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Characterization of [^{125}I]-PD164333, an ET_A selective non-peptide radiolabelled antagonist, in normal and diseased human tissues

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1 We have synthesized a new low molecular weight, non-peptide radioligand, [^{125}I]-PD164333, an analogue of the orally active butenolide antagonists of the endothelin ET_A receptor.

2 Analysis of saturation binding assays demonstrated that [^{125}I]-PD164333 bound with high affinity to a single population of receptors ($n \geqslant 3$ individuals $\pm$s.e.mean) in human aorta ($K_D = 0.26 \pm 0.08$ nM; $B_{max} = 8.8 \pm 3.95$ fmol mg^{-1} protein), left ventricle from the heart ($K_D = 0.16 \pm 0.02$ nM; $B_{max} = 34.2 \pm 3.02$ fmol mg^{-1} protein) and kidney ($K_D = 1.24 \pm 0.16$ nM; $B_{max} = 125.3 \pm 35.07$ fmol mg^{-1} protein). In each case Hill slopes were close to unity.

3 In kinetic experiments, the binding of [^{125}I]-PD164333 to ET_A receptors in sections of heart was time-dependent and rapid at 23°C. The data were fitted to a one site model, with an association rate constant (K_1 of $2.66 \pm 0.213 \times 10^8$ M^{-1} min^{-1}, and a half-time for association of 11 min. The binding was reversible at 23°C: analysis of the data indicated [^{125}I]-PD164333 dissociated from a single site, with a dissociation rate constant of 0.0031 ± 0.0004 min^{-1}, a half-time for dissociation of 216 min and a K_D calculated from these kinetic data of 0.01 nM.

4 Unlabelled PD164333 inhibited the binding of [^{125}I]-ET-1 to left ventricle (which expresses both subtypes) in a biphasic manner with a $K_D ET_A$ of 0.99 ± 0.32 nM and $K_D ET_B$ of 2.41 ± 0.22 μM, giving a selectivity of 2500 fold. ET_A-selective ligands competed monophasically for [^{125}I]-PD164333 binding in left ventricle, a one site fit was preferred to a two site model giving similar nanomolar affinities: BQ123, $K_D = 3.93 \pm 0.18$ nM; FR139317 $K_D = 3.53 \pm 0.69$ nM. In contrast, the ET_B selective agonists, BQ3020 and sarafotoxin S6c (1 μM) did not inhibit binding.

5 In human isolated saphenous vein, unlabelled PD164333 was a functional antagonist, producing parallel rightward shifts of the endothelin-1 (ET-1) concentration-response curve ($pA_2 = 8.84$) and a slope of unity.

6 In the human brain, autoradiography revealed high levels of [^{125}I]-PD164333 binding to the pial arteries of the cerebral cortex and to the numerous smaller intercerebral vessels penetrating the underlying grey and white matter. Conduit and resistance vessels contributing to the control of blood pressure from the heart, kidney, lungs and adrenal also displayed high densities of binding. In diseased vessels, binding of [^{125}I]-PD164333 was confined to the medial layer of both coronary arteries with advanced atherosclerotic lesions or occluded saphenous vein grafts. In contrast, little or no binding was detected in the proliferated smooth muscle of the intimal layer or occluded lesion.

7 These results show [^{125}I]-PD164333 is a specific, high affinity, reversible non-peptide radioligand for human ET_A receptors, which will facilitate the further characterization of this subtype, *in vitro* and *in vivo*.

Keywords: Endothelin ET_A radioligand; atherosclerosis; saphenous vein grafts; quantitative receptor autoradiography

Introduction

In man, two endothelin receptor sub-types, ET_A and ET_B have been cloned and shown to be present in varying ratios in different tissues (see Davenport, 1997). However, there is increasing evidence from ligand binding studies that the ET_A receptor is the principle (> 80%) subtype expressed by the medial smooth muscle of the human vasculature in both the large conduit (Davenport *et al*., 1993; 1995b), as well as the smaller resistance vessels contributing to the control of blood pressure (Davenport *et al*., 1995a). Although the snake venom toxin, sarafotoxin S6c, induces vasoconstriction in some of the human vessels studied to date, suggesting activation of ET_B receptors (Haynes *et al*., 1995), this response is frequently variable occurring in less than 50% of individuals (White *et al*., 1994; Maguire & Davenport, 1994) and is always less effective than endothelin-1 (ET-1; Haynes *et al*., 1995). Other human vessels from different vascular beds do not respond at all (Riezebos *et al*., 1994; Buchan *et al*., 1994). Furthermore, ET_A selective antagonists consistently fully reverse ET-1 induced vasoconstriction in human vessels (Adner *et al*., 1994; Fukuroda *et al*., 1994, Liu *et al*., 1994, Maguire & Davenport, 1994; Maguire *et al*., 1994; Opgaard *et al*., 1994; 1996). These results suggest that ET_A subtypes must be blocked in pathophysiological conditions where endothelin is over-expressed to produce a beneficial vasodilatation.

A number of antagonists that are highly selective for the native human ET_A receptor have been identified (Davenport, 1997) and a modified linear tetrapeptide radiolabelled ligand, [^{125}I]-PD151242 has been synthesized (Davenport *et al*., 1994a,b). However, owing to its peptidic structure, it is not orally active. The butenolides are a family of recently discovered non-peptide antagonists, with low molecular weight and oral bioavalability (Doherty *et al*., 1995; Reynolds *et al*., 1995; Patt *et al*., 1997). The lead compound in this series, PD156707 (CI-1020) has been shown to be effective in animal models of diseases including blocking acute pulmonary hypertension in the rat

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(Keiser *et al*., 1997), as well as reducing ischaemic injury in the feline brain (Patel *et al*., 1996) and rat lungs (Khimenko *et al*., 1996). We have shown that this compound is highly selective for human native ET_A receptors, and potently antagonizes ET-1-induced vasoconstriction in human isolated vessels, with pA_2 values greater than 8 (Maguire *et al*., 1997).

Our aim was to synthesize a low molecular weight, non-peptide ligand based on the butenolide series and to determine the affinity and selectivity of the resulting compound, [^{125}I]-PD164333 for human endothelin receptors.

Methods

With local ethical approval, human tissue was obtained at the time of operation, snap frozen immediately in liquid nitrogen and stored at −70°C until use. Cardiovascular and pulmonary tissues were from seven males (47–63 years) undergoing heart transplants for cardiomyopathy or heart-lung transplants. Samples of epicardial coronary artery containing advanced atheromatous lesions and occluded saphenous vein bypass grafts were from three males (51–59) undergoing cardiac transplantation for ischaemic heart disease. Drug therapy included angiotensin converting enzyme (ACE)-inhibitors, diuretics, calcium antagonists, vasodilators, digoxin and anticoagulants. Normal human brain (cerebral cortex) was from three male and female patients (19–46 years) all of whom were premedicated with dexamethasone before surgery for cerebral tumours. Regions of histologically normal adrenal gland were removed from three male and female patients (33–69 years) following adrenalectomy for phaeochromocytoma, who were being treated with phenoxybenzamine. Normal renal tissue (containing cortex and medulla) was obtained following nephrectomy from the opposite pole to the non-obstructing tumours from three male and female patients (32–74 years). Therapy included non-steroidal anti-inflammatory and analgesic drugs. Experiments described below were carried out with tissue from at least three individuals.

Saturation and competition assays

Ligand binding experiments were carried out as previously described (Davenport *et al*., 1994a). In saturation experiments, tissue sections were pre-incubated for 15 min in 50 mM HEPES buffer, pH 7.4 containing 5 mM $MgCl_2$, then incubated with increasing concentrations (8 pM–4 nM) of [^{125}I]-PD164333 for 2 h at 23°C. Non-specific binding was defined by incubating adjacent sections with 1 μM of unlabelled PD164333. Competition binding experiments were carried out under the same conditions. Sections were labelled with a fixed concentration of [^{125}I]-ET-1 (0.1 nM) and in the presence of increasing concentrations of unlabelled PD164333 in the range 20 pM to 100 μM, or a fixed concentration of [^{125}I]-PD164333 (0.2 nM) and increasing concentrations of either FR139317 or BQ123 in the range 20 pM to 100 μM. Non-specific binding was defined by incubating adjacent sections with 1 μM of unlabelled ET-1 or PD164333, respectively. The optimum conditions for separating tissue bound [^{125}I]-PD164333 from free ligand was determined empirically and found to be 3 × 5 min washes in Tris buffer, pH 7.4 at 4°C.

Kinetic experiments

Association experiments were carried out in left ventricular tissue as described above, except that sections were incubated for increasing time periods with 0.2 nM [^{125}I]-PD164333. For dissociation experiments, sections were incubated with [^{125}I]-PD164333 for two hours at 23°C before sections were washed in an excess of buffer at 23°C for increasing time.

Analysis

The results from binding experiments were analysed by use of the KINETIC, LIGAND and EBDA suite of programmes (Biosoft, Cambridge, U.K.). Hill slopes and initial estimates of binding parameters for saturation and competition experiments were obtained by use of EBDA (McPherson, 1985) and the resulting files from replicate experiments co-analysed by LIGAND (Munson & Rodbard, 1980). The presence of 1,2 or 3 sites was tested with the F-ratio test in LIGAND. The model adopted was that which provided the best fit ($P < 0.05$). Rates of association or dissociation of [^{125}I]-PD164333 were calculated by use of the non-linear curve fitting programme, KINETIC; the presence of more than one binding site was tested with the F-ratio test. The model adopted was that which provided the best fit ($P < 0.05$).

Reverse phase high performance liquid chromatography

The stability of [^{125}I]-PD164333 under the binding assay conditions described above was measured by collecting the supernatant exposed to sections of human left ventricle at the end of the experiments. samples were run on a reverse phase Spherisorb ODSII column (250 × 4.6 mm diameter) with an isocratic solvent mixture of 40% acetonitrile and 0.1% trifluoroacetic acid. The flow rate was 1 ml^{-1}. Sample fractions (0.5 ml) were collected over 30 min and counted.

Quantitative autoradiography

For autoradiography, sections were incubated with 0.2 nM [^{125}I]-PD164333 for 2 h under conditions described above. Dried sections were apposed to radiation sensitive film for 2 days, together with standards. The resulting autoradiograms were analysed by use of computer-assisted densitometry (Davenport *et al*., 1995a). Binding densities were compared in normal and diseased vessels by the Mann-Whitney U-test. Adjacent sections of coronary artery and occluded saphenous veins were stained for α-actin as well as with haematoxylin and eosin to facilitate histological identification.

In vitro *pharmacology*

Endothelium-denuded rings (2 mm) of saphenous veins were maintained in continuously oxygenated modified Krebs solution (with the following composition in mM: NaCl 90, KCl 5, $MgSO_4.7H_2O$ 0.5, Na_2HPO_4 1, $NaHCO_3$ 45, $CaCl_2$ 2.25, glucose 10, Na pyruvate 5, glutamic acid 5 and fumaric acid 5) at 37°C as previously described (Maguire & Davenport, 1994). Concentration-response curves were determined for ET-1 (100 pM–1 μM) and expressed as a percentage of the maximum contraction to 50 mM KCl used to terminate the experiment. The ability of PD164333 to antagonize the ET-1 response was determined and pA_2 values calculated by Schild analysis.

Drugs

PD164333 (2-benzo[1,3]dioxol-5-yl-4-(3-[2-(4-hydroxy-phenyl)-ethylcarbomoyl]-propoxy)-4,5-dimethoxy-phenyl) - 3 - (4-methoxy-benzoyl)-but-2-enoic acid Figure 1) was synthesized according to general methods previously described (Doherty

et al., 1995; Patt *et al*., 1997) and has a molecular weight of 801.

[^{125}I]-PD164333 (2000 Cimmol^{-1}) was synthesized by Amersham International plc (Amersham, U.K.) from the unlabelled material by direct iodination with sodium[^{125}I]-iodide by use of the lactoperoxidase method and purified to be carrier free by reverse phase high performance liquid chromatography (h.p.l.c) (3-[^{125}I]-iodotyrosyl)-ET-1 (2000 Cimmol^{-1}) was also from Amersham International plc.

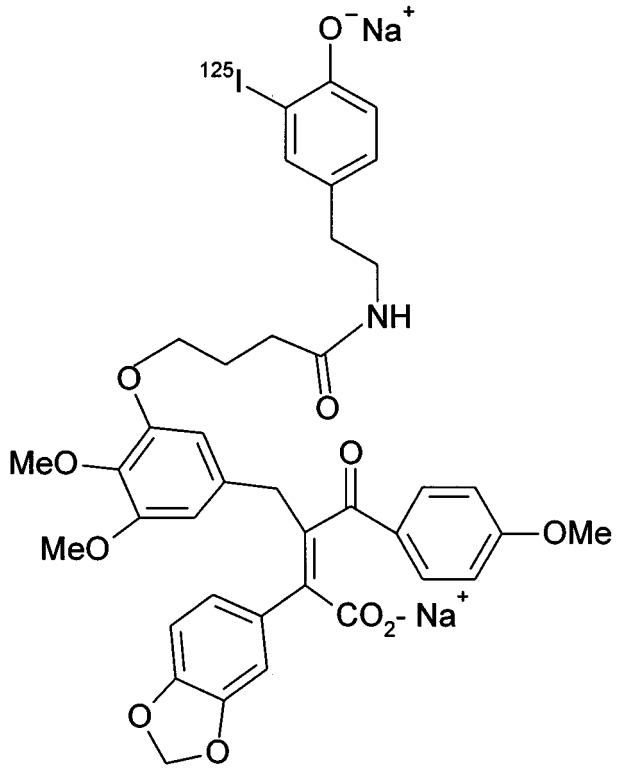

Figure 1 Structure of [^{125}I]-PD164333.

FR139317 (N-[(hexahydro-1-azepinyl)carbonyl])L-Leu(1-Me)D-Trp-3(2-pyridyl)-D-Ala); BQ123 (cyclo [D-Asp-L-Pro-D-Val-L-Leu-D-Trp-]) and BQ3020 ([Ala^{11,15}]Ac-ET-1$_{6\text{-}21}$) were synthesized by solid phase t-Boc chemistry. Other peptides were from the Peptide Institute (Osaka, Japan), Novabiochem (Nottingham, U.K.) or Peninsula (St. Helens, U.K.). Peptide concentrations were determined by u.v. spectrophotometry. Bosentan (4-tert-butyl-N-(6-(2-hydroxy-ethoxy)-5-(2-methoxyphenoxy)-2,2'-bipyrimidin-4-yl)-benzenesulphonamide) Clozel *et al*., 1994) and SB209670 ((+)-1**S**,2**R**,**S**-3-(2-carboxy-methoxy-4-methoxyphenyl)-1-(3,4-methyl enedioxyphenyl)-5-prop-1-yloxyindane-2-carboxylate) (Elliot *et al*., 1994) were synthesized by established methods.

Results

Binding characteristics

The binding of [^{125}I]-PD164333 was concentration-dependent and saturable to sections of human left ventricle, aorta and kidney (Table 1a). Equilibrium dissociation constants ranged from 0.2–1.2 nM. For each tissue, a one site was preferred to a two site model and the Hill coefficients were close to unity.

Table 1 Saturation binding experiments: dissociation constants (K_D), maximal density of receptors (B_{max}) and Hill coefficients (n_H) for [^{125}I]-PD164333 binding to human tissues

Tissue	n	K_D (nM)	B_{max} (fmol mg^{-1} protein)	n_H
Left ventricle	3	0.16±0.02	34.2±3.02	1.10±0.06
Aorta	4	0.26±0.08	8.8±3.95	0.89±0.04
Kidney	3	1.24±0.16	125.3±35.07	0.94±0.04

Values are the means±s.e.mean of at least three individuals.

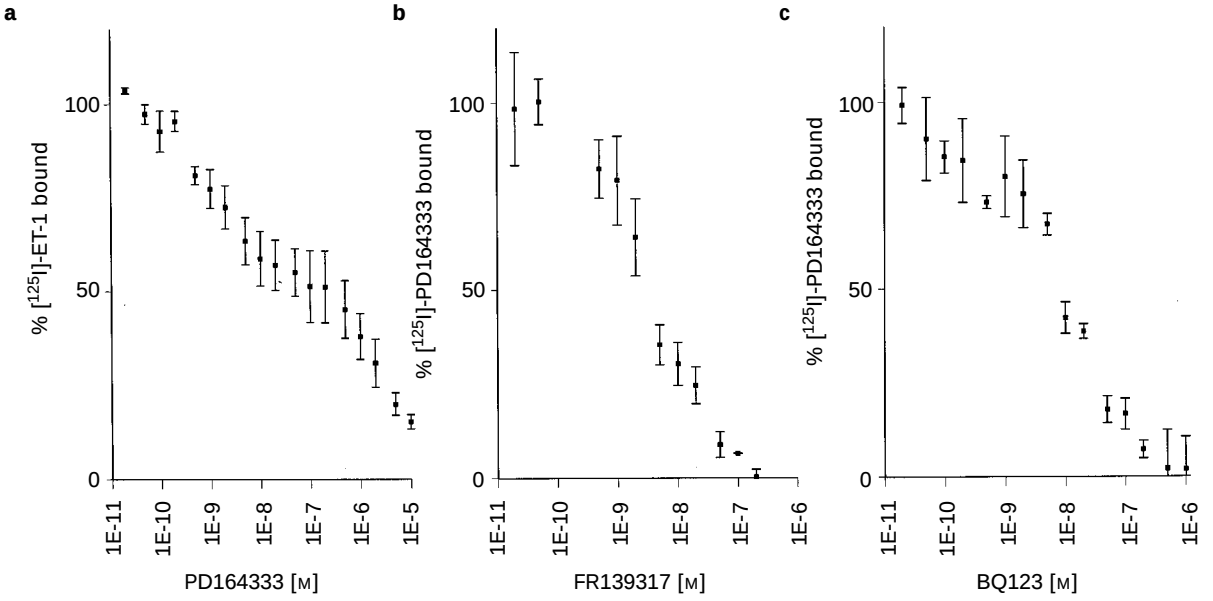

Figure 2 (a) Inhibition of 0.1 nM [^{125}I]-ET-1 binding by unlabelled PD164333 to slide mounted sections of human left ventricle. Over the concentration range tested, the antagonist competed in a biphasic manner and a two site fit was preferred to a one site or three site model. Inhibition of 0.2 nM [^{125}I]-PD164333 binding by (b) FR139317 and (c) BQ123. Both ET_A selective peptide antagonists competed monophasically. Each value represents the mean of three individuals; vertical lines show s.e.mean.

Binding of [^{125}I]-PD164333 to sections of human left ventricle was time-dependent and rapid at 23°C. The data were fitted to a one site model, with an association rate constant (K_1) of $2.66 \pm 0.213 \times 10^8$ M^{-1} min^{-1} ($n = 3 \pm$ s.e.mean). The half-time for association ($t_{1/2}$) was 11 min. The binding was reversible at 23°C and analysis of the data indicated [^{125}I]-PD164333 dissociated from a single site, with a dissociation rate constant of 0.0031 $\pm$0.0004 min^{-1} ($n = 3 \pm$ s.e.mean). The half-time for dissociation ($t_{1/2}$) was 216 min with a K_D calculated from these kinetic data of 0.01 nM.

Ligand selectivity

Unlabelled PD164333 competed for the binding of 0.1 nM [^{125}I]-ET-1 to the left ventricle as expected in a biphasic manner, with a high affinity site corresponding to the more abundant ET_A receptor but with low, micromolar affinity for the ET_B subtype (Figure 2a and Table 2). The unlabelled compound had a selectivity of about 2500 fold for the human ET_A receptor.

The ET_A selective peptide antagonists, FR139317 and BQ123 both competed monophasically, with similar high affinities, for the binding of a fixed concentration of [^{125}I]-PD164333 in left ventricle (Figure 2b,c and Table 2).

Selectivity was defined further by incubating 0.2 nM [^{125}I]-PD164333 with a series of compounds at a concentration of 1 μM in slide mounted sections of left ventricle. The ET_A/ET_B non-peptide antagonists SB209670 and bosentan, that have a different chemical structure, and the endogenous peptide agonists ET-1, ET-2 and sarafotoxin S6b inhibited binding to levels that were not significantly different from non-specific binding defined by 1 μM of unlabelled PD164333. ET-3 which has a lower affinity for the human ET_A subtype inhibited specific binding by 54 $\pm$5%. ET_B agonists, sarafotoxin S6c or BQ3020 showed little or no competition. Structurally-unrelated vasoactive peptides (angiotensin II, calcitonin gene-related peptide and atrial natriuretic factor) did not compete.

Stability

The stability of the radioligand was tested by incubating 0.2 nM of [^{125}I]-PD164333 with sections of human left ventricle under standard binding conditions. After two hours the assay buffer containing the ligand was aspirated off. Specific binding was still detected when the radiolabel was re-incubated with tissue sections ($n = 3$ individuals). The supernatant was also subject to fractionation by reverse-phase h.p.l.c. An example of the radiochemical profile for [^{125}I]-PD164333 is shown in Figure 3. A major peak eluted after 19 min, corresponding to the expected position for [^{125}I]-PD164333 and a minor peak after 4 min corresponding to the expected position for free iodine. Under these conditions, unlabelled, PD164333 detected by u.v. absorbance (254 nm) eluted after 17 min. Following exposure to tissue, the radioactivity recovered in the supernatant also eluted at the same time, although the magnitude of the peak was reduced as expected by tissue bound radioactivity. Exposure to tissue did not alter the peak corresponding to the free iodine nor were additional peaks detected, which might be expected if the ligand had been degraded.

In vitro *assays*

ET-1 potently constricted human isolated saphenous veins with an EC_{50} value of 0.3 nM. Unlabelled PD164333 produced parallel rightward shifts of the ET-1 concentration-response curve with a pA_2 value of 8.84, derived by Schild analysis. The slope of the regression was not significantly different ($P < 0.05$) from unity.

Anatomical localization

Autoradiography revealed that [^{125}I]-PD164333 bound predominantly to the vasculature in all sections of human tissue examined (Figure 4). In the brain (Figure 4a), high densities were localized to the pial arteries lying within the sulcus of the

Table 2 Competition binding experiments: inhibition of 0.1 nM [^{125}I]-ET-1 by unlabelled PD164333 and ET_A selective ligands competing for 0.2 nM [^{125}I]-PD164333 binding to human left ventricle

Tissue	n	$K_D ET_A$ (nM)	$K_D ET_B$ (μM)
[^{125}I]-ET-1 PD-164333	3	0.99 $\pm$ 0.32	2.41 $\pm$ 0.22
[^{125}I]-PD164333 ET_A selective:			
FR139317	3	3.53 $\pm$ 0.69	–
BQ123	3	3.93 $\pm$ 0.18	–

Values are the mean $\pm$ s.e.mean of three individuals. ET_B selective ligands did not inhibit [^{125}I]-PD164333 binding.

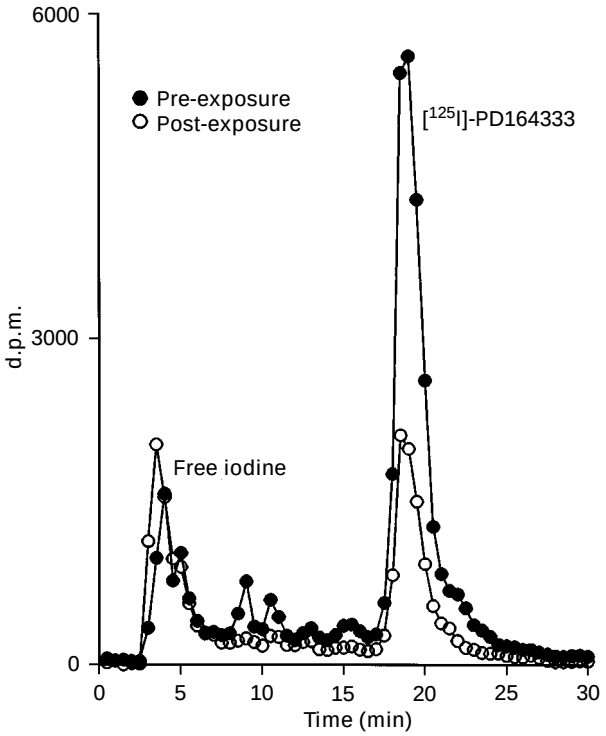

Figure 3 An example of the radiochemical profile obtained by reverse-phase high performance liquid chromatography for [^{125}I]-PD164333 following incubation for 2 h at room temperature, under the assay conditions described in the Methods section in the absence (pre-exposure) or presence (post-exposure) of ventricular tissue. A major peak of radioactivity eluted at the expected position for [^{125}I]-PD164333, which was reduced following exposure to tissue, representing receptor-bound ligand. A smaller peak was detected at the position corresponding to free iodine; this was unaltered by exposure to tissue. Chromatographic analyses were carried out on three separate tissues.

cortex and to the numerous smaller intercerebral resistance vessels penetrating the grey and white matter. In peripheral tissue, a similar pattern was observed in all tissues examined, with high densities of binding localized to larger arteries as well as smaller vessels of a size (diameter < 150 μm) likely to contribute to resistance. Binding was visualized to intramyocardial vessels in the heart (Figure 4C), arcuate arteries from the kidney (Figure 4E) as well as smaller intrarenal vessels. High densities were localized to the arteries surrounding the adrenal, to resistance vessels of the capsular plexus supplying the cortex and medulla (Figure 4G) and to pulmonary arteries of the lung (Figure 4I). Lower levels of binding were also detected to other cell types: myocytes in the ventricle, secretory cells of the zona glomerulosa in the adrenal, to bronchial epithelial cells and lung parenchyma. In epicardial coronary arteries containing advanced atherosclerotic lesions (Figure 5a), [^{125}I]-PD164333 binding was mainly localized to the medial layer, with an average density of 97.8 amol mm^{-1} (n = three sections from three individuals $\pm$s.e.mean) measured by quantitative autoradiography, but with little binding detectable on the intimal layer. There was no significant difference ($P = 0.5$) in the density of receptors measured in the intimal layer from normal coronary arteries (91.3 amol mm^{-1}) processed at the same time. Similarly, in saphenous veins used for bypass grafting and retrieved 2–8 years later following heart transplantation, highest binding was present in the vascular smooth muscle cells of the medial layer 38.0 $\pm$4.5 amol mm^{-1} ($n = 3$ individual $\pm$ s.e.mean), with little or no detectable binding present on the proliferated smooth muscle of the occlusive lesion (Figure 5c).

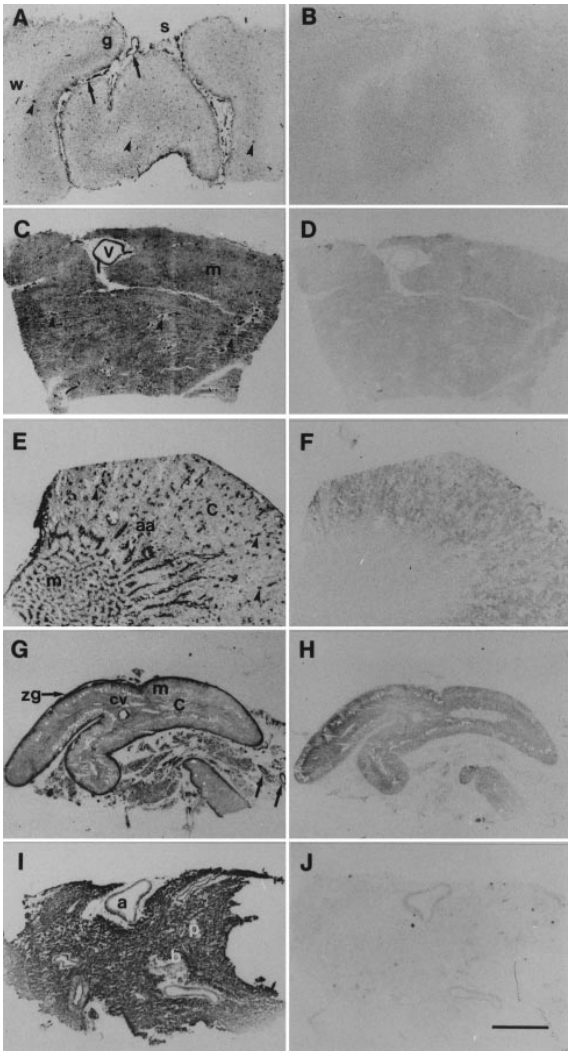

Figure 4 Bright-field autoradiographical images showing examples of the distribution of [^{125}I]-PD164333 binding to sections of normal human tissue. (A) Cerebral cortex showing high densities of binding to pial arteries (arrows) lying within the sulcus (s) and intercerebral vessels (arrowheads) within the grey (g) and white matter(w). Low densities of [^{125}I]-PD164333 binding were also visualized within the grey matter. (C) Left ventricle of the heart showing discrete binding to intramyocardial vessels of a size likely to contribute to resistance (arrowheads) and vascular smooth muscle of an artery (v). Lower densities were detected on myocytes (m). (E) In the kidney, binding was localized to the intrarenal vessels of the cortex (c) and medulla (m), including the arcuate arteries(aa) and adjacent veins. (G) High levels of binding were localized to the arteries surrounding the adrenal (arrows) shown in transverse section, the central vein (cv) and to secretory cells of the zona glomerulosa (zg) of the medulla (m) but not within the cortex (c). (I) Binding was present in the parenchyma (p) of the lung, smooth muscle of the arteries (a) and to the bronchus (b). (B,D,F,H, and J) Non-specific binding in adjacent control sections defined by incubation of the ligand in the presence of 1 μM unlabelled PD164333. Scale bar = 5 mm.

Discussion

This study has shown that [^{125}I]-PD164333 is a specific, high affinity, reversible non-peptide radioligand for human ET_A receptors. The radioligand bound with a single affinity to tissue expressing both subtypes where either the ET_A (aorta or heart) or ET_B subtype (kidney) predominated, since Hill slopes were close to unity and a one site fit was preferred. Binding affinities were within the same range ($K_D = 0.75$–1.07 nM) measured in these three tissues for the iodinated analogue of the endogenous ligand, [^{125}I]-ET-1 (Molenaar *et al*., 1993; Bacon & Davenport, 1996) or the ET_A selective peptide antagonist, [^{125}I]-PD151242 $K_D = 0.33$–0.97 nM (Davenport *et al*., 1994a,b; Peter & Davenport, 1995; Davenport *et al*., 1995a). B_{max} values measured with the new ligand (in fmol mg^{-1} protein) were within a similar range of densities for ET_A receptors previously described: left ventricle 34.2 versus 29.7 (Peter & Davenport, 1995), aorta 8.8 versus 9.4 (Davenport *et al*., 1995b) and kidney 125.3 versus 48.4 (Davenport *et al*., 1994b). Although both subtypes are present in the heart, at the concentration used in kinetic experiments, [^{125}I]-PD164333 was calculated to label only the ET_A receptors and the results represent kinetic constants for this subtype. In agreement with the saturation studies, these data showed that the radioligand bound to a single site, reaching equilibrium within 50 min at 23°C. Binding was also reversible, with the label dissociating from a single site.

Unlabelled PD164333 competed for [^{125}I]-ET-1 with high and low affinity corresponding to interaction with ET_A and ET_B subtypes. This compound was a competitive antagonist causing a parallel and rightward shift of the endothelin-1 concentration-response curve. All endothelin peptide agonists and antagonists competed in a manner consistent with [^{125}I]-PD164333 retaining this high selectivity and affinity for the human ET_A receptor. Both ET_A selective BQ123 and FR139317 competed for the binding monophasically, with no evidence for additional sites which might have suggested the presence of further subtypes for the ET_A receptor. Mutational analysis of this subtype has indicated that the pyrimidinylbenzenesulphonamide, bosentan and the indancarboxlic acid, SB-209670, have a similar binding domain (Webb *et al*., 1996). Both compounds inhibited [^{125}I]-PD164333 binding, suggesting that all three non-peptide antagonists interact with a similar region.

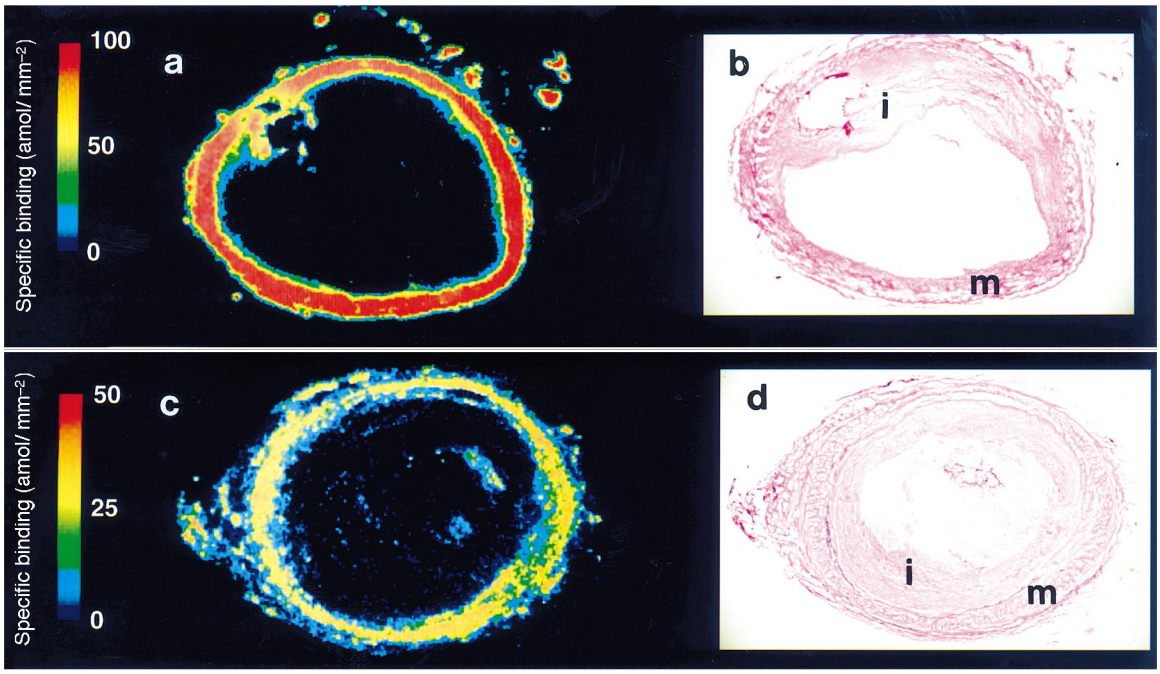

Figure 5 Colour-coded images showing the distribution of [^{125}I]-PD164333 binding in diseased vessels. Traverse sections of either epicardial coronary arteries containing an advanced atherosclerotic lesion (a) or an occluded saphenous vein used as a coronary artery bypass graft (c) were incubated with [^{125}I]-PD164333 and the resulting autoradiograms colour-coded to show the density of ET_A receptors. In both vessels, binding was mainly confined to the medial layer (m) with little or no binding to the intimal thickening (i) within the narrowed lumen of the coronary artery or occluded saphenous vein as shown by the sections stained with haematoxylin and eosin (b and d). The calcified core of the eccentric plaque adheres weakly to the slide and has been partly lost during tissue processing (b).

Selectivity for the ET_A receptor was confirmed by [^{125}I]-PD164333 binding to large conduit and smaller resistance vessels in a range of tissues that have previously been shown to express mainly or exclusively this subtype by molecular, ligand binding and functional assays (Davenport *et al*., 1994a,b; 1995a,b; Opgaard *et al*., 1996). Similarly, [^{125}I]-PD164333 only bound to ET_A receptors of the secretory cells of the zona glomerulosa and not to other regions of the adrenal that express the ET_B subtype (Davenport *et al*., 1996).

In diseased vessels, autoradiography allows the distinction between the medial layer of vessels (where the smooth muscle cells are expected to be predominantly of the contractile phenotype), and the intimal layer, comprising non-contractile proliferated smooth muscle. The latter is an important feature of atherosclerosis and a characteristic response of human saphenous vein to implantation as an arterial bypass graft. ET-1 is a potent vasoconstrictor of both epicardial coronary arteries (Franco-Cereceda, 1989; Godfraind, 1993; Opgaard *et al*., 1994; Bax *et al*., 1994) and saphenous veins (Costello *et al*., 1990; Seo *et al*., 1994). This peptide has been suggested as a factor contributing to the progression of atherosclerosis (Bacon *et al*., 1996) by promoting proliferation and contributing to vasospasm leading to plaque rupture. Whilst ET_A receptors were localized to the smooth muscle cell of the thinned media in atherosclerotic coronary arteries, little binding of [^{125}I]-PD164333 was detected within the intimal layer, in agreement with the lack of expression of ET_A mRNA in the thickened arterial intima of hypertensive patients (Hasegawa *et al*., 1994). We have previously shown that ET_B receptors cannot be detected either, indicating no switch to this subtype by the proliferated cells (Bacon *et al*., 1996) and non-peptide ET_A selective antagonists fully reverse ET-1-induced vasoconstriction (Maguire *et al*., 1997). The mediators of intimal thickening in saphenous veins may be different to those in native arterial disease.

However, in saphenous vein grafts, ET_A receptors were also difficult to detect compared with the media. Kanse *et al*., (1995) have shown that the mitogenic effect of ET-1 is mediated via the ET_A subtype, which may be involved in the initial proliferative response. At present it is unclear whether progression of smooth muscle cells through successive divisions is accompanied by down-regulation of ET_A receptors or whether absence reflects the undifferentiated nature of the smooth muscle cells.

Our results show [^{125}I]-PD164333 is a specific, high affinity, reversible non-peptide radioligand for human ET_A receptors. These results provide further evidence that the non-peptide antagonists, such as the butenolides, can bind to constrictor ET_A receptors in target vessels such as coronary arteries, and may be used to prevent vasospasm and the events underlying plaque rupture in coronary artery disease. The use of [^{125}I]-PD164333 will facilitate testing the intriguing hypothesis that antagonism of ET_A receptors may also inhibit the proliferative response of smooth muscle.

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References

ADNER, M., JANSEN, I. & EDVINSSON, L. (1994). Endothelin-A receptors mediate contraction in human cerebral, meningeal and temporal arteries. *J. Auton. Nerv. Syst.,* **49,** S117–121.

BACON, C.R., CARY, N.R.B. & DAVENPORT, A.P. (1996). Endothelin peptide and receptors in human atherosclerotic coronary artery and aorta. *Circ. Res.,* **79,** 794–801.

BACON, C.R. & DAVENPORT, A.P. (1996). Endothelin receptors in human coronary artery and aorta. *Br. J. Pharmacol.,* **117,** 986–992.

BAX, W.A., AGHAI, Z., VAN-TRICHT-C.L., WASSENAAR, C. & SAXENA, P.R. (1994). Different endothelin receptors involved in endothelin-1- and sarafotoxin S6B-induced contractions of the human isolated coronary artery. *Br. J. Pharmacol.,* **113,** 1471–1479.

BUCHAN, K.W., MAGNUSSON, H., RABE K.F., SUMNER M.J. & WATTS I.S. (1994). Characterisation of the endothelin receptor mediating contraction of human pulmonary artery using BQ123 and Ro 46–2005. *Eur. J. Pharmacol.,* **260,** 221–226.

CLOZEL, M., BREU, V., GRAY, G.A., KALINA, B., LOFFLER,B-M., BURRI, K., CASSAL, J-M., HIRTH, G., M., MULLER, M., NEIDHART, N. & RAMUZ, H. (1994). Pharmacological characterization of bosentan, a new potent orally active nonpeptide endothelin receptor antagonist. *J. Pharmacol. Exp. Ther.,* **270,** 228–235.

COSTELLO, K.B., STEWART, D.J. & BAFFOUR, R. (1990). Endothelin is a potent constrictor of human vessels used in coronary revascularization surgery. *Eur. J. Pharmacol.,* **186,** 311–314.

DAVENPORT, A.P. (1997). Distribution of endothelin receptors. In *Endothelins in Biology and Medicine*. ed. Miller, R., Pelton, J.T. & Huggins, J. pp.45–68. Florida: CRC Press Inc.

DAVENPORT, A.P., HOSKINS, S.L., KUC, R.E. & PLUMPTON, C. (1996). Differential distribution of endothelin peptides and receptors in human adrenal gland. *Histochem. J.,* **28,** 779–789.

DAVENPORT, A.P., KUC, R.E., FITZGERALD, F., MAGUIRE, J.J., BERRYMAN, K. & DOHERTY, A.M. (1994a). [^{125}I]-PD151242: a selective radioligand for human ET_A receptors. *Br. J. Pharmacol.,* **111,** 4–6.

DAVENPORT A.P., KUC, R.E., HOSKINS, S.L. & KARET, F.E. (1994b).[^{125}I]-PD151242: a selective ligand for endothelin ET_A receptors in human kidney which localises to renal vasculature. *Br. J. Pharmacol.,* **113,** 1303–1310.

DAVENPORT, A.P., KUC, R.E., MAGUIRE, J.J. & HARLAND, S.P. (1995a). ET_A receptors predominate in the human vasculature and mediate constriction. *J. Cardiovasc. Pharmacol.,* **26,** S265–267.

DAVENPORT, A.P., O'REILLY G. & KUC, R.E. (1995b). 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.

DAVENPORT, A.P., O'REILLY, G., MOLENAAR, P., MAGUIRE, J.J., KUC, R.E., SHARKEY, A., BACON, C.R. & FERRO, A. (1993). Human endothelin receptors characterised using reverse transcriptase-polymerase chain reaction, *in situ* hybridization and sub-type selective ligands BQ123 and BQ3020: evidence for expression of ET_B receptors in human vascular smooth muscle. *J. Cardiovasc. Pharmacol.,* **22,** S22–25.

DOHERTY, A.M., PATT, W.C., EDMUNDS, J.J., BERRYMAN, K.A., REISDORPH, B.R., PLUMMER, M.S., SHAHRIPOUR, A, LEE, C., CHENG, X.M., WALKER, D.M., HALEEN, S., KEISER, J., FLYNN, M., WELCH, K., HALLAK, H., TAYLOR, D. & REYNOLDS, E. (1995). Discovery of a novel series of orally active non-peptide endothelin-A (ETA) receptor-selective antagonists. *J. Med. Chem.,* **38,** 1259–1263.

ELLIOTT, J.D., LAGO, M.A., COUSINS, R.D., GAO, A., LEBER, J.D., ERHARD, K.F., NAMBI, P., ELSHOURBAGY, N.A., KUMAR, C., LEE, J.A., BEAN, J.W., DEBROSSE, C.W., EGGLESTON, D.S., BROOKS, D.P., FEUERSTEIN, G., RUFFOLO, R.R., WEINSTOCK, J., GLEASON, J.G., PEISHOFF, C.E. & OHLSTEIN, E.H. (1994). 1,3-Diarylindan-2-carboxylic acids, potent and selective non-peptide endothelin receptor antagonists. *J. Med. Chem.,* **37,** 1553–1557.

FRANCO-CERECEDA, A. (1989). Endothelin- and neuropeptide Y-induced vasoconstriction of human epicardial coronary arteries *in vitro*. *Br. J. Pharmacol.,* **97,** 968–972.

FUKURODA, T., KOBAYASHI, M., OZAKI, S., YANO, M., MIYAUCHI, T., ONIZUKA, M., SUGISHITA, Y., GOTO, K. & NISHIKIBE, M. (1994). Endothelin receptor subtypes in human versus rabbit pulmonary arteries. *J. Appl. Physiol.,* **76,** 1976–1982.

GODFRAIND, T. (1993). Evidence for heterogeneity of endothelin receptor distribution in human coronary artery. *Br. J. Pharmacol.,* **110,** 1201–1205.

HASEGAWA, K., FUJIWARA, H., DOYAMA, K., INADA, T., OHTANI, S., FUJIWARA, T,. HOSODA, K., NAKAO, K. & SASAYAMA, S. (1994). Endothelin-1-selective receptor in the arterial intima of patients with hypertension. *Hypertension,* **23,** 288–293.

HAYNES, W.G., STRACHAN, F.E. & WEBB, D.J. (1995). Endothelin ETA and ETB receptors cause vasoconstriction of human resistance and capacitance vessels *in vivo*. *Circulation,* **92,** 357–363.

KANSE, S.M., WIJELATH, E., KANTHOU, C., NEWMAN, P. & KAKKAR, V.V. (1995). The proliferative responsiveness of human vascular smooth muscle cells to endothelin correlates with endothelin receptor density. *Lab. Invest.,* **72,** 376–382.

KEISER, J., SCHROEDER, R.L., HALLAK, H., UPRICHARD, A.C.G., DOHERTY, A.M. & HALEEN, S.J. (1997). Pharmacodynamics of PD156707, a selective endothelin receptor-A (ET_A) antagonists in acute hypoxic pulmonary hypertension (PH). *FASEB J.,* **11,** A36 (Abstract).

KHIMENKO, P.L., MOORE, T.M. & TAYLOR, A.E. (1996). Blocked ETA receptors prevent ischemia and reperfusion injury in rat lungs. *J. Appl. Physiol.,* **80,** 203–207.

LIU, J.J., CASLEY, D., WOJTA, J., GALLICCHIO, M., DAUER, R., JOHNSTON, C.I. & BUXTON, B.F. (1994). Effects of calcium- and ETA-receptor antagonists on endothelin-induced vasoconstriction and levels of endothelin in the human internal mammary artery. *Clin. Exp. Pharmacol. Physiol.,* **21,** 49–57.

MAGUIRE, J.J. & DAVENPORT, A.P. (1994). ET_A receptors mediate the constrictor responses to endothelin peptides in human blood vessels *in vitro*. *Br. J. Pharmacol.,* **115,** 191–197.

MAGUIRE, J.J., KUC, R.E. & DAVENPORT, A.P. (1997). Affinity and selectivity of PD156707, a novel nonpeptide endothelin antagonist, for human ET(A) and ET(B) receptors. *J. Pharmacol. Exp. Ther.,* **280,** 1102–1108.

MAGUIRE, J.J., KUC, R.E., O'REILLY, G. & DAVENPORT, A.P. (1994). Vasoconstrictor endothelin receptors characterised in human renal artery and vein *in vitro*. *Br. J. Pharmacol.,* **113,** 49–54.

MOLENAAR, P., O'REILLY, G., SHARKEY, A., KUC, R.E., HARDING, D.P., PLUMPTON, P., GRESHAM, G.A. & DAVENPORT, A.P. (1993). Characterization and localization of endothelin receptor sub-types in the human atrioventricular conducting system and myocardium. *Circ. Res.,* **72,** 526–538.

MUNSON, P.J. RODBARD, D. (1980). Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.,* **107,** 220–239.

MCPHERSON, G.A. (1985). Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. *J. Pharmacol. Methods,* **14,** 213–228.

OPGAARD, O.S., ADNER, M., GULBENKIAN, S. & EDVINSSON, L. (1994). Localization of endothelin immunoreactivity and demonstration of constrictory endothelin-A receptors in human coronary arteries and veins. *J. Cardiovasc. Pharmacol.,* **23,** 576–583.

OPGAARD, O.S., CANTERA, L., ADNER, M. & EDVINSSON, L. (1996). Endothelin-A and -B receptors in human coronary arteries and veins. *Regul. Pept.,* **63,** 149–156.

PATEL, T.R., GALBRAITH, S., GRAHAM, D.I., HALLAK, H., DOHERTY, A.M. & MCCULLOCH, J. (1966). Endothelin receptor antagonist increases cerebral perfusion and reduces ischaemic damage in feline focal cerebral ischaemia. *J. Cereb. Blood Flow Metab.,* **16,** 950–958.

PATT, W.C., EDMUNDS, J.J., REPINE, J.T., BERRYMAN, K.A., REISDORPH, B.R., LEE, C., PLUMMER, M.S., SHAHRIPOUR, A., HALEEN, S., KEISER, J., FLYNN, M., WELCH, K., REYNOLDS, E.E., RUBIN, R., TOBIAS, B., HALLAK, H., & DOHERTY, A.M. (1997). Structure activity relationships in a series of orally active-hydroxy gamma-butenolide endothelin antagonists. *J. Med. Chem.,* **40,** 1063–1074.

PETER, M.G. & DAVENPORT, A.P. (1995). Selectivity of [^{125}I]-PD151242 for the human, rat and porcine endothelin ET_A receptors in the heart. *Br. J. Pharmacol.,* **114,** 297–302.

REYNOLDS, E.E., KEISER, J.A., HALEEN, S.J., WALKER D.M., OLSZEWSKI B., SCHROEDER R.L., TAYLOR D.G., HWANG O., WELCH, K.M. FLYNN, M.A., THOMPSON, D.M., EDMUNDS, J.J., BERRYMAN, K.A., LEE, C., REISDORPH, B.R., CHENG, X.M., PATT, W.C. '& DOHERTY, A.M. (1995). Pharmacological characterization of PD 156707, an orally active ETA receptor antagonist. *J. Pharmacol. Exp. Ther.,* **273,** 1410 – 1417.

RIEZEBOS, J., WATTS, I.S. & VALLANCE, P.J.T. (1994). Endothelin receptors mediating functional responses in human small arteries and veins. *Br. J. Pharmacol.,* **111,** 609 – 615.

SEO, B., OEMAR, B.S., SIEBENMANN, R., VON, SEGESSER, L. & LUSCHER, T.F. (1994). Both ET(A) and ET(B) receptors mediate contraction to endothelin-1 in human blood vessels. *Circulation,* **89,** 1203 – 1208.

WEBB, M.L., PATEL, P.S., ROSE, P.M., LIU, E.C., STEIN, P.D., BARRISH, J., LACH, D.A., STOUCH, T., FISHER, S.M., HADJI-LAMBRIS, O. LEE, H., SKWISH, S., DICKINSON, K.E. & KRYSTEK, S.R. (1996). Mutational analysis of the endothelin type A receptor (ETA): interactions and model of selective ETA antagonist BMS-182874 with putative ETA receptor binding cavity. *Biochemistry,* **35,** 2548 – 2556.

WHITE, D.G., GARRATT, H., MUNDIN, J.W., SUMNER, M.J., VALLANCE, P.J. & WATTS, I.S. (1994). Human saphenous vein contains both endothelin ET(A) and ET(B) contractile receptors. *Eur. J. Pharmacol.,* **257,** 307 – 310.

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