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SHORT COMMUNICATIONS

Cellular mechanism of thrombin on endothelin-1 biosynthesis and release in bovine endothelial cell

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Abstract—We have studied the cellular mechanism responsible for induction of preproendothelin (preproET)-1 mRNA and release of ET-1 by thrombin in cultured bovine endothelial cells (ECs). Thrombin induced an immediate and dose-dependent formation of inositol-1,4,5-trisphosphate (IP_3) with a concomitant increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The thrombin-induced ET-1 release was abolished either by a phospholipase C inhibitor, a protein kinase C (PKC) inhibitor, or an intracellular Ca^{2+}-chelator, whereas a Ca^{2+}-channel antagonist was ineffective. A selective thrombin inhibitor (argatroban) decreased IP_3 formation and the increase in $[Ca^{2+}]_i$ and ET-1 release stimulated by thrombin. Northern blot analysis revealed that thrombin-induced expression of preproET-1 mRNA was inhibited completely by a PKC inhibitor and partially by argatroban. These data suggest that thrombin is involved in the mechanism of preproET-1 mRNA expression and subsequent ET-1 release, possibly through activation PKC and mobilization of intracellular Ca^{2+} resulting from the receptor-mediated phosphoinositide breakdown in ECs.

Endothelin-1 was originally isolated and sequenced from the supernatant of cultured porcine endothelial cells (ECs*) [1]. The induction of preproET-1 mRNA in ECs is augmented by Ca^{2+} ionophore [1], phorbol ester [2], transforming growth factor-β [3] and cytokines [4]. Thrombin is also known as a potent inducer of preproET-1 mRNA and a secretagogue for ET-1 in EC [1], although its intracellular signaling has not been elucidated. We have recently shown that angiotensin II and arginine-vasopressin stimulate ET-1 release in cultured bovine EC by a common mechanism, involving receptor-mediated mobilization of intracellular Ca^{2+} and activation of PKC [5]. This study aims to find out whether the thrombin-induced expression of preproET-1 mRNA and ET-1 release in EC involves an intracellular signaling pathway similar to that of angiotensin II and vasopressin.

Materials and Methods

ECs from bovine carotid artery were prepared by enzymatic method and cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum [5]. To study the release of ET-1, confluent ECs (5–10th passage) were replaced with 1 mL fresh serum-free medium and incubated at 37° with the following compounds: thrombin (Midori-Juji Co., Ltd, Osaka, Japan), (2*R*,4*R*)-4-methyl-1-[N^2-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)-sulfonyl]-L-arginyl]-2-piperidinecarboxylic acid (argatroban: Tokyo-Tanabe Pharmaceutical Co., Ltd, Tokyo, Japan), 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-tetraacetoxy-methyl ester (BAPTA-AM: Dojin Chemicals, Kumamoto, Japan), nicardipine (Yamanouchi Pharmaceutical Co., Ltd, Tokyo, Japan), staurosporine (Kyowa Medex Co., Ltd, Tokyo, Japan), 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and neomycin (Sigma Chemical Co., St Louis, MO, U.S.A.).

ET-1-like immunoreactivity (LI) in medium was measured by a specific radioimmunoassay for ET-1 using rabbit anti-ET-1 serum, as described previously [5]. In brief, 0.1 mL standard or sample and 0.1 mL antibody (final dilution, 1:12,000) were preincubated at 4° for 24 hr, followed by the addition of 0.1 mL [^{125}I]ET-1 (sp. act. ~74 TBq/mmol, Amersham Japan, Tokyo, Japan) and further incubation for 24 hr. The sensitivity of ET-1 radioimmunoassay was 1 fmol/tube, and the 50% intercept was 14 fmol/tube.

Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured as described previously [5]. Subcultured ECs were trypsinized and incubated with 4 μM fura-2 acetoxymethyl ester (Dojin Chemicals) at 37° for 20 min. The Ca^{2+}-fura-2 fluorescence was measured in a spectrofluorimeter using excitation of 340 and 380 nm and emission of 500 nm. Values of $[Ca^{2+}]_i$ were determined according to the method of Grynkiewicz *et al.* [6].

IP_3 was determined as reported previously [5]. Confluent ECs were incubated at 37° in Hanks' medium with 10 mM LiCl for indicated times. After addition of ice-cold 15% trichloroacetic acid (TCA), the TCA extract was washed and neutralized. IP_3 levels were determined by a competitive protein binding assay.

Northern blot analysis using bovine preproET-1 as a probe was performed as described recently [7].

Analysis of variance with subsequent Wilcoxon rank-sum test was used to determine significant difference in multiple comparisons.

Results and Discussion

Thrombin stimulated ET-1-LI release from bovine cultured EC depending on time and dose; maximal response was induced with 2 U/mL thrombin after 24 hr incubation (not shown). Northern blot analysis showed a single band corresponding to the size (2.3 kb) of preproET-1 mRNA. Thrombin induced a transient expression (30–60 min) of preproET-1 mRNA (not shown).

It has been suggested that thrombin induces phospholipase C (PLC)-mediated phosphoinositide breakdown

* Abbreviations: preproET, preproendothelin; ET, endothelin; EC, endothelial cell; PKC, protein kinase C; argatroban, (2*R*,4*R*)-4-methyl-1-[N^2-[(3-methyl-1,2,3,4-tetrahydro-8-quinolinyl)-sulfonyl]-L-arginyl]-2-piperidinecarboxylic acid; BAPTA-AM, 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-tetraacetoxy-methyl ester; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; LI, like immunoreactivity; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; IP_3, inositol-1,4,5-trisphosphate; TCA, trichloroacetic acid; PLC, phospholipase C.

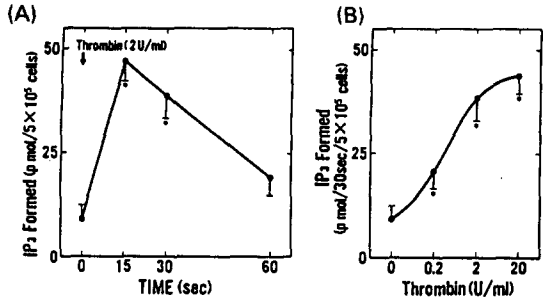

Fig. 1. Time- and dose-dependent effects of thrombin on formation of IP_3 in cultured bovine ECs. Confluent ECs were incubated with 2 U/mL thrombin in Hanks' medium containing 10 mM LiCl for indicated times (A) and with various doses (0–20 U/mL) of thrombin for 30 sec (B). IP_3 formed was determined by competitive protein binding assay. Each point is the mean of triplicate dishes; bar shows SE. * Statistically significant difference from the unstimulated control cells ($P < 0.05$).

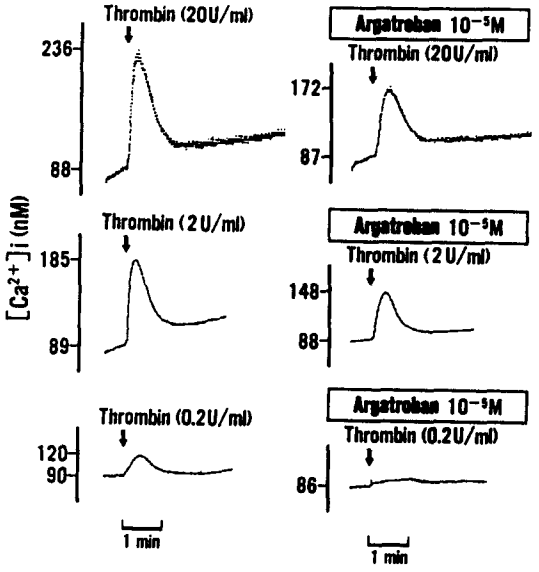

Fig. 2. Changes of Ca^{2+}-fura-2 fluorescence by thrombin in cultured bovine ECs. Cell suspensions loaded with fura-2 were challenged with various doses (0.2–20 U/mL) of thrombin in the absence (left panel) and the presence (right panel) of 10^{-5} M argatroban. Each panel shows typical tracing from representative experiments. Calculated values for $[Ca^{2+}]_i$ are shown on the ordinates.

in both platelets and EC [8], suggesting the putative thrombin receptor was functionally coupled to PLC in these cells. Cloning of the thrombin receptor from human megakaryocytes has elucidated its complete structure with seven transmembrane domains common to the rhodopsin-type G-protein-coupled receptor superfamily [9].

As shown in Fig. 1, thrombin induced immediate (within 30 sec) and dose-dependent (0.2–20 U/mL) IP_3 formation. Thrombin concomitantly increased in $[Ca^{2+}]_i$ in fura-2-loaded bovine EC (Fig. 2, left panel). The intracellular signaling by thrombin appears to be comparable to that by angiotensin and vasopressin [5]. As shown in Table 1, thrombin-induced ET-1-LI release was abolished either by a PLC inhibitor (neomycin), a PKC inhibitor (staurosporine), an intracellular Ca^{2+}-chelator (BAPTA), or pretreatment

Table 1. Effects of various compounds on thrombin-induced ET-1 release from cultured bovine EC

Drug (conc)	ET-1 released (fmol/4 hr/5 × 10^5 cells)
(a)	
Control	93.8 ± 2.4
Nicardipine (10^{-5} M)	93.6 ± 7.9
BAPTA-AM (10^{-8} M)	101.6 ± 4.4
Neomycin (10^{-4} M)	99.3 ± 5.7
Thrombin (2 U/mL)	172.5 ± 11.0*
Thrombin (2 U/mL) + nicardipine (10^{-5} M)	187.8 ± 18.1*
Thrombin (2 U/mL) + BAPTA-AM (10^{-8} M)	93.3 ± 6.4†
Thrombin (2 U/mL) + neomycin (10^{-4} M)	93.3 ± 6.3†
(b)	
Control	98.2 ± 3.9
Staurosporine (10^{-7} M)	96.5 ± 4.1
Thrombin (2 U/mL)	194.2 ± 6.0*
Thrombin (2 U/mL) + staurosporine (10^{-7} M)	126.8 ± 6.0*†
(c)	
Control	93.4 ± 2.6
TPA (10^{-7} M)	90.0 ± 3.6
Thrombin (2 U/mL)	172.0 ± 11.8*
TPA (10^{-7} M) + thrombin (2 U/mL)	121.1 ± 6.9*†

Confluent EC were incubated without (control) and with thromin in the absence and presence of various drugs: (a) nicardipine, BAPTA-AM, neomycin, (b) staurosporine, (c) cells were pretreated with or without 10^{-7} M TPA for 24 hr followed by the addition of thrombin and 4 hr incubation. ET-1-LI released into the medium was determined by radioimmunoassay. Each value is the mean ± SE (N = 6).

* $P < 0.05$, significantly different from control.

† $P < 0.05$, significantly different from thrombin alone.

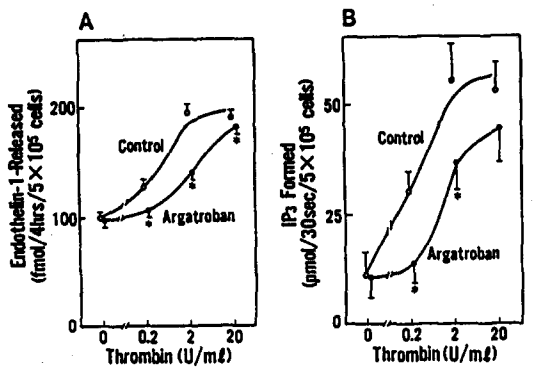

Fig. 3. Inhibitory effects of argatroban on thrombin-induced ET-1-LI release and IP_3 formation in cultured bovine ECs. ECs were incubated with various doses (0.2–20 U/mL) of thrombin in the absence (○) and presence of 10^{-5} M argatroban (●) (A) for 4 hr for ET-1-LI release and (B) for 30 sec for IP_3 formation. Each point is the mean of six experiments; bar shows SE. * Significant difference between cells in the presence and absence of argatroban ($P < 0.05$).

with TPA which down-regulated endogenous PKC activity, whereas a Ca^{2+}-channel antagonist (nicardipine) was ineffective. The thrombin-induced expression of preproET-1 mRNA was also completely inhibited by 10^{-7} M staurosporine (not shown). Since AP-1/*jun* binding sites are located in the 5′-noncoding region of the structural gene of preproET-1 [2], activation of PKC coupled with mobilization of intracellular Ca^{2+} by thrombin may induce transcription factors to induce the expression of preproET-1 mRNA and subsequent release of ET-1. Thus, both activation of PKC and intracellular Ca^{2+} increase resulting from the thrombin receptor-mediated phosphoinositide breakdown are required for biosynthesis and release of ET-1-LI as is the case for angiotensin and vasopressin [5].

Argatroban, an inhibitor of thrombin, binds to the hydrophobic binding pocket of thrombin via its carboxamide group [10]. The dose–response curves by the thrombin effects on ET-1-LI release (Fig. 3A) as well as on IP_3 formation (Fig. 3B) were shifted to the right in the presence of 10^{-5} M argatroban which also significantly inhibited the thrombin-induced $[Ca^{2+}]_i$ increment (Fig. 2, right panel). Furthermore, 10^{-5} M argatroban attenuated preproET-1 mRNA expression induced by thrombin (not shown). It has been suggested that thrombin may cleave the Arg^{41}–Ser^{42} bond of the amino-terminal extracellular binding domain of thrombin receptor [9]. Taken together, it is suggested that argatroban inhibits thrombin-induced cleavage of its receptor site to prevent it from interaction with its binding site.

In conclusion, our data strongly suggest that thrombin, like angiotensin and vasopressin, is involved in the mechanism of preproET-1 mRNA expression and subsequent ET-1 release, possibly through activation of PKC and mobilization of intracellular Ca^{2+} resulting from the receptor-mediated phosphoinositide breakdown in vascular endothelium.

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Second Department of Internal Medicine
Tokyo Medical and Dental University
Tokyo 113, Japan

TOSHIAKI EMORI
YUKIO HIRATA*
TAIHEI IMAI
KAZUKI OHTA
KAZUO KANNO
SATORU EGUCHI
FUMIAKI MARUMO

* Corresponding author: Yukio Hirata, M.D., Endocrine-Hypertension Division, Second Department of Internal Medicine, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113, Japan.

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