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A novel ET_A antagonist (BQ-123) inhibits endothelin-1-induced phosphoinositide breakdown and DNA synthesis in rat vascular smooth muscle cells

Satoru Eguchi[a], Yukio Hirata[a], Masaki Ihara[b], Mitsuo Yano[b] and Fumiaki Marumo[a]

[a]*Second Department of Internal Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyoku, Tokyo 113, Japan* and [b]*Central Research Laboratories, Banyu Pharmaceutical Co., 2-9-3 Shimomeguro, Meguroku, Tokyo 153, Japan*

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The effects of a novel cyclic pentapeptide (BQ-123), an endothelin (ET) antagonist selective for the ET_A receptor subtype, on phosphoinositide breakdown and DNA synthesis stimulated by ET-1 were studied in cultured rat vascular smooth muscle cells (VSMC). BQ-123 competitively inhibited the binding of [^{125}I]ET-1 to VSMC with the apparent K_i of 4×10^{-9} M. BQ-123 dose-dependently inhibited formation of inositol-1,4,5-trisphosphate and [^{3}H]thymidine uptake stimulated by ET-1. These data suggest that the ET-1-induced DNA synthesis in VSMC is mainly mediated by ET_A receptor subtype.

Endothelin-1; Receptor antagonist; Inositol trisphosphate; DNA synthesis; Vascular smooth muscle cell

1. INTRODUCTION

Endothelin-1 (ET-1) is a novel 21-residue vasoconstrictor peptide originally isolated from the supernatant of cultured porcine endothelial cells [1]. Subsequent cDNA cloning of the human genomic library revealed three ET isopeptides, ET-1, ET-2 and ET-3; these three isopeptides have different pharmacological profiles of pressor/vasoconstrictor activities, suggesting the heterogeneity of ET receptors [2]. Two distinct ET receptor subtypes have recently been cloned and sequenced [3,4]; one subtype shows selective affinity for ET-1 and ET-2 (ET_A), whereas the other subtype shows non-selective affinity for three isopeptides (ET_B). These receptors belong to the superfamily of G-protein-coupled receptors. In cultured rat vascular smooth muscle cells (VSMC) we have shown that ET-1 and ET-2 interact with ET receptors with almost the same affinity, while ET-3 had a far lower binding affinity, suggesting that VSMCs predominantly express ET_A receptors [5]. In addition to its role as a vasoconstrictor, ET-1 has been shown to stimulate DNA synthesis of VSMC, expression of proto-oncogenes (c-*fos*, c-*myc*), and increase in cell number [6–8], suggesting its possible involvement in the development of atherosclerotic vascular lesion.

Recently, a novel cyclic pentapeptide (BE-18257B), cyclo(-D-Glu-L-Ala-allo-D-Ile-L-Leu-D-Trp-), isolated from the fermentation products of *Streptomyces misakiensis*, has been shown to be a selective ET_A antagonist [9]. Very recently a more potent ET_A antagonist analog (BQ-123), cyclo(-D-Asp-L-Pro-D-Val-L-Leu-D-Trp-), has been synthesized [10]; BQ-123 is two orders of magnitude more potent than BE-18257B in antagonizing ET-1-induced vasoconstrictor/pressor actions. These reports prompted us to examine the effects of BQ-123 on receptor binding activity, phosphoinositide breakdown and DNA synthesis stimulated by ET-1 in cultured rat VSMC.

Correspondence address: Y. Hirata, Endocrine–Hypertension Division, Second Department of Internal Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyoku, Tokyo 113, Japan. Fax: (81) (3) 3818-7177.

2. MATERIALS AND METHODS

2.1. *Materials*

ET-1, ET-2 and ET-3 were purchased from Peptide Institute (Osaka, Japan), [^{125}I]ET-1 (spec. act. 2,000 Ci/mmol) from Amersham International (Tokyo, Japan), and [^{3}H]thymidine (spec. act. 6.7 Ci/mmol) from New England Nuclear (Boston, MA). BQ-123 was synthesized as recently reported [10].

2.2. *Cell culture*

VSMCs were prepared from the thoracic aorta of 15-week-old male Wistar rats by the explant method, and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum at 37°C in a humidified atmosphere of 95% air–5% CO_2 as previously described [11]. The cells thus obtained showed the 'hills-and-valleys' growth characteristic of cultured VSMCs in vitro; the expression of 20 kDa myosin light chain and its phosphorylation by angiotensin and vasopressine have been reported [12]. Subcultured VSMCs (10–20th passages) were used in the experiments.

2.3. *Binding experiments*

Confluent VSMCs (5×10^5 cells) were usually incubated with 6 pM [^{125}I]ET-1 at 37°C for 60 min in Hanks' balanced salt solution containing 0.1% bovine serum albumin, in the same manner as reported [13].

Specific binding was determined by subtracting non-specific binding in the presence of excess (10^{-7} M) unlabeled ET-1 from total binding.

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

Confluent VSMCs were incubated at 37°C for 30 s in Hanks' balanced salt solution containing 10 mM LiCl. Incubation was terminated by the addition of perchloric acid and IP_3 was measured by a radioreceptor assay kit (Du Pont, Boston, MA).

2.5. *DNA synthesis*

DNA synthesis was assessed by incorporation of [^{3}H]thymidine into cells as previously described [7]. In brief, subconfluent VSMCs (3×10^5 cells) that became quiescent after replacement with serum-free DMEM for 48 h, were incubated with or without ET-1 for 20 h, after which 1 μCi [^{3}H]thymidine was added and further incubated for 4 h. After completion, trichloroacetic acid-insoluble radioactivity was measured in a liquid scintillation counter.

3. RESULTS

A competitive binding study using [^{125}I]ET-1 as a radioligand to rat VSMC is shown in Fig. 1. ET-1 and ET-2 equipotently inhibited the binding of [^{125}I]ET-1, while ET-3 showed a far less potent inhibition than ET-1 and ET-2. BQ-123 also competitively inhibited [^{125}I]ET-1 binding to its receptor sites. The apparent inhibition constant (K_i) values for BQ-123, ET-1, ET-2 and ET-3 were 4.0×10^{-9} M, 1.0×10^{-10} M, 1.3×10^{-10} M and 7.7×10^{-8} M, respectively.

The effects of BQ-123 on ET-1-induced IP_3 formation in cultured rat VSMC are shown in Fig. 2. ET-1 dose-dependently (10^{-9}–10^{-7} M) stimulated IP_3 formation, while BQ-123 (10^{-6} M) almost completely inhibited IP_3 formation stimulated by ET-1 (10^{-9}–10^{-7} M) (Fig. 2A). BQ-123 (10^{-5}–10^{-6} M) alone did not affect basal IP_3 formation. BQ-123 dose-dependently (10^{-8}–10^{-5} M) inhibited IP_3 formation maximally stimulated by ET-1 (10^{-7} M) with the approximate dose for half-maximal inhibition (IC_{50}) of 2×10^{-8} M (Fig. 2B).

The effects of BQ-123 on DNA synthesis stimulated

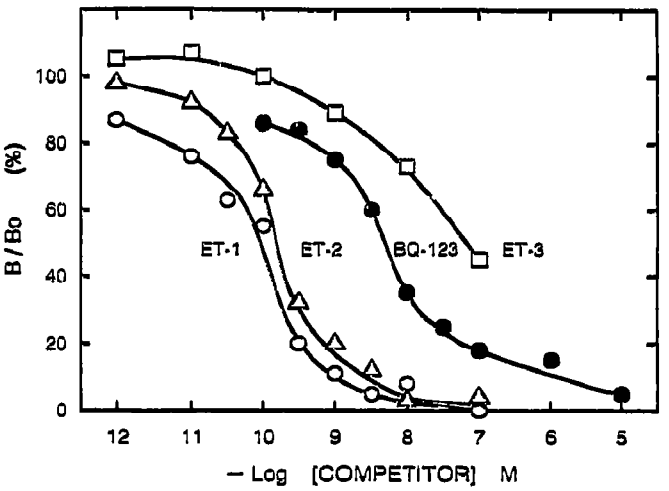

Fig. 1. Competitive binding of [^{125}I]ET-1 to rat VSMC. Confluent cells (5×10^5 cells/well) were incubated with 6 pM [^{125}I]ET-1 in the absence and the presence of BQ-123 (●), ET-1 (○), ET-2 (△) and ET-3 (□) in the indicated concentrations. Results are expressed as the percentage of specific binding in the absence of peptides (B_0); each point is the mean of three experiments.

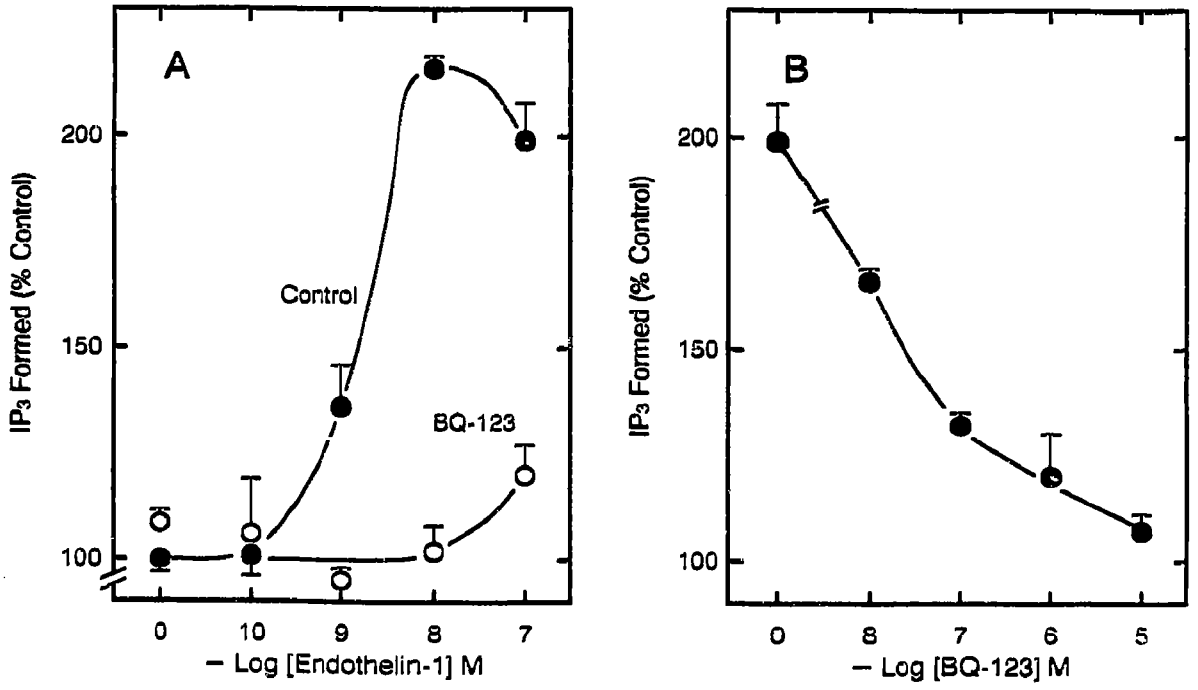

Fig. 2. Effects of BQ-123 on ET-1-induced IP_3 formation in rat VSMC. Confluent cells (2×10^6 cells) were incubated with various concentrations of ET-1 in the absence (●) and the presence (○) of 10^{-6} M BQ-123 (A), or with 10^{-7} M ET-1 in the absence and the presence of BQ-123 in concentrations as indicated (B). Each point is the mean of three samples and represents the percentage to the basal levels (8.3 ± 0.2 pmol); bars show S.E.M.

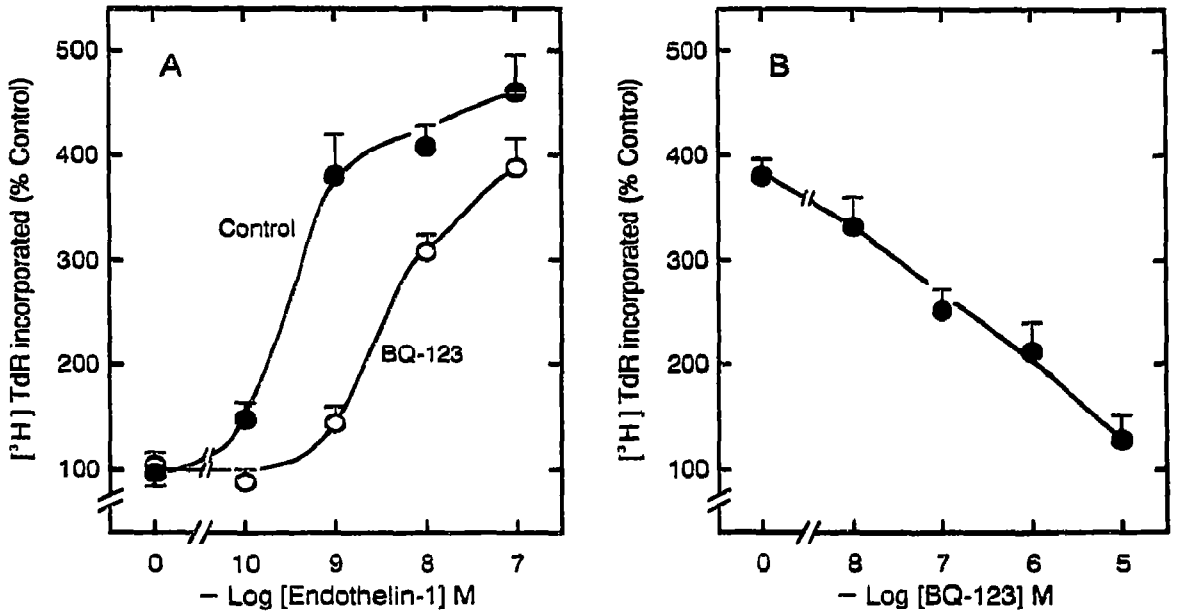

Fig. 3. Effects of BQ-123 on ET-1-induced DNA synthesis in rat VSMC. Quiescent VSMCs (3×10^5 cells) were incubated with various concentrations of ET-1 in the absence (●) and the presence (○) of 10^{-6} M BQ-123 (A), or with 10^{-9} M ET-1 in the absence and the presence of BQ-123 in concentrations as indicated (B). Each point is the mean of four samples and represents the percentage of the basal [³H]thymidine uptake ($15.6 \pm 0.8 \times 10^3$ dpm); bars show S.E.M.

by ET-1 in cultured rat VSMC are shown in Fig. 3. ET-1 dose-dependently (10^{-10}–10^{-7} M) stimulated [³H]thymidine uptake in quiescent VSMCs; BQ-123 (10^{-6} M) shifted the dose–response curve to the right, while BQ-123 alone dit not affect basal [³H]thymidine uptake (Fig. 3A). BQ-123 dose-dependently (10^{-8}–10^{-5} M) inhibited DNA synthesis maximally stimulated by ET-1 (10^{-9} M) with the approximate IC_{50} of 1×10^{-7} M (Fig. 3B).

4. DISCUSSION

The present study demonstrates for the first time that a novel ET_A receptor antagonist BQ-123 inhibits IP_3 formation and DNA synthesis stimulated by ET-1 in cultured rat VSMC. The present binding data are compatible with our previous observation that cultured rat VSMCs possess predominantly ET_A subtype receptors [5]. In the present binding study BQ-123 competitively inhibited the binding of [¹²⁵I]ET-1 to its vascular receptor with high-affinity (K_i 4×10^{-9} M), indicating that BQ-123 is highly selective for vascular ET_A receptor subtype. Our results are in good agreement with those of porcine VSMC as recently reported [10].

It has been shown that ET-1 stimulates phospholipase *c*-mediated phosphoinositide breakdown in VSMC to generate IP_3, which in turn mobilizes intracellular Ca^{2+} from its storage sites [13,14]. The present study clearly demonstrates that BQ-123 dose-dependently inhibits IP_3 formation stimulated by ET-1 in cultured rat VSMC. The approximate IC_{50} (2×10^{-8} M) of BQ-123 for IP_3 formation appears almost comparable to its IC_{50} (7.4×10^{-9} M) for the ET-1-induced vasoconstriction as recently reported [10].

In addition to its potent vasoconstrictor effect, ET-1 has been shown to stimulate DNA synthesis of rat VSMC in culture [6–8]. In the present study, we have demonstrated that BQ-123 has an inhibitory effect on DNA synthesis induced by ET-1 in cultured rat VSMC, as characterized by the rightward shift of the dose–response curve by BQ-123. However, the magnitude of the shift produced by 10^{-6} M BQ-123 on the ET-1 dose–response curve of DNA synthesis appears to be less potent than that of IP_3 formation. Such discrepancy may be accounted for by the difference in the affinity of the receptors mediating the two responses for the antagonist. In fact, the present binding study showed that about 15% of specific binding of [¹²⁵I]ET-1 to rat VSMC was not displaced by BQ-123 even at 10^{-6} M. The same result was also observed in porcine VSMC membranes [10]. Thus, it is probable that cultured rat VSMCs may possess small amount of non-isopeptide-selective ET_B receptors through which ET-1 may also stimulate DNA synthesis. Alternatively, the difference in the magnitude of the shifts by BQ-123 may be due to metabolism of the antagonist, altered biodistribution, and/or receptor modification, since it takes only 30 s for measurement of IP_3 but 24 h for measurement of DNA synthesis. Further study is needed to elucidate the molecular interaction between receptor subtypes and their signal transduction systems in VSMC.

It should be noted that circulating ET-1 levels were elevated in patients with atherosclerosis [15,16], impli-

cating the possible involvement of endogenous ET-1 in the development of atherosclerotic vascular lesions. Thus, a selective ET_A receptor antagonist (BQ-123) which inhibits the ET-1-induced phosphoinositide breakdown and DNA synthesis in rat VSMC will be a useful tool to know whether ET-1 has any pathophysiological role in the development of atherosclerotic vascular lesion.

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REFERENCES

[1] Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K. and Masaki, T. (1988) Nature 332, 411–415.
[2] Inoue, A., Yanagisawa, M., Kimura, S., Kasuya, Y., Miyauchi, T., Goto, K. and Masaki, T. (1989) Proc. Natl. Acad. Sci. USA 86, 2863–2867.
[3] Arai, H., Hori, S., Aramori, I., Ohkubo, H. and Nakanishi, S. (1990) Nature 348, 730–732.
[4] Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K. and Masaki, T. (1990) Nature 348, (732–735.
[5] Hirata, Y., Yoshimi, H., Takagi, Y., Kanno, K., Eguchi, S. and Marumo, F. (1990) Biomed. Res. 11, 195–198.
[6] Komuro, I., Kurihara, H., Sugiyama, T., Takaku, F. and Yazaki, Y. (1988) FEBS Lett. 238, 249–252.
[7] Hirata, Y., Takagi, Y., Fukuda, Y. and Marumo, F. (1989) Atherosclerosis 78, 225–228.
[8] Bobik, A., Grooms, A., Millar, J.A., Mitchell, A. and Grinpukel, S. (1990) Am. J. Physiol. 258, C408–415.
[9] Ihara, M., Fukuroda, T., Saeki, T., Nishikibe, M., Kojiri, K., Suda, H. and Yano, M. (1991) Biochem. Biophys. Res. Commun. 178, 132–137.
[10] Ihara, M., Noguchi, K., Saeki, T., Fukuroda, T., Tsuchida, S., Kimura, S., Fukami, T., Ishikawa, K., Nishikibe, M. and Yano, M. (1992) Life Sci. 50, 247–255.
[11] Hirata, Y., Tomita, M., Yoshimi, H. and Ikeda, M. (1984) Biochem. Biophys. Res. Commun. 125, 562–568.
[12] Takata, S., Hirata, Y., Furukawa, K., Tomita, M., Takagi, Y., Fujita, T. and Hidaka, H. (1988) Endocrinol. Japon. 35, 577–584.
[13] Hirata, Y., Yoshimi, H., Takata, S., Watanabe, T.X., Kumagai, S., Nakajima, K. and Sakakibara, S. (1988) Biochem. Biophys. Res. Commun. 154, 868–875.
[14] Resink, T.J., Scott-Burden, T. and Buhler, F.R. (1989) Biochem. Biophys. Res. Commun. 157, 1360–1368.
[15] Kanno, K., Hirata, Y., Numano, F., Emori, T., Ohta, K., Shichiri, M. and Marumo, F. (1990) JAMA 264, 2868.
[16] Lerman, A., Edwards, B.S., Hallett, J.W., Heublein, D.M., Sandberg, S.M. and Burnett, J.C. (1991) N. Eng. J. Med. 325, 997–1001.