Review

Endothelin in Vascular and Endocrine Systems: Biological Activities and Its Mechanisms of Action

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I. Introduction

SINCE ITS DISCOVERY IN 1988 [1], endothelin has been the subject of intensive investigation in the fields of cardiovascular biology, endocrinology and clinical medicine. In the past five years a huge number of reports describing pleiotropic biological actions of this novel bioactive peptide have rapidly been accumulated [2–4].

Endothelin was originally isolated as a potent vasoconstrictive activity from the culture supernatant of porcine aortic endothelial cells. Immediately thereafter, endothelin was found to exert complex actions on the vascular system, i.e. direct vasoconstriction and endothelium-dependent vasodilatation [5, 6]. Subsequent studies demonstrated that a wide variety of tissues outside the cardiovascular system possess high affinity specific binding sites for endothelin [7], and that endothelin induces diverse biological responses in these tissues, which include modulation of hormone release from various endocrine organs [8], regulation of the transport in the renal tubule [9, 10] and modulation of the central nervous activity [11]. One of the outstanding achievements in the progress of the endothelin research was the cloning of three distinct endothelin-related genes [12], which led to the discovery that mammals including human produce three members of this peptide family, endothelin-1 (the 'classical endothelin'), endothelin-2 and endothe-

lin-3. Binding studies using each of the radiolabeled endothelin isopeptides suggested the existence of receptor subtypes with different affinities for each isoform of the endothelin family peptides [13]. Indeed, subsequent molecular cloning of endothelin receptor cDNA identified two subtypes, i.e. a ET-1-selective ET_A subtype [14] and a non-selective ET_B subtype [15], which show distinct patterns of tissue specific expression. The existence of three endothelin isoforms and two subtypes of their receptors may in part underlie the diversity of biological activities of the endothelin family peptides. However, it remains to be elucidated whether the reported biological activities of endothelins, both within and outside the cardiovascular system, have physiological or pathophysiological significance, since most of them are based upon the data obtained with exogenously administered endothelin peptides. Recently discovered subtype-selective agonists [16] and antagonists [17] for endothelin receptors should serve as useful tools for addressing such questions and contribute to further understanding of the pathophysiological role of this novel peptide family.

In this article, I will briefly review the current status of knowledge concerning this novel peptide with special emphasis on its actions on the vascular and endocrine systems.

II. Vascular Actions

Pressor and Depressor Responses

Intravenous bolus injection of endothelin-1 causes an acute biphasic hemodynamic response in

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autonomically blocked rats: an initial transient (lasting 0.5–2 min) decrease in mean arterial pressure which is accompanied by systemic vasodilation (depressor response), followed by a remarkably sustained increase in mean arterial pressure which is accompanied by systemic vasoconstriction (pressor response) [1, 5]. Intravenous administration of endothelin-1 to experimental animals is also reported to induce moderate increases in plasma concentrations of vasoactive agonists such as adrenaline and vasopressin [8, 18]. Based upon the observations that the pressor response by endothelin-1 is barely affected by pretreatment with the receptor antagonists for these vasoactive agonists, and that endothelin-1 provokes a potent contraction of isolated arterial strips from various vascular regions [1], the acute pressor response to endothelin-1 infusion is considered to be mediated largely by its direct constrictive action on vascular smooth muscle. There are several exceptions reported including rat hepatic vascular bed [19], isolated canine basilar artery [20] and isolated aorta from spontaneously hypertensive rats [21], where endothelium-derived thromboxane A_2 is suggested to contribute significantly to contractions evoked by endothelins.

The endothelin-1-induced vasodilation is abolished by removal of endothelium, and is inhibited to various degrees, depending on vascular regions employed in the experiments, by cyclooxygenase inhibitors or endothelium-derived relaxing factor (EDRF) inhibitors such as methylene blue and oxyhemoglobin, indicating that the endothelin-1-induced vasodilation is mediated by release of vasodepressor prostanoids and EDRF from the endothelium [5, 6]. In the first report by Warner *et al.* [6] on the vasodilator effect of endothelins, it was already recognized that endothelin-3 with much less potency in vasoconstrictor activity was equipotent with endothelin-1 in inducing the vasodilator response, suggesting that endothelin receptors existent in vascular smooth muscle and the endothelium were different. In fact, subsequent molecular cloning of the receptor cDNA revealed the existence of two subtypes of endothelin receptors [14, 15]: one designated as ET_A subtype that prefers endothelin-1 over endothelin-3 and is expressed in vascular smooth muscle, and the other designated as ET_B subtype that shows an equal affinity for endothelin-1 and endothelin-3

and is expressed in endothelial cells. Thus, these findings are consistent with the idea that the vasoconstrictor activity of endothelin-1 is mediated through ET_A receptor and the vasodilator activity through ET_B receptor [2, 4].

However, a more complex view regarding the functional differences of the two types of endothelin receptors expressed in the vascular wall is emerging from recent studies in several laboratories employing newly discovered pharmacological probes. In these reports [22–26] they have shown that prior administration of a highly selective ET_A -receptor antagonist BQ-123 (cyclo [D-Glu-L-Ala-D-allo-Ile-L-Leu-D-Trp]) [17] to rats inhibits both the magnitude and duration of pressor response, but not depressor response by endothelin-1, confirming that the systemic vasoconstriction by endothelin-1 is indeed mediated by ET_A receptors. However, there were striking regional difference noted in the susceptibility to BQ-123 [24, 25]. Thus, endothelin-1-induced vasoconstrictions of the carotid and iliac beds were greatly inhibited by pretreatment with BQ-123. In contrast, endothelin-1-induced vasoconstrictions of the celiac, mesenteric and renal beds were much more resistant to BQ-123; especially, the celiac and renal vasoconstrictions were not significantly suppressed by BQ-123 at the dose which nearly completely blocked the carotid and iliac vasoconstrictions. Moreover, intravenous injection of a ET_B -selective agonist, [Ala^{1,3,11,15}] endothelin-1 induced an initial marked vasodilation followed by a very small vasoconstriction in the carotid and iliac vascular beds, whereas in the celiac, mesenteric and renal bed this agonist caused a weak vasodilation and a following potent vasoconstriction [25]. Pretreatment with BQ-123 did not affect either initial vasodilation or the second vasoconstriction in response to [Ala^{1,3,11,15}] endothelin-1 in these vascular beds [25]. Thus, the effects of endothelin-1 and its analogues on vascular beds vary in a complex manner depending upon the time and the vascular region. These observations strongly suggest that in rats, the endothelin-1-induced vasodilation is mediated by ET_B receptor in endothelial cells and that the endothelin-1-induced vasoconstriction is mediated in the carotid and iliac beds by ET_A receptors and in the celiac, mesenteric and renal beds largely by ET_B receptors. In accordance with this notion, it was shown that significant portions of the endothelin-1-

induced contraction of isolated vascular segments from various regions were resistant to BQ-123 [27, 28]. In particular, in rabbit jugular veins endothelin-1 and endothelin-3 were equipotent constrictors, and BQ-123 at up to 10 μ M was totally ineffective in inhibiting endothelin-1-induced contraction [28]. These composite findings suggest that vascular smooth muscle express both ET_A and ET_B receptors to various degrees depending on the vascular region [29, 30] and that stimulation of either subtype causes contraction. Especially, vascular smooth muscle of certain venous vascular regions including jugular and saphenous veins [31], and mesenteric and renal beds [24–26] appears to dominantly express ET_B-receptors. Thus, the heterogeneity of the distribution of two ET receptor subtypes in the vascular system may well account for the reported regional differences in the hemodynamic responses to BQ-123 and [Ala^{1,3,11,15}] endothelin-1.

Several previous reports [32, 33] suggested that there may exist an additional class (ET_C) of endothelin receptors which shows a higher affinity for endothelin-3 as compared to endothelin-1. It remains possible that the third receptor subtype which is stimulated by [Ala^{1,3,11,15}] endothelin-1 but insensitive to BQ-123 might mediate the endothelin-1-induced vasoconstriction in the mesenteric and renal vascular beds. However, the results of genomic Southern blot analysis [34] did not support this possibility. Southern blot analysis of human genomic DNA performed under a low stringency condition with either human ET_A or ET_B receptor cDNA as probe revealed the existence of two hybridizing genomic DNA fragments which probably corresponded to the ET_A and ET_B receptor genes. Therefore, the third endothelin receptor, if it actually exists, should have little similarity to ET_A and ET_B receptors.

Potential Role in Hypertension and Vasospasm

Several lines of evidence suggest the pathogenic role of endothelins in hypertension: first, endothelin infusion causes a sustained pressor response in experimental animals [1]; second, administration of antibodies raised against endothelin-1 lowers blood pressure in spontaneously hypertensive rats [35]; third, several reports demonstrate that plasma concentration of endothelin-1 is elevated in patients with essential hyperten-

sion [36, 37], although this observation is still controversial; fourth, patients with hemangioendothelioma showed changes in blood pressure concomitantly with changes in plasma endothelin-1 level before and after surgical resection of tumors [38]; and fifth, endothelin-1 is considered to be released mainly abluminally from the endothelium [39], which may allow released endothelin-1 to act on the underlying smooth muscle layer as a local regulator of vascular tone. However, a recent study [23] using a selective ET_A receptor antagonist, BQ-123, demonstrated that the blockade of ET_A receptors by intravenous administration of this antagonist at the dose which had a profound inhibitory effect on endothelin-1-induced pressor response had only a mild hypotensive effect in spontaneously hypertensive rats and DOCA-salt hypertensive rats, and no significant effect in two other hypertension models of two kidney-one clip rats and aortic ligated rats. These findings suggest that endothelin-1 is not a major contributing factor to the maintenance of high blood pressure in these four hypertension models. Further, several laboratories reported the effects of BQ-123 administration on the basal blood pressure in normal rats. Two groups [25, 26] demonstrated that bolus or continuous infusion of BQ-123 induced a significant but relatively small decrease in mean arterial pressure in anesthetized rats, whereas three other groups [22–24] showed that bolus or continuous infusion of BQ-123 at comparable doses did not alter basal arterial pressure in conscious or anesthetized rats. Very recently Kurihara *et al.* [40] reported the generation of mice deficient for endothelin-1 using the technique of homologous recombination of the gene. Unexpectedly, a small increase in the blood pressure was observed in mice heterologous for the mutated endothelin-1 gene compared with normal mice. Homologous mice died shortly after birth for unknown reasons. It awaits further investigation to determine the role of endothelin-1 as a local factor in the peripheral regulation of the basal vascular tone.

Administration of endothelin-1 and endothelin-3 into the cerebral ventricles of conscious rats induces the pressor response accompanied by increases in plasma levels of vasopressin, catecholamine and ACTH [41–43]. Intracisternal administration of endothelin-1 or endothelin-3 also evokes pressor response followed by depressor

response, and the responsible area for this response is localized to the ventral surface of the medulla [44–46]. Studies using ganglion blockers and receptor blockers indicate that the pressor response evoked by centrally administered endothelins is mediated by an increase in the sympathetic activity. Interestingly, central administration of brain natriuretic peptide attenuates endothelin-induced pressor response just as it antagonizes centrally administered angiotensin II-induced pressor response [43]. These observations suggest that endothelins locally produced in the central nervous system may participate in the central control of circulation. It remains to be elucidated whether the endothelin/receptor system in the central nervous system plays any pathogenetic role in the development or the maintenance of hypertension.

Cerebral vasospasm is one of the most serious complications of subarachnoid hemorrhage and exerts a profound deleterious effect on the cerebral circulation. Several studies [47–49] suggested a potential role of endothelins in vasospasm after subarachnoid hemorrhage. It was reported that the level of endothelin-1 in the cerebrospinal fluid is elevated in patients after subarachnoid hemorrhage and in canine models of subarachnoid hemorrhage [50]. Intracisternal injection of endothelin-1 (5–500 pmol) in dogs and cats evoked a long-lasting constriction of cerebral basilar artery while injection of endothelin-1 (up to 3000 pmol) into vertebral artery was without any vasoconstrictive effect [48]. Very recently, intracisternal injection of BQ-123 was shown *in vivo* to alleviate late spasm of basilar artery after subarachnoid hemorrhage in dogs [51]. Interestingly, however, administration of BQ-123 did cause a similar extent of vasodilation of normal basilar artery in control dogs, suggesting that ET_A receptors are involved in the maintenance of basal tone of basilar artery. This finding is not surprising because the cerebral vascular tone is thought to be relatively independent of the regulation by autonomic nerve and circulating vasoactive substances as compared to other regions of vascular beds, but rather strongly dependent on the inherent autoregulatory mechanism. Moreover, vasospasm after subarachnoid hemorrhage is different from endothelin-1-induced contraction of cerebral arteries in that vasospasm is quite resistant to a dihydropyridine Ca²⁺ channel antagonist unlike endothelin-1-

induced vasoconstriction [53]. From all these data, it appears to be reasonable to think that endothelin-1 does not play a major role in the pathogenesis of cerebral vasospasm at least in a canine model of subarachnoid hemorrhage.

Possible Role in Atherosclerosis and Vascular Smooth Muscle Proliferation

Atherosclerosis may be initiated by endothelial injury, which then leads to the migration and proliferation of intimal smooth muscle. A variety of cell types at the site of injury, including smooth muscle cells, macrophages, activated platelets and endothelial cells are capable of releasing a number of growth promoting molecules for smooth muscle such as PDGF, basic FGF, EGF and thrombin [53]. It is an intriguing possibility that endothelin-1 released from the endothelium could also serve as a causative factor for atherogenesis, since the expression of endothelin-1 in endothelial cells is stimulated by thrombin, TGF- β , increased shear stress and hypoxia which may accumulate in conditions associated with endothelial injury [2, 4]. Interestingly, endothelin-1-immunoreactivity was demonstrated in medial smooth muscle as well as the endothelium in atherosclerotic arteries, whereas in normal arteries it was detected only in the endothelium [54]. Moreover, growing evidence indicate that endothelins possess mitogenic potencies. Komuro *et al.* [55] first reported in cultured rat aortic smooth muscle cells that endothelin-1 stimulates DNA synthesis only when insulin was present together with endothelin-1, suggesting that endothelin-1 acts as a competence type of growth factor on vascular smooth muscle cells. Bobik *et al.* [56] showed that DNA synthesis started as early as 8 h after addition of endothelin-1 to quiescent rat aortic smooth muscle cells, suggesting that its activity is due to a direct mitogenic effect of endothelin-1 on vascular smooth muscle cells. Recently, Eguchi *et al.* [57] have reported that rat aortic vascular smooth muscle cells passaged more than 30 times express a higher level of ET_B endothelin receptor mRNA than younger cells and that endothelin-1 exhibits a much more potent mitogenic activity in older cells compared with younger cells, suggesting that ET_B endothelin receptors may mediate the mitogenic action of endothelin-1 more effectively in this cell type. On the basis of these observations, it is tempting to

speculate that endothelin-1 accumulated at the site of endothelial injury exerts a mitogenic effect on vascular smooth muscle in a paracrine or autocrine fashion and contributes to atherogenesis.

Mechanisms of Endothelin Action

(1) Vascular smooth muscle contraction

Endothelin-1 induces a dose-dependent contractions of isolated vascular segments from experimental animals and human mainly by directly acting on smooth muscle [1], with a few exceptions [19–21]. Like most of vasoactive receptor agonists including norepinephrine, angiotensin II, serotonin and histamine, the receptor activation by endothelins causes the gating of Ca^{2+} channels and the activation of phospholipase C which catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to give rise to inositol-1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) [58–60]. IP_3 acts as a second messenger to mobilize Ca^{2+} from an intracellular pool [61], and an increase in the DAG content activates protein kinase C [62]. Phospholipase D-catalyzed breakdown of phospholipids may also contribute to accumulation of DAG in cells stimulated with endothelin [63]. In many cases receptor stimulation by Ca^{2+} mobilizing agonists is coupled to phospholipase C activation via guanine nucleotide binding regulatory proteins (G proteins) [60]. It was first demonstrated, in membranes prepared from rat aortic smooth muscle A-10 cells which express abundant ET_A endothelin receptors, that endothelin-1 stimulates phospholipase C-catalyzed polyphosphoinositide hydrolysis in a manner strictly dependent on a nonhydrolyzable GTP analogue, $\text{GTP}\gamma\text{S}$ [64]. Further, $\text{GTP}\gamma\text{S}$ inhibits specific binding of ^{125}I -endothelin-1 to A-10 cell membranes in a dose-dependent manner [64]. The inhibitory modulation by guanine nucleotides of the binding of various agonists to their respective receptors is a well-known characteristic found in the receptors that interact with G proteins. These results strongly suggest that the ET_A endothelin receptor in vascular smooth muscle is coupled to phospholipase C via a G protein. Indeed the molecular cloning of the ET_A endothelin receptor revealed the transmembrane topology characteristic of the G protein-coupled receptor superfamily [14]. Pretreatment of A-10 cells with pertussis toxin which is known to inactivate Gi and

Go proteins does not significantly affect the endothelin-1-induced stimulation of phospholipase C [64]. Although further studies are required to conclusively identify the G protein species that couples ET_A endothelin receptors to phospholipase C, the members of Gq family (Gq, G_{11} , G_{14} , G_{16}) are likely candidates, as suggested for many other Ca-mobilizing G protein-coupled receptors [65].

As described above, both *in vitro* contraction studies using isolated vascular strips [22, 23] and hemodynamic studies in intact animals [24–26] suggest that the endothelin-induced vasoconstriction in certain vascular beds is mediated by ET_B endothelin receptors in vascular smooth muscle. Although the signal transduction mechanisms via ET_B endothelin receptors in vascular smooth muscle has not yet been defined, there are several non-smooth muscle cell types in which the signal transduction mechanism via ET_B endothelin receptor was characterized. In CHO cells stably transfected with the ET_B receptor cDNA [66], and bovine vascular endothelial cells [32] and rat osteosarcoma cells constitutively expressing ET_B receptors, endothelins stimulate phospholipase C like in cells expressing ET_A receptors. In ET_B receptor-expressing rat osteosarcoma cells endothelin-1-induced stimulation of phospholipase C is insensitive to pertussis toxin (unpublished observation). In contrast, in bovine endothelial cells it was reported that endothelin-3-induced stimulation of phospholipase C was totally abolished by pertussis toxin pretreatment [32]. These observations indicate that the endothelin receptor-G protein coupling may vary among cell types and receptor subtypes.

When the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is measured in intact porcine coronary smooth muscle strips with the photoprotein, aequorin, there is a biphasic increase in $[\text{Ca}^{2+}]_i$ upon endothelin-1 stimulation [60]. The $[\text{Ca}^{2+}]_i$ rapidly rises to a peak within 1 min, then falls to a plateau level slightly higher than the unstimulated baseline value. In the absence of extracellular Ca^{2+} , endothelin-1 stimulation causes a smaller initial transient increase in $[\text{Ca}^{2+}]_i$, which is most likely caused by IP_3 -induced mobilization of intracellular Ca^{2+} . These findings indicate that Ca^{2+} giving rising to the initial transient response comes from both intracellular and extracellular sources and that the sustained small increase in $[\text{Ca}^{2+}]_i$ is

dependent on extracellular Ca^{2+} , i.e. Ca^{2+} influx across the plasma membrane. Removal of extracellular Ca^{2+} or additions of inorganic Ca^{2+} channel blockers substantially but not completely inhibit endothelin-1-induced contraction in many vascular regions, indicating that the major portion of the endothelin-induced contraction is dependent on Ca^{2+} influx across the plasma membrane [59, 67]. Since endothelin-1-induced contraction of porcine coronary artery strips is markedly inhibited by a dihydropyridine Ca^{2+} channel antagonist [1, 60, 68], endothelin-1-induced Ca^{2+} influx appears to occur mainly through voltage-dependent Ca^{2+} channels of L-type. In fact, endothelin-1 has been shown to activate the dihydropyridine-sensitive, voltage-dependent Ca^{2+} channel in porcine coronary artery smooth muscle cells with a patch-clamp technique [60, 69]. Based upon the high sensitivity to a dihydropyridine Ca^{2+} channel antagonist observed in porcine coronary artery, it was initially hypothesized that endothelin-1 was an endogenous agonist of the dihydropyridine-sensitive Ca^{2+} channel [1]. However, it was later noted that isolated vascular segments from other vascular regions, for example rabbit aorta and porcine carotid artery, are not always inhibited by a dihydropyridine Ca^{2+} channel antagonist [67]. In addition, in coronary artery smooth muscle, serotonin- and histamine-induced contractions are also sensitive to a dihydropyridine Ca^{2+} channel antagonist [70]. On the other hand, in rabbit aorta serotonin- and histamine-induced contraction as well as endothelin-1-induced contraction are resistant to a dihydropyridine Ca^{2+} channel antagonist. These observations indicate that the high sensitivity to a dihydropyridine Ca^{2+} channel antagonist of the endothelin-1-induced contraction of porcine coronary artery smooth muscle represents the tissue-specific rather than the agonist-specific property. One of the factors which determine the region-specific sensitivity to a dihydropyridine Ca^{2+} antagonist may be a resting level of a membrane potential which affects the conformation of a L-type Ca^{2+} channel protein, a target molecule of a dihydropyridine drug, as suggested by Godfraind *et al.* [71]. Porcine coronary artery smooth muscle responds to endothelin-1 stimulation with only a small extent of membrane depolarization (8–15 mV) [68] which is brought about probably by stimulation of inward Na^+ current and/or outward Cl^- current, im-

plying that the activation of a L-type Ca^{2+} channel by endothelin-1 is not accounted for solely by the membrane depolarization [72, 73]. The electrophysiological study by Silverberg *et al.* [69] suggested that endothelin-1 acts via the second messenger system to induce the gating of a L-type Ca^{2+} channel in porcine coronary artery smooth muscle.

When endothelin-1 is added to porcine coronary artery smooth muscle, 20 kD myosin light chain (MLC) becomes maximally phosphorylated within 5 min by activation of a Ca^{2+} , calmodulin-dependent enzyme, MLC kinase, and stays at this level as long as the isometric contraction is sustained for at least 60 min [74, 75]. When the temporal changes in the extent of phosphorylation of 20 kD MLC is compared with that in $[\text{Ca}^{2+}]_i$, it is apparent that during the sustained phase of contraction the level of 20 kD MLC phosphorylation is maintained at a lower $[\text{Ca}^{2+}]_i$ compared to the $[\text{Ca}^{2+}]_i$ during the initial phase of contraction. The apparent sensitization of phosphorylation of 20 kD MLC during the sustained phase of endothelin-1-induced contraction may be caused by a GTP-dependent mechanism as shown in norepinephrine- and carbachol-induced smooth muscle contraction [76, 77]. Although the involvement of a small molecular weight G protein rho p21 [78], MLC-specific phosphatase [77] and protein kinase C [76, 79] in this sensitization phenomenon has been suggested, precise molecular mechanism underlying the sensitization remains to be clarified.

Several lines of evidence suggest that activation of the protein kinase C may also participate in the contractile mechanism activated by endothelin-1: first, stimulation of porcine coronary artery smooth muscle causes an increase in the tissue content of DAG [68], which is an endogenous activator of the protein kinase C, second, stimulation with endothelin-1 causes a sustained increase in the membrane-associated protein kinase C activity with a reciprocal decrease in the cytosolic protein kinase C activity [80], third, endothelin-1 induces a small but significant contraction even after strict Ca^{2+} depletion from both intra- and extracellular pools and this contraction is inhibited by a protein kinase C inhibitor, H-7 [68, 136], fourth, endothelin-1 induces phosphorylation of a thin filament associated protein, caldesmon and an intermediate filament protein, desmin [74, 75],

fifth, an activator of protein kinase C, phorbol ester causes a potent contraction and phosphorylation of caldesmon and desmin in porcine coronary artery smooth muscle [75]. Although the precise molecular mechanism underlying the protein kinase C-dependent contraction is not yet fully understood [77, 81], these observations suggest that the protein kinase C signalling pathway together with the sustained increase in the content of phosphorylated 20 kD MLC may mediate the endothelin-1-induced contraction (Fig. 1).

(2) Endothelium-dependent vasorelaxation

Endothelins stimulate release of vasodilator prostanoids and EDRF from the endothelium [5], which may mediate the vasodilator and the antiplatelet activities of the peptides. The demonstration of the binding property of ^{125}I -labeled endothelin isopeptides to endothelial cell membranes which is compatible with the existence of ET_B receptors [32, 82], the activity of a selective ET_B

agonist in stimulating EDRF production [25, 83], and the expression of the ET_B endothelin receptor message but not of the ET_A receptor message by Northern blot analysis [29, 30] is all consistent with the notion that ET_B endothelin receptor mediate the endothelin actions on the endothelium. The activation of ET_B endothelin receptors by endothelins causes activation of phospholipase C and phospholipase A_2 , and gating of a non-voltage-dependent Ca^{2+} channel [32]. The resulting increase in $[\text{Ca}^{2+}]_\text{i}$ activates a Ca^{2+} , calmodulin-dependent isoform (type III) of nitric oxide synthase and induces release of NO (EDRF) [84]. Released NO acts on the underlying smooth muscle layer and activates a soluble guanylate cyclase to generate cyclic GMP [85]. An increase in the intracellular cyclic GMP level induces relaxation of vascular smooth muscle by inhibiting agonist-induced activation of phospholipase C and Ca^{2+} channel gating and by activating Ca^{2+} extrusion across the plasma membrane [85]. The first

Fig. 1. Mechanisms of vascular smooth muscle contraction caused by endothelins. The binding of endothelins to either ET_A or ET_B endothelin receptors on vascular smooth muscle cells activates phospholipase C in a G protein (G)-coupled manner and causes gating of Ca^{2+} channels, leading to the generation of inositol-1, 4, 5-trisphosphate (IP_3) and 1, 2-diacylglycerol (DAG), a protein kinase C (PKC) activator, and to stimulation of Ca^{2+} influx. Increase in $[\text{Ca}^{2+}]_\text{i}$ activates a Ca^{2+} , calmodulin-dependent myosin light chain (MLC) kinase, resulting in phosphorylation (P) of 20 kD light chain subunit (MLC) of myosin which allows interaction with actin leading to the initiation of contraction. The receptor activation by endothelins may somehow stimulate the sensitizing mechanism for phosphorylation of myosin to an increase in $[\text{Ca}^{2+}]_\text{i}$. The activation of protein kinase C causes increases in phosphorylation levels of several proteins which may also contribute to the contraction. See the text for more detail.

step for the production of vasodilator prostanoids is cleavage and release of arachidonic acid by cytosolic type of phospholipase A₂ (cPLA₂) from the membrane phospholipids. An increase in [Ca²⁺]_i on the receptor activation by endothelins causes cPLA₂ to translocate from the cytosol to the membrane. Endothelins activate a cascade of serine/threonine kinases, leading to activation of mitogen activated kinase (MAP kinase) mainly in a protein kinase C-dependent manner. The activated MAP kinase then phosphorylate cPLA₂, causing an increase in cPLA₂ activity [86]. Released arachidonic acid is metabolized via the cyclooxygenase pathway to prostacyclin and other vasodilator prostanoids, which act on vascular smooth muscle to cause an increase in the intracellular cyclic AMP level and relaxation (Fig. 2).

(3) Mitogenic action

In addition to vascular smooth muscle cells, endothelins stimulate DNA synthesis in several types of cells [138], including fibroblasts [87], renal mesangial cells [88], osteoblastic cells [89, 90] and several cancer cell lines [138]. Molecular mechanisms underlying endothelin-1-induced mitogenesis were studied in Swiss mouse 3T3 fibroblasts, a well

characterized model system for the investigation of cell growth, in which activation of the protein kinase C induces synchronous DNA synthesis [91]. In this cell type, endothelin-1 activates phospholipase C via ET_A receptors with the production of two second messengers, IP₃ and DAG, leading to Ca²⁺ mobilization from both intra- and extracellular pools and activation of protein kinase C as evidenced by phosphorylation of a protein kinase C substrate, MARCKS protein. The mitogenic effect of endothelin-1 is markedly attenuated in phorbol ester-pretreated, protein kinase C-depleted cells, suggesting that protein kinase C activation is a necessary, if not sufficient, event in endothelin-1 induced DNA synthesis. Swiss 3T3 cells express at least four isoforms (α , δ , ϵ and ζ) of the protein kinase C, among which three of them (α , δ and ϵ) are subject to downregulation by phorbol ester-pretreatment, suggesting that either α , δ and/or ϵ isoform of the protein kinase C may mediate the mitogenic effect of endothelin-1 in this cell type. In Swiss 3T3 cells, the protein kinase C stimulation activates a cascade of intracellular serine/threonine kinases, leading to activation of MAP kinase. Activated MAP kinase, in turn, was recently found to phosphorylate, in addition to

Fig. 2. Mechanisms of action of endothelins in the endothelium. Endothelins act via ET_B receptors to cause activation of phospholipase C and gating of non-voltage-dependent Ca²⁺ channels, leading to an increase in [Ca²⁺]_i and PKC activation. The PKC, in turn, activates a cascade of serine/threonine kinases causing activation of mitogen activated protein kinase (MAPK). The MAPK phosphorylates a cytosolic type of phospholipase A₂ (PLA₂) liberating arachidonic acid (AA), which is metabolized to prostaglandin I₂ (PGI₂) and thromboxane A₂ (TxA₂). Increase in [Ca²⁺]_i activates a Ca²⁺, calmodulin-dependent isoform of NO synthase to lead to production of NO, endothelium-derived relaxing factor (EDRF). See the legend of Fig. 1. for the other abbreviations.

cPLA₂ as described above, Myc and Jun proteins, thereby enhancing their ability to stimulate gene transcription. This pathway may be involved in endothelin-1-induced expression of proto-oncogene *c-fos*, because 5' upstream region of *c-fos* gene contains a cis-element for Jun/Fos complex (AP-1). Moreover, it was found that stimulation of protein kinase C in serum-deprived, quiescent Swiss 3T3 cells results in activation of *cdc2*, a member of cell cycle-regulating cyclin-dependent protein kinases at a time point close to G1/S boundary of the cell cycle [92]. The activation of *cdc2* kinase is associated with a dramatic increases in *cdc2* protein level and its phosphorylation at serine/threonine residues. Cyclin dependent kinases including *cdc2* and *cdk2* have been shown to phosphorylate and to inactivate tumor suppressor RB gene product, which is now considered to be a crucial prerequisite for initiation of DNA synthesis by mammalian cells. In human vascular endothelial cells stimulation of protein kinase C in early G1 phase markedly potentiates growth factor-induced activation *cdc2* and *cdk2*, as well as expression of cyclin A and E [94]. These observations suggest that activation of protein kinase C through the inositol lipid signalling pathway converges to the common mitogenic signalling, which, although yet to be defined, eventually leads to the initiation of DNA synthesis. The mitogenic effect of endothelin-1 is dependent also on the extracellular Ca²⁺ concentration. Activation of *cdc2* and *cdk2* kinases and phosphorylation of RB protein are also dependent on the extracellular Ca²⁺ concentration [95]. Since *cdc2* and *cdk2* do not require Ca²⁺ for *in vitro* kinase activity, it is conceivable that there exists Ca²⁺-dependent pro-

cess(es) in the mitogenic signalling pathway at site(s) proximal to *cdc2* and *cdk2* activation. Although phospholipase C and protein kinase C are classical examples of Ca²⁺-dependent enzymes, activations of these enzymes are totally independent of extracellular Ca²⁺ [93], suggesting that the Ca²⁺-dependent process is located downstream of PKC action. Like many other mitogens which activate G protein-coupled receptors to stimulate phospholipase C-protein kinase C signalling pathway such as bombesin/GRP and thrombin, endothelin-1 is known to activate protein tyrosine kinase as well. This effect of endothelin may be the consequence of protein kinase C activation. Unlike bombesin/GRP, which synergizes with growth factors acting via the receptor tyrosine kinase activation but not with a phorbol ester, endothelin-1 shows synergistic stimulation of DNA synthesis when acting together with a phorbol ester or bombesin/GRP [96]. This observation may suggest the presence of thus far unrecognized mechanism of action of endothelin as a mitogen.

III. Actions on Endocrine System

Autoradiographic studies of rat tissues with ¹²⁵I-labeled endothelin-1 demonstrated the specific binding sites for endothelins in endocrine organs including hypothalami, pituitaries and adrenal glands. These studies suggest modulatory roles of endothelins for endocrine functions [1, 97]. Further, in these tissues the presence of endothelin-immunoreactivity and -mRNAs was also detected. Numerous studies actually demonstrated modulatory effects of endothelin peptides

Table 1. Modulation of endocrine secretion by endothelin peptides

Hormone/Substance	Effect	Tissue/cells	References
vasopressin	stimulation	hypothalamic slice	99
substance P	stimulation	hypothalamic slice	101
gonadotropin releasing hormone	stimulation	cultured hypothalamic neurons	102
prolactin	inhibition	cultured pituitary cells	105, 107–109
	biphasic (stimulation→inhibition)	cultured pituitary cells	104, 108
luteinizing hormone	stimulation	cultured pituitary cells	104, 107, 110, 111
follicle stimulating hormone	stimulation	cultured pituitary cells	107, 111
growth hormone	stimulation	cultured pituitary cells	104
thyrotropin	stimulation	cultured pituitary cells	107
atrial natriuretic peptide	stimulation	atrial cardiomyocytes	113–117
aldosterone	stimulation	zona glomerulosa cells	125–130
catecholamine	stimulation	adrenal chromaffin cells	132–134
renin	inhibition	juxtaglomerular cells	123, 124

on a variety of endocrine functions (Table 1); endothelins stimulate release of vasopressin and gonadotropin releasing hormones from hypothalamic neurons, several hormones from anterior pituitary cells, atrial natriuretic peptide from atrial cardiomyocytes, aldosterone and catecholamine from adrenal cells. On the other hand, endothelins inhibit release of prolactin from anterior pituitary cells and renin from juxtaglomerular cells.

Stimulation of Vasopressin, Substance P and Gonadotropin Releasing Hormone Secretion from Hypothalamus

Systemic intravenous administration of endothelin-1 causes an increase in plasma vasopressin level in anesthetized rats and dogs [8, 18]. This occurs despite of elevated level of plasma atrial natriuretic peptide and increased left atrial pressure, which both operate to inhibit vasopressin release, suggesting that endothelin-1 may have a central stimulatory effect on vasopressin release. In fact, subsequently, intracerebroventricular injection of endothelin-1 was demonstrated to cause an increase in plasma level of vasopressin [41–43]. Systemic administration of endothelin-1 enhances the activity of vasopressin- and oxytocin-secreting neurons in the paraventricular and supraoptic nuclei as well as of neurons in the subfornical organ which lack the blood-brain barrier and send efferent neural projections to both supraoptic and paraventricular nuclei of the hypothalamus [98]. The destruction of the subfornical organ abolishes the activation of vasopressin- and oxytocin-secreting neurones in response to systemic administration of endothelin-1, suggesting that the subfornical organ is a site at which endothelin-1 acts in the central nervous system to elicit vasopressin release [98]. It is controversial whether endothelins have any direct effect on vasopressin-secreting neurons in the hypothalamus [99, 100]. Yoshizawa *et al.* [103] reported that magnocellular neurones with projections to the median eminence and the posterior pituitary contained endothelin-1-immunoreactivity and that endothelin-1-immunoreactivity disappeared in the posterior pituitary of rats which were water-deprived for four days, suggesting a release of endothelin-1 under a physiological condition [11]. Endothelin-1 immunoreactivity was found to be co-localized with vasopressin or oxytocin in the neurosecretory granule of the axon. However, it is not known

whether endothelin-1 is released from the posterior pituitary to enter the systemic circulation exerting its action as a hormone or to act locally in a paracrine or autocrine manner. Interestingly, endothelin-1 and -3 were shown to act to antagonize the antidiuretic action of vasopressin in the renal collecting duct, suggesting the negative feedback interaction of endothelins with vasopressin at the level of the peripheral target organ of vasopressin [9, 135].

Endothelin-1 stimulates release of substance P from perfused isolated hypothalamic slice [101]. This effect was reported to be unaffected by removal of extracellular Ca^{2+} . On the basis of morphological studies showing that substance P-immunoreactive nerve terminals are localized in eminentia medialis of the hypothalamus [102], it is conceivable to postulate that substance P is thought to be secreted from neuroendocrine cells in the hypothalamus into portal vein to reach the anterior pituitary, where it may affect the pituitary function. The effect of endothelin-1 in the spinal cord was reported to be inhibited by a substance P-receptor antagonist, Spantide, implying that the action of endothelin-1 in the central nervous system might be, in part, mediated by substance P [11].

Endothelin-1 stimulates secretion of gonadotropin releasing hormone from cultured hypothalamic neurons derived from fetal rats [102]. Relative potencies of endothelin-1 and endothelin-3 in displacing ^{125}I -endothelin-1 binding to membranes, in activation of phospholipase C, and in the secretory response suggest that the ET_A receptor mediate the action of endothelin peptides on hypothalamic neurons. Interestingly, the same culture of hypothalamic cells was observed to secrete both endothelin-1 and endothelin-3 [102]. Since endothelins are also known to have a direct stimulatory action on gonadotroph cells as described below, endothelins appear to act at two sites to stimulate gonadotropin secretion.

Modulation of Secretion of Anterior Pituitary Hormones

Several groups reported conflicting data concerning the endothelin effects on release of prolactin (PRL), luteinizing hormone (LH), follicle stimulating hormone (FSH) and other anterior pituitary hormones from cultured rat pituitary cells. In particular, the reported data on PRL

secretion are quite confusing. Samson *et al.* [104] demonstrated that endothelin-3 (1–100 μM) induced a monophasic inhibition of PRL secretion in the static incubation system of cultured anterior pituitary cells from adult rats. When a more physiological perfusion system was employed, they found that endothelin-3 at 100 μM induced a biphasic response of PRL secretion: a transient stimulation followed by a longer-lasting inhibition. In the perfusion system, endothelin-3 also stimulated secretion of LH and growth hormone (GH). They showed that endothelin-1 was ineffective in eliciting the inhibition of PRL secretion in the static incubation system [105]. However, one of these authors reported later that the rank order of potency for the inhibition of PRL secretion was endothelin-1 = endothelin-2 > endothelin-3 and that the selective ET_A receptor antagonist, BQ-123, abolished the inhibitory effect of endothelins for PRL secretion, suggesting the role of ET_A receptors in this response [106]. Kanyicska *et al.* demonstrated that endothelin-3 dose-dependently inhibited PRL secretion and stimulated secretion of LH, FSH and thyrotropin, without affecting GH secretion in the static incubation system of cultured anterior pituitary cells from adult rats. Dymshitz *et al.* [108] showed that the cultivation of anterior pituitary cells in serum-containing medium caused a monophasic inhibition of PRL secretion upon endothelin stimulation in the static incubation system with endothelin-1 being a more potent inhibitor than endothelin-3, while the cultivation in serum-free medium caused a biphasic response, indicating that serum had a profound influence on the response of PRL release to endothelins. Domae *et al.* [109], however, demonstrated that endothelin-1 and endothelin-3 inhibited PRL release from isolated rat anterior pituitary slice with an almost identical potency, suggesting the involvement of ET_B receptors. Further, Stojilkovic *et al.* [110, 111] reported that endothelin-1 is more potent in eliciting $[\text{Ca}^{2+}]_\text{i}$ response and LH secretion and in binding to the membrane than endothelin-3 in cultured anterior pituitary cells from adult rats, suggesting that these responses by endothelins occurred via activation of ET_A receptors. The reasons for these discrepancies are not known at present.

It was reported that either intracerebroventricular injection or systemic intravenous administration of endothelin-3 at a pressor dose did not

significantly alter plasma levels of PRL, LH and GH in rats, suggesting that endothelins of peripheral origin are unlikely to act within the pituitary. As mentioned above, endothelin-1-immunoreactivity is detected in hypothalamic neurons which project to the median eminence [103]. This observation suggests that endothelin-1 secreted from neuroendocrine cells in the hypothalamus into portal vein reaches the anterior pituitary, where it may have a direct action on the functions of anterior pituitary cells. Alternatively, locally produced endothelins may act as a paracrine or autocrine regulator of pituitary functions, because both endothelin-1 and endothelin-3 are shown to be present in the pituitary. Particularly, the pituitary is unique in that it contains a larger amount of endothelin-3 than endothelin-1 [137].

Endothelins were shown to stimulate phospholipase C, generating IP_3 and DAG and to cause a biphasic increase in $[\text{Ca}^{2+}]_\text{i}$ in rat anterior pituitary cells, as in other types of cells [110, 111]. A dihydropyridine Ca^{2+} -channel antagonist, nifedipine, inhibits both the extracellular Ca^{2+} -dependent phase of the $[\text{Ca}^{2+}]_\text{i}$ response and LH secretion in gonadotroph [104, 111] cells. Pretreatment of anterior pituitary cells with pertussis toxin abolished the inhibitory effect of endothelin-3 on PRL release, indicating the involvement of pertussis toxin-sensitive G proteins (G_i or G_o) in the this action of endothelin-3 [112]. It is an interesting possibility that this inhibitory response is caused by inhibitions of adenylate cyclase activity and/or Ca^{2+} channel activity through endothelin receptor-G protein coupling, as demonstrated for dopamine and somatostatin actions on pituitary cells.

Stimulation of Atrial Natriuretic Peptide Secretion

Intravenous infusion of endothelin-1 causes elevation of plasma level of atrial natriuretic peptide (ANP) [8, 18]. ANP secretion from mammalian hearts is known to be augmented by a variety of stimuli, including elevation in left atrial pressure, tachycardia, and neurotransmitters and hormones such as norepinephrine and neuropeptide Y. One of the responsible mechanisms for ANP release caused by endothelin-1 infusion is a rise in left atrial pressure which occurs during endothelin-1 infusion. In addition to this, endothelin-1 directly acts on atrial cardiac muscle

to stimulate ANP release. This effect was demonstrated in cultured atrial cardiomyocytes [113] and isolated atria [114]. In rat atria, endothelin-1 enhances the ANP secretory response to stretch, implying that the direct action of endothelins on atrial muscle coordinately interacts with elevation of left atrial pressure to enhance atrial ANP secretion [117].

Endothelin-1 induces a dose-dependent increase in $[Ca^{2+}]_i$ in atrial cells. The releasing effect of endothelin-1 is inhibited by a reduction in extracellular Ca^{2+} and addition of either a blocker of voltage-dependent Ca^{2+} channels, a calmodulin antagonist calmidazolium or a protein kinase C inhibitor staurosporine [115, 116]. These observations suggest the importance of Ca^{2+} influx across the plasma membrane through a voltage-dependent Ca^{2+} channel, calmodulin and protein kinase C in endothelin-induced ANP secretion.

Interestingly, ANP antagonizes the actions of endothelins at several site. First, endothelin-1 is a potent vasoconstrictor while ANP is a vasodilator. Second, endothelin-1 has a potent antinatriuretic effect in the kidney while ANP causes natriuresis. Third, endothelin-1 stimulates aldosterone secretion from adrenal glomerulosa cells while ANP inhibits aldosterone secretion. Fourth, centrally administered natriuretic peptide antagonizes the pressor response and elevation of plasma vasopressin level caused by intracerebroventricular injection of endothelin-1. And lastly, ANP inhibits production and release of endothelin-1 from endothelial cells [118]. This antagonism between ANP and endothelins is reminiscent of that between ANP and angiotensin II, suggesting that ANP may act as a negative feedback regulator to oppose the overactivity of the pressor system composed of angiotensin II and endothelin-1.

Stimulation of Aldosterone and Catecholamine Secretion from Adrenals

Autoradiographic and binding studies demonstrated specific binding sites for ^{125}I -endothelin-1 in both adrenal cortex and medulla [7]. Adrenal medulla showed a higher density of binding of ^{125}I -endothelin-1 compared with cortex [7, 119]. Northern blot analysis revealed that adrenals express mRNAs for both ET_A and ET_B endothelin receptors [15, 29, 30]. Adrenal tissues (cortex plus medulla) were also reported to contain endothelin-

1 and to a lesser extent, endothelin-3, and Northern blot analysis detected mRNAs for both peptides [120–122]. A more detailed study demonstrated that adrenal cortical tissues express mRNAs for both receptor subtypes and peptides [122].

Systemic infusion of endothelin-1 induces elevation of plasma levels of aldosterone [8, 18]. Endothelin-1 infusion causes a rise in plasma renin activity, which is probably secondary to vasoconstriction of the renal vessels proximal to the juxtaglomerular cells because endothelins are shown to have a direct inhibitory effect on renin release from juxtaglomerular cells [123, 124]. In addition to this effect, endothelins were demonstrated to directly act on adrenal glomerulosa cells causing stimulation of aldosterone secretion [125–128]. The *in vitro* stimulatory effects of endothelins on aldosterone secretion is relatively weak compared to that of angiotensin II, a major regulatory factor for aldosterone secretion [125–128]. The endothelin receptor subtype which mediates the secretory effect of endothelins is not yet conclusively determined [129, 130]. Interestingly, aldosterone-secreting adenoma expresses endothelin receptors but do not respond to endothelin-1 with aldosterone secretion just as it is refractory to angiotensin II which acts as a Ca^{2+} -mobilizing peptide like endothelin-1 [128]. Endothelin peptides cause activation of phospholipase C and a biphasic increase in $[Ca^{2+}]_i$ in glomerulosa cells [127]. The aldosterone-secretory response by endothelin-1 is inhibited by either removal of extracellular Ca^{2+} or addition of a dihydropyridine Ca^{2+} channel antagonist, as in other cell types [125]. The target of a dihydropyridine Ca^{2+} channel antagonist is probably a voltage-dependent Ca^{2+} channels of T type in glomerulosa cells [131], since this type of Ca^{2+} channels are considered to be a major Ca^{2+} channel through which Ca^{2+} influx occurs in response to angiotensin II stimulation.

Systemic administration of endothelin-1 causes elevation of plasma levels of adrenaline and noradrenaline [8, 18]. Cultured bovine adrenal chromaffin cells were demonstrated to possess specific binding sites for endothelins with similar affinities for both endothelin-1 and endothelin-3, presumably ET_B receptors [132]. Addition of endothelin-1 to cultured adrenal cells induces release of adrenaline and noradrenaline [133, 134]. However, endothelin-1 is a very weak secre-

tagogue and does not alter secretion induced by high KCl or nicotine. Adrenal chromaffin cells express abundant voltage-dependent Ca^{2+} channels of L-type. However, the endothelin-1-induced catecholamine secretion is only partially inhibited by a dihydropyridine Ca^{2+} channel antagonist, which is distinct from high KCl and nicotine-induced responses [133]. In these studies endothelin-1 failed to stimulate phospholipase C [133, 134].

III. Summary

Endothelin family peptides are now known to exert diverse biological effects on a wide variety of tissues and cell types through at least two subtypes of receptors. In vascular systems, both ET_A and ET_B endothelin receptors present in vascular smooth muscle mediate the vasoconstrictor and mitogenic activities of the peptides, while ET_B receptors in the endothelium mediate the vasodila-

tor and antiplatelet activities. Endothelins also affect hormone secretion from a variety of endocrine organs including anterior and posterior pituitary, atria and adrenals. Endothelins activate the Ca^{2+} -messenger system which involves both calmodullin and protein kinase C to exert their biological activities in almost all cell types examined, and appear to work in a paracrine and autocrine fashion. However, physiological and pathophysiological roles of endothelins are still incompletely understood and further studies are clearly required for elucidation of biological significance of this peptide family.

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