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Presence of non-selective type of endothelin receptor on vascular endothelium and its linkage to vasodilation

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We studied the role of non-selective type (ET_B) of endothelin (ET) receptor in the vasculature, using a ligand specific to the ET_B receptor, [Glu^9]-sarafotoxin S6b ([Glu^9]SRTb). Endothelium-containing rat thoracic aorta possessed specific binding sites for ^{125}I-[Glu^9]SRTb, which were almost eliminated by removal of the endothelium, while ET-3-specific binding sites were not detected in the endothelium-intact rat aorta. Only ET_B receptor was detected in the membranes from the endothelium of porcine thoracic aorta. [Glu^9]SRTb exerted only vasodilation in rat aortic ring. These findings indicate that ET_B receptors are located on vascular endothelium and linked to vasodilation.

Endothelin; Sarafotoxin; Endothelium; Endothelial cell; Receptor; Vasodilation

1. INTRODUCTION

Endothelins (ETs) belong to a potent vasoactive peptide family consisting of three isopeptides, ET-1, ET-2 and ET-3 [1]. Sarafotoxins (SRTs), having chemical structures similar to those of ETs, also consist of a novel cardiotoxic peptide family [2] and these peptides interact with ET receptors [1]. A diverse set of pharmacological activities with different potencies exerted by ET/SRT family peptides [3–6] suggested the existence of ET/SRT receptor subtypes. We have reported that two distinct subclasses of ET receptors, namely, ET-1-specific and ET/SRT family-common receptors, are distributed in various proportions in human and porcine tissues [7]. Most recently, the structures of the two ET receptor subtypes have been determined by cloning cDNAs encoding those subtypes [8,9], and it was recommended that the ET-1 specific type be called ET_A and the non-selective one ET_B [10].

There have been accumulating observations suggesting that ET-1-secreting endothelial cells (ECs) themselves elicit ET-dependent vasodilation by releasing the endothelium-derived relaxing factor (EDRF) or prostacyclin (PGI_2) [4,11]. However, the precise mechanism is still ambiguous. Although it has been reported that the three ETs are almost equipotent in producing transient vasodilation [3,4], it was also reported that only an ET-3-specific receptor, which has no affinity for ET-1, is located on cultured bovine ECs [12].

The present study was designed to clarify the interrelationships between the ET receptor subtypes and the biological responses in the vasculature using an ET_B receptor-specific agonist, [Glu^9]SRTb.

2. MATERIALS AND METHODS

2.1. *Materials*

Synthetic ET-1, ET-2 and ET-3 were purchased from Peptide Institute (Osaka, Japan). Sarafotoxin S6b(SRTb), sarafotoxin S6c (SRTc) and [Glu^9]SRTb were synthesized by solid-phase method and the quality of the structures was verified by reverse-phase HPLC, amino acid analyses and FAB mass spectroscopy. Male Sprague-Dawley rats (300–350 g) were obtained from Charles River, Japan.

2.2. *Binding assays*

The microsomal fractions were prepared from porcine and rat thoracic aortas as previously described [13]. The endothelia of the aortas were removed mechanically by gentle scraping of the luminal surface with a small glass plate. Monoiodinated ET-1 (^{125}I-ET-1), ET-3 (^{125}I-ET-3) and [Glu^9]SRTb (^{125}I-[Glu^9]SRTb) were prepared by means of HPLC performed in essentially the same manner as previously described [7]. Each radioligand has a specific activity of 2.10–2.15 μCi/pmol. Binding studies were performed and the results were analyzed by a computer program for nonlinear curve-fitting as described [7]. Briefly, the membranes (0.1–0.3 mg) were incubated with 100 pM each of iodinated ligands for 90 min at 25°C in 0.5 ml of 50 mM Hepes buffer (pH 7.4) containing 0.15 M NaCl, 5 mM $MgCl_2$, 0.4 mM phenylmethylsulfonyl fluoride, 1 mg/ml of bovine serum albumin and 0.5 mg/ml of bacitracin. Bound radioligand was determined by trapping on Whatman GF/B filters. Nonspecific binding was determined using 1.0 μM each of the nonradioactive ligands and the values were 5–25% of the total binding.

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2.3. Vasoconstrictor and vasodilator activities in rat aortic rings

The endothelium-denuded rat aortic rings were mounted in 10 ml organ bath filled with Krebs-Henseleit solution, and contraction was measured isometrically using a force displacement transducer as previously described [5]. The resting tension was 1.0 g. The concentration/response relationships for [Glu^9]SRTb- and ET-1-induced vasoconstriction were determined by means of cumulative administrations. The contractile response to these peptides was expressed as a percentage of that elicited by 40 mM KCl which was taken as 100%. Vasodilator responses to these peptides were recorded using endothelium/intact aorta preparations preconstricted with 10^{-7} M phenylephrine, and were expressed as percentages of the vasodilation induced by 10^{-4} M papaverine in each preparation.

3. RESULTS

3.1. *Specific binding sites for ^{125}I-ET-1 and ^{125}I-[Glu^9]SRTb in rat and porcine aortas*

[Glu^9]SRTb displaced the bound ^{125}I-ET-1 with a potency comparable to that of ET-1 in rat cerebral membranes (Fig. 1A), which contain mainly ET_B receptor, whereas in the aortic membranes, which contain mainly ET_A receptor, the displacability of [Glu^9]SRTb was only slight even at 10^{-6} M (Fig. 1B). Specific and saturable bindings were observed not only for ^{125}I-ET-1 but also for ^{125}I-[Glu^9]SRTb in the membranes prepared from endothelia-containing aortas of rat (Fig. 2, solid line). The Scatchard analyses displayed a single component of high affinity binding sites for both ligands, and the apparent dissociation constant (K_d) values were 90 and 380 pM for ^{125}I-ET-1 and ^{125}I-[Glu^9]SRTb, respectively. The maximal binding capacity (B_{max}) for ^{125}I-[Glu^9]SRTb was approximately 5% of that for ^{125}I-ET-1. ET and SRT isopeptides were almost equipotent in displacing ^{125}I-[Glu^9]SRTb from the binding sites in the aortic membranes (Fig. 3A). Similarly, ^{125}I-ET-3 specifically bound to the endothelium-containing aorta and was displaced completely and equipotently by ET/SRT isopeptides including [Glu^9]SRTb (Fig. 3B). The B_{max} value for ^{125}I-[Glu^9]SRTb in the aorta was markedly reduced by scraping off the endothelium but the B_{max} for ^{125}I-ET-1 was not affected (Fig. 2, dotted line). A single class of high affinity binding sites for ^{125}I-ET-1 (K_d = 40 ± 11 pM, B_{max} = 175 ± 33 fmol/mg protein, mean ± S.E.M., n = 3) was detected in the membranes prepared from the intima (endothelium) of porcine aorta and the bound ^{125}I-ET-1 was non-selectively displaced by ET/SRT isopeptides (Fig. 4A), while ^{125}I-ET-1 bound to the membranes obtained from the media (smooth muscle) was displayed sensitively by ET-1 and ET-2 but not by [Glu^9]SRTb even at 10^{-6} M (Fig. 4B). Specific binding sites for ^{125}I-[Glu^9]SRTb and ^{125}I-ET-3 were also detected in the intima membranes, and those were displaced equipotently by each of the ET/SRT isopeptides (data not shown).

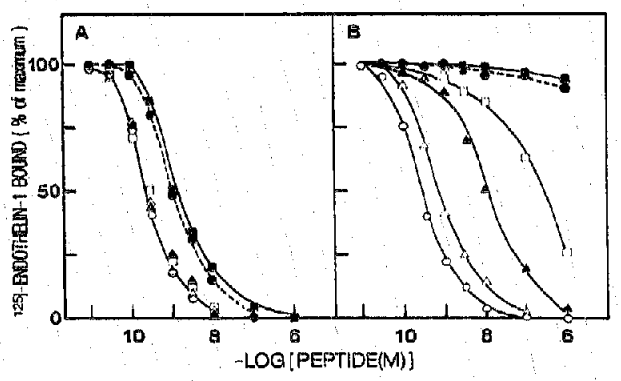

Fig. 1. Competitive binding of ^{125}I-ET-1 to the membranes (0.1 mg) obtained from rat cerebral cortex (A) and thoracic aorta (B) by [Glu^9]SRTb (%), ET-1 (○), ET-2 (▵), ET-3 (□), SRTb (▲) and SRTc (■). The maximal ^{125}I-ET-1 bindings for the controls were 260 and 290 fmol/mg protein for cerebral and aortic membranes, respectively.

3.2. *Effects of [Glu^9]SRTb and ET-1 on rat aortic rings*

In the endothelium-denuded rat aorta preparations, ET-1 caused slowly developing and concentration-

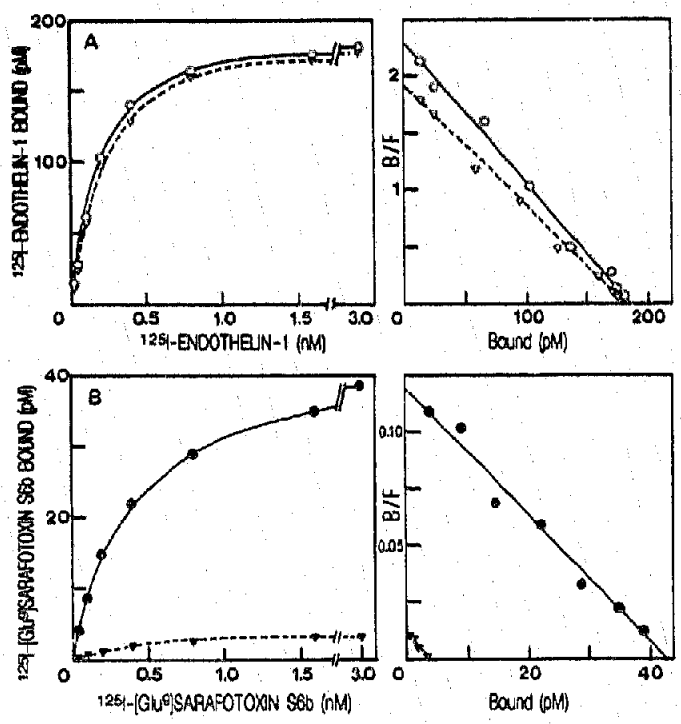

Fig. 2. Saturation bindings of ^{125}I-ET-1 (○, ▽) (A) and ^{125}I-[Glu^9]SRTb (●, ▼) (B) to the membranes obtained from endothelia/intact (solid line) and endothelia/denuded (dotted line) aortas of rat, and the Scatchard analyses. Binding data were obtained from one experiment which is representative of four independent experiments, and are plotted after correction for nonspecific bindings. The amounts of membranes used were 0.1 mg and 0.3 mg for ^{125}I-ET-1 and ^{125}I-[Glu^9]SRTb, respectively. The respective B_{max} values for ^{125}I-ET-1 and ^{125}I-[Glu^9]SRTb were 942 ± 49 and 45 ± 8 fmol/mg protein for the endothelium-intact aorta, and 930 ± 52 and 4 ± 1* fmol/mg protein for the endothelium-denuded aorta (*$P < 0.01$ vs B_{max} for ^{125}I-[Glu^9]SRTb in intact aorta). The respective K_d values for ^{125}I-ET-1 and ^{125}I-[Glu^9]SRTb were 86 ± 16 and 375 ± 43 pM for the endothelium/intact aorta, and 104 ± 18 and 340 ± 36 pM for the endothelium-denuded aorta (mean ± S.E.M., n = 4).

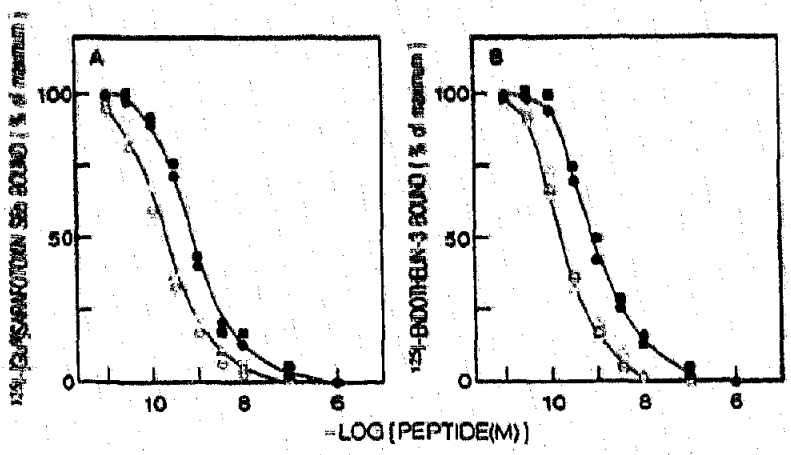

Fig. 3. Competitive bindings of ^{125}I-[Glu^9]SRTb (A) and ^{125}I-ET-3 (B) to the membranes (0.3 mg) obtained from endothelium/intact aorta of rat by [Glu^9]SRTb (•), ET-1 (○), ET-2 (▲), ET-3 (□) and SRTc (■). Each point is the mean of three experiments. The maximal bindings for the controls were 11 and 16 fmol/mg protein for ^{125}I-[Glu^9]SRTb and ^{125}I-ET-3, respectively. The B_{max} and K_d values for ET-3 were 52 fmol/mg protein and 90 pM, respectively.

dependent vasoconstriction at concentrations ranging from 10^{-11} M to 10^{-7} M, but [Glu^9]SRTb caused no vasoconstriction even at 10^{-7} M (Fig. 5A). By contrast, [Glu^9]SRTb produced a transient and concentration-dependent vasodilation at concentrations ranging from 10^{-8} M to 3×10^{-7} M in the endothelium/intact aorta preparations which were preconstricted with phenylephrine (10^{-7} M), but ET-1 produced no vasodilation; instead, a potent vasoconstriction was induced (Figs. 5B and 6). The vasodilator response to [Glu^9]SRTb was completely eliminated by removal of the endothelium or by pretreatment with methylene blue (10^{-5} M), an inhibitor for soluble guanylate cyclase, for 30 min even in the endothelium/intact aorta preparations (Fig. 6).

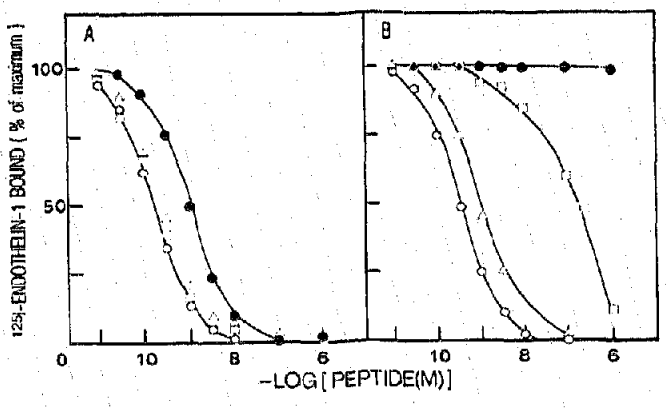

Fig. 4. Competitive binding of ^{125}I-ET-1 to the membranes (0.1 mg) obtained from the intima (A) and the media (B) of porcine thoracic aorta by [Glu^9]SRTb (•), ET-1 (○), ET-2 (▲) and ET-3 (□). Each point is the mean of three experiments. The maximal binding for the controls were 115 and 150 fmol/mg protein for the intima and the media, respectively. The B_{max} and K_d values for ET-1 in the media were 430 ± 55 fmol/mg protein and 130 ± 40 pM, respectively (mean ± S.E.M., $n = 3$).

4. DISCUSSION

The present study provided direct evidence that ET/SRT family-common receptors (ET_B) are located on vascular endothelium and are linked to vasodilation, and that ET-1-specific receptors (ET_A) on smooth muscle are linked to vasoconstriction.

SRT-c, which has the substitution of Glu^9 in a cluster of charged residues (Asp^8-Lys^9-Glu^{10}) common to the other ET/SRT family [1,2], has virtually no affinity for ET_A receptor [7], suggesting that Glu^9 may reduce the affinity of ET/SRT isopeptide for the ET_A receptor. Indeed, the synthetic radiolabelable ligand, [Glu^9]SRTb showed no affinity for the ET_A receptor but retained a full binding capability to the ET_B receptor. Rat thoracic aorta contained a significant amount of ^{125}I-[Glu^9]SRTb binding sites, namely, ET_B receptor, but contained no ET-3-specific receptor (Fig. 3). The binding sites for ^{125}I-[Glu^9]SRTb were almost eliminated by the removal of the endothelium from the aorta. Moreover, the membranes prepared from the porcine aortic endothelia possessed ET_B receptor, which was not due to contamination of the smooth muscle membranes, because the media of aorta had only ET_A receptor (Fig. 4). These observations clearly indicated that ET_A and ET_B receptors are localized on the media and the intima, respectively, in the vasculature. In contrast to the present study, Emori et al. have reported that cultured bovine ECS possess only ET-3-specific receptors [12]. However, their observation might be attributed to down-regulation of ET_B receptors by self-secreted ET-1 and by the aging process, because it has been shown that the incubation of ECs with anti-ET-1 serum significantly enhanced ET-1-induced increase in the intracellular Ca^{2+} level of ECs [14], and that ET_B receptors detected in cultured ECs were converted to ET-3-preferring ones with aging [15].

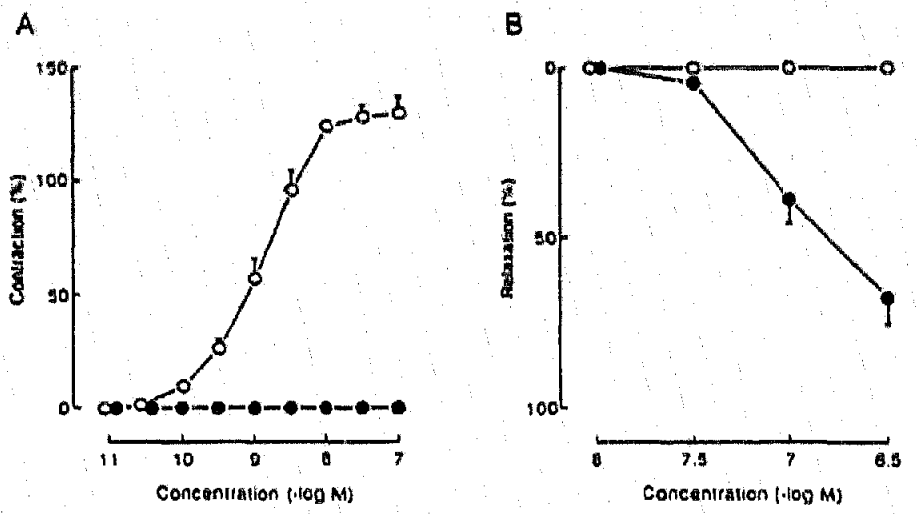

Fig. 5. Concentration/response curves for the vasoconstrictor (A) and vasodilator (B) effects of [Glu⁹SRTb (•) and ET-1 (○) on rat aortic rings. Contraction of the endothelium/denuded aorta and relaxation of the endothelium/intact aorta were assayed. Each point represents the mean ± S.E.M. ($n = 4$–5).

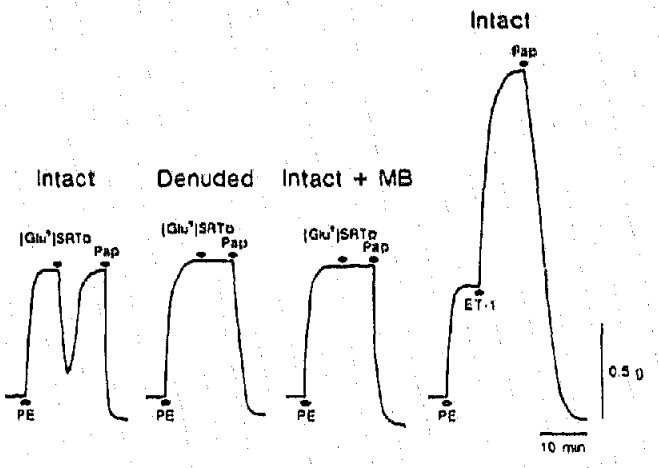

Fig. 6. A typical recording showing the effects of [Glu⁹]SRTb and ET-1 in rat aorta preparations preconstricted with phenylephrine as well as the influences of removal of the endothelium and pretreatment with Methylene blue. [Glu⁹]SRTb, [Glu⁹]SRTb 3×10^{-7} M; ET-1, ET-1 3×10^{-7} M; MB, Methylene blue 10^{-5} M; PE, phenylephrine 10^{-7} M; Pap, papaverine 10^{-4} M.

[Glu⁹[SRTb, which is thus considered to bind exclusively ET_B receptor in the vasculature, exerted only vasodilation, probably through the release of EDRF from the endothelium. A possible explanation of the fact that ET-1 did not induce dilation in the preconstricted aortic rings might be that, since ET-1 had access to smooth muscle from both the luminal and adventitial sides of the aorta, its constrictive effect overwhelmed its dilator effect. In fact, ET-1 injected into the lumen of isolated rat mesenteric artery exerted a transient vasodilation comparable to that of [Glu⁹]SRTb (unpublished data). Hence, the ET family-common receptor is differentiated functionally for vasodilation in the vasculature, while it has been suggested that the common receptor is linked to constriction in the bronchus and the small intestine [16].

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