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POSSIBLE ROLE OF ENDOTHELIN IN ENDOTHELIAL REGULATION OF VASCULAR TONE

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ABSTRACT

In vascular beds, a potent vasoconstrictor of endothelin-1 (ET-1) is produced by endothelial cells and released preferentially to the basal side of endothelial cells. It acts on endothelial cells and on the underlying smooth muscle cells, as a modulator of vascular tone, in an autocrine and paracine manner. ET-1 induces release of relaxing factors (nitric oxide and prostacycline) from endothelial cells. Whether relaxation or constriction is predominantly elicited by endogenous ET-1 may depend on the concentration of ET-1 in vascular beds, the density and mode of distribution of ET receptor subtypes on the endothelial and smooth muscle cells, the turnover of the receptors, and the existing conditions of each vascular beds. When ET-1 is overproduced by endothelial cells at pathological conditions, endogenous ET-1 acts as a vasoconstrictor. However, ET-1 may act as a vasodilator at physiologically low concentrations, depending on the existing condition.

Introduction

Endothelin (ET) is a potent vasoconstrictive 21–amino acid peptide originally isolated from the conditioned medium of cultured vascular endothelial cells (1). The peptide has attracted the attention of many investigators because of its unique amino acid sequence and pharmacological responses. When we published the first paper on endothelin (1), our search for a peptide with a

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similar sequence among the reported peptide sequences revealed that the amino acid sequence of ET is unique. It contains four cysteine residues forming two disulfide bonds in the N-terminal half and a cluster of hydrophobic amino acid residues at the carboxyl end of the peptide (1). The structure of the N-terminal domain determines the affinity of the binding to its receptor, while the C-terminal domain contains the binding site of the peptide to the receptor.

Subsequent analysis of the human endothelin genome revealed that in addition to the original ET found in cultured endothelial cells, two additional isoforms exist as well. Thus these three isoforms were designated endothelin-1 (ET-1), ET-2, and ET-3 (2). Results of further experiments have demonstrated that all three isoforms are expressed in various tissues and cells in different proportions, existing not only in vascular beds but also in noncardiovascular tissues such as neurons, the adrenal gland, and the kidney (3). This fact has considerably widened the field of ET research and has been discussed in a previous paper (3). The ET originally isolated from the medium of cultured endothelial cells was ET-1. Endothelial cells exclusively produce ET-1.

Soon after the discovery of ET, the structure of the rare snake venom (venom toxins of *Atractaspis engaddensis*) sarafotoxin (STX) was published (4). Surprisingly, its structure was very similar to those of the ETs. It also consisted of 21 amino acid residues, including four cysteine residues. Seven amino acid residues of sarafotoxin 6b (STXb) are different from those of ET-1 at the respective positions in the sequence. So far, four sarafotoxins (STXa, STXb, STXc, and STXd) have been reported (5). The structures of these ETs and STXs are summarized in Figure 1.

These endothelins and the related peptides can bind to the ET receptor, but they exhibit different potencies in their ability to elicit responses in different tissues and cells, including the vascular beds (6), suggesting that various subtypes of the ET receptor exist. These responses to ETs can be divided into two groups. In the first group of responses—which includes vasoconstriction

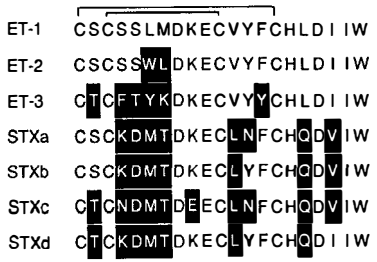

Figure 1 Structures of endothelins and sarafotoxins. Amino acids written in white boldface are different from those of ET-1. All of the endothelins and sarafotoxins have two disulfide bonds (between positions 1 and 15, and between positions 3 and 11).

Table 1 Subtypes of endothelin receptor

Subtypes	Rank order of affinity for endogenous agonists	Selective antagonists
ET_A	ET-1 = ET-2 >> ET-3	BQ-123, PD142893
ET_{B1}	ET-1 = ET-2 = ET-3	IRL1038, PD142893
ET_{B2}	ET-1 = ET-2 – ET-3	?
ET_C	ET-1 = ET-2 << ET-3	?

of most of the arteries, bronchoconstriction, and stimulation of aldosterone secretion—ET-1 and ET-2 are more potent than ET-3. In the second group, which includes vasodilation, the three isopeptides have similar potencies. In addition, the order of the affinities of ETs for the membrane fractions prepared from various tissues determined from ligand-binding studies demonstrate that there are at least two distinct subtypes of ET receptors.

Subsequently, two similar but distinct cDNA clones encoding ET receptors were isolated from bovine and rat lung cDNA libraries (7, 8). These two receptors, which correspond to the two pharmacologically distinct subtypes of the ET receptor mentioned above, are designated ET_A and ET_B (6–8) (Table 1). The differences in the ligand-binding properties and pharmacological characteristics between these two receptors have been important in the development of antagonists and in the analysis of the ET receptor.

Both receptors are also distributed in various tissues and cells in different proportions. In vascular beds, ET_B is found in the endothelial cells, and ET_A exists on the smooth muscle of most of the arteries. However, as discussed below, ET_B also exists on the smooth muscle of some vascular beds. This fact increases the complexity of differentiating among the ET-induced responses in vascular beds.

As mentioned above, ET-1 is a very potent vasoconstrictor. The EC_{50} value of the vasoconstrictive activity of porcine coronary artery in the first report on ET-1 was 3×10^{-10} M (1). This value is about 100-fold more potent than that of angiotensin II, which was the most potent vasoconstrictive peptide known until that time. In addition, the vasoconstrictive action of ET-1 is sustained for a long period of time. When a bolus ET dose, 10^{-9} g/kg, was injected intravenously into a rat, the rat's blood pressure initially decreased slightly and transiently but then began to increase. The pressor phase was sustained for more than an hour. This result naturally led many investigators in the field to the concept that ET may play an important role in the maintenance of blood pressure and the pathogenesis of essential hypertension or vasospasm.

A question may well arise as to whether ET is a circulating hormone that controls the vascular tone. To answer this question, methods to measure immunoreactive ET in plasma have been developed by several laboratories. Using a very sensitive and specific radioimmunoassay or sandwich-type immunoassay, numerous studies have reported the plasma concentrations of ET in various patients (9). The plasma concentration of ET-1 in a normal individual is very low, around 1 pg/ml. This amount is not sufficient to elicit vasoconstriction, suggesting that ET-1 is not a circulating hormone but actually a local hormone. The existence of ET receptors on the underlying smooth muscle of the endothelium supports this concept. ET-1 is produced by vascular endothelial cells and may act on the underlying smooth muscle cells in a paracrine manner. Thus, in concert with the eicosanoids and nitric oxide (NO) that endothelial cells produce, ET may play an important role in controlling the vascular tone. However, much of the physiology of the ETs in relation to the vascular tone remains to be elucidated.

Despite numerous efforts to discover the pathogenesis of hypertension, particularly with respect to possible involvement of the renin-angiotensin system and catecholamines, no definite answer has been found. Although the involvement of ET in the generation of essential hypertension has been suggested, no conclusive evidence has yet been obtained. Elevation of the plasma concentrations of ET-1 in patients with essential hypertension has not been clearly established. Although ET-1 may be an important factor in the generation of vasospasm after subarachnoid hemorrhage or in pulmonary hypertension, there are few vascular diseases in which alterations in ET-1 have been shown to play a major role (9).

Very recently, Kurihara et al (10) reported results in mice with a defective ET-1 gene. They concluded that ET-1 may serve as a depressor in the regulation of blood pressure rather than as a pressor in the physiological state in mice. This aroused a further controversy over the role of ET in the control of cardiovascular function. This study demonstrated an unexpected elevation of blood pressure in the animals in which the production of ET-1 decreased, apparently suggesting an involvement of ET-1 in the vasodilating mechanism of vascular beds.

In view of these complex results, the aim of the present article is to elucidate the possible role of ET-1 in the control of vascular tone.

Effect of ET-1 on Blood Pressure

As described above, exogenous ET-1 administered into vascular beds elicits a transient depressor response followed by a remarkable increase in blood pressure (1, 11). The transient depressor response is most apparent at a low dose of ET-1 and is counteracted by the pressor response at a high dosage (12). Since the vasodilation is blunted by methylene blue or hemoglobin in rat

isolated, perfused mesentery (13) and also attenuated in the presence of N^G-monomethyl-L-arginine (L-NMMA) (an inhibitor of NO synthase) in conscious rats (14), endothelium-derived NO is involved in this mechanism. The intravenously administered ET-1 may stimulate the endothelium from the luminal side, and the released NO acts on the underlying smooth muscle. At a high concentration, ET-1 is likely to penetrate the endothelium and act on the smooth muscle. Thus, the vasoconstriction occurs after the vasodilation when ET-1 is administered luminally.

However, it was suggested that endothelium has a higher sensitivity to ET-1 than smooth muscle cells. Low concentrations of ET-1 (10^{-11} to 10^{-9} M) induced relaxation rather than constriction in isolated rat aorta ring preconstricted with norepinephrine, while higher concentrations (more than 10^{-9} M) caused constriction (15). Since this response was inhibited by removal of the endothelium or in the presence of L-NMMA, oxyhemoglobin, or indomethacin, the ET-1-induced relaxation mechanism seems to be mediated by both cyclooxygenase products and NO in this case. Because ET_A and ET_B have similar affinities to ET-1, the apparent difference in sensitivity between endothelial and smooth muscle cells could be due to the different intracellular mechanisms involved in relaxation and constriction.

These results strongly suggest that endogenous ET-1 produced in the endothelium also produces both relaxation and constriction in vascular beds (Figure 2). Whether constriction or relaxation is predominantly elicited by endogenous ET-1 may depend on the concentration of ET-1 in the vascular wall, the number of ET receptors on endothelial cells and smooth muscle cells, and existing conditions in the vascular beds. It is generally accepted that administration of L-NMMA causes hypertension (16). L-arginine reverses the pressor response elicited by ET-1 (14). These results suggest that the pressor response caused by L-NMMA may be partly ascribable to endogenous ET-1 (14, 17). However, Gardiner et al also demonstrated that phosphoramidon, an inhibitor of endothelin converting enzyme (ECE), did not prevent the pressor response induced by L-NMMA (16), even though the amount of ET-1 was reduced by ECE. Furthermore, no studies demonstrated inhibition of normal levels of blood pressure by an ET-receptor antagonist. At this time, there is no direct evidence that ET-1 plays a role in the maintenance of the normal vascular tone.

Apart from the regional endothelial mechanism of modulation of blood pressure, ET-1 affects blood pressure via the nervous system (Figure 2). ET-1 may be a neurotransmitter or a neuromodulator in central cardiorespiratory control (18–21). Intracisternal bolus administration or intracerebroventricular infusion of a low dose of ET-1 elicits an increase in blood pressure (18, 19). The sympathetic nervous system was demonstrated to be involved in these ET-1-induced vasomotor responses. Further experiments revealed that ET-1

directly affects vasomotor neurons in the rostro-ventrolateral medulla and indirectly affects them through the medulla oblongata (20, 21). Topical application of a low dose of ET-1 to the medulla oblongata directly caused initial excitation and subsequent sustained inhibition of arterial pressure and heart rate. On the other hand, intrathecal administration of ET-1 elicited a decrease in blood pressure associated with vasodilation of the hindquarter in rats (22).

At a low concentration, ET-1 had no direct effect on baroreflex function (23), although a high concentration inhibited it (24). However, ET-1 is likely to potentiate the sensitivity of the baroreflex, because intracisternal administration of a high dose of ET-1 induced a significant increase in the vagal component of baroreflex sensitivity (25).

In contrast, Stewart et al demonstrated that a change in posture or exercise in a normal individual induced a rapid and transient increase in the plasma concentration of ET-1, followed by a second slower rise (26). The rapid and transient rise in plasma ET-1 concentration agrees well with the elevation of plasma ET-1 in response to cold stress that was reported previously (27), suggesting a nonendothelial mechanism, probably a neuroendocrine mechanism (26). This result also led to the concept that ET plays some role in blood-pressure regulation through an nonendothelial pathway in addition to the endothelial pathway. Accordingly, Kurihara et al suggested that the change of central cardiorespiratory control and the developmental insufficiency of the normal cardiovascular regulatory system might cause the hypertension observed in heterozygotes of ET-1-gene-disrupted mice (10).

Additionally, ET has a presynaptic neuromodulatory effect (Figure 2). It was demonstrated that ET elicited an increase in the concentration of intracellular free-calcium ions ($[Ca^{2+}]_i$) both in sympathetic and in parasympathetic peripheral autonomic neurons via ET_B receptors (28, 29). Smooth muscle in the vicinity the neurons in guinea pig trachea contracted following the ET-induced elevation of intracellular free-calcium level in the neurons (28). How-

Figure 2 Role of ET-1 in vascular tone. ET-1 is produced from a proform of ET-1 (pro ET-1) via an intermediate form, big ET-1. ET-1 is preferentially secreted from endothelial cells (ECs) towards the basal side. However, the concentration of ET-1 in interstitial space is low. The released ET-1 first binds to the ET_B receptor on the EC in an autocrine manner. The resulting activation of the ET_B receptor elicits production of nitric oxide (NO). The NO released from EC induces elevation of cGMP in smooth muscle cells. The remaining ET-1 binds to ET_A receptors on the underlying smooth muscle cells. Big ET-1 is released from EC. The released big ET-1 is converted by endothelin-converting enzyme (ECE) to ET-1 on the surface of smooth muscle cells and binds to ET_A receptors on smooth muscle cells. The activated ET_A receptor elicits elevation of intracellular free-calcium ions ($[Ca^{2+}]_i$) via activation of phospholipase C (PLC) and opening of ion channels that increases calcium influx. Diacylglycerol (DG) produced by activation of PLC and phospholipase D (PLD) may potentiate the contraction of smooth muscle via activation of protein kinase C (PKC). ET-1 plays some role in the vasomotor center in regulation of sympathetic tone. ET_B on peripheral autonomic nerves may regulate the release of transmitters.

ever, Wiklund et al have reported that norepinephrine release in guinea pig femoral artery (30) and acetylcholine release in guinea pig ileum (31) were both inhibited by ET-1. Aarnio et al reported that ET-1 released at the presynaptic level also inhibited norepinephrine release in isolated canine left anterior descending coronary artery (32). The results suggest that the constriction of vascular beds in response to ET-1 is modulated by the level of sympathetic and parasympathetic tone (32).

At present I do not have sufficient data to discuss the role of ET-1 in the control of blood pressure in the central or peripheral nervous system.

Endothelin-1 in Vascular Beds

To clarify further the role of ET-1 in vascular beds, the production, release, and fate of ET-1 in the vascular beds must be elucidated. Many endogenous chemical and physical stimuli have been reported to stimulate the production and release of ET-1 from endothelial cells, such as thrombin (1), calcium ionophore (1), tumor necrosis factor–α (TNF-α) (33), transforming growth factor–β (TGF-β) (34), activin (35, 36), insulin (37), angiotensin (38), arginine vasopressin (38), hypoxia (39, 40), and cyclosporine (41). In contrast, the production of ET-1 in the endothelium is inhibited by NO, natriuretic peptide, or heparin (42–45). All of these later factors increase the cytosolic cGMP level. However, these endogenous chemical factors may simply be modulators under various physiological or pathophysiological conditions.

The most important physiological factor regulating the production and release of ET-1 from endothelial cells may be shear stress (Figure 2). An increase in the rate of blood flow elicits vasodilation via the shear-stress receptor of endothelial cells. Activation of the shear-stress receptor at a high shear stress (more than about 5 dyn/cm^2) produces and releases NO from the endothelium and reduces the production and release of ET-1, resulting in vasodilation and reduction of the flow rate (46–48). In the rat leg skeletal microcirculation system, shear stress was independent of arterial diameter in the presence of endothelium. However, after damage to the endothelium, an increase in shear stress did not elicit vasodilation (49). The regulatory mechanism for the production and release of NO and ET-1 may have been destroyed in the damaged endothelium.

As described above, an increase in NO release inhibits the production of ET-1 (50). A decrease in NO synthesis inhibits the depression of ET-1 production (51). On the other hand, Suzuki et al reported that ET-1 augmented the release of the vasorelaxing factor (52). There is a reciprocal feedback mechanism of synthesis between these vasoconstrictors and vasodilators (51). Warner et al demonstrated that ET-3 but not ET-1 selectively released NO in bovine endothelial cells (51). If this is true, circulating or tissue ET-3 but not ET-1 regulates the production of NO and, in turn, the production of ET-1.

These results are compatible with the concept that ET-1 acts as a vasoconstrictor in the regulation of vascular tone.

However, an important question—whether the amount of ET-1 in vascular tissue is too small to regulate the vascular tone—remains unanswered. As a first step to answering this question, the concentration of ET-1 in the interstitial space between the endothelium and smooth muscle layer of the vascular beds must be determined. At present only a few reports describe the ET-1 content in vascular beds. According to the results published by Larivière et al, the concentration of immunoreactive ET-1 in vascular tissues was approximately 10 to 10^2 fmol/g tissue in normotensive rats, while the plasma ET-1 concentration was around 1 fmol/ml (53). Therefore, the concentration of ET-1 in vascular tissue can be assumed to be about 100 times higher than that in plasma (53, 54). However, ET-1 is assumed to be concentrated mostly in the endothelial cells. The concentration of ET-1 in the interstitial space between the endothelium and smooth muscle layer may not be high in comparison with that in plasma. Most of the ET-1 present in vascular tissues binds to receptors (55); therefore, free tissue ET-1 concentrations must be very low.

However, ET-1 produced in the endothelium was reportedly secreted preferentially to the basal side (56, 57). Using cultured human vascular endothelial cells, Wagner et al demonstrated that about 80% of the total amount of synthesized ET-1 was released into the basolateral compartment (57). This fact supports the hypothesis that ET-1 acts on the underlying smooth muscle layer in a local paracine manner, although the rate of ET-1 secretion to the basal side is only four times greater than that to the luminal side. The released ET-1 may not accumulate in the interstitial space and probably binds to the receptors on the endothelium and/or smooth muscle layer.

Another important factor that regulates the local concentration of ET-1 in vascular beds is a neutral endopeptidase, designated endothelin-converting enzyme (ECE). As described above, ET-1 is synthesized from a proform of ET-1 (pro ET-1) via an intermediate form, big ET-1 (1). Big ET-1 is converted into the mature peptide ET-1 by ECE. The endothelial ECE is a glycosylated, membrane-bound metalloproteinase (58–60). The vasoconstrictive activity of big ET-1 is much less than that of ET-1 (61). Therefore, the physiological significance of ECE is similar to that of angiotensin-converting enzyme (ACE) in this respect. Intravenous administration of big ET-1 into rats elicits an elevation of blood pressure as well as an increase in plasma ET-1 level (62), and this response is abolished by administration of phosphoramidon, an inhibitor of ECE (62, 63). Consequently, the ECE is considered to play an important role in situ in the generation of ET-1 in vascular beds and, in turn, regulation of vascular tone in response to ET-1. In vascular beds, however, ECE has also been detected in smooth muscle cells as well as in endothelial cells. The conversion of big ET-1 probably occurs on the surface of the smooth muscle

cells (62, 63). Therefore, big ET-1 released from endothelial cells reaches the smooth muscle layer and may be converted to ET-1 on the surface of the smooth muscle. Since big ET-1 does not bind to ET receptors, ECE on the smooth muscle is important in determining the availability of ET-1 for ET_A receptors on smooth muscle cells. Big ET-1 was also found to be secreted into the extracellular space (64). Both big ET-1 and ET-1 are secreted from endothelial cells along a constitutive pathway. A major site for the conversion of big ET-1 may be the intracellular vesicles of the endothelial cells. Therefore, when production of pro ET-1 increases, an increase in the concentration of ET-1 in the extracellular space may depend on the density of ECE on the vesicular and plasma membranes and the rate of secretion of the product. So far there have been only a few reports on the distribution of ECE in relation to the rate of ET-1 release.

The major question concerning the concentration of ET-1 in vascular beds is whether the elevation of ET-1 content leads to enhancement of vasoconstriction. Since endogenous ET-1 acts on vascular smooth muscle in a paracine manner, the circulating ET-1 concentration does not always reflect the increase in ET-1 production in the local vascular beds. Indeed, the plasma concentration of ET-1 in hypertensive patients or experimental animals is not always high (9). However, in some cases where the plasma ET-1 concentration increases, there is a good correlation between plasma ET-1 concentration and blood pressure (65, 66). In such cases, antagonists of the ET receptor reduced the blood pressure (66, 67). In several pathological conditions in which general impairment of the endothelium is assumed to occur, including atherosclerosis (68), cyclosporine-induced hypertension (69), renal insufficiency in a patient undergoing hemodialysis (65), and disseminated intravascular coagulation, the blood pressure increases parallel to the increase in plasma ET-1 concentration. Yokokawa et al reported that patients with hemangioendothelioma also showed a high plasma ET-1 concentration and hypertension (70). These results strongly suggest that the pathologically increased concentration of ET-1 in vascular beds, as a result of exaggerated production of ET-1, elicits an increase in vascular tone.

Endothelin Receptor of Vascular Beds

ET-induced vasoconstriction is considered to be mediated by ET_A receptor on vascular smooth muscle cells, while the relaxation by ET-1 is mediated by ET_B receptors on endothelial cells. However, recent reports have provided evidence that ET_B is also involved in the vasoconstriction of rabbit jugular vein (71) and rabbit saphenous vein (72). Subsequent experiments have demonstrated the existence of two pharmacologically distinct subtypes of ET_B receptors, named ET_{B1} and ET_{B2} (73, 74) (Table 1). Both ET_B receptor subtypes are not inhibited by the ET_A-specific receptor antagonist BQ-123. ET_{B1}

is inhibited by the ET_B antagonist IRL1038 and the nonselective ET-receptor antagonist PD142893, while ET_{B2} is not inhibited by either of these antagonists. The former receptor exists on endothelial cells and mediates release of the relaxing factors, while the latter exists on smooth muscle and mediates constriction. In addition, endothelial cells contain ET_C, a receptor subtype that has high affinity for ET-3 but low affinity for ET-1 or ET-2 (51, 75). As described above, Warner et al suggested that ET_C mediates NO release (51), although the existence and function of ET_C are still debatable. Further analysis of the agonist-antagonist interaction during contraction of the rabbit saphenous vein also suggested that at least two subtypes of ET_A exist (76). BQ-123-sensitive ET_A may possibly mediate release of prostacyclin from perfused rat lung (BQ-123 is a specific antagonist for the ET_A receptor) (77). ET-1 induces a variety of responses, depending upon the vascular beds. The differences between responses of the various vascular beds to ET-1 are ascribed to the different distributions of the receptor subtypes in these tissues. Nevertheless, it is generally accepted that, in most of the resistance vessels, ET_A receptors on smooth muscle mediate constriction and ET_B receptors on endothelial cells mediate relaxation.

The two responses to ET-1, relaxation and constriction, are in opposite directions and have the potential to counter each other. However, since the action of relaxing factors is of short duration while ET-1-induced vasoconstriction is long lasting, the ultimate response of the regional vascular bed to ET-1 is primarily constriction. The sustained constriction by ET-1 might be expected to be modulated by short-acting NO released by ET-1. However, the net response may depend greatly on the ratio of the number of ET_A receptors expressed on smooth muscle cells to that of ET_B receptors on endothelial cells. In fact, regional hemodynamic responses to ET-1 are different, depending upon the vascular beds (78).

An important factor that modulates the response to ET-1 in vascular beds is the density and turnover of ET_B receptors of endothelial cells and ET_A receptors of smooth muscle cells. The densities of these receptors are reportedly regulated by pathophysiological states. Increases in the plasma concentration of ET-1 in various pathological conditions, including acute renal failure and experimental congestive heart failure, reduce the response of the vascular beds to ET-1 (79, 80). The reduced response of small resistance arteries to ET-1 was also observed in patients with hypertension, despite their normal response to norepinephrine and other vasoconstrictor peptides such as vasopressin and angiotensin II (81, 82). The reduced responses to ET-1 in these conditions are considered to be due to a decrease in receptor density (83). For example, in DOCA-salt hypertensive rats, the binding of ET-1 to mesenteric arterial membranes was significantly lower, and the production of ET-1 in endothelial cells increased, while the plasma concentrations of ET-1 were

similar to those of the control animals (84). Since the decrease in constrictor effects of ET-1 was found in vascular beds without endothelium in this case, the decrease in receptor density observed in smooth muscle is probably the result of an overproduction of ET-1 in the vascular tissue.

Several papers provide direct evidence that ET-1 reduces the density of ET_A receptors on smooth muscle. Down-regulation of both subtypes of the receptor, i.e. ET_A receptors of smooth muscle cells and ET_B receptors of endothelial cells, occurs rapidly and similarly after the stimulation of the respective cells (85–88). In other words, at a high concentration of ET-1, ET-1 rapidly associates with the cell surface receptor and tightly binds to it, whether ET_A or ET_B receptors, and is rapidly internalized (85, 86, 89, 90). Total ET-receptor number decreased without any change in its binding affinity (85). For example, when 0.25-nM ^{125}I-ET-1 is incubated at 37°C, ET-1-binding to the cell surface receptor equilibrates within 15 min, while the completion of the internalization of ET-1 requires 60 min (84). About 70% of the bound ET-1 is internalized. Subsequent externalization of ET_A receptors after the down-regulation occurs within 30 min at 37°C (89). However, at a physiologically low concentration of ET-1, ET-1 binds to the receptor very slowly, and the binding is tight. Internalization probably occurs in a manner similar to that observed at high ET-1 concentrations.

ET-1 induces a slow-developing and long-lasting contraction (1). The contraction is assumed to be attributable to the ET-1 bound to surface receptors, including newly externalized ET_A (89), since BQ-123 inhibits the contraction when added any time after the administration of ET-1. The internalized ET-1-ET_A complex may not be responsible for the long-lasting constriction. In the presence of a high concentration of ET-1, ET-1 associates rapidly with its receptor. Therefore, rapid time-dependent accumulation of inositol 1,4,5-trisphosphate (IP_3) and the resulting increase in cytosolic free-calcium ions correspond to the time course of association of ET-1 to the ET receptor. Indeed, numerous reports suggest an involvement of the increase in cytosolic IP_3 production via stimulation of phospholipase C (PLC) in the mechanism of ET-induced vasoconstriction. The IP_3 produced releases free-calcium ions from the intracellular calcium pool and these ions, in turn, elicit constriction. At an ET-1 concentration higher than the threshold, a transient increase in cytosolic free-calcium ions occurs rapidly, with a peak around 30 s, and decreases to the initial level within a few minutes after the stimulation of ET-1 (91). Since released calcium ions are quickly taken up into the pool again, and IP_3 is also quickly metabolized, activation of PLC by ET-1 seems to occur only at a high concentration of ET-1 (92). Indeed, Muldoon et al demonstrated that in Rat-1 cells, ET-1 significantly increased the production of IP_3 at high concentration (92). The EC_{50} value of the inositol phosphate production stimulated by ET-1 was around 10^{-9} M, whereas ET-1 significantly increases

calcium influx at a low ET-1 concentration; peak stimulation occurs at about 2×10^{-11} M. In this case, the calcium influx was inhibited by elevation of the intracellular calcium concentration. Accordingly, calcium influx was inhibited at high ET-1 concentration. These results indicate that IP_3 is apparently not involved in the ET-induced sustained contraction.

ET-1 is well known to induce a transient increase, followed by a sustained increase in cytosolic free-calcium ions at a relatively high concentration of ET-1. The latter sustained phase is caused by the external calcium ion. Since the ET-1-induced constriction develops slowly, the calcium influx from the external medium may be important in the sustained constriction induced by ET-1. This leads to the next question, what mechanism is involved in the ET-induced calcium influx during the later phase at the physiologically low concentration of ET-1? The opening of L-type Ca^{2+} channels by ET-1 was proposed as a mechanism (93, 94). In support of this, ET-induced vasoconstriction is inhibited by a dihydropyridine calcium channel antagonist (93, 94) in porcine coronary artery. However, a number of papers have demonstrated that the inhibiton of ET-1-induced constriction as well as the sustained later elevation of cytosolic free-calcium ions was not always inhibited by the dihydropyridine calcium channel antagonists (95), suggesting an involvement of the another type of ion channel in this mechanism.

As described above, under physiological conditions, the concentration of released ET-1 in vascular tissue is very low. Consequently, association of ET-1 to its receptor is very slow. The stimulated receptor activates the second messenger transiently, but the response induced by the second messenger may be long lasting. At this low concentration of ET-1, activation of PLC apparently does not occur because released IP_3 is quickly metabolized, and the released calcium ions from the intracellular pool are also quickly taken back up into the pool. However, we cannot exclude the involvement of ET-induced PLC activation in the ET-induced vasoconstriction, even at the low concentration. The products of PLC may still directly or indirectly activate the ion channels. Thus, the ET-1-induced sustained response is mediated by activation of the cation channel, although the detailed mechanism is still unknown.

Because the number of ET-1-binding sites of the cultured 3T3 fibroblasts, rat mesangial cells, rat endothelial, or human endothelial cells increased by 1.4- to 17-fold over the control value during treatment with phosphoramidon, which is an inhibitor of ECE (88, 89), both ET_A and ET_B receptors may have been down-regulated by endogenous ET-1 released from the cells in an autocrine manner, even during normal incubation conditions. Increased ET-1 concentrations produced under some physiological conditions, or particularly in pathological conditions, induces down-regulation of ET_B receptors on endothelial cells and ET_A receptors on smooth muscle cells. In vascular beds, ET_B receptors on endothelial cells may respond more effectively to the endog-

enous ET-1 produced by the endothelium in an autocrine fashion than ET_A receptors on smooth muscle cells, resulting in the down-regulation of ET_B. Therefore, constriction may predominate over relaxation during the overproduction of ET-1. Conversely, a decrease in the production of ET-1 in the endothelium, as seen in the endothelium of heterozygotes of the ET-1-gene-disrupted mice (10), may induce up-regulation of ET_B receptors of the endothelium and ET_A receptors on smooth muscle cells. However, ET-1 binds very slowly to its receptor and probably produces almost no NO at the very low concentration seen in these mice because the release of NO by ET-1 via ET_B receptors is short acting. In contrast, the constrictor effect of ET-1 via ET_A receptors is long acting and cumulative. Therefore, constriction may also predominate over relaxation when production of ET-1 is reduced. Overall, ET-1 produced by the endothelium may be a vasodilator under normal physiological conditions and a vasoconstrictor under pathological conditions.

Interference of ET-Induced Response by Other Vasoactive Factors

Production of ET-1 in the endothelium and the action of ET-1 on vascular smooth muscle are affected by various preexisting conditions of the vascular beds. The presence of other vasoactive substances is particularly important. Interference with ET-induced responses by other vasoactive substances occurs both at the level of endothelial cells and smooth muscle cells.

NO and ET-1 mutually affect the production and action of the other, as discussed above. ET-1 is also known to elicit releases of prostacyclin and prostaglandin E_2 in some vascular beds. Both factors also counteract the constriction induced by ET-1. The relaxation of smooth muscle by NO requires that the smooth muscle be preconstricted. When an isolated rat aortic ring with endothelium was preconstricted with norepinephrine, ET-1 induced potent relaxation rather than constriction at a low concentration but elicited constriction at a higher concentration (14).

A low concentration of ET-1 enhanced the vasoconstriction in response to norepinephrine or 5-hydroxytryptamine (5-HT) in various types of arteries (96–100). This phenomenon may be relevant to hypertension and vasospasm. One explanation for this enhancement is potentiation of calcium influx by both agents, because both stimulate the cation channels. However, a recent report demonstrated that the ET-1-induced potentiaion of the norepinephrine-induced constriction is not caused by the potentiation of calcium uptake but by activation of the PKC-dependent pathway (98). To further elucidate this finding, more detailed experiments will be needed. It has also been suggested that the potentiation effect of 5-HT by ET-1 can be ascribed to the release of thromboxane A_2 or increase in the thromboxane A_2 receptor (100).

Interaction of the renin-angiotensin system with ET-1 is well known, although the detailed mechanism is still unclear. Angiotensin II is known to stimulate the production of ET-1 in cultured endothelial cells (101). Conversely, Dohi et al demonstrated that ET-1 stimulated the production of renin and angiotensin (101). Therefore, activation of the renin-angiotensin system can be assumed to potentiate ET-1-induced vasoconstriction.

Intravenous infusion of captopril, an inhibitor of angiotensin-converting enzyme, into rats inhibited the hypertension induced by exogeneously administered ET-1. This suggest that ET-1-induced hypertension involves stimulation of the renin-angiotensin system (102). However, confounding the interpreation of this study is the fact that captopril causes accumulation of endogenous bradykinin in the vascular beds that, in turn, stimulates production and release of NO (103) and attenuates the increase in blood pressure.

With regard to other relaxing factors, it has been reported that atrial natriuretic peptide and α-calcitonin-gene-related peptide (α-CGRP) attenuate the ET-1-induced vasoconstriction, probably via elevation of cytosolic cGMP (104, 105).

Finally, in the inact organism, the ET-induced vascular response is modulated by reflexes of the sympathetic nervous system. Infusion of ET-1 induced an increase in the mean circulatory filling pressure, an index of body venous tone, in addition to increasing systemic blood pressure in the rat. Waite & Pang suggested that the venous effect of ET-1 was mediated via modulation of sympathetic nerve activity and the activation of the α-adrenoreceptor (106). In human peripheral skin microcirculation, ET-1 elicits a flare response similar to the axon reflex, as well as a direct vasoconstrictor action (107). Involvement of the H_1-receptor was suggested in this study. However, since a local anesthetic inhibited this response, involvement of peripheral nerves (C fibers) was also suggested. In general, these results indicate that the ET-1-induced response in vascular beds is affected by the peripheral nervous system.

Conclusion

Vascular endothelial cells control vascular tone by producing vasodilators, including nitric oxide (NO) and prostacyclin, and vasoconstrictors, such as ET-1 and thromboxane A_2. ET-1 was initially isolated from the conditioned medium of cultured vascular endothelial cells as a potent vasoconstrictive peptide. However, ET-1 elicits both pressor and depressor responses. The pressor response seems to be chiefly mediated by ET_A receptors on smooth muscle cells, and the depressor response seems to be chiefly mediated by NO released from endothelial cells through ET_B receptors. Recent pharmacological analyses, however, have demonstrated that both ET_A and ET_B receptors are involved in the pressor response. Further detailed experiments will be needed

to clarify this problem as to how ET_B is involved in this pressor response. Whether constriction or relaxation is predominantly elicited by endogenous ET-1 depends on the concentration of ET-1 in the vascular wall, density of ET_B receptors on endothelial cells and of ET_A receptors on smooth muscle cells, and the preexisting state of the vascular beds.

There is a reciprocal feedback system influencing production of NO and ET-1 in endothelial cells. The ET-1 that is produced is preferentially secreted to the basal side rather than the lumincal side. However, the concentration of free ET-1 in the interstitial space of vascular tissue may be low. Most of the ET-1 is concentrated in the endothelial cells, and the released ET-1 binds tightly to the ET-receptors.

At nanomolar concentrations of ET-1, the ET_B receptor is rapidly occupied by ET-1, and PLC is activated. Thus ET-1 induces a transient increase in the intracellular free-calcium ion level and, in turn, elicits constriction. However, at the physiologically low concentration of ET-1, the rate of the receptor occupation by ET-1 is very slow, and ET-1 apparently does not activate PLC. Thus, the transient increase in the cytosolic free-calcium level apparently does not occur, because IP_3 is quickly metabolized and released calcium ions are immediately taken back up into the pool. However, ET-1 activates a calcium channel for a long period of time and enhances calcium influx from the external medium. The opening of the calcium channel by ET-1 is long lasting and persists even after the removal of ET-1 from the medium.

ET-1 therefore acts as a relaxing factor rather than as a constricting factor at physiologically low concentrations of ET-1, because the ET-1 released from endothelial cells binds preferentially to endothelial cells in an autocrine manner rather than to smooth muscles. Smooth muscle cells are located at some distance from the endothelium. However, the constrictive effect of ET-1 is long lasting and accumulating. In heterozygotes of ET-1-gene-disrupted mice, the amount of ET-1 released from the endothelial cells is decreased. Therefore, the relaxing activity of ET-1 may decrease. In contrast, in some pathological conditions in which production of ET-1 is exaggerated, such as the cases of general impairment of endothelial cells, ET-1 acts as a constricting factor.

In situ, the effect of ET-1 on vascular beds is also modulated by many other factors, such as norepinepherine, the renin-angiotensin system, and neuronal factors. It is well known that ET-1 affects blood pressure through endothelial and extra endothelial pathways. However, the mechanisms of these interactions are still unclear, and their elucidation must await further detailed experiments.

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