Biochemical Pharmacology, Vol. 55, pp. 697–701, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(97)00515-7

SHORT COMMUNICATION

In Vitro Enzymatic Processing of Radiolabelled Big ET-1 in Human Kidney

Fraser D. Russell, Alexander L. Coppell and Anthony P. Davenport

CLINICAL PHARMACOLOGY UNIT, UNIVERSITY OF CAMBRIDGE, ADDENBROOKE'S HOSPITAL, CAMBRIDGE CB2 2QQ, U.K.

ABSTRACT. We have investigated enzymatic processing of big ET-1 in sections of human renal cortex by examining selected binding characteristics of the radiolabelled precursor and cleaved peptide. Sections of histologically normal human kidney obtained from patients undergoing nephrectomy for hypernephroma (50–74 years, $N = 10$, male or female) were incubated with 0.1 nM [^{125}I]-ET-1, [^{125}I]-Tyr^{13} big ET-1 or [^{125}I]-Tyr^{31} big ET-1 in culture media at 37° to facilitate enzymatic activity. Specific binding measured from sections incubated with [^{125}I]-Try^{13} big ET-1 (which would yield [^{125}I]-ET-1 on enzymatic cleavage) was 39.7 ± 2.5%. This was significantly reduced to 19.0 ± 2.0% following co-incubation with 10 μM thiorphan, an inhibitor of neutral endopeptidase (NEP) but not the putative endothelin converting enzymes (ECE). No further reduction in specific binding was obtained with 100 μM thiorphan, indicating that this is a maximal effect. However phosphoramidon (100 μM), an inhibitor of ECE and NEP, almost abolished specific binding, indicating that both NEP and ECE cleave big ET-1 in the kidney. No specific binding was detected when sections were labelled with [^{125}I]-Tyr^{31} big ET-1 (which would be expected to yield [^{125}I] labelled C-terminal fragment). Binding of the product of processed [^{125}I]-Tyr^{13} big ET-1 was inhibited mainly by the ET_B selective antagonist (BQ788 = 75.1 ± 2.1% inhibition; FR139317 = 9.7 ± 7.3% inhibition), consistent with the predominance of this subtype in human kidney. We conclude that big ET-1 is processed by NEP and ECE in human kidney and that the cleaved product binds predominantly to the ET_B receptor subtype. ECE may be a therapeutic target in the attenuation of renal diseases in which ET-1 has been implicated. BIOCHEM PHARMACOL **55**;5:697–701, 1998.

KEY WORDS. endothelin; endothelin converting enzyme; human kidney

Endothelin (ET-1)† is a potent 21 amino acid vasoconstrictor peptide that is generated following proteolytic cleavage of its immediate precursor, big ET-1 by the putative endothelin converting enzymes (ECE-1 and ECE-2). Molecular studies have predicted that two isoforms of ECE-1 exist that differ only in their N-terminal sequence; ECE-1α and ECE-1β [1], also called ECE-1b and ECE-1a, respectively [2]. Neutral endopeptidase 24.11 (NEP) can also cleave big ET-1, but efficiently degrades ET-1 to produce small peptide fragments [3, 4]. ET receptors have low affinity for other peptide fragments, including ET-1 (1–20) [5], indicating a requirement for specific cleavage between the Trp^{21}-Val^{22} bond of big ET-1 for biological activity.

ET-1 regulates fluid and electrolyte levels in the kidney by activation of ET receptors located in tubules, collecting ducts and blood vessels [6]. NEP is highly expressed in the kidney and biochemical evidence in renal tubular epithelial cell lines suggests that this tissue may also express ECE [7]. Infusion of big ET-1 in humans produces a marked increase in the formation of ET-1 in the kidney [8, 9], indicating the importance of this tissue in the processing of the precursor.

The aim of this study was to exploit the requirement for the precise synthesis of the 21 amino acid peptide, ET-1 to characterise enzymatic processing of big ET-1. Human big ET-1 (1–38) contains two residues that are suitable for radio-iodination; Tyr^{13} and Tyr^{31}. Cleavage of [^{125}I]-Tyr^{13} big ET-1 at the Trp^{21}-Val^{22} bond will produce [^{125}I]-ET-1. ET receptors have high affinity for [^{125}I]-ET-1 and so binding of the radioligand can be used as a measure of ECE activity. In contrast, conversion of [^{125}I]-Tyr^{31} big ET-1 will produce an inactive radiolabelled C-terminal fragment.

MATERIALS AND METHODS

Materials

[^{125}I]-ET-1 (2200 Ci/mmol), [^{125}I]-Tyr^{13} big ET-1 (2200 Ci/mmol) and [^{125}I]-Tyr^{31} big ET-1 (2200 Ci/mmol) were from Amersham International Plc. FR139317 ((*N*-[(hexahydro-1-azepinyl)carbonyl]L-Leu-(1-Me)D-Trp-3-(2-pyridyl)D-Ala) and BQ788 (*N-cis*-2,6-dimethyl piperidinocarbonyl L-γ-MeLeu-D-Trp(COOCH$_3$)-D-Nle) were sup-

* Corresponding author: Fraser D. Russell, Clinical Pharmacology Unit, University of Cambridge, Level 2, F&G Block, Box 110, Addenbrooke's Hospital, Cambridge CB2 2QQ U.K. Tel. 44 (01223) 216894; FAX: 44 (01223) 216893; E-mail: FDR1001@MEDSCHL.CAM.AC.UK.

† *Abbreviations*: ECE, endothelin converting enzyme; ET, endothelin; MDCK, Madin–Darby canine kidney; NEP, neutral endopeptidase 24.11.

Received 2 June 1997; accepted 3 September 1997.

plied by Dr. A. M. Doherty, Parke–Davis Pharmaceutical Research Division. ET-1 and big ET-1 (human 1-38) were from Peptide Institute Inc. SB209670 [(+)-(1S,2R,3S)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methylenedioxy-phenyl)-5-(prop-1-yloxy)indane-2-carboxylic acid] was from Glaxo-Wellcome. Synthetic culture media, M199 was obtained from Life Technologies.

Tissue Collection

Histologically normal areas of human renal tissue were obtained from patients with blood pressure <160/100 undergoing nephrectomy for hypernephroma at Addenbrooke's Hospital, Cambridge, U.K. (50–74 years, $N = 10$, male or female). Tissues were immersed in liquid N_2 within 10 min of excision and stored at −70°. Tissue sections were cut on a cryostat (Bright) at −22°.

Enzymatic Activity in Kidney Sections

Sections of human renal cortex (10 μm) were preincubated with HEPES buffer (HEPES 50 mM, $MgCl_2$ 5 mM, BSA (fraction V) 0.3% (w/v); pH 7.4) for 15 min at 23° ($N = 10$). Sections were incubated with [^{125}I]-ET-1, [^{125}I]-Tyr^{13} big ET-1 or [^{125}I]-Tyr^{31} big ET-1 (0.1 nM), in M199 culture media (2 hr, 37°) that has previously been shown to facilitate ECE activity [10]. Neutral endopeptidase 24.11 (NEP) activity was inhibited by co-incubation of sections with thiorphan (10 or 100 μM) while the activity of both ECE and NEP was inhibited by co-incubation of sections with phosphoramidon (100 μM). Sections were washed in Tris buffer (Tris 0.05 M, pH 7.4 at 4°, 3 × 5 min) wiped from the slides and counted.

The receptor subtype(s) to which processed [^{125}I]-Tyr^{13} big ET-1 binds was determined by co-incubation of sections with ligands that have selectivity for either ET_A (FR139317, 0.1 μM) or ET_B receptors (BQ788, 0.1 μM) and that are predicted to inhibit >99% of ET_A and <1% of ET_B receptors and 91% of ET_B and 9% of ET_A receptors, respectively [11, 12].

Competition Binding Experiments

Renal sections were preincubated with HEPES buffer (15 min, 23°) and then labelled with [^{125}I]-ET-1 (0.1–0.15 nM, 2 hr, 23°) in the presence of increasing concentrations of big ET-1 (10 pM–10 μM) and 100 μM phosphoramidon to minimise enzymatic processing ($N = 3$). Affinity (K_D) of receptors for non-processed big ET-1 was determined using EBDA and LIGAND software [13, 14], in the KELL suite of programmes (Biosoft). Binding to one or more populations of sites was determined using the *pseudo* Hill coefficient and the *F* test [14]. Specific binding was expressed as a percentage of total binding (mean ± SEM) and significance, defined by a *P* value less than 0.05, was determined using the Mann–Whitney *U* test.

Immunocytochemistry

Renal sections (30 μm) were fixed in acetone (10 min, 4°) and blocked with swine serum (5%) in phosphate buffer solution (PBS; Na_2HPO_4, 0.1 M; NaCl, 0.16 M; pH 7.4) (30 min, 23°) ($N = 5$). The sections were incubated with antisera raised against a portion of the N-terminus of the ECE-1α sequence or with preimmune serum (1:100 fold dilution, 18 hr, 4°). The sections were washed (3 × 5 min, 4°) and incubated with swine anti-rabbit serum (1:200, 1 hr, 23°). After incubation of sections with rabbit peroxidase anti-peroxidase (1:400, 1 hr, 23°) sections were washed (3 × 5 min, 4°) and exposed to a diaminobenzidine solution (pH 7.4) for 5 min at 23°.

RESULTS AND DISCUSSION

We investigated enzymatic conversion of big ET-1 to the active 21 amino acid peptide, ET-1 in sections of human kidney. Processing of [^{125}I]-Tyr^{13} big ET-1 at the Trp^{21}-Val^{22} bond will generate radiolabelled mature peptide that has affinity for ET receptors and will be detected by ligand binding and an unlabelled C-terminal fragment. Indeed, sections labelled with [^{125}I]-Tyr^{13} big ET-1 produced 40% specific binding (expressed as a percentage of total binding). Co-incubation of [^{125}I]-Tyr^{13} big ET-1 with the NEP inhibitor, thiorphan (10 μM), caused a significant reduction ($P = 0.001$) in the level of specific binding indicating that NEP may, in part, convert big ET-1 (Fig. 1A). No further reduction in specific binding was obtained with 100 μM thiorphan (Fig. 2), indicating that this is a maximal effect of the inhibitor. However phosphoramidon (100 μM), an inhibitor of NEP and ECE (IC_{50} = 0.4 and 9.1 μM, respectively, Fig. 2), almost abolished specific binding (Fig. 1 and 2), indicating that both NEP and ECE cleave big ET-1 in the kidney.

A high concentration of unlabelled ET-1 (6,000-fold higher than the K_D) was used in binding experiments to determine non-specific binding. However, ET-1 is a substrate for NEP [3] and so we examined the possibility that ET receptor inhibition was reduced by degradation of the competing ligand. We used SB209670, a non-peptide antagonist [15] that is not a substrate for NEP as an independent measure of ET receptor inhibition. Comparable results were obtained with the two competing ligands (not shown) suggesting that receptor inhibition was not reduced by NEP activity.

Cleavage of [^{125}I]-Tyr^{31} big ET-1 would be expected to produce radiolabelled C-terminal fragment and unlabelled mature ET-1. ET receptors have low affinity for the C-terminal fragment, and so this peptide should not be detected under the conditions used in the ligand binding experiments. In agreement with this hypothesis, specific binding was not detected when [^{125}I]-Tyr^{31} big ET-1 was used (Fig. 1B).

To exclude the possibility that binding measured in our experiments is due to labelling of the unprocessed precur-

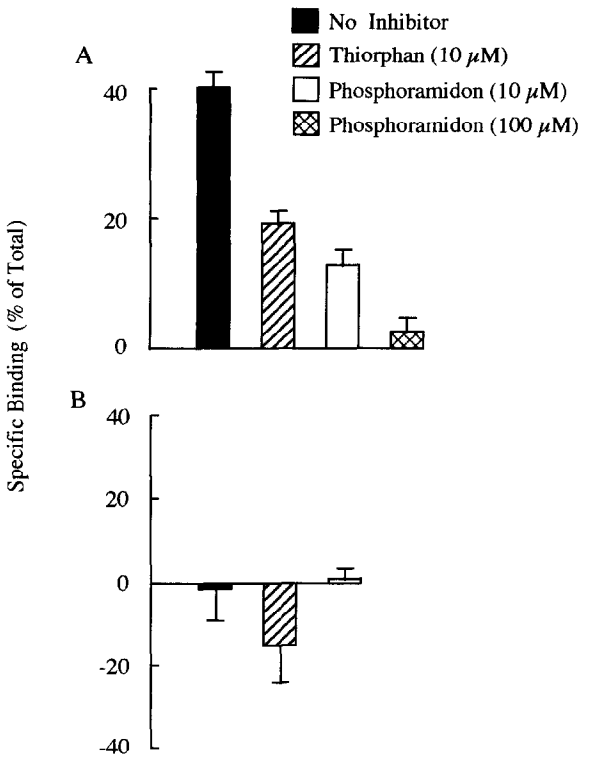

FIG. 1. Specific binding expressed as a percentage of total binding for the cleaved products of [^{125}I]-Tyr^{13} big ET-1 and [^{125}I]-Tyr^{31} big ET-1. Human renal cortical sections were incubated with 0.1 nM [^{125}I]-Tyr^{13} big ET-1 in the absence or in the presence of thiorphan (10 μM) or phosphoramidon (10 and 100 μM) (A). Conversion of [^{125}I]-Tyr^{13} big ET-1 to [^{125}I]-ET-1 was inhibited by thiorphan (P = 0.001, N = 5). Further inhibition was obtained using 100 μM phosphoramidon (P = 0.005, N = 5). No specific binding was detected when sections were labelled with 0.1 nM [^{125}I]-Tyr^{31} big ET-1, as expected (B, N = 7). Non-specific binding was determined in the presence of 1 μM ET-1. Values are expressed as mean ± SEM.

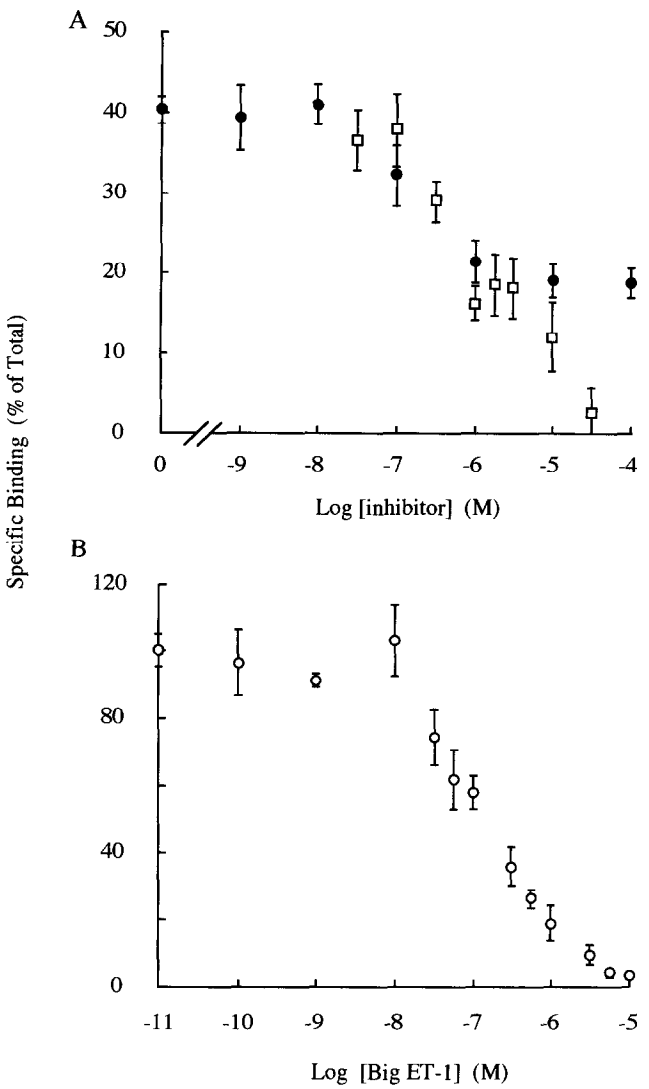

FIG. 2. Specific binding, expressed as a percentage of total binding, for the cleaved products of 0.1 nM [^{125}I]-Tyr^{13} big ET-1 when co-incubated with increasing concentrations of thiorphan (●, N = 5) or phosphoramidon (□, N = 4) (A). Thiorphan (10 μM) maximally inhibited NEP activity. B. Competition binding for human big ET-1 (1–38) against [^{125}I]-ET-1 (0.1–0.15 nM) in the presence of 100 μM phosphoramidon in sections of human renal cortex (N = 3). The binding curve was biphasic with high affinity ET_B receptors (K_D = 41.5 ± 18.3 nM) and low affinity ET_A receptors (K_D 0.73 ± 0.43 μM). The proportion of ET_B to ET_A receptors was 81% to 19%. All values are expressed as mean ± SEM.

sor, we examined the ability of big ET-1 to bind directly to ET receptors. Big ET-1 (10 pM–10 μM) competed against [^{125}I]-ET-1 in the presence of 100 μM phosphoramidon (used to minimise enzymatic activity by ECE and NEP) in a biphasic manner (K_{D1} = 41.5 ± 18.3 nM, N = 3 and K_{D2} = 0.73 ± 0.43 μM, N = 3) (Fig. 2B). The binding sites were expressed in a ratio of 81:19%. These findings are consistent with binding of big ET-1 to ET_B and ET_A receptors, respectively [16, 17]. Big ET-1 had 18 fold selectivity for ET_B over ET_A receptors and the affinity of ET_B and ET_A receptors for big ET-1 was 244 and 4,300 fold lower than for ET-1 (K_D = 0.17 nM) [16]. This indicates that ET receptors have low affinity for big ET-1 and that the proportion of receptors directly labelled by [^{125}I]-Tyr^{13} big ET-1 at the concentration used in the binding experiments (0.1 nM), is negligible (about 0.25% of ET_B and 0.02% of ET_A receptors, calculated using the mass action equation).

[^{125}I]-Tyr^{13} Big-ET-1 was co-incubated with either 0.1 μM BQ788 or 0.1 μM FR139317 to delineate the receptor subtype(s) to which the cleaved product of big ET-1 binds. Binding was predominantly inhibited by BQ788 (BQ788 = 75.1 ± 2.1% inhibition; FR139317 = 9.7 ± 7.3% inhibition; N = 5; mean ± SEM). This indicates that the product of processed [^{125}I]-Tyr^{13} big-ET-1 labels mainly ET_B receptors in the human renal cortex, consistent with the higher density and proportion of the ET_B subtype in this tissue [16].

Immunocytochemistry was used to determine the localisation of ECE in the human kidney. ECE-1α-like immu-

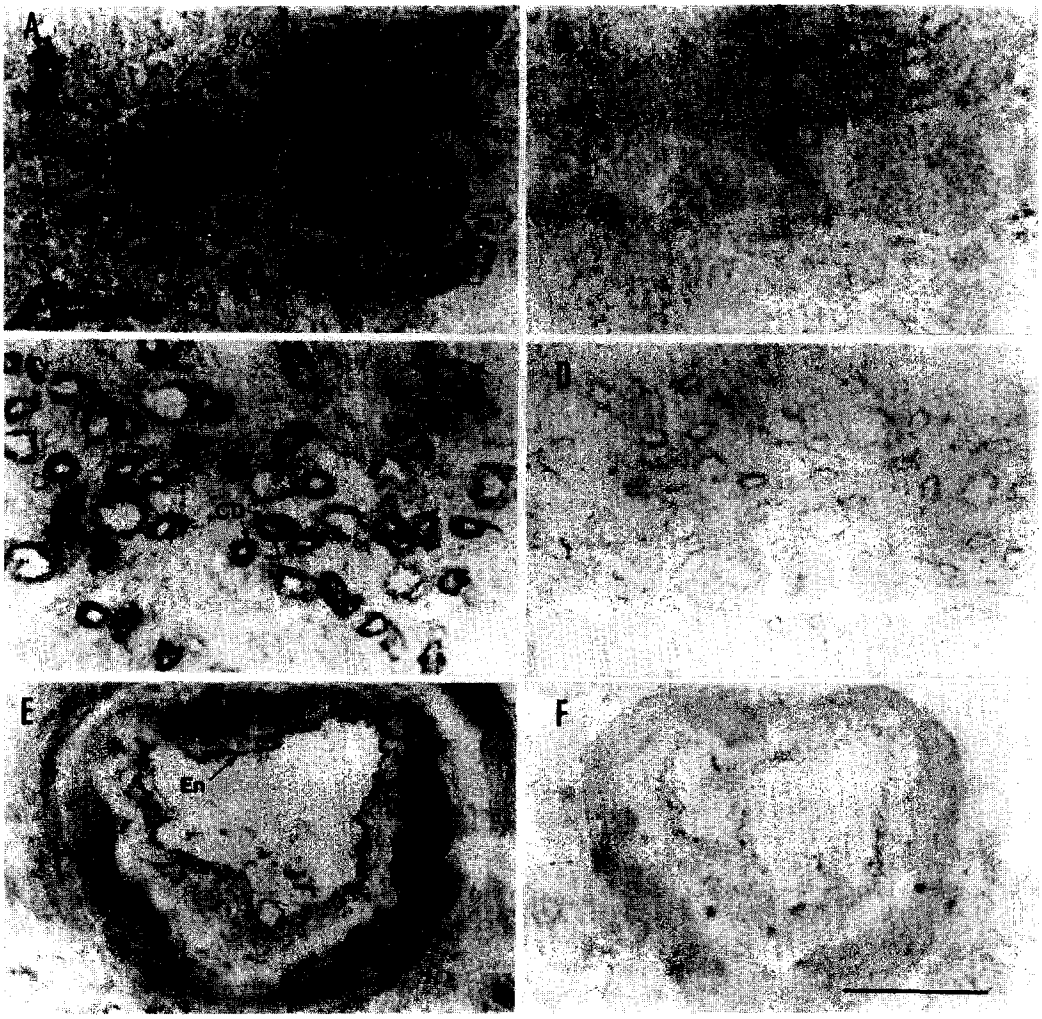

FIG. 3. Representative micrographs showing immunocytochemical localisation of antisera raised against a portion of the N-terminus of the ECE-1α sequence in sections of human kidney. Positive immunoreactivity was detected over: A. glomeruli (G) and distal convoluted tubules (DCT), C. collecting ducts (CD), and E. endothelial cells lining renal blood vessels (En). Adjacent sections incubated with preimmune serum showed negligible staining (B, D, F). Scale bar = 200 μm.

noreactive staining was detected over glomeruli, epithelial cells of the distal convoluted tubules and collecting ducts (Fig. 3). Our findings are consistent with the reported phosphoramidon-sensitive ECE activity determined in MDCK cells derived from distal tubules or collecting ducts [7]. Staining was also apparent over endothelial cells lining renal blood vessels. Infusion of big ET-1 in humans produces vasoconstriction with a decrease in renal blood flow and a concomitant increase in plasma ET levels [8, 9]. These findings suggest an important role of vascular endothelial cell ECE in the conversion of big ET-1 to the mature peptide.

ET-1 is thought to have a pathophysiological role in renal disorders such as ischaemic acute renal failure [18] and so ECE, located in renal epithelial cells and blood vessel endothelium, may be a therapeutic target in the attenuation of disease. Intravenous infusion of phosphoramidon suppresses tubular sodium wasting, and it was suggested that ECE blockade protects the tubular epithelium against ischaemic injury [19].

In conclusion, we have shown that big ET-1 is processed in the human kidney by both neutral endopeptidase and endothelin converting enzyme activity. The product of big ET-1 processing binds predominantly to the ET_B receptor subtype in this tissue. Big ET-1 also binds directly to ET receptors, but the affinity of the receptors for this precursor is lower than it is for ET-1. ECE-1α-like immunoreactivity was detected within tubule and collecting duct epithelial cells and blood vessel endothelium and so ECE may be involved in processing big ET-1 in these regions.

This work was supported by grants from the British Heart Foundation, Isaac Newton Trust, Royal Society and a Nuffied Foundation Undergraduate Research Bursary to A. L. Coppell. We thank Dr. Fiona Karet and the theatre staff at Addenbrooke's Hospital, Cambridge for collection of the renal tissue. Dr. F. D. Russell is a Glaxo-Wellcome Group Research Fellow.

References

1. Shimada K, Takahashi M and Tanzawa K, Cloning and functional expression of endothelin-converting enzyme from rat endothelial cells. *J Biol Chem* **269:** 18275–18278, 1994.
2. Valdenaire O, Rohrbacher E and Mattei M-G, Organization of the gene encoding the human endothelin-converting enzyme (ECE-1). *J Biol Chem* **270:** 29794–29798, 1995.
3. Murphy LJ, Corder R, Mallet AI and Turner AJ, Generation by the phosphoramidon-sensitive peptidases, endopeptidase-24.11 and thermolysin, of endothelin-1 and C-terminal fragment from big endothelin-1. *Br J Pharmacol* **113:** 137–142, 1994.
4. Turner AJ and Murphy LJ, Molecular pharmacology of endothelin converting enzymes. *Biochem Pharmacol* **51:** 91–102, 1996.
5. Huggins JP, Pelton JT and Miller RC, The structure and specificity of endothelin receptors: their importance in physiology and medicine. *Pharmacol Ther* **39:** 55–123, 1993.
6. Simonson MS, Endothelins: multifunctional renal peptides. *Physiol Rev* **73:** 375–411, 1993.
7. Takada J, Hata M, Okada K, Matsuyama K and Yano M, Biochemical properties of endothelin converting enzyme in renal epithelial cell lines. *Biochem Biophys Res Commun* **182:** 1383–1388, 1992.
8. Ahlborg G, Ottosson-Seeberger A, Hemsén A and Lundberg JM, Big ET-1 infusion in man causes renal ET-1 release, renal and splanchnic vasoconstriction, and increased mean arterial blood pressure. *Cardiovasc Res* **28:** 1559–1563, 1994.
9. Hemsén A, Ahlborg G, Ottosson-Seeberger A and Lundberg JM, Metabolism of big endothelin-1 (1–38) and (22–38) in the human circulation in relation to production of endothelin-1 (1–21). *Reg Peptides* **55:** 287–297, 1995.
10. Plumpton C, Kalinka S, Martin RC, Horton JK and Davenport AP, Effects of phosphoramidon and pepstatin A on the secretion of endothelin-1 and big endothelin-1 by human umbilical vein endothelial cells: measurement by two-site enzyme-linked immunosorbent assays. *Clin Sci* **87:** 245–251, 1994.
11. Russell FD and Davenport AP, Characterization of the binding of endothelin ET_B selective ligands in human and rat heart. *Br J Pharmacol* **119:** 631–636, 1996.
12. Russell FD, Skepper JN and Davenport AP, Detection of endothelin receptors in human coronary artery vascular smooth muscle cells but not endothelial cells by using electron microscope autoradiography. *J Cardiovasc Pharmacol* **29:** 820–826, 1997.
13. McPherson GA, A practical computer-based approach to the analysis of radioligand binding experiments. *Comput Programs Biomed* **17:** 107–113, 1983.
14. Munson PJ and Rodbard D, Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* **107:** 220–239, 1980.
15. Ohlstein EH, Beck GR, Douglas SA, Nambi P, Lago MA, Gleason JG, Ruffolo RR, Feuerstein G and Elliott JD, Nonpeptide endothelin receptor antagonists. II. Pharmacological characterization of SB 209670. *J Pharmacol Exp Ther* **271:** 762–768, 1994.
16. Karet FE, Kuc RE and Davenport AP, Novel ligands BQ123 and BQ3020 characterize endothelin receptor subtypes ET_A and ET_B in human kidney. *Kid Int* **44:** 36–42, 1993.
17. Cernacek P, Legault L, Stewart DJ and Levy M, Specific endothelin binding sites in renal medullary collecting duct cells: lack of interaction with ANP binding and cGMP signalling. *Can J Physiol Pharmacol* **70:** 1167–1174, 1992.
18. Lopez-Farre A, Gomez-Garre D, Bernabeu F and Lopez-Novoa JM, A role for endothelin in the maintenance of post-ischaemic renal failure in the rat. *J Physiol* **444:** 513–555, 1991.
19. Vemulapalli S, Chiu PJS, Chintala M and Bernardino V, Attenuation of ischemic acute renal failure by phosphoramidon in rats. *Pharmacology* **47:** 188–193, 1993.