

Themed Section: Endothelin

REVIEW

Endothelin-2, the forgotten isoform: emerging role in the cardiovascular system, ovarian development, immunology and cancer

Lowell Ling, Janet J Maguire and Anthony P Davenport

Clinical Pharmacology Unit, University of Cambridge, Cambridge, UK

Endothelin-2 [ET-2; also known as vasoactive intestinal contractor (VIC), in rodents] differs from endothelin-1 (ET-1) by only two amino acids, and unlike the third isoform, endothelin-3 (ET-3), it has the same affinity as ET-1 for both ET_A and ET_B receptors. It is often assumed that ET-2 would mimic the actions of the more abundant ET-1 and current pharmacological interventions used to inhibit the ET system would also block the actions of ET-2. These assumptions have focused research on ET-1 with ET-2 studied in much less detail. Recent research suggests that our understanding of the ET family requires re-evaluation. Although ET-2 is very similar in structure as well as pharmacology to ET-1, and may co-exist in the same tissue compartments, there is converging evidence for an important and distinct ET-2 pathway. Specifically it has been demonstrated that ET-2 has a key role in ovarian physiology, with ET-2-mediated contraction proposed as a final signal facilitating ovulation. Furthermore, ET-2 may also have a pathophysiological role in heart failure, immunology and cancer. Comparison of ET-2 versus ET-1 mRNA expression suggests this may be accomplished at the level of gene expression but differences may also exist in peptide synthesis by enzymes such as endothelin converting enzymes (ECEs) and chymase, which may allow the two pathways to be distinguished pharmacologically and become separate drug targets.

LINKED ARTICLES

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Abbreviations

ECE-1, endothelin converting enzyme 1; ECE-2, endothelin converting enzyme 2; ELISA, enzyme-linked immunosorbent assay; HIFs, hypoxia-inducible factors; MEK1/2, mitogen-activated protein kinase kinase; MMP-2, matrix metalloproteinase 2; MMP-9, matrix metalloproteinase 9; NEP, neutral endopeptidase; PRKO, progesterone receptor knockout; PMA, phorbol 12-myristate 13 acetate; VIC, vasoactive intestinal contractor

Introduction

The existence of a peptidic endothelium-derived constricting factor was originally proposed in 1985 by Hickey *et al.* (Hickey *et al.*, 1985) but was identified and named endothelin (ET) by Yanagisawa and colleagues in 1988 (Yanagisawa *et al.*, 1988). The peptide was renamed ET-1 to reflect the subsequent discovery a year later of human genes encoding two further members of the family, ET-2 and ET-3 (Inoue *et al.*, 1989). The ET peptides act by activating two G-protein coupled receptors, ET_A and ET_B. (Davenport, 2002; Alexander *et al.*, 2011) ET-3 is the only endogenous isoform that distin-

guishes between the two receptors, having the same affinity at ET_B as ET-1 and ET-2 but, at physiological concentrations, has much lower or little affinity for ET_A. The established dogma is that ET-2 has the same affinity for both ET_A and ET_B receptors as ET-1. It is often assumed that ET-2 would mimic the actions of the more abundant peptide, and current pharmacological interventions used to inhibit the ET system would also block the actions of ET-2. These assumptions have focused research on ET-1, leaving the ET-2 isoform much less studied. Recent research suggests that our understanding of the ET family requires re-evaluation. Evidence is emerging that ET-2 has a key role in ovarian physiology, as

Correspondence

Dr A. P. Davenport, Clinical Pharmacology Unit, University of Cambridge, Addenbrooke's Hospital, Cambridge, CB2 0QQ, UK. E-mail: apd10@medschl.cam.ac.uk

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ET-2-mediated contraction has been proposed as a final mechanical signal facilitating ovulation (Ko *et al.*, 2006; Palanisamy *et al.*, 2006). The peptide may also have a pathophysiological role in heart failure, immunology and in cancer. Furthermore, ET-2 knockout mice display a phenotype distinct from that of ET-1 or ET-3 (Chang *et al.*, 2009). Our aim is to discuss the mechanisms whereby ET-2 mediates its actions in separate spatiotemporal 'compartments' to ET-1, and to review the evidence that suggests a third and separate signalling pathway to the established ET-1/ET_A and ET-3/ET_B pathways.

ET_A/ET-1 pathway and disruption in knockout mice

ET-1 is the most abundant isoform in the human cardiovascular system and widely expressed, for example in endothelial cells lining every blood vessel (Plumpton *et al.*, 1996). The peptide is continuously released to cause vasoconstriction, by predominantly interacting with ET_A receptors present on smooth muscle, contributing to the maintenance of normal physiological vascular tone in men and mice (Haynes and Webb, 1994; Yanagisawa *et al.*, 1998). In contrast, mRNA encoding ET-3 has not been detected in human endothelial cells, and there is low expression of ET_B receptors on underlying smooth muscle (Davenport *et al.*, 1995; Maguire and Davenport, 1995). Genetically modified mice demonstrated the importance of ET-1/ET_A/ECE pathway in cardiovascular and craniofacial development. ET-1-deficient homozygous mice die at birth of respiratory failure secondary to severe craniofacial and cardiovascular abnormalities. In agreement, ET_A receptor and ECE-1 knockout mice have similar morphological abnormalities (Hosoda *et al.*, 1994; Clouthier *et al.*, 1998; Yanagisawa *et al.*, 1998).

ET_B/ET-3 pathway and disruption in knockout mice

ET-1 does not cross the blood-brain barrier under normal physiological conditions (Johnstrom *et al.*, 2005) and the presence of ET-1 and ECE have been detected in some neurons, glial cells and vascular endothelium indicating synthesis within the CNS (Davenport *et al.*, 1998). However, ET-3 is the major isoform in the rat brain (Matsumoto *et al.*, 1989) mainly restricted to neurons and glia, and being particularly abundant in the neostriatum, hypothalamic nuclei, hippocampus and Purkinje cells of the cerebellum and medulla oblongata (Giaid *et al.*, 1991). The ET_B subtype accounts for about 90% of the ET receptors in human normal cerebral cortex. The receptor is localized to neuronal regions particularly levels III and IV of the cortex, consistent with ET-3 functioning as a neuropeptide (Harland *et al.*, 1995). The ET-3/ET_B pathway also has a crucial role in development, but importantly homozygote ET_B knockout mice exhibit a different and non-overlapping phenotype to ET_A-deficient animals. ET_B knockout mice survive for up to 8 weeks but display aganglionic megacolon as a result of absence of ganglion

neurons, together with a pigmentary disorder in their coats. This is caused by the failure of enteric nervous system precursors and neural crest-derived epidermal melanoblasts to colonize the intestine and skin. ET-3 knockouts display an identical phenotype (Kurihara *et al.*, 2001).

Effect of ET-2 deletion in knockout mice

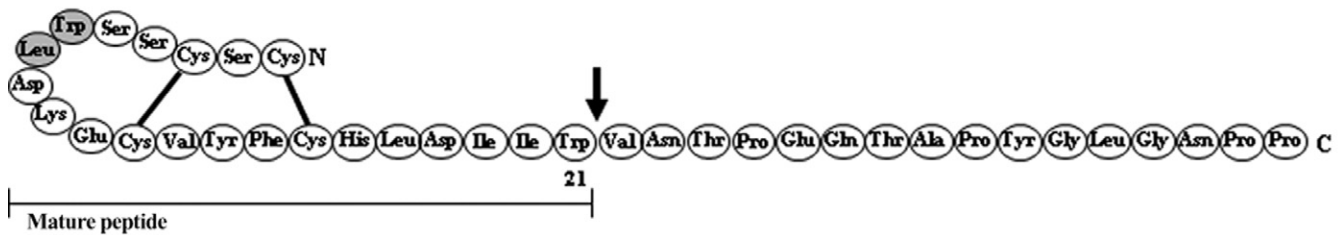
A global knockout of ET-2 in mice revealed a third, distinct phenotype to the phenotype of ET_A/ET-1 and ET_B/ET-3 knockout mice (Chang *et al.*, 2009). ET-2-deficient mice exhibited growth retardation with changes in energy homeostasis characterized by hypoglycaemia and ketonaemia. Mice were hypothermic leading to juvenile lethality. Changes in lung morphology and function were observed suggesting a key role in the development of the pulmonary system. This may be significant since the major current clinical application of ET receptor antagonists such as bosentan and ambrisentan is in the treatment of pulmonary arterial hypertension.

ET-2 and vasoactive intestinal contractor (VIC)

ET-2 differs from ET-1 by two amino acids with Trp⁶ and Leu⁷ substitutions for Leu⁶ and Met⁷, as shown in Figure 1. But this usually results in little or no effect on the binding affinity to ET receptors when compared with ET-1. This sequence difference is comparatively small and the solution structure of ET-2 is similar to ET-1 (Arvidsson *et al.*, 1998). Other mammals including monkeys, cats, dogs and cattle have the same sequence for ET-2 as humans do. An exception are mice and rats where the equivalent peptide to ET-2 is also called VIC, so named because of its contractile activity in the ileum and was originally identified in the mouse genome (Saida *et al.*, 1989; Bloch *et al.*, 1991). VIC differs from ET-1 by three amino acids (Asn⁴, Trp⁶ and Leu⁷), as shown in Figure 1 (Ishida *et al.*, 1989; Saida *et al.*, 1989; 2002). These authors describe a synthetic pathway for VIC that resembles that of ET-2, from a 175 amino acid precursor protein (prepro-VIC) via a 38 amino acid big VIC, which is cleaved to give the mature peptide and a C-terminal fragment (Saida *et al.*, 2000). Only limited studies have been carried out using VIC. Where detailed comparisons have been made VIC is reported to have the same affinity for both ET_A and ET_B receptors. For example, the ability of ETs to compete for [¹²⁵I]-VIC binding to ET_B receptors present on guinea pig longitudinal smooth muscle was compared, and no difference was found between unlabelled VIC, ET-1, ET-2 or ET-3. In addition, all four peptides were equally potent with a similar contractile response (Yoshinaga *et al.*, 1992). VIC was equipotent with ET-1 and ET-2 in other ET_B preparations (Cozza *et al.*, 1992; Lin and Lee, 1992) as well as predominantly ET_A tissues (Douglas and Hiley, 1990; Schoeffter and Randrianosa, 1993). However, discrepancies have been reported in cultured human smooth muscle cells where VIC had a lower potency than ET-1 (Iwashima *et al.*, 1997).

Mouse and rat genome databases retain the use of EDN2 for the gene encoding mouse VIC. This nomenclature is

Big Endothelin-2 (human, 1-37)



Big VIC (mouse, 1-38)



Figure 1

Amino acid sequence of Big ET-2, Big VIC and their respective mature peptides with disulfide bridges (—). Differences in amino acid sequence to Big-ET-1 and ET-1 are shaded in grey.

adopted by the majority of authors reporting on studies in rats and mice and is used in this review.

To date, the physiological and pathophysiological role of ET-2 has been less extensively studied than ET-1 partly because of the difficulty in distinguishing between the structure and function of the two peptides. Although not as ubiquitous as ET-1, mRNA encoding ET-2 has been detected in a range of human tissues (Table 1). Both ET-2 mRNA (O'Reilly *et al.*, 1993a) and Big ET-2 have been detected in the cytoplasm of endothelial cells (Howard *et al.*, 1992). In functional assays, ET-2 is also as potent a vasoconstrictor as ET-1 is (Maguire and Davenport, 1995), implying that on release from the endothelium, ET-2 may also contribute to physiological tone. The biologically inactive precursor Big ET-2 circulates in human plasma at a higher level than Big ET-1 does (Matsumoto *et al.*, 1994), although the level of the mature peptide at ~ 0.9 pmol·L⁻¹ is about one-fifth that of mature ET-1 level (Davenport and Maguire, 2006).

Regulation of ET genes

Regulation of ET peptide gene EDN1 has been reviewed in detail by Stow *et al.* (2011). The complexity of ET-1 regulation is a testament to the significant role of ETs in homeostasis. Briefly, most studies suggest that ET-1 regulation occurs at the transcription level, as various factors such as red wine, leptin and hypoxia act through transcription factors that interact with *cis*-acting elements in the EDN1 promoter regions (Lee *et al.*, 1990; Oliver *et al.*, 1991; Corder *et al.*, 2001; Chao *et al.*,

2007; Belaidi *et al.*, 2009). In addition, post-transcriptional modification, microRNA regulation and epigenetics may also explain tissue specific regulation of ET-1 expression (Val-lender and Lahn, 2006; Rodriguez-Pascual *et al.*, 2008; Yeligar *et al.*, 2009). In contrast, very little is known about EDN2, except that it is mapped to chromosome 1p34 (Arinami *et al.*, 1991). Although the evidence is sparse and incomplete, there are distinct mechanisms that regulate ET-2 production. For example, Tokito *et al.* (1991) demonstrated an inhibitory effect of epidermal growth factor in the production of ET-2 but not ET-1 in renal adenocarcinoma cells. Lambert *et al.* (1998; 2000) explored compounds regulating expression of ET-2 mRNA and secretion of the peptide in human renal adenocarcinoma cell lines. TNF- α as well as forskolin and dibutyryl cAMP (cell-permeable cAMP analog) stimulated concentration-dependent increases in ET-2 mRNA synthesis and release. The authors showed these were two distinct signalling pathways via TNF receptor-1 linked induction and a cAMP mechanism. As will be discussed in the following themed sections, various factors such as hypoxia and progesterone have been shown to affect ET-2 expression. However, the transcriptional control and mature peptide conversion underlying these factors are far from clear.

Does ET-2 have a separate synthesis from other ET peptides?

In addition to ET receptor antagonists, a second developing therapeutic strategy is to block the detrimental actions of ETs

Table 1

ET-2 distribution in humans

Distribution in humans	Form	Technique:	Reference
Heart			
Left atria, left ventricle	ET-2	radioimmunoassay, reverse phase high performance liquid chromatography	Plumpton <i>et al.</i> (1993)
Left ventricle	ET-2 mRNA	rt-PCR	O'Reilly <i>et al.</i> (1993b)
Left ventricle	ET-2 mRNA	rt-PCR	Plumpton <i>et al.</i> (1996)
Lung			
Submucosal glands	proendothelin-2	ELISA, immunohistochemistry	Marciniak <i>et al.</i> (1992)
Renal			
Medulla, cortex	ET-2 mRNA	rt-PCR	Karet and Davenport (1996)
Vasculature			
Saphenous vein, mesenteric artery/vein, internal mammary artery	proendothelin-2	ELISA, immunohistochemistry	Howard <i>et al.</i> (1992)
Reproductive organs			
Endometrium, myometrium, placenta	ET-2 mRNA	rt-PCR	O'Reilly <i>et al.</i> (1992; 1993b)
Endometrium	ET-2	radioimmunoassay, reverse phase high performance liquid chromatography	Cameron <i>et al.</i> (1993)
Ovarian follicles	mRNA	Northern blot with hybridization using 32P-labeled cDNA probes	Palanisamy <i>et al.</i> (2006)
Corpus luteum	mRNA	rt-PCR	Klipper <i>et al.</i> (2010)
Gastrointestinal			
Colon	preproET-2 mRNA	rt-PCR	McCartney <i>et al.</i> (2002)

in pathophysiological conditions by inhibition of the ET converting enzymes (ECE-1 and ECE-2), which have been established as the major synthetic pathway for ET-1. As the affinity of ET-2 is similar to ET-1 for both subtypes, it is important to understand whether this therapeutic strategy is also likely to block synthesis of ET-2 or if alternative synthetic pathways exist.

The ET-2 synthetic pathway is initially similar to that of ET-1 and ET-3, as shown in Figure 2. The first product of EDN2 gene transcription is preproendothelin-2. Following the removal of the signal sequence, the resulting proendothelin is cleaved by the enzyme furin, to yield the 37 amino acid peptide, Big ET-2. It is now well established that ET-1 is synthesized from Big ET-1 by an unusual hydrolysis of Trp²¹-Val²² bond, rather than the more frequent Arg-Arg or Arg-Lys as in other peptide precursors (Turner and Murphy, 1996). Synthesis is catalysed by a membrane-bound metalloprotease, ECE-1. ET-1 is also synthesized by ECE-2, a second metalloprotease with 59% homology with bovine ECE-1 but with an acidic pH for optimum activity in cleaving Big ET-1 to the mature peptide compared with a neutral range for ECE-1 (Emoto and Yanagisawa, 1995). ECE-2 also cleaves other peptides, including the vasodilator bradykinin. At least four isoforms exist for both enzymes that differ in their cellular and tissue distribution, possibly reflecting different functions.

Controversy currently exists in the literature about the efficiency of Big ET-2 conversion by ECE *in vitro* compared with *in vivo* studies. Although Big ET-2 shares the same scissile bond (Trp²¹-Val²²) with Big ET-1, the rate of conversion

of Big ET-2 by ECE-1 and ECE-2 was less than 10% of Big ET-1 when compared in the same assay (Emoto and Yanagisawa, 1995).

Although there are exceptions, a pattern is emerging using purified, cloned or expressed enzyme that Big ET-2 is poorly converted (in contrast to native enzyme) with no activity against Big ET-3 (D'Orléans-Juste *et al.*, 1991; Telemaque and D'Orléans-Juste, 1991; Shimada *et al.*, 1994; Turner and Murphy, 1996). The reason for this difference in specificity is that the scissile bond and the terminal sequence in Big ET-3 are different from the other big ET precursors. A zinc-dependent endopeptidase, Kell, with sequence similarity to ECE-1, efficiently cleaved Big ET-3 at Trp²¹-Val²², suggesting the existence of a specific converting enzyme for the synthesis of ET-3 (Lee *et al.*, 1999; Clapéron *et al.*, 2005). Big ET-1 and Big ET-2 also differ in key residues 27–29, which are crucial for efficient ECE-1 and ECE-2 activity. However, these results contrast with functional studies using native tissues that suggest Big ET-2 is converted mainly by ECE, and can be blocked as expected by dual inhibitors of ECE and neutral endopeptidase (NEP). Big ET-2 caused contraction of human isolated bronchus comparable with Big ET-1 and with the maximal response equivalent to that of ET-2, demonstrating full conversion of this precursor peptide (Yap *et al.*, 2000). The authors suggested ECE activity within human bronchi either has equal specificity for both precursors, or that there are multiple enzymes within this tissue, at least one having the same specificity for Big ET-2. In support of this hypothesis, CGS 26393 and CGS 26303 non-peptide dual ECE and

ET-2 Synthesis Pathway

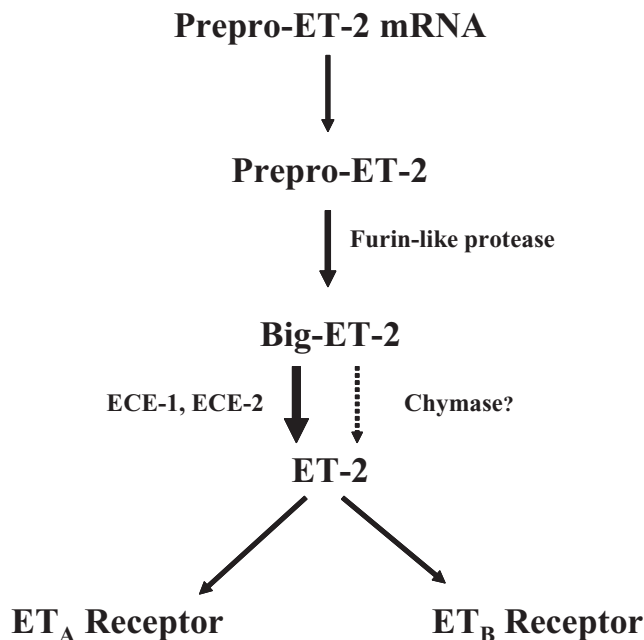


Figure 2

Synthesis pathway of ET-2 from mRNA through intermediates to mature peptide.

neutral NEP inhibitors with equivalent activity on ECE-1 and ECE-2 inhibits contractions to all three Big ETs (Yap *et al.*, 2000).

Detailed studies in chronically instrumented rats *in vivo* have shown that Big ET-2 is efficiently converted to produce cardiovascular actions in a similar way to Big ET-1, and can be blocked as expected by phosphoramidon (Gardiner *et al.*, 1992). These regional haemodynamic studies were carried out in conscious, chronically-instrumented rats to avoid the confounding effects of anaesthetics. Gardiner *et al.* (1992) demonstrated that administration of Big ET-2 in rats causes dose-dependent pressor, bradycardic, renal and, particularly, mesenteric vasoconstrictor effects. Phosphoramidon given as a $10 \mu\text{mol}\cdot\text{kg}^{-1}$ i.v. bolus completely abolished the pressor, bradycardic and hindquarters vasoconstrictor actions of Big ET-2. Phosphoramidon had no direct effects on the actions of ET-2, consistent at least in rats with Big ET-2 being converted by a phosphoramidon-sensitive enzyme such as ECE. Similar results were obtained in the anaesthetized, ganglion-blocked rat, where intravenous bolus doses of Big ET-2 were compared with Big ET-1. The onset of the vasopressor effect was more rapid (5–6 min) for the latter than that of Big ET-2 (10–13 min) but the actions of both peptides were blocked as expected by phosphoramidon but not thiorphan (Mattera *et al.*, 1993). In renal adenocarcinoma cells producing high levels of ET-2, the presence of ECE was confirmed by sequencing (Yorimitsu *et al.*, 1992; 1995).

In the anaesthetized rabbit, ET_A antagonist BQ-123 markedly reduced the pressor responses to ET-1, Big ET-1, ET-2 and

Big ET-2, whereas blockade of ET_B receptors with BQ-788 sharply potentiated the responses to ET-1, ET-2 and Big ET-1, but not to Big ET-2 (Gratton *et al.*, 2000). Systemically administered Big ET-1, but not Big ET-2, induced a phosphoramidon-sensitive increase in immunoreactive ET. The authors suggested that ET_B receptors played a significant limiting role on the pressor responses to Big ET-1. This receptor does not modulate the haemodynamic properties of Big ET-2 as it is poorly converted in the pulmonary or systemic circulation in anaesthetized rabbits. This is supported by human *in vivo* studies done by Bohm *et al.* (2003), which showed that ET_B functions as a clearance receptor and may modulate ET_A-mediated vasoconstriction by altering the plasma concentration of ET-1.

An alternative pathway for ET synthesis has been proposed, which involves chymase, a serine protease present in mast cells within the blood vessel wall that can cleave the Tyr³¹-Gly³² peptide bond of both Big ET-1 (Maguire *et al.*, 2001; Fecteau *et al.*, 2005; D'Orléans-Juste *et al.*, 2008) and Big ET-2 (Takai *et al.*, 1998) to generate the intermediate peptides ET-1(1–31) and ET-2(1–31), which is in turn converted to the mature peptides by an as yet uncharacterized mechanism. Interestingly, Takai *et al.* (1998) found the conversion of Big ET-2 could be blocked by chymase inhibitors but not that of Big ET-1 in monkeys. Because the number of mast cells increases with cardiovascular disease (Marone *et al.*, 1995) and chymase is highly expressed in these cells, it remains unclear if this synthesis pathway of either peptide is of pathological significance. Degranulation, which can occur under pathophysiological conditions, may release chymase into interstitial spaces with the potential to convert Big ETs.

The clinical strategy for inhibition of ECE is represented by SLV-306 (Daglutril), an orally active mixed enzyme inhibitor of both ECE and NEP. Therefore SLV-306 has the added advantage of blocking the NEP dependent breakdown of beneficial vasodilator atrial natriuretic factor. A Phase II trial was completed in 2010 by Solvay for the treatment of essential hypertension and congestive heart failure (Bayes *et al.*, 2003; Tabrizchi, 2003; Dickstein *et al.*, 2004). In agreement with animal studies of Gardiner *et al.* (1992), oral administration of SLV 306 to healthy volunteers resulted in a significant dose-dependent rise in circulating Big ET-1 levels. In addition, there was an expected parallel increase in the C-terminal fragment as mature ET-1 is also metabolized by NEP (Seed *et al.*, 2012). Because Big ET-2 circulates in human plasma at higher levels than Big ET-1 (Matsumoto *et al.*, 1994), it would be crucial to know whether SLV-306 also inhibits conversion to ET-2.

Distribution of ET-2 mRNA and mature peptide

Genetic techniques such as RT-PCR can provide robust assays for distinguishing between mRNA encoding ET-1 and ET-2 but a limitation is that tissue must be homogenized and it is not usually possible to identify cell types. Furthermore, the presence of mRNA in a specific tissue merely confirms that the tissue is capable of synthesizing mRNA. It does not shed

light on post-transcriptional modification, storage and functional secretion or give information about peptide levels. The majority of immunoassays (RIA, ELISA) that detect mature ET cross-react with both ET-1 and ET-2. To overcome this problem, HPLC can be used to separate the mature isoforms and their precursors, followed by RIA with antisera raised to the carboxy-terminal hexapeptide ET (15–21). This terminal is common to all three isoforms and detects the levels of the mature peptides but not the precursors in the resulting fraction. This method also relies on homogenates. A third strategy for cellular localization has been to use antisera raised against the variable C-terminal sequence of the precursor, Big ET-2(31–37), which does not cross react with Big ET-1 or Big ET-3. These antisera have been used to identify cells expressing Big ET-2-like immunoreactivity, and therefore these cells have the potential to synthesize and release the mature peptide.

In rats quantitative RT-PCR revealed expression of mRNA encoding ET-1 in all of 16 organs examined from rats (Uchida *et al.*, 2000). In contrast, ET-2 expression was more restricted, and had an organ specific distribution being detected in heart, lung, ovary, stomach and intestine. Particularly high levels were in ovary (but not testes) and all regions of the intestine (duodenum, jejunum, ileum, colon and rectum) (De La Monte *et al.*, 1995; Takizawa *et al.*, 2005). In contrast, the levels in cerebellum and cerebrum were below the level for mRNA detection (Saida *et al.*, 2002). Interestingly, ET-2 mRNA measured by QPCR was much higher than ET-1 mRNA in rat and mice pituitary gland and medulla oblongata (Masuo *et al.*, 2003). Moderate to low levels of ET-2 mRNA expression were observed in other brain regions compared with ET-1 suggesting a possible role in the pituitary. Similar to its wide distribution in rats, ET-2 expression in humans has been localized to different tissues, as shown in Table 1. ET-2 has been identified in human heart, lung, kidney, vasculature, intestine and ovaries (Howard *et al.*, 1992; Marciniak *et al.*, 1992; Plumpton *et al.*, 1993; Karet and Davenport, 1996; Palanisamy *et al.*, 2006).

Unexplained ET-2 pharmacology

Saturation binding studies comparing radiolabelled [¹²⁵I]-ET-2 with [¹²⁵I]-ET-1 provide 'gold standards' and accurate measures of affinity constants (K_D) and receptor densities (B_{max}). In cultured rat aortic smooth muscle cells that express ET_A receptors, the K_D values of [¹²⁵I]-ET-2 and [¹²⁵I]-ET-1 were identical at 0.1 nM and their B_{max} values were 46 and 54 fmol per million cells respectively. Saturation curves were monophasic with the same association rate constants (K_{obs}) of 0.01 min⁻¹ for [¹²⁵I]-ET-2 with [¹²⁵I]-ET-1 and similar slow dissociation rates (Roubert *et al.*, 1991). Intriguingly however, while [¹²⁵I]-ET-2 and [¹²⁵I]-ET-1 bound with the same affinity at ET_A receptors in Swiss 3T3 fibroblasts, ET-2 dissociated at a dramatically faster rate than ET-1 did, which may be important in determining pharmacological responses to ET-2 *in vivo* (Devesly *et al.*, 1990). It is not yet known whether kinetics of binding varies between cell types and can distinguish responses to the two isoforms *in vivo*.

Consistent with the results from transformed cultured cells, saturation experiments on tissue sections from the vas-

cular smooth muscle layer of human aorta and coronary artery yielded similar constants (Bacon and Davenport, 1996). These experiments demonstrated monophasic binding with Hill slopes close to unity with K_D values for [¹²⁵I]-ET-2 of 0.71 ± 0.17 nM and 0.43 ± 0.10 nM for [¹²⁵I]-ET-1. No differences were detected in competition binding studies using the ET_A selective antagonists FR139317. Inhibition of [¹²⁵I]-ET-2 and [¹²⁵I]-ET-1 binding in both arteries was monophasic with K_D values in the same range. In the human vasculature at least, there is no evidence for any differences in competition by antagonists such as BQ123 for labelled ET-2, and this is supported by vasoconstrictor studies in human isolated vessels (Maguire and Davenport, 1995) as well as animal preparations such as rat portal vein (Guimaraes *et al.*, 1992).

However, in other functional studies, there are reports of possible differences in the pharmacology of ET-2 compared with ET-1, which have not been explained and warrant further investigation. Bertelsen *et al.* (1992) reported in spinal cord of the rat using ligand binding assays, inhibition constants for ET-1 and ET-2 were found to be similar whereas in the cerebral cortex the values of ET-2 were found to be at least 6 times higher than ET-1. This suggests that in the rat brain, ET-2 has a higher affinity for ET receptors. In agreement, a proportion of neurones cultured from embryonic rat cerebral cortex responded selectively to ET-2 with an increase in intracellular calcium (Morton and Davenport, 1992). It is unclear whether these responses are due to differences in affinity compared with ET-1 or other factors such as the stability of peptides in this assay. Both ET-1 and ET-2 induced concentration-dependent tonic contraction of the human isolated oviduct by acting on ET_A receptors with similar EC₅₀ values (Jankovic *et al.*, 2009). However, ET-1 also caused a concentration-dependent inhibition of rhythmic contractions of the oviduct, with a similar potency whereas surprisingly ET-2 had no effect.

Neutrophils are important effector cells of tissue injury in several pathological conditions such as immune complex induced inflammation and tissue injury. In immune complex stimulated neutrophils, ET-1 but not ET-2 potentiated production of leukotriene B₄ (Paulino *et al.*, 2006). Similarly, ET-2 had differential actions to ET-1 on neutrophil migration and intracellular calcium (Elferink and de Koster, 1996). At low concentrations, ET-2 caused a chemotactic stimulation of migration whereas ET-1 induced a chemokinetic stimulation of migration. At higher concentrations, ET-2 inhibited N-formyl-methionyl-leucyl-phenylalanine (fMLP)-activated migration with both actions dependent on extracellular Ca²⁺. Unlike ET-1, which caused an increase in cytosolic free Ca²⁺ at a concentration that stimulated migration, ET-2 caused a measurable increase of cytosolic free Ca²⁺ at a concentration that did not stimulate migration (Elferink and de Koster, 1996). Comparisons have also been made between ET-2 and other agonists such as sarafotoxin 6b (S6b), where Gardiner *et al.* (1990) showed S6b is a more potent vasoconstrictor than ET-2.

ET-2 in cardiovascular system

ETs were originally discovered as a vasoconstrictor peptide produced by endothelial cells (Yanagisawa *et al.*, 1988). Not

only is ET-1 the most studied isoform in cardiovascular disease, but it is one of the most powerful vasoconstrictors (Poli *et al.*, 1991b; Davenport and Maguire, 2006). But studies suggest ET-1 may not be the only ET isoform that regulates the vasculature. Genetic studies have identified a specific EDN2 polymorphism that is associated with essential hypertension (Sharma *et al.*, 1999) whereas other studies have shown associations between rare ET-1 and ET-2 polymorphisms with lower diastolic blood pressures (Brown *et al.*, 2000). In the kidney, human ET-2 (hET-2) transgenic rat studies suggests that overexpression of ET-2 contributes to the development of glomerulosclerosis (Hoche *et al.*, 1996). Apart from its effects on the vasculature, ET-2 is also a strong positive inotrope on the human myocardium (Poli *et al.*, 1991a). Although the benefits of ET antagonism in initial experimental models of heart failure showed promise, clinical trials have thus far not demonstrated the desired outcomes (Sakai *et al.*, 1996; Borgeson *et al.*, 1998; Kalra *et al.*, 2002; Packer *et al.*, 2005). More encouragingly, ET antagonism with bosentan and ambrisentan have been shown to improve exercise tolerance and slow down clinical deterioration in pulmonary hypertension (Channick *et al.*, 2001; Rubin *et al.*, 2002; Barst *et al.*, 2006). Most of the biological evidence that led to these trials stemmed from knowledge in ET-1 signalling. There are, however, notable differences between ET-1 and ET-2 expression in the cardiovascular system. In rat left coronary artery ligation cardiac failure models, ET-1 expression in the failing heart is almost four times higher than in control hearts (Kakinuma *et al.*, 1999). In contrast, ET-2 expression is 88% lower in the failing heart compared with control hearts. Interestingly, this reciprocal differential expression is reversed in hypoxic rat cardiomyocyte cell cultures, where ET-1 expression is decreased by 38% and ET-2 expression is increased by almost fourfold compared with controls. Recently, hET-2 rat studies showed that elevated ET-2 expression increases myocardial fibrosis, particularly in the presence of diabetes (Liefeldt *et al.*, 2010). Long-term ET-2 overexpression in diabetic rats also resulted in marked coronary artery wall hypertrophy compared with diabetic wild-type rats. However, Liefeldt *et al.* (2010) acknowledges that in their experiments, ET-1 levels were higher in hET-2 transgenic rats compared with wild-type, thus ET-1 may be mediating, at least in part, the remodelling seen in hET-2 rats.

In addition to its effects on the myocardium, ET-2 may also modulate cardiac conduction. In a single centre trial with a small number patients, Nagai *et al.* (2007) showed that a significant association of the A985G polymorphism of the ET-2 gene with increased incidence of atrial fibrillation (the most common cause of tachyarrhythmia) in patients with hypertrophic cardiomyopathy. The functional consequences are not yet known of the A985G polymorphism but the authors speculate that transcription and translation of EDN2 could be altered. The cumulative evidence from these studies suggests ET-2 may modulate vascular tone and also exert control on vascular tissue morphology and remodelling.

ET-2 in ovarian physiology

The ET pathway has been shown to play an important role in ovarian physiology (Meidan and Levy, 2007). Most studies

have focused on ET-1 as an important regulator of follicular development and terminating luteal phase (Haq *et al.*, 1996; Flores, 2000; Shirasuna *et al.*, 2006). However, expression of ET-2 is actually higher than ET-1 expression in ovaries (Uchide *et al.*, 1999). Over the last 5 years, there has been growing interest in the role ET-2 may have in ovulation (Ko *et al.*, 2006). Using a rat ovarian gene expression database and subsequent RT-PCR, Ko *et al.* (2006) found that both ECE-1 and ET-2 were transiently expressed in rat ovaries during ovulation. In particular, ET-2 is expressed in the granulosa cells of periovulatory follicles and not during other stages of follicular development. In mice, induced superovulation results in a dramatic increase in ET-2 mRNA expression (Palanisamy *et al.*, 2006). At 11 h after gonadotropin was given, ET-2 expression surged and quickly declined by 13 h, which coincided with the time of follicular rupture. However, ET-2 expression seems to be different in bovine ovaries compared with rat or mice ovaries (Klipper *et al.*, 2010). Using gonadotropin induced superovulation, Klipper *et al.* (2010) found that follicular ET-2 mRNA expression transiently increased at 4 h post-gonadotropin, although this result was not statistically significant. Otherwise, ET-2 expression remained low prior to follicular rupture. Interestingly, a surge in ET-2 expression occurred at 60 h post-gonadotropin, corresponding to early corpus luteum phase, which was not previously demonstrated in rat or mice studies. How do we reconcile the differences between bovine and rats/mice ovarian ET-2 expression? Firstly, the discrepancy may be due to interspecies differences. Secondly, it was not clear if the investigators in the rat/mice studies measured ET-2 expression beyond follicular rupture. Thirdly, the difference in approaches used to achieve superovulation induction may account for the differences in ovulation mechanism. This leads to the next question: what causes the alteration in ovarian ET-2 expression?

Studies on progesterone receptor knockout (PRKO) mice have shown that progesterone signalling is critical in ovulation, as PRKO mice not only failed to ovulate but also resulted in accumulation of mature unruptured follicles (Lydon *et al.*, 1995; Chappell *et al.*, 1997). PRKO mice lose the transient ET-2 expression that wild-type mice have when superovulation is induced by gonadotrophin (Palanisamy *et al.*, 2006; Kim *et al.*, 2009). In cultured granulosa cells, forskolin and phorbol 12-myristate 13 acetate (PMA) stimulation has been used to mimic and model the *in vivo* pre-ovulation LH surge that normally results in increased progesterone receptor expression. Palanisamy *et al.* (2006) found that forskolin and PMA stimulation in cultured granulosa cells also resulted in increased ET-2 expression 12 h post-exposure, similar to the *in vivo* gonadotropin challenge. Blocking progesterone receptor function in these cells inhibits this ET-2 expression response to forskolin and PMA. However, a subsequent *in vivo* study found that indomethacin and a PR antagonist, RU-486 had no effect on ET-2 expression in superovulated mice (Na *et al.*, 2008). This study found that it was hypoxia that ultimately determines follicular ET-2 expression in the periovulatory phase. Continuing on this theme, it has been suggested that signal transduction between progesterone receptor and ET-2 expression is due to a set of hypoxia-inducible factors (HIFs) that regulate cell transcriptional responses to hypoxia (Kim *et al.*, 2009). Blocking progesterone induced HIF gene expression with echinomycin results in

reduced ET-2 expression in granulosa cells. The conflicting results from different studies may be explained by the inadequate and inaccurate modelling of *in vivo* conditions with cultured cells, but may also be a reflection that progesterone signalling and hypoxia reflect different convergent pathways on ET-2 expression during ovulation (Na *et al.*, 2008).

Although the mechanism of follicular rupture and ovulation are still undetermined, ET-2 has been shown to cause contraction of the smooth muscle layer surrounding each follicle (Ko *et al.*, 2006). Intriguingly, this smooth muscle layer is absent at the site of impending follicular rupture, which suggests that the follicle may rupture at this point because of the relatively weak surface tension during muscle contraction (Wallis *et al.*, 1990; Ko *et al.*, 2006). The concept that mechanical contraction is a key step in ovulation is relatively new. But this is consistent with the finding that tezosentan, a mixed ET receptor antagonist, reduces ovulation and results in mature unruptured follicles despite gonadotropin superovulation. This is reminiscent of the PRKO mice phenotype, supporting the idea that ET-2 expression is a downstream signal of the progesterone pathway. ET-2-mediated contraction may play a key role in follicular rupture, without which normal ovulation cannot proceed. In particular, it has been shown that ET_B antagonism with BQ-788 alone results in 85% reduction in ovulation in mice PMSG/hCG induced superovulation (Palanisamy *et al.*, 2006). This may be because ET_B expression is much higher than ET_A expression in the ovary. However, these *in vivo* results differ from *in vitro* isometric tension experiments done by Bridges *et al.* (2010), which has shown that blocking ET_A with BQ123 reduces the rat ovarian contractile response to ET-2. In addition, these authors have not been able to repeat the same reduction of ovulation with ET antagonists (BQ123, BQ788) in rats as those observed from earlier mice studies.

While the ET pathway in ovarian physiology remains to be elucidated, it is clear that ET-2 has a temporal and spatial role in ovulation. Furthermore, ET-2 has also been shown to be a potent oviductal constrictor (Al-Alem *et al.*, 2007). Better understanding of ET signalling in ovulation is exciting as this is a potential drug target that may ultimately treat causes of infertility such as luteinized unruptured follicle syndrome.

ET-2 in immunology

ET-2 has recently been recognized as a chemokine and modifier of leukocyte function, which may have far reaching implications for cancer and inflammatory diseases (Wright *et al.*, 1994; Elferink and de Koster, 1996; Grimshaw *et al.*, 2002a). It was first shown that ET-2 is a chemoattractant for neutrophils at low concentrations, but inhibits neutrophil migration at high concentrations (Elferink and de Koster, 1996). Subsequent studies demonstrated that ET-2 is also chemotactic for macrophages and the effects are mediated via ET_B receptors (Grimshaw *et al.*, 2002b). Using Boyden chambers, Grimshaw *et al.* (2002b) showed that the chemotactic response of macrophages to ET-2 is as strong as their response to CCL2, a well-established inflammatory chemokine. Moreover, they found that ET-2 exerts chemotactic response in a similar way to CCL2, through the MAPK pathway (Yen *et al.*, 1997; Grimshaw *et al.*, 2002b). But unlike CCL2, ET-2 exerts

no chemotactic effects on monocytes. Interestingly, chemotaxis of macrophages towards both CCL2 and ET-2 are inhibited by hypoxia as it induces MAPK phosphatase 1 resulting in dephosphorylation of MAPK (Negus *et al.*, 1998; Turner *et al.*, 1999; Grimshaw and Balkwill, 2001; Grimshaw *et al.*, 2002b). In addition to its role as a chemoattractant, ET-2 increases the activation of macrophages (Grimshaw *et al.*, 2002b). As discussed in the following cancer section, the specific effects of ET-2 on macrophage function may have particular consequences for cancer pathogenesis and represents a tangible target for cancer therapy.

ET-2 in cancer pathogenesis

There is growing evidence that ETs are involved in the pathogenesis of many types of cancer (Nakamuta *et al.*, 1993; Kojima and Nihei, 1995; Bagnato *et al.*, 1999; 2011; Eberle *et al.*, 2002). ET-1 has been the main ET studied in cancer, as it has been shown to affect multiple stages of disease development including proliferation, angiogenesis, migration, invasion and apoptosis (Grant *et al.*, 2003; Bhalla *et al.*, 2009). But actually, it was ET-2 and not ET-1 that was first isolated in renal adenocarcinoma cell lines (Ohkubo *et al.*, 1990). More recently, growing interest in ET-2 within cancer pathogenesis started when it was reported that ET-2 expression was increased in human breast cancer (Grimshaw *et al.*, 2002a). ET-2 is not normally expressed in breast tissue, but Grimshaw *et al.* (2002a) found that hypoxic areas of breast tumour from HTH-K mice were particularly associated with increased ET expression. To elucidate this further, they simulated hypoxic conditions in 0.1% oxygen for 24 h in HTH-K mice cell culture and human breast tumour cell lines, and found that ET-2, ET_A and ET_B expressions were all increased. As ET-1 and ET-3 expression were unaffected by hypoxia, it is likely that ET-2 is the main subtype responsible for the increased ET expression in hypoxic tumours. Elevated ET-2 is also reported in other cancers such as those found in the skin, where ET-2 expression is three times higher in basal cell carcinoma compared with normal skin (Tanese *et al.*, 2010). Moreover, the authors showed that ET-2 expression was a downstream effect of hedgehog pathway signalling.

But what is the significance of enhanced ET-2 expression in cancer? In hypoxic conditions, addition of an ET_B antagonist, BQ788 decreases the survival of HTH-K mice and human breast cell lines (Grimshaw *et al.*, 2002a). Moreover, addition of ET-2 to these cells under hypoxia increases cell survival rates, but the protective effect are reversed by BQ-788. Consistent with these *in vitro* studies, HTH-K mice given 5 days of BQ-788 had reduced tumour size and increased necrotic area in breast tumour compared with controls.

In addition to regulating cancer cell apoptosis, ET-2 has been shown to affect invasive and metastatic potential (Grimshaw *et al.*, 2004). Similar to the chemotaxis of macrophages towards ET-2 discussed earlier, cancer cells are also chemotactic and more invasive in the presence of ET-2. Using the matrigel invasion assay, Grimshaw *et al.* (2004) showed that breast cancer cell lines show a chemotaxis in a dose response relationship towards ET-2. This effect was dependent on activation of both ET_A and ET_B receptors since BQ-123 (ET_A antagonist) or BQ-788 (ET_B antagonist) when used on their

own reduced invasion of cells but did not abolish it completely. Again, similar to macrophage chemotaxis, the chemotactic response of cancer cells is mediated via the MAPK pathway downstream of ET_B, and inhibition of MEK1/2 blocked this response. Interestingly, co-culture of breast tumour cells with macrophages increased the invasive potential of tumour cells when compared with tumour cells alone (Grimshaw *et al.*, 2004; Hagemann *et al.*, 2004). This effect was further accentuated when ET-1 or ET-2 was added to the culture. Importantly, the increased invasiveness of ET-2 was shown to be due to increases in expression of MMP-2 and MMP-9 by macrophages and a marked increase in invasion of tumour cells. Up-regulated ET-2 expression in hypoxic tumours may explain why these tumours have been shown to have higher invasive potential and worse prognosis than their well-oxygenated counterparts (Watanabe, 1975). However, these results were not replicated in BCC cell cultures, where ET-2 and ET antagonism with BQ-123 or BQ-788 had no effects on cell proliferation and invasiveness (Tanese *et al.*, 2010). It is tempting, however to infer from *in vitro* studies that show ET-2 having an effect on regulating apoptosis and invasion, as these fit conveniently to *in vivo* studies where there are observed increases of ET and ET_B expression from *in situ* carcinoma to metastatic cancer cells, coinciding with the increasing invasiveness of progressive cancer stages.

How do we incorporate the factors of hypoxia, ET-2 expression, cancer cell apoptosis, chemotaxis and invasion? The trigger may well start with hypoxia, which is a consequence of the tumour cells reaching a critical volume where diffusion becomes the limiting factor for growth (Harris, 2002). This can be interpreted as a switch for the cancer to move on to the next stage of development, leading to increased ET-2 expression, which prevents tumour apoptosis and increases their ET receptor expression. In the meantime, the tumour may release chemokines that attract monocytes and macrophages to the tumour site (Grimshaw *et al.*, 2002b). Once in the tumour, areas of hypoxia expressing ET-2 attract macrophages to the site, and further migration is inhibited. These macrophages are activated and express matrix metalloproteinases to prepare for local tissue invasion. In the presence of activated macrophages, tumour cells with up-regulated ET receptors then chemoattract towards ET-1 and ET-2, and invades tissue and metastasize distally. In terms of therapeutics, the difference between ET-1 and ET-2 may ultimately not matter if the targets are the ET receptors that are under investigation in trials such as a Phase III randomized placebo controlled trial on Zibotentan in prostate cancer. Nonetheless, it is still important to appreciate the different contributions of the ET subtype on cancer pathogenesis as it may provide novel targets for upstream intervention.

Conclusion

The three ET genes are thought to have evolved from a common ancestor by exon and gene duplications (Bloch *et al.*, 1989; Landan *et al.*, 1991; Saida *et al.*, 2000) with ET-1 and ET-3 mediating their actions particularly in development through separate pathways. Although ET-2 is very similar in structure as well as pharmacology and may co-exist in the same tissue compartments as ET-1, there is converging evi-

dence for an important and distinct ET-2 pathway. It is clear from knockout mice and studies in cardiovascular and ovarian system that ET-2 does not simply amplify or duplicate the physiological actions ET-1 and may have a separate pathophysiological role in conditions such as cancer. Comparison of ET-2 versus ET-1 mRNA suggests this may be accomplished at the level of gene expression, but differences may also exist in synthesis by enzymes such as ECE and chymase, which may allow the two pathways to be distinguished pharmacologically and become separate drug targets.

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Conflicts of Interest

None.

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