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Plasticity of contractile endothelin-B receptors in human arteries after organ culture

[1]Mikael Adner, Leonor Cantera, *Frederick Ehlert, Leif Nilsson & Lars Edvinsson

Division of Experimental Vascular Research, Department of Internal Medicine, Lund University Hospital, Lund, Sweden and *Department of Pharmacology, University of California, Irvine, U.S.A.

1 The pharmacology and mRNA expression of endothelin (ET) receptors in human omental arteries were characterized by use of functional contractile assays and the reverse transcriptase-polymerase chain reaction (RT-PCR).

2 In freshly obtained segments of human omental arteries, ET-1 and ET-3 induced concentration-dependent contractions which were normalized to the response produced by 60 mM K^+. ET-1 produced a maximum contraction (E_{max}) amounting to $151 \pm 17\%$ of the K^+ response. The pEC_{50} for this agonist was 8.64 ± 0.17. The effect of ET-3 was less pronounced (E_{max}: $71 \pm 22\%$ and pEC_{50}: 6.69 ± 0.17) than that of ET-1. The ET receptors involved were characterized with FR139317 (a selective ET_A receptor antagonist), PD 145065 (a mixed ET_A and ET_B receptor antagonist) and BQ 788 (an ET_B receptor antagonist). A high concentration of these antagonists (10 μM) abolished the contractile responses to ET-3, and produced a parallel rightward shift of the ET-1 concentration-response curve without changing the maximal effect. FR139317 and PD 145065 were equally effective while BQ 788 was much less effective. This is consistent with ET_A receptors mediating contraction in human omental arteries.

3 Arterial segments cultured for 5 days in serum-free Dulbecco's medium at 37°C under sterile and humidified conditions retained contractility although responses to 60 mM K^+ were somewhat reduced. ET-3 was significantly more potent in the cultured arteries (pEC_{50}: 8.56 ± 0.15) and achieved a greater maximum effect (E_{max}: $116 \pm 19\%$). Responses were not antagonised by FR139317 but were competitively blocked by PD 145065 and BQ 788 with the latter antagonist being the more potent. In contrast E_{max} ($179 \pm 17\%$) and pEC_{50} (8.66 ± 0.23) values for ET-1 were not significantly different from those obtained with fresh arteries. PD 145065 still demonstrated a rightward shift of the ET-1-induced concentration-response curve, whereas FR139317 and BQ 788 caused non-significant shifts. These findings suggest that functional ET_B receptors contribute significantly to the endothelin contractile response in cultured arteries.

4 Two-site analysis of the ET-1 induced concentration-response curve from cultured arteries suggests that ET_B receptors, at the high potency component, and ET_A receptors, at the low potency component, contribute both to the contractile response in relative proportion of 70% and 30%, respectively. Further analysis suggested that the ET_A receptor would be capable of evoking at least 75% of the ET-1 contraction in the absence of ET_B receptors, although with a lower potency as compared to fresh arteries.

5 Electrophoresis of RT-PCR products from the smooth muscle layer of freshly obtained human arteries indicated the presence of mRNA for both ET_A and ET_B receptors. Arteries cultured for 1 and 5 days demonstrated an increase of mRNA for the ET_B receptor as compared to the ET_A receptor. The identities of the PCR products were verified by restriction enzyme digestion.

6 In freshly obtained human omental arteries, the contractile effects of endothelins appear to be mediated predominantly by the ET_A receptor subtype, with a negligible contribution by ET_B receptors. Cultured arterial segments, however, exhibited a substantial ET_B receptor mediated contractile response and an increase in ET_B receptor mRNA content, consistent with an upregulation of functional ET_B receptors. These *in vitro* data suggest plasticity in the smooth muscle cell expression of contractile ET_B receptors.

Keywords: BQ 788; ET_A receptor; ET_B receptor; FR139317; *in vitro* pharmacology; organ culture; PD 145065; RT-PCR; upregulation

Introduction

Three isoforms of the endothelin (ET) peptide family are known in man; ET-1, ET-2 and ET-3 (Inoue *et al.*, 1989). The actions of the endothelin peptides have been found to be mediated through two distinct receptor subtypes: ET_A and the ET_B receptors (Arai *et al.*, 1990; Sakurai *et al.*, 1990). ET-1 and ET-2 have higher affinity for the ET_A receptor than does ET-3, whereas all the isopeptides have the same affinity for the ET_B receptor (Masaki *et al.*, 1994). ET-1 is the most potent endogenous vasoconstrictor yet described (Yanagisawa *et al.*, 1988) and is proposed to be released primarily from the abluminal side of endothelial cells towards the smooth muscle cells (Wagner *et al.*, 1992) where it tightly binds to its receptors (Hirata *et al.*, 1988). Thus, the function and regulation of the endothelin receptors are of considerable interest for the understanding of the physiological and pathophysiological roles of the ET peptides.

Both ET_A and ET_B receptors are present in vascular tissue. Smooth muscle ET_A receptors have been shown to mediate the potent vasoconstrictor effect of ET-1 in human arteries (Wenzel *et al.*, 1994; Riezebos *et al.*, 1994; Maguire & Davenport, 1995). In contrast, ET_B receptors have been found to produce transient vasodilatation resulting from activation of endothelial receptors and subsequent release of nitric oxide or

[1]Author for correspondence at: Department of Cellbiology 1, EB-blocket, Lund University Hospital, S-221 85 Lund, Sweden.

prostacyclin (De Nucci *et al.*, 1988). However, the smooth muscle layer of human vessels expresses mRNA for both ET_A and ET_B receptor subtypes (Davenport *et al.*, 1995) and a number of studies in animals (Clozel *et al.*, 1992; Moreland *et al.*, 1994; Ekelund *et al.*, 1994) and man (Seo *et al.*, 1994; Tschudi & Lüscher, 1994; Haynes *et al.*, 1995) have now demonstrated ET_B receptor-mediated vasoconstriction. In addition, further subtypes of contractile ET_A and ET_B receptors with different binding characteristics have been suggested (Karaki *et al.*, 1994). The function of the different endothelin receptor subtypes in vascular smooth muscle is therefore unclear, but it has been proposed that the ET_A receptor is the major subtype mediating constriction on the high pressure side of the circulation while contractile ET_B receptors are more abundant in low pressure systems such as the pulmonary and venous circulation (Moreland *et al.*, 1994).

It is also possible that the relative contributions of contractile ET_A and ET_B receptors vary according to the physiological or pathological state of the vessel. To study endothelin receptor plasticity, we have cultured arterial segments in serum-free medium for a time period up to 5 days. A preliminary study demonstrated that the selective ET_B agonist IRL 1620 produced a negligible contractile effect in fresh arterial segments (Adner *et al.*, 1995). However, after one day in organ culture a contractile effect of IRL 1620 appeared, which increased after 5 days in culture. Thus, the aim of the present study was to characterize further the pharmacology of ET receptors in fresh and cultured arteries by use of ET-1 and ET-3 alone and in the presence of either the selective ET_A receptor antagonist FR139317 (Sogabe *et al.*, 1993), the mixed ET_A/ET_B receptor antagonist PD 145064 (Cody *et al.*, 1993) or the selective ET_B receptor antagonist BQ 788 (Ishikawa *et al.*, 1994). In addition, the reverse transcriptase-polymerase chain reaction (RT-PCR) was used to examine the expression of mRNA for the ET_A and ET_B receptors in the vascular smooth muscle layer in fresh arteries and after 1 and 5 days in organ culture.

Methods

Tissue preparation and organ culture procedure

Omental arteries with connecting fat were removed from patients during abdominal surgery and immersed in cold sterile Dulbecco's modified Eagle's medium (DMEM; Sigma, U.S.A.). Within 4 hours vessels with a resting diameter between 0.2 and 0.5 mm were dissected free from adherent tissue under sterile conditions and cut into circular segments, 1 mm long for *in vitro* pharmacology and 20–40 mm long for RNA extraction and RT-PCR. Segments from each vessel were divided in two groups; one for immediate experimental analysis (fresh) and the other for organ culture. The segments for culture were placed in a 48 well plate, one segment in each well, containing 750 μl DMEM and incubated at 37°C in humidified 5% CO_2 in air. Serum-free DMEM (4500 mg l^{-1} D-glucose) contained sodium pyruvate (110 mg l^{-1}), L-glutamine (584 mg l^{-1}), and was supplemented with penicillin (100 u ml^{-1}) and streptomycin (100 μg ml^{-1}). The wells were inspected daily and if a change in pH occurred, the segments were placed in fresh medium.

The protocol was approved by the Ethical Committee of University Hospital (Lund, Sweden).

In vitro *pharmacology*

Experimental set-up For contractile experiments, the arteries were immersed in temperature-controlled (37°C) tissue baths containing a buffer solution of the following composition (mM): NaCl 119, $NaHCO_3$ 15, KCl 4.6, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.5 and glucose 11. The solution was continuously aerated with 5% CO_2 in O_2 resulting in a pH of 7.4. Each segment was mounted on two L-shaped metal prongs, one of which was connected to a Grass FT-03 transducer attached to a MacLab unit for continuous recording of isometric tension. A tension of 2 mN was applied to the segments. The segments were allowed to stabilize at this level of tension for one hour and were thereafter exposed to an isotonic potassium (K^+)-rich buffer solution (60 mM). The K^+-induced contraction was used as a reference for tissue contractile capacity (Högestätt *et al.*, 1983). Concentration-response curves for the endothelin agonists were obtained by cumulative application of the peptides. The antagonists were added 15–20 min before an agonist. Integrity of the endothelium was assessed histologically at the end of the experiment.

Molecular biology

RNA preparation Human omental arteries were prepared as open ring segments. A scalpel was used to remove the endothelium and adventitia (checked by histology) before the segments were frozen in liquid nitrogen. Total cellular RNA was isolated by the method of acid guanidinium thiocyanate/phenol/chloroform extraction (Chomczynski & Sacchi, 1987). Frozen tissues were homogenized with 4 M guanidinium isothiocyanate containing 0.1 M 2-β-mercaptoethanol and 0.5% sarcosyl and further extracted with phenol and chloroform-isoamyl alcohol. RNA was precipitated at −20°C with isopropanol and dissolved in 20 μl diethylpyrocarbonate-treated water. The amount and the purity of RNA was evaluated by use of a DU-64 spectrophotometer (Beckman Instruments AB, Sweden) at absorption wavelengths of 260 and 280 nm. The ratio of absorbance peaks (260:280 nm) of all preparations was between 1.6 and 1.8.

Reverse transcriptase-polymerase chain reaction (RT-PCR) The reverse transcription of total RNA to cDNA and subsequent PCR was carried out by use of the GeneAmp RNA PCR kit (Perkin Elmer AB, Sweden) in a Perkin Elmer DNA Thermal Cycler. First strand cDNA was synthesized from 1 μg total RNA in a 20 μl reaction volume following the standard reverse transcription protocol with random hexamers as primers. The reaction was incubated at 42°C for 15 min, heated to 99°C for 5 min, and chilled to 5°C for 5 min. Resultant cDNA was amplified by PCR in a final volume of 100 μl following the standard PCR protocol. AmpliTaq DNA polymerase (Perkin-Elmer) was used as the thermostable enzyme. The PCR was carried out by using four linked files as follows: file 1, 2 min at 95°C for 1 cycle; file 2, 1 min at 95°C and 1 min at 60°C for 25 or 45 cycles; file 3, 7 min at 72°C for 1 cycle; file 4, incubation at 4°C for 5 min.

Oligonucleotide design Reverse transcriptase-polymerase chain reaction assays for ET_A and ET_B receptor mRNA were performed with the following primers (Scandinavian Gene Synthesis AB, Sweden): ET_A forward, 5′-TGGCCTTTTGATCACAATGACTTT-3′ (bases 436–459); ET_A reverse, 5′-TTTGATGTGGCATTGAGCATACAGGTT-3′ (bases 737–711); ET_B forward, 5′-ACTGGCCATTTGGAGCTGAGATGT-3′ (bases 497–521); ET_B reverse, 5′-CTGCATGCCACTTTTCTTTCTCAA-3′ (bases 924–901). The primers were designed to span one or more introns so that PCR amplification of the cDNA could be interpreted to reflect tissue mRNA expression rather than contamination by genomic DNA (O'Reilly *et al.*, 1992; Hosoda *et al.*, 1992; Arai *et al.*, 1993). The PCR products obtained after 45 cycles were verified by restriction enzyme digestion. The product corresponding to the ET_A receptor mRNA was digested with BamHI (MBI Fermentas, Lithuania) and the product corresponding to the ET_B receptor mRNA was digested with EcoRV (Boehringer Mannheim, Germany). After incubation for 2 h at 37°C, the products were electrophoresed (see below).

Electrophoretic analysis of PCR products After RT-PCR, a 10 μl aliquot from each PCR product was electrophoresed on a 1.5% Seakem LE agarose gel (FMC Bioproducts, U.S.A.),

containing 1.0 $\mu g\ ml^{-1}$ ethidium bromide, in TBE buffer (89 mM TRIS-borate, 2 mM EDTA, pH 8.0) at 5 V cm^{-1} for 1.5 h. The DNA ladder 100 basepairs (Pharmacia Biotech, Sweden) was used as the molecular weight marker. This analysis was performed with a GNA 200 Electrophoresis Apparatus (Pharmacia Biotech).

Drugs

The sources of the agonists and antagonists were: ET-1, ET-3 (Auspep, Parkville, Australia), FR139317 ((**R**)2-[(**R**)-2-[(**S**)-2-[[1-(hexahydro-1H-azepinyl)]carbonyl]amino-4-methylpentanoyl] amino-3-[3-(1-methyl-1H-indoyl)]-propionyl] amino-3-(2-pyridyl)propionic acid; Fujisawa Pharmaceuticals Co., Osaka, Japan), PD 145065 (Ac-D-Bhg[16]-Leu-Asp-Ile-Ile-Trp[21]; Parke-Davies Pharmaceutical Research, Ann Arbour, MI, U.S.A.) and BQ 788 (N-*cis*-2,6-dimethyl-piperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine; Neosystem, Strasbourg, France). All other reagents were purchased from Sigma Chemical Co., U.S.A. The peptides were dissolved in 0.1% bovine serum albumin (Sigma). In the antagonist experiments, FR139317, PD 145065 and BQ 788 were tested at the same concentration (10 μM) to obtain maximal blocking effects. However, since BQ 788 also showed agonist properties in the cultured arterial segments, a concentration-response curve for this compound was obtained.

Calculation and statistics

Data are expressed as mean values ± s.e.mean and n refers to the number of patients from which the vessel segments were obtained. Contractile responses in each segment were expressed as a % of the contraction induced in that segment by 60 mM K^+ and the maximum contractile effects of an agonist were indicated as E_{max} values. pEC_{50}, the negative logarithm of the molar concentration that produced half maximum contraction, was calculated from the line between the concentration above and below the midpoint of the concentration-response curve. A shift in the concentration-response curves was calculated as a ratio of the EC_{50} values obtained with the agonist in the presence of an antagonist and alone. The Hill-slopes for the one-site analyses were calculated by use of GraphPad Prism.

In some cases, the contractile response data were analyzed according to a two-site model by fitting the following equation to the data by nonlinear regression analysis:

$$y = \max\left(\frac{Max_H[X]^{n_{H1}}}{[X]^{n_{H1}} + EC_{50H}^{n_{H1}}} + \frac{(1 - Max_H)[X]^{n_{H2}}}{[X]^{n_{H2}} + EC_{50L}^{n_{H2}}}\right) \quad (1)$$

In this equation, Max denotes the overall maximum contractile response, Max_H denotes the proportion of the response mediated by the high potency component, EC_{50H} and EC_{50L} denote the high and low potency EC_{50} values, and n_{H1} and n_{H2} denote the Hill-slopes of the high and low potency components, respectively. Following organ culture, the contractile response data to ET-1 were analyzed by fitting equation (1) to data obtained in the absence and presence of an antagonist, sharing estimates of Max, Max_H, n_{H1} and n_{H2} between the two sets of data. The shift in the EC_{50} value caused by an antagonist was calculated as the ratio of EC_{50} values measured in presence and absence of antagonist. The least squares fit to the equation was derived by the Gauss-Newton method (Hartley, 1961) by use of an algorithm written in Microsoft QuickBASIC. In order to determine if the regression equation provided an adequate fit to the data, analysis of variance was applied as described by Hanin and colleagues (1966). With this technique, the variance estimate based on deviations between the mean data points and the regression equation (SS^2) is compared with that estimated by replicate measurements of contraction (s^2). The ratio of these two variance estimates was calculated as F:

$$F = \frac{m \times SS^2}{n \times s^2} \quad (2)$$

in which n denotes the number of drug concentrations, m denotes the number of times the experiments was repeated, and s^2 denotes the average variance for replicate measurements of contraction. The value (F) was then compared with the critical value of $F_{n,n(m-1)}$ (0.05).

Wilcoxon-signed rank test was used for paired analysis within the group of fresh segments or within the group of cultured arteries. The Mann-Whitney U test was used for unpaired analysis between the two groups. Differences were considered significant at P values <0.05.

Results

In vitro *pharmacology*

Potassium induced contraction The K^+ induced contraction appeared to decline after culture of the arteries for 5 days, but the decline did not reach statistical significance at the 5% level (10.96 ± 3.07 mN vs. 4.63 ± 1.24 mN, $n=9$; $P=0.058$). In addition, the K^+ induced contraction did not significantly vary between the groups in the fresh arteries or between the groups of cultured arteries.

Contractile effects of ET-1 and ET-3 ET-1 produced a strong and potent contraction of fresh segments from human omental artery. ET-3 induced a significantly weaker and less potent contraction (Figure 1, Table 1). The contraction elicited by the highest concentration of ET-3 tested probably does not reflect the true maximum, thus the pEC_{50} estimate for ET-3 is most probably overestimated. In cultured arteries, ET-3 elicited contractions with greater E_{max} and significantly higher pEC_{50} (Figure 2, Table 1), whereas the contractile responses to ET-1 showed no significant change in E_{max} (in relation to the K^+ induced contraction) or pEC_{50} in cultured arteries. However, at the lowest concentrations (0.1–0.3 nM), significantly stronger contractions were observed in the cultured segments as compared to the fresh segments (Figure 3a). There was no significant difference in potency between ET-1 and ET-3 in the cultured group; however, the E_{max} of ET-3 was 67% that of ET-1. The Hill-slopes for the experiments performed on fresh arteries were between 2.8 and 4.6 and were all higher than the Hill-slopes in the cultured arteries, which were between 1.1 and 2.1. In addition, there was a significant difference between the Hill-slope of ET-1 in fresh arteries compared to the Hill-slope of ET-3 in cultured arteries (Table 1).

Effects of the antagonists in fresh segments In the fresh segments the ET_A receptor antagonist FR139317 (10 μM) induced a 73 fold parallel rightward shift of the ET-1 concentration-response curve. A shift of about the same magnitude (61 fold) was produced by the mixed ET_A/ET_B receptor antagonist PD 145065 (10 μM), while the ET_B receptor-selective antagonist BQ 788 (10 μM) caused a significant 4 fold parallel rightward shift of the concentration-response curve (Figure 1a, Table 1). The E_{max} of the different concentration-response curves did not significantly differ from the effect of ET-1 alone. All three antagonists abolished the ET-3-induced contraction (Figure 1b). None of the antagonists showed any contractile effects by themselves.

Effects of the antagonists in vessel segments after 5 days of organ culture The effects of the antagonists (10 μM) were altered in arteries cultured for 5 days. When tested alone, FR139317 and PD 145065 had no contractile effects in the incubated segments; however, BQ 788 produced a small transient contraction (E_{max}: 40 ± 14%, pEC_{50}: 5.86 ± 0.22, $n=4$). In some cases, the contractile response to BQ 788 was still present

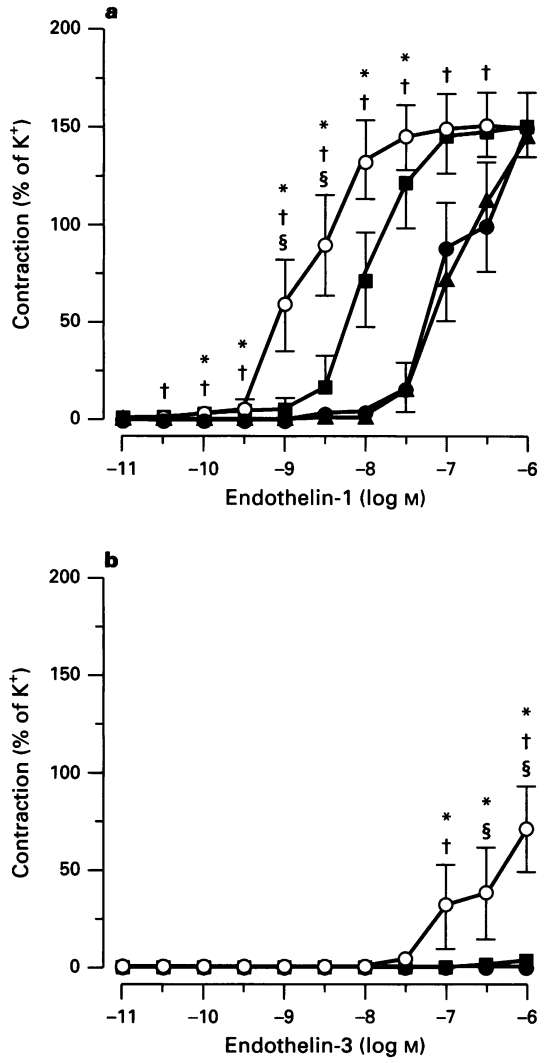

Figure 1 Contraction of fresh human omental arteries induced by cumulative concentrations of endothelin-1 (ET-1) (a) and ET-3 (b) without (○) and with either the ET_A receptor antagonist FR139317 (●), the mixed ET_A and ET_B receptor antagonist PD 145065 (▲) or the ET_B receptor antagonist BQ 788 (■). Each antagonist was added to the bath 15–20 min before the agonists for a final concentration of 10 μM. The contraction of each segment tested was calculated as a percentage of the potassium (K^+)-induced contraction in the same segment, and each point represents the mean of all segments tested with error bars representing s.e.mean. $P<0.05$ when the effect of agonist alone is compared to the effect of the agonist with FR139317 (*), PD 145065 (†) or BQ 788 (§); $n=6-9$ in each group.

before the agonists were applied. FR139317 did not significantly affect the contractile effect of ET-3. However, the ET-3 induced concentration-response curve was shifted in a parallel manner to the right by PD 145065 and BQ 788 (Figure 2). BQ 788 induced a 70 fold shift and was more potent than PD 145065, which caused a 25 fold shift. FR139317 caused a non-significant two fold shift of the ET-1-induced concentration-response curve (Table 1). However, the effects of a high concentration of ET-1 (30 nM) were significantly attenuated by FR139317 indicating a biphasic shape of the concentration-response curve (Figure 3a). This antagonizing effect of FR139317 became apparent at contractile responses that were equal to or greater than the E_{max} of the ET-3 concentration-response curve (Figures 2 and 3a). PD 145065 caused a significant 10 fold parallel rightward shift of the ET-1-induced concentration-response curve (Figure 3b), which was 6 fold less compared to that in fresh segments. BQ 788 caused a non-significant 4 fold rightward shift of the ET-1 induced concentration-response curve (Figure 3c), which was only observed in the low potency part of the curve because the contractile effect of BQ 788 itself in these experiments was high (49±26% of K^+-induced contraction). None of the E_{max} values were significantly altered.

Because both the ET_A receptor selective antagonist, FR139317, and the ET_B selective antagonist, BQ 788, only caused minor shifts in the ET-1 concentration-response curve for the arteries cultured for 5 days, it is possible that both ET_A and ET_B receptors are present, and that either receptor, by itself, can trigger enough contraction to generate a nearly maximal contraction. Consequently, we analyzed the mean concentration-response curve for ET-1 alone and with FR139317 or PD 145065 according to a two-site model as described in the Methods section (Table 2). The data obtained in the presence of BQ 788 were not analyzed by this method because BQ 788 elicited a small contractile response by itself which complicated the analysis. The Hill-slope of the high potency component was fixed to the same value as that of the mean ET-3-induced concentration-response curve on day 5 (1.3) and the Hill-slope of the low potency component was fixed to the same value as that of the mean ET-1-induced concentration-response curve in fresh arteries (2.8). The *F* test demonstrated that the data from the concentration-response curves were adequately described by the two-site model. FR139317 did not cause a shift of the high potency component of the concentration-response curve, but caused a 108 fold shift of the low potency component (Figure 3a). PD 145065 caused a 8 fold shift of the high potency component, and a 51 fold shift of the low potency component (Figure 3b). The pEC_{50} values for ET-1 at the high and low potency components (8.79 and 8.14, respectively) represent the mean values calculated from the analyses with the different antagonists. The proportion of the high potency component of

Table 1 Contractile effects of ET-1 and ET-3 in fresh or cultured segments (5 days) of human omental arteries without or with antagonists (10 μM)

	Fresh arteries					*Arteries cultured for 5 days*				
	n	E_{max} (%)	pEC_{50}	*Shift*	*Hill-slope*	n	E_{max} (%)	pEC_{50}	*Shift*	*Hill-slope*
ET-1 (control)	9	151±17	8.64±0.17		2.8±0.4	9	179±17	8.66±0.23		1.8±0.2
+FR 139317	7	149±11	6.96±0.18*	73±2	4.0±0.9	7	152±22	8.36±0.24	2±2**	1.1±0.2
+PD 145065	8	145±10	6.96±0.16*	61±2	4.0±0.7	8	172±17	7.66±0.21*	10±2	2.1±0.3
+BQ 788	7	150±19	7.99±0.18*	4±2	4.6±0.6	7	179±35	8.12±0.27	4±2	1.9±0.3
ET-3 (control)	8	⩾71±22†	⩽6.69±0.17†		3.3±0.8	9	116±19†	8.56±0.15**		1.3±0.2§§
+FR 139317	7	>1±1*	ND	ND	ND	8	124±29	8.63±0.09	<1	1.8±0.4
+PD 145065	7	>3±2*	ND	ND	ND	8	135±22	7.21±0.29*	25±1	2.0±0.3
+BQ 788	6	>3±2*	ND	ND	ND	6	103±19	6.80±0.25*	70±2	1.4±0.1

Responses were characterized by E_{max} values (maximum contractile effect expressed as a % of 60 nM K^+-induced contraction) and pEC_{50} values (negative logarithm of the molar concentration that produced half maximum contraction). The shift is calculated from the ratio of the EC_{50} values obtained in the presence and absence of antagonist. Hill-slopes are calculated with one-site analysis. *$P<0.05$ between effect of antagonist and of agonist alone. †$P<0.05$ compared to ET-1 in arteries with the same treatment (i.e. fresh or cultured). **$P<0.01$ compared to fresh arteries. §§$P<0.01$ when compared to ET-1 in fresh arteries. ND, not determined. *n* refers to the number of patients from which the vessel segments were obtained.

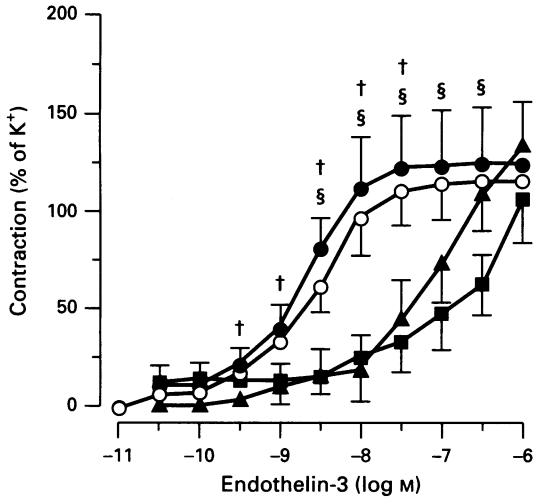

Figure 2 Contraction of human omental arteries which had been in organ culture for 5 days induced by cumulative concentrations of endothelin-3 without (○) and with either the ET_A receptor antagonist FR139317 (●), the mixed ET_A and ET_B receptor antagonist PD 145065 (▲) or the ET_B receptor antagonist BQ 788 (■). All the antagonists were added to the bath 15–20 min before the agonists for a final concentration of 10 μM. The contraction of each segment tested was calculated as a percentage of the potassium (K^+)-induced contraction in the same segment, and each point represents the mean of all segments tested with error bars representing s.e.mean. $P<0.05$ when the effect of agonist alone is compared to the effect of the agonist with FR139317 (*), PD 145065 (†) or BQ 788 (§); $n=6-9$ in each group.

the concentration-response curves was in agreement with the proportion of the E_{max} elicited by ET-3 compared to ET-1 in the same arteries.

Molecular biology

Endothelin receptor cDNA In order to maximize the possibility of detecting mRNA from the vessel segments, the first PCR reaction was performed for 45 cycles. Agarose gel electrophoresis demonstrated RT-PCR products of expected size corresponding to mRNA encoding the human ET_A receptor (302 base pairs) and the ET_B receptor (428 base pairs) (Figure 4). The signals were intense in both fresh and cultured arteries. After 25 cycles of PCR, the signals were of lower intensity than after 45 cycles, but were still visible. In fresh segments there was a similar intensity of the mRNA products from both receptors. However, after 25 cycles PCR, the ET_B receptor mRNA products for the incubated segments demonstrated a more intense signal than the corresponding ET_A receptor mRNA product. This pattern was seen after both one and five days in culture. Absence of contaminants was checked by negative control samples, in which the RNA samples were replaced with sterile water (not shown).

Restriction enzyme analysis The PCR products obtained after 45 cycles of the ET_A receptor mRNA were digested with BamH1 into two bands (108 and 194 base pairs) whereas the product of ET_B receptor mRNA was digested with EcoRV into two bands (100 and 328 base pairs). The electrophoresis showed two single bands of each product, from both fresh and cultured arteries, with expected size of each product (not shown).

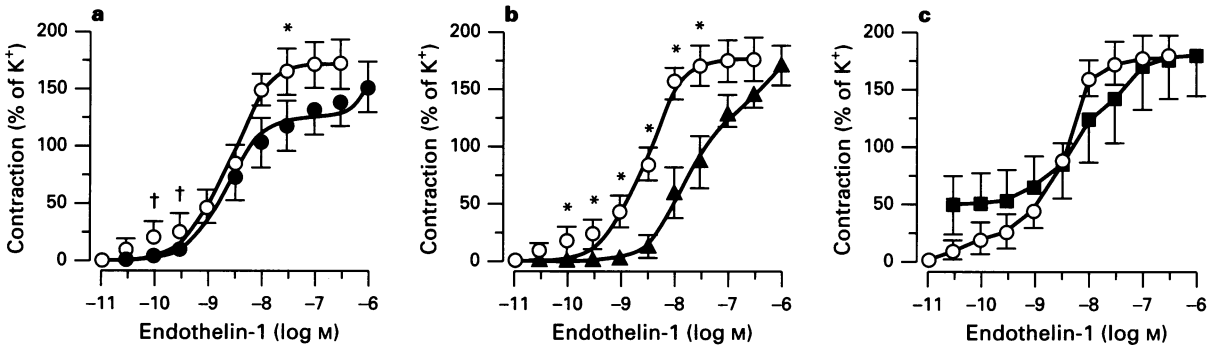

Figure 3 Contraction induced by cumulative concentrations of endothelin-1 (ET-1) without (○) and with either (a) the ET_A receptor antagonist FR139317 (●) ($n=7$), (b) the mixed ET_A and ET_B receptor antagonist PD 145065 (▲) ($n=8$) or (c) the ET_B receptor antagonist BQ 788 (■) ($n=7$) in human omental arteries which had been in organ culture for 5 days. All the antagonists were added to the bath 15–20 min before the agonists for a final concentration of 10 μM. The contraction of each segment tested was calculated as a percentage of the potassium (K^+) induced contraction in the same segment, and each point represents the mean of the segments tested with error bars representing s.e.mean. Lines in (a) and (b) represent the least squares fit of a two-site model to the data (see Methods). *$P<0.05$ when the effect of agonist alone is compared to the effect of the agonist with the antagonist. †$P<0.05$ when the compiled effect on all the ET-1-induced concentration-response curves achieved from arteries cultured for 5 days are compared to the effect of ET-1 in fresh arteries ($n=9$ in both groups).

Table 2 Two-site analysis of the paired mean concentration-response curves for ET-1 with the selective ET_A receptor antagonist FR139317 and the mixed ET_A and ET_B receptor antagonist PD 145065 (10 μM) in arteries cultured for 5 days

	High potency component			*Low potency component*		*E_{max} of ET-3*
	pEC_{50}	*Shift*	*Proportion (%)*	*pEC_{50}*	*Shift*	*(% of ET-1)*
ET-1 (control)	8.79			8.14		
+FR 139317	8.67	1	74	6.08	108	77
+PD 145065	7.87	8	75	6.45	51	69

The concentration-response curves to ET-1 in the absence and presence of FR 139317 or PD 145065 were analysed simultaneously sharing the same estimate of the maximum response and the proportion of the high potency component between the two curves. The high potency slope was fixed to the same value as the mean of ET-3 in cultured arteries (1.3) and the low potency slope was fixed to the same value as the mean of ET-1 in fresh arteries (2.8).

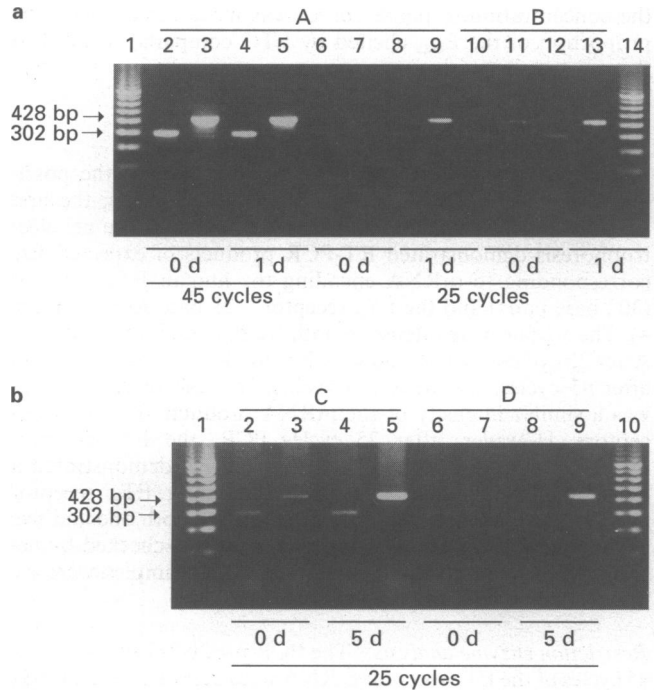

Figure 4 Electrophoresis of reverse transcriptase-polymerase chain reaction (RT-PCR) products corresponding to mRNA encoding human ET_A and ET_B receptors in omental arteries without endothelium and adventitia. The amplified products were of the predicted size for ET_A (302 base pairs) and ET_B (428 base pairs) receptors. Samples were analysed on a 1.5% Seakem LE agarose/ethidium bromide gel at $5\,V\,cm^{-1}$. (a) Lane 1: DNA size marker; lanes 2–5 demonstrate the products from arteries of patient A after 45 cycles and lanes 6–9 after 25 cycles; lanes 10–13 from patient B after 25 cycles; lanes 2, 3, 6, 7, 10 and 11 from fresh arteries (0 d); lanes 4, 5, 8, 9, 12 and 13 from arteries cultured for one day (1 d) and lane 14; DNA size marker. bp, base pairs. (b) Lane 1: DNA size marker; lanes 2–5 demonstrate the products after 25 cycles from arteries of patients C; lanes 6–9 from patient D after 25 cycles; lanes 2, 3, 6 and 7 from fresh arteries (0 d); lanes 4, 5, 8 and 9 from arteries cultured for five days (5 d) and lane 10: DNA size marker. bp, base pairs.

Discussion

In this study we have compared the contractile effects of ET-1 and ET-3 in human omental arteries that were tested within 4 hours of removal from the patients (fresh) and arterial segments that had been cultured for up to 5 days in serum-free DMEM at 37°C. Relatively high doses of endothelin receptor antagonists FR139317, PD 145065 and BQ 788 were used as tools for characterizing the receptors. In addition, the expression of mRNA for ET_A and ET_B receptors in the vascular smooth muscle layer with RT-PCR were examined. Our data demonstrate that fresh segments express mRNA for both ET_A and ET_B receptors; however, the contraction is only due to the ET_A receptor. After organ culture, an increase in mRNA for the ET_B receptor occurs and there is a corresponding appearance of a contractile effect which appears to be mediated via the ET_B receptor.

Compared to ET-3, ET-1 induced a much stronger and more potent contraction of fresh human omental arterial segments. This is consistent with an effect mediated predominantly by the ET_A receptor, at which the affinity of ET-1 is much greater than that of ET-3 (Masaki *et al.*, 1994). FR139317, which in binding studies has IC_{50} values for the ET_A receptor of 2 nM and for the ET_B receptor of 3.2 μM (Doherty *et al.*, 1993), produced a surmountable rightward shift of the concentration-response curve for ET-1. This further supports the presence of a contractile ET_A receptor. The mixed antagonist PD 145065, with IC_{50} values for the ET_A receptor of 4 nM and for the ET_B receptor of 15 nM (Doherty *et al.*, 1993), caused a parallel rightward shift of the concentration-response curve for ET-1 of the same magnitude as that caused by FR139317, in agreement with the similar binding affinities of these antagonists for the ET_A receptors. The relatively lower apparent potency of the selective ET_B receptor antagonist BQ 788 can be explained by its poor affinity for the ET_A receptor (IC_{50} values for the ET_A receptor of 280 nM and for the ET_B receptor of 1.2 nM; Ischikawa *et al.*, 1994). In addition, BQ 788 used at the same concentration as in our study, has earlier been shown to antagonize weakly the ET-1-induced contraction in rat aorta (Karaki *et al.*, 1994). This may explain why all antagonists, including BQ 788, blocked the ET-3 induced ET_A receptor-mediated contraction in this preparation. The binding data (Doherty *et al.*, 1993; Ischikawa *et al.*, 1994) give a picture of the relative potency of the antagonists, but appear to be higher than those measured in functional assays, perhaps due to hypotonic buffers used in the binding assays.

After organ culture for 5 days, ET-3 induced a contraction that was about 100 fold more potent than that observed in fresh arteries. The pEC_{50} of ET-3 for eliciting contractions was similar to that of ET-1 but the E_{max} was smaller. These observations suggest that a contractile receptor with high affinity for ET-3 is present. FR139317 did not antagonize the ET-3-induced contraction, whereas PD 145065 and BQ 788 caused clear-cut shifts to the right in a parallel manner, the latter being the most potent antagonist in accordance with the known profile for the ET_B receptor (Ischikawa *et al.*, 1994). These data suggest that the contractile response to ET-3 is mediated by the ET_B receptor. Another possibility is that the contraction is mediated by activation of an ET_C receptor, which according to the endothelin receptor classification has higher affinity for ET-3 than ET-1 (Masaki *et al.*, 1994). As yet, there are no binding data available for BQ 788 or PD 145065 at this receptor. In contrast, FR139317 did not cause any significant shift of the concentration-response curve of ET-1 after organ culture, indicating that a receptor with approximately the same affinity for both ET-1 and ET-3 is present (i.e. the ET_B receptor). Interestingly, the Hill-slope of the concentration-response curve for ET-3 in cultured segments differed significantly from the slope of ET-1 in fresh segments, which may be due to different intracellular mechanisms for the different receptor subtypes. For example it has been shown that the increase in intracellular Ca^{2+} elicited by the ET_B receptor in cultured aortic smooth muscle cells from hypertensive rats is due solely to mobilization of intracellular Ca^{2+}, whereas stimulation of the ET_A receptor also generates an additional extracellular Ca^{2+}-influx (Batra *et al.*, 1993).

The high potency component of the ET-1 concentration-response curve in cultured arteries was antagonized by PD 145065 but not by FR139317, indicating that this major component (about 70%) was elicited by a receptor having properties similar to that which elicited the ET-3 response. In accordance with the lack of effect of FR139317 on the contractile response to ET-3, two-site analyses of the concentration-response curve of ET-1 after organ culture indicated no shift of the high potency component. However, a major shift was observed at the low potency component which was of approximately the same magnitude as that observed with ET-1 in fresh segments (108 vs 73). Two-site analysis of the data for PD 145065 showed that the shift of the high potency component of the ET-1 concentration-response curve was not much different from the corresponding shift of the ET-3 concentration-response curve in cultured arteries (8 vs. 25). Moreover, the shift of the low potency component was similar to PD 145065-induced shift of the ET-1 concentration-response curve in fresh segments (48 vs. 61). These analyses agree with the hypothesis that the ET_B receptor mediates the high potency component of the concentration-response curve of ET-1, whereas an ET_A receptor mediates the low potency component.

The contractile effect of BQ 788 in the cultured arteries complicated the analyses of the antagonistic effect of this compound, but nevertheless suggested the appearance of active

ET_B receptors which were previously absent in fresh arteries. In the presence of BQ 788, the concentration-response curve of ET-1 was shifted by the same amount as that observed in fresh segments indicating that essentially all of the response to ET-1 above the basal BQ 788-induced contraction was mediated through the ET_A receptor. A potential shift of the high potency component was precluded by the persistent contractile action of BQ 788. Collectively our data suggest that the ET_B receptor has the capacity to elicit about 70% of the maximum contractile response to ET-1, and that by itself the ET_A receptor has the capacity to elicit at least 75% of the contraction. However, in the absence of antagonist, the maximum contractile capacity of the smooth muscle is exceeded before the ET_A receptor can fully manifest its contractile activity. This hypothesis can explain why PD 145065 is the most potent antagonist of the ET-1 concentration-response curve following culture. The potency of the ET_A receptor seemed to be attenuated which may be due to a decrease in the number of receptors after organ culture and may explain why ET-3 did not show a biphasic contraction over the concentration-range. Taken together, these data suggest an appearance of contractile ET_B receptors after organ culture, and that ET_A receptors, which are present in fresh arteries, continue to persist throughout organ culture.

The presence of mRNA for ET_A and ET_B receptors in the smooth muscle cells from human omental arteries is in accordance with results from earlier studies of other human vascular beds (Davenport *et al.*, 1995). Even though mRNA for both receptors has been detected, binding studies have demonstrated that the ET_A receptor is the major subtype present in smooth muscle cells (Davenport *et al.*, 1995) and was found to be the subtype mediating contraction in the fresh omental arteries. The ET_B receptor-induced contraction seen following organ culture could be due to receptor induction or post-transcriptional events, e.g. phosphorylation, translocation or decreased degradation. However, in our study, RT-PCR analysis revealed an increase in ET_B receptor mRNA relative to ET_A receptor mRNA after organ culture. This relative increase was better seen after 25 cycles, because 45 cycles probably comes close to a plateau of the PCR amplification. Iwasaki and colleagues (1994) have shown that the optimum number of amplification cycles in the rat kidney is 27 for the ET_A and 25 for the ET_B receptor and that a plateau for both the receptors was reached in about 35 cycles. The difference between the ET_A and ET_B receptor mRNA products was seen after both one and five days of culture. These results are consistent with our previous observation that a contractile response to the selective ET_B receptor agonist, IRL 1620, appears spontaneously in the human omental artery after one day in organ culture and persists for at least five days (Adner *et al.*, 1995). Even though earlier studies suggest a poor correlation between the number of receptors and either the concentration of mRNA or the contractile effect (Davenport *et al.*, 1995; Maguire & Davenport, 1995), this study demonstrates an increase of both mRNA and function of the ET_B receptor in the same preparation suggesting an upregulation of functional ET_B receptors. However, further studies with receptor binding are required to confirm this hypothesis.

During organ culture, the vessel segments are exposed to an inactive environment for a period of days during which there is no pulsating blood flow and no nervous or humoral stimulation, which may contribute to the upregulation of the ET_B receptor. During this period, it is possible that the smooth muscle cells start to differentiate and lose their contractile capacity, which is a problem with dispersed smooth muscle cells in culture (Chamley-Campbell *et al.*, 1979). However, light and electron microscopy showed a virtually normal morphological appearance together with a maintained Na^+-K^+ exchange in rat tail arterial segments after organ culture for up to 2 weeks in serum-free medium (Todd & Friedman, 1978). In accordance with the previous study (Adner *et al.*, 1995), we observed that the depolarization responses to 60 mM K^+, a reference of the contractile capacity, tended to decrease after organ culture, whereas the contractile response to ET-1 was unaffected (in relation to the K^+-induced contraction). Collectively, the results described above provide clear evidence for an increase in ET_B receptor-mediated contractions relative to those elicited by the ET_A receptor following organ culture. Whether this phenomenon represents a process of differentiation or a response to the lack of certain stimuli is still unknown. However, it does not appear to be limited to the human omental artery, because an increased effect of contractile ET_B receptors has been seen after organ culture in rat mesenteric and femoral arteries and femoral veins (unpublished observations).

Contraction due to ET_B receptor activation has recently been demonstrated in human internal mammary artery, pulmonary artery and internal mammary vein (Seo *et al.*, 1994), and in human resistance and capacitance vessels (Tschudi & Lüscher, 1994; Haynes *et al.*, 1995). It has been suggested that the ET_B receptor-mediated contraction is mainly present in low pressure vascular systems; this has been demonstrated in animal studies with veins, pulmonary circulation and skeletal microcirculation (Moreland *et al.*, 1994; Ekelund *et al.*, 1994). Therefore, the upregulation of ET_B receptors in the cultured segments could be due to the loss of pressure in the vascular wall. However, it is also possible that denervation or loss of blood flow may cause this event. One functional role proposed for the vascular ET_B receptor is that it may participate in the clearance of circulating ET-1 which has been demonstrated in rat (Fukuroda *et al.*, 1994). In the organ chamber situation, the levels of ET-1 reaching the smooth muscle layer may be diminished, perhaps by a decrease in endothelial ET-1 secretion, and this condition may cause a blunted sequestering of the ligand-receptor complex leading to an accumulation of free ET_B receptors in the smooth muscle membrane.

An upregulation of ET_B receptors has also been demonstrated under pathological conditions. Recently, it has been shown that the relative density of ET_B receptors is higher in human atherosclerotic coronary arteries compared to coronary arteries obtained from patients with cardiomyopathy or obtained from a normal heart (Dagasson *et al.*, 1996). Furthermore, the dog basilar artery exhibited an increase in ET_B receptor binding sites after experimental subarachnoid haemorrhage (Roux *et al.*, 1995). Increased intracellular calcium due to ET_B receptor activation has been seen in aortic smooth muscle cells from spontaneous hypertensive rats as compared with controls (Batra *et al.*, 1993), and rat kidney medulla demonstrated an upregulation of the ET_B receptor mRNA after treatment with cyclosporin (Iwasaki *et al.*, 1994). These data together with the present study suggest that the ET_A receptor is more static as compared to the ET_B receptor, and consequently the regulation of the ET_B receptor may have physiological or pathophysiological implications.

In conclusion, ET_A receptors are the predominant ET receptor subtype that mediates contraction in human omental arterial segments, even though mRNA for both the ET_A and the ET_B receptor are present in the smooth muscle. After organ culture there is an enhanced ET_B receptor-mediated response and an augmentation of ET_B receptor mRNA content, which indicates an upregulation of functional ET receptors. Thus, these *in vitro* data suggest plasticity in the vascular smooth muscle cell expression of ET_B receptors. The physiological significance of these findings has yet to be evaluated, but they illustrate the complex regulation of responses to endothelin in the cardiovascular system.

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