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[Ala^{1,3,11,15}]ENDOTHELIN-1 ANALOGS WITH ET_B AGONISTIC ACTIVITY

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SUMMARY: A linear peptide analog of endothelin (ET)-1, [Ala^{1,3,11,15}]ET-1 (4AlaET-1), and its truncated peptide analogs were synthesized to study the structural requirements of ET-1 for the recognition of ETs-nonselective ET_B receptors. ET-1 exhibited sub-nanomolar binding to two distinct ET receptor subtypes (ET_A and ET_B), but 4AlaET-1 bound to ET_B with an affinity 1,700 times higher than that seen during binding to ET_A. The truncated linear peptides 4AlaET-1(6-21), 4AlaET-1(8-21) and N-acetyl-4AlaET-1(10-21) still had high affinity for ET_B, whereas 4AlaET-1(6-20) and 4AlaET-1(11-21) displayed remarkably reduced affinity for ET_B. Therefore, ET-1 requires the Glu^{10}-Trp^{21} sequence for ET_B binding, but not the disulfide bridges. These ET_B-binding peptides elicit endothelium-dependent vasorelaxation of porcine pulmonary arteries in parallel with the binding affinity for ET_B, suggesting that they are ET_B agonists.

Endothelin (ET)-1, a potent vasoconstrictor isolated from cultured porcine aortic endothelial cells, consists of 21 amino acid residues with two disulfide bridges (1). Subsequent studies have revealed the presence of two other related peptides, ET-2 and ET-3, in mammalian species (2,3). Another class of structurally-related peptides, the sarafotoxins (SRTX) S6 consisting of at least three isopeptides, SRTX-S6a, S6b and S6c, were isolated from the venom of the Israeli snake *Atractaspis eingadensis* (4). These isopeptides of the ET/SRTX family produce numerous biological responses, such as constriction of various smooth muscles, vasodilation, and pressor and depressor actions, *via* multiple ET receptor subtypes that are widely distributed in many tissues (5). Recently, two ET receptor subtypes selective to ET-1 (ET_A) and nonselective to ET/SRTX isopeptides (ET_B) have been cloned from the cDNA library (6,7). The structure-activity relationships of ET-1 have previously been investigated by determining the ET_A-mediated vasoconstrictive activity of synthetic ET-1 analogs (8). However, there has been little research on the structural requirements for ET_B receptor binding and function. It was reported that a linear ET-1 analog, [Ala^{1,3,11,15}]ET-1 (4AlaET-1), in which four cysteine residues of ET-1 are replaced by alanines, was as potent as ET-1 and ET-3 in inhibiting [^{125}I]ET-1 binding to rat cerebellar homogenate (9). It has also been shown that the cerebellar membrane is rich in ET_B (10), thereby suggesting that 4AlaET-

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1 may have a high affinity for ET_B. We report here the structural requirements of ET-1 for ET_B-selective binding and agonistic activity using 4AlaET-1 and its truncated peptide analogs.

MATERIALS AND METHODS

Materials: ET-1, ET-2, ET-3 and SRTX-S6b were purchased from Peptide Institute Inc. (Osaka, Japan). SRTX-S6a, SRTX-S6c and ET C-terminal hexapeptide (ET-1(16-21)) were purchased from Peninsula Laboratories, Inc. (California, U.S.A.). [^{125}I]ET-1 was obtained from Amersham Japan.

Synthesis of 4AlaET-1 analogs: Fully-protected peptide-resins for the 4AlaET-1 analogs were synthesized using HMP resin (Applied Biosystems, California, U.S.A.) and Fmoc-amino acids (Kokusan Chemical Works, Tokyo, Japan) in an peptide synthesizer (Applied Biosystems Model 431A). For the synthesis of [N-acetylAla^1]4AlaET-1 and N-acetyl-4AlaET-1(10-21), Fmoc-deprotected and other side chain-protected peptide-resins were obtained with the peptide synthesizer and acetylated with acetic anhydride in pyridine for 1 hour at 25°C. To ensure complete acetylation, the acetylated peptides were checked by the ninhydrin test (11). These protected peptide-resins were all deprotected and removed from the resin by the trifluoracetic acid cleavage method, and were purified by reverse-phase preparative high-pressure liquid chromatography (HPLC). The purity and molecular mass of the synthesized peptides were checked by analytical HPLC and fast atom bombardment mass spectrometry (FAB-MS), respectively.

Binding experiments in membranes: Porcine aortic smooth muscle and cerebellar membranes were prepared as previously described (12). The membranes were incubated at 25°C with 10pM [^{125}I]ET-1 in the presence of increasing concentrations of the peptides in 50mM Tris-HCl buffer (pH 7.4) containing 0.1mM phenylmethylsulfonyl fluoride, 1μM pepstatin, 2μM leupeptin, 1mM 1,10-phenanthroline, 1mM EDTA, 10μM $CaCl_2$, 10μM $MgCl_2$, and 0.1% BSA. After 4 hours of incubation, 2ml of cold 5mM Hepes/Tris buffer (pH 7.4) containing 0.3% BSA (Buffer A) was added to the incubation mixture, followed by a rapid filtration through GF/C glass fiber filters (Whatman, England). After washing with Buffer A, the radioactivity remaining on the filters was determined with a gamma counter (PACKARD: COBRA 5002). Nonspecific binding was defined by adding 200nM ET-1 to the assay mixture.

Vasorelaxation study: Intrapulmonary arteries (approx. 1mm o.d.) were isolated from fresh porcine lungs. The connective tissues and adherent fats were removed, and the cleaned arteries with intact endothelium were cut into strips about 10mm long x 1mm wide. Each strip was placed in a 5ml organ bath containing modified Krebs-Henseleit solution bubbled with 95% O_2 - 5% CO_2 at 37°C, and was allowed to equilibrate for at least 1 hour under a 0.6g-resting load before the experiments were initiated. Tension was recorded on a polygraph (Nihon Kohden RMP-6018, Tokyo) *via* isometric transducers (Nihon Kohden TB-651T). The presence of intact-endothelium was confirmed by the ability of 1μM acetylcholine to relax the strips preconstricted with 1μM norepinephrine. The peptides were cumulatively added to endothelium-intact preparations preconstricted with 1μM norepinephrine, and the resulting vasorelaxant activities were measured and expressed as percentages of the norepinephrine-induced contraction in each preparation.

RESULTS

Table 1 shows the structure and binding affinity of the ET/SRTX isopeptides, 4AlaET-1 and its peptide analogs. The N-terminal Glu of 4AlaET-1(10-21) was acetylated to avoid pyroglutamate formation. The IC_{50} values of ET-1, ET-2 and ET-3 for the inhibition of [^{125}I]ET-1 binding to porcine aortic smooth muscle membranes were determined to be 0.16, 0.22 and 5.7nM, respectively, and those to cerebellar membranes were 0.11, 0.11 and 0.07nM, respectively. These results indicate that porcine aortic membranes are rich in ET_A (ET-1 and ET-2 selective) and cerebellar membranes are rich in ET_B (ETs nonselective). The IC_{50} values of SRTX-S6a, SRTX-S6b and SRTX-S6c for the inhibition of [^{125}I]ET-1 binding to

Table 1. Inhibition of [125I]ET-1 binding by ET/SRTX isopeptides and 4AlaET-1 analogs

Peptides	Structures	IC_{50}(nM) ET_A[1]	IC_{50}(nM) ET_B[2]	Selectivity (A/B)
	1 5 10 15 21			
ET-1	CSCSSLMDKECVYFCHLDIIW	0.16[3]	0.11	1.5
ET-2	CSCSSWLDKECVYFCHLDIIW	0.22	0.11	2.0
ET-3	CTCFTYKDKECVYYCHLDIIW	5.7	0.07	81
SRTX-S6a	CSCKDMTDKECLNFCHQDVIW	9.4	0.41	23
SRTX-S6b	CSCKDMTDKECLYFCHQDVIW	0.95	0.13	7.
SRTX-S6c	CTCNDMTDEECLNFCHQDVIW	>200	3.0	>67
4AlaET-1	ASASSLMDKEAVYFAHLDIIW	570	0.33	1700
N-Ac-4AlaET-1	Ac-ASASSLMDKEAVYFAHLDIIW	600	0.34	1800
4AlaET-1(6-21)	LMDKEAVYFAHLDIIW	330	0.32	1000
4AlaET-1(6-20)	LMDKEAVYFAHLDII	>100μM	22μM	——
4AlaET-1(8-21)	DKEAVYFAHLDIIW	1400	1.2	1200
N-Ac-4AlaET-1(10-21)	Ac-EAVYFAHLDIIW	9900	12	830
4AlaET-1(11-21)	AVYFAHLDIIW	2600	1200	2.
ET-1(16-21)	HLDIIW	56μM	>100μM	——

1) Inhibition of [125I]ET-1 binding to porcine aortic smooth muscle membranes.

2) Inhibition of [125I]ET-1 binding to porcine cerebellum membranes.

3) The values in the table represent averages of more than three experiments.

4) Underlined amino acid residues are different from the corresponding ET-1 residues.

ET_A were 9.4, 0.95 and >200nM, respectively, and those to ET_B were 0.41, 0.13 and 3.0nM, respectively. The IC_{50} values of 4AlaET-1 were 570nM for ET_A and 0.33nM for ET_B, indicating that 4AlaET-1 was 1,700 times more selective for ET_B than ET_A (Fig.1, Table 1). A similar ET_B-selective binding affinity was also seen with [N-acetylAla1]4AlaET-1(N-Ac-4AlaET-1) (IC_{50}s for ET_A and ET_B: 600 and 0.34nM, respectively). The truncated peptides such as 4AlaET-1(6-21), 4AlaET-1(8-21) and N-Ac-4AlaET-1(10-21) maintained a high affinity and selectivity for ET_B (IC_{50}: 0.32, 1.2 and 12nM, respectively), whereas 4AlaET-1(11-21), 4AlaET-1(6-20) and ET-1(16-21) had a remarkably reduced affinity for ET_B (IC_{50}: 1.2, 22 and >100μM, respectively) (Fig.1, Table 1).

Whether the peptides with high affinity for ET_B are ET_B agonists was subsequently determined in isolated endothelium-intact pulmonary arteries, since ET_B receptors on the endothelium reportedly induce the release of the vasorelaxant EDRF (13,14,15). None of these linear peptides induced vasorelaxation in endothelium-denuded preparations (data not shown). In the endothelium-intact preparations preconstricted with norepinephrine, 4AlaET-1, N-Ac-4AlaET-1, 4AlaET-1(6-21), 4AlaET-1(8-21) and N-Ac-4AlaET-1(10-21) evoked vasorelaxation in a dose-dependent manner (ED_{40}: 4.4, 1.1, 2.1, 11 and 180nM, respectively),

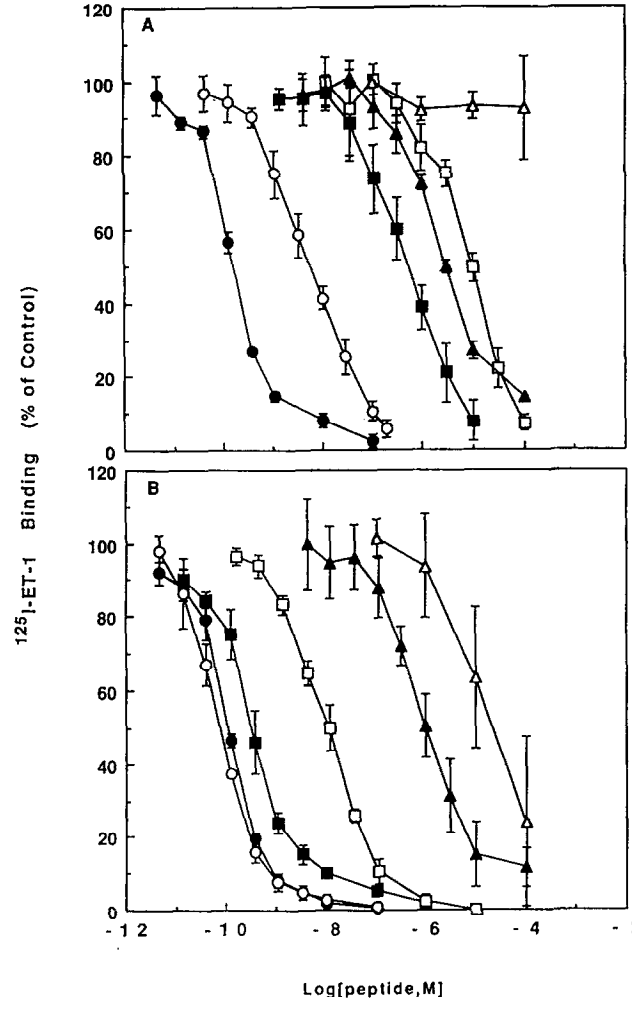

Fig.1. Inhibition of [^{125}I]ET-1 binding to (A) porcine aortic smooth muscle membranes and (B) cerebellar membranes by ET-1(●), ET-3(○), 4AlaET-1(■), N-Ac-4AlaET-1(10-21) (□), 4AlaET-1(11-21)(▲) and 4AlaET-1(6-20)(△). All points represent the average of more than three experiments.

whereas 4AlaET-1(11-21) and 4AlaET-1(6-20) scarcely elicited vasorelaxation at 10μM (Fig.2).

DISCUSSION

The structure-activity relationship of ET-1 analogs for vasoconstriction and binding to ET_A has been discussed in several reports (8,16,17). At the least, the disulfide bridges and the C-terminal Trp^{21} seem to be essential for vasoconstriction and binding to ET_A. However, there is little information about the structure-activity relationship of ET-1 for ET_B binding and function. Hiley *et al.* reported that the linear ET-1 analog 4AlaET-1 was nearly equipotent to ET-1 and ET-3 in inhibiting [^{125}I]ET-1 binding to rat cerebellar homogenate (9). Furthermore, 4AlaET-1 is a weak vasoconstrictor (18), and cerebellar membrane fraction is rich in ET_B (10,12).

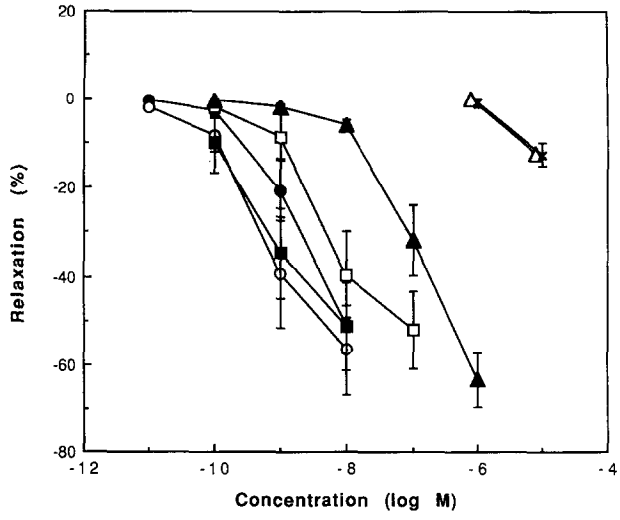

Fig.2. Endothelium-dependent relaxation of norepinephrine-tensioned porcine pulmonary artery by 4AlaET-1(●), N-Ac-4AlaET-1(○), 4AlaET-1(6-21)(■), 4AlaET-1(8-21)(□), N-Ac-4AlaET-1(10-21)(▲) ,4AlaET-1(11-21)(△) and 4AlaET-1(6-20)(×). All points represent the mean ± SE values of more than three experiments.

Taking both of these factors into account, 4AlaET-1 is likely to be an ET_B-selective ligand that may serve as a useful tool for understanding the structural requirement of ET-1 to recognize ET_B.

In the present study, we demonstrated that 4AlaET-1 binds to ET_B 1,700 times more selectively than to ET_A, while ET-1 has a high affinity for both ET_A and ET_B. Furthermore, N-Ac-4AlaET-1 was as potent as 4AlaET-1 for ET_B-selective binding and function, although the N-acetylation of ET-1 ([N-acetylCys^1]ET-1) resulted in a great loss of ET-1-selective vasoconstrictor activity (8). These findings indicate that 4AlaET-1 selectively binds to ET_B, and that a free α-amino group of the N-terminal residue and the rigid tertiary structure formed by the disulfide bridges are not required for the binding of ET-1 to ET_B, different from its ET_A binding requirements.

4AlaET-1 analogs with shortened sequences also provided us with further information. Deletion of five N-terminal amino acid residues, Ala^1-Ser^2-Ala^3-Ser^4-Ser^5 (4AlaET-1(6-21)), did not alter ET_B-selective binding affinity. The further deletion of Leu^6-Met^7 (4AlaET-1(8-21)) and Asp^8-Lys^9 (N-Ac-4AlaET-1(10-21)) resulted in slight decreases in ET_B-selective binding affinity. However, the further deletion of Glu^{10} (4AlaET-1(11-21)) or the removal of the C-terminal Trp^{21} (4AlaET-1(6-20)) results in complete loss of the ET_B-selective binding affinity. In addition, ET-1(16-21) which reportedly discriminates between different ET receptors (19) scarcely showed any binding affinity to ET_A and ET_B in this study. These data indicate that the Glu^{10}-Trp^{21} sequence of ET-1 may be the minimal requirement for ET_B binding. Furthermore, 4Ala-ET-1, N-Ac-4AlaET-1 and 4Ala-ET-1(6-21), which were the most

potent ET_B-selective ligands in this study, were almost as potent as ET-1 and ET-3, and were 10 times more active than the previously reported ET_B-selective ligand, SRTX-S6c (20).

All of the ET/SRTX isopeptides that we tested showed high affinity for ET_B. ET isopeptides are highly homologous in the Glu^{10}-Trp^{21} sequence, but are largely different in the Ser^2- Lys^9 sequence. Furthermore, in the Glu^{10}-Trp^{21} region, seven amino acid residues including Glu^{10} and Trp^{21} residues are completely conserved in the ET/SRTX isopeptides (Table 1). These data suggest that all ET/SRTX isopeptides bind to ET_B due to recognition of the homologous Glu^{10}-Trp^{21} sequence, and that some isopeptides such as ET-1, ET-2 and SRTX-S6b also bind to ET_A due to recognition of their tertiary structure formed by the disulfide bridges and their amino acid residues in the loop region.

Are these synthesized peptides ET_B agonists? The peptides induced endothelium-dependent vasorelaxation in norepinephrine-preconstricted pulmonary arterial strips. This response is reportedly mediated by ET_B receptors on the vascular endothelial cells, which release the vasorelaxant EDRF (13,14,15). The ET_B binding activity of these peptides almost parallels their vasodilative activity, suggesting that they are ET_B agonists.

The present study revealed that ET_B binding and function do not need the rigid tertiary structure formed by the disulfide bridges and the full peptide sequence of ET-1, but require only the truncated Glu^{10}-Trp^{21} peptide sequence. It has been suggested that ETs play important roles in normal as well as disease states such as hypertension, heart failure, asthma, renal failure and vasospasm (21). However, it is difficult to determine which receptor subtype is involved in these biological effects. We already had an ET_A antagonist (12), and now we have ET_B agonists. Both of these ligands with strict selectivity for either ET_A or ET_B will provide powerful tools for the elucidation of the pharmacological and pathophysiological roles of ET peptides.

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