Transcriptional down-regulation of the rabbit pulmonary artery endothelin B receptor during phenotypic modulation

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1 We confirmed that endothelium-independent contraction of the rabbit pulmonary artery (RPA) is mediated through both an endothelin A (ET_AR) and endothelin B (ET_{B2}R) receptor.

2 The response of endothelium-denuded RPA rings to endothelin-1 (ET-1, pD₂ = 7.84 ± 0.03) was only partially inhibited by BQ123 (10 μM), an ET_AR antagonist.

3 Pretreatment with 1 nM sarafotoxin S6c (S6c), an ET_BR agonist, desensitized the ET_{B2}R and significantly attenuated the response to ET-3 (pD₂ = 7.40 ± 0.02 before, < 6.50 after S6c).

4 Pretreatment with S6c had little effect on the response to ET-1, but BQ123 (10 μM) caused a parallel shift to the right of the residual ET_AR-mediated response to ET-1 (pD₂ = 7.84 ± 0.03 before S6c, 7.93 ± 0.03 after S6c, 6.81 ± 0.05 after BQ123).

5 Binding of radiolabelled ET-1 to early passage cultures of RPA vascular smooth muscle cells (VSMC) displayed two patterns of competitive displacement characteristic of the ET_AR (BQ123 pIC₅₀ = 8.73 ± 0.05) or ET_{B2}R (S6c pIC₅₀ = 10.15).

6 Competitive displacement experiments using membranes from late passage VSMC confirmed only the presence of the ET_AR (ET-1 pIC₅₀ = 9.3, BQ123 pIC₅₀ = 8.0, S6c pIC₅₀ < 6.0).

7 The ET_AR was functionally active and coupled to rises in intracellular calcium which exhibited prolonged homologous desensitization.

8 Using a reverse transcriptase polymerase chain reaction for the rabbit ET_{B2}R, we demonstrated the absence of mRNA expression in phenotypically modified VSMC.

9 We conclude that the ET_{B2}R expressed by VSMC which mediates contraction of RPA is rapidly down-regulated at the transcriptional level during phenotypic modulation *in vitro*.

Keywords: endothelin receptor; pulmonary artery; vascular smooth muscle; phenotypic modulation; intracellular calcium; mRNA

Abbreviations: ET, endothelin; ETR, endothelin receptor; FBS, foetal bovine serum; GRGESP, Gly-Arg-Gly-Glu-Ser-Pro hexapeptide; RPA, rabbit pulmonary artery; S6c, sarafotoxin 6c; VSMC, vascular smooth muscle cells

Introduction

Three distinct endothelin receptors (ETR) have been sequenced and characterized (Bax & Saxena, 1994). The ET_AR has greater affinity for endothelin-1 (ET-1) than ET-3, whereas the ET_BR has similar affinity for all ET isoforms and sarafotoxin S6c (S6c). A receptor with highest affinity for ET-3, the ET_CR, has been identified in *Xenopus laevis* (Karne *et al.*, 1993) and a response with similar ligand specificity has been described in rabbit saphenous vein (Douglas *et al.*, 1995). On the basis of functional assays and ligand selectivity, subtypes of ET_BRs have been described. The ET_{B1}R on endothelial cells mediates endothelium-dependent relaxation of vascular smooth muscle cells (VSMC) while the ET_{B2}R on VSMC mediates contraction (Warner *et al.*, 1993). Rabbit pulmonary artery (RPA) preparations were among the first described examples of ET_{B2}R-mediated vasoconstriction (Fukuroda *et al.*, 1994; La Douceur *et al.*, 1993; Panek *et al.*, 1992; Warner *et al.*, 1993) but others have also been identified (Lodge & Halaka, 1993; Moreland *et al.*, 1992; Shetty *et al.*, 1993; Sudjarwo *et al.*, 1994; Webb *et al.*, 1993). Whilst BQ788

antagonizes effects attributable to both B receptor subtypes (Ishikawa *et al.*, 1994) the antagonists PD142893 and IRL 1038 have been used to discriminate between them (Sudjarwo *et al.*, 1994; Warner *et al.*, 1993).

The structural basis for the pharmacological differences between ET_BR subtypes is uncertain. All of the sequenced ETRs are members of the G protein-coupled receptor superfamily and low stringency screening has failed to identify multiple genes. The interpretation of standard pharmacological assays is complicated by 'cross-talk' between different receptors in preparations where both the ET_AR and ET_{B2}R are expressed (Clozel & Gray, 1995). Post-translational modifications or alternative mRNA splicing could account for different ligand binding characteristics. However, the only reported splice variant of the ET_BR does not affect the coding region (Cheng *et al.*, 1993) and the binding characteristics of alternatively spliced ET_AR products have not yet been reported (Miyamoto *et al.*, 1996). The inferred protein sequence of a putative human ET_{B2}R was not significantly different from that of the human endothelial ET_{B1}R (Webb *et al.*, 1995). However, a recent study using the endothelin B receptor gene knockout mouse suggests that both ET_{B1}R- and ET_{B2}R-mediated contractions may be due to ET_B receptors derived from the same gene (Mizuguchi *et al.*, 1997).

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Many studies of second messenger systems have been performed using cell lines, including those stably transfected with cloned receptors. Although facilitating biochemical characterization of the responses, they need not have a bearing on the behaviour of normal cells, many of which express more than one receptor (including RPA VSMC). While the events in contractile cells which are coupled to signalling through the ET_AR are relatively well described, particularly calcium transients (Pollock *et al.*, 1995), those activated through the ET_BR have not been well characterized. In attempting to identify second messenger systems coupled to ETRs on normal VSMC from the RPA and the nature of 'cross-talk' between them, we have confirmed that both the ET_B2R and ET_AR mediate RPA contraction. We have shown that the ET_AR expressed in both early and late passage cells is functional and that ligand binding stimulates a rise in intracellular calcium. However, during phenotypic modulation of VSMC early in culture (Campbell *et al.*, 1988), expression of ET_B2R mRNA is rapidly down-regulated, leading to the loss of ET_B2R expression at the cell membrane. We have correlated the down-regulation of the ET_B2R with that of smooth muscle high molecular weight caldesmon, a marker of the contractile phenotype (Birukov *et al.*, 1993; Kashiwada *et al.*, 1997).

Methods

Animals

Main pulmonary arteries were obtained from heparinized (500 U, i.v. male and female New Zealand White rabbits (<1.5 kg) after Nembutal anaesthesia (120 mg pentobarbitone Na, Boehringer Ingelheim, Sydney) according to a protocol approved by the Garvan/St. Vincent's Hospital Animal Experimentation and Ethics Committee.

Organ bath experiments

Endothelium was removed by gentle rubbing of the luminal surface with a wooden swab stick. Rings (2 mm) were suspended at 37°C in 10 ml jacketed organ baths containing freshly prepared Krebs-bicarbonate solution (in mM) NaCl 118, KCl 4.7, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.18, glucose 5, NaHCO₃ 25, CaCl₂·2H₂O 2.54 continuously gassed with 5% CO₂ and 95% O₂. The basal tension was 2.00±0.05 g and isometric tension was measured using Grass FT03 force transducers (Quincy, MA, U.S.A.) coupled to a MacLab data acquisition system (Analog Digital Instruments, Sydney, Australia). In cumulative concentration-response experiments, drugs were administered sequentially after the tension had stabilized (≥2 min). In some experiments rings were pre-treated with 1 nM S6c, an ET_BR-selective agonist, for 30 min prior to the concentration-response protocols.

Tissue culture

VSMC from RPA were obtained from vessels which were washed several times in Hanks balanced salt solution (GIBCO, Life Technologies, Melbourne) and incubated in 1000 u ml⁻¹ collagenase type II (Sigma Chemical Co, Sydney) for 30 min at 37°C to remove endothelium. Strips of vessel wall media were peeled off using watchmakers' forceps, diced and incubated in a solution containing collagenase and 60 u ml⁻¹ porcine pancreatic elastase (Calbiochem-Novabiochem, Sydney) for 2–3 h at 37°C with periodic pipetting. Either the tissue explants or dispersed cells were seeded in

Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (100 u ml⁻¹), streptomycin (100 µg ml⁻¹), fresh L-glutamine (4 mM, all from GIBCO) and 10% foetal bovine serum (FBS, PA Biologicals, Sydney). Cultured cells expressed smooth muscle actin but not Factor VIII:RAG, an endothelial cell marker, using an indirect immunofluorescence assay.

Expression of high molecular weight caldesmon

At subculture between passages 2–4, a small portion of cells from the main culture batch were seeded into 8-well chamber slides (Nunc, Medos, Sydney). The cells were rinsed twice with phosphate-buffered saline (PBS), and fixed for 10 min in 3% formaldehyde/0.1% glutaraldehyde in PBS. After three washes in PBS, the cells were permeabilized with 0.5% Triton X-100 (Rohm & Haas) in PBS for 30 min. After a further three washes with PBS, mouse monoclonal anti-high molecular weight caldesmon (clone hCD, Sigma Australia) was applied to the cells for 30 min at room temperature in 0.1% bovine serum albumin (BSA, Sigma Australia) in PBS. Antibody binding was detected using FITC-rabbit anti-mouse antibody (Silenus Laboratories, Hawthorn, Australia) for 30 min at room temperature in 0.1% BSA/PBS. Slides were mounted in glycerol containing 1 mg ml⁻¹ paraphenylenediamine (BDH Sydney) and fluorescence visualized using a laser confocal microscope (Molecular Dynamics, Melbourne, Australia).

Whole cell binding assays

Passage 2 cells were seeded into 24-well plates (Corning, Sydney) at 4 × 10⁴ cells well⁻¹ and cultured to confluence (6–8 × 10⁴ cells well⁻¹). The cells were washed twice with binding buffer (phosphate-buffered saline containing CaCl₂ 1 mM, MgCl₂ 0.5 mM and 0.1% BSA) at 30°C (Serradeil-Le Gal *et al.*, 1991). Wells were incubated at 22°C with 1 ml binding buffer containing 10 pM [¹²⁵I]-ET-1 (Amersham, Sydney) ± unlabelled ET-1, BQ123, S6c or an unrelated control hexapeptide of sequence GRGESP (all American Peptide Co, Sunnyvale, CA, U.S.A.). Non-cell-associated (free) counts were determined in the aspirated supernatant which was pooled with two 1 ml washes of binding buffer. Cell associated (bound) counts were determined following solubilization of the cells with two 0.5 ml aliquots of NaOH (0.5 M). Tubes were counted for 1 min in a Packard Cobra Auto Gamma counter (Meriden, CT, U.S.A.).

Membrane preparation and binding assays

Passage 6–8 cells in 150-cm² flasks were rinsed with ice-cold phosphate-buffered saline and scraped from the flask. After two further washes the cell pellet was lysed with HEPES (5 mM) lysis buffer (5 × 10⁸ cells 20 ml⁻¹) according to published protocols (Lagny-Pourmir *et al.*, 1989; Servin *et al.*, 1987). The lysate was pelleted at 24,500 × g for 15 min at 4°C in a Heraeus Sepatech Supafuge (Osterode, Germany) with a HFA20.16 fixed angle rotor. The pellets were resuspended at a cell equivalent concentration of 2 × 10⁷ ml⁻¹ in RBM buffer (Servin *et al.*, 1987) containing (in mM) Tris HCL (pH 7.5) 50, NaCl 100, MgCl₂ 5, EDTA 1; bacitracin 100 µg ml⁻¹, leupeptin 10 µg ml⁻¹ (both Calbiochem, Sydney) and phenylmethylsulphonyl fluoride (100 µM) (Sigma). The crude receptor preparations were then gently syringed with a 23 gauge needle and stored at –70°C in 50 µl aliquots for up to 1 week. Aliquots were thawed and sonicated on ice using a Branson Sonifier 250 (6 × 10 s cycles at 50% power setting). Protein concentration was determined using a bicinchoninic

acid assay kit (Pierce, Lab Supply, Sydney). After the optimum protein concentration had been defined, competitive receptor binding assays were performed according to published protocols (Servin *et al.*, 1987). In a final volume made up to 200 μ l with KRB buffer (see below), membrane protein was added to 15 μ l [^{125} I]-ET-1 (final concentration 30 pM) and 20 μ l of 10 fold dilutions (range 10^{-6} – 10^{-12} M final concentration) of unlabelled competitors ET-1, ET-3, BQ123 and sarafotoxin 6c or control hexapeptide GRGESP (American Peptide Co.). Ligands were diluted in KRB buffer (in mM) (NaCl 68, KCl 2.4, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.6, KH_2PO_4 0.6, NaHCO_3 2.5, glucose 2.8, HEPES 10, CaCl_2 1, 0.2% BSA, 1 mg ml $^{-1}$ bacitracin (Boehringer Mannheim, Sydney), pH 7.4) in Eppendorf microfuge tubes. The tubes were incubated for 2 h in a 37°C waterbath, after which each incubation mix was underlaid with 500 μ l FBS using a 1 ml tuberculin syringe fitted with a 25G needle. The tubes were spun for 10 min at $13,000 \times g$ at 4°C to separate receptor-bound radioactivity in the pellet from unbound radioactivity in the supernatant. The supernatants were aspirated and kept for counting the unbound radioactivity, and the tips of the tubes containing the pellets were cut off for counting the bound radioactivity. Results were expressed as percentage bound/control as a function of ligand concentration.

Intracellular calcium assays

At least 3 days before intracellular calcium assays, passage 2–3 cells (2×10^5 /well) were plated onto glass coverslips and serum-deprived for 24 h in DMEM containing 4% Monomed (Commonwealth Serum Laboratories, Melbourne). They were incubated for 30 min with Fura-2 acetoxymethyl ester (5 μ M) (Fura-2 AM; stock solution 1 mM in dimethyl sulphoxide; Molecular Probes, BioScientific, Sydney) in 4% Monomed and washed twice in perfusion solution (in mM) NaCl 145, KCl 5, HEPES 10, MgCl_2 1.2, CaCl_2 1 and 0.1% bovine serum albumin; pH 7.45). The loaded cells were mounted on the stage of a Nikon Diaphot microscope, modified for microfluorescence studies as previously described (Gibb *et al.*, 1994) and superfused with perfusion solution at 37°C using a peristaltic pump (Ismatech, Extech Equipment, Melbourne). The ratio of fluorescence emission at 510 nm by Fura-2 at excitation wavelengths of 340 nm and 380 nm were used to measure $[\text{Ca}^{2+}]_i$ as previously described (Gryniewicz *et al.*, 1985):

$$[\text{Ca}^{2+}]_i = K \frac{(R - R_{\min})}{(R_{\max} - R)} \cdot \frac{(S_{f2})}{(S_{b2})}$$

where $K = 224$ nM (Fura-2 at 37°C); $R_{\min}$ = ratio value in Ca^{2+} free conditions; $R_{\max}$ = ratio value at a maximal Ca^{2+} concentration; $S_{f2} = 380$ nm reading in low Ca^{2+} conditions (corrected for background) and $S_{b2} = 380$ nm reading in high Ca^{2+} conditions (corrected for background). $R_{\min}$ and S_{f2} were determined at the end of an experiment by perfusing the cells with Ca^{2+} -free perfusion solution containing EGTA (5 mM, Calbiochem) and a divalent cation ionophore, ionomycin (2 μ M, Calbiochem). $R_{\max}$ and S_{b2} were then determined by the addition of CaCl_2 (5 mM) in ionophore-containing perfusion solution.

Ligands and inhibitors

Stock solutions of human ET-1, human ET-3, BQ123, S6c (all American Peptide Co.), adenosine 5'-triphosphate (ATP, disodium salt) and uridine 5'-triphosphate (UTP, monosodium

salt, both from Sigma) were stored at -20°C . For organ bath experiments, drugs were freshly diluted in Krebs bicarbonate solution and for intracellular calcium experiments in perfusion solution.

Data analysis

Organ bath results from at least four experiments are presented as means $\pm$ s.e.mean. Graphs were obtained by fitting the results to a four parameter logistic function (SigmaPlot, Jandel Corporation, San Rafael, CA, U.S.A.). The IC_{50} from binding experiments was calculated using the Inplot program (Graphpad Software, San Diego, CA, U.S.A.).

cDNA preparation

RNA from RPA (four rabbit donors) and cultured VSMC derived from the same RPA (primary through to passage 4 cells) was prepared according to standard procedures (Chomczynski & Sacchi, 1987). RNA pellets were resuspended in 50 μ l diethylpyrocarbonate (Sigma)-treated water. cDNA was prepared from 2 μ g RNA in a 50 μ l reaction volume containing dNTPs (0.25 mM each, Boehringer Mannheim), 200 ng oligo(dT), 4 U AMV reverse transcriptase and 2 U RNasin (all Promega, Sydney).

Reverse Transcriptase-Polymerase chain reaction (RT-PCR)

The rabbit ET_BR was cloned from a rabbit lung cDNA library screened with a PCR product derived from RPA VSMC using human primers (N. Yang *et al.*, in preparation). Specific primers based on the rabbit DNA sequence (forward 5'-ACTGGCCATTTGGAGCTGAGAT and reverse 5'-TTGCATTCCACTCTTCTTTCTTAA) were used to amplify a 430 base pair product, which spans an intron, from cDNA prepared from each RPA and cultured VSMC cells at each passage derived from the same RPA. Rabbit GAPDH was used as a control (forward 5'-GATGCTGGTGCCGAGTACGTGG and reverse 5'-TCAGCAACGCATCCTGCAC-CAC) for the integrity of the cDNA preparation. Reactions (50 μ l) were performed using 5 μ l cDNA, dNTPs (0.25 mM), Mg^{2+} for GAPDH (1.5 mM), or Mg^{2+} for ET_BR (2.5 mM and 1–2 U Taq polymerase (Boehringer Mannheim). The cycle conditions for ET_BR were: 95°C, 3 min; 57°C, 30 s, 75°C, 30 s, for 1 cycle; 95°C, 1 min; 57°C, 30 s; 75°C, 30 s for 28 cycles; 95°C, 1 min; 57°C, 30 s; 75°C, 7 min for 1 cycle final extension. The cycle conditions for GAPDH were 95°C, 1 min; 62°C, 30 s; 72°C, 30 s for 1 cycle; 95°C, 30 s; 62°C, 30 s; 72°C, 30 s for 32 cycles; 95°C, 30 s; 62°C, 30 s; 72°C, 5 min for 1 cycle final extension. RT-PCR products were run on 1.8% agarose gels (Amresco, Astral Scientific, Sydney) in Tris-borate-EDTA buffer and stained with ethidium bromide for visualization under UV light. Gels were photographed using a Polaroid MP-4 camera and type 665 film.

Results

ET-mediated contraction of rabbit pulmonary artery

Cumulative concentration-response experiments using ET-1 and ET-3, the latter being relatively selective for the ET_BR , confirmed that they both stimulated endothelium-independent contraction via ET_AR or ET_BR (ET-1 $\text{pD}_2 = 7.84 \pm 0.03$, $n = 8$; ET-3 $\text{pD}_2 = 7.4 \pm 0.02$, $n = 9$, Figure 1A). Pretreatment of

Figure 1 Contraction of endothelium-denuded RPA rings with increasing concentrations of (A) ET-1 ($n=8$), ET-3 ($n=9$) and ET-1 + 10 μM BQ123 ($n=8$), and (B) ET-1 ($n=4$), ET-3 ($n=3$) and ET-1 + 10 μM BQ123 ($n=4$) after pretreatment with S6c.

endothelium-denuded rings with the ET_B R-selective agonist S6c (1 nM) abolished the contractile response to ET-3 ($\text{pD}_2 < 6.50$, $n=3$, Figure 1B). However, pretreatment with S6c had no effect on the response subsequently induced by ET-1 ($\text{pD}_2 = 7.93 \pm 0.03$ after S6c, $n=4$, Figure 1B). The dose-response curve to ET-1 was bi-sigmoidal in the presence of the ET_A R-specific antagonist BQ123 (Ihara *et al.*, 1992; Figure 1A, $n=8$), suggesting that the contractile response to ET-1 was mediated *via* activation of both ET_A R and ET_B R. A parallel shift to the right of the response to ET-1 in the presence of BQ123 followed desensitization with S6c ($\text{pD}_2 = 6.81 \pm 0.05$, $n=4$, Figure 1B), indicating a pure ET_A R-mediated response.

Expression of high molecular weight caldesmon

Due to the small numbers of cells available at early passages, RPA VSMC were not examined immunohistochemically until passage 2 (Figure 2). At this stage, approximately 30% of cells stained positively for high molecular weight caldesmon, the staining being associated with cytoplasmic filaments running the length of the cells (Figure 2A). Thus, by passage 2, most of these cells had down-regulated their expression of high molecular weight caldesmon. In passage 3 and 4 cells (Figure 2B and C, respectively), caldesmon staining was localized around the nuclei, with little cytoplasmic staining present.

Down-regulation of ET_B R expression in cultured vascular smooth muscle cells

Small scale binding studies were used to identify the receptors expressed on low numbers of early passage adherent cells. Binding of radio-iodinated ET-1 increased over 1.5–2 h and

Figure 2 Expression of high molecular weight caldesmon. (A) Caldesmon immunofluorescence is associated with cytoplasmic filaments. Only 30% of cells exhibit positive staining at passage 2. Caldesmon expression is only perinuclear at passages 3 and 4 (arrows, B and C, respectively). Note that the perinuclear staining in the passage 4 cells extends slightly into the extended pseudopodia (arrows). Scale bar = 10 μm .

was specifically inhibited by unlabelled ET-1 (results not shown). Competition binding studies were performed using ET-1 and the ET_A R-selective ligands, BQ123 and S6c respectively. Representative experiments are shown in Figure 3 and the results of six experiments are summarized in Table 1. Individual cultures from single rabbits displayed two patterns of binding which were typical of either the ET_A R or ET_B R (Figure 3). Figure 3A illustrates competitive displacement by ET-1 ($\text{pIC}_{50} = 10.2$) and BQ123 ($\text{pIC}_{50} = 8.7$) but not S6c ($\text{pIC}_{50} < 6.0$) while Figure 3B illustrates competitive displacement by ET-1 ($\text{pIC}_{50} = 10.4$) and S6c ($\text{pIC}_{50} = 8.6$) but not BQ123 ($\text{pIC}_{50} < 6.0$). In experiments demonstrating ET_A R binding ($n=4$, preparations 2 and 4–6,

Table 1) the mean pIC_{50} for BQ123 was 8.73 ± 0.05 , while in experiments demonstrating ET_B R binding ($n=2$, preparations 1 and 3, Table 1) the mean pIC_{50} for S6c was 10.15 ($n=2$). The mean pIC_{50} s for ET-1 were 9.95 ± 0.25 and 10.25 respectively.

ET_AR expression in late passage vascular smooth muscle cells

Given the small number of cells obtained from a single rabbit, we were unable to follow the pattern of binding sequentially in cultured cells derived from the same animal. However, there were sufficient cells from late passages (6–8) to obtain

satisfactory membrane preparations for competitive binding studies ($n=3$). These cells were clearly 'synthetic' as judged by their morphological characteristics (transition from spindle-shaped to polygonal cells, cellular hypertrophy) and decreased expression of high molecular weight caldesmon isoforms by immunofluorescence. These binding studies demonstrated concentration-dependent inhibition of radiolabelled ET-1 binding by unlabelled ET-1 ($pIC_{50}=9.3$) and BQ123 ($pIC_{50}=8.0$) but not S6c (Figure 4). This was consistent with the binding characteristics of an ET_A R. Thus phenotypic modulation of RPA SMC is associated with down-regulation of ET_B R ligand binding.

ET_AR-mediated $[Ca^{2+}]_i$ responses in cultured vascular smooth muscle cells

In passage 2 and 3 cultured cells ET-1 (200 nM) induced a typical biphasic rise in $[Ca^{2+}]_i$ (Figure 5A). The resting $[Ca^{2+}]_i$ in RPA VSMC was 60–100 nM with a peak response up to ~500 nM. Oscillations with a periodicity of ~3 min were occasionally observed, particularly in cultures approaching confluence (data not shown). In keeping with the binding studies which suggested that the ET_A R was the predominant receptor expressed by cultured RPA VSMC, the ET_B R-selective agonists S6c and ET-3 failed to induce a significant rise in $[Ca^{2+}]_i$ (Figure 5B). Whereas the ET-1 response demonstrated homologous desensitization, the responses to P_{2Y} purinoceptor agonists (ATP and UTP) were unaffected by pre-exposure to ET-1 (Figure 5A and B, respectively). We have shown previously that the P_{2Y2} receptor on RPA does not demonstrate homologous desensitization (Qasabian *et al.*, 1997). The $[Ca^{2+}]_i$ response to 200 nM ET-1 was completely abolished by pretreatment and co-perfusion with 1 μ M BQ123 (Figure 6). This confirms that the ET-1 induced rise in $[Ca^{2+}]_i$ is mediated through the ET_A R. There was no significant difference in the responses of early versus late passage cells (data not shown).

Mechanism of ET_BR down-regulation during phenotypic modulation

A reverse transcriptase-PCR assay was developed to examine mRNA expression on small numbers of cells using primers designed from the sequence of the cloned RPA ET_B R (N. Yang *et al.*, in preparation). In preliminary experiments we

Figure 3 Inhibition of $[^{125}I]$ -ET-1 binding to whole, early passage VSMC in the presence of increasing concentrations of unlabelled ET-1, BQ123, or S6c. Representative experiments are illustrated which demonstrate binding typical of the ET_A R (A) and ET_B R (B).

Table 1 Competitive displacement of $[^{125}I]$ -ET-1 (10 pM) by unlabelled ligands in six early passage whole cell binding experiments

Expt #	ET-1 (pIC_{50})	BQ123 (pIC_{50})	S6c (pIC_{50})
1	10.4	<6.0	8.6
3	10.1	<6.0	11.7
mean	10.25	<6.0	10.15
2	10.2	8.7	<6.0
4	9.6	8.7	<6.0
5	10.0	8.8	<6.0
6	10.0	8.7	<6.0
mean	9.95 ± 0.25	8.73 ± 0.05	<6.0

Experiments 1 and 3 show evidence for ET_B R in early passage RPA VSMC; experiments 2 and 4–6 show evidence for ET_A R only.

Figure 4 Inhibition of $[^{125}I]$ -ET-1 (30 pM) binding to late passage (P6) VSMC membranes ($n=3$) by increasing concentrations of unlabelled ET-1, and BQ123 but not S6c.

Figure 5 Homologous desensitization of the intracellular calcium response in passages 2 and 3 RPA VSMC to ET-1 (A,B) and no response to the ET_B R-selective agonists, S6c and ET-3 (B). Responses to the P_2 purinoceptor agonists ATP and UTP were unaffected by previous exposure to 200 nM ET-1.

Figure 6 Abolition of the intracellular calcium response in passage 2 and 3 RPA VSMC to ET-1 (200 nM) following preincubation and co-perfusion with the ET_A R-selective antagonist, BQ123 (1 μM).

demonstrated that there was no significant contamination with genomic DNA and that the exponential phase of the reaction extended to 30–35 cycles. We examined ET_B R expression in RPA from four rabbits and series of cultured VSMC derived from these. ET_B R mRNA was present in RNA prepared from RPA, and rabbit lung (used as positive control) but was not detected in cultured VSMC (primary culture through to 4th passage) derived from the RPA (Figure 7). GAPDH PCR confirmed the integrity of the cDNA in these samples. Down-regulation of the ET_B R during phenotypic modulation thus appears to be at the level of mRNA transcription.

Figure 7 Reverse transcription-polymerase chain reactions for rabbit ET_B R (upper panel) and GAPDH (lower panel) using RPA ($n=4$) and derived VSMC. Lane 1: RPA; lane 2: primary cultured VSMC; lanes 3–6: passages 1–4 of VSMC; lane 7: negative control; lane 8: positive control.

Discussion

In this paper we describe several series of experiments which provide evidence for the rapid transcriptional down-regulation of the ET_B R and its functional consequences upon initiation of *in vitro* culture of RPA VSMC. By several overlapping lines of evidence we show that: (i) both ET_A R and ET_B R are present and functional in intact RPA (organ bath studies, RT-PCR); (ii) ET_B R is present in a minority of early passage (passage 2) RPA VSMC cultures (ligand binding) and (iii) ET_B R is absent in late passage (passage 6, synthetic) cultured RPA VSMC (ligand binding, RT-PCR). This represents a spectrum of ET_B R expression, ranging from its functional presence in VSMC of the contractile phenotype, to its absence in synthetic or proliferative VSMC. Given that significant differences between similar rabbit vascular preparations have been reported in different laboratories (Douglas *et al.*, 1995; Gray *et al.*, 1994), it was important for us to confirm the characteristics of ET_A R- and ET_B R-mediated contraction of the RPA. The maximum tension generated by ET-1, a non-selective agonist, was similar whether or not the ET_B R had been desensitized by prior exposure to S6c (Figure 1). Blockade and/or desensitization of both receptors was necessary to block the action of ET-1 in the RPA. Similar functional responses to ET-1-induced contraction have been reported in rat trachea which is also known to express both ET_A R and ET_B R (Clozel & Gray, 1995; Henry, 1993). Activation of either receptor in the RPA produces a similar contractile response, which is compatible with a model of receptor 'cross-talk' (Clozel & Gray, 1995).

In attempting to define the second messenger system(s) coupled to the ET_B R, we found that the ET_A R mediated increases in $[Ca^{2+}]_i$ in cultured cells at passage 2 and 3. Whereas ET_B R ligand binding patterns were observed in 2/6 membrane preparations from passage 2 cultured cells, neither of the ET_B R-selective agonists S6c nor ET-3 elevated $[Ca^{2+}]_i$ in Fura-2 loaded cells from early or late passage indicating that the ET_B R was rapidly down-regulated. Previous binding studies on RPA membrane preparations have estimated the proportions of ET_B R and ET_A R to be 60:40 (La Douceur *et*

et al., 1993) or 77:23 (Fukuroda *et al.*, 1994). Since both the ET_AR and ET_BR are biochemically and functionally demonstrable on the RPA, this implied that in cultured VSMC either the ET_BR was not coupled to [Ca²⁺]_i responses or that it was down-regulated *in vitro*. Therefore we systematically examined its expression *in vitro* using small scale binding assays and microfluorescence techniques in order to study individual animals. Although we did demonstrate the predominant expression of the ET_BR in binding experiments in some cultures of early passage RPA VSMC, the majority demonstrated competition binding typical of an ET_AR. These differences arose despite careful control of assay conditions. We never saw a mixed pattern of ET receptor responses and we inferred that there must have been a relatively rapid down-regulation of ET_BR expression during early culture. In keeping with this, membrane preparations from later passage cells whose phenotype had clearly become synthetic only expressed ET_AR. Although data from the RT-PCR indicated that ET_BR transcription was rapidly down-regulated after initiation of *in vitro* culture, we attributed the presence of the ET_BR in some of the early passage binding studies to the persistence or slow turnover of the ET_BR at the cell surface in the absence of transcription.

Two previous studies have addressed changes in ET receptor expression in cultured VSMC. Contractile rabbit aortic VSMC express a high affinity ET_AR which is down-regulated in parallel with phenotypic modulation, but differences in the expression of receptor subtype as found in this study were not identified (Serradeil-Le Gal *et al.*, 1991). Cultured rat aortic VSMC express predominantly the ET_AR during passages 10–15, but later passage cells (>30) expressed both the ET_AR and ET_BR (Eguchi *et al.*, 1994). However, phenotypic modulation of VSMC occurs within days, rather than weeks of culture (Campbell *et al.*, 1988). Therefore, these observations on late passage cells are unlikely to be relevant to the process of phenotypic modulation.

Because the ET_BR is down-regulated rapidly in culture, we have not been able to study the second messenger system(s) to which it is coupled. However, we have shown that the ET_AR is coupled to intracellular calcium transients (Pollock *et al.*, 1995). The rise in [Ca²⁺]_i seen in response to ET-1 would be sufficient to activate intracellular calcium-dependent protein kinases and calmodulin. The ET_AR characteristically demonstrated rapid and prolonged homologous desensitization, the mechanism of which probably involves receptor phosphorylation (Cyr *et al.*, 1993). In this study, the mechanism of ET_BR down-regulation *in vitro* occurs at the transcriptional level, and in parallel with VSMC phenotypic modulation, suggesting the existence of a local factor maintaining expression of ET_BR *in*

vivo. This phenomenon is distinct from the well-described process of agonist-mediated down-regulation of G protein linked receptors modelled by other groups using β -adrenergic receptors (Karoer *et al.*, 1996), and resembles the phenotype-related alteration of atrial natriuretic peptide receptor expression in rat aortic SMC (Suga *et al.*, 1992). In this study we made no attempts to re-induce the contractile phenotype in these cells through manipulation of culture conditions in order to maintain expression of the ET_BR.

Phenotypic modulation of VSMC *in vitro* from the 'contractile' to the 'synthetic' phenotype is thought to reflect the changes seen in certain pathophysiological states, such as atherosclerosis and vascular remodelling. However, experimental evidence concerning the role of ETR subtypes in these conditions is scant and conflicting. Winkles *et al.* (1993) found that ET_BR mRNA was down-regulated in 6/7 human aortic atherosclerotic plaque specimens compared to normal aortas, however the authors acknowledged that the cell types involved were not known. In contrast, Dagassan *et al.* (1996) found that ET_BR were up-regulated in human atherosclerotic coronary arteries, but again, no cell type was implicated in this up-regulation. Bacon *et al.* (1995) found that ET_BR was expressed only in areas of neovascularization in human atherosclerotic coronary arteries, co-localizing with von Willebrand factor staining, suggesting that ET_BR expression was found only on endothelial cells. The increase in pulmonary vascular resistance in patients with pulmonary hypertension (Wagenvoort & Mooi, 1989) and in animal models of the disease is associated with structural changes in the resistance vessels. Phenotypic modulation of the VSMC and their migration into the intima contribute to luminal narrowing in pulmonary hypertension and local production of ET-1 may play a role in this process (Giaid *et al.*, 1993; Stewart *et al.*, 1991). Given the potential therapeutic applications of selective ET receptor antagonists in such conditions, it is important to recognize that the target receptor may be different from that expressed in normal cells. The identification of factors which regulate ET_BR expression in this system should facilitate further studies on the interaction between the ETRs.

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