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Antagonist activity of [Thr^{18},γ-methylleucine^{19}]endothelin-1 in human endothelin receptors

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Abstract

Receptor binding and antagonist properties of endothelin-1 analogues, [Thr^{18},γ-methylleucine^{19}]endothelin-1, [Thr^{18},Leu^{19}]endothelin-1 and [Thr^{18},cyclohexylalanine^{19}]endothelin-1, were investigated using cloned human endothelin ET_A and ET_B receptors expressed in Chinese hamster ovary cells. Among them, [Thr^{18},γ-methylleucine^{19}]endothelin-1 had a high affinity for endothelin ET_A and ET_B receptors with respective K_d values of 300 and 110 pM and had no agonist activity on the stimulation of arachidonic acid release in endothelin ET_A and ET_B receptor-expressing cells. [Thr^{18},γ-methylleucine^{19}]Endothelin-1 had potent antagonist activity in endothelin-1-induced arachidonic acid release in endothelin ET_A and ET_B receptor-expressing cells with respective pA_2 values of 8.2 and 8.5. In an inositol phosphates accumulation assay, [Thr^{18},γ-methylleucine^{19}]endothelin-1 also exhibited potent antagonist activity for endothelin ET_A and ET_B receptors with respective pA_2 values of 8.0 and 8.4. In conclusion, [Thr^{18},γ-methylleucine^{19}]endothelin-1 acts as a potent and nonselective antagonist with no agonist activity for cloned human endothelin ET_A and ET_B receptors.

Keywords: Endothelin receptor antagonist; [Thr^{18},γ-methylleucine^{19}]Endothelin-1

1. Introduction

Endothelin-1, consisting of 21 amino-acid residues, is a highly potent vasoconstrictive peptide isolated from a conditioned medium of porcine aortic endothelial cells (Yanagisawa et al., 1988). Two mammalian endothelin isopeptides, endothelin-2 and endothelin-3, have been identified using a molecular cloning approach (Inoue et al., 1989). Endothelins have a wide variety of effects on both vascular and non-vascular tissues (Masaki et al., 1992). These actions are thought to be mediated by at least two receptor subtypes, endothelin ET_A and ET_B receptors (Masaki et al., 1994). The endothelin ET_A receptor has a high affinity for endothelin-1 and endothelin-2, but a lower affinity for endothelin-3 (Arai et al., 1990; Hosoda et al., 1991). On the other hand, the endothelin ET_B receptor has an equal affinity for all endothelin isopeptides (Ogawa et al., 1991; Sakurai et al., 1990). Both receptor subtypes belong to a superfamily of G-protein-coupled receptors with seven putative transmembrane domains (Arai et al., 1990; Sakurai et al., 1990). Both receptors are coupled via G-proteins to phospholipase C with the production of inositol triphosphate and an increase in intracellular Ca^{2+} (Aramori and Nakanishi, 1992). Stimulation of arachidonic acid release via phospholipase A_2 activated by both receptor subtypes has also been reported in different cell types (Aramori and Nakanishi, 1992; Resink et al., 1989).

In recent years, much progress has been made in the development of peptide endothelin agonists and antagonists with distinct receptor subtype selectivities. A cyclicpentapeptide BQ-123 (Ihara et al., 1991) and the linear tripeptides FR139317 (Aramori et al., 1993) and TTA-386 (Kitada et al., 1993) are endothelin ET_A receptor-selective antagonists. Sarafotoxin S6c (Williams et al., 1991), [Ala^{1,3,11,15}]endothelin-1 (Saeki et al., 1991), BQ-3020 (Ihara et al., 1992), and IRL 1620 (Takai et al., 1992) are endothelin ET_B receptor-selective agonists and IRL 1038 (Urade et al., 1992), RES-701-1 (Tanaka et al., 1994), and BQ-788 (Ishikawa et al., 1994) are endothelin ET_B receptor-selective antagonists. These endothelin receptor subtype-specific agonists and antagonists support the receptor subtype classification (Masaki et al., 1994).

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Furthermore, more pharmacological evidence is accumulating to suggest the existence of additional endothelin receptor subtypes. It has been proposed that the endothelin ET_B receptor can be subdivided into two pharmacologically distinct subtypes, named endothelin ET_{B1} and ET_{B2} (Douglas et al., 1994; Karaki et al., 1994). The endothelin ET_{B1} receptor, located on vascular endothelium, mediates vasorelaxation through the release of nitric oxide and is sensitive to RES-701-1 and BQ-788. On the other hand, the endothelin ET_{B2} receptor is found on smooth muscle and mediates vasoconstriction; this subtype is sensitive to BQ-788, but insensitive to RES 701-1. It has been proposed that the endothelin ET_A receptor can also be subclassified into BQ-123-sensitive ET_{A1} and BQ-123-insensitive ET_{A2} (Sudjarwo et al., 1994).

Previously, we reported the structure-activity relationship of endothelin-1 analogues substituted at positions 18 and 19 (Kikuchi et al., 1993). We found that [Thr^{18},γ-methylleucine^{19}]endothelin-1 is an antagonist of the endothelin-1-induced vasoconstriction of porcine coronary artery, of the sarafotoxin S6c-induced vasoconstriction of rabbit pulmonary artery (Kikuchi et al., 1993), and of the [$Ala^{1,3,11,15}$]endothelin-1-induced vasoconstriction of porcine cardiac vein (Shimamoto et al., 1993). Vasoconstriction stimulated by endothelins is now believed to be mediated not only by endothelin ET_A, but also by endothelin ET_B receptors. The proportion of the endothelin receptor subtypes differs in each blood vessel. This sometimes makes it difficult to determine the potencies of the antagonists for the individual receptors. Thus far, no precise data on pharmacological profiles of [Thr^{18},γ-methylleucine^{19}]endothelin-1 for a single receptor subtype have been provided. Functional and separate expression of the cDNAs for the endothelin ET_A and ET_B receptors is helpful for the study of the precise pharmacological profile of the endothelin antagonist for each receptor (Masuda et al., 1996). As such, we investigated the receptor binding and antagonist properties of [Thr^{18},γ-methylleucine^{19}]endothelin-1, using cloned human endothelin ET_A and ET_B receptors expressed separately in Chinese hamster ovary (CHO) cells, to extend the pharmacological characterization of [Thr^{18},γ-methylleucine^{19}]endothelin-1 for the individual receptor subtype. The present study describes the pharmacological characterization of [Thr^{18},γ-methylleucine^{19}]endothelin-1 as a highly potent antagonist for both cloned human endothelin ET_A and ET_B receptors.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium was obtained from Nissui (Tokyo, Japan). [^{125}I]Endothelin-1 (2000 Ci/mmol) and *myo*-[2-^{3}H]inositol (19 Ci/mmol) were obtained from Amersham (Arlington Heights, IL, USA). [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (100 Ci/mmol) was obtained from New England Nuclear (Boston, MA, USA). [Thr^{18},γ-methylleucine^{19}]Endothelin-1, [Thr^{18},Leu^{19}]endothelin-1 and [Thr^{18},cyclohexylalanine^{19}]endothelin-1 were synthesized as previously described (Kikuchi et al., 1993). Endothelin-1, E-64, leupeptin and pepstatin A were purchased from the Peptide Institute (Osaka, Japan). Phenylmethylsulfonyl fluoride was purchased from Wako (Osaka, Japan), and 3-[(cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) was purchased from Dojindo Laboratories (Kumamoto, Japan).

2.2. Receptor binding experiments

Establishment of the CHO cell lines with stable expression of cloned human endothelin ET_A or ET_B receptors, membrane preparation of the receptor-expressing cells, and receptor binding experiments were performed as previously described (Masuda et al., 1996). Membranes (10 μg protein/ml for the endothelin ET_A receptor, 20 μg protein/ml for the endothelin ET_B receptor) suspended in a binding assay buffer (20 mM Tris, 5 mM $(CH_3COO)_2Mg$, 2 mM EGTA, 0.1% bovine serum albumin, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 20 μg/ml leupeptin and 4 μg/ml E-64, pH 7.4) were incubated with 100 pM of [^{125}I]endothelin-1 and various concentrations of unlabeled peptide at 25°C for 60 min. The reaction was terminated by the addition of 1.5 ml of ice-cold binding buffer supplemented with 0.05% of CHAPS followed by rapid filtration through a Whatman GF/F glass fiber filter. The filter was then washed with 1.5 ml of ice-cold binding buffer. Radioactivity trapped on the filter was measured using a gamma-ray counter. The nonspecific binding was determined in the presence of 0.3 μM of unlabeled endothelin-1. The K_d values were calculated by computer analysis (LIGAND; Munson and Rodbard, 1980).

2.3. Measurement of arachidonic acid release

The release of [^{3}H]arachidonic acid was measured as previously described (Masuda et al., 1996). The receptor-expressing cells, seeded in 24-well plates at a density of 5×10^4 cells/well, were cultured for 1 day. The cells were then labeled with [^{3}H]arachidonic acid (0.25 μCi/well) for 1 day. The cells were washed twice with Dulbecco's modified Eagle's medium supplemented with 20 mM Hepes and 0.2% bovine serum albumin (pH 7.4) and were incubated in this medium for 30 min at 37°C. After preincubation with fresh medium for 5 min at 37°C, the cells were treated with the peptides for 30 min at 37°C. Radioactivity in the conditioned medium was measured using a liquid scintillation counter. The concentration for half-maximal response (EC_{50}) was calculated from a concentration-response curve. The response was expressed as

a percentage of the maximal response elicited by 100 nM endothelin-1. The pA_2 values were determined by Schild plot analysis (Arunlakshana and Schild, 1959). The results are expressed as the mean ± S.E.M.

2.4. Measurement of inositol phosphates accumulation

Accumulation of total inositol phosphates (inositol mono-, bis- and triphosphates) was measured according to a previously described method (Masuda et al., 1996). The receptor-expressing cells seeded in 24-well plates at a density of 5×10^4 cells/well were cultured for 1 day. The cells were then labeled with *myo*-[2-^{3}H]inositol (2.5 μCi/well) for 1 day. The cells were washed twice with assay medium (140 mM NaCl, 4 mM KCl, 1 mM Na_2HPO_4, 1 mM $MgCl_2$, 1.25 mM $CaCl_2$, 10 mM LiCl, 10 mM glucose, 0.2% bovine serum albumin, and 20 mM Hepes, pH 7.4) and were incubated for 30 min at 37°C. After preincubation with fresh medium for 5 min at 37°C, the cells were treated with the peptides for 15 min at 37°C. The reaction was terminated by the addition of 10% perchloric acid. The reaction mixture was neutralized with 60 mM Hepes solution containing 1.5 M KOH and was centrifuged for 15 min at 4°C. Separation of ^{3}H-labeled inositol phosphates in the resulting supernatant was performed as previously described (Masuda et al., 1996). The pA_2 values were determined as described above. The results are expressed as the mean ± S.E.M.

3. Results

3.1. Effects of the endothelin-1 analogues on [^{125}I]endothelin-1 binding to the membranes prepared from human endothelin ET_A and ET_B receptor-expressing cells

Competitive binding experiments indicated that [Thr^{18},γ-methylleucine^{19}]endothelin-1, [Thr^{18},Leu^{19}]endothelin-1, and [Thr^{18},cyclohexylalanine^{19}]endothelin-1 potently inhibited binding of [^{125}I]endothelin-1 to the membranes prepared from endothelin ET_A and ET_B receptor-expressing cells in a concentration-dependent manner (Fig. 1A and B). The K_d values and Hill coefficients of endothelin-1, [Thr^{18},γ-methylleucine^{19}]endothelin-1, [Thr^{18},Leu^{19}]endothelin-1 and [Thr^{18},cyclohexylalanine^{19}]endothelin-1 are listed in Table 1. The K_d values of the analogues for the two receptor subtypes were only 2–5-fold larger than that of endothelin-1. The results demonstrated that all of the analogues maintained a high affinity for human endothelin ET_A and ET_B receptors and exhibited no receptor subtype selectivity. The rank order of binding affinity of the analogues for human endothelin ET_A and ET_B receptors was [Thr^{18},Leu^{19}]endothelin-1 > [Thr^{18},γ-methylleucine^{19}]endothelin-1 > [Thr^{18},cyclohexylalanine^{19}]endothelin-1.

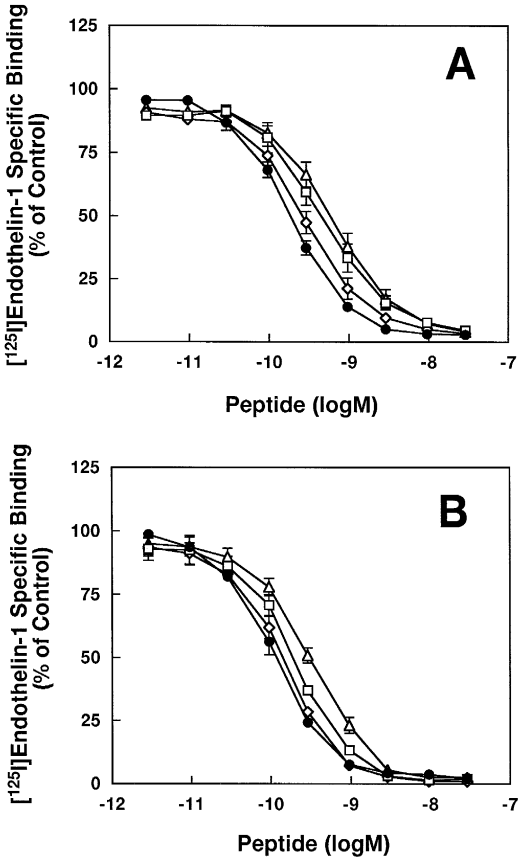

Fig. 1. Competitive binding experiments using membranes prepared from human endothelin ET_A and ET_B receptor-expressing cells. Specific binding of 100 pM [^{125}I]endothelin-1 to endothelin ET_A (A) and to ET_B (B) receptors in the membranes were competitively inhibited by various concentrations of unlabeled endothelin-1 (●), [Thr^{18},γ-methylleucine^{19}]endothelin-1 (□), [Thr^{18},Leu^{19}]endothelin-1 (◇) and [Thr^{18},cyclohexylalanine^{19}]endothelin-1 (△). The results are expressed as a percentage of control specific binding. Each point represents the mean ± S.E.M. (n = 3–5).

Table 1
The K_d values and Hill coefficients (n_H) of the endothelin-1 analogues for human endothelin ET_A and ET_B receptors

Ligand	ET_A		ET_B	
	K_d	n_H	K_d	n_H
Endothelin-1	76 ± 11	1.1 ± 0.02	44 ± 3	1.1 ± 0.03
[Thr^{18},γ-methylleucine^{19}]Endothelin-1	300 ± 74	0.94 ± 0.08	110 ± 11	1.1 ± 0.02
[Thr^{18},Leu^{19}]Endothelin-1	160 ± 37	0.95 ± 0.02	62 ± 3	1.1 ± 0.05
[Thr^{18},cyclohexylalanine^{19}]Endothelin-1	370 ± 100	0.93 ± 0.03	190 ± 34	1.1 ± 0.08

The values are represented as mean values ± S.E.M. (n = 3–4).

3.2. Effects of the endothelin-1 analogues on arachidonic acid release in human endothelin ET_A and ET_B receptor-expressing cells

The effects of endothelin-1 analogues on arachidonic acid release in endothelin ET_A and ET_B receptor-expressing cells were investigated to determine whether the endothelin-1 analogues had agonist activity for human endothelin ET_A and ET_B receptors. Endothelin-1 stimulated arachidonic acid release in human endothelin ET_A and ET_B receptor-expressing cells with respective pD_2 values of 9.4 and 9.6 (Fig. 2A and B; Masuda et al., 1996). None of the analogues stimulated arachidonic acid release in endothelin ET_A receptor-expressing cells up to a concentration of 1 μM, indicating that all of the analogues lost agonist activity for human endothelin ET_A receptor (Fig. 2A). On the other hand, [Thr[18],Leu[19]]endothelin-1 stimulated arachidonic acid release in endothelin ET_B receptor-expressing cells with a pD_2 value of 8.9 ± 0.05 ($n = 3$); however, the maximal release was 40% of the maximal release induced by endothelin-1 (Fig. 2B). In contrast, neither [Thr[18],γ-methylleucine[19]]ET-1 nor [Thr[18],cyclohexylalanine[19]]ET-1 stimulated arachidonic acid release in endothelin ET_B receptor-expressing cells up to a concentration of 1 μM (Fig. 2B). These results indicated that [Thr[18],γ-methylleucine[19]]endothelin-1 and [Thr[18],cyclohexylalanine[19]]endothelin-1 were devoid of agonist activity for both human endothelin ET_A and ET_B receptors and that [Thr[18],Leu[19]]endothelin-1 had no agonist activity for human endothelin ET_A receptor but exhibited partial agonist activity for human endothelin ET_B receptor. [Thr[18],γ-methylleucine[19]]Endothelin-1 has the desirable feature of a peptide antagonist of possessing a higher affinity and no agonist activity for both receptor subtypes.

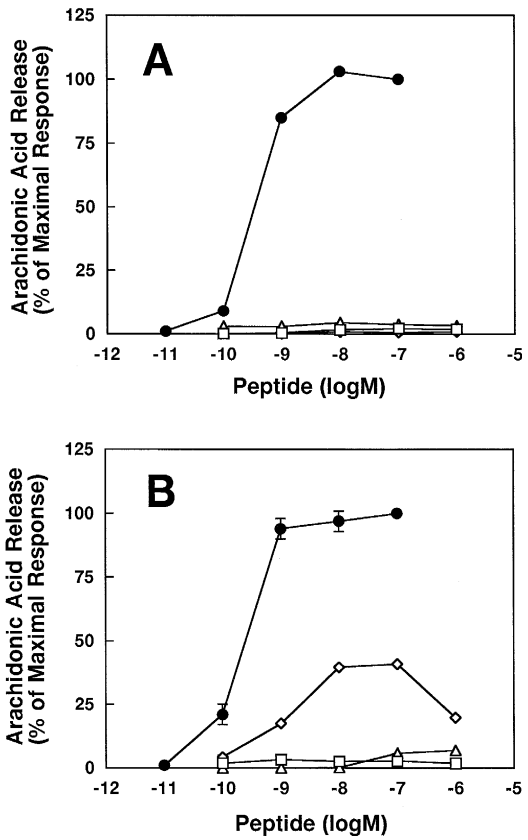

Fig. 2. Effects of endothelin-1 and its analogues on arachidonic acid release in human endothelin ET_A and ET_B receptor-expressing cells. The endothelin ET_A (A) or the ET_B (B) receptor-expressing cells labeled with [^{3}H]arachidonic acid were challenged with indicated concentrations of endothelin-1 (●), [Thr[18],γ-methylleucine[19]]endothelin-1 (□), [Thr[18],Leu[19]]endothelin-1 (◇), and [Thr[18],cyclohexylalanine[19]]endothelin-1 (△). After incubation for 30 min at 37°C, radioactivity in the conditioned medium was measured. The results are expressed as percentage of maximal release with 100 nM endothelin-1. Each point represents the mean ± S.E.M. ($n = 5$).

3.3. Effects of the endothelin-1 analogues on phosphatidyl inositol hydrolysis in human ET_A and ET_B receptor-expressing cells

Endothelin-1 induced inositol phosphates accumulation in a concentration-dependent manner in human endothelin ET_A and ET_B receptor-expressing cells with pD_2 values of 9.2 (ET_A) and 9.3 (ET_B; Masuda et al., 1996). To ascertain whether the endothelin-1 analogues had agonist activity for human endothelin ET_A and ET_B receptors, the effects of the analogues on accumulation of total inositol phosphates were also investigated. None of the analogues stimulated accumulation of total inositol phosphates in endothelin ET_A receptor-expressing cells at a concentration of 1 μM (data not shown). In the endothelin ET_B receptor-expressing cells, [Thr[18],Leu[19]]endothelin-1 stimulated accumulation of total inositol phosphates in a concentration-dependent manner with a pD_2 value of 9.1 ± 0.2 ($n = 6$, Fig. 3). The maximal response with [Thr[18],Leu[19]]endothelin-1 was 10% of the maximal response induced by endothelin-1. On the other hand, [Thr[18],γ-methylleucine[19]]endothelin-1 and [Thr[18],cyclohexylalanine[19]]endothelin-1 had no effects even at 1 μM (Fig. 3). The results verified that from the arachidonic acid release assay.

3.4. Effects of the endothelin-1 analogues on endothelin-1-induced arachidonic acid release in human endothelin ET_A and ET_B receptor-expressing cells

The effects of the endothelin-1 analogues on endothelin-1-induced arachidonic acid release in the endothelin ET_A and ET_B receptor-expressing cells were examined to assess the antagonist activity for human endothelin ET_A and ET_B receptors. Fig. 4A shows that [Thr[18],γ-methylleucine[19]]endothelin-1 shifted the concentration-response curve to the right and did not alter the maximal response elicited by endothelin-1 in endothelin ET_A receptor-expressing cells, indicating competitive antagonism for human endothelin ET_A receptor. The pA_2 value of [Thr[18],γ-

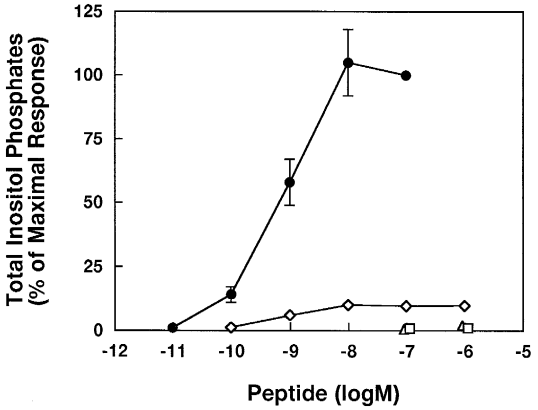

Fig. 3. Effects of endothelin-1 and its analogues on inositol phosphates accumulation in human endothelin ET_B receptor-expressing cells. The ET_B receptor-expressing cells labeled with [^{3}H]inositol were challenged with indicated concentrations of endothelin-1 (●), [Thr^{18},γ-methylleucine^{19}]endothelin-1 (□), [Thr^{18},Leu^{19}]endothelin-1 (◇), and [Thr^{18},cyclohexylalanine^{19}]endothelin-1 (△). After incubation for 15 min at 37°C, total inositol phosphates were extracted with perchloric acid, separated using anion exchange column, and then radioactivity was measured. The results are expressed as percentage of maximal accumulation with 100 nM endothelin-1. Each point represents the mean ± S.E.M. (n = 3–6).

Table 2
The pA_2 values of the endothelin-1 analogues in inhibiting endothelin-1-induced arachidonic acid release in human endothelin ET_A and ET_B receptor-expressing cells

Ligand	ET_A	ET_B
[Thr^{18},γ-methylleucine^{19}]Endothelin-1	8.2 ± 0.04	8.5 ± 0.08
[Thr^{18},Leu^{19}]Endothelin-1	8.3 ± 0.04	8.0 ± 0.01
[Thr^{18},cyclohexylalanine^{19}]Endothelin-1	7.9 ± 0.02	8.1 ± 0.08

The pA_2 values are represented as mean values ± S.E.M. (n = 3–5).

methylleucine^{19}]endothelin-1 was 8.2 ± 0.04 and the slope of the Schild plot was 1.2 ± 0.05 (n = 5; Fig. 4C). In endothelin ET_B receptor-expressing cells, [Thr^{18},γ-methylleucine^{19}]endothelin-1 also potently inhibited endothelin-1-induced arachidonic acid release without changing the maximal response with a pA_2 value of 8.5 ± 0.08 (slope of the Schild plot = 1.2 ± 0.07, n = 5; Fig. 4B and D). The results demonstrated that [Thr^{18},γ-methylleucine^{19}]endothelin-1 is a potent and competitive antagonist for both human endothelin ET_A and ET_B receptors. The pA_2 values of [Thr^{18},cyclohexylalanine^{19}]endothelin-1 and [Thr^{18},Leu^{19}]endothelin-1 are listed in Table 2.

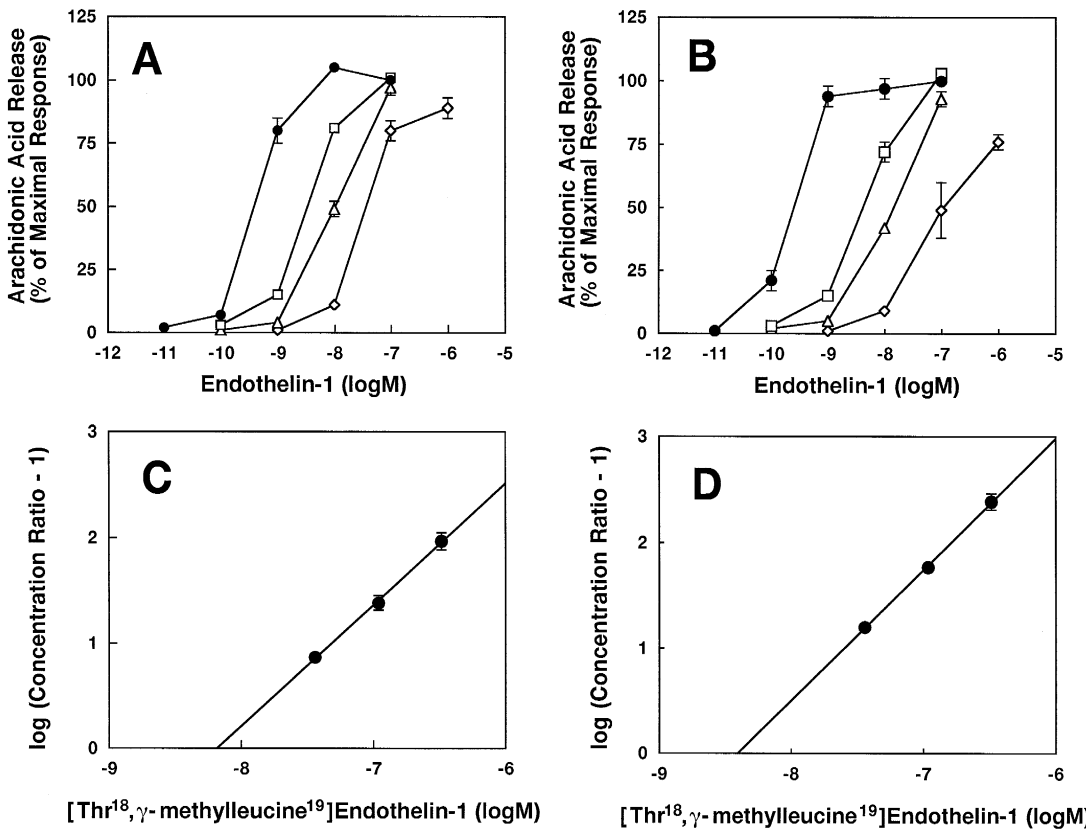

Fig. 4. Effects of [Thr^{18},γ-methylleucine^{19}]endothelin-1 on endothelin-1-induced arachidonic acid release in human endothelin ET_A and ET_B receptor-expressing cells. The ET_A (A) or the ET_B (B) receptor-expressing cells labeled with [^{3}H]arachidonic acid were challenged with increasing concentrations of endothelin-1 in the absence (●) or presence of [Thr^{18},γ-methylleucine^{19}]endothelin-1 (□, 36 nM; △, 110 nM; ◇, 330 nM). After incubation for 30 min at 37°C, radioactivity in the conditioned medium was measured. The results are expressed as percentage of maximal release with 100 nM endothelin-1. Schild plot for inhibition of endothelin-1-induced arachidonic acid release by [Thr^{18},γ-methylleucine^{19}]endothelin-1 in endothelin ET_A (C) or ET_B (D) receptor-expressing cells. Each point represents the mean ± S.E.M. (n = 3).

3.5. Effects of the endothelin-1 analogues on endothelin-1-induced phosphatidylinositol hydrolysis in human endothelin ET_A and ET_B receptor-expressing cells

Antagonist activities of the analogues were assessed by their ability to inhibit endothelin-1-induced inositol phosphates accumulation in endothelin ET_A and ET_B receptor-expressing cells. Fig. 5A shows that [Thr[18],γ-methylleucine[19]]endothelin-1 shifted the concentration-response curve to the right and did not change the maximal response of total inositol phosphates accumulation to endothelin-1 in endothelin ET_A receptor-expressing cells, indicating competitive antagonism. The pA_2 value of [Thr[18],γ-methylleucine[19]]endothelin-1 was 8.0 ± 0.02 (slope of the Schild plot $= 1.2 \pm 0.06$, $n = 5$; Fig. 5C). Similarly, antagonist activity for human endothelin ET_B receptor was examined in endothelin ET_B receptor-expressing cells. [Thr[18],γ-methylleucine[19]]Endothelin-1 also inhibited endothelin-1-induced inositol phosphates accumulation in a competitive manner with a pA_2 value of 8.4 ± 0.12 (slope of the Schild plot $= 1.1 \pm 0.06$, $n = 4$; Fig. 5B and D). The results confirmed that [Thr[18],γ-methylleucine[19]]endothelin-1 is a potent and competitive antagonist for both human endothelin ET_A and ET_B receptors. The pA_2 values of [Thr[18],cyclohexylalanine[19]]endothelin-1 and [Thr[18],Leu[19]]endothelin-1 are listed in Table 3.

Table 3
The pA_2 values of the endothelin-1 analogues in inhibiting endothelin-1-induced inositol phosphates accumulation in human endothelin ET_A and ET_B receptor-expressing cells

Ligand	ET_A	ET_B
[Thr[18],γ-methylleucine[19]]Endothelin-1	8.0 ± 0.02	8.4 ± 0.12
[Thr[18],Leu[19]]Endothelin-1	8.0 ± 0.04	8.4 ± 0.03
[Thr[18],cyclohexylalanine[19]]Endothelin-1	7.8 ± 0.13	8.1 ± 0.07

The pA_2 values are represented as mean values ± S.E.M. ($n = 3$–5).

4. Discussion

Several isolated tissues contain both endothelin ET_A and ET_B receptors in different proportions. Accurate determination of antagonist potencies for individual receptors has

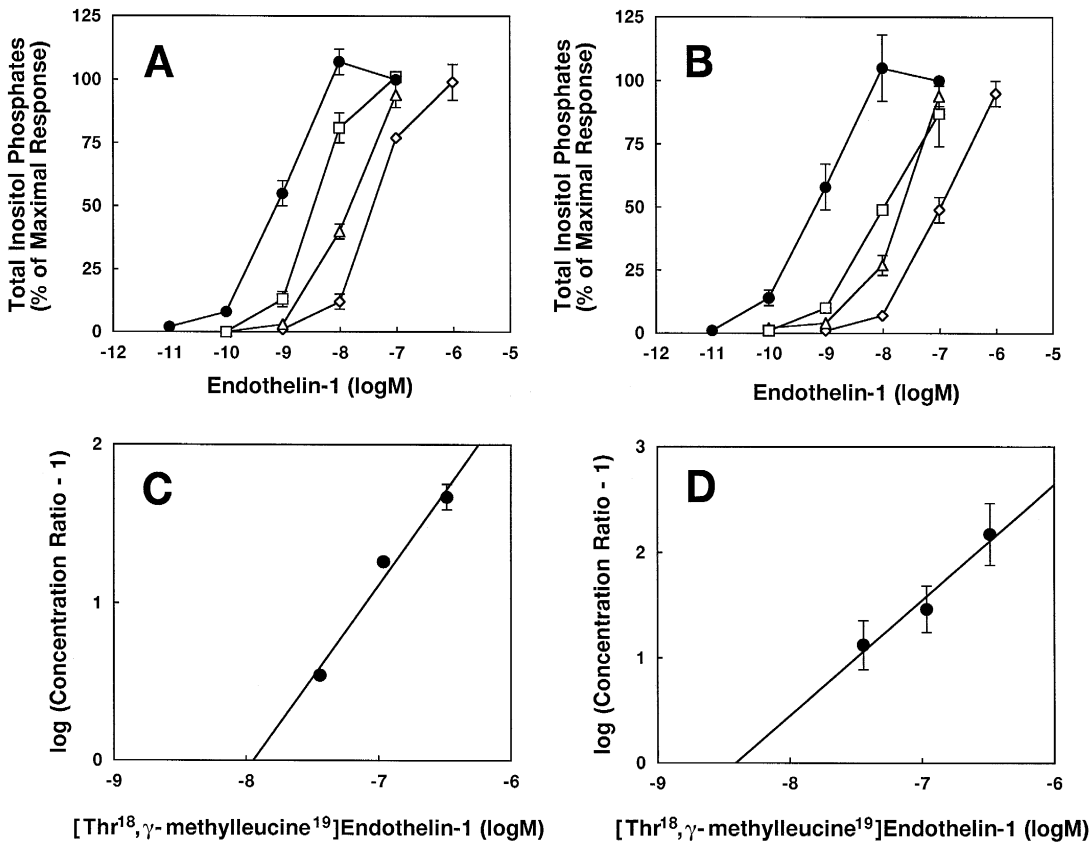

Fig. 5. Effects of [Thr[18],γ-methylleucine[19]]endothelin-1 on endothelin-1-induced inositol phosphates accumulation in human endothelin ET_A and ET_B receptor-expressing cells. The ET_A (A) or the ET_B (B) receptor-expressing cells labeled with [^{3}H]inositol were challenged with increasing concentrations of endothelin-1 in the absence (●) and presence of [Thr[18],γ-methylleucine[19]]endothelin-1 (□, 36 nM; △, 110 nM; ◇, 330 nM). After incubation for 15 min at 37°C, total inositol phosphates were extracted with perchloric acid, separated using anion exchange column, and then radioactivity was measured. The results are expressed as percentage of maximal accumulation with 100 nM endothelin-1. Schild plot for inhibition of endothelin-1-induced inositol phosphates accumulation by [Thr[18],γ-methylleucine[19]]endothelin-1 in endothelin ET_A (C) or ET_B (D) receptor-expressing cells. Each point represents the mean ± S.E.M. ($n = 5$).

been hampered by the coexistence of multiple receptor subtypes. Functional expression of the cDNA for the individual receptor subtypes in the same cell line resolves these difficulties. In the present study, we evaluated the binding affinity and antagonist activity of the three endothelin-1 analogues substituted at positions 18 and 19 on cloned human endothelin ET_A and ET_B receptors stably expressed in CHO cells. Receptor binding experiments with membranes prepared from the receptor-expressing cells demonstrated that the three endothelin-1 analogues had a high affinity for the cloned human endothelin ET_A and ET_B receptors. The IC_{50} values of the three analogues for human endothelin ET_A receptor and for endothelin ET_B receptor (data not shown) were comparable to those of porcine cardiac ventricular endothelin ET_A and to those of bovine cerebral endothelin ET_B receptors, respectively (Kikuchi et al., 1993).

To determine whether the endothelin-1 analogues have agonist activity for human endothelin ET_A and ET_B receptors, the effects of the analogues on arachidonic acid release and inositol phosphates accumulation were examined in cells expressing a single endothelin receptor subtype. Among the analogues, [Thr^{18},γ-$methylleucine^{19}$]endothelin-1 has the desirable antagonist profile of lacking agonist activity in the two functional assay for either receptor subtype. However, [Thr^{18},Leu^{19}]endothelin-1 exhibited a significant partial agonist activity for human endothelin ET_B receptor and no agonist activity for human endothelin ET_A receptor.

The results were consistent with those obtained in other animal species. It was shown that [Thr^{18},γ-$methylleucine^{19}$]endothelin-1 is devoid of agonist activity for the endothelin ET_A receptor in rat aortic smooth muscle A10 cells, and for the endothelin ET_B receptor in mouse peritoneal macrophage (Kikuchi et al., 1993; Shimamoto et al., 1993). [Thr^{18},Leu^{19}]Endothelin-1 has a partial agonist activity for the endothelin ET_B receptors in mouse peritoneal macrophage and rabbit pulmonary artery (Kikuchi et al., 1993). The results indicate that combined substitution with Thr^{18} and γ- $methylleucine^{19}$ is essential to abolish the agonist activity irrespective of species differences.

It may be possible to deduce the underlying mechanism of the disappearance of agonist activity from a previous study on the structure-activity relationship of endothelin analogues. Single substitution of Asp^{18} with Thr did not significantly affect the binding activity and agonist activity (Kikuchi et al., 1993). Further substitution, however, of Ile^{19} with a more bulky aliphatic amino acid resulted in the loss of agonist activity. For the ET_A receptor, the loss of agonist activity was observed with [Thr^{18},Leu^{19}], [Thr^{18},$cyclohexylalanine^{19}$], and [Thr^{18},γ-$methylleucine^{19}$]endothelin-1 (Kikuchi et al., 1993). For the ET_B receptor, complete inhibition of the agonist activity was found only with [Thr^{18},γ-$methylleucine^{19}$]endothelin-1 (Kikuchi et al., 1993). The substitution with Ile^{19} with a more bulky residue presumably causes steric hindrance that changes fitting of bound ligand and results in no agonistic interaction. The ET_B receptor apparently has a larger binding site than the ET_A receptor, which permits a more bulky residue at position 19 as an agonist. Studies elucidating the interaction between Asp^{18} and Ile^{19} of endothelin-1 and of specific residues of the endothelin receptors would contribute to an understanding of the molecular mechanism of the receptor activation. The endothelin-1 analogues reported here should prove useful in studying the structural requirement for endothelin-1 binding and the mechanisms of activation of endothelin receptors.

[Thr^{18},γ-$methylleucine^{19}$]Endothelin-1 had a potent antagonist activity for human endothelin ET_A and ET_B receptors. [Thr^{18},γ-$methylleucine^{19}$]Endothelin-1 inhibited endothelin-1-induced arachidonic acid release in human endothelin ET_A and ET_B receptor-expressing cells with pA_2 values of 8.2 (ET_A) and 8.5 (ET_B). Similar pA_2 values were obtained with a functional assay of inositol phosphates accumulation. [Thr^{18},γ-$methylleucine^{19}$]Endothelin-1 inhibited phosphatidylinositol hydrolysis elicited by endothelin-1 in endothelin ET_A and ET_B receptor-expressing cells with pA_2 values of 8.0 (ET_A) and 8.4 (ET_B). Therefore, [Thr^{18},γ-$methylleucine^{19}$]endothelin-1 is classified as a potent, subtype-nonselective antagonist.

The pA_2 value of [Thr^{18},γ-$methylleucine^{19}$]endothelin-1 for human endothelin ET_B receptor is consistent with that obtained in sarafotoxin S6c-induced contraction of rabbit pulmonary artery ($pA_2 = 8.4$; Kikuchi et al., 1993), which is believed to be mediated by the endothelin ET_B receptor. The pA_2 values is less, however, than that obtained in the porcine endothelin ET_B receptor of [$Ala^{1,3,11,15}$]endothelin-1-induced contraction of coronary vein ($pA_2 = 9.2$; Shimamoto et al., 1993). On the other hand, the pA_2 value for human endothelin ET_A receptor is larger than that obtained in the porcine endothelin ET_A receptor of endothelin-1-induced contraction of the coronary artery ($pA_2 = 7.4$; Kikuchi et al., 1993). Karaki et al. (1995) recently reported that [Thr^{18},γ-$methylleucine^{19}$]endothelin-1 inhibited contraction of the rabbit saphenous vein mediated by all the types of pharmacologically distinct endothelin receptors, ET_{A1}, ET_{A2}, ET_{B1}, and ET_{B2}. Therefore, [Thr^{18},γ-$methylleucine^{19}$]endothelin-1 is an antagonist that is nonselective for both subtypes of the cloned receptor, and also has antagonist properties for all the pharmacological subtypes of the endothelin receptor. Moreover, [Thr^{18},γ-$methylleucine^{19}$]endothelin-1 inhibited the transient depressor and sustained pressor response caused by exogenous endothelin-1 in rats (Kubo et al., unpublished result). An antagonist with such a profile may be useful in investigating the involvement of endothelin receptors. In conclusion, the present study indicated that [Thr^{18},γ-$methylleucine^{19}$]endothelin-1 has a high affinity and potent antagonist activity, but no agonist activity, for both human endothelin ET_A and ET_B receptors.

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