

Endothelin Receptor Subtype B Mediates Synthesis of Nitric Oxide by Cultured Bovine Endothelial Cells

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Abstract

Endothelins (ET) produce endothelium-dependent vasodilation through nitric oxide (NO) synthesis. The present study was designed to elucidate the cellular mechanism by which ET induces synthesis and release of endothelium-derived NO by cultured bovine endothelial cells (EC). Binding studies revealed that bovine EC membrane had the binding sites of a novel agonist (BQ3020) for non-isopeptide-selective receptor subtype (ET_B). Affinity labeling studies showed a major labeled band with the apparent molecular mass of 50 kD. Northern blot analysis demonstrated the expression of mRNA for ET_B receptor. BQ3020 rapidly and dose dependently induced formation of inositol-1,4,5-triphosphate and increased intracellular Ca²⁺ concentrations in fura-2-loaded cells. Concomitantly, BQ3020 dose dependently stimulated production of both nitrate/nitrite (NO_x) and cyclic GMP; a highly significant correlation existed between NO_x and cGMP production. The stimulatory effect on NO_x and cGMP production by ET_B agonist was inhibited by NO synthase inhibitor monomethyl-L-arginine; this effect was reversed by coaddition of L-arginine, but not D-arginine. NO_x and cGMP production stimulated by BQ3020 was inhibited by pretreatment with pertussis toxin. ET_B agonist-induced NO_x production was blocked by a calmodulin inhibitor and an intracellular Ca²⁺ chelator, but not by an extracellular Ca²⁺ chelator or a Ca²⁺ channel blocker. These data suggest that endothelins stimulate ET_B receptor-mediated phosphoinositide breakdown via pertussis toxin-sensitive G-protein(s), which triggers release of intracellular Ca²⁺, thereby activating Ca²⁺/calmodulin-dependent NO synthase in EC. (*J. Clin. Invest.* 1993. 91:1367-1373.) Key words: endothelin • receptor subtype B • nitric oxide • Ca²⁺/calmodulin • endothelial cell

Introduction

Endothelin-1 (ET-1)¹ is a potent endothelium-derived vasoconstrictor peptide originally isolated from culture media of porcine vascular endothelial cells (EC) (1). Subsequent human genomic cDNA cloning revealed three distinct ET isopeptides, designated ET-1, ET-2 and ET-3 (2). Compared with the equipotent vasoconstrictor/pressor activities of ET-1 and ET-2, ET-3 has less vasoconstrictor/pressor activity but the most potent initial depressor response (2), suggesting the existence of receptor subtypes. Recently, cDNA cloning of ET receptors

from bovine and rat lung has revealed at least two distinct subtypes (3, 4): one selective for ET-1 and ET-2 (ET_A) and the other non-isopeptide-selective (ET_B). Both receptor subtypes comprise seven transmembrane domains common to the G-protein-coupled receptors. It has been suggested that vascular smooth muscle has ET_A receptors that mediate contraction, while vascular EC has ET_B receptors that may be involved in the vasodilation (3, 4). Very recently, several agonists selective for ET_B receptors have been developed (5). One of these, BQ3020, a linear ET analog (*N*-acetyl-Leu-Met-Asp-Lys-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp), is highly selective for the ET_B receptors (6); BQ3020 binds to the ET_B receptors several thousands times more selectively than to the ET_A receptors.

Endothelium-derived relaxing factor (EDRF) or nitric oxide (NO) (7, 8) is a potent vasodilator with anti-platelet aggregation property produced by a variety of cells including ECs, vascular smooth muscle, macrophages, and neuronal cells (9). The formation of NO is catalyzed from L-arginine by a NADPH-dependent dioxygenase, termed NO synthase (NOS). There exist at least two distinct isoforms of NOS (9); one is a constitutive, Ca²⁺/calmodulin-dependent enzyme present mainly in ECs and brain, while the other is an inducible, Ca²⁺/calmodulin-independent enzyme activated by cytokines that is found predominantly in macrophages and vascular smooth muscle cells.

It has been shown that ET-3 is a vasodilator rather than a vasoconstrictor in the isolated perfused rat mesentery via production and release of EDRF/NO (10). We have recently shown that ET-3-induced vasodilation is ascribed mainly to EDRF/NO in isolated porcine pulmonary artery in vitro (11). In cultured bovine ECs, we have demonstrated that ET-3, but not ET-1, stimulates receptor-mediated phosphoinositide breakdown, which increases cytosolic free Ca²⁺ concentrations ([Ca²⁺]_i) (12) and activates soluble guanylate cyclase, possibly by the generation of EDRF/NO (13). However, the cellular mechanism by which ET stimulates the synthesis and release of EDRF/NO remains largely unknown. In the present study, we have attempted to ascertain whether vascular ECs predominantly express ET_B receptors using receptor binding study with a novel ET_B receptor agonist and Northern blot analysis, and

1. *Abbreviations used in this paper:* BAPTA, *N,N,N',N'*-tetraacetic acid; [Ca²⁺]_i, cytosolic free Ca²⁺ concentration; cNOS, Ca²⁺/calmodulin-dependent constitutive enzyme; DSS, disuccinimidyl suberate; EC, endothelial cell; EC₅₀, half maximal stimulation; EDRF, endothelium-derived relaxation factor; ET, endothelin; ET_A, ET receptor selective for ET-1 and ET-2; ET_B, non-isopeptide-selective receptor subtype; H-7, 1-5-isoquinolinesulfonyl-2-methylpiperazine; IBMX, 3-isobutyl-1-methylxanthine; iNOS, Ca²⁺/calmodulin-independent cytokine-inducible enzyme; IP₃, inositol-1,4,5-triphosphate; LNMMA, *N*^G-monomethyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; NO_x, nitrite/nitrate; PTX, pertussis toxin; W-5, *N*-(6-aminoethyl)-1-naphthalene-sulfonamide; W-7, *N*-(6-aminoethyl)-6-chloro-1-naphthalenesulfonamide.

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to elucidate the intracellular mechanism(s) responsible for NOS activation using an ET_B receptor agonist in cultured bovine ECs.

Methods

Drugs. Synthetic BQ3020 (10) was generously provided by Dr. M. Ihara and Dr. M. Yano, Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd. (Tsukuba, Japan). ET-1, ET-3, leupeptin, and pepstatin D were purchased from Peptide Institute (Osaka, Japan); N^G-monomethyl-L-arginine (LNMMMA) and 3-isobutyl-1-methylxanthine (IBMX) from Calbiochem Corp. (La Jolla, CA); EDTA, EGTA, 1,2-bis(2-aminophenoxy)ethane *N,N,N',N'*-tetraacetic acid (BAPTA), *N*-(6-aminohexyl)-1-naphthalene-sulfonamide (W-5), *N*-(6-aminohexyl)-6-chloro-1-naphthalenesulfonamide (W-7), 1-5-isoquinolinylnsulfonyl 2-methylpiperazine (H-7), PMSF, disuccinimidyl suberate (DSS), and D- and L-arginine from Sigma Chemical Co. (St. Louis, MO); nicardipine from Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan); pertussis toxin from Kaken Chemical (Tokyo, Japan); methylene blue from Schmidt GmbH (Stuttgart-Unterturkeim, Germany); and fura-2 acetoxymethyl ester from Dojin Chemical (Kumamoto, Japan).

Cell culture and incubation. ECs from bovine carotid artery were prepared and cultured in DME containing 10% FCS and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) at 37°C in a humidified atmosphere of 95% air and 5% CO₂ (5, 6). The cells were identified as ECs by the contact-inhibited monolayer growth with a "cobblestone" appearance and the presence of Factor VIII antigen detected immunocytochemically. After serial subculture with 0.05% trypsin and 0.02% EDTA, ECs harvested between the 8th and 12th passage were used in the experiments. Confluent cells were washed with HBSS that was replaced with fresh HBSS unless otherwise stated.

Binding study. Membranes from ECs were prepared by the same method as previously reported (14). Briefly, bovine ECs and rat cerebellum were homogenized by a Polytron homogenizer (Kinematica AG Littau, Switzerland) in 20 mM phosphate buffer (130 mM NaCl, 1 mM EDTA, 0.2 mM PMSF, 10 µg/ml pepstatin, 10 µg/ml leupeptin), pH 7.5; then the mixture was washed, centrifuged, and resuspended in the same buffer. The membrane suspension (15–30 µg protein) was usually incubated with 25 pM [¹²⁵I]BQ3020 (specific activity 2,000 Ci/mmol) (Amersham Japan, Tokyo, Japan) in the presence or absence of unlabeled peptides at 30°C for 30 min. After incubation, free radioactive ligand was removed by centrifugation. Radioactivity was measured with gamma counter (ARC-300; Aloca, Tokyo, Japan). Specific binding was determined by subtracting nonspecific binding in the presence of excess (10⁻⁶ M) unlabeled BQ3020 from total binding.

Affinity labeling. Affinity labeling of [¹²⁵I]BQ3020 to the EC membrane was performed as described previously (14). Briefly, the membrane suspension (10 µg protein) was incubated with 750 pM [¹²⁵I]-BQ3020 at 30°C for 30 min in the presence or absence of excess amounts (10⁻⁶ M) of unlabeled peptides, and DSS was added to a final concentration of 1 mM. The mixture was then incubated for 20 min at 25°C, followed by the addition of 2 M ammonium acetate to stop the reaction. The reaction mixture was subjected to SDS-PAGE in the presence of 5% 2-mercaptoethanol according to the method of Laemmli (15). After electrophoresis, the gels were dried and exposed to film (X-Omat AR; Kodak, Rochester, NY) at -70°C for 5 d.

Northern blot analysis. RNA was extracted from bovine ECs by the acid guanidinium thiocyanate-phenol-chloroform method (16). Briefly, ECs were homogenized in 10 ml guanidinium solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, and 1% 2-mercaptoethanol) using a Polytron homogenizer and 1 ml 2 M sodium acetate (pH 4), 10 ml water-saturated phenol, and 2 ml chloroform-isoamyl alcohol mixture (49:1) were added with through mixing and vortexing; the solution was cooled on ice for 15 min. After centrifugation at 1,000 g for 20 min, an aqueous phase containing RNA was transferred and precipitated with isopropyl alcohol at -20°C for 20 min. RNA was pelleted by centrifugation, washed with 70% ethanol,

dissolved in diethylpyrocarbonate-treated water, and subjected to poly(A)⁺ RNA selection. Poly(A)⁺ RNA (10 µg) was fractionated by formaldehyde/1.1% agarose gel electrophoresis and transferred to a nylon membrane (MagnaGraph; Micron Separations Inc., Westboro, MA). Probe for bovine ET_B receptor cDNAs (17) was labeled with [³²P]-dCTP (Amersham International, Amersham, UK) by the random-primed method, and incubated at 42°C for 16 h with the membranes in a hybridization buffer containing 1 M NaCl/50% (vol/vol) formamide, 1% SDS, and 250 µg/ml salmon sperm DNA. The membranes were washed with 0.3 M NaCl, 30 mM sodium citrate, and 1% SDS at 60°C, and they were autoradiographed on film (X-Omat AR; Rochester) at -80°C for 16 h.

Determination of nitrite/nitrate (NO_x). Confluent ECs were usually incubated in Hanks' medium without phenol red at 37°C for 1 min unless otherwise specified. Medium was removed and NO_x concentration was determined by an autoanalyzer (TCI-NOX 1000; Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) as recently described (18). In brief, sample premixed with the carrier solution (0.07% EDTA, 0.03% NH₄Cl) was passed through a copperized cadmium reduction column, where NO₃⁻ was reduced to NO₂⁻, which reacts with Griess reagent (one part 1% sulfonamide and one part 0.1% *N*-1-naphthylethylenediamine dihydrochloride/5% HCl) to form a purple azo dye (19). Absorbance at 540 nm was detected by a flow-through visible spectrophotometer (model S/3250; Soma-Kogaku Co., Ltd., Tokyo, Japan) connected to a strip chart recorder. The limit of detection of NO₂⁻ was 0.2 µM (99% confidence) and intraassay and interassay coefficients of variation were 1.6% and 0.7%, respectively.

Determination of inositol-1,4,5-triphosphate (IP₃). For measurement of IP₃, confluent ECs were incubated at 37°C in HBSS containing 10 mM LiCl as reported (12, 13). Incubation was terminated by the rapid removal of medium and the addition of ice-cold 15% TCA and the cells were placed on ice for 30 min. The TCA extract was washed with ethylether and neutralized with 1N sodium acetate. IP₃ levels were determined with an IP₃ assay kit (New England Nuclear, Boston, MA).

Determination of intracellular cyclic guanosine monophosphate (cGMP). For measurement of cGMP, confluent cells were incubated at 37°C for 10 min in HBSS containing 0.5 mM IBMX as described previously (13). After extraction with 6% TCA, the concentration of intracellular cGMP was determined with a cGMP RIA kit (New England Nuclear).

Measurement of cytosolic free Ca²⁺ concentration ([Ca²⁺]_i). Measurement of [Ca²⁺]_i was measured by the Ca²⁺-fura-2 fluorescence method as described previously (20). After incubation in serum-free DME for 24 h, ECs were trypsinized and incubated with 4 µM fura-2 acetoxymethyl ester at 37°C for 20 min in buffered physiological salt solution, essentially as reported previously (20). The Ca²⁺-fura-2 fluorescence of the suspended cells (~2 × 10⁶ cells/ml) was measured by a spectrofluorometer (CAF-100; JASCO Co., Ltd., Tokyo, Japan) using excitation at 340 and 380 nm and emission at 500 nm. Values of [Ca²⁺]_i were determined according to the method of Grynkiewicz et al (21). [Ca²⁺]_i was calculated by the following equation:

$$[Ca^{2+}]_i (\text{nM}) = K_d \times (R - R_{\min}) / (R_{\max} - R) \times \beta$$

where K_d is the dissociation constant of fura-2 for Ca²⁺ at 37°C (224 nM), R represents the ratio of fluorescence of the sample at 340 nm to that at 380 nm, R_{\max} and R_{\min} are the ratios of fura-2 free acid at the same wavelengths in the presence of saturating Ca²⁺ to those in nominally zero Ca²⁺ by adding Triton X and EGTA, respectively, and β is the ratio of fluorescence of fura-2 at 380 nm in zero Ca²⁺ to that in saturating Ca²⁺.

Results

Binding study. [¹²⁵I]-labeled BQ3020 specifically bound to bovine ECs and rat cerebellar membranes as a function of time; the maximal binding at 30°C was obtained after 30 min, and

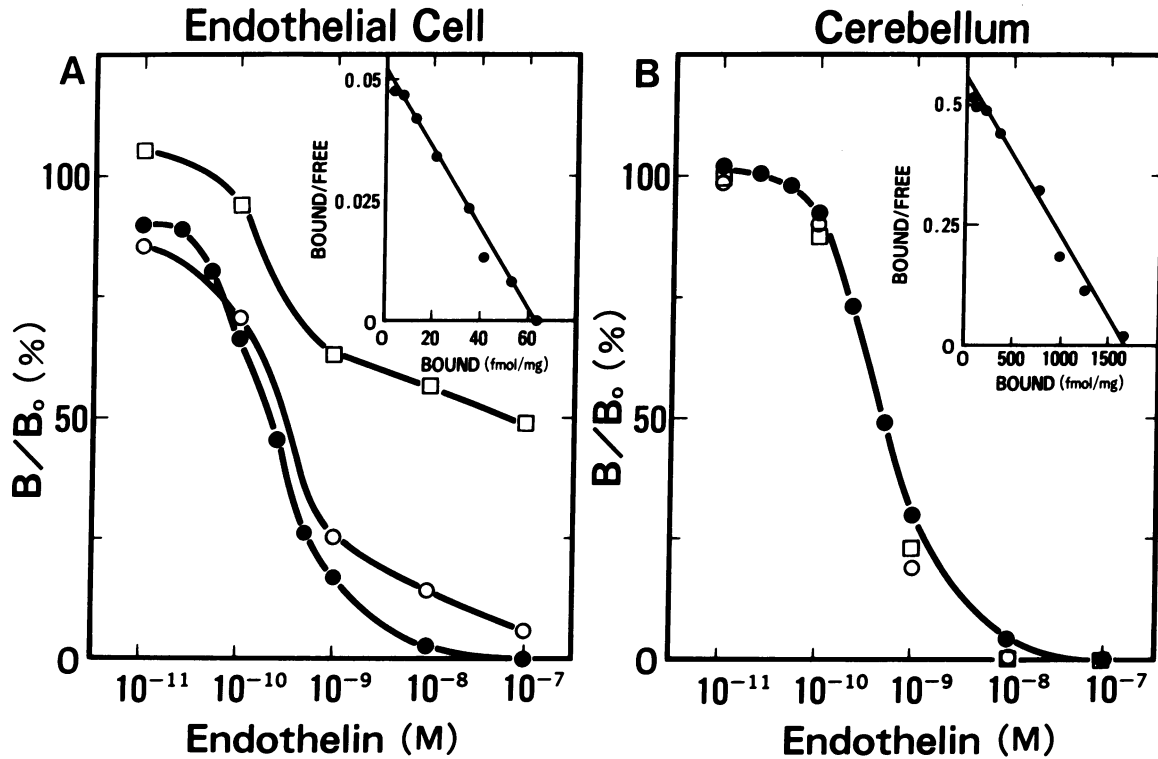


Figure 1. Competitive binding of [¹²⁵I]ET_B agonist (BQ3020) to membranes of cultured bovine endothelial cells and rat cerebellum. Membranes (15–30 μg protein) were incubated with [¹²⁵I]BQ3020 at 37°C for 30 min in the presence or absence of unlabeled ET-1 (□), ET-3 (○), and BQ3020 (●). Each point shows the mean of triplicate samples. (Inset) Scatchard plot of BQ3020 binding.

the nonspecific binding was < 10% of the total binding (data not shown). As shown in Fig. 1 A, unlabeled BQ3020 and ET-3 equally displaced radioligand binding to EC membranes, while ET-1 was less potent; the apparent K_d was 2×10^{-10} M and the B_{max} was 62 fmol/mg protein. In contrast, the binding of [¹²⁵I]-BQ3020 to rat cerebellar membranes was equally inhibited by unlabeled BQ3020, ET-3, and ET-1 (Fig. 1 B); the apparent K_d and B_{max} were 2×10^{-10} M and 1.6 pmol/mg protein, respectively.

SDS-PAGE after affinity labeling of ¹²⁵I-labeled BQ3020 to EC membranes by bifunctional crosslinker DSS revealed one major labeled band with an apparent molecular mass of 50 kD (Fig. 2); this band was completely abolished by an excess amount (10^{-6} M) of unlabeled BQ3020 and ET-3, but incompletely by ET-1.

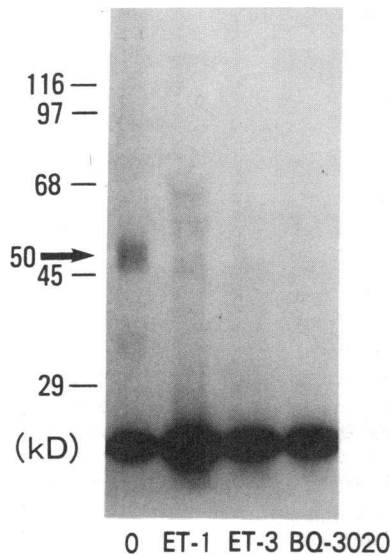


Figure 2. SDS-PAGE after affinity labeling of ET_B agonist cross-linked to bovine endothelial cell membrane. Cell membrane suspensions were incubated with [¹²⁵I]BQ3020 in the presence or absence of 10^{-6} M each of ET-1, ET-3, and BQ3020, cross-linked by disuccinimidyl suberate, and subjected to SDS-PAGE followed by autoradiography. Arrow indicates position of 50 kD. Molecular markers are indicated by bars on the left.

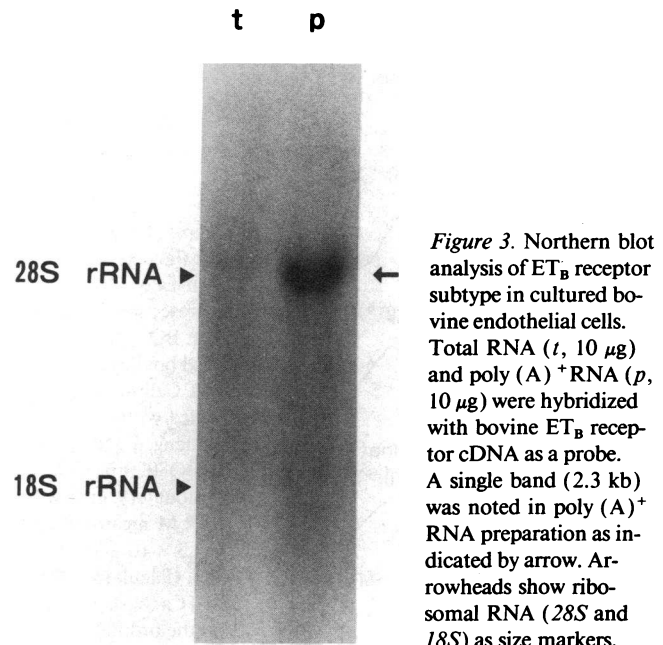


Figure 3. Northern blot analysis of ET_B receptor subtype in cultured bovine endothelial cells. Total RNA (t, 10 μg) and poly (A)⁺ RNA (p, 10 μg) were hybridized with bovine ET_B receptor cDNA as a probe. A single band (2.3 kb) was noted in poly (A)⁺ RNA preparation as indicated by arrow. Arrowheads show ribosomal RNA (28S and 18S) as size markers.

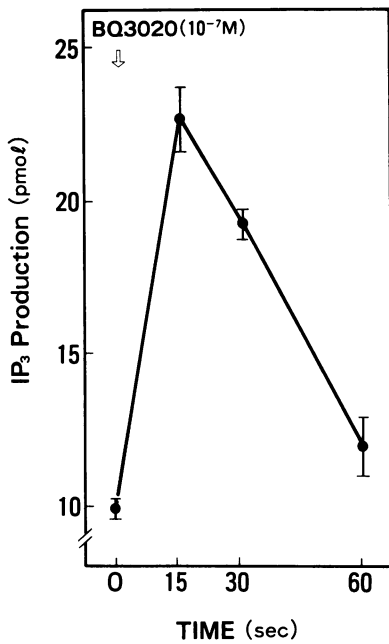


Figure 4. Effect of ET_B agonist BQ3020 on IP_3 production as a function of time in cultured bovine endothelial cells. After addition of 10^{-7} M BQ3020, confluent cells were incubated for indicated times. IP_3 generation was determined. Each point with bar shows the mean \pm SEM ($n = 6$).

Northern blot analysis. Northern blot hybridization of poly (A)⁺ RNA from bovine ECs with cloned cDNA for bovine ET_B receptor as a probe revealed a distinct band (4.8 kb) corresponding to the size of ET_B receptor mRNA (Fig. 3).

IP_3 production and $[Ca^{2+}]_i$ increase by ET_B agonist. BQ3020 rapidly stimulated IP_3 production, which peaked at 15 s and then declined almost to basal levels by 1 min (Fig. 4). BQ3020 also induced an immediate increase in $[Ca^{2+}]_i$, which peaked at 15 s and returned to basal levels after 1 min (Fig. 5); the ET_B agonist-induced increase in $[Ca^{2+}]_i$ was not affected by pretreatment with nicardipine or EGTA. As shown in Fig. 6, BQ3020 similarly stimulated IP_3 production and $[Ca^{2+}]_i$ increase in a dose-dependent fashion (10^{-9} – 10^{-7} M); the approximate doses of BQ3020 for half maximal stimulation (EC_{50}) and maximal stimulation were 5×10^{-9} and 10^{-7} M, respectively.

Production of NOx and cGMP by ET_B agonist. BQ3020 rapidly stimulated production and release of NOx, which

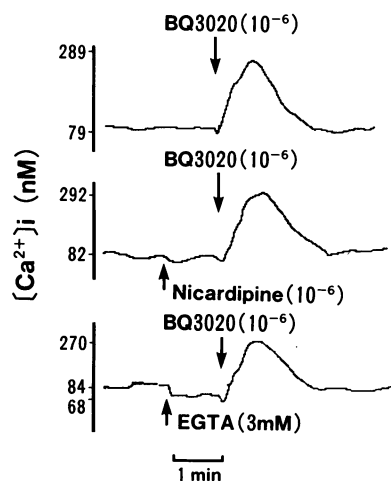


Figure 5. Representative tracings show changes of Ca^{2+} -fura-2 fluorescence by ET_B agonist BQ3020 in cultured bovine endothelial cells. Cell suspensions loaded with fura-2 were challenged with 10^{-6} M BQ3020 pretreated without (A), and with 10^{-6} M nicardipine (B) and 3×10^{-3} M EGTA (C). Calculated values for $[Ca^{2+}]_i$ are shown on the ordinates.

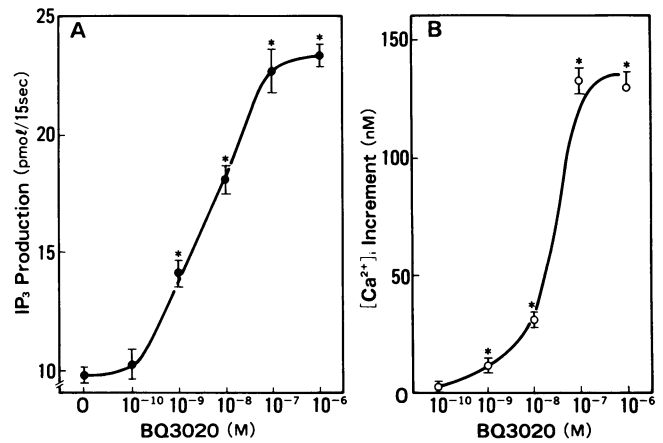


Figure 6. Effect of ET_B agonist (BQ3020) on IP_3 production and increase in $[Ca^{2+}]_i$ in cultured bovine endothelial cells. (A) Confluent cells were incubated at $37^\circ C$ with various doses (10^{-10} – 10^{-6} M) of BQ3020 for 15 s and IP_3 generation was determined. (B) Fura-2-loaded cells were incubated with various doses of BQ3020; agonist-induced increment of $[Ca^{2+}]_i$ was calculated from basal levels (79 ± 5.9 nM). Each point with bar shows the mean \pm SEM ($n = 4$). * $P < 0.05$ vs. control.

peaked at 1 min and lasted for ≥ 20 min, and similarly stimulated cGMP generation, which peaked at 5–10 min in the presence of the phosphodiesterase inhibitor IBMX (data not shown). As shown in Fig. 7, BQ3020 dose dependently (10^{-9} – 10^{-7} M) stimulated production of both NOx and cGMP with almost comparable EC_{50} value ($\sim 2 \times 10^{-8}$ M). There was a highly significant ($P < 0.001$) correlation ($r = 0.93$) between NOx and cGMP production. Methylene blue (10^{-3} M), a soluble guanylate cyclase inhibitor, completely blocked BQ3020-induced cGMP generation (data not shown).

Arginine as a substrate for NOx production. To ascertain whether ET_B agonist-induced NOx production is mediated by NOS, the effect of the stereospecific NOS inhibitor, LNMMA, on NOx production was studied. The BQ3020-induced NOx production was completely inhibited by 4×10^{-4} M LNMMA,

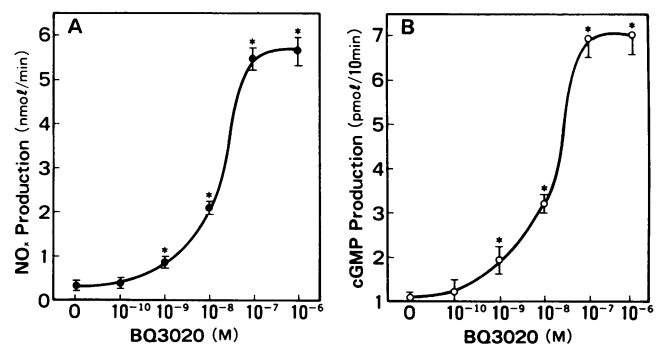


Figure 7. Effect of ET_B agonist BQ3020 on production of NOx and cGMP in cultured bovine endothelial cells. (A) Confluent cells were incubated with or without various doses (10^{-10} – 10^{-6} M) of BQ3020 at $37^\circ C$ for 1 min; NOx released into medium was measured. (B) Confluent cells were incubated with or without the same doses of BQ3020 at $37^\circ C$ for 10 min in the presence of 0.5 mM methylisobutylxanthine; intracellular cGMP produced was determined. Each point with bar shows the mean \pm SEM ($n = 6$). * $P < 0.05$ vs. control.

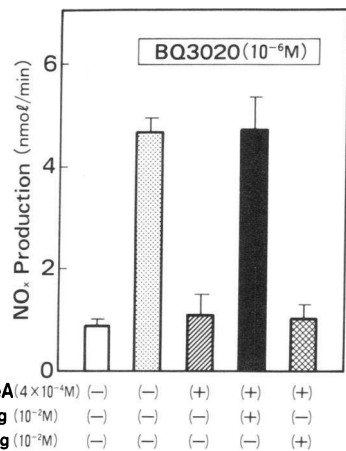


Figure 8. Effects of *N*^G-monomethyl-L-arginine (L-MeA), and L- and D-arginine (Arg) on ET_B agonist BQ3020-induced production of NO_x in cultured bovine endothelial cells. Confluent cells were incubated without (□) or with 10⁻⁶ M BQ3020 at 37°C for 1 min in the absence (□) or presence of 4 × 10⁻⁴ M L-MeA alone (■), L-MeA plus 10⁻² M L-Arg (▨) and L-MeA plus 10⁻² M D-Arg (▩); NO_x released

into medium was measured. Each column represents the mean; bar shows SEM (*n* = 6).

and this effect was reversed by the simultaneous addition of 10⁻² M L-arginine, but not by 10⁻² M D-arginine (Fig. 8).

Involvement of pertussis toxin (PTX)-sensitive G-protein(s). To determine whether PTX-sensitive G-protein(s) is involved in ET_B receptor-mediated NO synthesis, cultured bovine ECs were pretreated with PTX (100 ng/ml) for 6 h, and the effect of the ET_B agonist on NO_x and cGMP production was studied (Fig. 9). BQ3020-induced production of both NO_x and cGMP was abolished in a similar fashion by pretreatment with PTX, while PTX alone had no effect on the basal production of NO_x and cGMP.

Role of Ca²⁺/calmodulin system in NO_x production. To elucidate whether ET_B receptor-mediated NO_x synthesis requires Ca²⁺ from an intracellular and/or extracellular source, the effects of an intracellular Ca²⁺ chelator (BAPTA) and an extracellular Ca²⁺ chelator (EGTA) on NO_x production were studied (Table I). Removal of an intracellular Ca²⁺ by

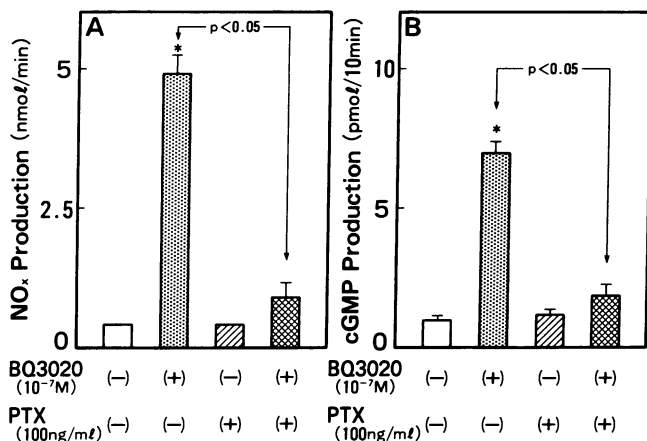


Figure 9. Effect of PTX on ET_B agonist BQ3020-induced production of NO_x and cGMP in cultured bovine endothelial cells. After pretreatment without (□, □) or with (■, ■) PTX (100 ng/ml) for 6 h, cells were incubated in the absence (□, ■) or the presence (□, ■) of 10⁻⁷ M BQ3020 for measurement of (A) NO_x production and (B) cGMP generation, as in Fig. 7. Each column represents the mean; bar shows SEM (*n* = 6). **P* < 0.05 vs. control.

Table I. Effects of Various Compounds on ET_B Agonist (BQ3020)-induced NO_x Production by Cultured Bovine Endothelial Cells

Drugs (concentration)	NO _x production nmol/min
None	0.90 ± 0.11
BQ3020 (10 ⁻⁶ M)	4.80 ± 0.12*
BQ3020 (10 ⁻⁶ M) + BAPTA (3 × 10 ⁻³ M)	1.19 ± 0.09
BQ3020 (10 ⁻⁶ M) + EGTA (3 × 10 ⁻³ M)	4.56 ± 0.19*
BQ3020 (10 ⁻⁶ M) + W-7 (5 × 10 ⁻⁵ M)	2.02 ± 0.17*
BQ3020 (10 ⁻⁶ M) + W-5 (5 × 10 ⁻⁵ M)	4.84 ± 0.22*
BQ3020 (10 ⁻⁶ M) + H-7 (10 ⁻⁶ M)	5.01 ± 0.34*

Confluent bovine endothelial cells (10⁵ cells/dish) were incubated at 37°C for 1 min in the absence and presence of 10⁻⁶ M BQ3020 alone and in combination with either an intracellular Ca²⁺ chelator (BAPTA), an extracellular Ca²⁺ chelator (EGTA), a relatively selective calmodulin inhibitor (W-7), a nonselective calmodulin inhibitor (W-5), and a protein kinase C inhibitor (H-7) in concentrations as indicated. NO_x released into medium was measured as described in Methods. Each value is the mean ± SEM (*n* = 6). **P* < 0.05 vs. control.

3 × 10⁻³ M BAPTA completely abolished NO_x production stimulated by 10⁻⁶ M BQ3020, whereas removal of extracellular Ca²⁺ by 3 × 10⁻³ M EGTA had no effect.

The effects of calmodulin inhibitors and a protein kinase C inhibitor on ET_B agonist-induced NO_x production were also examined (Table I). The BQ3020-induced NO_x production was significantly decreased by a relatively selective calmodulin inhibitor (W-7), but not by either a nonselective calmodulin inhibitor (W-5) or a protein kinase C inhibitor (H-7).

Discussion

Recent cDNA cloning studies have revealed the existence of at least two subtypes of ET receptors, termed ET_A and ET_B (3, 4); ET_A receptor shows selective affinity for ET-1, whereas ET_B receptor shows nonselective affinity for ET isopeptides. It has been suggested that vascular smooth muscle expresses ET_A receptors that mediate contraction, while vascular endothelium expresses ET_B receptors that mediate relaxation, possibly via production of EDRF/NO and prostacyclin (3, 4). Recently, several analogs selective for ET_B receptors have been synthesized (5), among which BQ3020 has been shown to be the most potent, stable, and selective agonist for ET_B receptor thus far reported (6); BQ3020 displaces [¹²⁵I]ET-1 binding to the ET_B receptor in the porcine cerebellar membranes with a selectivity 4,700 times greater than that to the ET_A receptor in vascular smooth muscle. We have confirmed that [¹²⁵I]BQ3020 binding to rat cerebellar membrane is inhibited equipotently by BQ3020, ET-1, and ET-3 with high affinity similar to that of porcine cerebellar membrane (6).

The present binding study clearly demonstrated the presence of binding sites for ET_B agonist in cultured bovine ECs with high affinity (*K*_D = 2 × 10⁻¹⁰ M) comparable to that of rat cerebellar membranes. In contrast to the complete and equal binding inhibition by ET isopeptides in cerebellar membranes, unlabeled ET-1 failed to displace [¹²⁵I]BQ3020 completely from its binding site in EC membranes. The same apparent

lack of complete displacement by unlabeled ET-1 has already been shown in [¹²⁵I]ET-3 binding to cultured bovine ECs (12) and human ECs (22). Several possible mechanisms may account for the anomalous behavior of ET-1 in cultured ECs. First, this may be caused by differences in the dissociation rates of ET isopeptides. Indeed, it has been reported that the dissociation of [¹²⁵I]ET-1 bound to Swiss 3T3 fibroblasts is much slower than that of [¹²⁵I]ET-2 (23). Second, this may be ascribable to downregulation of the ET_B receptor by endogenous ET-1 constitutively secreted by ECs as previously suggested (14). It has recently been shown that pretreatment of cultured human ECs with phosphoramidon, a putative ET-converting enzyme inhibitor, led to an increase in the binding capacity of the ET_B receptor that interacts equally with ET-1 and ET-3 (24), suggesting upregulation of endothelial ET_B receptor after blocking processing from big ET-1 to ET-1. We have confirmed these observations in that bovine endothelial ET_B receptor exhibits equal binding affinity for ET-1 and ET-3 in phosphoramidon-treated cells (unpublished observation). Third, there may be another receptor subtype in addition to the ET_A and ET_B receptors that prefers ET-3 over ET-1. However, there have been no reports thus far on the isolation of cDNA clones encoding an ET-3 selective receptor (ET_C) (25); Southern blot analysis of human genomic DNA using ET_A and ET_B receptor cDNA probes revealed only two different genomic DNA fragments even under low stringency conditions (26), suggesting that mammalian genomes contain only two closely related ET receptor genes, probably for the ET_A and ET_B receptors. Should a third novel receptor gene exist in mammalian genomes, it has little if any similarity to the two known receptors. Whatever the mechanism involved, our study shows that ET-3 preferentially interacts with ET_B receptor in cultured bovine ECs.

Affinity labeling experiments to visualize receptor molecules on SDS-PAGE after covalent binding of BQ3020 to EC membranes by a bifunctional cross-linker revealed one major labeled band with the apparent molecular mass of 50 kD, whose size appears to be comparable to that of the ET_B receptor (4, 14, 17). Northern blot analysis of poly(A)⁺ RNA from cultured bovine EC verified the expression of the ET_B receptor gene in these cells.

In our previous study (12), we showed that ET-3 stimulates receptor-mediated phosphoinositide breakdown, which triggers intracellular Ca²⁺ mobilization in cultured bovine ECs. Subsequent studies have shown that ET-3 stimulates soluble guanylate cyclase activity, possibly resulting from NO formation in cultured bovine ECs (13). The present study clearly demonstrated that BQ3020, a more selective ET_B agonist than ET-3, rapidly and dose dependently stimulated phosphoinositide breakdown and [Ca²⁺]_i increase in cultured bovine ECs. These findings are consistent with our previous observation that ET-3 induces receptor-mediated phosphoinositide breakdown and [Ca²⁺]_i increase in the same cell preparation (12). The coincident times of peak IP₃ formation and [Ca²⁺]_i transient elicited by BQ3020 and the failure of the response, either by removal of extracellular Ca²⁺ with EGTA or by blockade of voltage-dependent Ca²⁺ channels with nifedipine, strongly suggest that the [Ca²⁺]_i increase derives mainly from IP₃-mediated intracellular Ca²⁺ release rather than Ca²⁺ influx from an extracellular source.

Furthermore, the present study showed that an ET_B agonist immediately and dose dependently stimulated NOx and cGMP production in cultured bovine ECs. The highly signifi-

cant correlation between NOx production and cGMP generation and the inhibitory effect of methylene blue on cGMP generation in bovine ECs suggest that ET_B agonist-induced NO production led to the stimulation of soluble guanylate cyclase within the same cells. However, the physiological function(s) of NO-induced cGMP generation in ECs remains unknown.

There appears to be a discrepancy between the apparent K_d for BQ3020 binding (~ 2 × 10⁻¹⁰ M) and the EC₅₀ values for IP₃ formation and [Ca²⁺]_i transient (~ 5 × 10⁻⁹ M) and the corresponding values for NOx and cGMP production (~ 2 × 10⁻⁸ M). Such a discrepancy may be partly accounted for by the use of different incubation conditions (temperature and time) and preparations (cell membranes, dispersed cells, and monolayers). Alternatively, different cellular responses to the ET_B agonist may be mediated via activation of different subpopulations of ET_B receptors, although Scatchard analysis of BQ3020 binding revealed a single class of binding sites in EC membranes. It should be noted that there is also a discrepancy between the binding affinity of ET_A receptor and ET-1-induced cellular responses, including IP₃ formation, [Ca²⁺]_i increase, and contraction in rat glomerular mesangial cells (27).

Both ET_A and ET_B receptor subtypes have seven hydrophobic membrane-spanning domains belonging to the superfamily of G-protein-coupled receptors (3, 4, 17). Several types of G-proteins have been identified in cardiovascular tissues (28). Pertussis toxin induces NAD-dependent ADP ribosylation of the α-subunit of several G-proteins (G_i, G_o, and G_q). The present study revealed that pertussis toxin completely blocked ET_B agonist-induced NOx and cGMP production in bovine ECs. In our previous study (13), pertussis toxin was shown to block phosphoinositide breakdown and the [Ca²⁺]_i increase stimulated by ET-3 in bovine ECs. These data are in contrast to findings with the ET_A receptor in vascular smooth muscle, which is functionally coupled to phospholipase C-mediated phosphoinositide breakdown via pertussis toxin-insensitive G-proteins (29). Taken together, these observations suggest that the ET_B receptor in vascular endothelium is functionally coupled to phospholipase C, possibly via pertussis toxin-sensitive G-protein(s) involved in synthesis and/or release of EDRF/NO.

EDRF/NO is produced from L-arginine by NADPH-dependent NOS (8, 9). The inhibition by LNMMA of ET_B agonist-induced NOx production, as well as its reversal by L-arginine, but not by D-arginine, as demonstrated in this study, is consistent with the notion that NOS requires stereoselectivity for the L-enantiomer of arginine as a substrate to generate NO (30). At least two isoforms of NADPH-dependent NOS have been found in a variety of tissues and cells (9). Brain NOS is a constitutive Ca²⁺/calmodulin-dependent enzyme (cNOS), the entire structure of which has been elucidated by cDNA cloning of rat brain (31). Macrophage NOS, on the other hand, is a Ca²⁺/calmodulin-independent cytokine-inducible enzyme (iNOS), of which the entire structure has recently been determined by cDNA cloning of murine macrophages (32). Both isoforms share 50% sequence homology and comprise binding sites for NADPH, flavin mononucleotide, and flavin adenine dinucleotide. Very recently, a distinct cNOS isoform from bovine ECs has been cloned (33), which shows 50–60% sequence identity with murine macrophage iNOS and rat brain cNOS isoforms.

The present study clearly showed that ET_B receptor-mediated NO synthesis in bovine EC requires Ca²⁺ and calmodu-

lin, based on the observations that BQ3020-induced NO_x production is completely blocked by an intracellular Ca²⁺ chelator (BAPTA), but not by an extracellular Ca²⁺ chelator (EGTA), and by a relatively selective calmodulin inhibitor (W-7), but not by a less selective calmodulin inhibitor (W-5) or a protein kinase C inhibitor (H-7). It has been suggested that endothelial NOS is constitutive and dependent on Ca²⁺/calmodulin (9, 34). In fact, cNOS isoforms from bovine ECs (33) and rat brain (31), but not iNOS from murine macrophages (32), contain binding sites for calmodulin within the molecule. From these findings, we conclude that vascular endothelium contains cNOS that is activated by a Ca²⁺/calmodulin-dependent pathway resulting from ET_B receptor-mediated phosphoinositide breakdown and Ca²⁺ mobilization via pertussis toxin-sensitive G-protein(s).

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