British Journal of Pharmacology (1996) 119, 311-314

The increase in human plasma immunoreactive endothelin but not big endothelin-1 or its C-terminal fragment induced by systemic administration of the endothelin antagonist TAK-044

Christopher Plumpton, *Charles J. Ferro, *William G. Haynes, *David J. Webb & [1]Anthony P. Davenport

Clinical Pharmacology Unit, University of Cambridge, Box 110, Addenbrooke's Hospital, Cambridge, CB2 2QQ, and *Department of Medicine, University of Edinburgh, Western General Hospital, Edinburgh, EH4 2XU

1 We examined the effects of systemic infusion, in healthy human volunteers, of the endothelin antagonist TAK-044 on the plasma concentrations of mature endothelin, big endothelin-1 and the C-terminal fragment of big endothelin-1, by selective solid-phase extraction and specific radioimmunoassays.

2 Unlabelled TAK-044 competed with specific [^{125}I]-endothelin-1 binding to human left ventricle tissue in a biphasic manner giving K_D values of 0.11 nM and 26.8 nM at the ET_A and ET_B receptor subtypes, respectively, indicating a 244 fold selectivity for the ET_A receptor subtype.

3 A 15 min intravenous infusion of placebo or 30 mg TAK-044 (giving a serum concentration of 2 nM, calculated to block >95% of ET_A but <5% ET_B receptors) had no effect on the immunoreactive plasma concentrations of the three peptides.

4 At the higher dose of 750 mg TAK-044 (giving a serum concentration of 80 nM, calculated to block >99% of ET_A and >75% ET_B receptors), the immunoreactive plasma endothelin concentrations were increased 3.3 fold over basal levels ($P<0.01$). The concentrations of big endothelin-1 or C-terminal fragment of big endothelin-1 were unchanged.

5 At both doses of TAK-044, there were significant decreases in diastolic blood pressure, and peripheral vascular resistance, with corresponding increases in cardiac index and stroke index. There were no changes in systolic or mean arterial blood pressures or heart rate.

6 Since only the concentrations of the mature peptide were increased, we conclude that the most likely sources of endothelin contributing to the observed rise were displacement of receptor-bound peptide and reduction in plasma clearance rather than peptide synthesis.

Keywords: Endothelin; big endothelin; C-terminal fragment; human vasodilatation; selective solid-phase extraction; radioimmunoassay

Introduction

The endothelins are a family of three potent vasoconstrictor peptides (endothelin-1 (ET-1), ET-2 and ET-3, Inoue *et al.*, 1989). ET-1 and a biologically inactive C-terminal fragment (CTF, big $ET\text{-}1_{(22-38)}$) are generated from an intermediate big ET-1, following an unusual cleavage, by one or more endothelin-converting enzymes (Battistini *et al.*, 1995). In functional assays the endothelins exert their actions through two distinct receptor subtypes, ET_A and ET_B, distinguished by the rank order of potency of the three endothelin isoforms (Arai *et al.*, 1990, Sakurai *et al.*, 1990).

We have previously demonstrated that local infusion of the ET_A-selective antagonist BQ123 causes vasodilatation suggesting that endothelin contributes to basal vascular tone in man (Haynes & Webb, 1994). In addition, we have previously shown that local infusion of exogenous big ET-1 results in an increase in immunoreactive (IR) plasma CTF as well as IR endothelin (Plumpton *et al.*, 1995a, b). All three peptides can be detected in the conditioned medium from cultured endothelial cells (Plumpton *et al.*, 1996a), suggesting that any *de novo* synthesis of endothelin would be accompanied by a rise in CTF.

Animal and human studies have suggested that endothelin is important in a range of clinical conditions and have demonstrated the potential therapeutic beneficial effects of endothelin receptor antagonism (Ferro & Webb, 1996). However, plasma concentrations of IR endothelin are increased following non-selective endothelin receptor antagonism (Löffler *et al.*, 1993; Donckier *et al.*, 1995; Kiowski *et al.*, 1995; Teerlink *et al.*, 1995). Our aim was to determine whether the systemic infusion of the cyclic hexapeptide endothelin receptor antagonist TAK-044 (cyclo[D-αc-aspartyl-3-[(4-phenylpiperazin-1-yl) carbonyl]-L-analyl-L-αc-aspartyl-D-2-(2-thienyl) glycyl-L-leucyl-D-tryptophyl] disodium; Kikuchi *et al.*, 1994) altered the concentrations of IR endothelin in human plasma. Secondly, to distinguish between possible sources of IR plasma endothelin, we also measured the concentrations of the precursor big ET-1, and by-product CTF. A preliminary account of this work has been presented to the British Pharmacological Society (Plumpton *et al.*, 1996b).

Methods

Competition binding experiments

The selectivity of TAK-044 was determined for native human endothelin receptors as previously described (Davenport *et al.*, 1995). The human left ventricle was used as this expresses both endothelin receptor subtypes and allows the selectivity of competing ligands to be determined in the same assay. Frozen sections of left ventricle (10 μM, $n=3$) were thaw mounted onto gelatine coated microscope slides and pre-incubated for

[1] Author for correspondence.

15 min at 23°C in 50 mM HEPES, 5 mM $MgCl_2$, 0.3% BSA pH 7.4. Sections were then incubated for 2 h at 23°C in the same buffer containing 0.1 nM [^{125}I]-ET-1 (2000 Ci mmol^{-1}) and increasing concentrations of TAK-044 (20 pM – 100 μM). Total ET-1 binding was determined in the absence of TAK-044 and non-specific binding was defined by inclusion of 1 μM unlabelled ET-1. Sections were washed three times in ice-cold Tris-HCl (pH 7.4) for a total of 15 min then wiped from the slides and counted using a gamma counter. Protein estimations were made by use of a modified Lowry assay (Bio-Rad) by reference to bovine serum albumin (BSA) standards. Pseudo Hill coefficients were determined by use of EBDA (McPherson, 1983) from which the final values of equilibrium dissociation constant (K_D) were calculated with the iterative curve fitting program LIGAND (Munson & Rodbard, 1980).

Clinical procedures

Healthy male volunteers (25–60 years of age, mean age 40) participated in the study with the approval of the local ethical committee. Each subject had an antecubital vein of the left and right arm cannulated for administration of infusate and withdrawal of blood samples, respectively. TAK-044 was synthesized and supplied by Takeda Chemical Industries Ltd, Osaka, Japan. Blood pressure and heart rate were measured by semi-automated oscillometric monitors, cardiac function (stroke volume, cardiac output and heart rate) was measured by a non-invasive bioimpedance methodology (Haynes *et al.*, 1996). Eight subjects received either 50 ml sucrose placebo, 30 mg or 750 mg TAK-044 dissolved in saline over 15 min on separate occasions in a single-blind, three-phase, randomised, placebo-controlled, crossover study. The two doses of TAK-044 resulted in serum concentrations of 2 and 80 nM TAK-044 after 15 min of infusion, respectively as determined by high performance liquid chromatography (h.p.l.c.). In six subjects, venous blood samples were taken before and 15 min after dosing. Blood was collected into EDTA tubes and separated immediately. The resulting plasma was stored at −70°C until assayed.

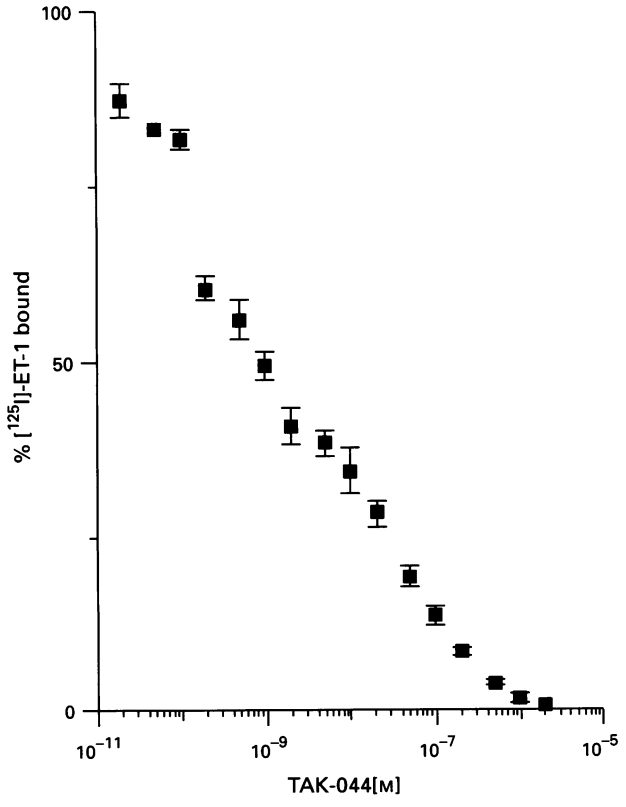

Figure 1 Competition assays showing the inhibition of [^{125}I]-endothelin-1 (ET-1) binding to sections of human left ventricle by TAK-044. Results are expressed as percentages of the specific binding. Mean values ($n=3$) are shown and vertical lines indicate s.e.mean.

Radioimmunoassays for endothelin peptides

After being thawed, 5 ml plasma samples were assayed for IR endothelin, big ET-1 and CTF by selective solid-phase extraction and radioimmunoassay as previously described (Plumpton *et al.*, 1995a; 1996a) using rabbit antisera raised against the C-termini of endothelin (ET-$1_{(15-21)}$) and big ET-1 (big ET-$1_{(31-38)}$). For both assays, ED_{50} values were 20–25 fmol/tube, inter- and intra-assay coefficients of variation were <13% in the range 6–30 fmol/tube and the sensitivities of detection (defined as two s.d. above zero standard) were <1.25 fmol/tube. The recoveries of ET-1, big ET-1 and CTF were 57.5%, 39.8% and 76.6%, respectively ($n=4$). Plasma IR peptide concentrations are shown uncorrected for extraction recovery.

Under the present conditions, the mature endothelin RIA cross-reacted 100% with ET-1, ET-2 and ET-3. Cross-reactivity with ET-$1_{(1-20)}$, big ET-$1_{(22-38)}$, big ET-1, big ET-2 and big ET-3 were <0.02%. The big ET-1 RIA showed <0.007% cross-reactivity with the mature endothelins, big ET-2 and big ET-3, and cross-reacted 143% with big ET-$1_{(22-38)}$ thus allowing the quantification of CTF following fractionation. Neither of the assays showed any detectable cross-reactivity (<0.000002%) with TAK-044. Furthermore, TAK-044 did not interfere with either assay as indicated by superimposable standard curves at concentrations five orders of magnitude greater than the serum TAK-044 levels achieved. No cross-reactivity was detected (<0.005%) at the highest concentrations tested with unrelated vasoactive peptides such as angiotensin II, atrial natriuretic factor, and α-calcitonin gene-related peptide.

Results

TAK-044 competed biphasically for the binding of [^{125}I]-ET-1 giving K_D values of 0.11 nM and 26.8 nM at the ET_A and ET_B receptor subtypes, respectively (Figure 1). Based on these affinities, the concentration of TAK-044 measured in the serum following the low dose was calculated to block >95% of the ET_A but <5% of the ET_B receptors. However, at the high dose, the majority (>75%) of the ET_B receptors were calculated to be blocked in addition to >99% of the ET_A receptors.

At both doses of TAK-044, there were significant decreases in diastolic blood pressure, and peripheral vascular resistance, with corresponding increases in cardiac index and stroke index. There were no changes in systolic or mean arterial blood pressures or heart rate in any of the phases (Table 1). These findings are in agreement with an action of TAK-044 as an arteriolar vasodilator. Although most of the changes in the haemodynamic parameters appeared greater after 750 mg than 30 mg TAK-044, none of these differences reached statistical significance. TAK-044 was well tolerated in all volunteers as assessed by direct questions, visual analogue scales, routine electrocardiography, biochemistry and haematology. In particular, no serious or clearly drug-related adverse effects were detected.

When placebo or TAK-044 at the 30 mg dose was infused there were no significant changes in the concentration of IR peptide compared with basal levels (Figure 2). However, at the higher dose of 750 mg TAK-044, although there were no detectable changes in the concentrations of IR big ET-1 or CTF, the IR endothelin increased from 4.0±1.5 pM to 13.3±2.2 pM ($P<0.01$, compared to pre-infusion).

Table 1 Baseline values and mean changes (Δ) over 15 min for systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate, stroke index, cardiac index and peripheral vascular resistance (PVR) after dosing with TAK-044

	Placebo			*TAK-044*					
				30 mg			*750 mg*		
	Baseline value	*Mean Δ over 15 min*	*95% CI*	*Baseline value*	*Mean Δ over 15 min*	*95% CI*	*Baseline value*	*Mean Δ over 15 min*	*95% CI*
SBP (mmHg)	127±5	4±2	−3 to +10	128±5	1±1	−2 to +5	129±5	−8±4	−18 to +2
DBP (mmHg)	75±2	1±1	−2 to +5	73±2	−3±1*	−5 to −1	74±2	−4±2*	−8 to 0
MAP (mmHg)	92±3	2±1	−2 to +6	91±1	−2±1	−4 to +1	92±3	−6±2	−9 to +1
Heart rate (beats min^{-1})	58±3	1±1	-2 to +3	57±4	1±2	−5 to +6	59±2	1±1	−2 to +4
Cardiac index ($l\,min^{-1}\,m^{-2}$)	3.2±0.3	−0.1±0.04	−0.2 to +0.1	3.0±0.2	0.3±0.1*	+0.1 to +0.5	2.8±0.2	0.4±0.1*	+0.1 to +0.6
Stroke index ($ml\,m^{-2}$)	56±5	0±1	−2 to +2	51±3	3±1**	+1 to +5	47±5	5±1**	+2 to +8
PVR (dyn s $cm^{-5}\,m^{-2}$)	2392±237	77±57	−66 to +220	2472±117	−259±61**	−413 to −105	2719±248	−452±41***	−554 to −350

For the placebo column, mean change from predose is shown. For the active treatment columns, placebo corrected changes from predose are shown. Mean values ($n=8$) are shown ± s.e.mean and 95% confidence intervals (CI). Statistical differences were analysed by ANOVA followed by paired Student's t tests. *$P<0.05$; **$P<0.01$; ***$P<0.0001$ compared to basal.

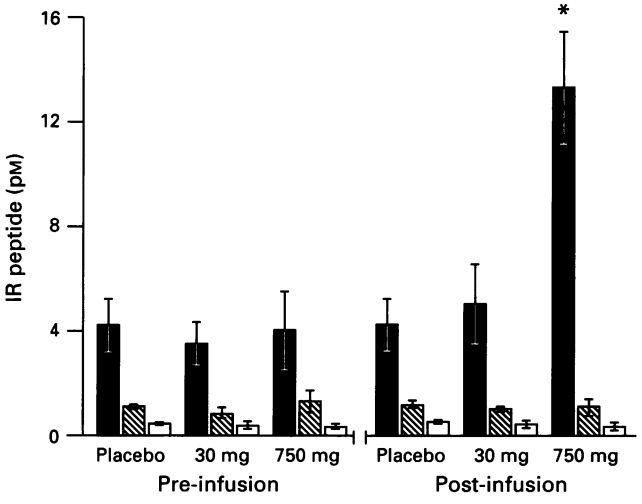

Figure 2 Effect of intravenous infusion of placebo or TAK-044 (30 mg or 750 mg) for 15 min on immunoreactive plasma endothelin (solid columns), big endothelin-1 (hatched columns) and C-terminal fragment of big endothelin-1 (open columns). Mean values ($n=6$) are shown and vertical lines indicate s.e.mean. Statistical differences were analysed by ANOVA followed by Student's t tests. *$P<0.01$ compared to basal.

Discussion

This study shows that in healthy human volunteers, systemic infusion of 750 mg TAK-044, but not placebo or 30 mg TAK-044, results in a significant increase in venous plasma IR endothelin compared to basal levels, with no detectable changes in the corresponding concentrations of either big ET-1 or CTF.

Local infusion of big ET-1 in healthy human volunteers results in significant increases of IR plasma endothelin and CTF compared to basal (Plumpton *et al.*, 1995a, b). Therefore, if the present rise in IR endothelin were due to an increased synthesis, we would predict altered concentrations of big ET-1 and/or CTF, but no change from basal levels was detected. Taken together with the rapid rate of increase in plasma IR endothelin, the effects are unlikely to be due to *de novo* synthesis, although the processing of stored precursors cannot be excluded. These data support other endothelin peptide measurements obtained following endothelin receptor antagonism in animals (Löffler *et al.*, 1993; Donckier *et al.*, 1995; Teerlink *et al.*, 1995) and patients with chronic heart failure (Kiowski *et al.*, 1995).

At the serum concentrations achieved with both doses of TAK-044, it was calculated that virtually all of the ET_A receptors are blocked and may therefore produce the observed haemodynamic changes, since ET-1 predominantly mediates vasoconstriction via ET_A receptors (Davenport & Maguire, 1994; Maguire & Davenport, 1995). In contrast, only at the higher dose are the majority of the ET_B sub-type antagonised. This suggests that the increase in human plasma IR endothelin following the infusion of 750 mg TAK-044 could be associated with displacement of ET_B receptor-bound peptide and/or blockade of clearance receptors. This is consistent with the identification of a high density of non-vascular ET_B receptors, possibly clearance receptors, in human kidney (Karet *et al.*, 1993) and their efficient regional extraction of mature endothelins (Weitzberg *et al.*, 1991; Gasic *et al.*, 1992).

We conclude that the increase in IR plasma endothelin following infusion of TAK-044 under the present conditions, is most likely due to the displacement of mature endothelin from binding sites and/or blockade of clearance from the circulation.

This work was supported by the British Heart Foundation, Newton Trust, Royal Society, Scottish Home and Health Department, Takeda Euro R&D Centre and The Wellcome Trust.

References

ARAI, H., HORI, S., ARAMORI, I., OHKUBO, H. & NAKANISHI, S. (1990). Cloning and expression of a cDNA encoding an endothelin receptor. *Nature*, **348**, 730–732.

BATTISTINI, B., BOTTING, R. & WARNER, T.D. (1995). Endothelin: a knockout in London. *Trends Pharmacol. Sci.*, **16**, 217–222.

DAVENPORT, A.P. & MAGUIRE, J.J. (1994). Is endothelin-induced vasoconstriction mediated only by ET_A receptors in humans? *Trends Pharmacol. Sci.*, **15**, 9–11.

DAVENPORT, A.P., O'REILLY, G. & KUC, R.E. (1995). Endothelin ET_A and ET_B mRNA and receptors expressed by smooth muscle in the human vasculature: majority of the ET_A sub-type. *Br. J. Pharmacol.*, **114**, 1110–1116.

DONCKIER, J., STOLERU, L., HAYASHIDA, W., VAN MECHELEN, H., SELVAIS, P., GALANTI, L., CLOZEL, J-P., KETELSLEGERS, J-M. & POULEUR, H. (1995). Role of endogenous endothelin-1 in experimental renal hypertension in dogs. *Circulation*, **92**, 106–113.

FERRO, C.J. & WEBB, D.J. (1996). The clinical potential of endothelin receptor antagonists in cardiovascular medicine. *Drugs*, **51**, 12–27.

GASIC, S., WAGNER, O.F., VIERHAPPER, H., NOWOTNY, P. & WALDÄUSL, W. (1992). Regional hemodynamic effects and clearance of endothelin-1 in humans: renal and peripheral tissues may contribute to the overall disposal of the peptide. *J. Cardiovasc. Pharmacol.*, **19**, 176–180.

HAYNES, W.G., FERRO, C.J., O'KANE, K.P.J., SOMMERVILLE, D., LOMAX, C.C. & WEBB, D.J. (1996). Systemic endothelin receptor blockade decreases peripheral vascular resistance and blood pressure in humans. *Circulation*, **93**, 1860–1870.

HAYNES, W.G. & WEBB, D.J. (1994). Contribution of endogenous generation of endothelin-1 to basal vascular tone. *Lancet*, **344**, 852–854.

INOUE, A., YANAGISAWA, M., KIMURA, S., KASUYA, Y., MIYAUCHI, T., GOTO, K. & MASAKI, T. (1989). The human endothelin family: Three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 2863–2867.

KARET, F.E., KUC, R.E. & DAVENPORT, A.P. (1993). Novel ligands BQ123 and BQ3020 characterize endothelin receptor subtypes ET_A and ET_B in human kidney. *Kidney Int.*, **44**, 36–42.

KIKUCHI, T., OHTAKI, T., KAWATA, A., IMADA, T., ASAMI, T., MASUDA, Y., SUGO, T., KUSUMOTO, K., KUBO, K., WATANABE, T., WAKIMASU, M. & FUJINO, M. (1994). Cyclic hexapeptide endothelin antagonists highly potent for both receptor subtypes ET_A and ET_B. *Biochem. Biophys. Res. Commun.*, **200**, 1708–1712.

KIOWSKI, W., SÜTCH, G., HUNZIKER, P., MÜLLER, P., KIM, J., OECHSLIN, E., SCHMITT, R., JONES, R. & BERTEL, O. (1995). Evidence for endothelin-1-mediated vasoconstriction in severe chronic heart failure. *Lancet*, **346**, 732–736.

LÖFFLER, B-M., BREU, V. & CLOZEL, M. (1993). Effect of different endothelin receptor antagonists and of the novel non-peptide antagonist Ro 46–2005 on endothelin levels in rat plasma. *FEBS Lett.*, **333**, 108–110.

MCPHERSON, G. (1983). A practical computer-based approach to the analysis of radioligand binding experiments. *Comput. Prog. Biomed.*, **17**, 107–114.

MAGUIRE, J.J. & DAVENPORT, A.P. (1995). ET_A receptor-mediated constrictor responses to endothelin peptides in human blood vessels *in vitro*. *Br. J. Pharmacol.*, **115**, 191–197.

MUNSON, P.J. & RODBARD, D. (1980). LIGAND: A versatile computerized approach for the characterization of ligand binding systems. *Analyt. Biochem.*, **107**, 220–239.

PLUMPTON, C., ASHBY, M.J., KUC, R.E., O'REILLY, G. & DAVENPORT, A.P. (1996a). Expression of endothelin peptides and mRNA in the human heart. *Clin. Sci.*, **90**, 37–46.

PLUMPTON, C., FERRO, C.J., HAYNES, W.G., WEBB, D.J. & DAVENPORT, A.P. (1996b). The endothelin antagonist TAK-044 increases human plasma immunoreactive endothelin but not big endothelin or C-terminal fragment of big endothelin-1. *Br. J. Pharmacol.*, **117**, 32P.

PLUMPTON, C., HAYNES, W.G., WEBB, D.J. & DAVENPORT, A.P. (1995a). Phosphoramidon inhibition of the *in vivo* conversion of big endothelin-1 to endothelin-1 in the human forearm. *Br. J. Pharmacol.*, **116**, 1821–1828.

PLUMPTON, C., HAYNES, W.G., WEBB, D.J. & DAVENPORT, A.P. (1995b). Measurement of C-terminal fragment of big endothelin-1: A novel method for assessing the generation of endothelin-1 in humans. *J. Cardiovasc. Pharmacol.*, **26**, s34–36.

SAKURAI, T., YANAGISAWA, M., TAKUWA, Y., MIYAZAKI, H., KIMURA, S., GOTO, K. & MASAKI, T. (1990). Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature*, **348**, 732–735.

TEERLINK, J.R., CARTEAUX, J-P., SPRECHER, U., LÖFFLER, B-M., CLOZEL, M. & CLOZEL, J-P. (1995). Role of endogenous endothelin in normal hemodynamic status of anaesthetised dogs. *Am. J. Physiol.*, **268** *(Heart Circ. Physiol.*, **37)**, H432–H440.

WEITZBERG, E., AHLBORG, G. & LUNDBERG, J.M. (1991). Long-lasting vasoconstriction and efficient regional extraction of endothelin-1 in human splanchnic and renal tissues. *Biochem. Biophys. Res. Commun.*, **180**, 1298–1303.

(Received May 8, 1996
Revised June 20, 1996
Accepted June 26, 1996)