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VENOUS SMOOTH MUSCLE CONTAINS VASOCONSTRICTOR ET_B-LIKE RECEPTORS

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Summary: Two endothelin (ET) receptor subtypes have been identified to date: the ET_A receptor which preferentially binds ET-1 over ET-3, and the ET_B receptor which is non-selective. This study characterized the ET receptor subtypes present in several vascular smooth muscle preparations using standard *in vitro* techniques. In all but one of the arteries tested, ET-3 was significantly less potent than ET-1. In contrast, the potency of ET-3 was very similar to that of ET-1 in all of the veins. The selective ET_A receptor antagonist BQ-123 blunted the ET-1 contractions in rabbit carotid artery, but not in saphenous vein. The selective ET_B receptor ligand sarafotoxin S6c contracted the rabbit saphenous vein, but not the carotid artery. These data suggest that vascular smooth muscle cells express ET_A and ET_B receptors. Stimulation of either receptor subtype can result in force development.

In 1988, Yanagisawa *et al.* (1) discovered endothelin (ET), a potent vasoconstrictor peptide. Since then, a family of peptides has been described including the snake venom sarafotoxins, vasoactive intestinal constrictor polypeptide (ET-ß), and three distinct isoforms of ET named ET-1, ET-2, and ET-3 (2). All members of the ET family consist of 21 amino acid peptides joined with two intrachain disulfide bridges from Cys^1 to Cys^{15} and from Cys^3 to Cys^{11}. Most of the members of the ET family are potent pressor agents and constrictors of isolated vascular smooth muscle (for review, see 3).

In a general survey of smooth muscles, we found that ET-1 was equipotent to ET-2 and significantly more potent than ET-3 as a constrictor of isolated rabbit iliac artery. In contrast, ET-1, ET-2, and ET-3 displayed very similar potencies in the isolated dog saphenous vein. This finding prompted us to examine whether the difference lay in the species or in the blood vessel. In the present study, we examined the constrictor effects of ET-1, ET-2, and ET-3 on iliac arteries and saphenous veins from dogs, rabbits, and monkeys. A more in-depth pharmacological characterization, using ET_A and ET_B selective ligands, was conducted on the ET receptors in the rabbit carotid artery and rabbit saphenous vein. Our results suggest that the rabbit carotid artery

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contains primarily receptors which resemble the ET_A subtype and the receptors in the rabbit saphenous vein contains mainly ET_B-like receptors. Stimulation of either receptor subtype elicits vasoconstriction. To our knowledge, this is the first demonstration of vasoconstriction resulting from stimulation of ET_B-like receptors in venous smooth muscle.

Materials and Methods

Mongrel dogs, African green monkeys, and New Zealand white rabbits were anesthetized and vascular tissues of interest were removed and mounted for isometric force recording. Circumferential strips of dog saphenous vein were mounted between two gold clips. One clip was connected to a micrometer for control of tissue length, the other to a Grass FT 03 force transducer and Grass model 7D polygraph. All other vessels were studied as rings with two stainless steel self-closure wires (0.011 - 0.013 inch diameter) inserted through each ring; the wires were held by gold clips. The mounted preparations were placed in individual water jacketed muscle chambers in one of two physiological salt solutions (PSS) at 37° C. MOPS-PSS was aerated with 100% O_2 and contained (in mM): 140 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.6 $CaCl_2$, 1.2 NaH_2PO_4, 5.6 D-glucose, 2 morpholinopropane-sulfonic acid (MOPS, pH 7.4), and 0.02 Na_2-ethylenediamine tetraacetic acid (Na_2-EDTA). Bicarb-PSS was aerated with 95% O_2/5% CO_2 (pH 7.4) and contained (in mM): 118.4 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.9 $CaCl_2$, 1.2 KH_2PO_4, 10.1 D-glucose, and 25 $NaHCO_3$.

During the equilibration period, preparations were stretched to a maintained preload of 4 g (dog, monkey, and rabbit iliac artery and rabbit carotid artery), 2 g (dog and monkey saphenous vein), or 0.5 g (rabbit saphenous vein). The preparations were contracted twice with 110 mM K^+PSS, prepared by equimolar substitution of KCl for NaCl, then returned to PSS. Cumulative concentration response curves were obtained for ET-1, ET-2, ET-3, and sarafotoxin S6c in individual preparations and the EC_{50} value, the concentration of agonist causing half-maximal force, was determined. In some experiments, the tissues were exposed to various concentrations of ET_A or ET_B receptor antagonists for 20 min prior to and during the concentration-response curves.

ET-1, ET-2, and ET-3 were purchased from Peptides International and sarafotoxin S6c from Peninsula Laboratories. Stock solutions were prepared immediately before use by dissolving the peptides in a small amount of 1 M acetic acid which was subsequently adjusted to the appropriate volume with water containing 0.05% bovine serum albumin. BQ-123 and two linear analogs of ET-1 were synthesized in the Department of Chemistry, Cardiovascular Diseases at the Bristol-Myers Squibb Pharmaceutical Research Institute.

In all graphs, the points represent the mean force ± SEM. The EC_{50} value was calculated by linear regression analysis. K_B values were determined by analysis of the dose-ratios (4). Significant differences were determined by the Student's t-test; $p < 0.05$ was taken as significant.

Results and Discussion

Cumulative concentration curves were obtained to ET-1, ET-2, and ET-3 in iliac arteries and saphenous veins from dogs, monkeys, and rabbits. All three peptides were of similar efficacy; there were no significant differences in the maximal force attained. With the exception of the rabbit iliac artery and the monkey saphenous vein, the force developed in response to ET-1 was not significantly different from that developed in response to depolarization with KCl (Table 1). In all vascular preparations examined, with the exception of the monkey iliac artery, ET-1 and ET-2 were statistically equipotent (Table 2). In the dog and monkey saphenous vein, there was a small but significant rightward shift of the concentration-response curve for ET-3 as compared with ET-1; the rabbit saphenous vein responses to ET-1 and ET-3 were not significantly different (Figure 1). The EC_{50} values for ET-3 ranged from 1.3 to 3.8 times higher than those for ET-1 in the

Table 1. Maximal force development in isolated tissue preparations

Vessel	Species	KCl	ET-1	ET-2	ET-3
Carotid Artery					
	Rabbit	7.2 ± 0.6	8.4 ± 1.1	7.4 ± 0.9	6.2 ± 1.7
Iliac Artery					
	Dog	24.2 ± 2.5	22.3 ± 7.1	22.5 ± 7.1	21.6 ± 6.3
	Monkey	11.2 ± 0.9	12.7 ± 1.7	15.2 ± 1.8	12.4 ± 2.0
	Rabbit	7.2 ± 0.5*	10.1 ± 0.9	9.2 ± 1.2	9.0 ± 0.7
Saphenous Vein					
	Dog	9.9 ± 0.6	9.5 ± 0.9	8.9 ± 0.6	10.2 ± 1.0
	Monkey	5.6 ± 0.5*	8.8 ± 1.2	6.5 ± 1.4	7.9 ± 1.7
	Rabbit	2.5 ± 0.2	3.1 ± 0.2	3.0 ± 0.5	4.3 ± 0.8

Mean peak force (in g) ± SEM. *Significantly different from ET-1.

saphenous veins. In contrast, in the dog and rabbit iliac arteries, the concentration-response curves for ET-3 were consistently and significantly shifted to the right of the curves for both ET-1 and ET-2 (Figure 2) such that the EC_{50} value for ET-3 was at least 13-fold higher than that for ET-1 in each vessel. Thus, in general, the responses of the iliac arteries to the ET peptides appeared different from the responses of the saphenous veins suggesting that veins and arteries may contain different subclasses of ET receptors. The exception was the monkey iliac artery in which ET-1 and ET-3 showed only a 5-fold difference in potency, however, the EC_{50} values for both ET-1 and ET-2 were significantly lower than that for ET-3. Thus, the ET receptor population in the monkey iliac artery more closely resembled the receptor in the saphenous veins than in the other iliac arteries. It is important, then, to fully characterize each individual tissue with respect to its ET receptor subtype(s).

Two different isoforms of the ET receptor have been cloned to date (5-9). The ET_A receptor binds ET-1 with greater affinity than ET-3 (5). ET_A receptors are generally thought to be present in the membranes of vascular smooth muscle cells (10) and their stimulation produces vasoconstriction via the G-protein mediated phosphoinositide cascade (for review, see 11). It is likely that the ET receptors responsible for vasoconstriction in the dog, rabbit, and monkey iliac arteries are the ET_A isoform. ET_B receptors in the vasculature, in contrast, are believed to be present in the membranes of endothelial cells (12). The ET_B receptors are non-selective; ET-1, ET-2, and ET-3 bind with equal affinity (6). Stimulation of ET_B receptors on vascular endothelial cells

Table 2. Potency of ETs in isolated tissue preparations

Vessel	Species	ET-1	ET-2	ET-3
Carotid Artery				
	Rabbit	0.94 ± 0.31	0.92 ± 0.12	48 ± 17*†
Iliac Artery				
	Dog	3.5 ± 2.2	5.6 ± 2.9	52 ± 10*†
	Monkey	13 ± 2.9	37 ± 7.4*	65 ± 5*†
	Rabbit	5.0 ± 1.9	5.2 ± 1.1	65 ± 16*†
Saphenous Vein				
	Dog	1.8 ± 0.3	2.2 ± 0.6	6.8 ± 1.3*†
	Monkey	14 ± 5.9	31 ± 10	44 ± 8.3*
	Rabbit	2.7 ± 1.7	3.9 ± 1.4	3.5 ± 1.7

Mean EC_{50} values (in nM) ± SEM. *Significantly different from ET-1. †Significantly different from ET-2.

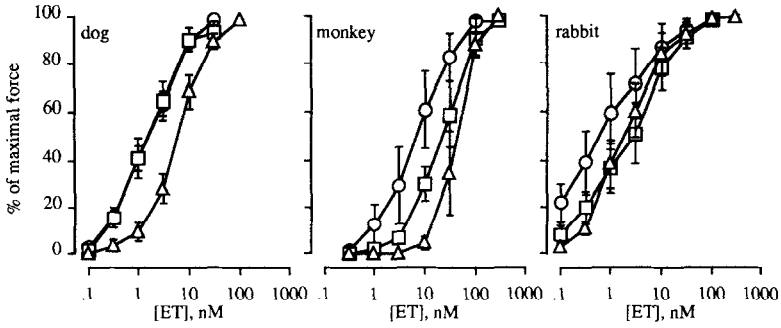

Figure 1. Cumulative concentration-response curves obtained in isolated saphenous veins from the dog, monkey, and rabbit in response to ET-1 (○), ET-2 (□), and ET-3 (Δ). Data are plotted as a percent of the maximum force developed in response to each agonist. Values shown are mean ± SEM; n = 7 strips from different dogs, 7 rings from different monkeys, and 6 rings from different rabbits.

results in vasodilation (12) presumably by the release of NO. Thus, the finding that saphenous veins contain non-selective ET receptors, based on the similarity in potencies for ET-1, ET-2, and ET-3, suggests that they express a novel vasoconstrictor ET_B-like receptor. To our knowledge, this is the first demonstration of an ET_B-like receptor in venous tissue which is coupled to vasoconstriction.

In order to pharmacologically characterize the vasoconstrictor ET receptor in the saphenous vein, the following series of experiments was conducted using rabbit saphenous vein and rabbit carotid artery. We determined that the rabbit carotid artery contained ET_A receptors by showing that ET-1 was 15-fold more potent than ET-3 (Table 2). The rabbit carotid artery was used for further characterization instead of an iliac artery because it provided a longer segment of vessel for study, yet still expressed a large statistically significant difference in ET-1 and ET-3 potency. Sarafotoxin S6c displays high affinity binding to the ET_B receptor, but binds with low affinity to

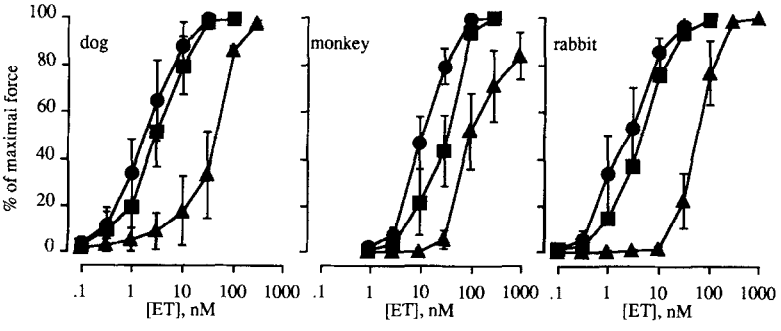

Figure 2. Cumulative concentration-response curves obtained in isolated iliac arteries from the dog, monkey, and rabbit in response to ET-1 (●), ET-2 (■), and ET-3 (▲). Data are plotted as a percent of the maximum force developed in response to each agonist. Values shown are mean ± SEM; n = 3 rings from different dogs, 6 rings from different monkeys, and 8 rings from different rabbits.

the ET_A receptor; thus, sarafotoxin S6c binds selectively to ET_B receptors (13,14). As expected, at concentrations as high as 300 nM, sarafotoxin S6c had no effect on the rabbit carotid artery whether or not the endothelium was present and functional (acetylcholine relaxation of phenylephrine contraction). Sarafotoxin S6c (0.1 - 300 nM) was unable to relax phenylephrine-contracted rabbit carotid arteries containing functional endothelium. This was surprising in light of our expectations that sarafotoxin S6c would bind to ET_B receptors on the vascular endothelium and thereby cause vasodilation. In the rabbit saphenous vein, sarafotoxin S6c produced a marked, concentration-dependent contraction ($EC_{50} = 0.8 \pm 0.05$ nM). The selective vasoconstrictor action of sarafotoxin S6c for the saphenous vein with no apparent effect on the carotid artery supports our hypothesis that the ET receptors responsible for contraction of the saphenous vein are similar to the ET_B isoform. Recently, sarafotoxin S6c was shown to increase blood pressure in pithed rats following intravenous injection (14). In light of the present findings, it is possible that this pressor response is the result of stimulation of vasoconstrictor ET_B-like receptors in the vasculature.

The truncated linear peptide analogs of ET-1, [$Ala^{11,15}$]ET-1 (6-21) and [$Ala^{11,15}$]ET-1 (8-21), have been reported to bind selectively to the ET_B receptor and produce an endothelium-dependent relaxation of norepinephrine contractions in porcine pulmonary artery (15). In the present study, we used [$Ala^{11,15}$]ET-1 (8-21) (Asp-Lys-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp) and [$Ala^{11,15}$]ET-2 (6-21) (Trp-Leu-Asp-Lys-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp) which are 7800- and 15000-fold selective for ET_B receptors, respectively (Dr. Maria Webb, Bristol-Myers Squibb). At 1 μM, neither linear peptide affected ET-1 induced contractions in the rabbit carotid artery. In contrast, 100 nM concentrations of either linear analog completely blocked the vasoconstrictor effects of sarafotoxin S6c in the saphenous vein (data not shown). Thus, the analogs acted as ET_B receptor antagonists in the saphenous vein, although they apparently activated ET_B receptors in the porcine pulmonary artery (15). These data provide additional support for our hypothesis that the rabbit saphenous vein contains a vasoconstrictor ET_B-like receptor. The data are also consistent with the presence of vasoconstrictor ET_A receptors in the rabbit carotid artery.

Ihara *et al.* (16) isolated a selective ET_A receptor antagonist from *Streptomyces misakiensis*, then developed a more potent analog BQ-123 (cyclo-D-Trp-D-Asp-Pro-D-Val-Leu-) which was recently disclosed in a patent from Banyu Pharmaceuticals .[+] We have confirmed the observation of Ihara *et al.* (17) that BQ-123 is a selective ET_A receptor antagonist by examining its effects on concentration-response curves to ET-1 in the rabbit carotid artery (Figure 3). Increasing concentrations of BQ-123 caused parallel rightward shifts in the concentration-response curves to ET-1 without depressing the maximum force suggesting it is a competitive antagonist ($K_B = 35 \pm 14$ nM). Further evidence of competitive antagonism was provided by analysis of the slope of the Schild plot which was not significantly different from -1 (slope = -0.99). In the saphenous vein, however, 300 nM BQ-123 had no effect on the concentration-response curves to either ET-1 or sarafotoxin S6c. These data support the presence of vasoconstrictor ET_A receptors

[+]European patent number EP436,189, issued July 10, 1991.

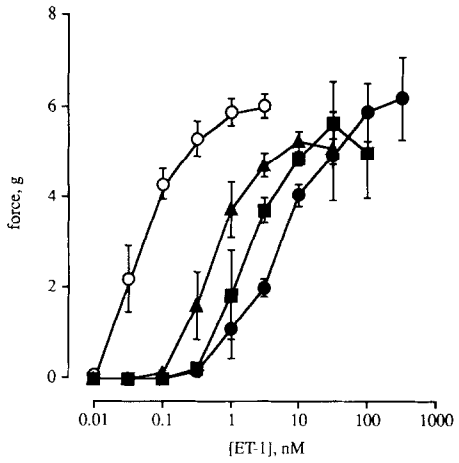

Figure 3. Cumulative concentration-response curves obtained in isolated rabbit carotid arteries in response to ET-1 in the absence (○) and presence of 0.3 (▲), 1 (■), and 3 (●) μM BQ-123. Data are plotted as the actual force (in g) developed in response to ET-1. Values shown are mean ± SEM; n = 4 rings from different rabbits.

in the carotid artery, but indicate that the vasoconstrictor action of ET in the saphenous vein was not due to activation of ET_A receptors.

In this study, we have pharmacologically characterized the ET receptors in seven different blood vessels from three different species. The results suggest that both ET_A and ET_B receptors can be expressed in vascular smooth muscle. In arterial smooth muscle the ET receptor responsible for vasoconstriction appears to be the ET_A isoform, whereas ET_B-like receptors are vasoconstrictor in venous smooth muscle. ET_B receptors have recently been implicated in contractile responses to ET-1 in isolated porcine coronary artery (17). Our data show for the first time that stimulation of ET_B receptors in venous tissue can induce force development.

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