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Review

Subcellular mechanisms of endothelin action in vascular system

Tomoh Masaki [a,*], Soichi Miwa [b], Tatsuya Sawamura [a], Haruaki Ninomiya [b], Yasuo Okamoto [b]

[a] *National Cardiovascular Center Research Institute, 5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan*
[b] *Department of Pharmacology, Faculty of Medicine, Kyoto University, Kyoto, Japan*

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Abstract

To elucidate the role of endothelin in the regulation of vascular function, the cellular and subcellular mechanisms for the synthesis of endothelin and the function of endothelin-receptors have been studied extensively. In this article, recent results regarding these problems are reviewed. (1) Oxidatively modified low-density-lipoprotein (LDL) reduces nitric oxide (NO) release via inhibition of the high-affinity arginine transporter of endothelial cells. (2) Endothelin-1-induced vasoconstriction is mediated by Ca^{2+} influx through a non-selective cation channel sensitive to 1-[β-[3-(4-methoxyphenyl) propoxyl]-4-methoxyphenethyl]-1*H*-imidazole HCl (SK & F96365). (3) A distinct domain of the endothelin-receptor is required for the coupling of different G_{α}-proteins. (4) Endothelin ET_A receptor-mediated mitogenic activity is mediated by two pathways, one classical protein kinase C(PKC)-dependent, and the other phosphoinositide 3-kinase dependent. Both stimulate mitogen-activated protein kinase (MAPK). Endothelin ET_B receptor-mediated mitogenic activity is also mediated by the PKC-dependent pathway. In contrast, endothelin ET_B receptor-mediates differentiation and apoptosis via G_{α^i} coupling.

Keywords: Endothelin; Endothelin receptor; Endothelial oxidized low density lipoprotein receptor; Cationic amino acid transporter

1. Introduction

Endothelin, which was originally found in cultured porcine aortic endothelial cells, is a potent vasoconstrictor peptide with 21 amino acid residues (Yanagisawa et al., 1988). There are three endogenous isoforms of endothelin named endothelin-1, endothelin-2 and endothelin-3 (Inoue et al., 1989). They are produced and released from a variety of cells in different proportions. Vascular endothelial cells exclusively produce endothelin-1.

The production of endothelin-1 in endothelial cells is regulated by several factors. Hemodynamic forces such as the shear stress of blood flow, stretch of the vascular bed or blood pressure have been implicated in the expression of endothelin-1 mRNA. Several chemical factors such as angiotensin and cytokines, which are produced locally, also modulate the expression and release of endothelin-1 from the endothelium.

Endothelin-1 is synthesized initially as a long peptide precursor, i.e., prepro endothelin-1. After removal of the signal peptide, the resulting pro-endothelin-1 is further processed by a furin-like convertase to produce an intermediate form, big endothelin-1 (Yanagisawa et al., 1988; Kido et al., 1997). Big endothelin-1 is further cleaved by an endopeptidase, endothelin-converting enzyme-1, to produce mature endothelin-1 (Yanagisawa et al., 1988; Shimada et al., 1994; Xu et al., 1994). Another isoform endothelin-2 or endothelin-3 is also produced through a similar pathway from its own precursor. The processing of endothelin-1 occurs intracellularly in constitutive secretory vesicles.

So far, two types of endothelin-receptors, named endothelin ET_A and endothelin ET_B receptors, have been reported in mammalian tissues (Arai et al., 1990; Sakurai et al., 1990). Both receptors belong to the family of heptahelical G-protein-coupled receptors. Endothelin ET_A receptor has a high affinity for endothelin-1 and endothelin-2 but a low affinity for endothelin-3, while endothelin ET_B receptor has equal affinity for all three isoforms of endogenous endothelin. These two receptors are also distributed in a variety of cells and tissues in different proportions, which suggests that endothelins have a variety of physiological functions (Masaki et al., 1994). In the vascular bed, endothelin ET_A receptors located on smooth mus-

* Corresponding author. Tel.: +81-6-6833-5012/2501; Fax: +81-6-6833-1421; E-mail: masaki@ri.ncvc.go.jp

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cle cells and mediates contraction, while endothelin ET_B receptor exists on endothelial cells and mediates the release of relaxing factors such as nitric oxide(NO) and prostacyclin. Nowadays, many peptide and non-peptide antagonists for endothelin ET_A, endothelin ET_B or both endothelin $ET_{A/B}$ receptors are available (Ruffolo, 1995; Miyauchi and Masaki, 1999), some of which discriminate pharmacologically between two subtypes of endothelin ET_B receptor, endothelin ET_{B1} and endothelin ET_{B2} receptors (Douglas et al., 1994). Endothelin ET_{B1} receptor exists on the endothelium and mediates the release of relaxing factor. Endothelin ET_{B2} receptor exists on smooth muscle, such as rabbit saphenus vein smooth muscle, and mediates contraction. Another type of endothelin-receptor, named endothelin ET_C receptor, has been cloned from the dermal melanophores of *Xenopus laevis* and has high affinity for endothelin-3, but low affinity for endothelin-1 and endothelin-2 (Karne et al., 1993). The existence of a similar type of endothelin-receptor in rabbit saphenus vein is suggested by pharmacological experiments (Douglas et al., 1995). However, to date, this type of receptor has not been detected in mammalian tissues by molecular biology.

It has been suggested that endothelin-1 participates in the regulation of cardiovascular homeostasis as a local humoral factor. With regard to the mechanism of maintenance of vascular tone, the shear stress of blood flow or local humoral factors stimulate endothelial cells to release endothelin-1. The released endothelin-1 acts on its own and neighboring endothelial cells to release relaxing factor(s) in a paracrine and autocrine manner. It also acts directly on the underlying smooth muscle to induce contraction. These results suggest the versatile nature of endothelin-1 as a modulator of vascular tone (Masaki, 1995). In fact, it was demonstrated that infusion of an antagonist of both endothelin $ET_{A/B}$ receptors decreased peripheral resistance and also blood pressure in human, suggesting that endogenous endothelin-1 regulates basal vascular tone (Haynes et al., 1996). Furthermore, a recent study of heterozygote endothelin-1 deficient mice and normal mice indicated that blood pressure was elevated despite a low plasma level of endothelin-1 (Kurihara et al., 1994). This unexpected result is explained by an increased sympathetic tone as a consequence of hypoxia in the heterozygous mouse (Ling et al., 1998) or by a decrease in the release of relaxing factor from the endothelium as a result of the decreased expression of endothelin-1. The latter possibility is supported by the observation that selective endothelin ET_B receptor antagonists increased the blood pressure of normal mice but not that of he endothelin ET_B-deficient mice, and that the arterial blood pressure of endothelin ET_B-deficient mice was also significantly higher than that of normal mice (Ohuchi et al., 1999).

There is consensus that endothelin has an aggravating role in several vascular diseases including chronic heart failure, pulmonary hypertension, cerebrovascular spasm after subarachnoid hemorrhage, acute renal failure and essential hypertension (Miyauchi and Masaki, 1999). In these diseases, the plasma endothelin-1 level is increased, and endothelin receptor antagonists have beneficial effects on pathological features. For example, in chronic heart failure, endothelin receptor antagonists improve the survival rate and various parameters of cardiac function (Sakai et al., 1996).

Despite these results, the physiological and pathophysiological significance of endothelin in the cardiovascular system still remains to be solved. Therefore, in this article, we focus on the recent progress made in the study of the cellular and subcellular mechanisms underlying the vascular action of endothelin-1, i.e., (1) the relationship between NO and endothelin-1 in their production by the endothelium and their action on target cells; (2) the structure and function of endothelin-receptor; and (3) the intracellular mechanisms of contraction and cell-growth activity of endothelin-1. The elucidation of these mechanisms will throw new light on local vascular regulatory mechanisms.

2. Interaction of endothelin and NO in their synthesis

Under physiological conditions, the shear stress of blood flow and a variety of receptor agonists regulate the production of endothelin and NO. When shear stress is increased, the expression of endothelial NO synthase is enhanced. The expression of prepro endothelin-1 is increased by a low level or brief exposure to shear stress, but decreased by sustained exposure to a higher shear stress. Since an increase in the cyclic GMP (cGMP) level inhibits the release of endothelin-1, the decrease in endothelin-1 release induced by high shear stress is ascribed to the increase in cGMP level occurring as a consequence of increased levels of NO (Kuchan and Frangos, 1993).

In endothelial dysfunction, the release of endothelin-1 from endothelial cells increases, while the release of NO decreases. Vascular tone increases under this condition. Endothelial dysfunction is promoted by risk factors for atherosclerosis. Oxidation is an important process induced by risk factors. The level of oxidatively modified low-density-lipoprotein (oxLDL) reportedly increases in the vascular bed under the endothelial dysfunction. It has been reported that oxLDL impairs endothelium-dependent arterial relaxation induced by acetylcholine, suggesting that oxLDL impairs NO release (Kugiyama et al., 1990). The expression of endothelial NO synthase (eNOS) is not impaired under hypercholesterolemic conditions, but there is increased oxidative breakdown of NO, probably due to the enhanced formation of super oxide radicals (Forstermann et al., 1993).

We hypothesized that oxLDL impairs the activity of the arginine-transporter, thereby decreasing the production of NO, because an increase in the concentration of arginine in the extracellular medium reversed the impairment of NO production in hypercholesterolemic animals. To test this

hypothesis, we examined the effect of lysophophatidylcholine, the major component of oxLDL, on the incorporation of ^{14}C-labeled arginine into bovine endothelial cells and on NO release from endothelial cells. Arginine incorporation was suppressed by lysophosphatidylcholine in a dose- and time-dependent manner (Kikuta et al., 1998). Accordingly, the production of NO decreased.

Analysis of the dose dependence of arginine transport from the extracellular space into endothelial cells in the presence and absence of lysophosphatidylcholine revealed that lysophosphatidylcholine inhibited the incorporation of arginine, but that at a high concentration of arginine, the incorporation was restored. Further, Eadie-Hoffstee's plot analysis showed that arginine was transported by two systems, and that lysophosphatidylcholine impaired selectively the high-affinity arginine transporter, i.e., cationic amino acid transporter-1(CAT-1) (Kikuta et al., 1998). These results suggest that, under physiological conditions, extracellular arginine is transported usually by a high-affinity transporter, probably CAT-1. When oxLDL impairs the high-affinity transporter, NO production is inhibited, and the release of endothelin-1 is increased, probably partly due to the decreased cGMP level. However, once the concentration of extracellular arginine increases, arginine may be transported by the low-affinity transporter. Accordingly, the production of NO appears to be restored at high concentrations of arginine, under hyperlipidemic conditions.

An endothelial receptor specific for oxLDL has been cloned (Sawamura et al., 1997). Structurally, the new receptor belongs to the C-type lectin family. Therefore, it was named lectin-like oxLDL receptor (LOX-1). It differs from the oxLDL receptor of macrophages. The impairment of CAT-1 is mediated probably by LOX-1. OxLDL also stimulates the expression of prepro endothelin-1 via LOX-1. In addition to the decrease in the cGMP level of endothelial cells when the NO level decreases, oxLDL induces upregulation of the expression of LOX-1 (unpublished data). Accordingly, the release of ET-1 from endothelial cells is enhanced.

Under hyperlipidemic conditions, the plasma endothein-1 level is increased, and production of NO in endothelial cells is decreased. LOX-1 is expected to be upregulated in the endothelium under these conditions. Indeed, LOX-1 is expressed in the endothelium under hyperlipidemic conditions and in atheroma tissues in atherosclerosis (unpublished data). At present, we have no information on the intracellular signal for the regulation of the production of NO and endothelin-1 after the activation of LOX-1.

3. Structure and function of ET-receptor

To elucidate the mechanism of action of endothelin-1 on target cells, analysis of the function of the endothelin-receptor is required. Receptor antagonists are useful tools for this purpose. Numerous agonists and antagonists for endothelin-receptors have been developed recently (Ruffolo, 1995). The carboxy-terminal amino acid sequences of these peptide ligands and endogenous endothelins are very similar, suggesting that the binding sites of the endothelin-receptor for these ligands are similar. Nevertheless, the two receptors show a clear difference in ligand binding selectivity. A series of experiments on the subtype-specific determinant of the selectivity of endothelin ET_A and endothelin ET_B receptors and the binding domain of the receptor demonstrated that the endothelin-receptor consists of two distinct domains, i.e., ligand-selection and ligand-binding domains (Sakamoto et al., 1993). Each ligand-selection domain of the two receptors selects the ligand specific for its own receptor. Thus the ligand-selection domain of the endothelin ET_A receptor differs from that of the endothelin ET_B receptor. In the ligand-binding domain, the endothelin receptor binds the ligand and transmits the message to intracellular signal transduction systems.

Both endothelin ET_A and endothelin ET_B receptors can be coupled to $G\alpha s$, $G\alpha i$ and $G\alpha q$ (Takigawa et al., 1995), depending on the cell type, when they are activated by ligands. When the endothelin ET_A or endothelin ET_B receptor is expressed in CHO cells, endothelin ET_A is coupled to $G\alpha s$ and $G\alpha q$ and endothelin ET_B is coupled to $G\alpha i$ and $G\alpha q$ (Takagi et al., 1995). Different domains of the endothelin receptor structure are required for the coupling of different Gα-proteins. $G\alpha s$ is coupled to the second loop and to some extent to the third loop. In both endothelin ET_A and endothelin ET_B receptors, carboxy-terminal cysteine residues are palmitoylated and form the fourth loop. For the activation of $G\alpha i$, the third intracellular loop, the fourth intracellular loop and the carboxy-terminal tail sequence of endothelin ET_B receptor are critical (Takagi et al., 1995; Okamoto et al., 1997). For the $G\alpha q$ coupling of endothelin ET_A and endothelin ET_B receptors the fourth intracellular loop is essential.

4. Mechanism of endothelin-1-induced contraction

Endothelin-1 activates phospholipase C and regulates adenylate cyclase activity (Fig. 1). It increases the cyclic AMP (cAMP) level via endothelin ET_A receptor and decreases it after forskolin stimulation via endothelin ET_B receptor. Activation of phospholipase C is probably mediated by $G\alpha q$. Upregulation and downregulation of the cAMP level are mediated by $G\alpha s$ and $G\alpha i$, respectively. Previously, endothelin ET_A receptor-mediated smooth muscle contraction was believed to be mediated by activation of phospholipase C and opening of voltage-operated calcium channels (Rubanyi and Polokoff, 1994). However, these effects were observed at a relatively high concentration of endothelin-1. Under a physiological concentration of endothelin-1, endothelin-1-induced contraction was me-

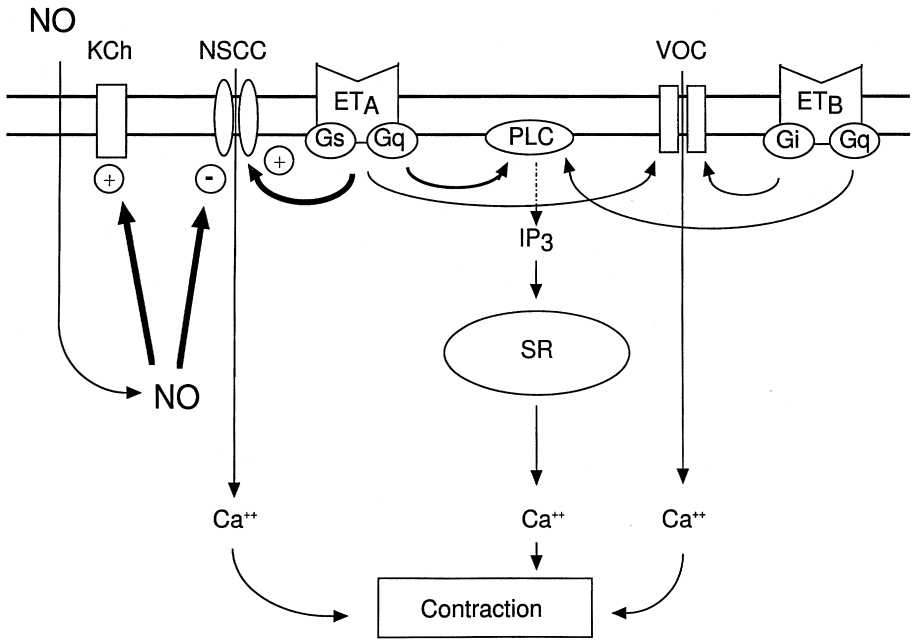

Fig. 1. Mechanism of ET-1-induced vasoconstriction. NSCC: non-selective cation channel, Kch: calcium sensitive potassium channel, PLC: phospholipase C, VOC: coltage operated calcium channel, IP3: inositol tris-phosphate, SR: sarcoplasmic reticulum. + potentiation, − inhibition.

diated by the activation of non-selective cation channels (Iwamuro et al., 1999; Zhang et al., 1999). When smooth muscle cells isolated from rat aorta were stimulated by 10^{-8} M endothelin-1, the intracellular free calcium level increased transiently and then remained at an elevated level. However, at low concentration of endothelin-1, only the sustained phase was observed. The first transient phase was due to Ca^{2+} ions from the intracellular free calcium pool. The sustained phase was due to extracellular Ca^{2+} ions because this phase was abolished after removal of Ca^{2+} ions from the medium. We measured simultaneously the intracellular free calcium ion level and contraction of isolated smooth muscle evoked by endothelin-1. The sustained contraction evoked by endothelin-1 required the persistent entry of extracellular Ca^{2+} ions. In the case of rat aorta smooth muscle cells, nifedipine had no effect on the endothelin-1-evoked contraction or on the Ca^{2+} ion level. The phospholipase C-inhibitor U-73122 had no effect on the endothelin-1 evoked contraction either. In contrast, an inhibitor of non-selective cation channels, SK & F96365, inhibited both endothelin-1-induced contraction and the increase in intracellular free Ca^{2+} ion concentration, suggesting that a non-selective cation channel sensitive to SK & F96365 was involved in the endothelin-1-induced contraction.

Interestingly, NO donors such as sodium nitroprusside and 3-morpholinosydnonime (SIN-1) inhibit the endothelin-1-induced contraction and the endothelin-1-induced increase in intracellular free calcium ion simultaneously. Further, the whole cell patch-clamp method with isolated smooth muscle cells showed that NO and endothelin acted on the same non-selective cation channel (Minowa et al., 1997; Zhang et al., 1998; Iwamuro et al., 1999). Interestingly, in guinea pig tracheal smooth muscle, endothelin ET_B- receptor-mediated contraction but not endothelin ET_A receptor-mediated contraction was inhibited by an inhibitor of the voltage-operated calcium channel, nifedipine (Inui et al., 1999), suggesting a different mechanism of endothelin-1-induced contraction via endothelin ET_B receptor.

5. Cell growth activity of ET-1

Endothelin-1 also affects the growth of many kinds of cells including endothelial, smooth muscle and glia cells. The cell-growth activity evoked by endothelin-1 is generally mediated by endothelin ET_A receptor via activation of MAPK. The activity is mediated by two independent pathways (Sugawara et al., 1996). One is classical PKC dependent, via the activation of phospholipase Cβ, leading to MAPK activation. Gαq mediates the signal. The other way depends on phosphoinositide-3-kinase(PI3K) activation. $G_{\beta\gamma}$ probably mediates this pathway. [15-(1α, 6bα, 9αβ, 11α, 11bβ)]-11-(Acetyloxy)-1, 6b, 7, 8, 9α, 10, 11, 11b-octahydro-1-(methoxymethyl)-9 α, 11b-dimethyl-3H-furo[4, 3, 2-de]indeno[4, 5-h]-2-benzopyran-3, 6, 9-trione (wortmannin), an inhibitor of PI3K, significantly abolishes the endothelin-1-induced mitogenic response while it rarely affects that induced by phorbol 12-myristate 13-acetate (Sugawara et al., 1996). Endothelin-1 stimulates tyrosine phosphorylation of Shc and association with Grb2 and Sos1, resulting in Ras activation (Foschi et al., 1997). As a result, MAPK is activated. The activation of PI3K evoked by endothelin-1 may not be part of this pathway. There-

fore, it is possible that the endothelin-receptor is partly linked to another tyrosine kinase receptor. Indeed, the epidermal growth factor receptor was shown to become phosphorylated in response to activation of the endothelin-receptor (Daub et al., 1996). The mechanism of the activation of PI3K is not fully understood.

Only a few reports have provided evidence that endothelin ET_B receptor-mediates cell growth activity. In a human melanoma cell line A375, endothelin-1 dose dependently suppressed cell growth activity. This inhibitory effect was abolished by the endothelin ET_B receptor-antagonist [*N-cis*-2, 6-dimethylpiperidinocarbonyl-L-γ-methyl-Leu-D-1-methoxycarbonyl-Trp-D-norLeu] (BQ788), but not by the endothelin ET_A receptor-antagonist cyclo (DTrp-DAsp-Pro-DVal-Leu)(BQ-123). However, endothelin ET_B receptor mediated differentiation and apoptosis, and suppressed cell growth in a cell cycle-dependent manner (Okazawa et al., 1998). This response was associated with an enhanced expression of P53 and was mediated by Gαi.

6. Conclusion

To elucidate the multiple roles of endothelins in the regulation of vascular function, the regulation of endothelin-1-production and the function of the endothelin-receptor and its signal transduction need to be investigated. In this article, we have described the inhibitory effect of oxLDL on the arginine transporter in NO synthesis and the subsequent increase in endothelin-1 synthesis, the role of non-selective cation channels sensitive to SK and F96365 in endothelin-1-induced contraction, and the signal transduction systems involved in the cell growth activity of endothelin ET_A receptor. We suggest that the versatile action of the endothelin-receptor is regulated by the activation of various kinds of G-proteins and their signal transduction systems.

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