Vasoconstrictor endothelin receptors characterized in human renal artery and vein *in vitro*

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1 We have identified the endothelin receptors present in the media of human main stem renal artery and vein and characterized the subtypes mediating vasoconstriction in these blood vessels *in vitro*.

2 Messenger RNA encoding both ET_A and ET_B receptors was identified in the smooth muscle layer of human renal artery and vein by reverse transcriptase-polymerase chain reaction assay. In cryostat-cut cross-sections of both vessels autoradiographical visualisation suggested a majority of ET_A receptors. Intense binding was obtained to the non-selective ligand [¹²⁵I]-ET-1 and the ET_A-selective [¹²⁵I]-PD151242 but only weak labelling of sites by the ET_B-selective [¹²⁵I]-BQ3020.

3 ET-1 potently constricted renal artery and vein preparations with EC₅₀ values of 4.06 nM and 1.00 nM, respectively. Sarafotoxin 6b was approximately ten times less potent than ET-1 with EC₅₀ values of 36.3 nM and 13.8 nM respectively. In the renal artery, ET-3 and sarafotoxin 6c showed little or no activity up to 300 nM. Responses to these peptides were more variable in the renal vein. Preparations from three individuals did not respond to ET-3 but in three further cases, although ET-3 was much less potent than ET-1, full dose-response curves were obtained. S6c elicited dose-related contractions in vein preparations from 5/6 individuals and although more potent than ET-1, the maximum response was 30–60% of that obtained to ET-1.

4 ET-1-induced vasoconstriction of renal artery and vein was antagonized by the ET_A-selective, BQ123 (3–10 μM). The dose-response curves to ET-1 were displaced in a parallel rightward fashion with no attenuation of the maximum responses. pA₂ values were estimated to be 6.8 ± 0.1 and 6.8 ± 0.4 for artery and vein respectively.

5 These data suggest that mRNA encoding both ET_A and ET_B receptors is present in the media of human main stem renal artery and vein. However, autoradiographical studies indicate that the majority of ET receptors expressed are of the ET_A subtype. The relative potencies of ET-1 and ET-3 as vasoconstrictors of renal blood vessels *in vitro* is consistent with this being an ET_A-mediated response, and therefore whilst responses to S6c indicate that constrictor ET_B receptors may be present in renal veins from some individuals these are likely to be of less importance in these blood vessels.

Keywords: Endothelin; ET_A receptor; ET_B receptor; human renal artery; human renal vein; vasoconstriction; mRNA detection; *in vitro* pharmacology

Introduction

The precise role of the endothelin (ET) peptides in human renal vascular disease remains to be elucidated, but elevated plasma ET levels have been reported in conditions of acute renal failure (Tomita *et al.*, 1989), chronic renal failure (Stockenhuber *et al.*, 1992), cyclosporine-induced nephrotoxicity (DeRay *et al.*, 1991) and following haemodialysis of uraemic patients (Koyama *et al.*, 1989; Totsune *et al.*, 1989; Suzuki *et al.*, 1990). Cyclosporine treatment in rats is also associated with an increase in the density of renal endothelin binding sites (Nambi *et al.*, 1990).

Subtypes of endothelin receptors have been proposed, characterized according to the relative potencies of the three endogenous endothelin isoforms. ET-1 and ET-2 are more potent than ET-3 at the ET_A receptor whereas all three have equal affinity for the ET_B receptors (Sakurai *et al.*, 1992; Watson & Girdlestone, 1993). The endothelin receptors which mediate the pathophysiological effects of ET in the human kidney have not yet been defined; indeed the physiological role of the endothelins and their receptors in the kidney remains to be clarified. In human kidney, subtype-selective ligands have identified the presence of both ET_A and ET_B receptors. Although ET_B receptors comprise 70% of the total ET receptor population, microautoradiographical studies indicate that it is the ET_A receptors which localize to the media of the renal resistance vessels (Karet *et al.*, 1993).

This contrasts with findings in the rat, in which renal vasoconstriction *in vivo* may be mediated via activation of both receptor subtypes. This has been determined not only by the profound pressor effects of selective ET_B agonists (Davis *et al.*, 1991; Clozel *et al.*, 1992; Gardiner *et al.*, 1993) but also by the inability of selective ET_A antagonists to antagonize completely the vasoconstrictor response to ET-1 (Bigaud & Pelton, 1992; Cristol *et al.*, 1993; Pollock & Opgenorth, 1993). Similar data have been obtained in the rat isolated perfused kidney (Battistini *et al.*, 1993; Wellings *et al.*, 1994).

The relative density of vasoconstrictor ET_A to ET_B receptors appears to be dependent on both the vascular bed and the species studied (Davenport & Maguire, 1994), therefore it is important to determine the relative contribution made by each receptor subtype to the regulation of blood flow through the human kidney. We have obtained segments of human main stem renal artery and vein and as an initial step to characterizing vasoconstrictor ET receptors in the human kidney, we have identified those endothelin receptors present in these vessels using reverse transcriptase polymerase chain reaction (RT-PCR) assays and autoradiographical visualisation. Endothelin-induced vasoconstriction in rings of isolated renal artery and vein was characterized by use of the non-selective agonists, ET-1 and sarafotoxin 6B (S6b), the ET_B-selective agonists, ET-3 and sarafotoxin 6c (S6c) and the ET_A-selective antagonist, BQ123 (cyclo-(D-Trp-D-Asp-L-Pro-D-Val-L-Leu)).

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Methods

Tissue collection

Histologically normal sections of main branch renal artery and vein were obtained from nine individuals (6 male and 3 female aged 29–73 years) undergoing nephrectomy for non-obstructive carcinoma, and were immediately transferred to cold oxygenated Krebs solution. Excised kidneys were functional and of normal size. Biochemical functions were normal and renal ultrasound showed no evidence of hydronephrosis.

Reverse-transcriptase polymerase chain reaction assays

The media of renal arteries and veins from three individuals was separated from the intima and the endothelial layer removed by scraping. Extraction of total RNA, cDNA synthesis and RT-PCR assays were carried out using previously described methods (O'Reilly *et al.*, 1992; 1993a,b; Davenport *et al.*, 1993; Molenaar *et al.*, 1993). Briefly, RNA was extracted by the guanidinium isothiocyanate method and cDNA synthesized using avian myoblastosis virus reverse transcriptase enzyme. The following nested oligonucleotide primers were designed from published nucleotide sequences:

- (A) ET_A, ET_B forward TATCACAGAACTGAAAGTGC-TATG (Bases 379–396)
- (B) ET_A, ET_B reverse CAAGCAAGCAACGTAAG-AGCA (Bases 1214–1198)
- (C) ET_A forward CCTTTTGATCACAATGACTTT (Bases 439–459)
- (D) ET_A reverse TTTGATGTGGCATTGAGCATACAG (Bases 737–714)
- (E) ET_B forward ACTGGCCATTTGGAGCTGAGAT (Bases 497–519)
- (F) ET_B reverse CTGCATGCCACTTTTCTTTCTCAA (Bases 924–901)

PCR amplification was carried out using 1 µl cDNA, 5 µl reaction buffer (100 mM Tris-HCl, pH 8.3 at 25°C, 500 mM KCl and 15 mM MgCl₂), 5 µl deoxyNTPs (2 mM), 5 µl of each oligonucleotide primer A and B (10 µM) and 2.5 u Taq polymerase in a Techne PHC-3 programmable thermocycler (Techne, Cambridge). One µl of this reaction mixture was used in the second round of amplification using the internal primer pairs C and D and E and F. PCR products were separated by agarose gel electrophoresis and the bands stained with ethidium bromide. These PCR products have been previously cloned and sequenced and show 100% homology with published sequences for ET_A and ET_B receptor mRNA respectively.

Autoradiography

ET_A and ET_B receptors were visualized using published autoradiographical techniques (Molenaar *et al.*, 1992). Consecutive 10 µm thick cross-sections of renal artery and vein from three individuals were cut on a cryostat microtome (Bright Instruments, Huntingdon) and thaw mounted onto gelatine subbed slides. Sections were pre-incubated for 15 min at room temperature (23°C) in incubation buffer (50 mM HEPES containing 5 mM MgCl₂, bovine serum albumin, 0.3% w/v). The sections were then incubated in the same buffer for 2 h containing either 0.1 nM [¹²⁵I]-ET-1 (~2000 Ci mmol⁻¹) to detect all ET receptors or the ET_A-selective [¹²⁵I]-PD151242 (~2000 Ci mmol⁻¹) (Davenport *et al.*, 1994) or ET_B-selective [¹²⁵I]-BQ3020 (~2000 Ci mmol⁻¹) (Molenaar *et al.*, 1992). Non-specific binding was determined by incubating adjacent sections in the presence of 1 µM of the corresponding unlabelled peptide. At the end of the incubation period, sections were rinsed in three successive 5 min washes of ice cold Tris buffer (pH 7.4) and dried under a

stream of cold air. Sections were exposed to radiation sensitive film (Hyperfilm βmax).

In vitro pharmacology experiments

Segments of vessels ($n = 9$) were cut into rings, 2 mm in width, and the endothelium removed by gently rubbing with a metal seeker. Verification of this procedure was made histologically (see Figure 1). Rings were attached to isometric transducers (Swema, Stockholm, Sweden) and mounted under an initial resting tension of 1–2 g in 25 ml organ baths containing Krebs solution. The bathing medium was continuously gassed with 95% O₂:5% CO₂ and maintained at 37°C. Contractile responses were recorded on a Graphtec chart recorder (Linton Instruments, Diss, Norfolk). The preparations were allowed to equilibrate for 90 minutes before control responses were obtained to a maximal concentration of KCl (50 mM). After washing, cumulative dose-response curves (DRCs) were constructed to ET-1, ET-3, S6b and S6c (10^{-10} M– 3×10^{-7} M) with only one agonist tested per preparation. The experiments were terminated by addition of 50 mM KCl to the organ bath to determine the maximum contractile response possible, and agonist contractions were subsequently expressed as a percentage of this. In a further series of experiments the effect of BQ123 (3 and 10 µM) on ET-1-mediated contractions was determined. ET-1 DRCs were constructed in the absence (control) and presence of BQ123 added to the bathing medium 30 min prior to ET-1.

Materials

Reagents for RT-PCR were purchased from Pharmacia Biotech (St. Albans, Herts.) and H.T. Biotechnology Ltd. (Cambridge). [¹²⁵I]-ET-1, [¹²⁵I]-PD151242 and [¹²⁵I]-BQ3020 were from Amersham International (Amersham, Bucks.), BQ3020 ([Ala^{11,15}]Ac-ET-1_(6–21)), BQ123 (Davenport *et al.*, 1993) and PD151242 (N-[(hexahydro-1-azepinyl) carbonyl]L-Leu(1-me)-D-Trp-D-Tyr; Davenport *et al.*, 1994) were synthesized using solid phase t-Boc chemistry. ET-1, ET-3, S6b, S6c and BQ123 were purchased from Novabiochem, (Nottingham). Stock solutions (10^{-4} M) of ET-1, ET-3, S6b and S6c were dissolved in 0.1% acetic acid; BQ123 (10^{-3} M) was dissolved in dimethylsulphoxide (DMSO) and used directly or diluted in distilled water. The concentration of unlabelled peptides was determined by u.v. spectrophotometry. All other reagents were purchased from Sigma Chemical Co. (Poole, Dorset) or BDH (Lutterworth, Leics.), and were of analar grade or better. Krebs solution had the following composition (mM): NaCl 90, KCl 5, MgSO₄·7H₂O 0.5, Na₂HPO₄ 1, NaCO₃ 45, CaCl₂ 2.25, glucose 10, Na pyruvate 5, fumarate 5.

Figure 1 Agarose gel showing cDNA from the media of renal arteries and veins amplified with ET_A and ET_B specific primers. In lane 1, the marker is a 100 base pair ladder; in lane 2, the DNA template has been omitted as the negative control. The results show the presence of bands corresponding to size predicted for ET_A (299 base pairs) and ET_B (428 base pairs) in lanes 3 and 4 (renal arteries from two individuals) and 5 and 6 (renal veins from two individuals). These results are typical of arteries and veins from four individuals.

Figure 2 Autoradiographical localization of ET receptors in cross-sections of human renal artery following incubation with ligands for 2 h, washing and apposition to radiation sensitive film. (a) Total [125 I]-ET-1 binding showing the distribution of all ET receptors. (c) ET_A receptors localized using [125 I]-PD151242. (e) ET_B receptors localised with [125 I]-BQ3020. Non-specific binding determined by incubating adjacent sections in the presence of $1 \mu\text{M}$ of unlabelled ET-1 (b), PD151242 (d), or BQ3020 (f). Scale bar = 2 mm.

Results

Detection of ET_A and ET_B receptor mRNA

The amplified products of cDNA from the media of human renal artery and vein taken from four individuals, separated on an agarose gel (Figure 1), were of the size predicted for ET_A (299 base pairs) and ET_B (428 base pairs) receptors.

Localization of ET_A and ET_B receptors

In sections of renal artery and vein from three individuals, intense staining was obtained to both [125 I]-ET-1, binding to both receptor subtypes, and the ET_A -selective [125 I]-PD151242. In contrast lower levels of binding were detected with the ET_B agonist, [125 I]-BQ3020 (Figure 2).

Figure 3 Dose-response curves to ET-1 in rings of human isolated renal artery (■) and vein (●). Cumulative dose-response curves were constructed to ET-1 (10^{-10} M– 3×10^{-7} M) with each response expressed as a % of the maximal contraction elicited by 50 mM KCl. Data are the mean $\pm$ s.e.mean from 8–10 experiments.

Relative potencies of ET/sarafotoxin peptides

Maximum contractions elicited by 50 mM KCl were 1.36 ± 0.25 g tension in renal artery and 1.46 ± 0.32 g tension in renal vein (mean $\pm$ s.e.mean, $n = 9$). ET-1 produced dose-dependent long lasting contractions of renal artery and vein with EC_{50} values of 4.06 (1.84 – 8.95) nM ($n = 9$) and 1.00 (0.56 – 2.04) nM ($n = 8$, geometric mean $\pm$ 95% confidence interval), respectively. The DRCs to ET-1 in each preparation were monophasic (Figure 3). The structurally related S6b was approximately ten times less potent with EC_{50} values in artery of 36.3 (18.8 – 70.3) nM ($n = 5$) and in vein of 13.8 (2.36 – 81.07) nM ($n = 3$). The maximum responses to both ET-1 and S6b were comparable, approximately 50–60% of that to 50 mM KCl in the renal artery and 90–100% in the

renal vein. ET-3 was inactive in 6/6 and S6c in 5/6 artery preparations at concentrations up to 300 nM (Figure 4a). Responses in veins were more variable. ET-3 failed to elicit any contractile response in veins from three individuals at concentrations up to 300 nM whereas three further preparations did respond. In these cases ET-3 was much less potent than ET-1 (EC_{50} 51.9 (25.8 – 104.5) nM). S6c potently contracted 3/4 veins (EC_{50} 0.2 (0.07 – 0.54) nM) but the maximum response obtained was always less than 60% of that to ET-1 in the same experiment (Figure 4b).

Effect of BQ123

BQ123 (3 or 10 μ M) produced parallel dose-related rightward shifts of the ET-1 DRC in the renal artery (Figure 5a). The

Figure 4 Cumulative dose-response curves to ET-1 (●), S6b (■), ET-3 (○) and S6c (◆) in (a) renal artery and (b) renal vein *in vitro*. Agonist responses were expressed as a % of the maximal contraction to 50 mM KCl. Only one agonist dose-response curve was constructed per preparation and data are the mean $\pm$ s.e.mean from 3–10 experiments per agonist. For ET-3 and S6c in renal vein only data from those preparations which responded are included.

Figure 5 The effect of 3 μ M BQ123 on the vasoconstrictor responses to ET-1 in human renal artery (a) and vein (b) *in vitro*. ET-1 dose-response curves were constructed in the absence (●) and presence (■) of 3 μ M BQ123 added to the bathing medium 30 min prior to ET-1. ET-1 responses are expressed as a % of the maximal contraction to 50 mM KCl. Data are the mean $\pm$ s.e.mean from 3–10 experiments.

pA_2 value estimated from the Gaddum-Schild equation was 6.8 ± 0.1 ($n = 6$). Similar results were obtained using $3 \mu M$ BQ123 against ET-1 in renal vein with the pA_2 estimated as 6.8 ± 0.4 ($n = 3$) (Figure 5b). In all cases no attenuation of the maximum response to ET-1 was observed.

Discussion

We have shown that ET_A and ET_B mRNA can be detected in the media of renal arteries and veins in concordance with previous results in other human blood vessels including aorta, pulmonary and coronary arteries (Davenport *et al.*, 1993; Davenport & Maguire, 1994). *In situ* hybridization has confirmed the localization of mRNA encoding both receptors to the media of these vessels (Davenport *et al.*, 1993).

Autoradiography using [^{125}I]-ET-1 identified ET receptors localized to the media and intima of renal vessels. Use of the ET_A -selective [^{125}I]-PD151242 confirmed the presence of the ET_A sub-type, with a lower density of ET_B receptors detected using the ET_B -selective [^{125}I]-BQ3020. These results are in agreement with previous studies which showed that ET_A and ET_B selective compounds competed for iodinated ET-1 in a biphasic manner, suggesting the expression of both endothelin receptor sub-types, for example in the media of coronary arteries (Davenport *et al.*, 1993), with ET_A receptors comprising more than 80% of the total. The relative potency of the endogenous endothelins, ET-1 and ET-3, in both artery and vein are consistent with the presence of constrictor ET_A receptors. This was particularly striking in the renal artery in which ET-3 was without effect. As expected the non-selective compound S6b produced similar responses to ET-1 although it was ten times less potent. In agreement with these observations was the lack of effect of the ET_B -selective agonist, S6c in the renal artery confirming that vasoconstriction in this blood vessel, at least *in vitro*, is exclusively an ET_A -mediated phenomena. In common with other human blood vessels such as the coronary artery, mammary artery and saphenous vein (Maguire & Davenport, 1993) S6c did, on occasion, elicit small responses in the renal vein. These were variable and the magnitude of the responses were always considerably less than those to ET-1. However, these data do suggest that constrictor ET_B receptors are present in human renal vein although vasoconstriction by the endothelin peptides appears to be via ET_A receptors. BQ123 antagonized the effect of ET-1 in both preparations with estimated pA_2 values comparable with those obtained for blockade of ET-1 responses in the rat aorta (exclusively ET_A). This serves to confirm the involvement of ET_A receptors in ET-1-mediated vasoconstriction of human renal vascular smooth muscle. The apparent lack of ET_B response to either ET-1 or ET-3 may reflect the relatively small number of ET_B receptors in these vessels and suggests that the snake venom toxin S6c exhibits higher efficacy for this receptor subtype than either of the naturally occurring mammalian ET peptides. As far as is possible to determine in this limited study, there was no obvious correlation between the age or sex of an individual and whether or not responses to S6c were obtained.

A recent study of ET-1 and ET-3 vasoconstriction in rat main branch artery *in vitro* reported ET-3 to be 12 times less potent than ET-1 (Pierre & Clark, 1994). In agreement with

our own findings this suggests the presence of vasoconstrictor ET_A receptors in large diameter renal blood vessels. In contrast to our own observations, the effect of ET-1 could not be antagonized by $3 \mu M$ BQ123, although they were able to antagonize responses to ET-3 with a low concentration ($0.3 \mu M$) of this peptide. The authors suggested the additional presence of a vasoconstrictor BQ123-insensitive receptor for which we have no supporting evidence in human renal blood vessels.

Regulation of blood flow through the normal and diseased human kidney is determined by the small resistance blood vessels rather than the large capacitance vessels used in this study. It is possible that these smaller diameter vessels express both ET_A and ET_B receptors as predicted, for example, from *in vivo* experiments in rats. Further experiments on human renal resistance vessels are required to confirm or refute this. However, microautoradiographical visualisation of sections of human kidney using the ET_A -selective [^{125}I]-BQ123 and the ET_B -selective [^{125}I]-BQ3020 clearly indicate that intrarenal vascular endothelin receptors are labelled only by the ET_A -selective [^{125}I]-BQ123 but not by the ET_B -selective [^{125}I]-BQ3020 (Karet *et al.*, 1993). Whilst data obtained for one vascular bed cannot be extrapolated to another, it is interesting that experiments carried out using human small resistance arteries and veins *in vitro* suggest that endothelin-induced vasoconstriction in the resistance vessels of the heart (Godfraind, 1993) and omentum (Riezebos *et al.*, 1994), as in the larger human blood vessels, is mediated via the ET_A receptor.

If human renal vasoconstriction is an ET_A -mediated event then antagonism of this receptor may provide a novel therapeutic target in a number of pathophysiological conditions. Even if some of the constrictor effects of endothelin intrarenally are mediated via ET_B receptors, ET_A -selective compounds have already proved effective not only in antagonizing the haemodynamic responses in rats to infused ET-1 and big ET-1 (Pollock & Opgenorth, 1994) but also in limiting tissue necrosis and biochemical changes in at least two rat models; a cyclosporine-induced model of nephrotoxicity (Fogo *et al.*, 1992) and a model of chronic renal failure associated with reduced renal mass (Benigni *et al.*, 1993). This is despite reports that, in rats at least, some part of renal vasoconstriction is ET_B -mediated. It would be very interesting to know whether non-selective endothelin antagonists are more effective than ET_A -selective antagonists such as BQ123 in either of these models. If not, selective blockade of ET_A receptors in the kidney would have the beneficial 'side effects' of leaving unopposed ET_B -mediated vasodilatation and those non-vascular ET_B receptors whose function remains to be determined.

In summary we have demonstrated the presence of both ET_A and ET_B receptors in the media of human renal artery and vein but endothelin-mediated vasoconstriction *in vitro* is due to ET_A receptor activation.

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