-induced vasoconstriction and pulmonary hypertension *in vivo* (Staub, 1985). Hence the aim of this study was to examine the responsiveness of PRAs to ET-1 from the fetal and neonatal rabbit and examine the effect of ET antagonists on ET-1-mediated vasoconstriction.

We examined the functional responses to ET-receptor stimulation in rabbit PRAs from fetal and neonatal rabbits at three age points during the first week of life, using ET-1 and sarafotoxin S6c (S6c; an ET_B-selective agonist). The ET receptor antagonists used to characterise the endothelin receptors were the ET_A-selective antagonist FR139317 (Sogabe *et al*., 1993) and BQ-788, a potent and selective ET_B receptor antagonist (Ishikawa *et al*., 1994). The non-selective ET_A/ET_B receptor antagonist SB 209670 is an effective antagonist in rat, rabbit and human PRAs and its potency depends on the agonist studied (Ohlstein *et al*., 1994; Hay *et al*., 1996; McCulloch *et al*., 1998; Docherty & MacLean, 1998). We also, therefore, examined its ability to inhibit responses to ET-1 and S6c in the post-natal vessels.

Methods

Rabbit PRAs

Fetal (2 days pre-term) and neonatal New Zealand White rabbit pups were studied at 0–24 h, 4 days and 7 days after birth. They were killed by sodium pentobarbitone (60 mg kg^{-1} i.p.) and the lungs were removed. Small intralobar PRAs ($\sim$250 μm i.d., all 2nd order) were dissected out and cleaned of surrounding parenchyma. The average internal diameters (μm) were as follows ($n \geqslant 15$): fetal: 334$\pm$12.6; 0–24 h: 323$\pm$55.4; 4 day: 333.9$\pm$15.6 and 7 day: 315.8$\pm$26.9. These were mounted as ring preparations ($\sim$2 mm long) on a wire myograph, bathed in Krebs solution at 37°C. Preliminary studies have shown that bubbling with 95%O_2 inhibits responses to vasoconstrictors in the rabbit PRAs so we bubbled with 3%O_2/5%CO_2 balance N_2 for fetal rabbit vessels as this is the condition *in utero*, and 16%O_2/5%CO_2 balance N_2 for all others, to give values similar to those found *in vivo*. Control experiments conducted previously compared the effect of 3%O_2 and 16%O_2 on vasoconstrictor responses to ET and S6c in these vessels and found that responses were not altered. These muscular vessels have medial layers of $\sim$2 μm width and therefore provide minimum barrier to diffusion.

Vessels were tensioned to give a transmural pressure equivalent to approximately 12–14 mmHg, similar to *in vivo* pressures of rabbit pulmonary arterioles after birth. Fetal vessels were also tensioned to this degree as this was the minimum tension at which contractile responses could be observed. The average equivalent transmural pressures (mmHg) were as follows ($n \geqslant 15$): fetal: 13.0$\pm$0.8; 0–24 h: 12.2$\pm$0.6; 4 day: 13.0$\pm$0.6 and 7 day: 12.5$\pm$0.9.

Experimental protocol

After a 1 h equilibration period, response to 50 mM KCl was determined twice. The response to 1 μM noradrenaline was established. In vessels exhibiting a significant contraction to noradrenaline, once this contraction had stabilised, the

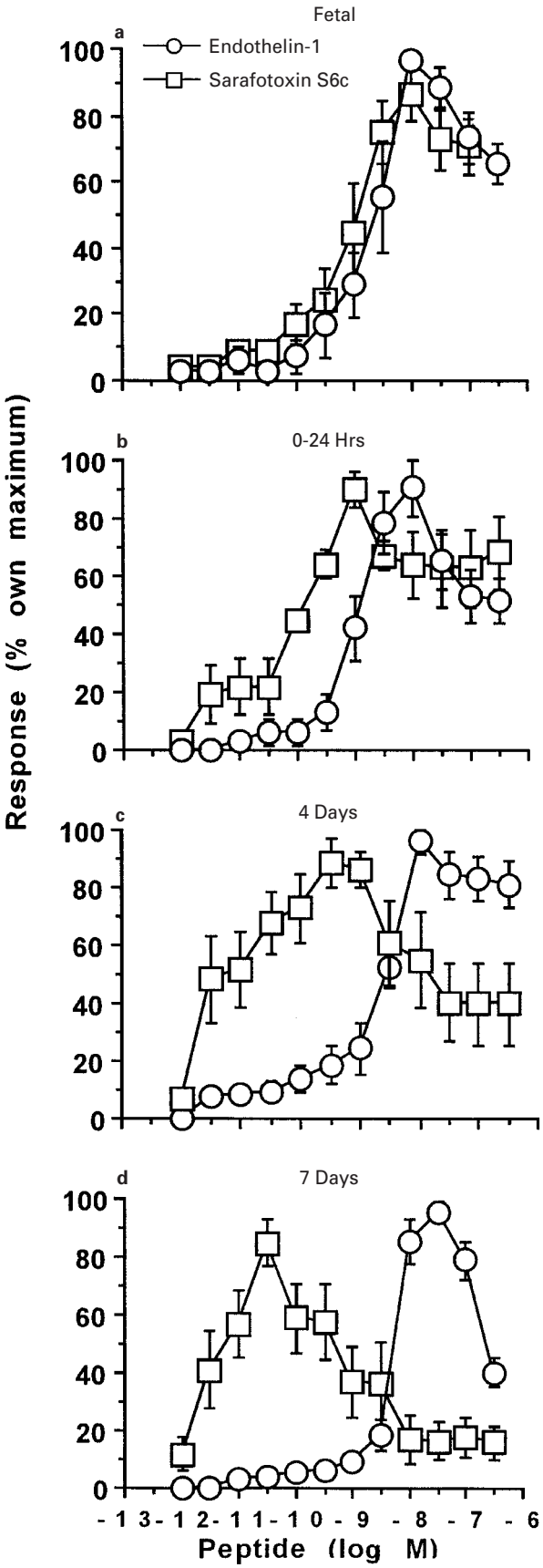

Figure 1 Cumulative concentration-response curves to endothelin-1 and sarafotoxin S6c in pulmonary resistance arteries (PRAs) from (a) fetal rabbits, (b) 0–24 h rabbits, (c) 4 day-old rabbits, (d) 7 day-old rabbits. Data are expressed as a percentage of the maximum response in each preparation. Each point represents mean$\pm$s.e.mean. $n=6$ animals in all cases.

response to 1 μM acetylcholine was investigated. In some vessels, in separate experiments, this was repeated after administration of the nitric oxide synthase inhibitor L-N$^{\omega}$-nitroarginine methylester (L-NAME). We were unable to mechanically remove the vascular endothelium without damaging the fragile underlying smooth muscle. Cumulative concentration-response curves (CCRCs) were constructed to ET-1 or S6c (1 pM – 0.3 μM). Some vessels were preincubated with ET receptor antagonists for 45 min prior to the construction of the CCRC. Concentrations of antagonists studied were chosen due to their calculated pA_2/pK_b values in other vascular preparations. The antagonists studied were FR139317 (1 μM) (pA_2 vs ET-1 in rabbit aorta: 7.2, Sogabe *et al*., 1993); BQ-788 (1 μM) (pA_2 vs BQ-3020 in rabbit pulmonary artery: 8.4, Ishikawa *et al*., 1994) and SB 209670 (0.1 μM) (pK_b vs ET-1 in adult rabbit PRA: 6.8, Docherty & MacLean, 1998). In some vessels the response to 1 nM – 10 μM noradrenaline was also examined.

Drugs and solutions

The composition of the Krebs/bicarbonate buffer (pH 7.4) was as follows (in mM): NaCl 118.4, $NaHCO_3$ 25, KCL 4.7, KH_2PO_4 1.2, $MgSO_4$ 0.6, $CaCl_2$ 2.5, glucose 11, EDTA 23. The following drugs were used: ET-1 (Thistle Peptides, Glasgow, Scotland), BQ-788 (N-cis-2,6-dimethylpiperidinocarboxyl-L-g-methylleucyl-D-I-methocarbonyltrypophanyl-D-norleucine) (Peptide International, Kentucky, U.S.A.), FR139317 ((R)2-[(R)-2-[(S)-2-[[1-(hexahydro-1H-azepinyl)]-carbinyl]amino-4-methylpertanoyl]amino-3-[3-(1-methyl-1H-indoyl)]propionyl]amino-3-(2-pyridyl)propionic acid; Neosystems, France), SB 209670 ((+)-(1S,2R,3S)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methylenedioxyphenyl)-5-(prop-1-yloxy)indane-2-carboxylic acid; gifted by SmithKline Beecham Pharmaceuticals, King of Prussia, U.S.A.), sarafotoxin S6c, acetylcholine chloride, 5-hydroxytryptamine creatinine sulphate and L-N$^{\omega}$-nitroarginine methylester (Sigma, Poole, U.K.). Stock solutions of S6c were prepared in 0.1% acetic acid and those of BQ-788 in 0.1% DMSO. All other drugs and dilutions were prepared in distilled water.

Data analysis

When a maximum response to ET-1 or S6c was obtained, pEC_{50} values were calculated by computer interpolation from individual CCRCs and expressed as $-\log$ M concentration. However, due to the biphasic nature of the CCRCs, we acknowledge that this can only be an estimate of the pEC_{50} values. For this reason also we could not use standard curve fitting programs. Statistical comparison of the means of groups of data were made by one way analysis of variance (ANOVA); $P<0.05$ was considered statistically significant. Throughout, data are expressed as mean $\pm$ s.e.m. and n = number of animals. CCRCs are shown as responses expressed as a % of maximum response to agonist. Wherever possible, pK_b values were estimated but it is noted that in many cases only an estimate can be determined as maximum responses are often not achieved to the ET peptide concentrations used in the presence of antagonists.

Results

Responses to ET-1 and S6c

ET-1 and S6c, were potent vasoconstrictors of rabbit PRAs at all age points studied (Figure 1(A – D), Table 1). ET-1 potency was in the following order: fetal = 0 – 24 h = 4 day > 7 day (Table 1). ET-1 evoked a similar maximum response at all age points studied (Table 2). The CCRC to ET-1 was 'bell-shaped' with responses decreasing around 10 nM (Figure 1(A – D)).

The order of potency for S6c was 7 days = 4 days > 0 – 24 h > fetal (Table 1). Responses to S6c from 1 pmol – 1 nmol increased with developmental age whilst responses to higher concentrations decreased. For example, in the fetus there was little contraction to 10 pmol S6c whilst in the 7 day vessels, the maximum response to S6c was observed at this concentration; in the fetal vessels, 10 nmol S6c produced a maximum response whilst this concentration produced little response at seven days of age (Figure 1(A – D)). The CCRC to S6c was also 'bell-shaped'. The concentration at which the 'drop off' in response occurred varied with age, being noted over 0.1 nM in fetal PRAs and then progressively lower concentrations with increasing age. Maximal contractions to S6c were similar in 0 – 24 h and 4 day-old vessels (Table 2) and were significantly greater than that observed in fetal and 7 day-old rabbit PRAs ($\sim$40%).

ET-1 and S6c were equipotent in the fetal PRAs (Figure 1(A)) however the magnitude of the S6c maximum response was significantly smaller ($P<0.01$; Table 2). In comparison, S6c was significantly more potent that ET-1 at 0 – 24 h following birth (Figure 1(B)) and this difference in potency was even more pronounced at 4 (Figure 1(C)) and 7 days (Figure 1(D)) old. The magnitude of the S6c maximum response was similar to that noted to ET-1 in the 0 – 24 h and 4 day-old preparation but as was seen in the fetus, was

Table 1 pEC_{50} values for endothelin-1 and sarafotoxin S6c, in the absence and presence of antagonists, in pulmonary resistance arteries from fetal and neonatal rabbits

	ET-1 control	n	*ET-1 + 1 μM FR139317*	n	*ET-1 + 1 μM BQ-788*	n	*S6c control*	n	*S6c + 1 μM BQ-788*	n
Fetal	8.7 ± 0.2^{a}	6	7.9 ± 0.4	6	7.6 ± 0.1**	6	8.6 ± 0.3	6	7.1 ± 0.2*	7
0 – 24 h	8.8 ± 0.2^{a}	6	8.2 ± 0.2*	6	8.0 ± 0.1**	6	$9.7\pm0.1^{++b}$	6	7.0 ± 0.1***	5
4 days	8.6 ± 0.1^{a}	6	7.7 ± 0.2**	6	8.2 ± 0.0**	6	$10.8\pm0.3^{+++bbc}$	6	7.8 ± 0.1***	7
7 days	8.0 ± 0.2	6	7.7 ± 0.3	6	8.3 ± 0.2	7	$11.1\pm0.2^{+++bbcc}$	6	7.3 ± 0.3***	4

Statistical comparisons were made by ANOVA; presence of antagonist compared to ET-1 or S6c control *$P<0.05$; **$P<0.01$; ***$P<0.001$; ET-1 control compared to S6c control at same age point $^{++}P<0.01$, $^{+++}P<0.001$; 7 day-old control ET-1 compared to other age points. $^{a}P<0.05$; fetal control S6c compared to other age points; $^{b}P<0.01$, $^{bb}P<0.001$; 0 – 24 h old S6c control compared to other age points; $^{c}P<0.01$, $^{cc}P<0.001$. Values are mean $\pm$ s.e.m. ET-1,endothelin-1; S6c, sarafotoxin S6c. n, number of animals.

Table 2 Maximal contractile responses to endothelin-1 and sarafotoxin S6c expressed as % of contraction to 50 mM KCl, in pulmonary resistance arteries from fetal and neonatal rabbits

	Fetal	*0–24 h*	*4 days*	*7 days*
ET-1	95.3±9	84±14	101.7±16.8	92.7±11.1
S6c	39.5±13.2**^{c}	97.8±27.7	86.1±8.9	41.3±7.1**^{cc}
ET-1 & FR139317	47.4±9.9^{aa}	65±21.3	114.1±21.6	82.2±3.5
ET-1 & BQ-788	140.1±15.8^{b}	169.6±17.7^{bb}	163.9±13.4^{b}	115.4±19.9
SXS6c & BQ-788	109.3±27.1	88.2±20	117.2±12.8	78.2±24.2

Statistical comparisons were made by ANOVA; control ET-1 compared to control S6c $^{**}P<0.01$; control ET-1 compared to presence of FR139317 $^{aa}P<0.01$; control ET-1 compared to presence of BQ-788 $^{b}P<0.05$, $^{bb}P<0.01$, $^{bbb}P<0.001$; 4 day-old control S6c compared to other age points, $^{c}P<0.05$, $^{cc}P<0.01$. Values are mean±s.e.m. ET-1, endothelin-1; S6c, sarafotoxin S6c. (n=4–7 animals).

significantly smaller than the ET-1 maximum by 7 days (Table 2).

Effect of antagonists on ET receptor mediated responses

FR139317 vs ET-1 The selective ET_A receptor antagonist FR139317 (1 μM) did not inhibit the ET-1-induced vasoconstriction of fetal PRAs (Figure 2A). In comparison, ET-1 responses in PRAs from 0–24 h (Figure 2B) and 4 day-old (Figure 2C) rabbits were significantly inhibited by this antagonist. The estimated pK_b was 6.41±0.16 for 0–24 h PRAs. An estimated pK_b of 6.84±0.30 was calculated for 4 day PRAs but this can only be an estimate as responses to the range of ET-1 concentrations studied, in the presence of the antagonist, did not reach a maximum. In 7 day old rabbit PRAs, FR139317 caused an inhibition of the ET-1-induced responses to concentrations above 3 nM (Figure 2D). FR139317 produced a marked reduction in the maximum ET-1 response of the fetal PRAs but had no affect on the magnitude of the maximal contraction to ET-1 at any of the postnatal age points studied (Table 2).

BQ-788 vs ET-1 BQ-788 (1 μM) significantly inhibited ET-1 responses in PRAs from fetal, 0–24 h and 4 day old rabbits (Figure 2 (A–C), Table 1). The estimated pK_b values were 6.71±0.13 and 6.23±0.08 for 0–24 h and 4 day old PRAs respectively. An estimated pKb of 7.06±0.25 was calculated for the fetal vessels PRAs but this can only be an estimate as responses to the range of ET-1 concentrations studied, in the presence of the antagonist, did not reach a maximum. BQ-788 increased the maximum response to ET-1 at these age points (Table 2). BQ-788 did not, however, inhibit ET-1-induced responses in the 7 day vessels and did not significantly alter the maximum contractile response in these vessels (Figure 2 (D), Table 1, Table 2).

BQ-788 vs S6c BQ-788 produced a dramatic inhibition of the S6c-induced responses at all age points studied (Figures 3 (A–D), Table 1). Inhibition was most pronounced in the neonate PRA response as the potency of S6c increased. The estimated pK_b value was 7.49±0.50, 8.70±0.2, 8.49±0.20 and 9.80±0.45 for fetal, 0–24 h, 4 day and 7 day-old vessel responses respectively. It should be noted, however, that a maximum response to the concentration of ET-1 studied, in the presence of BQ-788, was not obtained in the vessels from the fetal, 0–24 h and 7 day-old rabbits.

SB 209670 vs ET-1 SB 209670 inhibited ET-1 vasoconstrictions in the vessels tested (0–24 h and 4 days, Figure 4 (A and B), Table 3). The estimated pK_b values were 8.3±0.1 (0–24 h, $n=5$) and 8.0±0.1 (4 day, $n=6$). SB 209670 did not significantly affect the maximum response to ET-1 (Table 3).

SB 209670 vs S6c SB 209670 was extremely potent against S6c in the vessels tested (0–24 h and 4 days, Figure 4 (C and D), Table 3). The estimated pK_b values were 9.2±0.1 (0–24 h, $n=3$) and 10.2±0.2 (4 day, $n=5$). SB 209670 did not significantly affect the maximum response to S6c (Table 3). The pK_b values were significantly greater in the 0–24 h vessels ($P<0.05$). The pK_b values for SB 209670 were also significantly greater with S6c as the agonist than with ET-1 at both the 0–24 h ($P<0.001$) and 4 day ($P<0.0001$) time points.

Responses to 50 mM KCl, noradrenaline, 5-hydroxytryptamine and acetylcholine

50 mM KCl induced the following contractions (mg wt tension). In fetal vessels: 168.8±24.3 ($n=11$); 0–24 h vessels: 175.7±24.3 ($n=18$); 4 day vessels: 283.6±38.5 ($n=13$) and 7 day vessels: 411.5±67.2 ($n=13$). The degree of contraction in the fetal and 0–24 h vessels was significantly smaller than in the 7 day vessels ($P<0.01$ and $P<0.001$ respectively).

The 1 μM bolus of noradrenaline induced significant contractions in the fetal, 0–24 h and 4 day vessels. The magnitude of the contractions (expressed as a % of the contraction to 50 mM KCl) were as follows: In fetal vessels: 26±16 ($n=15$); 0–24 h vessels: 81±21 ($n=12$); 4 day vessels: 22±7 ($n=7$) and 7 day vessels: 2±1 ($n=6$). The degree of contraction in the 0–24 h vessels was significantly greater than in the other vessels ($P<0.05$). We examined the full CCRC to noradrenaline in the postnatal vessels. The CCRCs to noradrenaline were bell-shaped with the 'fall off' in contraction occurring at 0.3 μM and responses to noradrenaline were significantly enhanced at 0–24 h (Figure 5). The response to 1 μM acetylcholine was examined in the fetal, 0–24 h and 4 day vessels, i.e. where there was a maintained contraction to a single bolus dose of 1 μM noradrenaline. Acetylcholine relaxed these vessels and the degree of relaxation was significantly reduced in the 0–24 h vessels compared with both the fetal and 4 day vessels (Figure 6). To examine if acetylcholine-induced relaxation was preserved at 7 days, we preconstricted 7 day vessels with 1 μM 5-hydroxytryptamine ($n=6$, contraction: 60% of response to 50 mM KCl). Acetylcholine relaxed these vessels by 75±10%. All responses to acetylcholine were inhibited totally by L-NAME (1 μM) [data not shown].

Discussion

The results demonstrate a marked alteration in ET-receptor mediated vasoconstriction in rabbit PRAs, in the first week of life. The potency of ET-1 and S6c varied with developmental

age with the potency order of ET-1 being: fetal = 0–24 h = 4 day > 7 day whilst the potency order for S6c was: fetal < 0–24 h < 4 days = 7 days. These changes in potency resulted in S6c and ET-1 being equipotent in the fetal vessels whereas S6c was increasingly more potent that ET-1 with development. In classical ET_A receptor preparations such as the rat aorta, S6c is

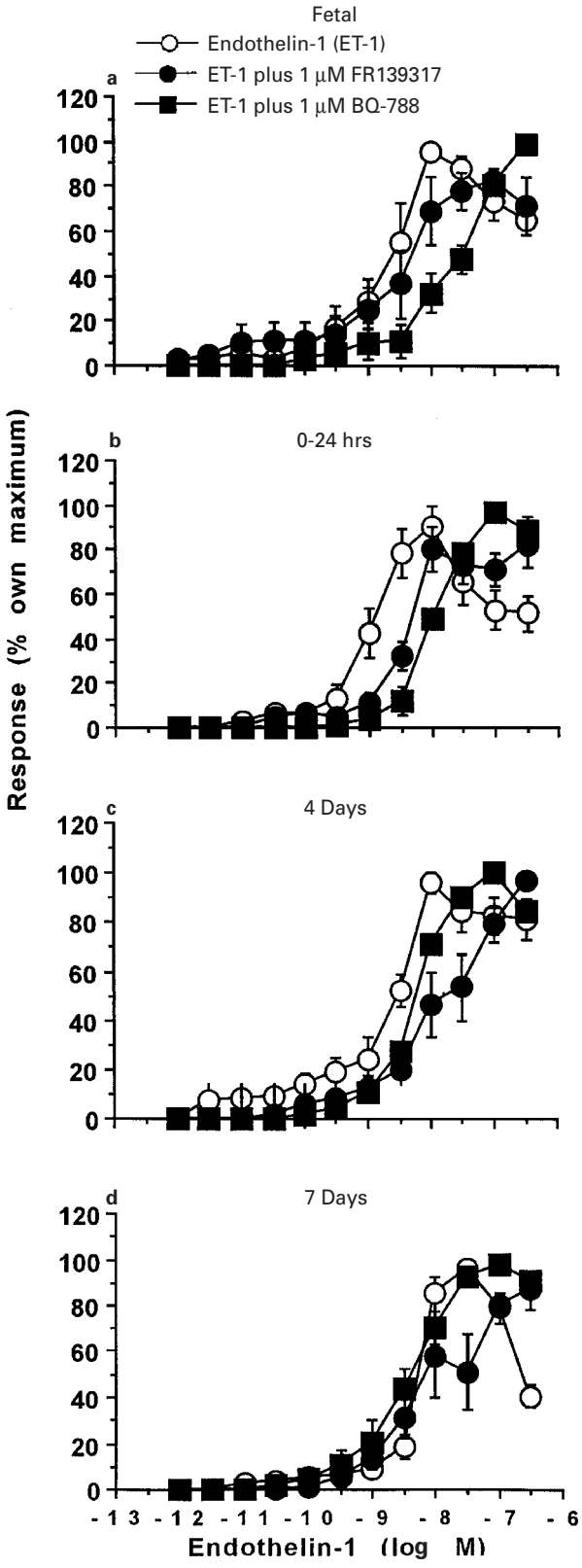

Figure 2 Cumulative concentration-response curves to endothelin-1 from (a) fetal rabbits (b) 0–24 h rabbits (c) 4 day-old rabbits (d) 7 day-old rabbits: effect of the ET_A receptor antagonist FR139317 and the ET_B receptor antagonist BQ-788. Data are expressed as a percentage of the maximum response in each preparation. Each point represents mean ± s.e.mean. $n = 6$ rabbits in all cases.

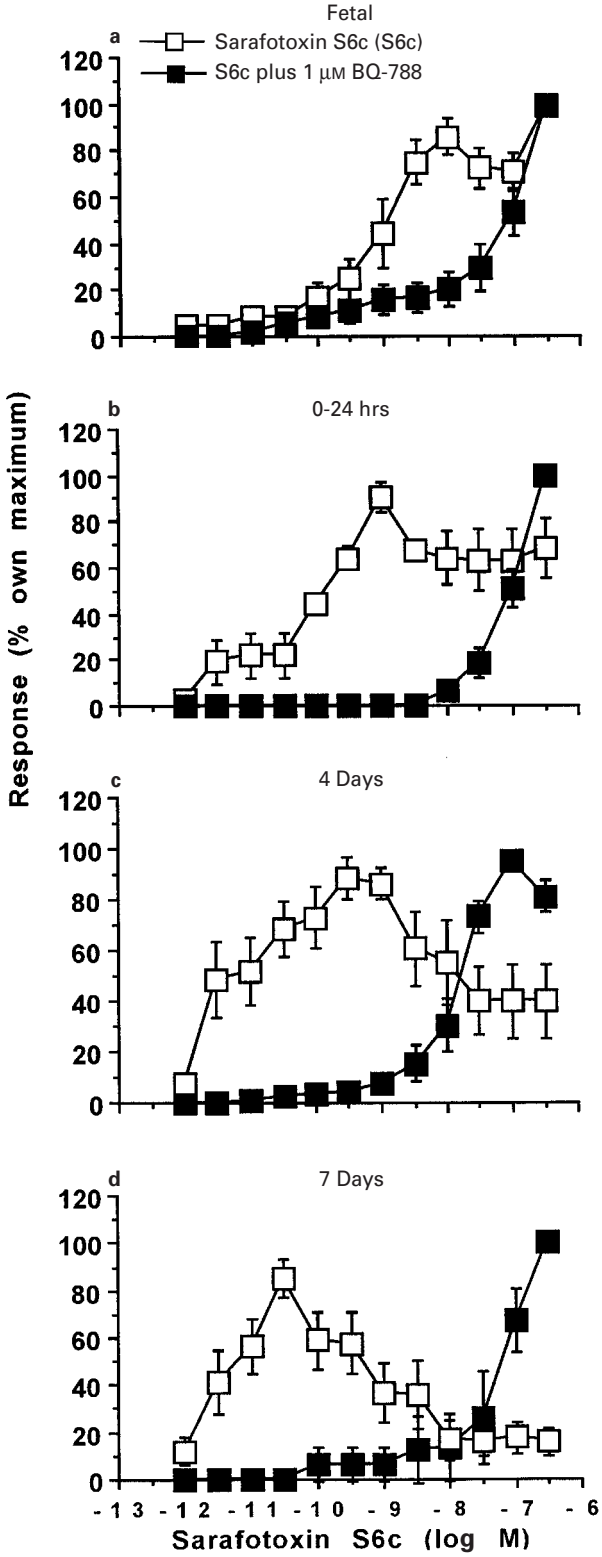

Figure 3 Cumulative concentration-response curves to sarafotoxin S6c in pulmonary resistance arteries from (a) fetal rabbits (b) 0–24 h rabbits (c) 4 day-old rabbits (d) 7 day-old rabbits: effect of the ET_B receptor antagonist BQ-788. Data are expressed as a percentage of the maximum response in each preparation. Each point represents mean ± s.e.mean. $n = 6$ rabbits in all cases.

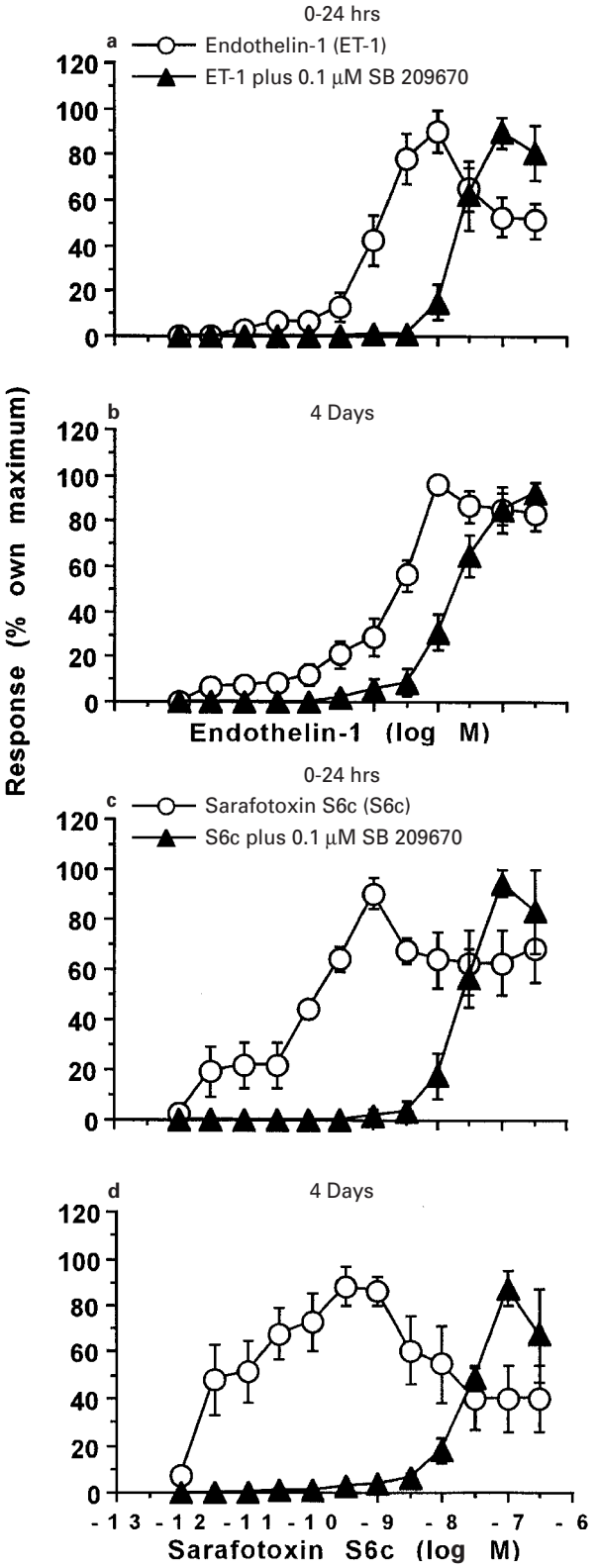

Figure 4 The effect of 0.1 μM SB 209670 on response curves to endothelin-1 (a, b) and sarafotoxin S6c (c, d) in rabbit pulmonary resistance arteries. (a) Endothelin-1 ($n=6$), vs SB 209670 ($n=4$) in vessels from 0–24 h old rabbits. (b) Endothelin-1 ($n=6$) vs SB 209670 ($n=6$) in vessels from 4 day-old rabbits. (c) sarafotoxin S6c ($n=6$) vs SB 209670 ($n=3$) in vessels from 0–24 h old rabbits. (d) Sarafotoxin S6c ($n=6$) vs SB 209670 ($n=5$) in vessels from 4 day-old rabbits. Data are expressed as a percentage of the maximum response in each preparation. Each point represents mean ± s.e.mean.

considerably less potent that ET-1 (Williams *et al*., 1991). The order of potency in the perinatal vessels, therefore suggests that an ET_B-like receptor population prevails. In the postnatal vessels, the receptor has the characteristics of an 'ET_C' receptor where S6c is more potent than ET-1 (Masaki *et al*., 1994). This is in accordance with previous reports where S6c is a more potent vasoconstrictor that ET-1 in rabbit and human saphenous veins, rabbit conduit pulmonary arteries and adult rabbit and rat PRAs (Moreland *et al*., 1994; LaDoucer *et al*., 1993; White *et al*., 1994; Douglas *et al*., 1995; Hay *et al*., 1996; McCulloch *et al*., 1998; Docherty & MacLean, 1998). In these reports, the ET receptor has been variably referred to either as 'ET_C-like', 'ET_B-like' or as an 'atypical' ET receptor. Here we show that the characteristics of this receptor develop in the first week of life in the rabbit PRA.

CCRCs to S6c, ET-1 and noradrenaline were all bell-shaped. This was particularly evident for S6c and noradrenaline. ET_B receptors stimulated by S6c are known to be rapidly desensitised (Sharifi & Schiffrin, 1996). Indeed, we have observed this phenomenon in adult rat and rabbit PRAs and noted that this effect is not dependent on nitric oxide but due to receptor densensitisation (MacLean *et al*., 1994; Docherty & MacLean, 1998; MacLean & McCulloch 1998). It can be seen that the rising phase of the CCRC to ET-1 in the rabbit PRA is actually biphasic in nature in that there is a shallow component up to $\sim$1 nM followed by a steeper component (i.e. Figure 1). This is an effect that we have observed and discussed previously both in the rabbit and rat (Docherty & MacLean, 1998; MacLean & McCulloch, 1998). In adult rabbit PRAs, using an ET binding assay, we have also demonstrated ET binding which best fitted a two-site model (MacLean *et al*., 1998). Pharmacological analysis suggests that there may be a heterogeneous population of ET_B-like receptors in this preparation contributing to the biphasic nature of the CCRC to ET-1 (Docherty & MacLean, 1998).

It has also been known for some time that repeated application of noradrenaline onto vascular smooth muscle causes desensitisation of the α-adrenoceptor (Bobik *et al*., 1984). Recently, this has been proposed to be due to G-protein uncoupling (Seasholtz *et al*., 1997). From the present results, it is apparent that ET and α-adrenoceptor receptors in immature pulmonary smooth muscle are rapidly desensitised. The mechanisms behind this are currently unclear and beyond the scope of the current investigation. Noradrenaline does not cause vasoconstriction in adult rabbit pulmonary resistance arteries (Docherty & MacLean, unpublished observations). Absence of contractile responses to noradrenaline from 7 days of age suggest that these rapidly desensitised α-adrenoceptor receptors either become permanently uncoupled from their intracellular signalling apparatus or diminish in number. Presynaptic β-adrenoceptors exist in the rabbit and human pulmonary artery and these receptors may be upregulated in pulmonary hypertension (Majewski, 1984; Lopes *et al*., 1991). Hence, changes in these receptors may also account for the observed changes in noradrenaline-induced contraction. Curiously, responses to noradrenaline (via both α_1-adrenoceptor and α_2-adrenoceptors) are evident in adult rabbit large pulmonary arteries, another example of regional differences in receptor distribution within the pulmonary arterial tree (MacLean *et al*., 1993).

In the human neonate plasma, ET-1 levels are high at birth but decline in the first week of life (Malamitsi Puchner *et al*., 1993; Endo *et al*., 1996). Similar alterations in plasma levels have also been demonstrated in various animals, such as pig (Levy *et al*., 1995). We have also observed parallel increases in levels of BigET-1 and ET-1 in neonates with pulmonary

Table 3 pEC$_{50}$ values and maximum responses to endothelin-1 and sarafotoxin S6c, in the absence and presence of 0.1 μM SB 209670, in pulmonary resistance arteries from 0–24 h and 4 day-old rabbits

	0–24 h			*4 days*		
	n	p*EC$_{50}$*	*max*	n	p*EC$_{50}$*	*max*
ET-1 control	6	8.8±0.2	85.0±12.1	6	8.6±0.1	102.0±15.9
ET-1+0.1 μM SB 209670	5	7.5±0.1***	65.1±9.4	6	7.6±0.2***	nma
S6c control	6	9.7±0.1	98.1±27.5	6	10.8±0.3	86.4±7.0
S6c+0.1 μM SB 209670	3	7.5±0.1***	50.2±16.5	5	7.5±0.1***	105.1±10.0

Statistical comparisons were made by ANOVA; presence of antagonist compared to ET-1 or S6c control; ***$P<0.001$. Values are mean±s.e.m. Max, maximum response expressed as % of the response to 50 mM KCl. ET-1, endothelin-1; S6c, sarafotoxin S6c. *n*, number of animals; nma, no maximum achieved.

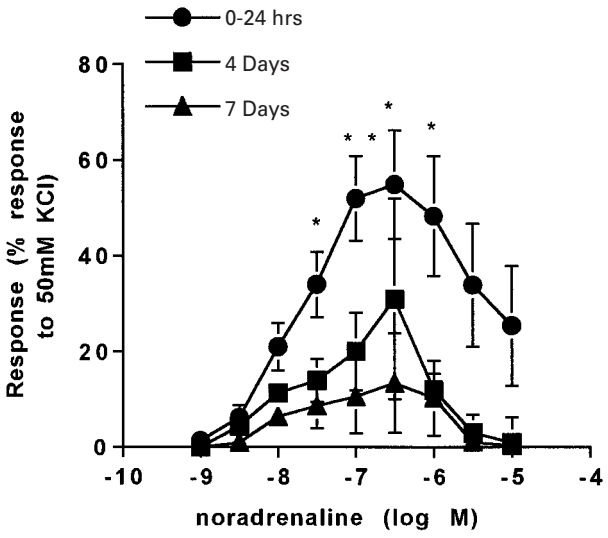

Figure 5 Cumulative concentration-response curves to noradrenaline in pulmonary resistance arteries from 0–24 h rabbits ($n=8$), 4 day-old rabbits ($n=3$) and 7 day-old rabbits ($n=5$). Data are expressed as a percentage of the maximum response to 50 mM KCl in each preparation. Each point represents mean±s.e.mean. Statistical comparisons of 0–24 h cf 7 day were by ANOVA; *$P<0.05$, **$P<0.01$.

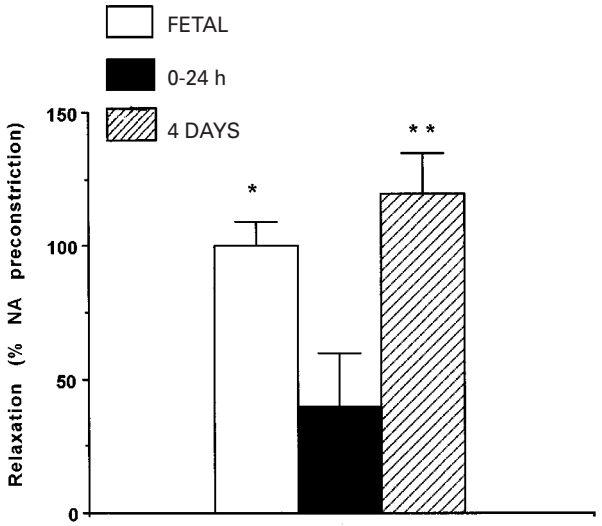

Figure 6 Relaxation to 1 μM acetylcholine in pulmonary resistance arteries from fetal rabbits ($n=17$), 0–24 h rabbits ($n=11$) and 4 day rabbits ($n=11$). Data is expressed as the percentage of preconstriction to 1 μM noradrenaline removed by acetylcholine. Statistical comparisons cf. 0–24 h were by ANOVA; *$P<0.05$, **$P<0.01$.

hypertension (Sabharwal *et al*., 1997). Hence we observe an increase in the potency of ET-1 at a time where we might expect high plasma and tissue levels of ET-1 to prevail, despite evidence for ET receptor desensitisation in our studies. This increased sensitivity may be due to a comparatively greater number of ET receptors present on the pulmonary vasculature in the new-born compared to adult lung. Indeed, Hislop *et al.* (1995b) have demonstrated a reduction in I^{125} ET-1 binding in isolated porcine pulmonary arteries between birth and adulthood.

Responses to S6c were inhibited by BQ-788 which had an estimated pK$_b$ value of ~8.5 in the neonatal PRAs. However in adult rabbit PRAS we have calculated a pK$_b$ value of 6.8 for S6c (Docherty & MacLean, 1998). Hay *et al.* (1996) also showed that BQ-788 has a pK$_b$ value of 6.2 in rabbit large pulmonary arteries. Hence BQ-788 appears to be extremely potent against S6c-induced vasoconstrictions in perinatal PRAs which suggests that further developmental changes to ET ligand binding and/or efficacy must occur after 7 days of age. This is consistent with our observations on the potency of S6c with development. The pEC$_{50}$ values for ET-1 and S6c in the 7 day vessels were 8.0 and 11.1 respectively. Separate studies in our laboratory have provided values for ET-1 and S6c in adult rabbit PRAs of ~7.9 and 8.6 respectively (Docherty & MacLean, 1998). Hence PRAs from 7 day neonatal and adult rabbits are similar in sensitivity to ET-1, whereas sensitivity to S6c in the 7 day-old rabbit is markedly greater than that observed in the adult rabbit PRAs. It therefore appears that ET-1 potency decreases over the first week of life to that observed in the adult. A decrease in S6c potency to that existing in adult vessels must occur at a later age. This divergence in the development of responses to ET-1 and S6c suggests that these peptides either activate different receptor populations or have different binding domains on the same receptor as suggested by Sakamoto *et al.* (1993). There is a growing body of pharmacological evidence that novel ET_B receptors may exist in the lung (Ohlstein *et al*., 1994; Hay *et al*., 1996; Hay & Luttman, 1997; Hay *et al*., 1998; McCulloch *et al*., 1998; Docherty & MacLean, 1998). Only one ET_B receptor has been cloned however, although there is evidence for at least two ET_B receptor splice variants (Shyamala *et al*., 1994; Elshourbagy *et al*., 1996). Up to 4 days of age, BQ-788 inhibited ET-1-evoked response. By 7 days the ET-1-mediated response is clearly of a different character from that mediated by S6c in that it has developed resistance to BQ-788 as observed in PRAs from adult rabbits (Docherty & MacLean, 1988). This suggests that this receptor has similar characteristics to the 'mature' receptor by 7 days.

BQ-788 had no significant effect on the magnitude of the maximum responses to S6c whilst it augmented the ET-1

maximum contraction in the fetal, 0–24 h and 4 day vessels. The reason for this is unknown but may possibly be due to the blockade of ET clearance receptors which have been shown to be ET_B in nature (Fukuroda *et al*., 1994a). BQ-788 can also inhibit the endothelial ET_B receptor which mediates endothelium-dependent relaxation (Douglas *et al*., 1995). We can only find evidence for vasoconstrictor ET_B receptors in rabbit PRAs (Docherty & MacLean, 1998). Nevertheless, we cannot rule out the presence of endothelial ET_B receptors in the perinatal rabbit PRAs given the ability of BQ-788 to increase in the maximum contractile responses to ET-1. It is clear from the present results, however, that the dominant effect of ET-1 in the perinatal vessels is an ET_B-receptor-mediated vasoconstriction.

Evidence for a significant role of ET_A receptors was provided by the inhibitory effect of FR139317 on ET-1-induced vasoconstriction of PRAs from 0–24 h and 4 day-old rabbits. A pK_b of ~6.4 was estimated for the new-born ET-1 responses. This is similar to values obtained for FR139317 in typical ET_A receptor preparations. e.g. rat aorta (Sumner *et al*., 1992). FR139317 did not significantly inhibit the fetal and 7 day rabbit PRA responses to ET-1 and at 7 days, inhibition was only apparent at the higher ET-1 concentrations. FR139317 also caused a significant reduction in ET-1 maximum vasoconstrictions in fetal vessels. This is indicative of ET-1 acting via ET_A receptors at high concentrations but at another receptor at low concentrations in these tissues. A similar effect of ET_A receptor blockade has been observed in human PRAs and larger rabbit PRAs where ET_A and ET_B receptors coexist (McCulloch *et al*., 1996; LaDouceur *et al*., 1993). There is evidence for vasoconstriction via ET_A as well as ET_B receptors in newborn piglets whilst ET_A receptors maintain basal fetal vascular tone in sheep (Wong *et al*., 1994a; Ivy *et al*., 1994; Perreault & Baribeau, 1995).

Neither FR139317 nor BQ-788 were able to inhibit ET-1-induced responses in PRAs from 7 day-old rabbits. This phenomenon has been observed in PRAs from adult rabbits (Docherty & MacLean, 1998). Other investigators have found it necessary to block both ET_A and ET_B receptors in larger rabbit PA and human bronchi in order to antagonise responses to ET-1 fully (LaDouceur *et al*., 1993; Fukuroda *et al*., 1994c, 1996). In comparison, ET_B blockade alone was sufficient to inhibit response to ET-1 in fetal vessels. In 0–24 h and 4 day vessels, ET-1-induced vasoconstrictions were effectively antagonised by inhibition of ET_B receptors or ET_A receptors alone. Thus in the newborn, it appears only necessary to block either receptor in order to inhibit ET-1-mediated responses whereas with increasing age, dual blockade is necessary. Such synergy between ET_A and ET_B receptors is thought to be due to intracellular cross-talk (Ozaki *et al*., 1997). Hence it is likely that development of intra-cellular signalling pathways and cross-talk occurs during the first week of life in the rabbit PRA.

The non-selective ET_A/ET_B receptor antagonist SB 209670 virtually abolished responses to ET-1 in PRAs from 0–24 h and 4 day-old rabbits. A comparatively greater inhibition was noted in the presence of this dual antagonist compared to either FR139317 or BQ-788 alone at the same concentration. For example, FR139317 and BQ-788 resulted in a 6 and 8 fold increase respectively, in the ET-1 pEC_{50} in 0–24 h old rabbit PRAs, whereas SB 209670 caused a significantly greater 18 fold shift when present at the same concentration. We have shown in adult rabbit PRAs that SB 209670 only antagonises vasoconstriction induced by high concentrations of ET-1 (Docherty & MacLean, 1998). This further indicates an alteration in ET receptor subtype population with developmental age. In the PRAs, SB 209670 was even more potent at inhibiting S6c-evoked responses than those evoked by ET-1. This phenomenon is preserved in large and small pulmonary arteries from adult rabbits (Ohlstein *et al*., 1994; Docherty & MacLean, 1998). In addition, co-administration of both FR139317 and BQ-788 does not mimic the effects of SB 209670 in adult rabbit pulmonary resistance arteries (Docherty & MacLean, 1998). The explanation for these phenomena is not clear but there is growing pharmacological evidence for the existence of multiple ET_B-like receptor subyptes in pulmonary tissues which could explain the differences in antagonist affinity (Ohlstein *et al*., 1994; Hay *et al*., 1996, 1998; Hay & Luttman, 1997). Ligands may also bind to different binding domains within a single ET_B-receptor population (Hiley *et al*., 1992; Sakamoto *et al*., 1993).

ET-1 caused similar maximal responses at all age points studied (~94% response to KCl) but the magnitude of the maximum contractile response to S6c was significantly greater at 0–24 h and 4 days (~92%) compared to 7 days (~40%) after birth. These functional differences may be related to alterations in the muscularisation of the arteries with postnatal age (Haworth, 1995). Responses to KCl were, however, significantly smaller at 0–24 h and 4 days than at 7 days, making this unlikely. This is consistent with there being rapid changes in pulmonary artery smooth muscle phenotypes, combined with transient depolymerisation of contractile and cytoskeletal filaments immediately after birth (Haworth, 1995). We have previously shown, in adult rat and rabbit PRAs, that contractile responses to S6c are markedly potentiated by inhibition of nitric oxide synthesis (Docherty & MacLean, 1998; MacLean & McCulloch, 1998). To investigate if the maximum responses to S6c may be affected by protection afforded by the endothelium, we examined acetylcholine-induced vasodilation. This was observed in all the perinatal vessels but was significantly reduced at 0–24 h, recovering by 4 days. This suggests that endothelial protection will be deficient immediately after birth. Responses to acetylcholine were fully inhibited with the L-NAME suggesting that acetylcholine-induced vasodilation was due to the release of nitric oxide. We have previously shown that adult rabbit PRAs do not exhibit acetylcholine- or endothelin-induced vasodilation (Docherty & MacLean, 1998). Hence, acetylcholine-induced vasodilation is present at birth, declines just after birth, recovers over the first week and subsequent development leads to a reduction in this response. Similar findings have been reported in the postnatal pulmonary arteries from the pig (Liu *et al*., 1992). The reason for the decline in acetylcholine-induced relaxation in the immediate postnatal period is not fully understood. Hislop *et al*. (1995a) have shown that nitric oxide synthase is present in the newborn pig pulmonary arteries at this time, despite a reduction in endothelium-dependent vasodilation and so is unlikely to be due to an absence of this enzyme. We also demonstrate that, in the 0–24 h vessels, simultaneous with the decline in acetylcholine-mediated relaxations, there was an increase in the response to noradrenaline. This may, indeed be a consequence of the decline in endothelial protection as we have previously shown that responses to noradrenaline are enhanced by inhibition of nitric oxide or endothelium removal in adult rabbit large pulmonary arteries (MacLean *et al*., 1993). In addition, we have demonstrated restoration of acetylcholine-mediated relaxations in 0–24 h rabbit neonates with superoxide dismutase, suggesting that the activity of nitric oxide may be compromised by superoxide anion in these vessels (Morecroft & MacLean, 1996). If this situation exists in human PRAs, these results may explain why human neonates are extremely

susceptible to pulmonary hypertension after birth, when the pulmonary circulation is faced with elevated vascular responses to circulating agents such as noradrenaline and endothelin-1 in the face of reduced endothelial protection.

In conclusion, the results of this study indicate a rapid alteration in ET-receptor-mediated contraction in rabbit PRAs during the first week of life. There is a significant population of ET_B-like receptors mediating vasoconstriction which coexist alongside ET_A receptors in fetal and neonatal rabbit PRAs. The contribution of these receptor subtypes to the overall ET-induced responses varies with developmental age and a marked hypersensitivity to ET_B receptor stimulation is apparent in new-born rabbit PRAs. Synergy between ET_A and ET_B receptors is not present at birth but develops by 7 days. There is also a reduction in endothelium-dependent vasodilation immediately after birth and an increase in responsiveness to noradrenaline. Hence increased potency of ET-1 mediated by ET_B-like receptors, combined with changes in endothelial function, may contribute to the enhanced pulmonary reactivity often observed at birth.

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