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Influence of ETR-p_1/f1 antisense peptide on endothelin-induced constriction in rat renal arcuate arteries

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1 This study set out to examine the endothelin receptor subtypes mediating vasoconstriction in the rat renal arcuate artery. This was done in isolated vessels 120–200 μm in diameter, incubated with a selective agonist and the novel 'antisense' peptide to part of the human endothelin$_A$ receptor.

2 Groups of vessels ($n=6$) were incubated with increasing concentrations of endothelin-1 (ET-1), from 1 to 100 nM, which caused a 65% maximal contraction at the highest dose with an pEC_{50} of 8.16 ± 0.11 M. By contrast, in six other vessels sarafotoxin 6c over the same dose range gave a minimal contraction (around 5% of maximum).

3 Preincubation of six vessels with the antisense peptide ETR p_1/f1 at 1 μM had no effect on the ET-1 induced vasoconstriction, in terms of displacement of the concentration-response curve or the maximal tension achieved by the agonist. In the six vessels exposed to 4 μM ETR p_1/f1, there was a significant shift of the concentration-response curve and a lower pEC_{50} at 7.78 ± 0.09 M ($P<0.05$). At the highest concentrations of ETR p_1/f1, there was a marked suppression of all responses to ET-1, which at the maximal concentrations tested, 0.1 μM, only reached some 10% of the maximal achievable contraction.

4 Increasing ET-1 concentrations up to 2 μM in vessels incubated with 40 μM ETR-p_1/f1 showed that the blockade could be overcome and that the relationship was shifted to the right ($P<0.001$) by approximately one log unit with a pEC_{50} of 7.13 ± 0.11 M. A Schild plot of the data indicated the antagonist to be acting competitively at a single population of receptors.

5 At the highest concentrations tested, 40 μM, ETR-p_1/f1 had no effect on noradrenaline-induced contractions, indicating a lack of non-specific actions.

6 Together, these data suggest that at the rat renal arcuate artery the endothelin$_A$ receptor is the predominant functional receptor mediating contraction. Furthermore, this study has shown the potential usefulness of this novel type of 'antisense' peptide in blocking receptor activation.

Keywords: Renal arcuate arteries; antisense peptides; endothelins; endothelin receptors

Introduction

Yanagisawa *et al.* (1988) were amongst the first to show that a vasoconstrictor peptide was produced by the endothelial cells of the vasculature and it became apparent from later work that the endothelins represent a family of peptides, 21 amino acids in length, which comprise of three isoforms, endothelin-1 -2 and -3 (ET-1, ET-2 and ET-3) which have both autocrine and paracrine actions to modify vascular tone. Activation of the endothelin gene results in the production of the protein prepro-endothelin which undergoes intracellular cleavage to produce the mature endothelins which are released (Rubanyi & Polokoff, 1994). A body of evidence has now been generated which supports the existence of two main receptor subtypes, termed ET_A and ET_B which have been cloned and expressed (Arai *et al.*, 1990; Sukuri *et al.*, 1990; Elshourbagy *et al.*, 1993). Following the development of selective antagonists, radio-ligand binding and pharmacological studies (Clozel *et al.*, 1993; Eglezos *et al.*, 1993; Bodelsson & Stjernquist, 1993) indicated that several receptor subtypes may be present in different tissues. There is now a concensus that the ET_A receptor subtype is activated primarily by ET-1 and ET-2 while the ET_B receptor subtype is stimulated most effectively by ET-3 and the related peptide, sarafotoxin 6c. Nonetheless, there is some evidence and indications that further subtypes of the endothelin receptor may exist. It is now recognised that ET_A receptors are present on vascular smooth muscle cells and cause vasoconstriction. The ET_B receptors are also found on the endothelial cells, whereas their activation causes a dilatation which may be mediated by the generation of nitric oxide (NO) or unidentified factors (EDHF, endothelial derived hyperpolarizing factor) which act in a paracrine fashion at the vascular smooth muscle (Simonson & Dunn, 1993). Interestingly, there are studies showing that ET_B receptors are expressed on vascular smooth muscle but in this cell type cause vasoconstriction (Sumner *et al.*, 1992) and these differing sites and actions may help explain the biphasic action which has been found (Kuwahara *et al.*, 1996).

At the level of the kidney, there are a number of studies that have demonstrated that endothelin receptors are present along most of the vascular tree, from renal artery to vasa recta (Brown *et al.*, 1996), as well as the tubular elements (Marson *et al.*, 1994) of the organ. The renal vasoconstriction induced by endothelins appears to be mediated via ET_A receptors as ET-1 is very potent compared with the ET_B receptor agonists, ET-3 or sarafotoxin 6c (Clark & Pierre, 1995) which is in contrast to other tissues where ET_A and ET_B receptors contribute more equally to the endothelial-induced vasoconstriction. A further feature of the endothelin-mediated vasoconstriction within the kidney is that it is very long lasting and in many ways, is difficult to study. The reason for this difference between the kidney and other tissues is unclear but it is apparent that the contribution and density of the endothelin receptor subtypes, from conducting artery, to large resistance artery, to resistance

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arteriole may vary considerably (Godfraind, 1993). Furthermore, there is evidence that there may be differences in endothelin receptor subtype composition not only between organs but also across species (Fuchs *et al*., 1992).

The function of the endothelins and studies of their distribution has been undertaken with a range of potent and selective pharmacological agonists and antagonists of both peptidic and non-peptidic nature. Recently, Baranyi *et al*. (1995) utilized a somewhat different approach, whereby they took a fragment of the human ET_A receptor and constructed an antisense peptide which would interact with the sense sequences on the receptor protein. They showed that this peptide was able to overcome the endothelin-induced vasoconstriction of carotid and femoral arteries. Furthermore, they found that LPS-induced endotoxaemic shock was prevented by prior administration of the peptide. This evidence suggested that this peptide could be a potent, selective and very effective antagonist. The aim of this study was two fold. Firstly, to compare the effectiveness of ET-1 and sarafotoxin 6C in causing constriction of the rat renal arcuate artery with the view to determining the contribution of ET_A as against ET_B receptor activation. Secondly, to determine the effectiveness of the ET_A antisense peptide in blocking the action of ET-1 on this segment of the renal vascular tree.

Methods

Preparation of blood vessels

Male Wistar rats (300–400 g) were killed by CO_2 asphyxia. The left kidney was removed into iced physiological salt solution (PSS) containing (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, EDTA 0.026, glucose 5.5, pH 7.4, bubbled with 5% CO_2 in O_2. Renal arcuate arteries, internal diameter of 120–220 μm, were identified and isolated with a dissecting microscope. The vessels were mounted on two 40 μm tungsten wires in a small vessel myograph (Cambustion, Cambridge, U.K.) capable of measuring isometric tension.

After an equilibration period of 1 h in PSS, the passive tension/internal circumference characteristics were determined (Mulvany & Halpern, 1977). The vessels were then set to an internal circumference equivalent to 90% of that they would have had when relaxed *in situ* under a transmural pressure of 100 mmHg. The maximum active tension is developed at approximately this circumference (Mulvany & Halpern, 1977). Maximal potassium activation was achieved by KPSS (PSS with equimolar substitution of KCl for NaCl resulting in a final potassium concentration of 125 mM). After a further equilibration period of half an hour, the arteries were then maximally contracted for 2 min every 10 min on five occasions. The first two and the last contraction being with KPSS and 5 μM noradrenaline while the third was with 5 μM noradrenaline alone following KPSS. Any vessel that failed to develop a maximum tension equivalent to a pressure of 100 mmHg was excluded. The maximum tension developed was taken as 100% and used as the value against which the endothelin-induced contraction was measured.

Experimental protocol

Cumulative concentration-response curves were constructed to ET-1, 1–100 nM, for each artery. Each concentration of agonist was left in the bath for 10 min, the tension being recorded at the end of the period. The above concentration-response curves to ET-1 were undertaken in further sets of vessels in the presence of the antisense peptide receptor antagonist, ETR-p_1/f1 (Baranyi *et al*., 1995) at 1 μM, 4 μM and 40 μM. Another set of vessels were exposed to the 40 μM ETR-p_1/f1 but challenged with higher concentrations of ET-1, that is up to 1 μM, to achieve a maximal contraction. In a further study, sarafotoxin 6c was tested over the concentration range 0–5 μM in another set of vessels. The selectivity of ETR-p_1/f1 was investigated in a set of vessels which were exposed to noradrenaline, 10 nM to 10 μM, before and following incubation with 40 μM of the antisense peptide.

Drugs

ET-1 was purchased from Sigma, (Poole, Dorset), noradrenaline from (Winthrop Laboratories, (Guildford, Surrey). All other reagents were obtained from BDH (Poole, Dorset U.K.). All reagents were of Analar grade and solutions were prepared fresh daily. ETR-p_1/f1 peptide was prepared by Dr H. Okada (Nagoya Medical School, Nagoya, Japan). The detailed design of the antisense peptide has been described previously (Baranyi *et al*., 1995) and was based on the antisense homology-box derived peptide ETR-$_{p1}$: NH_2-CALSVDRYRAVASW-COOH which is a fragment of human $endothelin_A$ receptor and may have inhibitory properties. In order to increase the specific activity of ETR-p_1, a simplistic approach was taken of adding flanking amino acids to correct for the change in hydropathic profile to produce ETR-p_1/f1: NH_2-VLNLCALSVDRYRA-VASWSRVI-COOH. The peptide was synthesized by an automated peptide synthesizer followed by h.p.l.c. purification and the final product was freeze dried for storage.

Statistical analysis

Concentration-response curves were compared by two-way analysis of variance (SuperANOVA) followed by Bonferroni all means *post hoc* test. A Hill plot was constructed for each concentration-response relationship where possible. The $-\log$ concentration of agents required to produce 50% of the maximum response (pEC_{50},M) was calculated by use of a computerized curve fitting software package (Graphpad Inplot Version 3.14).

Tension was expressed as mN mm^{-1} artery length and the maximum tension generated was that obtained during incubation with KPSS and 5 μM noradrenaline. The vasoconstrictor responses to ET and sarafotoxin were calculated as a percentage of the maximum contraction. All values are presented as mean $\pm$ s.e.mean. Differences between means were assessed by use of Student's t test for paired and unpaired observations. $P<0.05$ was taken as statistically significant.

Results

In Figure 1 the percentage of the maximum contraction induced by a pre-conditioning vasoconstrictor concentration of noradrenaline is plotted against the log concentration of agonist after 10 min of exposure, when a stable situation had been reached. It can be seen (Figure 1a) that increasing concentrations of ET-1 gave progressively greater degrees of contraction, reaching a peak at 50 nM which was some 65–70% of the maximal attainable, which in this set of vessels was 4.05 ± 0.81 mNmm^{-1} and gave a pEC_{50} of 8.16 ± 0.11 M. Administration of the higher concentration of ET-1 resulted in a smaller constriction, to 59% of the maximal response. By contrast, in a second set of vessels having a maximal tension of 5.15 ± 1.02 mNmm^{-1} (Figure 1a) it was clear that there was no response to sarafotoxin 6c even at the highest concentration tested, 0.2 μM, and in a few vessels, but not included here, even 5 μM was ineffective in inducing a reliable vasoconstriction.

In Figure 1b, the ET-1 concentration-response curve is replotted and included for comparative purposes. A third set of vessels with a maximal tension of 2.48 ± 0.48 mNmm^{-1} was exposed to the ETR-p_1/f1 peptide at 1 μM and, although there was a slight rightward shift of the curve at the lower concentrations of ET-1, at higher concentrations the peak contraction was similar to that obtained when the peptide was not present. The two curves could not be distinguished statistically and ETR-p_1/f1 peptide gave a pEC_{50} of 7.82 ± 0.13 M versus

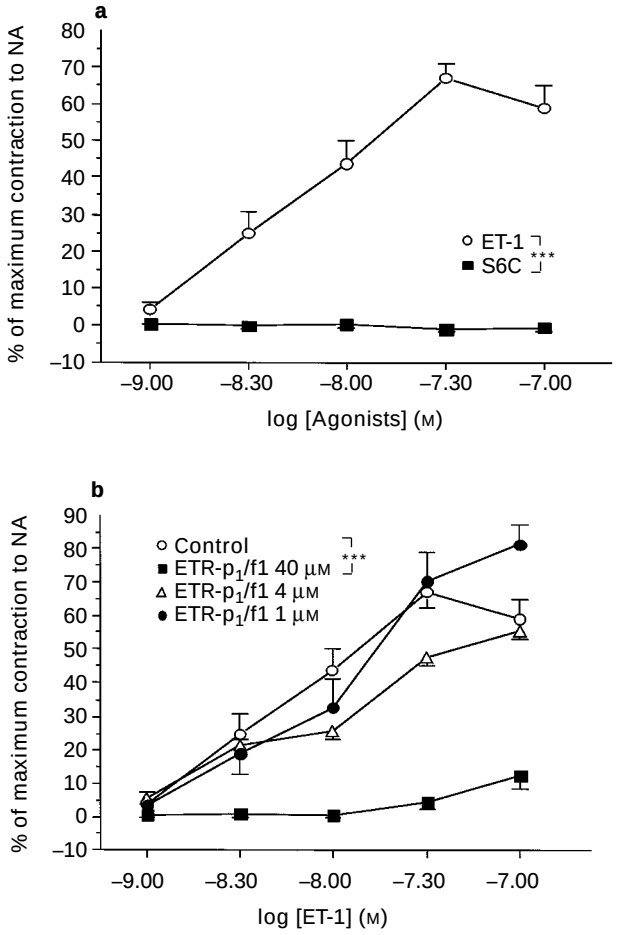

Figure 1 The maximum contraction (expressed as % of that induced by noradrenaline) in response to increasing doses of agonists. (a) The dose-response relationship to endothelin-1 (ET-1) in 6 vessels from 6 rats, and sarafotoxin 6c, in 4 vessels from 4 rats. (b) The vasoconstrictor responses to increasing doses of ET-1 in the absence and presence of 1, 4 and 40 μM of the endothelin receptor antagonist (ETR-p_1/f1) after 10 min of exposure, obtained in 6 vessels from 6 rats in each group. ***$P<0.001$, by use of ANOVA to compare the two curves.

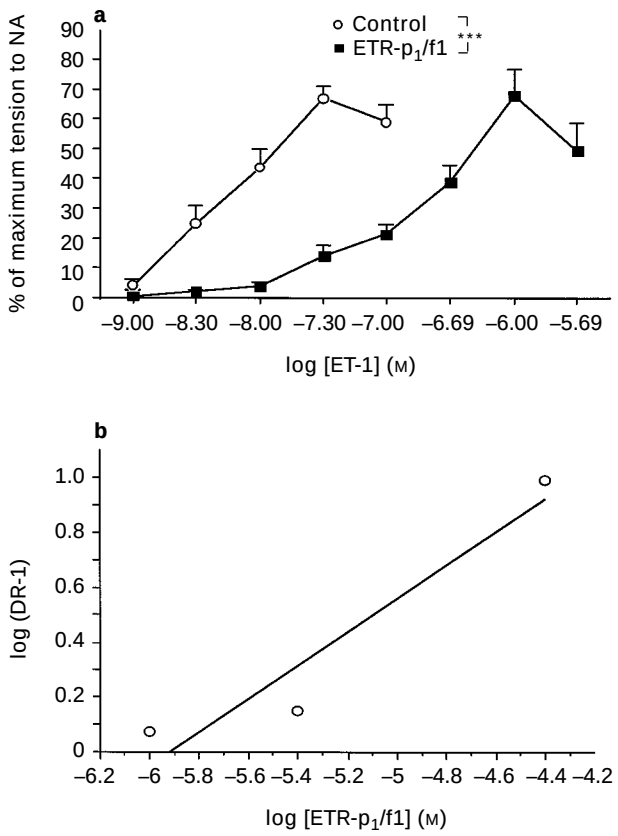

Figure 2 (a) The concentration-response curve, generated in 6 vessels from 6 rats, for ET-1, 1 nM to 2 μM, in the presence of 40 μM ETR-p_1/f1. The same control data shown in Figure 1a are included for comparative purposes. (b) The Schild plot, log (DR − 1) agonist against log ETR-p_1/f1 concentrations (where DR = the ratio of the pEC_{50} in the presence of ETR-p_1/f1 to that in its absence), for each of the sets of vessels demonstrates that a linear relationship exists with a slope not significantly different from one. ***$P<0.001$, by use of ANOVA to compare the two curves.

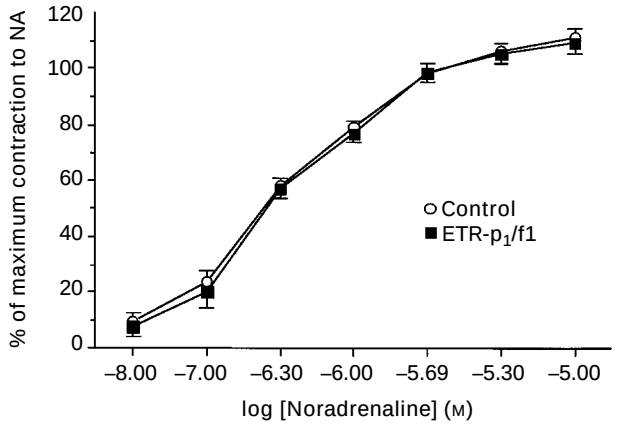

Figure 3 The plot of percentage of the maximum contraction, in 6 vessels, caused by increasing doses of noradrenaline before (control) and following exposure to 40 μM ETR-p_1/f1. It was evident that the two curves are virtually identical.

8.16 ± 0.11 M in the absence of the antagonist. In the presence of 4 μM ETR-p_1/f1 the maximal tension generated by the vessels was 5.51 ± 0.34 mNmm^{-1} and there was a significant ($P<0.001$) rightward shift of the curve compared with the 1 μM concentration of antagonist, with the peak tension only being achieved at the highest concentration of ET-1, 100 nM and there was a significant ($P<0.05$) reduction in the pEC_{50} to 7.78 ± 0.09 M. A further study was done in 6 vessels exposed to 40 μM ETR-p_1/f1. The mean maximal tension achieved was 3.49 ± 0.79 mNmm^{-1} and it was apparent that over the whole of the concentration range studied, the vasoconstrictor effect of ET-1 was markedly attenuated ($P<0.001$ compared to all other groups). At the highest concentrations of ET-1 a small contraction was obtained, indicative of a marked rightward shift of the curve. A second set of vessels when incubated with 40 μM ETR-p_1/f1 (Figure 2a) had a mean maximal tension of 3.49 ± 0.79 nNmm^{-1}. This set was exposed to ET-1 up to a concentration of 2 μM, which induced a peak contraction and caused the curve to be shifted to the right compared to the control vessels ($P<0.001$), with a pEC_{50} of 7.13 ± 0.11 M which was over one log unit greater ($P<0.001$) than that obtained in the absence of the ETR-p_1/f1. The data from the control vessels and those exposed to the differing doses of antisense peptide were used to construct a Schild plot (Figure 2b). This gave a straight line with a slope of 0.987 which was not significantly different from 1, indicative of competitive antagonism involving a single receptor. Figure 3 presents concentration-response curves to noradrenaline from 10 nM to 10 μM, in a set of vessels with a mean maximal tension of 4.13 ± 0.33 nNmm^{-1} before and following incubation with 40 μM ETR-p_1/f1. It can be seen that the peptide had no effect on the relationship with the pEC_{50} being 5.97 ± 0.17 M, in the absence, and 6.27 ± 0.11 M, in the presence of the antisense antagonist.

Discussion

This study set out to examine the role of differing endothelin receptor subtypes present in the rat isolated renal arcuate vessel and to explore the potential role of the synthetic antisense peptide, ETR-p_1/f1 (Baranyi *et al*., 1995) to block ET-1 induced vasoconstriction. The particular strategy applied in generating this class of peptides has been to design a sequence of amino acids, of approximately 15 units in length, which form an 'antisense' molecule to the corresponding peptide sequence which exists within a large protein molecule. The ETRp_1/f1 peptide was formulated to be an antisense peptide targeted to a particular intramembrane-intracellular region of the human ET_A endothelin-receptor subtype (Baranyi *et al*., 1995). In these initial studies the authors were able to show that the peptide was very effective at low concentrations in blocking endothelin-mediated contraction in the large femoral and carotid arteries from the rat, and ameliorated the endothelin-mediated components of endotoxin-induced shock.

One aim of the present study was to use the endothelin antisense peptide as an experimental pharmacological tool, as at present the selectivity of other non-peptide antagonists for ET_A and ET_B receptors is relatively limited. It is also evident that knowledge of the ET receptor subtype composition in various organs and between species is sparse and it is becoming apparent that it may change along the vascular tree in any one organ. Thus, in the present study it is necessary to emphasize that a selected part of the vascular tree of the kidney was used, that is, the rat renal arcuate artery. This vessel can be considered to be a conduit artery which contributes to a relatively small extent to the total renal vascular resistance. It is likely that the major sources of vascular resistance in the kidney resides at the afferent and efferent arterioles, which probably make up to 70 to 80% of the total renal resistance.

The administration of ET-1 clearly showed that the arcuate vessels were sensitive to the peptide over a concentration range which was similar to that reported by others (Simonson & Dunn, 1993), and demonstrated that the preparation was sensitive and viable. The question arises as to whether this vasoconstriction might be mediated by ET_A or ET_B receptors. This was partially answered by the study with sarafotoxin 6c in that over a very large concentration range it was ineffective. Furthermore, over this same concentration range, ET-1 caused a vasoconstriction which was very clearly concentration-related. Because sarafotoxin 6c acts primarily on ET_B receptors (Simonson, 1993), the conclusion from this study would be that ET_B receptors are either not present at this segment of the vascular tree or the receptor density is so low that they are unable to participate in the contractile response. This would lead to the suggestion that the ET-1 mediated contraction must be as a consequence of activation of ET_A receptors. Thus, had significant numbers of ET_B receptors been available, the endothelin-1 would have acted upon them to induce a vasoactive response. Interestingly, in an *in vitro* study in a large conducting vessel, the rat renal artery (Clarke & Pierre, 1995), no evidence was obtained that ET_B receptors are involved in the ET-1-dependent contraction of the conducting artery.

To support this contention, studies were undertaken in which the novel antisense peptide ETR-p_1/f1, which should selectively block ET_A receptors, was used. An important first step was to establish that the antisense peptide had no non-specific action to depress contractility and to this end, the effect of the peptide on noradrenaline-induced contractions was tested. It was evident that at the highest concentration of ETR-p_1/f1 there was no influence on the noradrenaline-induced contractions. Thus, this would suggest that the antisense peptide was unlikely to be interfering with other agonist-receptor interactions. The ETR-p_1/f1 proved to be an effective antagonist to the ET-1 induced contractions which were suppressed in a concentration dependent manner up to 40 μM of the ETR-p_1/f1. It was of interest that with the higher concentrations of ET-1, the ETR-p_1/f1 inhibition was overcome and a maximal contraction was achieved indicating that there had been a marked shift to the right. Importantly, generating a Schild plot from the available data, demonstrated that there was a linear relationship between the antagonist-induced blockade of agonist contractions, having a slope with a value of one. This finding is compatible with the inhibition being competitive in nature with only one receptor being involved. Together, these two studies showed that the ETR-p_1/f1 peptide was selective and acting as a competitive antagonist on ET_A receptors in the rat arcuate artery.

The intracellular signalling initiated by endothelin receptor activation is under investigation and appears to be diverse. Most work has examined the consequence of ET_A activation and it is apparent that the phosphoinositide cascade is initiated and leads to an increase in intracellular calcium which mediates vasoconstriction (Masaki *et al*., 1990); phospholipase A, arachidonic acid metabolism and adenosine 3′:5′-cyclic monophosphate cyclic AMP) production may also be stimulated. However, there are data (Goto *et al*., 1989; Inoue *et al*., 1990) that show endothelin can increase the opening probability of voltage-gated calcium channels, but whether ligand gated channels may also be activated remains to be resolved. There may also be some involvement of the ATP-sensitive potassium channels (Haynes *et al*., 1993), but the underlying relationships between these responses remain unclear.

There are apparently conflicting views over the contribution of ET_A and ET_B receptors mediating the action of ET-1 in the kidney. There are two studies (Wellings *et al*., 1993; Pollock & Opgenorth, 1993) in which ET-1 was given into the whole animal and the renal vascular responses were found to involve, in part, a contribution from ET_B receptors. Furthermore, in other studies performed in this laboratory (Marshall & Johns, 1996), by use of infusion of endothelin directly into the rat kidney *in vivo*, it was demonstrated that sarafotoxin 6c could decrease renal blood flow, indicative of increased resistance. However, in all these studies the changes in resistance would have been the result of constriction of the more distal parts of the vascular tree, that is the interlobular arteries and afferent arterioles. It is therefore entirely feasible that at these sites ET_B receptors may be present in greater density and are able to participate in the constrictor response. The next logical step would be to use a selective ET_A receptor antagonist, such as ETR p_1/f1 infused into the renal artery of these *in vivo* preparations to clarify the contribution of the ET_B receptors at this level.

The present study has shown that the rat renal arcuate arteries are sensitive to endothelin-1, which induces a concentration-related vasoconstriction. By contrast, the ET_B receptor selective agonist, sarafotoxin 6c, was relatively insensitive over a similar concentration range, suggesting ET_B receptors were not present. The antisense peptide ETR-p_1/f1 was shown to block selectively endothelin-induced contractions and the block was competitive in nature and appeared to involve only one receptor type. The antisense peptide ET_A receptor antagonist effectively blocked the endothelin-1-induced contraction suggesting that only ET_A receptors were involved. Whether a similar situation pertains further down the vascular tree at the level of the resistance vessels remains to be resolved.

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