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Specific receptors for endothelin-3 in cultured bovine endothelial cells and its cellular mechanism of action

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Among three endothelin (ET) isopeptides, ET-3 shows the most potent initial depressor response through the endothelium-dependent mechanism. We studied the presence of specific binding sites for ET-3 in cultured bovine endothelial cells (EC) and its cellular mechanism of action. Binding studies revealed the presence of two distinct subclasses of ET-3 receptors with high and low affinities. ET-3 dose-dependently (10^{-10}–10^{-7} M) increased both intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) and inositol trisphosphate (IP_3) formation. The ET-3-induced increase in $[Ca^{2+}]_i$ was not affected by either removal of extracellular Ca^{2+} or Ca^{2+}-channel blockers. These data suggest that ET-3 induces phosphoinositide breakdown and increase in $[Ca^{2+}]_i$ in ECs, possibly resulting from intracellular Ca^{2+} mobilization, thereby leading to vasodilatation.

Endothelin-3; Endothelial cell; Vasodilatation

1. INTRODUCTION

Endothelin (ET) is a novel potent vasoconstrictor peptide with 21 amino acid residues, originally characterized from the supernatant of cultured porcine endothelial cells (EC) [1]. Recently, three distinct ET genes have been demonstrated in the human, porcine and rat genomes [2], and cloning and sequence analysis of these genes revealed three ET isopeptides (ET-1, ET-2, ET-3). ET-1 is identical to human/porcine ET and ET-2 is [Trp^6,Leu^7]ET-1, while ET-3 is identical to ET originally deduced from cDNA cloning of rat genome. These three isopeptides show different pharmacological profiles of pressor/vasoconstrictor activities [2]; ET-3, despite its least potency in pressor response, exerts the most potent initial depressor response among three ET isopeptides.

Warner et al. have recently demonstrated that ET-3 is more selective than ET-1 as a vasodilator through the release of EDRF at doses 100-fold lower than those required to induce vasoconstriction [3], suggesting its physiologcal role as a vasodilator rather than a vasoconstrictor. To our knowledge, there has been no direct evidence that ET-3 directly acts on EC. Therefore, the present study was designed to investigate whether there exists a specific receptor for ET-3 in cultured bovine EC and to elucidate its intracellular responses.

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2. MATERIALS AND METHODS

2.1. *Binding experiments*

Subcultured bovine carotid artery ECs (7–9th passage) were used in the experiments. Binding studies were performed essentially in the same manner as [^{125}I]ET-1 binding to vascular smooth muscle cells (VSMC) as described [4]. Briefly, confluent ECs (~6×10^5 cells) were incubated with 2.5×10^{-13} M [^{125}I]ET-3 (spec. act. ~74 TBq/mmol, Amersham Japan, Tokyo) in 1 ml Hanks' balanced salt solution containing 0.1% bovine serum albumin at 37°C for 60 min. After completion, cells were extensively washed and the cell-bound radioactivity was determined. Specific binding was defined as total binding minus nonspecific binding in the presence of an excess (10^{-7} M) of unlabeled ET-3 (Peptide Institute Inc., Osaka, Japan).

2.2. *Measurement of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$)*

After incubation in serum-free Dulbecco's modified Eagle's medium (DMEM) for 36 h, ECs were trypsinized and incubated with 4 μM fura-2 acetoxymethyl ester (Dojin Chemicals, Kumamoto, Japan) at 37°C for 20 min in Hepes-buffered physiological salt solution essentially in the same way as reported [4]. The fluorescence of Ca^{2+}-fura-2 of the suspended cells (~5×10^6 cells/ml) was measured by a spectrofluorimeter (CAF-100; Jasco Co., Ltd., Tokyo, Japan) using excitation of 340 and 380 nm, and emission of 500 nm. Values of $[Ca^{2+}]_i$ were calculated according to the method of Grykiewicz et al. [5].

2.3. *Measurement of inositol-1,4,5-trisphosphate (IP_3)*

Confluent ECs preincubated in serum-free DMEM were incubated at 37°C with or without ET-3 in Hanks' medium containing 10 mM LiCl for the indicated times. Incubation was terminated by rapid removal of medium and addition of ice-cold 15% trichloroacetic acid (TCA), and the cells were placed on ice for 30 min. TCA extract was washed with ethylether and neutralized with 1 N sodium acetate. IP_3 levels were determined by a competitive protein binding assay kit (Amersham Japan, Tokyo).

3. RESULTS

Specific binding of [^{125}I]ET-3 to bovine EC was a

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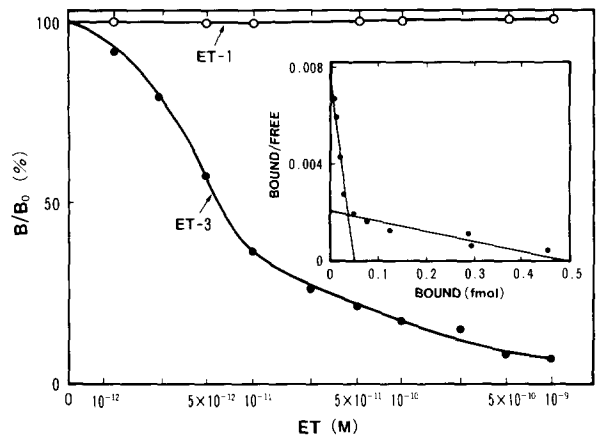

Fig. 1. Competitive binding of [^{125}I]ET-3 by unlabeled ET-3 and ET-1 to cultured bovine EC. Confluent cells were incubated at 37°C for 60 min with 2.5 × 10^{-13} M [^{125}I]ET-3 in the absence and the presence of unlabeled ET-3 (•) or ET-1 (○) in concentrations as indicated. The bound [^{125}I]ET-3 was 1.8 fmol/10^6 cells in the absence of unlabeled ET-3. Nonspecific binding was 38% of total binding and subtracted from all values. Each point is the mean of two experiments. (Inset) Scatchard plot of binding data.

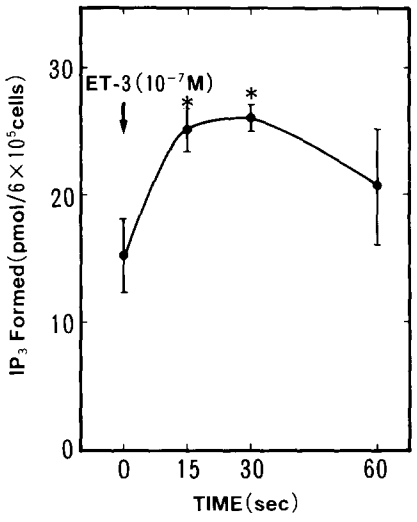

Fig. 3. Effect of ET-3 on IP_3 formation in cultured bovine EC as a function of time. Confluent cells were incubated with ET-3 (10^{-7} M) in Hanks' medium containing 10 mM LiCl for the indicated times. Each point is the mean of triplicate dishes; bars show SE. Asterisks show statistically significant differences from the unstimulated control cells ($P < 0.05$).

time- and temperature-dependent process; binding at 37°C was more rapid and greater than at 4°C, reaching an equilibrium after 60 min. Nonspecific binding at 37°C was ~40% of total binding throughout the incubation. As shown in Fig. 1, unlabeled ET-3 competitively inhibited the binding of [^{125}I]ET-3 to bovine EC, whereas ET-1 did not affect the binding in concentrations up to 10^{-7} M. Scatchard analysis of the binding data (Fig. 1, inset) suggested the presence of two distinct subclasses of binding sites for ET-3: one with high-affinity (K_d: 6.6 × 10^{-12} M) and low capacity (B_{max}: 50 sites/cell), and the other with low-affinity (K_d: 2.5 × 10^{-10} M) and high-capacity (B_{max}: 490 sites/cell).

As shown in Fig. 2, ET-3 dose-dependently (10^{-10}–10^{-7} M) induced an immediate and transient increase in $[Ca^{2+}]_i$; $[Ca^{2+}]_i$ increased significantly ($P < 0.01$) from 88 ± 2.4 nM (mean ± SE, n = 12) to 101 ± 1.3 (n = 3, 10^{-10} M), 127 ± 2.3 (n = 3, 10^{-9} M), 171 ± 3.5 (n = 3, 10^{-8} M) and 219 ± 3.0 (n = 3, 10^{-7} M) 5 s after the addition of ET-3. The increase in $[Ca^{2+}]_i$ stimulated by 10^{-7} M ET-3 was not affected by

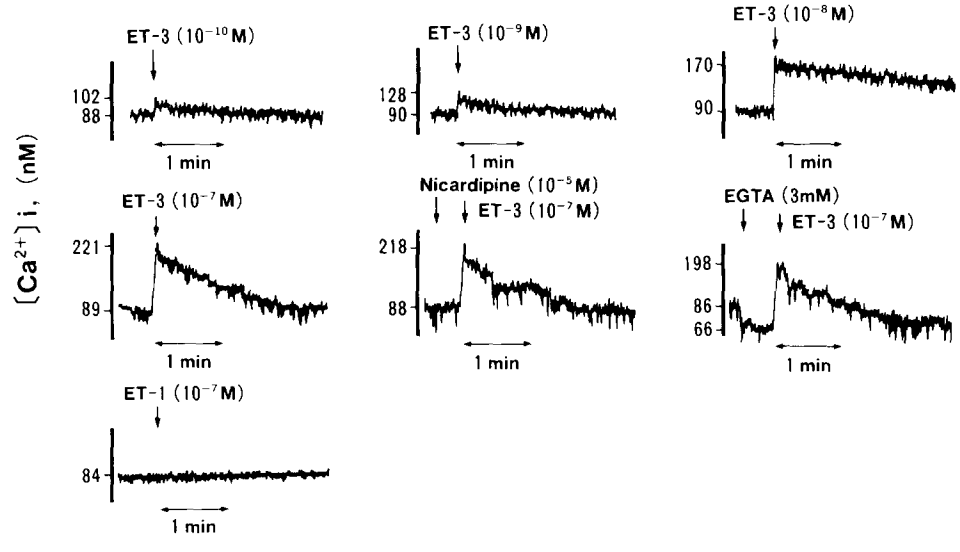

Fig. 2. Changes of Ca^{2+}-fura-2 fluorescence by ET-3 and ET-1 in cultured bovine EC. Cell suspensions loaded with fura-2 were challenged with various doses (10^{-10}–10^{-7} M) of ET-3 and ET-1 (10^{-7} M). The basal $[Ca^{2+}]_i$ were 88 ± 2.4 nM (mean ± SE, n = 12). Each panel shows a typical tracing from representative experiments. Calculated values for $[Ca^{2+}]_i$ are shown on the ordinates.

pretreatment with either 10^{-5} M nicardipine or 3 mM EGTA. However, ET-1 (10^{-7} M) had no effect.

ET-3 (10^{-7} M) induced an immediate (15–30 s) formation of IP_3, followed by a gradual decline by one min (Fig. 3). ET-3 dose-dependently (10^{-9}–10^{-7} M) stimulated formation of IP_3 during 30 s incubation, whereas ET-1 (10^{-7} M) was without effect (not shown).

4. DISCUSSION

It has originally been reported that ET-1 causes a transient decrease in systemic arterial blood pressure accompanied by regional vasodilating responses in anesthetized rats [1,2]. Furthermore, De Nucci et al. have reported that ET-1 induces transient depressor response in isolated perfused rat mesenteries mediated by the release of EDRF [6]. A subsequent study by the same group has shown that ET-3 is far more potent than ET-1 in vasodilating perfused rat mesenteries [3], suggesting that ET-3 functions as a vasodilator rather than a vasoconstrictor.

This is the first report demonstrating the presence of specific receptors for ET-3 in cultured bovine EC, with which ET-1 does not interact. The present binding study clearly shows two distinct subpopulations of binding sites for ET-3 with higher (K_d: 7×10^{-12} M) and lower (K_d: 2.5×10^{-10} M) affinities in cultured bovine EC. Recent studies have reported that at least two subtypes of binding sites for ET isopeptides exist in the membranes of chick heart [7] and rat lung [8]; one subtype with preferential binding affinity to ET-1 and ET-2, and the other to ET-3, suggesting that the population of ET receptor subtypes may differ from one tissue to another. Our data indicate that ECs express predominantly ET-3 receptor subtype.

The present study also demonstrates that ET-3, but not ET-1, dose-dependently (10^{-10}–10^{-7} M) induces profound increases in $[Ca^{2+}]_i$ in fura-2-loaded EC. These data are compatible with those of the ET-1-induced $[Ca^{2+}]_i$ increase in cultured rat VSMC [4]. However, the failure of chelating extracellular Ca^{2+} by EGTA and inhibiting voltage-dependent Ca^{2+}-channels by nicardipine on ET-3-induced $[Ca^{2+}]_i$ increase, strongly suggests that the rise of $[Ca^{2+}]_i$ stimulated by ET-3 resulted from intracellular Ca^{2+} release rather than Ca^{2+} influx from extracellular source.

In fact, the present study shows that ET-3 induces an immediate (15–30 s) and dose-responsive effect on IP_3 formation, suggesting that ET-3 stimulates phosphoinositide breakdown in EC in a similar manner as does ET-1 in VSMC [9]. Since IP_3 serves as a second messenger for mobilizing Ca^{2+} from intracellular store sites [10], the concomitant rapid responses in both IP_3 formation and $[Ca^{2+}]_i$ increase by ET-3 indicate that the ET-3-induced increase in $[Ca^{2+}]_i$ derives mainly from intracellular Ca^{2+} mobilization in EC.

However, the dose-dependency of ET-3 (10^{-10}–10^{-7} M) on increases in $[Ca^{2+}]_i$ and IP_3 formation does not appear to correspond to the apparent K_d values from the binding data. The apparent discrepancy may be partly accounted for by different cell preparations and incubation conditions (temperature, time) used for the binding study and measurements of $[Ca^{2+}]_i$ and IP_3 formation. Alternatively, the ET-3-induced $[Ca^{2+}]_i$ increase and IP_3 formation in EC may be a consequence of the activation of the lower-affinity ET-3 receptors. Indeed, it has recently been reported that there also exist two subpopulations of ET-1 receptors in cultured rat glomerular mesangial cells and the ET-1-induced IP_3 formation, $[Ca^{2+}]_i$ increase and subsequent contraction may be mediated through the low-affinity ET-1 receptors [11]. If that is the case, the nature of the biological response by the higher-affinity ET-3 receptors in EC needs to be elucidated.

Our data are consistent with the contention that mobilization of intracellular Ca^{2+} is required for the production and/or release of endothelium-derived vasodilators, such as EDRF and prostacyclin [12]. Thus, the presence of specific ET-3 receptors in EC strongly suggests that ET-3 plays an important role in the regulation of vasodilatation, possibly via receptor-mediated release of EDRF and/or prostacyclin from EC. The precise interaction between the ET-1-induced vasoconstriction and the ET-3-induced vasodilatation in the regulation of vascular tonus remains open to question.

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