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SECRETORY MECHANISM OF IMMUNOREACTIVE ENDOTHELIN IN CULTURED BOVINE ENDOTHELIAL CELLS

Toshiaki Emori, Yukio Hirata*, Kazuki Ohta,
Masayoshi Shichiri and Fumiaki Marumo

Second Department of Medicine,
Tokyo Medical and Dental University,
Yushima 1-5-45, Bunkyo-ku,
Tokyo 113, Japan

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Summary: To elucidate the cellular mechanism by which endothelin (ET) is secreted, we have studied the effects of a variety of vasoactive agents on the secretion of immunoreactive (IR)-ET from cultured bovine endothelial cells (EC). Confluent bovine EC cultured in serum-free medium secreted IR-ET as a function of time. Not only thrombin, but also vasoconstrictive hormones, such as arginine-vasopressin (AVP) and angiotensin (ANG) II, dose-dependently stimulated IR-ET secretion, and these effects were completely abolished by V_1-receptor antagonist and [Sar^1,Ala^8]-ANG II, respectively. Protein kinase C (PKC)-activating phorbol ester and Ca^{2+} ionophore ionomycin had stimulatory effects on IR-ET seretion, and the combination of both compounds had a synergistic effect. These data suggest that AVP and ANG II, like thrombin, stimulate ET secretion from EC by a mechanism possibly involving receptor-mediated mobilization of intracellular Ca^{2+} and activation of PKC.

Endothelin (ET), a novel vasoconstrictive peptide with 21-amino-acid residues, has been isolated from the supernatant of cultured porcine endothelial cells (EC) (1). ET induces a potent vasoconstrictive effect on a variety of blood vessels from various species (1,2), positive inotropic action on isolated atria in guinea pigs (3), and the secretion of atrial natriuretic peptide (ANP) from cultured rat atrial myocytes (4). These effects are dependent on extracellular Ca^{2+} and inhibitable by Ca^{2+}-channel antagonists, suggesting the importance of Ca^{2+} influx in the mechanism of its actions.

*To whom correspondence should be addressed.

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It has been reported that the expression of the ET gene in porcine EC is augmented by several agents, such as adrenaline, thrombin and Ca^{2+} ionophore (1), which stimulate the release of endothelium-derived relaxing factor (EDRF) (5). However, the detailed mechanism of the release of ET from vasculature remains elusive. Therefore, the present study was undertaken to determine what agents affect ET release and to investigate the cellular mechanism by which ET is secreted from cultured bovine EC in vitro.

Materials and Methods

EC from bovine carotid arteries was prepared by the enzymatic method and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and antibiotics, as previously described (6). After serial subculture by trypsin, EC harvested between the 15th and 20th passage was used in the experiments. To study the effects of various agents on immunoreactive (IR)-ET secretion, a confluent ($\sim 5 \times 10^5$) EC monolayer was replaced with 1 ml of fresh serum-free DMEM and preincubated for 2 hrs. The following agents were then added and incubated at 37°C for 4 hrs: thrombin (Midori-Juji, Osaka, Japan); ionomycin (Behring Diagnostics, La Jolla, CA); 12-O-tetradecanoyl phorbol-13-acetate (TPA; Sigma Chemical, St. Louis, MO); arginine-vasopressin (AVP); angiotensin (ANG) II and [Sar^1,Ala^8]-ANG II (Peptide Institute, Osaka, Japan); and [1-(β-mercapto-β, β-cyclopentamethylene propionic acid), 2-O-methyltyrosine] (POMT)-AVP (Peninsula Laboratories, Belmont, CA). After incubation, the media were removed and subjected to radioimmunoassay (RIA).

RIA for porcine (p) ET was performed as described (7) by using rabbit anti-pET serum; the antibody mainly recognizes the C-terminal Trp^{21} residue of pET with no cross-reactivity with AVP, ANG II, or other polypeptide hormones. In brief, 0.2 ml standard or sample and 0.1 ml antibody (final dilution, 1:12,000) were preincubated at 4°C for 24 hrs, followed by the addition of 0.1 ml [^{125}I]pET (Amersham Japan, specific activity: 2000 Ci/mmol) and further incubation for 24 hrs. The bound ligands were separated from the free ones by the double antibody method. The sensitivity of pET RIA was 2.5 pg/tube, and the 50% intercept was 35 pg/tube. The coefficients of intra- and inter-assay variations were 3.2% (n=6) and 8.6% (n=5), respectively.

Results

As shown in Fig. 1, the dilution curve of the conditioned medium from bovine EC was parallel to that of standard pET. IR-ET was released from the confluent bovine EC in a serum-free

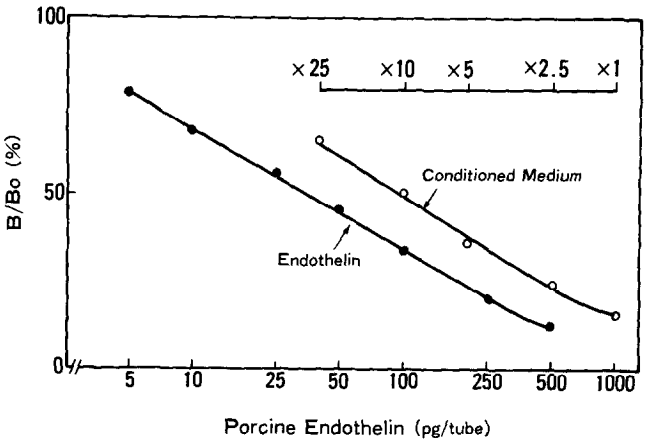

Fig.1. Serial dilution of conditioned medium from bovine EC in ET radioimmunoassay.
The dilution curve of the conditioned medium from bovine EC (o) is compared to that of standard pET (●).

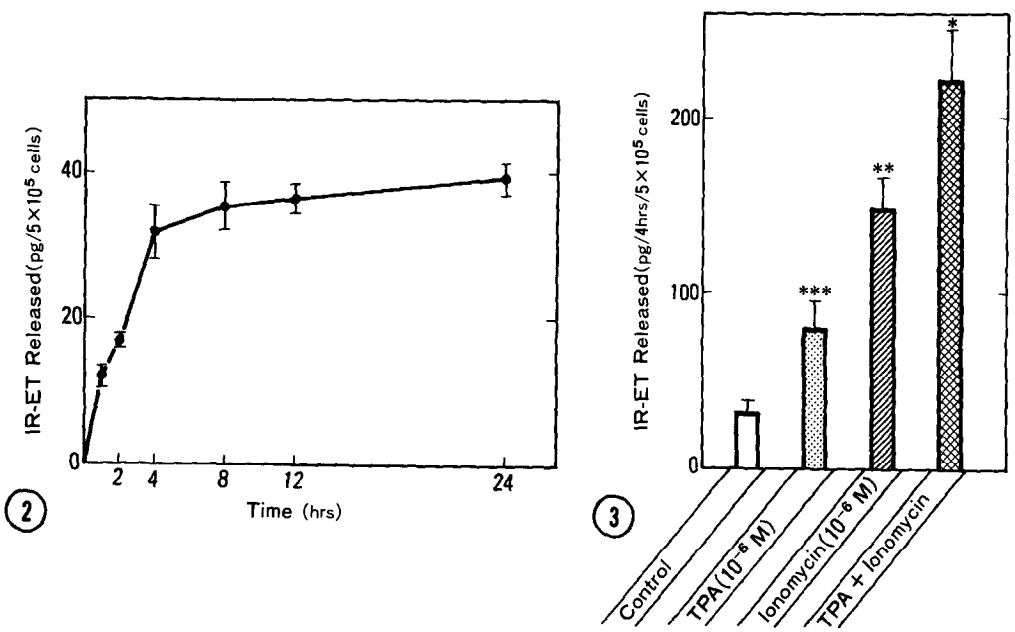

Fig.2. Release of immunoreactve endothelin (IR-ET) from cultured bovine EC as a function of time.
IR-ET released into medium was determined at different times, as indicated. Each point is the mean of triplicate dishes; bars indicate SE.

Fig.3. Effects of TPA and ionomycin on IR-ET secretion from cultured bovine EC.
Confluent bovine EC was incubated at 37° C for 4 hrs in the absence (□) and presence of 10^{-6} M TPA (▨), 10^{-6} M ionomycin (▨), and TPA plus ionomycin (▩). Each column represents the mean of triplicate dishes; bars indicate SE. Asterisks show a statistically significant difference from control (*$p<0.005$, **$p<0.01$, ***$p<0.05$).

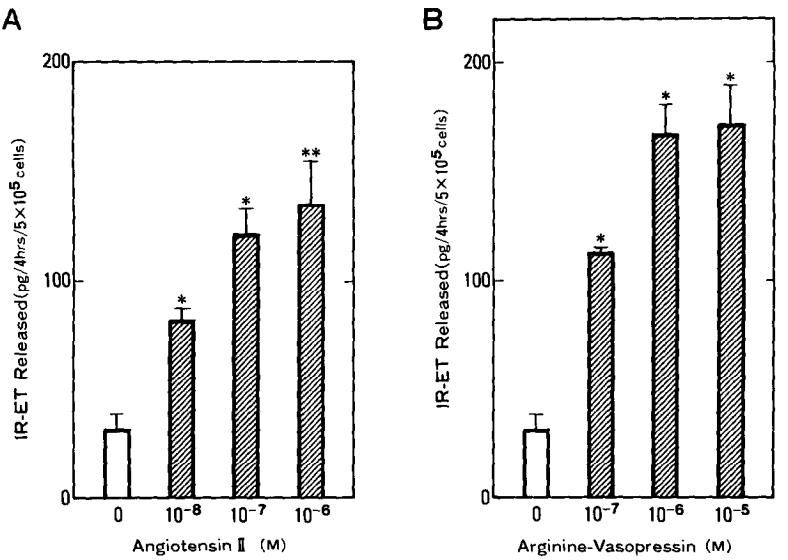

Fig.4. Effects of angiotensin II and arginine-vasopressin on IR-ET secretion from cultured bovine EC.

Confluent bovine EC was incubated with various doses of (A) angiotensin II, and (B) arginine-vasopressin. Each column represents the mean of triplicate dishes; bars indicate SE. Asterisks show a statistically significant difference from control (*p<0.005, **p<0.01).

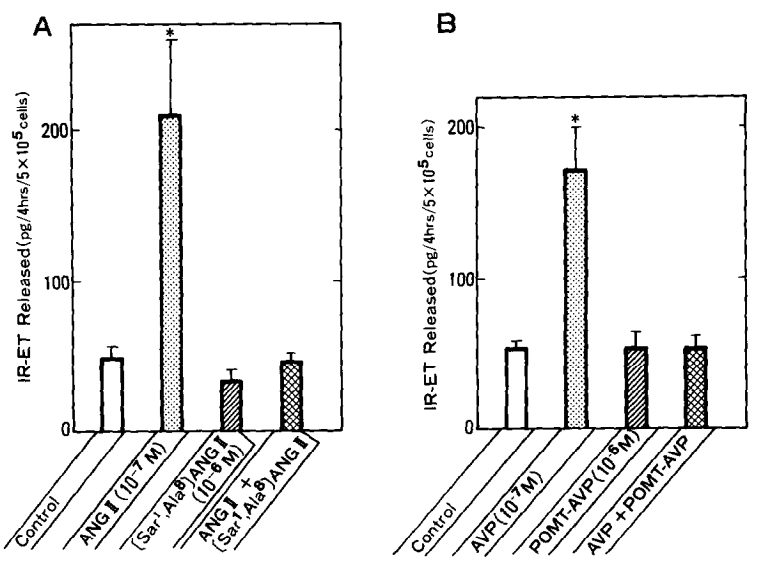

Fig.5. Effects of receptor antagonists on ANG II- and AVP-induced IR-ET secretion.

Confluent bovine EC was incubated without (□) and with (A) ANG II (⊡), [Sar^1,Ala^8]ANG II (▨), ANG II plus [Sar^1,Ala^8]ANG II (▩), (B) AVP (⊡), POMT-AVP (▨), and AVP plus POMT-AVP (▩). Each column represents the mean of triplicate dishes; bars indicate SE. Asterisks show a statistically significant difference from control (p<0.005).

condition as a function of time (Fig. 2); IR-ET increased linearly during 4 hrs incubation, reaching a plateau level thereafter. Therefore, subsequent experiments were performed by incubating the cells for 4 hrs at 37°C.

TPA (10^{-6} M) and ionomycin (10^{-6} M) had stimulatory effects on IR-ET seretion, while the coaddition of each agent induced a synergistic effect (Fig. 3). Thrombin (0.2-20 IU/ml) induced a profound increase in IR-ET secretion (data not shown), while ANG II (10^{-8}-10^{-6} M) and AVP (10^{-7}-10^{-6} M) also stimulated IR-ET secretion in a dose-dependent manner (Fig. 4). The stimulatory effects of ANG II and AVP on IR-ET secretion were completely abolished by their specific receptor antagonists: [Sar^1,Ala^8]-ANG II and POMT-AVP, respectively (Fig. 5).

Discussion

Although the complete amino-acid sequence of bovine ET has not yet been elucidated, the present results with the apparent parallelism of serial dilutions between the conditioned medium and synthetic pET in ET RIA, as well as the time-dependent increase of IR-ET accumulated in the cultured media, strongly suggest that ET and/or related peptide(s) immunologically indistinguishable from pET is/are released from bovine EC in culture.

Yanagisawa et al. originally reported that expression of the ET gene of porcine EC is regulated by several compounds, such as thrombin, Ca^{2+} ionophore, and adrenaline (1); these compounds caused a marked induction of mRNA coding for preproET within 1 hr by Northern blot analysis. We have also shown that both thrombin and ionomycin stimulate IR-ET secretion from cultured bovine EC, thus confirming their data. Since these compounds are known as potent releasers of EDRF (5), there may

be a close interaction between ET and EDRF release for the regulation of vascular tonus. It should also be noted that thrombin stimulates phosphoinositide breakdown in human EC (8).

Recently, Yanagisawa et al. demonstrated that TPA, a potent protein kinase C (PKC) activator, as well as ionomycin, caused a marked and immediate induction of preproET mRNA in human EC, and further that two TPA-responsive elements are located in the 5'-flanking region of the structural gene of human preproET (9). These cis-acting nucleotide sequences have been shown to be the binding sites for protooncogene products, such as c-jun and c-fos (10), suggesting that activation of PKC by TPA is functionally coupled to activation of these transcription factors, thereby affecting the preproET gene. It is generally believed that the Ca^{2+}-messenger system consists of two branches, one being mediated by a rise in the cytosolic free Ca^{2+} concentrations with a subsequent calmodulin-dependent response, and another by a phospholipase C-mediated phosphoinosite breakdown, thereby generating diacylglycerol (DG), which activates PKC, while these two branches interact synergistically to induce full biologic responses (11,12). In the present study, we have shown that TPA has a stimulatory effect on IR-ET secretion and a synergistic effect in the presence of ionomycin. Our data complement results obtained by Yanagisawa et al. (9) and further lend support to the notion that the two branches of the Ca^{2+}-messenger system are closely involved in the mechanism of ET synthesis and/or secretion.

The present study further demonstrates that both ANG II and AVP are potent secretagogues for ET in cultured bovine EC; these effects appear to be receptor-mediated, because ANG II- and AVP-induced IR-ET secretion was completely abolished by

specific ANG II and V_1-receptor antagonists, respectively. It is well documented that these vasoconstrictive hormones induce a receptor-mediated breakdown of phosphoinositide in vascular smooth muscle cells (VSMC), thereby generating DG and inositol triphosphate, which activate PKC and mobilize intracellular Ca^{2+}, respectively (13-15). Taken together, it is strongly suggested that these vasoconstrictors cause cellular responses in EC similar to those in VSMC, finally leading to the release of ET from EC, possibly via the putative two branches of the Ca^{2+}-messenger system. Therefore, the possible involvement of ET induced by endogenous vasoconstrictive hormones in the hormone-induced contractile response of blood vessels needs to be considered.

Acknowledgments

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