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STRUCTURE-ACTIVITY RELATIONSHIP OF ENDOTHELIN: IMPORTANCE OF CHARGED GROUPS

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Endothelin (ET)-related peptides including ET-1 (1-39) were synthesized, and their constricting activity in rat pulmonary artery rings and pressor activity in unanesthetized rat were measured to elucidate their structure-activity relationship. The vasoconstrictor activities of ET-2, ET-3 and sarafotoxin S6b were one-half, one-60th and one-third that of ET-1, respectively. Such differences in biological activities should mainly arise from sequence heterogeneity at the N-terminal portion, especially at positions 4 to 7. All of the blocked ETs at the amino or carboxyl termini showed greatly decreased activities. A monocyclic analog, in which Cys^{3} and Cys^{11} were replaced by Ala, showed one-third the activity of ET-1; however, its deamino dicarba analog was almost completely inactive. Significant activities were retained even with replacement of amino acids at positions Ser^{4}, Ser^{5}, Leu^{6}, Met^{7}, Lys^{9}, Tyr^{13}, and Trp^{21} by Ala, Ala, Gly, Met(O), Leu, Phe and Tyr, or Phe, respectively. On the other hand, replacement of Asp^{8}, Glu^{10} and Phe^{14} by Asn, Gln and Ala, respectively, resulted in complete loss of the biological activity. These results indicated that two disulfide bonds in ET molecule were not essential for the expression of vasoconstricting activity. Both terminal amino and carboxyl groups, carboxyl groups of Asp^{8} and Glu^{10}, and the aromatic group of Phe^{14} seemed to be contributing, more or less, to the expression of the biological activities.

Endothelin (ET), originally isolated from culture media of porcine aortic endothelial cells, is a 21-amino acid peptide having two intramolecular disulfide bonds and shows potent vasoconstrictor activities (1). The structures of human and rat ETs have also been deduced from their cDNA sequences; human ET was identical with porcine ET, but distinct from rat ET (2,3). Recently, the structures of three types (ET-1, ET-2 and ET-3) of ET were deduced from human genomic DNA, in which ET-1 and ET-3 were found to be identical with porcine (human) and rat ET, respectively (4). ET-2 only differs from ET-1 at position 6 and 7; the Leu-Met sequence was replaced by Trp-Leu

ABBREVIATIONS: Asu, α-aminosuberic acid; TFA, trifluoroacetic acid; WSCI, water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; HOObt, 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine; HOBT, 1-hydrozybenzotriazole; DIEA, diisopropylethylamine; BOP, benzotriazolyloxy-tris (dimethylamino) phosphonium hexafluorophosphate; BDT, butanedithiol.

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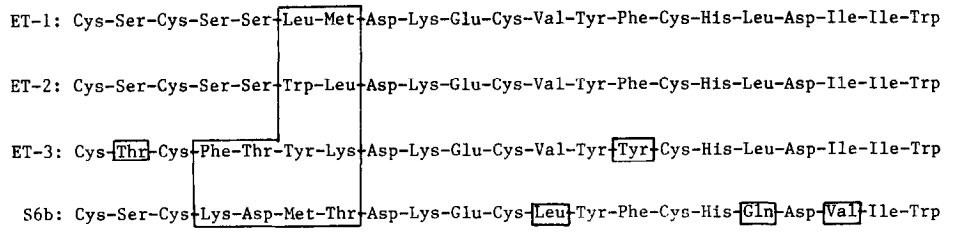

Fig. 1. Structures of ET-1, ET-2, ET-3 and sarafotoxin S6b.

(Fig. 1). Furthermore, sarafotoxin S6b, a similar vasoconstrictor peptide isolated from the venom of the snake Atractaspis engaddensis, was shown to have a high degree of homology to ETs in the primary structure (5). Previously, we reported the synthesis of ET-1, and determined the disulfide structure, which was confirmed to be located at positions 1-15 and 3-11 (6). Successively, we have reported the synthesis of ET-3 and sarafotoxin S6b, and found that the whole molecule forming the proper double cyclic structure was important for the expression of their biological activities (7).

In the present study, we synthesized various ET analogs including ET-1 (1-39) and ET-2 to further elucidate the relationship between the structure and activity of ET.

MATERIALS AND METHODS

Synthesis of peptides ET-1 analogs were synthesized by solid phase procedure using an Applied Biosystems Model 430A peptide synthesizer on a Boc strategy. Details of the synthesis have been reported previously (6). ET-1, ET-2, ET-3, sarafotoxin S6b and ET-1 (1-39) were synthesized by the segment condensation procedure in solution applying our maximum protection strategy; details were reported previously(6). The synthesis of ET-1 (1-39) will be reported elsewhere. The deamino-dicarba (Asu) analog of $Ala^{3,11}$-ET-1 was synthesized as outlined in Fig. 2. After removal of the Pac groups by zinc powder in AcOH, each segment was coupled by WSCI in the presence of HOObt as additive from the C-terminus. The fully protected peptide was treated with TFA to remove the N-terminal Boc group and then treated with Zn powder in TFA to remove the Pac group attached to the Asu residue. Next, the linear product was cyclized using BOP reagent (8) in the presence of DIEA. The cyclized product was treated with HF in the presence of 5% p-cresol and 15% BDT to remove all the protecting groups. The crude product thus obtained was purified by reversed phase HPLC. All other synthetic peptides were purified in the same manner and their homogeneities were confirmed by analytical HPLC and amino acid analysis. The net contents of the peptides for measuring the biological activities were determined by amino acid analysis after acid hydrolysis.

Smooth muscle constricting activity in vitro The vasoconstrictor activity of ET-1 analogs, ET-2, ET-3, sarafotoxin S6b and ET-1 (1-39) were determined using rat pulmonary artery ring preparations as reported previously (6,7). Pulmonary arteries were removed from decapitated male Sprague-Dawley rats (250-350 g) and placed in Krebs-Henseleit solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4.7H_2O$ 0.6, KH_2PO_4 1.2,

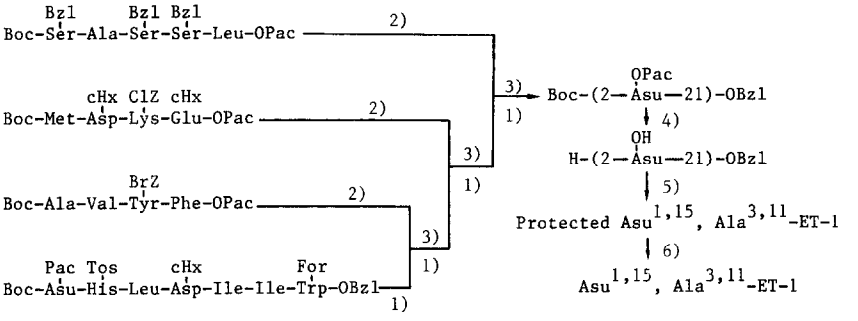

Fig. 2. Scheme for the synthesis of ($Asu^{1,15}$, $Ala^{3,11}$)-ET-1
1) TFA, 2) Zn/AcOH, 3) WSCI/HOObt, 4) Zn/TFA,
5) BOP/DIEA, 6) HF/p-cresol/BDT.

$NaHCO_3$ 25 and glucose 11. These arteries were cleaned of extraneous connective tissue and ring preparations (about 3 mm) were isolated from the right branch. The ring segments were mounted on stainless steel wires and suspended from force displacement transducers in tissue baths with water-jackets (37°C) and containing 10 ml Krebs-Henseleit medium gassed with 95% O_2-5% CO_2. The rings were equilibrated for 1 hr under the optimal resting load of 0.3 g, maximally constricted with 80 mM KCl, then washed and allowed to dilate to baseline tension. The changes in isometric tension 20-30 min after addition of ET-related peptide were recorded. Muscle tension was measured with an isometric recording system (Star-Medical SUL2GR, PA-011, San-Ei 8K21, Tokyo, Japan). Constrictor responses were expressed as the percentage of the response to 80 mM KCl (% KCl max). The potency ratio for each peptide was calculated from the ED_{50} values of the peptides and ET-1.

Pressor activity The aortic blood pressure was determined directly without anesthesia or restraint 2-10 days after a cannula had been inserted into the abdominal aorta via the left femoral artery (3). Blood pressure was determined using an electronic system (Century Technology CP-01, Star Medical PA-011 and San-Ei 8K21, Tokyo, Japan). ET-1, ET-2, ET-3 and sarafotoxin S6b were injected as a bolus (1 ml/kg) through the other aortic cannula via the right femoral artery. The tip of this cannula was about 5 mm peripheral to the cannula for blood pressure determination. Pressor potencies of the peptides were calculated from the vasopressor doses of ET-1 and analogs that elicited a 30 mm Hg rise in mean blood pressure.

RESULTS AND DISCUSSION

In the previous studies, we determined that the disulfide structures of ET-1, ET-3 and sarafotoxin S6b have two disulfide bonds between position 1 to 15 and 3 to 11 based on our precise synthesis of the disulfide analogs (6,7). Sarafotoxin S6b was isolated from snake venom, but found to have the same biological activities as those of ETs. Therefore, sarafotoxin S6b is considered to be a homologous analog of ETs. The vasoconstrictor activities of ET-1, ET-3 and sarafotoxin S6b having the natural disulfide structure was the most potent among the other disulfide analogs; the potency difference of each peptide was in the following order: ET-1>sarafotoxin S6b>ET-3. Such

Table 1. Smooth muscle constricting activity in vitro and pressor activity in vivo of ET-1, ET-2, ET-3 and sarafotoxin S6b

Peptide	Potency Ratio	
	Pulmonary artery	Blood Pressure
ET-1	100[a] (n=7)	100[b] (n=7)
ET-2	49.0 (n=6)	60.2 (n=6)
ET-3	1.9 (n=6)	53.0 (n=6)
Sarafotoxin S6b	35.1 (n=6)	53.4 (n=6)

The biological activity of each peptide was expressed as % of the standard ET-1.
a: ED_{50}= 0.65 ± 0.05 nM.
b: Vasopressor dose that elicited a 30 mmHg rise in blood pressure was 151 ± 46.7 pM/kg.

differences in the biological activities should arise from the sequence heterogeneity at the N-terminal region, especially at the region between 4 and 7. The only difference in the structure of newly determined ET-2 from that of ET-1 is at positions 6 and 7; the Leu-Met sequence in ET-1 is replaced by Trp-Leu in ET-2. The vasoconstrictor activity of ET-2, ET-3 and sarafotoxin S6b on rat pulmonary artery ring preparations were one-half, one-60th, and one-third that of ET-1, respectively, and the potency order was ET-1>ET-2> sarafotoxin S6b>ET-3, which was coincident with the previous findings in porcine coronary artery reported by Inoue et al (4). These potency orders were also observed in pressor activity in unanesthetized rat as shown in Table 1. On the other hand, the time required for the recovery of blood pressure after ET-2 administration was almost the same as that of ET-1 (data not shown), which was not agreement with the data reported previously (4); the recovery time of blood pressure in anesthetized rat was ET-2>ET-1>ET-3. This discrepancy in biological activities among different ETs might be due to the difference in the preparations for measuring the activities.

The vasoconstrictor activity of ET-1-related peptides including ET-1(1-39) on pulmonary artery ring preparations are summarized in Table 2. As reported previously, the opening of any disulfide bond in ET-1 caused marked decrease in the activity; however, a monocyclic analog, $Ala^{3,11}$-ET-1 having a disulfide bond between positions 1 and 15 was the most active among those analogs, indicating that two disulfide bonds in ET molecules are not essential for activity expression. On the other hand, a similar analog, ($Asu^{1,15}$, $Ala^{3,11}$)-ET, was practically inactive, which suggests that the terminal NH_2 group in the ET-1 molecule is important for the expression of the biological activity. These results were confirmed by the fact that amino terminal-blocked and -extended peptides, such as Ac-ET and Lys-Arg-ET, showed only markedly decreased activities.

The importance of the terminal COOH group was also indicated by the diminished potencies of ET-NH_2 and ET-1(1-39). Previous work by us (7) and

Table 2. Smooth muscle constrictor activity of ET-1 analogs on rat pulmonary artery ring preparations

Peptide	Potency ratio	
(Cys^{1-15}, Cys^{3-11})-ET:(ET-1)	100	(n=7)*
(Cys^{1-11}, Cys^{3-15})-ET	0.8	(n=8)*
$(Cys^{1-15}, Cys(Acm)^{3,11})$-ET	0[a]	(n=6)*
$(Cys^{3-11}, Cys(Acm)^{1,15})$-ET	0[b]	(n=8)*
$(Cys(Acm)^{1,3,11,15})$-ET	0	(n=5)*
$(Cys^{1-15}, Ala^{3,11})$-ET	29.6	(n=6)
$(Ala^{1,15}, Cys^{3-11})$-ET	0.4	(n=6)
$(Asu^{1,15}, Ala^{3,11})$-ET	0	(n=4)
ET(1-15)-NH_2	0	(n=6)*
ET(16-21)	0	(n=6)*
Ac-ET	0.5	(n=8)
Lys-Arg-ET	0.2	(n=5)*
Des-Trp^{21}-ET	0	(n=5)*
ET-NH_2	5.6	(n=6)*
ET-1 (1-39)	1.8	(n=8)
Ala^4-ET	42.7	(n=4)
Ala^5-ET	24.2	(n=4)
Gly^6-ET	78.1	(n=6)
$Met(O)^7$-ET	69.1	(n=6)*
Asn^8-ET	0.8	(n=7)
Leu^9-ET	54.8	(n=5)
Gln^{10}-ET	0	(n=4)
Phe^{13}-ET	63.3	(n=4)
Ala^{14}-ET	0[b]	(n=4)
Tyr^{21}-ET	32.8	(n=5)
Phe^{21}-ET	18.4	(n=5)

The biological activity of each analog was expressed as % of the standard ET-1.
* Previous work (7).
a: ED_{25}= ca300 nM, b: ED_{25}= ca1000 nM.

Kimura et al.(9) showed that removal of the Trp residue from the C-terminus resulted in complete loss of the activities. However, our present study indicated that the presence of the C-terminal carboxyl group rather than of the Trp side chain is important for the biological activity, as deduced from the fact that the analogs Tyr^{21}-ET and Phe^{21}-ET were both somewhat biologically active. ET-1(1-39), a precursor form of ET-1, should be useful for monitoring the proteolytic cleavage of the Trp-Val bond in biological systems.

Analogs Ala^4-, Ala^5-, Gly^6-, $Met(O)^7$-, Leu^9- and Phe^{13}-ET-1, substituted at the positions of Ser^4, Ser^5, Leu^6, Met^7, Lys^9 and Tyr^{13} respectively, still

retained one-fifth to four-fifths of the activity. However, replacement of Asp^{8}, Glu^{10} and Phe^{14} by Asn^{8}, Gln^{10}, and Ala^{14}, respectively, resulted in greatly decreased activity. From these results, we concluded that, although formation of the inner disulfide bond in ET molecules is not necessary for its expression of activity, terminal NH_2 and COOH groups, COOH groups of Asp^{8} and Glu^{10} and the aromatic moiety of Phe^{14} are important for binding of the ET molecule to its receptor.

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