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Putative precursors of endothelin have less vasoconstrictor activity in vitro but a potent pressor effect in vivo

Tomoko Kashiwabara, Yoshimasa Inagaki, Hideo Ohta, Akihiro Iwamatsu, Motoyoshi Nomizu, Akihito Morita and Koji Nishikori

Pharmaceutical Laboratory, Kirin Brewery Co., Ltd, Soujamachi 1-chome 2-2, Maebashi, Gunma 371, Japan

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Endothelin (ET-21) induced a sustained contraction of rat thoracic aortae ($EC_{50} = 2.65 \times 10^{-10}$ M) in vitro, and caused a potent pressor effect in vivo after intravenous administration to rats. In contrast, the precursor deduced from porcine cDNA coding ET-21 (pET-39) had 100-fold less contractile activity in vitro ($EC_{50} = 3.26 \times 10^{-8}$ M), and so did the precursor from human cDNA (hET-38) ($EC_{50} = 1.48 \times 10^{-8}$ M). However, both pET-39 and hET-38 caused almost the same dose-dependent pressor effects as ET-21 in vivo. After intravenous bolus injection at 1 nmol/kg, ET-21 caused an initial transient drop of the arterial pressure, and then induced a gradual pressor effect. On the other hand, hET-38 caused only a gradual rise of the arterial pressure. There may be different mechanism(s) for ET-21 and hET-38 which induce changes in the arterial pressure in vivo.

Endothelin; Precursor; Arterial pressure; Vasoconstriction; (Rat aorta)

1. INTRODUCTION

Endothelin (ET-21), isolated from the cultures of porcine aortic endothelial cells, is a novel vasoconstrictor peptide consisting of 21 amino acid residues with two disulfide bonds [1]. It has been reported that ET-21 causes the contraction of various vessels such as porcine coronary artery [1] and rat aorta [2], and induces a pressor effect after intravenous administration to rats [1–3].

Sequence analysis of porcine cDNA encoding ET-21 showed the existence of a 203-residue prepro-endothelin [1]. Based on the deduced amino acid sequence, Yanagisawa et al. [1] postulated a possible pathway for the production of mature ET-21. Thus, the dibasic-pair-specific endopeptidase, which processes the prepro-form of many other peptide hormones [4], may convert prepro-endothelin to the putative 39-amino acid residue precursor (pET-39). For the production of ET-21, pET-39 may be processed by a unique endopeptidase, named 'endothelin converting enzyme (ECE)'.

Recent analysis of human cDNA has shown that ET-21 is also present in human prepro-endothelin [5]. The dibasic-pair-specific endopeptidase may convert human prepro-endothelin to a putative 38-amino acid residue precursor (hET-38: see fig.1).

It is of interest to know what role hET-38 and pET-39 may play during the maturation process of ET-21. The first approach to this problem is to look for the vasoconstrictor activity of hET-38 and pET-39. In this study, hET-38 and pET-39 were synthesized by the solid-phase method. The pharmacological activity of synthetic precursors was determined in vitro by measuring the contraction of rat thoracic aorta, and in vivo by recording the

Correspondence address: K. Nishikori, Pharmaceutical Laboratory, Kirin Brewery Co., Ltd, Soujamachi 1-chome 2-2, Maebashi, Gunma 371, Japan

Abbreviations: ET-21, endothelin; hET-38, precursor deduced from human cDNA coding ET-21; pET-39, precursor deduced from porcine cDNA coding ET-21; ECE, endothelin converting enzyme; MBP, mean blood pressure; EDRF, endothelium-derived relaxing factor; EC_{50}, the half effective concentration of peptides for vasoconstrictor activity

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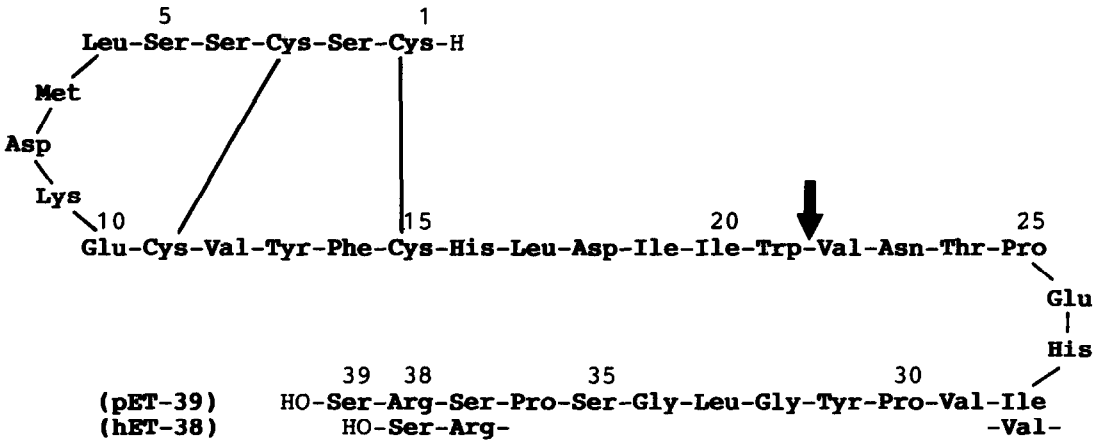

Fig.1. Schematic sequences of endothelin and its putative precursors. The sequence of pET-39 is illustrated. ET-21 is the N-terminal 21-residue moiety of pET-39. The sequence of hET-38 is only illustrated where it differs from pET-39. The arrow indicates the putative site which may be cleaved by ECE.

arterial pressure in anesthetized, chemically denervated rats. Both putative precursors (hET-38 and pET-39) have less vasoconstrictor activity in vitro than ET-21, but have a potent pressor effect in vivo comparable to ET-21.

2. MATERIALS AND METHODS

2.1. *Synthesis of putative precursors of endothelin*

Synthetic ET-21 was purchased from Peptide Institute (Osaka, Japan). The other peptides were synthesized by the solid-phase method, using an automatic peptide synthesizer. Details of this synthesis will be described elsewhere. Obtained peptides were purified by a preparative reverse-phase HPLC. The purity and the position of two disulfide bridges of the synthesized products were estimated on an analytical reverse-phase HPLC. The amino acid sequence of synthesized peptides was confirmed using a gas-phase sequencer, followed by isocratic reverse-phase HPLC [6].

2.2. *Measurement of the contraction of rat thoracic aortae*

The 3-mm long ring of endothelium-denuded thoracic aortae from male Wistar rats (250–300 g) was mounted in an organ bath filled with 10 ml of Krebs-Ringer solution (mM): 112 NaCl, 4.7 KCl, 2.2 $CaCl_2$, 25 $NaHCO_3$, 1.2 $MgCl_2$, 1.2 KH_2PO_4, and 14 glucose. The tension of the ring connected with a transducer was measured isometrically. The concentration-response relationship for ET-21, pET-39, and hET-38 was determined by means of cumulative administration. The response of each aortic ring to the peptides was expressed as a percent of its maximum response to K^+ (40 mM).

2.3. *Measurement of the arterial pressure in rats*

Male Wistar rats (220–280 g) were anesthetized with urethane (1 g/kg, i.p.) and α-chloralose (25 μg/kg, i.p.), and denervated with atropine (0.25 mg/kg, i.v.), propranolol (1 mg/kg, i.v.), and bunazosin (1 mg/kg, i.v.). The catheter connected with a pressure transducer was inserted into the right carotid artery for the measurement of blood pressure, and the catheter for drug administration was placed in the jugular vein.

3. RESULTS AND DISCUSSION

Fig.2 shows the concentration-response curves for the contractile activity of ET-21, hET-38, and pET-39. ET-21 caused strong contraction of the rat aortic ring (EC_{50} = 2.65 × 10^{-10} M), as reported previously [1]. In contrast, the contractile potency of both hET-38 and pET-39 was less than that of ET-21 by approximately 2 orders of magnitude (fig.2), but was higher than that of the other vasoconstrictors, such as angiotensin II [7]. The EC_{50} values of hET-38 and pET-39 are 1.48 × 10^{-8} M and 3.26 × 10^{-8} M, respectively. The maximal tensions of the aortic ring at 3 × 10^{-7} M of hET-38 and pET-39 (125.9 ± 3.2 and 132.8 ± 6.0%, respectively) were almost identical with that of ET-21 at 10^{-8} M (121.5 ± 1.5%). Kimura et al. [8] have previously postulated that the removal of C-terminal Trp^{21} is the inactivation mechanism of ET-21. This removal reduced the potency of the contractile activity by 3 orders of magnitude, and led to the loss of the long-lasting activity. In our experiments, the putative precursors induced slow- and long-lasting contraction of rat aortic rings similarly to ET-21 (not shown). These results support the hypothesis that conversion of the putative precursor to ET-21 is the activation process during biosynthesis of ET-21.

Fig.3 shows the potency of the pressor effect in

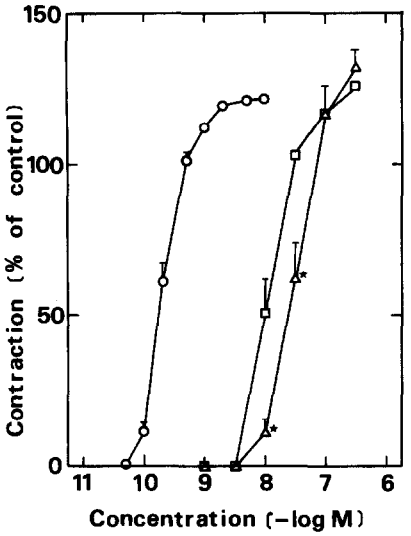

Fig.2. Concentration-response curves for the contraction of rat aortic rings following cumulative administration of ET-21 (○, $n = 8$), hET-38 (□, $n = 9$) and pET-39 (△, $n = 8$). The data are given as means ± SE. Stars indicate a significant difference between the activities of hET-38 and pET-39 ($p < 0.01$).

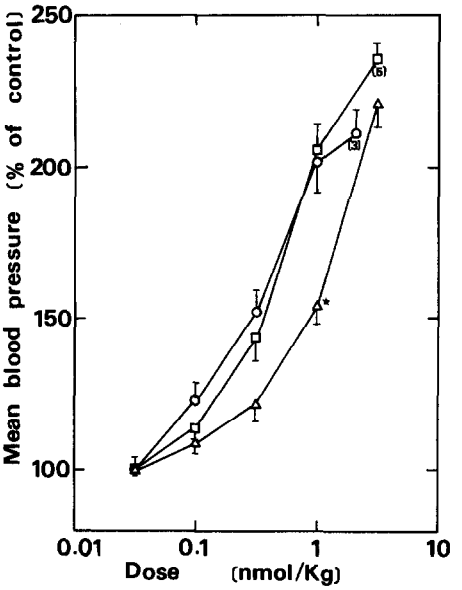

Fig.3. Dose-response curves for the arterial pressure following cumulative administration to rats of ET-21 (○, $n = 5$), hET-38 (□, $n = 5$) and pET-39 (△, $n = 9$). The data are expressed as a percent of the basal level of MBP and given as means ± SE. The number of rats which survived at a dose of 3 nmol/kg is presented in parentheses. The star indicates a significant difference between the activities of hET-38 and pET-39 ($p < 0.01$).

rats after cumulative administration of ET-21, hET-38, and pET-39. ET-21 caused a dose-dependent elevation of the arterial pressure (fig.3). The half effective dose of ET-21 could not be calculated, because administration of ET-21 to the animals at doses higher than 3 nmol/kg caused death. Takasaki et al. [9] indicated that there were functional and structural similarities between ET-21 and sarafotoxin S6 (snake venom toxin). ET-21 may have the properties of an endogenous toxin.

As shown in fig.3, hET-38 caused almost the same dose-dependent pressor effect in vivo as ET-21. The MBP value increased after administration of doses higher than 0.1 nmol/kg. At doses higher than 3 nmol/kg, hET-38 also caused death. The effect of pET-39 in vivo was also similar to that of hET-38 (fig.3), but its potency was somewhat less than that of hET-38. At a dose of 1.0 nmol/kg, the rise in MBP for pET-39 was significantly less than that for hET-38. The constrictor activity in vitro of pET-39 was also significantly smaller than that of hET-38 at concentrations of 10^{-8} M and 3×10^{-8} M (fig.2). Our preliminary experiments indicate that pET-39 is more sensitive to nonspecific proteases when compared with hET-38. It is possible that the lesser activity of pET-39 may be due to the higher sensitivity to the proteases.

Although ET-21 may be the most active form converted from the precursor, the potency of the precursors in vivo was almost the same. In order to check the difference between the pressor effects of ET-21 and hET-38, the time-dependent effects on the arterial pressure of rat after the bolus administration (1 nmol/kg i.v.) were observed. As shown in fig.4, the response to ET-21 was biphasic, as described previously [1–3,10]. The initial effect of ET-21 was a transient drop of the arterial pressure within the first minute. The average of MBP at 0.5 min for 5 rats (45.6 ± 3.0 mmHg) was much smaller than the basal level (62.4 ± 5.1 mmHg). This effect may be due to vasodilation caused by the release of eicosanoids and/or endothelium-derived relaxing factor (EDRF) from endothelial cells, as described by De Nucci et al. [10]. Then, ET-21 produced a prolonged pressor effect lasting for over 1 h. Heart rate

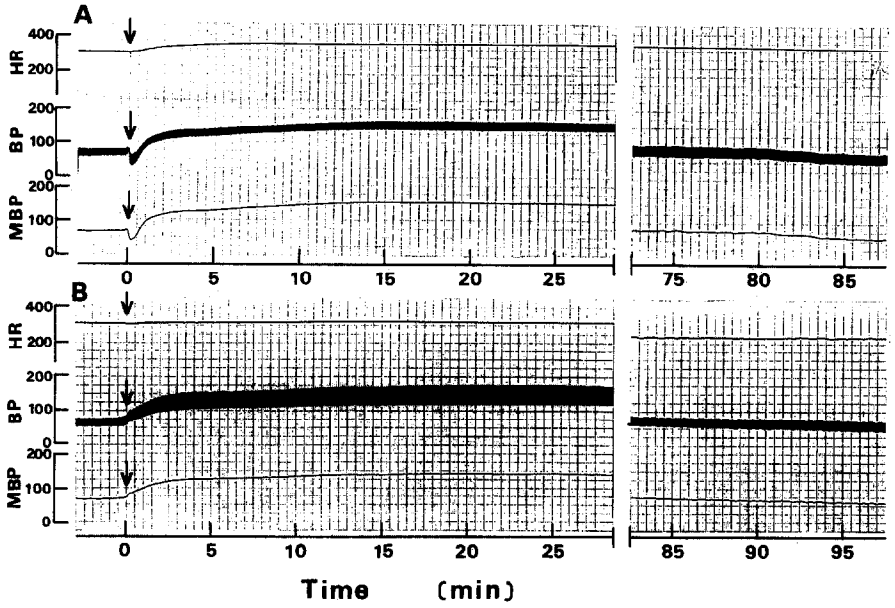

Fig.4. A typical chart of the arterial pressure following administration of ET-21 (A) and hET-38 (B) at a dose of 1 nmol/kg (i.v. bolus), to an anesthetized, chemically denervated rat. Arrows show the time of administration. BP, blood pressure (mmHg); MBP, mean blood pressure (mmHg); HR, heart rate (beats/min).

was slightly increased by ET-21, but the magnitude of this increase was varied in each experiment.

On the other hand, the effect of hET-38 was different from that of ET-21 (fig.4). There was no initial drop in blood pressure, although hET-38 causes a sustained rise in blood pressure lasting for over 1 h similar to ET-21. Even at 0.5 min the average of MBP for 4 rats (65.5 ± 5.4 mmHg) was higher than the basal level (60.8 ± 5.1 mmHg). This finding indicates that the mechanism(s) that induce changes in the arterial pressure is (are) different for ET-21 and hET-38. The response of pET-39 was similar to that of hET-38 (not shown).

Considering the finding that ET-21 caused an initial drop in blood pressure caused by the release of eicosanoids and/or EDRF, it is likely that hET-38 does cause the release of eicosanoids and/or EDRF. The other possibility is that hET-38 may be gradually converted to ET-21 at the site of activation in vivo, and that the amounts of precursors converted to ET-21 were not be enough to cause a drop in blood pressure but may only cause a pressor effect. It is necessary to make clear the mechanisms of activation of endothelin in vivo, because this process may play an important role in the regulation of blood pressure.

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