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A novel non-peptide endothelin antagonist isolated from bayberry, *Myrica cerifera*

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A potent non-peptide ET receptor antagonist, myriceron caffeoyl ester (50-235), was isolated from the bayberry, *Myrica cerifera*. This compound selectively antagonized specific binding of [^{125}I]ET-1, but not of [^{125}I]ET-3, to rat cardiac membranes, ET-1-induced increase in the intracellular free calcium concentration in Swiss 3T3 fibroblasts, and ET-1-induced contraction of rat aortic strips. Thus, 50-235 is the first non-peptide ET_A receptor antagonist. This compound can be useful for studying the physiological role of endothelin and exploring its role in various diseases.

Endothelin antagonist; Myriceron caffeoyl ester; Receptor binding; Cytosolic free calcium; Vasoconstriction

1. INTRODUCTION

Endothelin (ET) is a peptide family consisting of three peptides (ET-1, ET-2 and ET-3) which were discovered by Yanagisawa et al. [1–3]. They found ET to be one of the most potent vasoconstrictors known of porcine coronary artery strips. The existence of the receptor for ET in most tissues [4,5] suggests that ET contributes to many regulatory functions in the body. Although ET has been demonstrated to possess a spectrum of pharmacological activities much wider than initially inferred [6] the physiological and pathological roles have been unclear because of the lack of specific antagonists. Recently, Ihara et al. [7] isolated a competitive peptide ET antagonist, cyclo(-D-Glu-L-Ala-allo-D-Ile-L-Leu-D-Trp-) (BE-18257B), from the fermentation products of *Streptomyces misakiensis*, and produced its potent synthetic analogs [8]. We have been searching for ET receptor antagonist activities in plant extracts, because plants have been a good source for medicines, most of which interact with the corresponding specific receptors for neurotransmitters or neuropeptides in mammals. Here we report on a novel non-peptide ET_A receptor antagonist, myriceron caffeoyl ester (50-235), which was discovered in bayberry, *Myrica cerifera*.

2. MATERIALS AND METHODS

2.1. *Materials*

50-235 was purified from the methanol extract of branches of *Myrica cerifera*, as described elsewhere (Sakurai et al., in preparation).

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[^{125}I]ET-1 and [^{125}I]ET-3 were obtained from New England Nuclear. ET-1, ET-2, ET-3, neuropeptide Y, bradykinin, angiotensin II, leupeptin and pepstatin A were obtained from the Peptide Institute (Osaka, Japan). Fura-2-AM was obtained from Dojin (Kumamoto, Japan). Phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor and bacitracin were obtained from Sigma Chemical Co. (St. Louis, MO), and aprotinin from Boehringer (Mannheim, Germany).

2.2. *Cell culture*

Swiss 3T3 fibroblast cells were obtained from the American Type Culture Collection through Dainippon Seiyaku (Osaka, Japan), and cultured in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum (Gibco), 50 μg/ml streptomycin, and 50 U/ml penicillin G (Gibco) in a 5% CO_2/95% air incubator at 37°C.

2.3. *Binding studies*

The ventricles from rat hearts were minced with scissors and homogenized in 7 vols. of ice-cold 20 mM $NaHCO_3$ containing 0.1 mM PMSF (pH 7.4) with a Polytron homogenizer (Bringmann Instruments Inc., Westberg, NY). The homogenates were centrifuged at 1,000 × *g* for 10 min, and then the pellet was discarded. The supernatant was centrifuged at 30,000 × *g* for 30 min. The pellet was washed once and resuspended in Tris buffer (50 mM, pH 7.4 at 25°C) containing 0.1 mM PMSF, and stored at −80°C until use. Binding studies were performed according to the method described by Gu et al. [9]. In brief, cardiac membranes (0.21 mg/ml as protein) were incubated with 25 pM [^{125}I]ET-1 or [^{125}I]ET-3, unless otherwise stated, in a final assay volume of 0.1 ml, in borosilicated glass tubes, containing 50 mM Tris-HCl, 0.1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 250 μg/ml bacitracin and 10 μg/ml soybean trypsin inhibitor (pH 7.4). Binding was performed for 60 min at 37°C. The binding reaction was terminated by the addition of 2.5 ml of ice-cold 50 mM Tris-HCl (pH 7.4), followed by rapid filtration through a Whatman GF/C glass fiber filter (pre-soaked in 1% polyethylenimine) under reduced pressure. The filters were then quickly washed 4 more times with 2.5 ml of the buffer. Radioactivity retained on the filter was counted. Non-specific binding was defined in the presence of 10^{-7} M ET-1. Specific binding is the difference between total and non-specific binding.

2.4. *Measurement of cytosolic free calcium concentration ($[Ca^{2+}]_i$) in Swiss 3T3 fibroblast cells*

$[Ca^{2+}]_i$ was fluorometrically measured using the Ca^{2+}-sensitive fluorescent dye, fura-2. Cells were dispersed with 0.025% trypsin/1 mM EDTA. Cell suspensions were washed once with the growth medium. Cells were counted and resuspended in HEPES (20 mM)-buffered Hanks' solution (pH 7.4) to a final concentration of 1×10^6 cells/ml. The cell suspensions were incubated with 2 μM fura-2-AM at 37°C for 30 min. The fura-2-loaded cells thus obtained were resuspended in 0.3 ml of HEPES (20 mM)-buffered Hanks' solution at 1×10^6 cells/ml in a cuvette (50 × 7 mm diameter) and continuously stirred. Peptides were supplied in 3 μl of phosphate-buffered saline containing 0.1% bovine serum albumin. 50-235 in 1 μl dimethylsulfoxide was added 1 min before the addition of the peptides. Fluorescence measurements were made with a spectrofluorometer (CAF-100, Japan Spectroscopy Inc., Tokyo, Japan) as described previously [10].

2.5. *Vasoconstriction experiments*

The measurement of mechanical response was done as previously described [11]. In brief, the thoracic aorta was isolated from a male Wistar rat. Adhering fat and connective tissues were removed and transverse strips (2 mm wide and 4–5 mm long) were prepared. The endothelium was removed by gently rubbing the interior surface of the aorta. The strips were suspended in an organ bath containing modified Locke–Ringer solution (pH 7.4), which was bubbled with 95% O_2/5% CO_2 at 37°C. The composition of the solution was as follows (mM): NaCl 120, KCl, 5.6, $NaHCO_3$ 25, $CaCl_2$ 2.2, $MgCl_2$ 1.0 and glucose 5.6. The resting tension was adjusted to 1.5 g and each strip was allowed to equilibrate for 90 min, during which time the solution was replaced every 10–15 min. Isometric contractions were recorded on a polygraph system (Nihon Koden RM-6000) with a force–displacement transducer (Nihon Koden TB-611T).

The contraction induced by 30 mM KCl was determined first. After the KCl had been washed out, the strips were pre-incubated for 5 min with various concentrations of 50-235, and then were contracted with ET-1. The concentration–response curves for ET-1 obtained in the absence and presence of 50-235 were compared. Contractions induced by 30 mM KCl were taken as 100%, and relative values to these contractions were obtained.

3. RESULTS

We screened the extracts from approximately 400 kinds of plants for ET receptor ligands. The result of this effort was the discovery of myriceron caffeoyl ester (50-235, Fig. 1), a potent inhibitor of [^{125}I]ET-1 binding to rat cardiac membranes. It was isolated from the methanol extract of the bark of the bayberry *Myrica cerifera*. The structure was determined by X-ray crystallographic analysis and NMR spectroscopy (Sakurai et al., manuscript in preparation). Rat cardiac membranes had high affinity-binding sites, not only for [^{125}I]ET-1 (K_d = 31 ± 3 pM and B_{max} = 106 ± 11 fmol/mg protein), but also for [^{125}I]ET-3 (K_d = 48 ± 18 pM and B_{max} = 25 ± 4 fmol/mg protein). As shown in Fig. 2A and B, ET-1 was 60-times more potent than ET-3 in displacing [^{125}I]ET-1 binding, whereas ET-1 and ET-3 were equipotent in displacing [^{125}I]ET-3 binding. These results indicate that the [^{125}I]ET-1 and [^{125}I]ET-3 binding are the ET_A-type and ET_B-type, respectively, according to the nomenclature of Sakurai et al. [12]. 50-235 displaced [^{125}I]ET-1 binding with a K_i value of 78 ± 18 nM, but this compound at 10^{-6} M inhibited only 25% of [^{125}I]ET-3 binding (Fig. 2A and B). No action of 50-235 was noted on [^{125}I]neuropeptide Y binding to porcine hippocampal membranes [13], nor on [^{125}I]angiotensin II binding to porcine aortic smooth muscle membranes [14] (data not shown). Scatchard analysis revealed that the presence of 10^{-7} M 50-235 reduced the affinity of [^{125}I]ET-1 binding from 36 to 100 pM, with no change on the maximal density of the binding sites (Fig. 2C). This apparent competitive type of inhibition indicates that 50-235 interacts with the ET-1 receptor binding sites themselves.

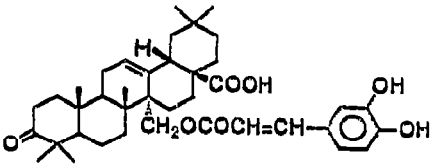

Fig. 1. Structure of myriceron caffeoyl ester (50-235).

The antagonistic effect of 50-235 for the ET receptor was further evaluated in functional assays. Swiss 3T3 fibroblasts respond to ET isopeptides, bradykinin and bombesin, with increasing $[Ca^{2+}]_i$. 50-235 inhibited the ET-1-induced increase in $[Ca^{2+}]_i$ but did not substantially affect the $[Ca^{2+}]_i$ increase induced by bradykinin or bombesin at 10^{-6} M (Fig. 3). Prolonging the pre-incubation time enhanced the inhibitory effect of 50-235 on the ET-1 response (Fig. 3A). However, when the cells were washed twice in HEPES-buffered Hanks' solution after a 30 min incubation with 10^{-8} M 50-235 the ability of ET-1 to increase $[Ca^{2+}]_i$ was partially recovered (Fig. 3B). Adding 10^{-7} M 50-235 simultaneously with ET-1 increased the EC_{50} value of ET-1 for increasing $[Ca^{2+}]_i$ (from 9 to 16 nM), as shown in Fig. 3C. The dissociation constant (K_d) of 50-235 was 130 nM, which was similar to its K_i value for inhibiting [^{125}I]ET-1 binding (78 nM), when calculated according to the equation [15]:

$$(A'/A) - 1 = B/K_d$$

where A' and A are EC_{50}'s of ET-1 in the presence and absence of 50-235, respectively, and B is the concentration of 50-235 (10^{-7} M). When the cells were challenged with 10^{-8} M ET-1 after a 1-min pre-incubation with 50-235 the IC_{50} value of 50-235 was 14 ± 1 nM (Fig. 3D). Furthermore, 50-235 inhibited the ET-1-induced $[Ca^{2+}]_i$ increase in rat aortic smooth muscle A7r5 cells, rat C6 glioma, a rat neuronal cell line RT4-D, and a human fibroblast cell line CCD19LU, but not in human Girardi heart cells (data not shown).

ET-1 induced contraction of the transverse strip prepared from rat thoracic aorta in a concentration-dependent manner. 50-235 antagonized this ET-1-induced vasoconstriction at 10^{-7} and 10^{-6} M without reducing the maximum response (Fig. 4), while it had no direct

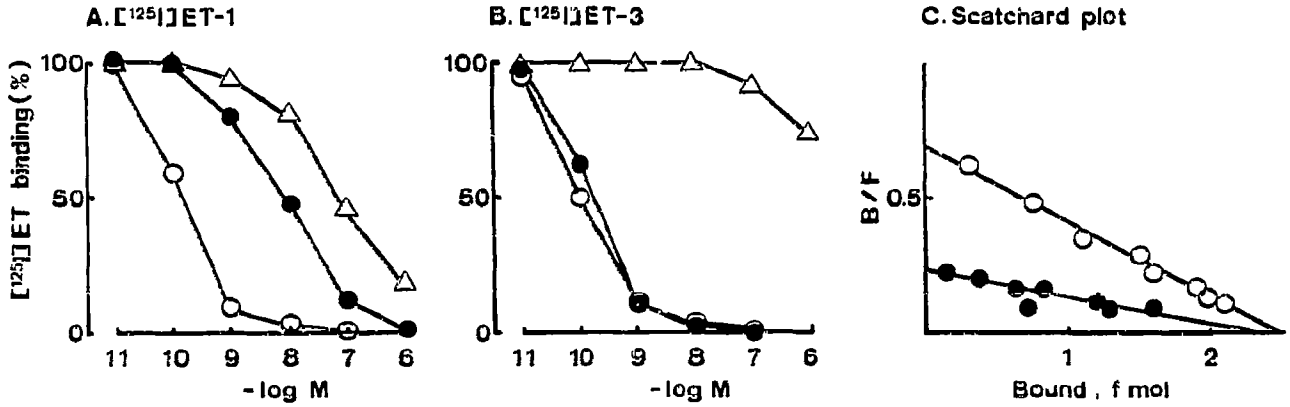

Fig. 2. (A,B) Inhibition by ET-1 (○), ET-3 (●) and 50-235 (△) of specific [125I]ET-1- (A) and [125I]ET-3 bindings (B) to rat cardiac membranes. (C) Scatchard analysis of specific [125I]ET-1 binding in the absence (○) or presence of 10^{-7} M 50-235 (●). The figure shows the results of one representative experiment which was repeated 3 times.

vasocontractile activity at 10^{-6} M. The pA_2 value of 50-235 analyzed by Schild plot was 6.65 ± 0.13. 50-235 had no effect on the contraction induced by KCl and norepinephrine (data not shown).

4. DISCUSSION

The ET isopeptides exert diverse effects on the mammalian body via different receptors [3,16,17]. Recently, complementary DNAs encoding two different types of ET receptors have been cloned and expressed [12,18]. They have been designated either as ET_A, which displays a high affinity for ET-1 and ET-2, or as ET_B, which shows the same affinity for all the ET isopeptides. 50-235 appeared to be a selective antagonist for the ET_A receptor, regardless of animal species, based on the following findings: (i) 50-235 displaced [125I]ET-1 binding, but not [125I]ET-3 binding, to rat cardiac membranes with a K_i value of 78 nM. 50-235 had no effect on [125I]neuropeptide Y and [125I]angiotensin II binding at 10^{-6} M; (ii) 50-235 inhibited the ET-induced $[Ca^{2+}]_i$ increase in Swiss 3T3 fibroblasts, which express the ET_A-receptor [19]. Furthermore, 50-235 at 10^{-6} M blocked the effect of ET isopeptides on $[Ca^{2+}]_i$ in rat aortic smooth muscle A7r5 cells, rat C6 glioma, rat neuronal cell line RT4-D, and a human fibroblast cell line, CCD19LU, but not at all in human Girardi heart cells, which express the ET_B-receptor [20] (Mihara et al., manuscript in preparation); (iii) 50-235 antagonized vasoconstriction induced by ET-1 (Fig. 4) but had no effect on that induced by KCl or norepinephrine. We are now studying the in vivo effect of 50-235.

ET-1 induces contraction of isolated arterial and venous strips, including cerebral arteries and coronary arteries, from various mammalian species [6]. In in vivo experiments intracisternal injections of ET-1 produced significant vasospasm in cats and dogs [21,22]. Furthermore, plasma and cardiac tissue concentrations of endogenous ET increased after the coronary ligation–reperfusion procedure in the infarction model of rats [23]. The development of a highly specific and sensitive radioimmunoassay for ET-1 revealed that plasma ET-1 concentrations were significantly higher in patients with certain diseases, such as acute myocardial infarction, subarachnoid haemorrhage, essential hypertension and end-stage renal failure [24–29]. Although such findings suggested that ET-1 is a candidate for critical mediators of these symptoms it is difficult to judge how much ET-1

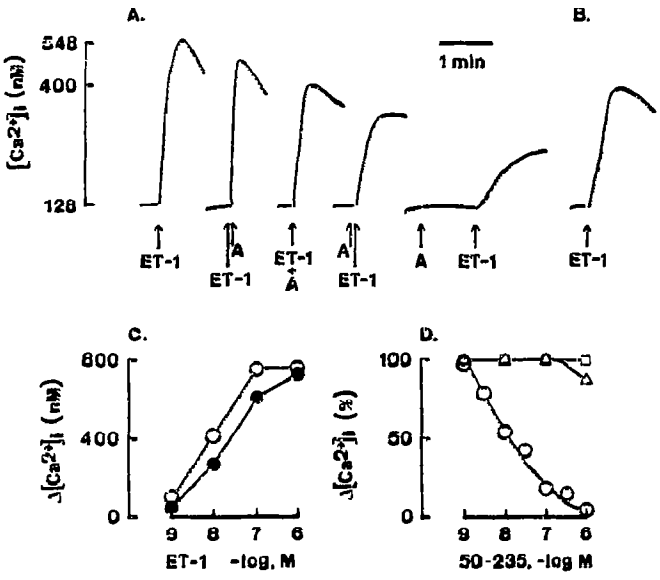

Fig. 3. Effect of 50-235 on the agonist-induced increases in $[Ca^{2+}]_i$ in mouse Swiss 3T3 fibroblast cells. (A) Time-dependent inhibition by 50-235 (10^{-7} M) of the ET-1 (10^{-8} M)-induced increases in $[Ca^{2+}]_i$. (B) Reversibility of the 50-235 effect. Cells were treated with 10^{-7} M 50-235 during the fura-2 loading (30 min) and then washed twice in HEPES-buffered Hanks' solution. (C) Concentration–response curves for ET-1 in the absence (○) or presence of 10^{-7} M 50-235 (●). 50-235 was added simultaneously with ET-1. (D) Effects of 50-235 on the $[Ca^{2+}]_i$ increase induced by ET-1 (○), bradykinin (□) and bombesin (△) (10^{-8} M each). 50-235 was given 1 min before the addition of the peptides. The figure shows the results of one representative experiment which was repeated 3 times.

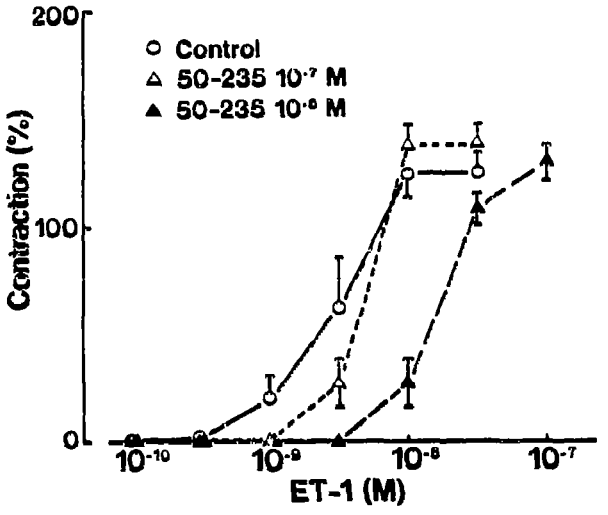

Fig. 4. Concentration–contractile–response curves to ET-1 under various concentrations of 50-235 (○, none; △, 10^{-7} M; ▲, 10^{-6} M) in isolated rat thoracic aorta. 50-235 was given 5 min before the addition of ET-1. Contractions induced by 30 mM KCl, measured before the start of the experiment, were taken as 100%. $n = 4$.

contributes to the cause of such disorders. 50-235, as well as BE-18257B and its analogs, can be used to elucidate the physiological and pathological roles of ET, and might be therapeutically useful in the treatment of diseases involving ET-1.

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