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Perspective

Endothelin: A New Challenge

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Introduction

Endogenous vasoactive peptides act through a variety of mechanisms to control vascular tone and peripheral blood flow.[1-3] Some of these peptides, such as angiotensin II, vasopressin, neuropeptide Y (NPY), and endothelin are potent vasoconstrictors acting on smooth muscle and in the central nervous system (CNS).[2-5] A wide range of endogenous peptidic vasodilators such as atrial natriuretic peptide (ANP), bradykinin and related neurokinins, substance P (SP), and calcitonin-gene-related peptide (CGRP), presumably act in concert with the vasoconstrictor peptides to maintain homeostasis.[6-8] It is interesting that the vasoconstrictor substances are usually mitogenic, while the vasodilatory peptides inhibit cell growth. Endothelial cells are known to be capable of releasing vasoactive substances that regulate smooth muscle tone and platelet function.[9,10] Indeed many endothelium-dependent vasodilators act by formation of endothelium-derived relaxing factor (EDRF)[11,12] and other endogenous vasodilators, such as prostanoids, particularly prostacyclin. There is considerable interest in vascular control mechanisms and especially in the vasomotor role of endogenous peptides. The endothelium has been proposed to mediate vasoconstriction via production of endothelium derived vasoconstrictor factor(s) (EDCF) in response to various chemical and physical stimuli. The nature of EDCF(s) is the subject of much current discussion and research. The recent discovery of endothelin-1 (ET-1), a potent vasoconstrictor peptide released from endothelial cells, has attracted great interest as one possible candidate for EDCF.[13,14] ET-1, now known to belong to a new peptide class, is some 10-fold more potent than the vasoconstrictor angiotensin II, and has extremely long-lasting pressor effects. There are differing opinions as to whether ET-1 may be secreted to fill some crucial physiological role, as either a short-term or a long-term regulator, or whether its actions are purely pathological in nature. It seems hard to believe that such a potent series of peptides synthesized in all endothelial and many other types of cells would not play some important physiological role. Indeed the expression of this peptide has been highly conserved during the course of vertebrate evolution and may perform similar homeostatic functions in a variety of

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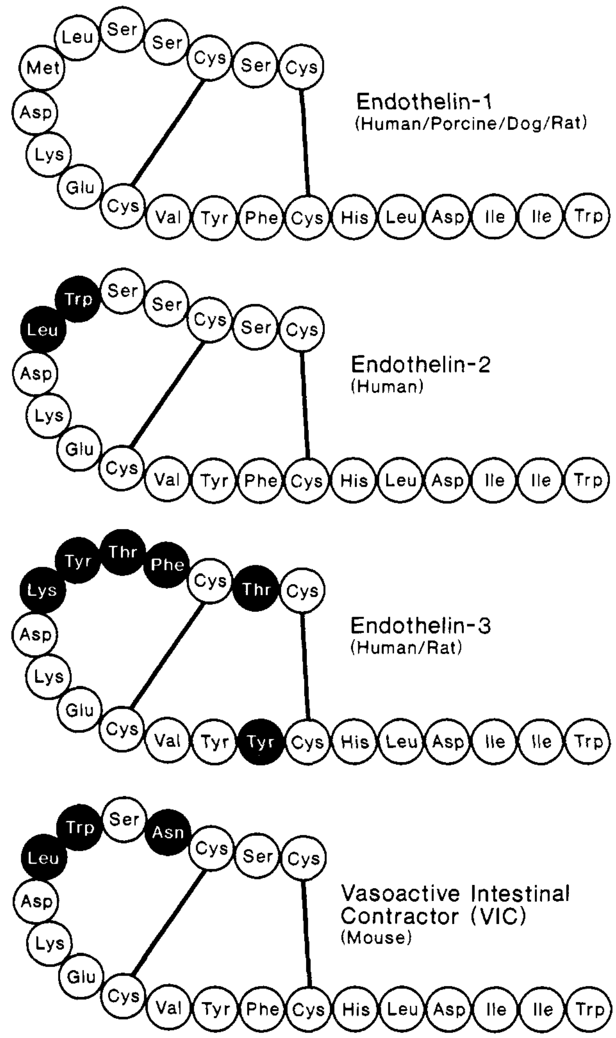

Figure 1. Amino acid sequences of the endothelin peptide family. Filled circles: residues different from those in ET-1.

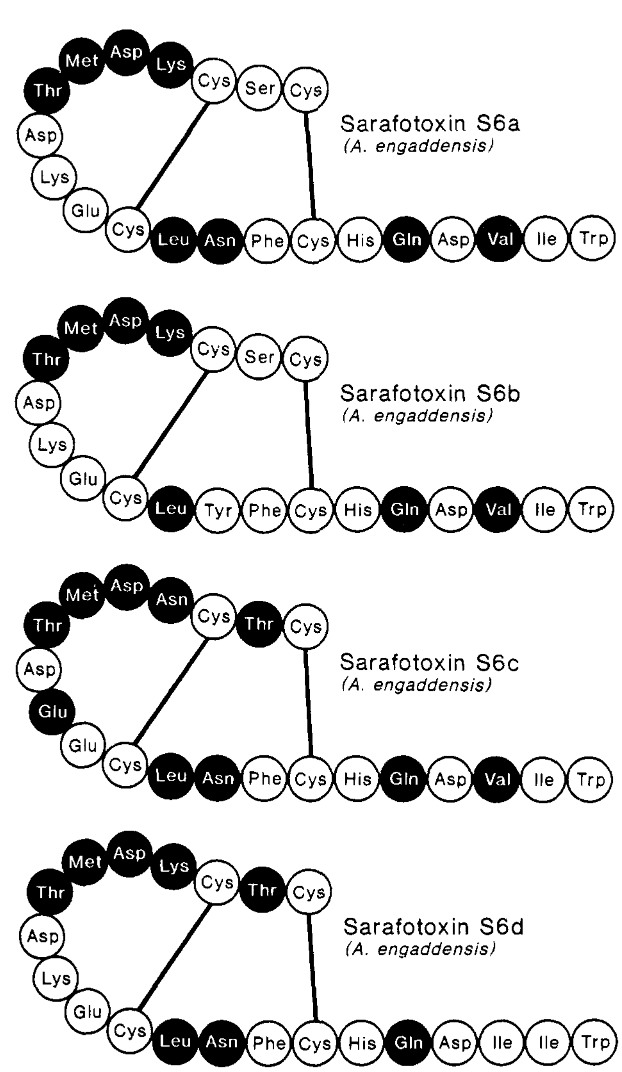

Figure 2. Amino acid sequences of the sarafotoxin peptide family. Filled circles: residues different from those in ET-1.

mammalian and nonmammalian species. Over the last 3 years this peptide has drawn the attention of many investigators because of its unique structure (Figure 1) and numerous biological actions (Table I).

In this Perspective some of the biological actions of the ET's (Table I) are described and the evidence for a possible involvement in a variety of diseases are discussed (Table II). The final sections of this article discusses two possible methods to mediate the effects of ET and its isopeptides. Clearly the development of selective receptor antagonists and/or processing inhibitors is eagerly awaited and may provide novel therapeutic agents for the treatment of a variety of human diseases.

Identification and Characterization

Endothelin is a 21-amino acid vasoconstrictor belonging to a new class of peptides. It was originally discovered in the supernatant of cultured bovine aortic endothelial cells, and subsequently isolated from cultured porcine aortic endothelial cells.[15,16] The primary sequence of human endothelin has been deduced from a human placental cDNA library and found to be identical to that of porcine endothelin, now referred to as endothelin-1 (ET-1).[17] Since the initial identification of ET-1, two other related peptides have been reported and designated endothelin-2 (ET-2) and endothelin-3 (ET-3), differing by 2 and 6 amino acid residues, respectively.[18,19] All three forms appear to be distinct gene products.[20] Two endothelin-related genes

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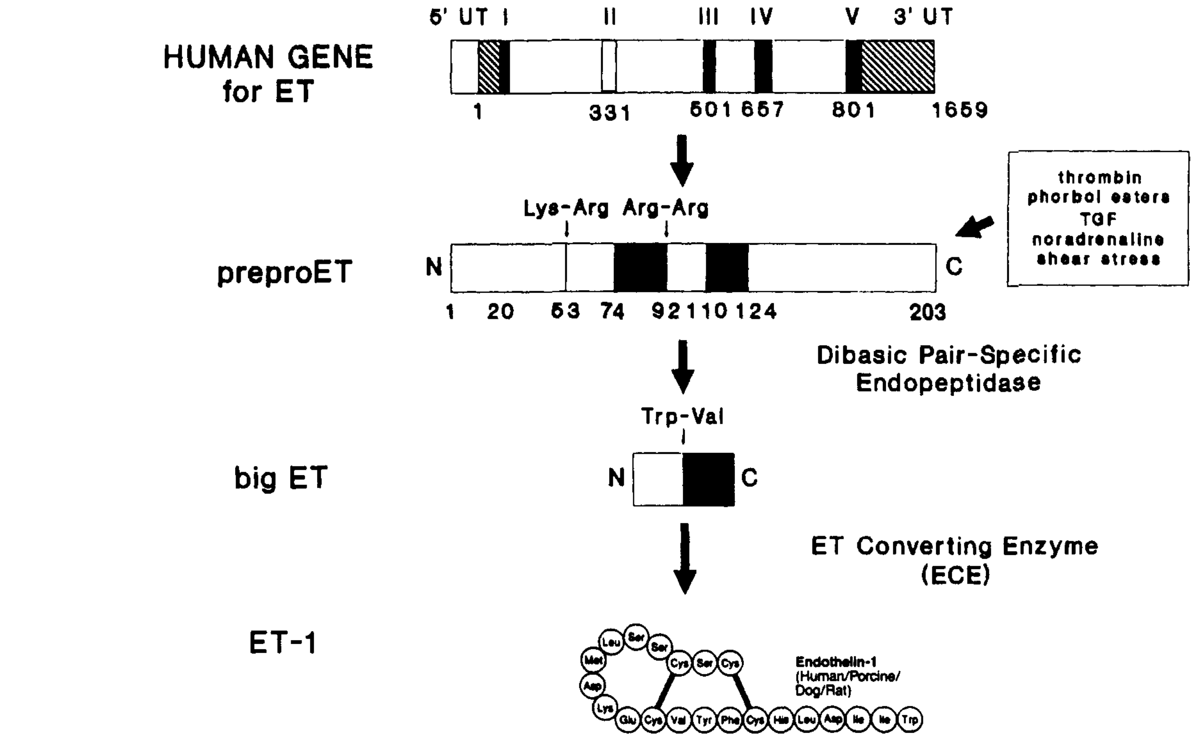

Figure 3. Biosynthesis of endothelin.

were identified by cloning and sequence analysis of the mouse genome.[21] One encoded the peptide ET-1, while the other encoded a new peptide differing by three amino acid residues. The gene for this novel peptide is only expressed in the intestine and has been referred to as "vasoactive intestinal contractor" (VIC).[21] The structures of these peptides, endothelin-1, -2, and -3 and the related peptide, VIC, are shown in Figure 1.

A related group of cardiotoxic peptides, known as the sarafotoxins (SRTX's) isolated from the venom of the Israeli burrowing asp, *Atractaspis engaddensis*, show a remarkable sequence homology with the endothelin peptides, suggesting an ancient and common evolutionary origin (Figure 2).[22,23]

Biosynthesis and Tissue Distribution of ET-1 and Related Peptides

ET-1 is derived from a 203 amino acid peptide precursor known as preproendothelin, which is cleaved after translation by endopeptidases specific for the paired dibasic residues to form a 38 (human) or 39 (porcine) amino acid peptide, proendothelin or big ET (Figure 3).[24] The biological significance of differences in the amino acid sequence between the prepropeptides and the presence of an ET-like peptide within preproET are presently unclear. The identity of the dibasic endopeptidase is not currently known. Big ET is then converted to active ET by a putative endothelin converting enzyme (ECE) (Figure 3).[16,25] The physiological importance of cleavage of ET(1–39) is indicated by the reported 140-fold increase in vasoconstrictor activity upon cleavage to ET-1.[26] There has been some speculation that the biosynthetic pathway may be tissue and possibly species specific. A further complication is the possibility that the endothelin isopeptides may be processed by different pathways[27] although there is no conclusive evidence at the present time.

ET-1 mRNA is widely expressed in rat, porcine, guinea pig, and human tissues.[28] The distribution of the proppeptide, big ET and immunoreactive (ir) ET-1, has been compared in porcine tissues.[29] The concentration of ir big ET was highest in the aortic intima and lung, while the highest concentration of ir ET-1 was found in the kidney inner medulla.[30] The broad range of binding sites indicates that ET may function in the regulation of a variety of organ systems.

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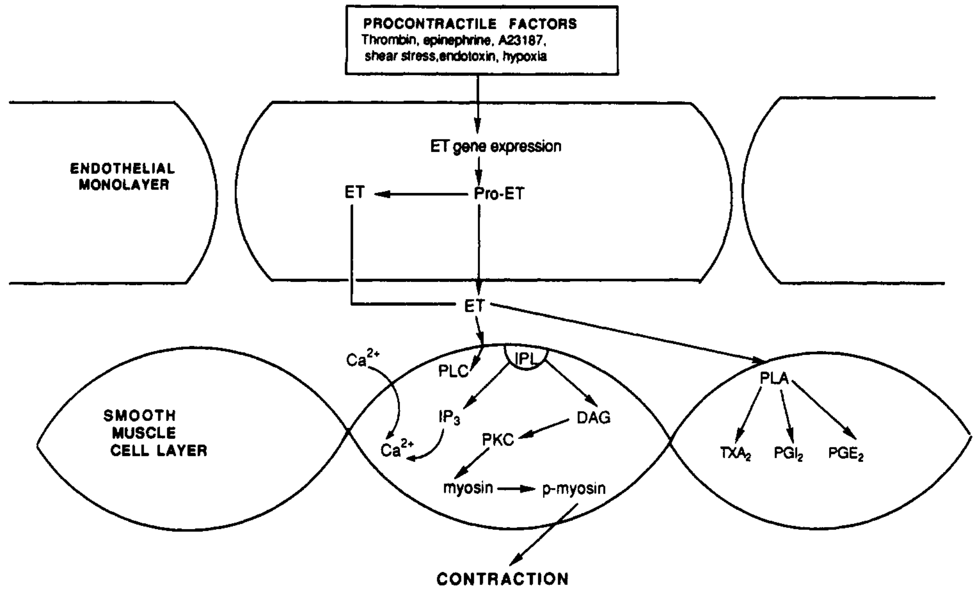

Figure 4. Pathways of intracellular transmembrane signalling.

Table I. Biological Actions of Endothelin

tissue/organ	effect
vascular smooth muscle	long-lived constriction of isolated vascular muscle[13,67,68] mitogenic actions in cultured smooth muscle cells[69] release of endothelium-derived relaxing factor[70] coronary arterial vasoconstriction, increased perfusion pressure[71]
nonvascular smooth muscle	constriction of intestinal, tracheal and uterine smooth muscle[72]
heart	increased contractility[73] increased heart rate[74] stimulation of ANP release[75]
nervous tissue	enhanced neurotransmitter release[76]
kidney	inhibition of renin release[77] decrease in renal blood flow, decrease in glomerular filtration rate and urinary Na^+ and K^+ excretion[30]
adrenal glands	stimulation of aldosterone biosynthesis[78] release of catecholamines[79]

In addition to endothelial cells, from which endothelin obviously derives its name, ET-1 is produced by mesangial, kidney, and epithelial cells and also by various human cancer cell lines and human macrophages.[31-35] ET gene transcription occurs in a variety of functional regions in the human brain, especially the hypothalamus.[36] Evidence for transcription and expression of the ET-3 gene in the human placenta and the ET-2 gene in human tumor cells has only recently been reported.[37,38]

The expression of the preproendothelin gene in cultured cells is stimulated by thrombin, TGF-β, epinephrine, vasopressin, phorbol esters, and the calcium ionophore A23187 (Figure 4).[25] Other conditions that cause its release include increased shear stress, hypoxia, oxyhemoglobin, elevated glucose levels, and endogenous digitalis-like factor.[39-43] In the intact circulation, thrombin

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Table II. Therapeutic Implications: Possible Physiological Roles and Beneficial Actions

Cardiovascular diseases	Renal disease
myocardial ischemia	acute/chronic renal failure
congestive heart failure	
arrhythmia	Vascular disorders
unstable angina	atherosclerosis
hypertension	other vascular disorders, e.g. Buergers disease, Takayasu's arteritis
	Raynaud's phenomenon
Bronchoconstriction	complications in diabetes
pulmonary hypertension	
asthma	Cancer, esp. pulmonary carcinoma
Neuronal action	Gastric mucosal damage
cerebral vasospasm	gastrointestinal disorders
subarachnoid hemorrhage	
Endocrine	Other
pre-eclampsia	endotoxic shock, septicemia
Beneficial actions of ET?	
physiological regulation of blood pressure	
neuroendocrine regulation	
closure of umbilical vessels	
wound healing	
control of menstruation	

and A23187 have been demonstrated to enhance ET-1 release, while EDRF inhibits its production.[44]

An improved understanding of the regulation of ET secretion is likely to lead to novel pharmacological approaches to diseases associated with altered production of ET.

Cardiovascular Actions

During the last 3 years the literature on the actions and possible physiological and pathological roles of the ET peptides has been expanding rapidly.[45-47]

The ET's elicit a long-lasting vasoconstriction in almost all arteries and veins.[48] Numerous reports have described the effects of ET on the cardiovascular system in vitro and in vivo. Some of these actions are summarized in Table I and have been reviewed recently.[49]

Intravenous infusion to normotensive and spontaneously hypertensive rats causes a transient hypotensive effect, followed by a sustained pressor response with a reduced cardiac output.[50,51] The pressor responses of ET-1 are reduced by Ca^{2+} channel blockers and the K^+ channel opener, cromakalim.[51] Interestingly, low concentrations of ET-1 in mammary artery rings potentiated contractions to norepinephrine and serotonin, suggesting that ET may play an important role in acute ischemic disorders associated with platelet activation.[52] The cardiovascular responses to VIC, ET-2, and S6b are similar to those of ET-1, eliciting biphasic changes in arterial pressure and increased central venous pressure, cardiac output, and pulmonary arterial pressure.[53,54] In addition, S6b and ET-1 are reported to have potent inotropic and negative chronotropic effects on isolated perfused hearts and to induce coronary vasospasm, severe arrythmia, atrioventricular block, and lethal ventricular fibrillation.[55]

In contrast, low-dose infusions of ET-1 and -3 have elicited only a vasodilatory action.[56] There have been several reports describing the initial transient but potent vasodilation of ET that appears to be selective for certain arterial beds.[57,58] The effect has been observed to occur

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in vivo in ganglionic-blocked animals and therefore cannot be due to a reflex response to the vasoconstrictor component.[57] Low doses of ET-3 have been reported to cause continuous vasodilation of mesenteric arteries preconstricted with norepinephrine and to be accompanied by elevation of cyclic nucleotides.[59] It is possible that the vasodilation elicited by the ETs is mediated via a single receptor subtype[60] in certain tissue beds, although definitive proof for this is not available. If the two activities of ET can be separated pharmacologically, then selective agonists that mediate this vasodilator response might be of interest for the development of diseases associated with vascular dysfunction.

Intravenous infusions of ET-1 to humans (1, 2.5, and 5.0 ng/kg per min) caused increases in mean blood pressure and serum potassium concentration,[61] while plasma concentrations of renin, ANP, and aldosterone were unchanged. ET monoclonal antibodies have been shown to attenuate ET-induced contraction of rat aortic rings and the pressor effects of ET-1 in pithed rats.[62]

The many potent effects of the ET's on the cardiovascular system have implicated this peptide class in a variety of human diseases (Table II). There have been several reports implicating ET in the pathogenesis of congestive heart failure and myocardial ischemia.[63-65] Left coronary-artery ligation and reperfusion to induce myocardial infarction in the rat heart caused a 4–7-fold increase in endogenous endothelin levels.[64] Administration of ET–antibody was reported to reduce the size of the infarction in a dose-dependent manner.[64]

In the anesthetized dog with congestive heart failure, a significant 2–3-fold elevation of circulating ET levels has been reported,[65] and studies in humans have shown similar increases.[66] In hypertension the story is less clear regarding levels of endothelin in the plasma and there really has been no consensus. Conclusions that ET is not involved in hypertension thus appear premature if one postulates a paracrine-like long-term regulation in the control of blood pressure. ET plasma levels may then be of no consequence. Studies with monoclonal antibodies may be a powerful preliminary tool to investigate a pathophysiological significance for ET in essential hypertension.

Mechanism of Action

A direct involvement of endothelin with the slow calcium channel was originally postulated by Yanagisawa and coworkers.[16] However, subsequent studies have demonstrated that the vasoconstrictor response to endothelin can be observed in calcium-free conditions, and that in certain tissues calcium channel blockers have little effect on the response to endothelin.[80,81] Thus although extracellular calcium appears to be important, direct activation of the voltage-sensitive Ca^{2+} channels does not seem to be in-

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volved. The response to endothelin is also not blocked by a variety of receptor antagonists and enzyme inhibitors, including phentolamine, nordihydroguaiaretic acid, atropine, methysergide, diphenhydramine, tetrodotoxin, and indomethacin.[16] The sodium-calcium exchange inhibitor dichlorobenzamil totally blocked the constrictor response to ET in isolated rat aortic rings, suggesting that endothelin plays a role in stimulating sodium movement into the cell.[82]

ET-1 binds to its G-protein coupled receptor to activate phospholipase C (PLC), resulting in increased formation of inositol tris- and bisphosphates (IP) and 1,2-*O*-diacylglycerol (DAG), with subsequent stimulation of protein kinase C (PKC) (Figure 4).[83,84] These events have been implicated in the initial rise in intracellular calcium ($[Ca^{2+}]_{int}$) and phosphorylation of myosin light chains leading to the vascular contractile responses of ET. Preliminary experiments suggest that a pertussis toxin sensitive G-protein couples ET receptors to PLC (Figure 4). Further work is needed to understand the pathways of calcium influx activated by ET. PKC appears to inhibit ET-induced Ca^{2+} signalling, thereby serving as a negative feedback signal. There is some evidence that ET activates phospholipase A_2 (PLA_2) in cultured smooth muscle and mesangial cells, causing stimulation of the arachidonic acid cascade.[85] It is not known whether ET activates PLA_2 directly via a G protein or indirectly by increasing intracellular Ca^{2+}. The initial transient vasodilator action of the ET's has been attributed to the release of PGI_2 (prostaglandin I_2) and/or EDRF.[9] As discussed previously, the vasodilator and vasoconstrictor effects may be mediated via different receptors.

Clearly the study and elucidation of the signal transduction pathways involved in the biological actions of the ET's will be an area that continues to be studied intensely. Intervention at the ET receptor signalling level could provide novel therapeutic agents to study its role in the pathophysiology of various disease states. Conceptionally one might ask how would it be possible to develop agents acting at a stage beyond the receptor that might attain sufficient specificity? However, there are several possible ways in which this might be achieved. Molecular cloning has revealed the existence of isozymes of both PKC and PLC. In addition the heterogenous distribution, both tissue and intracellular localization, of IP_3 receptors and PKC isozymes, offers an opportunity for development of selective therapeutic agents. Intervention of the PLA_2/arachadonic acid signalling pathway or in the modulation of cell responsiveness at the G-protein level are other possibilities. Finally there may be an excess or lack of second messengers associated with ET-derived disease states.

The Control of Renal Function

Several studies have shown that ET-1 has an important impact on the kidney, causing reduction in renal blood flow and urinary sodium excretion.[86] Very low concentrations of ET-1 cause intense long-lasting renal vasoconstriction. In contrast to ET-1, bolus injection of big ET to conscious rats did not change renal blood flow or renal resistance. Both peptides caused a dose-dependent diuretic and natriuretic response. These effects seem to indicate different mechanisms of action for the two peptides.[87] In DOCA-salt hypertensive rats (treated with deoxycorticosterone acetate), ET clearance is reduced, resulting in a lower renal blood flow compared with normotensive controls.[88] The reported inhibition of renin release both in vitro and in vivo is not antagonized by nicardipine or nifedipine; however, the presence of extracellular calcium is clearly important. Platelet activating factor antagonists have been reported to inhibit the effects of ET on renal function and mesangial cell contraction.[89]

In the kidney, mRNA for ET has been detected in the cortical and medullary regions. Autoradiographic studies in rats have localized ET receptors in the renal artery and vein, glomerulus, arcuate artery, interlobular artery, vascular bundle, and renal papilla. The vasoconstrictor effects of ET on renal hemodynamics are significantly modified by its ability to enhance production of vasodilators, including prostacyclin.[30]

ET-1 and -3 levels have been shown to be elevated in patients undergoing hemodialysis treatment, implicating their involvement in the pathogenesis of chronic renal failure. There is also evidence that ET participates in cyclosporine-induced renal failure since cyclosporine increases ET synthesis and release.[90] Studies by Kon and colleagues using anti-ET antibodies in an ischemic kidney model to deactivate endogenous ET indicated the peptide's involvement in acute renal ischemic injury.[91]

Mitogenic Actions. Involvement in Vascular Disorders

ET-1 has been reported to be a potent mitogen in fibroblasts and rat aortic smooth muscle cells and has been implicated in the pathophysiology of atherosclerosis.[69,92] Other studies have indicated that ET is in fact a co-mitogen in the presence of platelet derived growth factor.[93]

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ET-1 has also been shown to stimulate mitogenesis in glial cells in the rat brain, and may play a role in wound healing after neuronal injury.[94] In addition, ET stimulates hypertrophy of neonatal rat cardiac myocytes that can be reversed completely by the protein kinase C inhibitor H-7. The growth factor activity of ET-1 and ET-3 on vascular smooth muscle can be inhibited by atriopeptin III (ANP 5-28).[95] The broad spectrum neuropeptide antagonist [D-Arg^1, D-Phe^5, D-Trp^{7,9},Leu^{11}]substance P previously reported as an ET/VIC antagonist blocking the binding of ^{125}I-ET-1 (IC_{50} = 40 μM), and its Ca^{2+} mobilizing and mitogenic effects in mouse 3T3 cells, appears not to be characterized as a competitive inhibitor.[96] Several groups have found that ET-1 does not directly induce or modulate the aggregation of human platelets in vitro.[97] However, ET can inhibit platelet aggregation in vivo, presumably via release of EDRF or prostacyclin.[98] Interestingly, it has recently been reported that platelets can directly stimulate ET expression and biosynthesis in cultured bovine and human endothelial cells.[99] Thus platelets appear to regulate endothelium-dependent constricting factors and indeed there may be a feedback mechanism that exists from endothelial cells to platelets.

Abnormal proliferation of the vascular smooth muscle underlying endothelial cells is observed in a number of diseases including atherosclerosis. The co-mitogenic properties of ET have suggested an involvement in these vascular disorders. It is intriguing that EDRF inhibits mitogenesis in vascular smooth muscle cells while ET promotes growth. Thus a sensitive regulatory mechanism exists within the endothelial cell and imbalance of these factors may cause vascular dysfunction. Structural vascular changes may be of importance in the long-term determination of vascular resistance and of large artery compliance, both of which are important factors in hypertension.

Chemotactic Effects. Involvement in Wound Healing?

The ET's have been demonstrated to possess chemoattractant activity for human neutrophils which was inhibited by the FMLP (formyl-Met-Leu-Phe) antagonist ^{t}Boc-Phe-Leu-Phe-Leu-Phe.[100] Interestingly, the monocyclic loop Ac-ET-1(3-11)-NH_2 was equipotent with ET-1. Since it has been shown that the loop region of ET exhibits no binding to rat heart receptors,[101] the chemotactic effects are clearly unrelated to receptor affinity in this tissue.

There have been few reports that ET agonists may be beneficial, but its chemoattractant properties suggest a possible role in wound-healing processes.

Bronchopulmonary Effects

The effects of ET-1 on the bronchopulmonary system have been reviewed recently.[102] ET-1 is one of the most potent contractile agonists known in human isolated bronchus (EC_{50} = 18.3 nM) and pulmonary artery (EC_{50} = 3.2 nM). A significant inhibitory effect of the platelet activating factor antagonist, BN 52021 (ginkgolide B), or specific thromboxane receptor antagonists on the bronchopulmonary response induced by ET-1 has been reported in a variety of species, supporting a role for the involvement of cyclooxygenase products in its effects.[103,104] However, in the human lung these contractile effects do not seem to be effected by extracellular Ca^{2+} concentration nor to the release of prostaglandins or thromboxane A_2. A recent report, demonstrating the vasodilatory properties of ET-1, -2, and -3 in vivo, indicates that the pulmonary vasodilation observed depends, in part, on the potassium channel activation.[105]

Human bronchial smooth muscle cells possess specific binding sites for ET-1 and the human bronchial epithelial cells have been shown to secrete an ET-like material.[106] ET immunoreactivity has been localized to pulmonary endocrine cells, especially in the fetal lung and in non-small carcinomas of the lung. It is tempting to postulate that the mitogenic activity of ET may play a role in the embryological development of the lung or in the pathology of pulmonary tumours. There appear to be significant interspecies differences in airway responsiveness of ET-1 in a variety of smooth muscle preparations.

The role of the lung in the clearance of ET is controversial. There have been suggestions of a clearance receptor, similar to the well-known ANP clearance receptor; however, there is little evidence for this at present.

Increased levels of ET-1 in bronchoalveolar lavage fluid during asthmatic attacks may suggest its involvement in the control of bronchial tone.[107]

Gastrointestinal Effects

In the gastrointestinal tract, autoradiographic studies have shown that ET receptors are present in the mucosal

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layer of rat colon, intestine, and stomach.[108] Both ET-1 and ET-3 cause contraction of rat stomach strips, rat colon, and guinea pig ileum. Local intraarterial infusion of ET causes hemorrhagic and necrotic damage in rat mucosa, and thus ET has been implicated in the pathogenesis of ulcerative diseases of the stomach.[109] However, there appear to be few binding sites for ET in the antrum of the stomach and duodenum of the rat, where ulcers are likely to occur. Further studies in the human gastrointestinal tract are required to elucidate whether ET has any physiological or pathological role in the control of gastrointestinal function.

Is ET a Neuroendocrine Modulator?

There have been many studies indicating the widespread occurrence of ET receptors in the brain of both animals and humans.[110] Immunoreactive ET-1 has been detected in cerebral cortex, cerebellum, brain stem, basal ganglia, and hypothalmus of the human brain, with a much lower abundance in the pituitary gland. Colocalization studies in the cortex have shown that ET-1 mRNA and immunoreactivity are present in cells that express NPY mRNA and immunoreactivity.[111] Astrocytes from brain cortex have the ability to produce and release ET-3 and possess a single class of binding sites with comparable affinity for ET-1, -2, and -3.[112] This ET receptor differs from that characterized from endothelial cells in brain microvessels that appears to be selective for ET-1, while recognizing ET-3 with only low affinity. ET peptides and their precursors have been detected in the human cerebrospinal fluid in addition to big ET-1, -2, and -3. As a result ET-1 and/or ET-3 have been suggested to act as neuropeptides, playing an important role in the control of neuronal function.

ET-1 has been shown to release vasopressin from perfused rat hypothalmus and SP from perfused rat hypothalmus and anterior pituitary gland.[113] Intracerebroventricular (icv) injection of ET-1 to conscious rats caused dose-dependent elevation of arterial pressure and increases in heart rate that could be significantly attenuated by the icv pretreatment with nicardipine and nicorandil. This indicates that ET may elevate $[Ca^{2+}]_{int}$ of sympathetic nerve activity regulatory neurons of the brain.[114] ET-1 has been reported to stimulate gonadotropin release in perfused pituitary cells.[115] ET-3 inhibits prolactin secretion and stimulates the release of luteinizing hormone, follicle stimulating hormone, and thyroid stimulating hormone from primary monolayer cultures of rat anterior pituitary cells.[116] In fact, ET stimulates pituitary gonadotropin release as efficiently as gonadotropin-releasing hormone. Thus both ET-1 and ET-3 are suggested to act as neuroendocrine modulators.

The pathogenesis of cerebral ischemia, often observed following subarachnoid hemorrhage, is a subject of intense study. Endothelin levels in the cerebrospinal fluid of such patients (both ET-1 and ET-3) are indeed substantially elevated, indicating ET's possible involvement in cerebral vasospasm and the subsequent neurologic deterioration.[117]

Diabetes

ET is a potent agonist in the liver eliciting both a sustained vasoconstriction of the hepatic vasculature and a significant increase in hepatic glucose output.[118] Moreover, it has recently been reported that insulin stimulates ET-1 gene expression in endothelial cells.[119] These results indicate that ET may be an important factor contributing to vascular complications associated with diabetes.

Endocrinological Effects

There have been some interesting theories that the ET peptides may be involved in the formation of life in addition to its destruction although these seem a little dramatic! An approximately 5-fold increase in amniotic fluid ET-like immunoreactivity was measured at term as compared to mid-trimester values, suggesting a possible involvement in the closure of the umbilical vessels occurring at delivery.[120] Interestingly micro-autoradiography has revealed high densities of iodinated ET-1, -2, and -3 in the human uterus localized to glandular epithelial cells and blood vessels, leading the authors to suggest a possible role in the control of menstruation.[121] Endothelin is also thought to play a role in pregnancy-induced hypertension.

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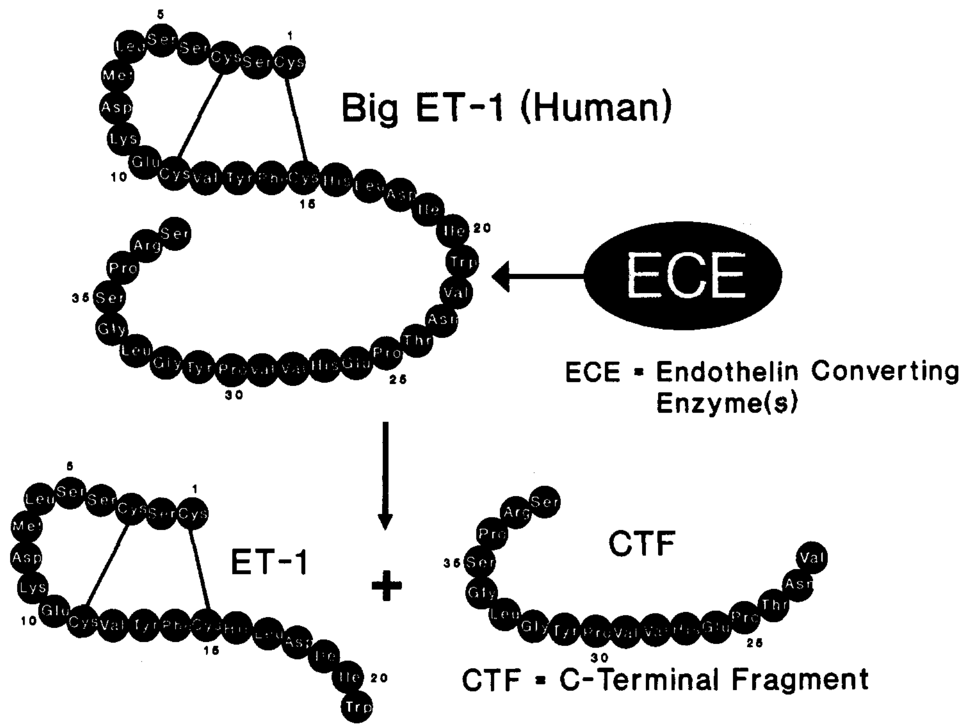

Figure 5. Conversion of big ET-1 to ET-1.

Therapeutic Implications

Reading the literature on the ET peptide family over the last 3 years, might bring one to the conclusion that they are involved in every disease known to man! Plasma concentrations of ET have been reported to be elevated in many cardiovascular disorders and in an amazing variety of other diseases (Table II). The relevance of these data is unclear at present, since it is not known whether ET acts as a local regulatory peptide or as a circulating hormone. Indeed many of the reported elevations are marginal and there are several conflicting results from different groups. The levels of extraction of ET from the plasma have been found to be low and variable and thus the accuracy of plasma ET measurements reported may also be in question. If ET acts as a local regulator on the vascular smooth muscle (Figure 4), then measurement of ET blood levels may be a fruitless exercise. Alternatively, slight elevations of ET in the bloodstream may represent a "spill-over" that could be a marker for some disease states. Interestingly, Burnett and co-workers recently demonstrated that exogenous infusion of ET (2.5 ng/kg per mL) to anesthetized dogs, producing a doubling of the circulating concentration, did have biological actions.[122] Thus heart rate and cardiac output decreased in association with increased renal and systemic vascular resistances and antinatriuresis. These studies support a role for endothelin in the regulation of cardiovascular, renal, and endocrine function. There has been little attempt to study circulating ET-2 and -3 levels probably because plasma levels are so low, although the presence of ET-3 in the brain has suggested a role in neuronal function.

The development of transgenic models expressing elevated quantities of ET would be of great interest but the likelihood of success is questionable since such a model may not survive very long!

ET may be involved in some of the diseases listed in Table II although it should be reemphasized that elevated circulating levels certainly do not prove a causal relationship. It would be premature to speculate in which disease states ET will eventually be proven to play an important role. Indeed there are different proponents according to the bias of individual research groups! I do believe however that ET modulators will find a central role in various cardiovascular diseases such as congestive heart failure and myocardial infarction. A list of possible other (beneficial) functions of the ET's is also included in Table II to indicate possible physiological roles of ET or potential uses for ET agonists.

Endothelin Processing. Design of ECE Inhibitors

Much effort has been expended over the last couple of years to identify ECE(s) (Figure 5). The main difficulty has been that several classes of enzymes appear to cleave big ET to ET in vitro. In many of the isolation procedures designed to elucidate ECE, several irrelevant enzymes may have been suggested. Initial reports from endothelial cell cultures implicated the involvement of an aspartic proteinase.[123,124] Specifically, pepsin and cathepsin D were shown to cleave big ET to ET in vitro. However, it was subsequently shown that cathepsin D causes further rapid degradation of ET-1, and thus it seems unlikely to be involved in its formation in vivo.[125,126] Human cathepsin

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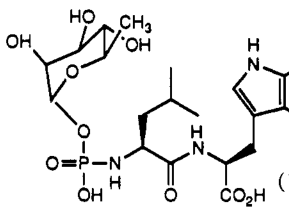
(123) Ikegawa, R.; Matsumura, Y.; Takaoka, M.; Morimoto, S. Evidence for pepstatin-sensitive conversion of porcine big endothelin-1 to endothelin-1 by the endothelial cell extract. *Biochem. Biophys. Res. Commun.* **1990**, *167*, 860–866.

(124) Sawamura, T.; Kimura, S.; Shinmi, O.; Sugita, Y.; Kobayashi, M.; Mitsui, Y.; Yanagisawa, M.; Goto, K.; Masaki, T. Characterization of endothelin converting enzyme activities in soluble fraction of bovine cultured endothelial cells. *Biochem. Biophys. Res. Commun.* **1990**, *169*, 1138–1144.

E, a closely related aspartic proteinase, has been shown to specifically cleave big ET at the Trp21–Val22 bond in vitro to produce ET-1 with no further degradation.[126] In addition, cathepsin E immunoreactivity has been detected in endothelial cells where ET is known to be synthesized. However there are no reports of studies with specific human cathepsin E inhibitors in vivo to verify the significance of these data. Other reports of attempted isolation of ECE from cultured endothelial cells and vascular smooth muscle have implicated a phosphoramidon-sensitive neutral metalloprotease.[127-129] It has recently been reported that ET-converting activity was detected in both the membranous and cytosolic fractions of cultured bovine endothelial cells.[130] In addition, relatively high concentrations of phosphoramidon (1) have been shown to block the

(1) Structure of phosphoramidon



(1)

pressor response of big ET in vitro and in vivo.[131-133] One might ask the question "what else is phosphoramidon affecting?" It is a potent neutral endopeptidase (NEP) inhibitor [EC 3.4.24.11] and would be expected to potentiate the actions of a number of other vasoactive peptides including ANP. However, kelatorphan, a specific NEP inhibitor did not inhibit the biological effect, indicating that this enzyme is not ECE.[133] Indeed it is curious that the actions of ET were not potentiated by phosphor-

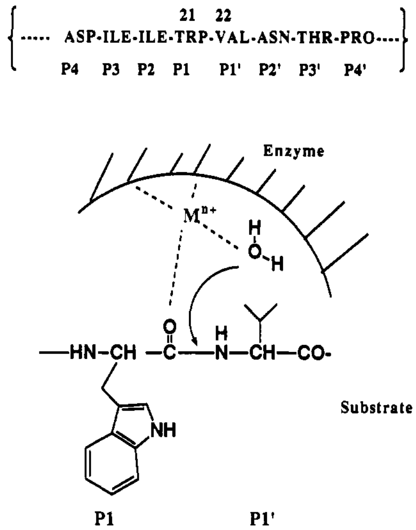


Figure 6. Enzymic hydrolysis of the P1–P1′ peptide bond.

Table III. Receptor Subtype Selectivity in a Variety of Rat Tissues[144]

	receptor binding: K_i, nM			
ligand	aorta	atrium	cerebellum	hippocampus
ET-1	0.11	0.034	0.015	0.010
ET-3	2.50	1.60	0.021	0.036
SRTX-6b	0.24	0.087	0.013	0.012
SRTX-6c	>5000	4200	0.016	0.023

amidon, because NEP 24:11 has been shown to be involved in its degradation in vitro. As with many other endogenous peptides, neutral endopeptidase 24:11 (enkephalinase) is thought to be involved in the degradation of the ET's and sarafotoxins. Cleavage at the Ser5–Leu6 bond occurs first (in vitro), followed by rapid cleavage between Asp18 and Ile19.[134] Phosphoramidon has been shown to potentiate the ET-1 induced bronchopulmonary response in guinea pigs, indicating the involvement of endopeptidase-like enzymes, present in airway tissue, in the modulation of ET-1.[135] Interestingly it has recently been reported that phosphoramidon is able to inhibit vasoconstrictor effects evoked by intravenous injections of big ET-1 in anesthetized pigs, but did not have any effect on the plasma ET-1 level.[136]

Polymorphonuclear leukocytes also effect the conversion of big ET to ET that is inhibited by 1, but not by inhibitors of serine-, cysteine-, aspartate-, or leucine-specific proteases.[137] Ultimately it may be difficult to identify the relevant ECE in vivo and will require the discovery of specific ECE inhibitors for verification. The design of substrate-based inhibitors, where the P1–P1′ peptide bond cleaved in the enzymic hydrolysis (Figure 6), is replaced by a noncleavable transition state isostere is one possible

(125) Sawamura, T.; Shinmi, O.; Kishi, N.; Sugita, Y.; Yanagisawa, M.; Goto, K.; Masaki, T.; Kimura, S. Analysis of big endothelin-1 digestion by cathepsin D. *Biochem. Biophys. Res. Commun.* **1990**, *172*, 883–889.
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approach to ECE inhibition. Another possible approach might be to modify phosphoramidon, in an attempt to increase its specificity for ECE over other metalloproteinases.

Receptor Studies

One of the most exciting advances in ET research in the last year has been the cloning and expression of two distinct receptor subtypes.[138,139] Both are G-protein coupled and belong to the rhodopsin family, with seven transmembrane domains. One, isolated from bovine lung, highly specific for ET-1 and ET-2 (MW = 48.5 kDa), is located in the periphery and CNS, and has been suggested to be the vascular smooth muscle (VSM) type.[138,140] The proposed nomenclature, based on the relative affinity of the agonists for the receptors, has termed this receptor as the ET_A type [the Second International Symposium Japan, December 1990]. The other is a "nonselective" subtype that binds ET-1, -2, and -3 with similar affinity[139,141] and has been termed the ET_B receptor. It was first thought not to be located in VSM, and reported to be localized in endothelial cells and in many rat tissues including brain, kidney, and liver.[141] There is some further evidence for an ET-3 specific receptor subtype that is located primarily in brain and in endothelial cells although this putative subtype has not been cloned.[142,143]

There are many studies ongoing in a variety of animal tissues attempting to elucidate the existence and distribution of ET receptor subtypes. Comparison of the receptor affinities of various ET's and SRTX's in rat aorta and atria (ET_A) or cerebellum and hippocampus (ET_B) indicates that SRTX-c is a selective agonist for the cerebellum/hippocampus receptor(s), i.e. an ET_B selective ligand (Table III).[144] A recent study indicated that this ligand exerted only vasodilation in the rat aortic ring, possibly through the release of EDRF from the endothelium.[145] Other selective ET_B ligands, for example, the linear analogue ET[1,3,11,15-Ala] and truncated analogues ET[6–21, 1,3,11,15-Ala], ET[8–21, 11,15-Ala] and *N*-acetyl-ET[10–21], have been reported to cause vasorelaxation in isolated, endothelium-intact porcine pulmonary arteries.[146] However, we have found that some of these analogues, ET[1,3,11,15-Ala] and ET[8–21, 11,15-Ala], are potent ET_B agonists causing vasoconstriction in the rabbit pulmonary artery.[147] We have found that this tissue appears to possess an ET_B nonselective type of receptor. Thus the present evidence available indicates that the physiological response mediated by the ET_B receptor in certain tissue beds cannot be solely described by vasodilation. Indeed it appears that vascular smooth muscle can possess the an ET_B-like or nonselective receptor subtype. It is possible that the situation will become more complicated in the future with the discovery of further receptor subtypes.

Clearly, in view of the tissue and species differences observed to date, it will be important to determine the relevance of these reports to the distribution of receptor subtypes in the human. The use of specific antibodies to the ET_A and ET_B receptors should enable useful receptor localization studies to be performed. This may enable us to make a more informed decision on the most valuable therapeutic target for drug therapy.

Structure–Activity Relationships (SAR). Design of ET Receptor Antagonists

Initial attempts have been made to define the regions of the endothelins and sarafotoxins essential for receptor binding and vasoconstrictor activity. The discovery of selective ET receptor antagonists will facilitate identification of the physiological and pathological roles of the various ET isopeptides. To date many of the peptide structure–activity studies have been carried out in tissues and species that express unknown receptor subtype populations. Clearly, it will be important in future studies to profile these tissues and to evaluate compounds in specific receptor binding assays, a task that has been facilitated by the cloning and expression of two of the ET receptor subtypes.

Obviously it would be highly desirable to discover selective, non-peptide ligands for the ET receptor subtypes. When one reviews the renin–angiotensin system and the methods that have been attempted to modulate the vasoactive properties of angiotensin II (AII), it becomes clear that random screening followed by "rational design" played an important part in the discovery of lead structures that eventually led to the ACE (angiotensin converting enzyme) inhibitors and more recently to some non-peptidic AII antagonists. The renin inhibitor problem has been much more difficult to solve, perhaps due to the size of the substrate and specificity of the enzyme. Clearly there are many examples of progress made in approaching peptidomimetic leads from peptides, but the transition from peptidomimetic structures to a truely non-peptide lead, using "rational design", is still difficult to solve, poorly understood and a time-consuming endeavour.

Structural Studies. The 3-dimensional structure of ET has been studied by NMR, molecular dynamics simulation (with energy minimization), and circular dichroism (CD)[148–150] in an attempt to determine specific conforma-

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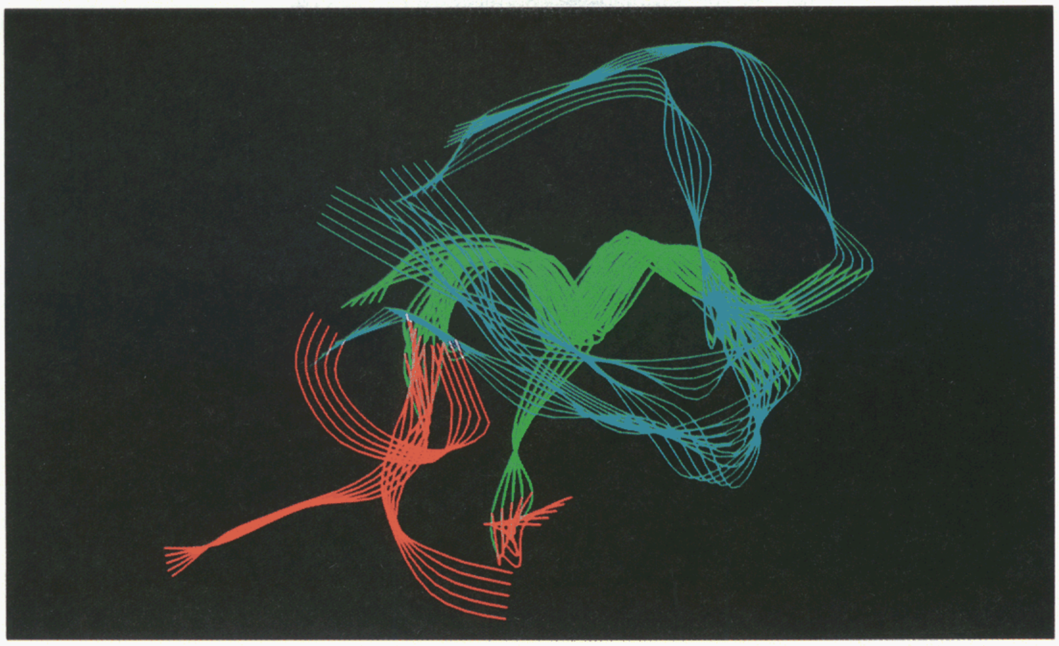

Figure 7. Ribbon diagrams of representative structures of ET-1 consistent with the NMR data.[151] These were chosen to illustrate the range of possible conformers. The blue ribbons show residues 1–8, green ribbons are residues 9–15. The yellow is the transition from the helical area to the C-terminal residues 16–21 which are in red.

tional features that may be important to receptor binding and vasoconstrictor activity. CD studies indicate that ET-1 is about 30–35% helical, and although there are some variations, the helical region is generally considered to exist between residues Lys9 and Cys15. The fully linear peptide ET-1[1,3,11,15-Ala] shows no helical character and thus the disulfide arrangement seems to induce this helicity. There have been conflicting reports of the conformation of the biologically important C-terminal hexapeptide, but most studies are unable to define this apparently flexible region. Figure 7 provides an illustration of the backbone conformation of six low-energy structures of ET-1 derived from NOE constraints and molecular mechanics calculations.[151] Of course in the receptor environment the conformation derived from solution structure, in a variety of different solvents, may not be relevant and the structural studies carried out by some research groups[149] indicating that the C-terminal region is located close to the bicyclic portion of ET-1 may then become a reality.

Structure–Activity Studies. (a) Full-Length Analogues. The different biological potencies of the endothelins and sarafotoxins have largely been attributed to the sequence heterogeneity in the N-terminal region of the peptides, specifically between residues 4–7.[152,153] The four cysteine residues at positions 1, 3, 11, and 15, the carboxyl-terminal hexapeptide region [16–21], the aromatic dipeptide at positions 13 and 14, and the charged loop region, Asp8-Lys9-Glu10, are highly conserved among the endothelins (Figure 1).

ET-3 appears to be the weakest vasoconstrictor in the ET family and even acts as a vasodilator in some vascular beds. The substitution of serine with threonine at position 2 in ET-3 is shared by two weak constrictor peptides in the sarafotoxin family, SRTX-c and SRTX-d, suggesting its importance. However structure–activity studies of various substituted sarafotoxins have indicated that the Lys9 to Glu9 substitution results in a much larger loss of biological activity than either the Ser2 to Thr2, Lys4 to Asn4, or Tyr13 to Asn13 substitutions.[154] Thus, the low lethality and vasoconstrictor activity of SRTX-d is somewhat unexpected in view of its structure, which only has the Ser2 to Thr2 and Val19 to Ile19 differences from SRTX-b (Figure 2).[23] It has been proposed that the net charge within the Cys3-Cys11 loop may be important for biological activity, although SRTX-d, which possesses extremely low biological activity, indicates that this is not the case.[152]

The cyclic structure of these peptides would appear to be essential for binding and functional activity only in certain tissues,[155,156] for example, in the rat and porcine aorta (ET_A), where the outer disulfide bond Cys1-Cys15 would appear to be much more important than the inner Cys3-Cys11 bond.[157] In other studies, reduction and

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Table IV. C-Terminal-Containing ET Peptides[167]

peptide	IC_{50}, μM binding affinity[a]	IP$_3$ accum[b]
His-Leu-Asp-Ile-Ile-Trp	44	<50
Ac-His-Leu-Asp-Ile-Ile-Trp	58	<50
Ac-His-Leu-Asp-Ile-D-Ile-Trp	13	<50
Ac-D-His-Leu-Asp-Ile-Ile-Trp	3.7	1.4

[a] Binding affinity in rat heart ventricle. [b] Inositol phosphate accumulation in rat skin fibroblasts.

carboxymethylation of the cysteine residues caused a complete loss of agonist activity in rat isolated perfused mesentery and also the tetraalanyl analogue ET-1-[1,3,11,15-Ala] was functionally inactive in the rat mesenteric bed and rat isolated aorta.[158,159] However, the structural requirements for binding to rat cerebellum (thought to contain the ET_B receptor) do not require the presence of the disulfide linkages as illustrated by the equipotent binding of the tetraalanyl-substituted analogue ET-1[1,3,11,15-Ala] and ET-1.[159] These results in various tissues clearly indicate a different distribution of receptor subtypes in the brain (ET_B) and in cardiac tissues (ET_A) (also see Table III).[159]

Removal of the C-terminal tryptophan residue reduces the vasoconstrictor potency in porcine coronary artery strips by 3 orders of magnitude, and in a rat pulmonary artery preparation substitution with other aromatic residues such as Phe and Tyr is only poorly tolerated.[160,161] L-Stereochemistry of the C-terminal Trp is clearly important.[160] Progressive deletion of the C-terminal residues decreases receptor binding and vasoconstrictor activity, with ET[1–15] being essentially inactive.[155,161]

Receptor binding results in cultured rat smooth muscle cells (presumably ET_A) revealed that ET[1–23], ET[1–26], and ET-1 were equipotent, although functional studies demonstrated that these C-terminal elongated peptides were weaker agonists.[162] This series could yield receptor antagonists.

(b) Linear and Monocyclic Fragment Analogues. Several reports describing receptor binding and functional activities for the C-terminal hexapeptides,[163–165] using a variety of tissue preparations for evaluation, have appeared. Maggi and co-workers have reported the ET[16–21] is a full agonist on guinea pig bronchial tissue with 33 times lower potency than ET-1.[165] In contrast, ET[16–21] was devoid of any agonist or antagonistic activity in most other tissues.[160,166] Functional studies in the guinea pig bronchus, indicate that the Trp21, Asp18, His16, and Leu17 residues are important for biological activity in this tissue. D-Amino acid substitutions at His16 and Ile20 resulted in increased binding affinity for rabbit aorta, pulmonary artery, and rat heart receptors[165] compared with their corresponding L-amino acid containing analogues. In a functional biochemical assay measuring intracellular levels of second messengers (IP$_3$ accumulation) we have found that compounds with D stereochemistry at position 16 are indeed ET receptor antagonists (Table IV).[167] In a binding assay to rabbit cardiac tissue, ET[1–20], ET[1–15]-NH$_2$, or the C-terminal hexapeptide ET[16–21] were completely inactive at concentrations up to 10 μM.[168]

Binding (A10 vascular smooth muscle cell membranes, ET_A) and vasoconstrictor activities (rabbit carotid rings) of a range of monocyclic analogues, ET-1[Ala3,11,Nle7], containing Ala substitutions at each position, have indicated that Glu10, Phe14, Leu17, and Asp18 may be important for agonist activity, while Asp8, Tyr13, Ile20, and Trp21 are important for binding.[169] The residues Ser2, Val12, His16, and Ile19 were less important for binding or agonist activity in this series. It is interesting to note that the SAR is clearly different for linear ET fragments and monocyclic or bicyclic analogues. The binding affinity of various monocyclic fragments of ET-1 in rabbit pulmonary artery (ET_B like)[147] and aorta (ET_A) have indicated that the loop region 3–11 does not bind at concentrations up to 100 μM. A monocyclic analogue without the 3–11 region (i.e. disulfide [Cys-Ser-Aoc-Val-Tyr-Phe-Cys]-His-Leu-Asp-Ile-Ile-Trp where Aoc = 8-aminooctanoic acid) binds with μM affinity but shows no functional activity up to 30 μM.[101] However it should be pointed out that these monocyclic analogues have 1000-fold less binding affinity than ET-1 itself, clearly indicating the importance of the loop region.[100,168]

A number of ET-1 analogues were synthesized to investigate their effect on the pulmonary vasodilator response when compared to ET-1 itself.[170] Intralobar injections of ET[16–21], big ET-1[22–39], and ET-1-

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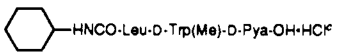
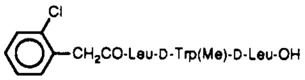
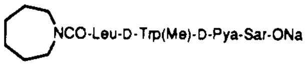
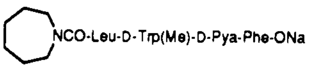
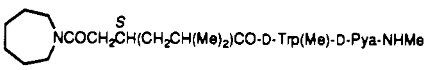
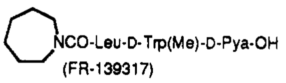
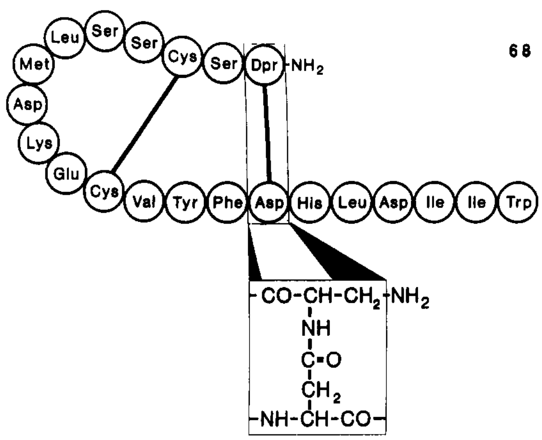
Table V. Endothelin Antagonists[177]

compound structure	binding:[a] IC_{50}, nM	functional[b] IC_{50}, nM
	2.3	230
	32	d
	7.6	d
	21	d
	7.6	d
 (FR-139317)	2.5[e]	d

[a] Binding affinity in porcine aortic membranes. [b] Inhibition of the contractile response of endothelin in rabbit thoracic aorta. [c] D-Pya = D-(2-pyridyl)alanine. [d] Not reported. [e] Human aorta.

[1,3,11,15-Ala] to the intact cat did not alter arterial blood pressure while ET-1, ET[1–15], and big ET caused a decrease in lobar arterial blood pressure.[170] These results indicate that only the intact amino terminus and intrachain disulfide bridges are necessary for pulmonary vasodilation.

The first reports of a variety of receptor antagonists have appeared over the last few months. A full-length ET analogue, ET-1[Dpr1-Asp15] (2), has been reported as a



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(2) ET selective antagonist

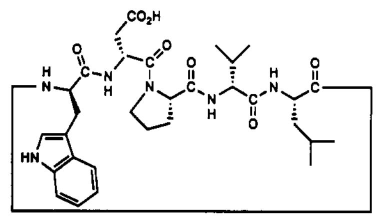
specific ET antagonist by Spinella and co-workers.[171] This compound may be a nonselective ET_A/ET_B receptor antagonist; however, binding results were not reported at a known ET_B tissue preparation (rat cerebellum). An exciting report of a cyclic pentapeptide ET_A receptor antagonist discovered by random screening of fermentation products from *Streptomyces misakiensis* has recently appeared.[172] Structure–activity studies around this peptide BE-18257B (3) have led to a more potent analogue

(3) **BE 18257B**

cyclo(-D-Glu-L-Ala-allo-D-Ile-L-Leu-D-Trp-)

BINDING

ET_A (porcine aortic smooth muscle)	IC_{50} = 1.4 μM
ET_B (rat cerebellum)	IC_{50} = > 100 μM



D-TRP-D-ASP-PRO-D-VAL-LEU

BQ-123

BINDING

ET_A (porcine aortic smooth muscle)	IC_{50} = 22 nM
ET_B (porcine cerebellum)	IC_{50} = 23 uM

(4) ET_A selective cyclic pentapeptide receptor antagonist

known as BQ-123 (4).[173,174] Binding affinity in cardiac tissue (ET_A) was reported as IC_{50} = 22 nM with a 1000-fold selectivity over binding to rat cerebellum (ET_B). Presumably studies of the conformation of this peptide should aid in the development of non-peptide antagonists and a better understanding of the structural requirements at the ET_A receptor. Thus, by comparing biological activities within this series, with 3-dimensional conformation elucidated by NMR and molecular dynamics techniques, it may be possible to elucidate those interactions important for binding to the ET_A receptor.

A non-peptide series of ET receptor antagonists, discovered by random screening from a *Streptomyces* strain, have been disclosed in a recent patent from Fujisawa although the specificity of these compounds for the ET receptors is not known.[175] The most potent compound reported (5), known as FR901367, possesses a binding affinity in porcine aorta of IC_{50} = 0.67 μM (presumably

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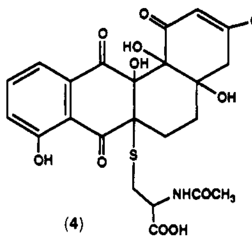

(5) Anthraquinone ET receptor antagonist from *Streptomyces* sp. No. 89009.

ET_A) and inhibits the contractile responses of ET-1 in rabbit thoracic aorta only weakly (75% at 10^{-4} M). Interestingly a very similar series of cyclic pentapeptide antagonists to those covered in the Banyu patent was very recently reported by this group.[176] Smaller tripeptidic compounds are reported by the Fujisawa group to possess ET antagonist activity.[177] Binding and functional data for the few examples are shown in Table V. Whether these compounds act selectively at the ET_A receptor is not reported.

It is clear from the results obtained to date that many of the analogues and fragments differentiate between tissues and species, and it will be important to define localization of receptor subtypes in order to develop specific and hopefully non-peptidic receptor antagonists. However, it should be remembered that the physiological responsibilities of the two receptor subtypes (ET_A and ET_B) are not clear at present, and whether it will eventually prove beneficial to block both ET_A and ET_B receptors, in certain pathological situations, is currently not known.

(176) New peptide prepared by culturing *Streptomyces* spp.—used as endothelin antagonist. Hashimoto, M.; Nishikawa, M.; Esaki, M.; Kiyoto, S.; Okuhara, M.; Takase, S.; Henmi, K.; Neya, M.; Fukami, N.; Hashimoto, M. JO 3130-299-A, filed August 8, 1990.

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Future Prospects

Since its discovery, endothelin has attracted considerable interest because of its concerted actions on the heart, vascular smooth muscle, and kidney, as well as its ability to alter the release of other hormones and neurotransmitters. Although much information has been obtained regarding the inotropic, vasoconstrictor, and mitogenic actions of endothelin, its involvement in modulating the activity of the cardiovascular system under normal conditions has not been elucidated. A better understanding of the role of endothelin isopeptides in the pathogenesis of a variety of diseases is required. Among the wealth of information that we have gained on the ET's over the last few years, there are still several key questions that need to be answered in order to understand how the ET peptides may regulate many diverse physiological and pathophysiological events. Is ET a circulating hormone or paracrine regulator? Can we develop in vivo models that relate to human diseases causing increased secretion of endothelin? Can we learn more about the control of preproET-1 gene expression? What physiological actions do the individual receptor subtypes mediate? Can we develop selective/nonselective agonists and antagonists to the ET receptor subtypes? Are there any further receptor subtypes yet undiscovered? What are the roles of ET-2 and ET-3? What is the true identity of ECE and is there a single relevant ECE in vivo? The list of questions continues and it is clear that ET will occupy the minds of many researchers worldwide for several years to come.

The discovery of pharmacological agents which either block the generation of endothelin from its precursor or antagonize its binding to cellular receptors should provide a means to assess the physiological role for endothelin, and also provide useful therapy for conditions associated with altered production or responsiveness to endothelin.

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Registry No. Endothelin, 116243-73-3.