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Evidence for a differential location of vasoconstrictor endothelin receptors in the vasculature

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1 There are at least two subtypes of vascular endothelin (ET) receptors. Stimulation of the ET_A receptors on vascular smooth muscle cells leads to vasoconstriction, whereas activation of the ET_B receptors on endothelial cells elicits vasodilatation. Several reports in the literature have suggested the presence of a vasoconstrictor non-ET_A receptor on vascular smooth muscle which has pharmacological similarities to the ET_B receptor. The present study was undertaken to determine the location of this ET_B-like receptor within the vascular system.

2 Fourteen vascular smooth muscle preparations from six species were used to determine the effect of the ET_A receptor antagonist, BQ-123, on concentration-response curves elicited by ET-1 and the ability of the ET_B receptor agonist, sarafotoxin S6c, to cause contraction. The vessels fell into two categories. One group was sensitive to BQ-123 and insensitive to sarafotoxin S6c and, thus, probably contained ET_A receptors. The other group, with vasoconstrictor ET_B-like receptors, was insensitive to BQ-123 and sensitive to sarafotoxin S6c.

3 Vessels from cynomolgus monkeys, when studied *in vitro*, appeared to contain primarily ET_A receptors, although the potency of BQ-123 was quite variable, suggesting the possibility of ET_A receptor subtypes. In contrast, both ET-1 and sarafotoxin S6c, given as intravenous injections in conscious monkeys, produced transient, equipotent, and dose-related increases in blood pressure. The highest dose of sarafotoxin S6c (1 nmol kg^{-1}, i.v.) also caused a marked secondary depressor response (-80 ± 6 mmHg) that lasted approximately 10 min. The pressor responses suggest that the vasoconstrictor ET_B-like receptors are present in cynomolgus monkeys.

4 The data suggest the presence of two distinct vasoconstrictor ET receptor subtypes on smooth muscle cells. The ET_A receptors are primarily located on the high pressure side of the circulation. The vasoconstrictor ET_B-like receptors appear to be concentrated on the low pressure side.

Keywords: Vascular smooth muscle; endothelin; blood pressure; ET receptors

Introduction

Three isoforms of the endothelin (ET) receptor have been cloned. ET_A receptors preferentially bind ET-1 over ET-3 (Arai *et al.*, 1990) and are present on vascular smooth muscle cells (Lin *et al.*, 1991) where their stimulation leads to vasoconstriction. The ET_B receptors are non-selective for ET-1 and ET-3 (Sakurai *et al.*, 1990) and are located on endothelial cells where their stimulation leads to vasodilatation (Saeki *et al.*, 1991; Takayanagi *et al.*, 1991). The ET_C receptor binds ET-3 with a higher affinity than ET-1 and was recently cloned from *Xenopus* melanophores (Karne *et al.*, 1993). It remains to be established whether this amphibian ET_C receptor has a mammalian counterpart.

Although it is well accepted that ET_A receptors mediate contraction in vascular smooth muscle, recent data have suggested that activation of a non-ET_A receptor subtype constricts some vascular and non-vascular smooth muscles. Both Williams *et al.* (1991) and Clozel *et al.* (1992) reported that administration of the selective ET_B receptor agonist, sarafotoxin S6c, to pithed rats elicited a pressor response. We (Moreland *et al.*, 1992b) and others (Sumner *et al.*, 1992; Panek *et al.*, 1992) have shown that ET_B-selective agonists contract certain isolated smooth muscle preparations and that ET_A-selective antagonists have little or no effect in those tissues. These data support the hypothesis that an ET_B-like receptor subtype is present on vascular smooth muscle obtained from certain locations in the vasculature and that stimulation of this receptor leads to contraction. The present study was undertaken to map the location of vasoconstrictor ET receptors in the vasculature from a variety of species.

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Methods

In vitro *studies*

Tissues were prepared by a modification of techniques described previously (Moreland *et al.*, 1992b). Hog carotid arteries were obtained at slaughter from Hatfield Packing Company. Male Sprague-Dawley rats (250–300 g), New Zealand white rabbits (2–3 kg), and mongrel dogs (12–16 kg) were used in these studies. Male and female cynomolgus monkeys (2.5–4 kg) were purchased from Charles River and Hazelton. Before the surgical removal of the necessary tissues, the rats were killed with CO_2 and the rabbits were anaesthetized with sodium methohexitone. Dogs were anaesthetized with sodium pentobarbitone, treated with heparin, then intubated and ventilated. Monkeys were sedated with ketamine (10 mg kg^{-1}, i.m.), then a peripheral venous catheter was placed in the forearm and an overdose of Nembutal was administered. All tissues were exposed gently, then removed and quickly placed in ice-cold MOPS-buffered physiological salt solution (PSS) where they were cleaned of connective tissue.

Dog left anterior descending coronary arteries, dog saphenous veins, monkey iliac arteries, monkey jugular veins, monkey saphenous veins, rat aortae, rabbit carotid arteries, rabbit renal arteries, and rabbit saphenous veins were cut into rings 3–4 mm wide. The endothelium was removed by gently rubbing the intimal surface. Two stainless steel self-closure wires (0.001–0.003 inch diameter) were inserted through each ring. Rat portal veins, approximately 1 cm in length, were mounted intact. Rabbit pulmonary arteries and monkey vena cavae were prepared as circumferential strips with the endothelium removed as described above. Hog carotid thin medial strips were prepared by removing the

intima and adventitia (Moreland *et al.*, 1992a), then cut into 1.5–2.5 mm widths.

All preparations were mounted for isometric force recording 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. The mounted preparations were placed in individual water-jacketed muscle chambers in bicarbonate-buffered PSS at 37°C, aerated with 5% CO_2 in O_2. During an equilibration period of at least 1 h, the tissues were brought to the following preloads (in g): dog coronary artery (10), dog saphenous vein (2), monkey iliac artery (5), monkey jugular vein (2), monkey saphenous vein (1.5), monkey vena cava (1), rabbit carotid artery (4), rabbit pulmonary artery (1), rabbit renal artery (1), rabbit saphenous vein (0.5), rat aorta (2), and rat portal vein (2). The hog carotid artery was adjusted to L_o, the optimal length for active contraction. Next the effectiveness of the endothelium removal was determined by confirming the absence of the characteristic relaxation of a phenylephrine (10 μM)-induced contraction by acetylcholine (10 μM). If any relaxation occurred, the preparation was re-rubbed to complete the removal. No attempts were made to remove the endothelium from the human saphenous veins or rat aortae.

Bicarbonate-buffered PSS contained, in mM: NaCl 118.4, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25 and D-glucose 10.1. The pH was maintained at 7.4 by aeration with 5% CO_2 in O_2. MOPs-buffered PSS contained, in mM: NaCl 140, KCl 4.7, $NaHPO_4$ 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.6, 2 MOPS (3-[N-morpholino]propane sulphonic acid), Na_2EDTA 0.2 (ethylenediamine tetraacetic acid), and D-glucose 5.6. The pH was adjusted to 7.4 with 5 N NaOH.

In all tissues except rat portal veins, cumulative concentration-response curves were obtained for ET-1 in the absence and presence of the ET_A receptor antagonists BQ-123 (cyclo(D-tryptophyl-D-aspartyl-L-prolyl-D-valyl-L-leucine); Ihara *et al.*, 1992) or FR139317 ((**R**)2-[(**R**)-2-[(**S**)-2-[[1-hexahydro-1H-azepinyl)]carbonyl]amino-4-methyl pentanoyl] amino-3-[3-(1-methyl-1H-indoyl)]propionyl]amino-3-(2-pyridyl) propionic acid; Sogabe *et al.*, 1993). A 20 min incubation time was allowed with the antagonists before exposure of the tissue to the first concentration of ET-1. Three to five concentrations of ET_A antagonist were tested. Concentrations were chosen in half-log increments between 0.3 and 100 μM BQ-123 and between 1 and 100 μM FR139317. The antagonists were also tested for their ability to block spontaneous contractions in rat portal veins. A separate group of tissues was exposed to sarafotoxin S6c (100 nM) and, if force developed, full concentration-response curves were obtained and EC_{50} values determined.

Data are shown as mean ± s.e. Agonist potency is expressed as an EC_{50} value, the concentration of agonist producing half-maximal force, which was calculated by linear regression analysis for each tissue. Antagonist activity (apparent K_B) was calculated with multiple concentrations of antagonist from the following equation: apparent $K_B = B/[(A'/A)-1]$, where B is the concentration of antagonist, A' is the EC_{50} value for the agonist in the presence of antagonist, and A is the EC_{50} value of the agonist alone. $n = 3-6$ tissues from separate animals for each concentration of antagonist.

In vivo *studies*

Male and female cynomolgus monkeys were selected at random from a colony of trained animals implanted with chronic catheters in the abdominal aorta and vena cava as described by Scalese *et al.* (1990). The routine care and maintenance of our primate colony was described in a previous publication (Scalese *et al.*, 1990) and is in accordance with the principles of humane animal care and use established by the American Association for the Accreditation of Laboratory Animal Care. On the morning of an experiment, the conscious monkeys were seated in restraining chairs and the arterial catheters were connected via Gould-Statham strain gauge transducers to a Beckman recorder for measurement of mean arterial blood pressure. The monkeys were left undisturbed for at least 30 min in order to establish the baseline mean arterial pressure.

ET-1 and sarafotoxin S6c were dissolved in saline so that each dose was administered in a volume of 1 ml kg^{-1}. The blood pressure responses to increasing doses of 0.03, 0.1, 0.3 nmol kg^{-1} i.v. of ET-1 and 0.03, 0.1, 0.3 and 1 nmol kg^{-1} i.v. of sarafotoxin S6c were determined in two groups of monkeys (n = 4/peptide). Enough time was allowed between doses to permit blood pressure to return to baseline values before a higher dose was delivered. The maximal pressor and depressor responses were measured after each dose of sarafotoxin S6c to generate dose-response curves. In addition, the time course of the blood pressure changes produced by 1 nmol kg^{-1} i.v. of sarafotoxin S6c was determined.

Results

In vitro *studies*

ET-1 contracted all the isolated smooth muscles examined in a concentration-dependent fashion. The EC_{50} values ranged from 0.32 nM in the monkey vena cava to 3.3 nM in the rabbit saphenous vein (Table 1). Concentrations of the ET_B agonist, sarafotoxin S6c, as high as 100 nM were unable to elicit contraction in any of the preparations from the high pressure side of the circulation, with the exception of one monkey iliac artery. However, sarafotoxin S6c did produce contractions in several of the isolated smooth muscles on the low pressure side. This finding suggests that the vasoconstrictor ET_B-like receptor is localized primarily on the venous side of the circulation. The EC_{50} values for sarafotoxin S6c ranged from 0.4 nM in the dog saphenous vein to 2.1 nM in the rabbit saphenous vein.

The selective ET_A receptor antagonists were tested for their ability to depress contractions in the isolated smooth muscle preparations elicited by ET-1 (Figure 1). Generally, the ET_A receptor antagonists were effective in tissues that did not contract in response to sarafotoxin S6c and ineffective in those that did (Table 1). The potency (apparent K_B) of BQ-123 ranged from 35 nM in the rabbit carotid artery to 2.4 μM in the dog saphenous vein. The potency of FR139317 was similar to that of BQ-123 in the tissues in which it was studied. With the exception of the dog saphenous vein, tissues which responded to sarafotoxin S6c were unaffected by the ET_A receptor antagonists. The peak force of the sarafotoxin S6c contractions in the dog saphenous vein was a small percentage (<10%) of the force elicited by ET-1 (Table 1). Likewise, a minor component of ET-1-induced force was unaffected by the ET_A receptor antagonists. These data suggest that there is a mixed population of ET_A and ET_B-like receptors in the dog saphenous vein.

The effect of BQ-123 on spontaneous activity in the rat portal vein was also examined. At concentrations as high as 10 μM, BQ-123 had no effect on the peak force or rate of spontaneous contractions suggesting that the ET_A receptor is not an important mediator of spontaneous contractions in the rat portal vein.

The vessels isolated from the cynomolgus monkeys did not fit the simple pattern of effects observed in the dog, hog, rabbit, or rat. In the monkey iliac artery, 100 nM sarafotoxin S6c elicited a contraction in only one specimen which contracted to 32% of the maximal ET-1-induced force. In the monkey jugular vein, 100 nM sarafotoxin S6c elicited small (10% of ET-1-induced force) contractions in most preparations. Sarafotoxin S6c (100 nM) did not contract the monkey saphenous vein. At 100 nM, sarafotoxin S6c produced variable amounts of force ranging from 0–78% of the force elicited by ET-1 in the monkey vena cava. The relative lack

Table 1 Potency of ET receptor agonists and antagonists in isolated smooth muscle preparations

Tissue	*ET-1* EC_{50} (nM)	*Sarafotoxin S6c* EC_{50} (nM)	*BQ-123* K_B (nM)	*FR139317* K_B (nM)
Dog				
coronary artery	0.44 ± 0.071 (8.2 ± 1.4 g)	>100	70 ± 16	55 ± 14
saphenous vein	1.3 ± 0.42 (16 ± 2.0 g)	0.4 ± 0.12 (1.3 ± 0.4 g)	2400 ± 1100	780 ± 440
Hog				
carotid artery	1.3 ± 0.45 (8.8 ± 1.8 g)	>100	77 ± 16	NT
Monkey				
iliac artery	0.55 ± 0.22 (16 ± 1.8 g)	>100	290 ± 55	NT
jugular vein	0.34 ± 0.25 (4.0 ± 0.5 g)	>100	>1000	NT
saphenous vein	1.1 ± 0.70 (7.6 ± 1.5 g)	>100	710 ± 430	NT
vena cava	0.32 ± 0.23 (2.0 ± 0.1 g)	>100	44 ± 17	NT
Rabbit				
carotid artery	0.59 ± 0.16 (6.0 ± 0.3 g)	>100	35 ± 14	51 ± 10
pulmonary artery	1.8 ± 0.22 (3.4 ± 0.3 g)	1.5 ± 0.40 (2.9 ± 0.3 g)	>300	NT
renal artery	2.8 ± 0.60 (10 ± 1.2 g)	>100	>300	NT
saphenous vein	3.3 ± 0.98 (5.2 ± 0.8 g)	2.1 ± 1.0 (5.3 ± 0.8 g)	>300	NT
Rat				
aorta	0.52 ± 0.11 (6.1 ± 0.4 g)	>100	64 ± 10	NT

K_B = apparent K_B, calculated as described in the Methods. Maximum force for contractions elicited by ET-1 or sarafotoxin S6c is shown in parentheses below the corresponding EC_{50} values. Data are shown as mean ± s.e. NT: not tested.

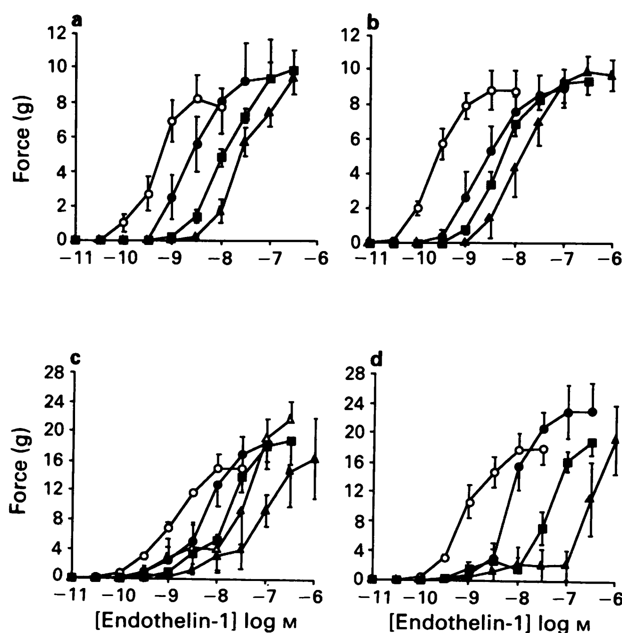

Figure 1 Cumulative concentration-response curves for endothelin-1 (ET-1) in various isolated vascular smooth muscle preparations: (a) shows data collected in dog coronary artery in the absence (○) and presence of 0.3 (●), 1 (■), or 3 (▲) μM BQ-123; n = 4 animals, (b) shows data obtained in rabbit carotid artery in the absence (○) and presence of 0.3 (●), 1 (■), or 3 (▲) μM FR139317; n = 3 animals. Panel (c) shows results collected in dog saphenous vein in the absence (○) and presence of 3 (●), 10 (■), 30 (△), or 100 (▲) μM BQ-123; n = 4 animals; (d) shows curves obtained in dog saphenous vein in the absence (○) and presence of 3 (●), 10 (■), or 100 (▲) μM FR139317; n = 3–4 animals.

of effect of sarafotoxin S6c on the monkey isolated vessels compared with the other preparations suggested that the vasoconstrictor ET_B-like receptor might not play an important role in the primates. Therefore, sarafotoxin S6c was tested in the human isolated saphenous vein where it was found to cause a concentration-related contraction (EC_{50} = 1.4 ± 0.21 nM).

In vivo studies

The effects of sarafotoxin S6c were also determined in conscious cynomolgus monkeys. Mean arterial pressure was 112 ± 3, 110 ± 4, 109 ± 4 and 114 ± 2 mmHg before administration of 0.03, 0.1, 0.3 and 1 nmol kg^{-1} i.v. of sarafotoxin S6c in the monkeys. The lower doses of sarafotoxin S6c (0.03–0.3 nmol kg^{-1}, i.v.) produced predominantly pressor responses in the conscious monkeys (Figure 2). This activity reached its peak within 1 min then gradually waned during the next 10 to 15 min. A higher dose of 1 nmol kg^{-1} i.v. of sarafotoxin S6c caused an initial pressor response followed within minutes by a profound reduction in mean arterial pressure (Figures 2 and 3). Blood pressure then returned toward pretreatment levels within 10 min. All monkeys were observed to cough and gag after administration of the highest dose of sarafotoxin S6c, perhaps due to the activation of airway ET_B-like receptors (Hay, 1992).

ET-1 was equipotent with sarafotoxin S6c and also produced dose-related increases in mean arterial pressure in conscious cynomolgus monkeys (Figure 2). Mean arterial pressures were 116 ± 7, 114 ± 6 and 116 ± 7 mmHg before administration of 0.03, 0.1 and 0.3 nmol kg^{-1} i.v. of ET-1. The magnitudes of the responses to ET-1 were similar to the activities of equimolar doses of the ET_B agonist sarafotoxin S6c in the conscious monkeys. Because 1 nmol kg^{-1} of sarafotoxin S6c elicited a large depressor response in the

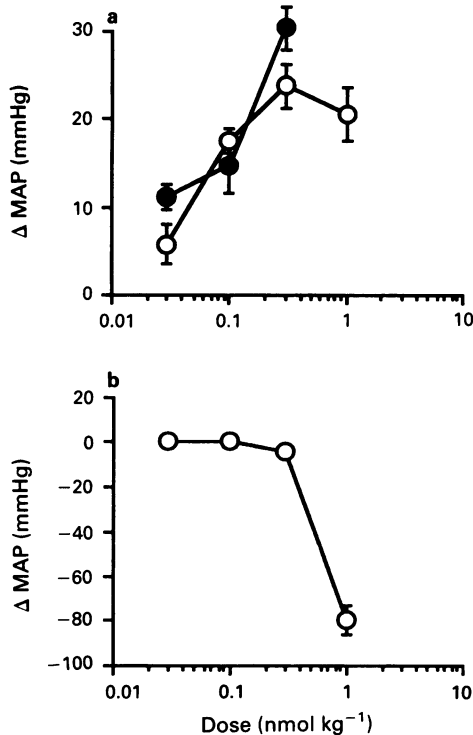

Figure 2 Effects of sarafotoxin S6c (○) and endothelin-1 (ET-1) (●) on mean arterial pressure (MAP) in conscious monkeys. Peak pressor (a) and depressor (b) effects are plotted as mean ± s.e. $n = 4$ animals/peptide.

primates, doses > 0.3 nmol kg^{-1} of ET-1 were not tested. The coughing produced by sarafotoxin S6c was not evident with ET-1. However, ET-1 induced emesis in one monkey.

Discussion

This survey of tissues was undertaken to map the location of functional ET_A and ET_B-like receptors within the vasculature and across species. The isolated smooth muscles used in this study can be divided into two major groups. Those which were sensitive to the effects of BQ-123 and FR139317, and did not contract in response to sarafotoxin S6c were defined as containing ET_A receptors. This group included the dog coronary artery, hog carotid artery, monkey saphenous vein, rabbit carotid artery, and rat aorta. The preparations which were not affected by BQ-123 or FR139317, but contracted in response to sarafotoxin S6c were defined as containing the vasoconstrictor ET_B-like receptors. Tissues with the ET_B-like receptors included the rabbit pulmonary artery and rabbit saphenous vein. The human saphenous vein probably also contains ET_B-like receptors which were responsible for the contraction induced by sarafotoxin S6c. A few of the tissues appeared to contain a variable mixture of both types of receptors, such as the dog saphenous vein, monkey iliac artery, and monkey jugular vein. The rabbit renal artery did not contract in response to sarafotoxin S6c nor were the contractions elicited by ET-1 antagonized by BQ-123 suggesting that the ET receptor in rabbit renal artery is functionally distinct from either the ET_A or ET_B-like isoforms. A similar ET receptor, insensitive to BQ-123 and to sarafotoxin S6c, was described in rat vas deferens (Eglezos *et al.*, 1993).

To date, we have not found functional evidence for ET_B-like receptors on the high pressure side of the circulation, although Harrison *et al.* (1992) have suggested their presence in the pig coronary artery. In contrast, ET_A receptors are apparently present on both high and low pressure sides of the circulation. This appears to be particularly true in the vessels

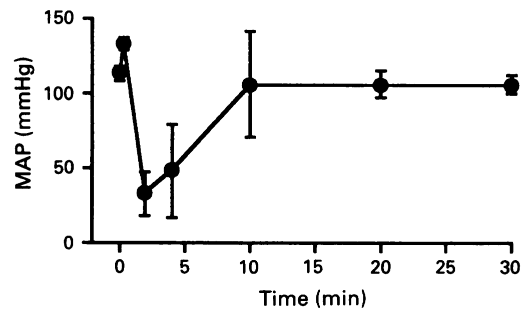

Figure 3 Time course of effects of 1 nmol kg^{-1} i.v. of sarafotoxin S6c on mean arterial pressure (MAP) in conscious monkeys. Data are plotted as mean ± s.e. $n = 4$ animals.

isolated from the cynomolgus monkey. However, intravenous injection of sarafotoxin S6c produced substantial increases in mean arterial pressure in conscious cynomolgus monkeys. Thus, the lack of evidence for ET_B-like receptors in the isolated tissues probably resulted from choosing large vessels for study. It is possible that the resistance vessels responsible for the control of blood pressure contain ET_B-like receptors.

Sarafotoxin S6c causes a hypertensive response upon intravenous injection, presumably by activation of ET_B-like receptors which would also be stimulated by ET-1. Thus, activation of the ET_B-like receptors may be important in pressor responses elicited by ET-1. It is difficult to imagine how substantial increases in blood pressure would result from stimulation of the ET_B-like receptor present relatively rarely on only the low pressure side of the circulation. It may be that functional ET_B-like receptors are also on large vessels on the high pressure side of the circulation or that they occur with more frequency in the resistance vessels. If this were true, we would have missed sampling the arterial ET_B-like receptors by chance. An alternative possibility is that sarafotoxin S6c activation of endothelial ET_B receptors releases a constrictor factor. However, neither a thromboxane receptor nor an angiotensin II receptor antagonist affected contractions elicited by sarafotoxin S6c in the rabbit isolated saphenous vein (data not shown).

The responses to sarafotoxin S6c and ET-1 in the monkeys differed from the biphasic activity of ET-1 reported previously in rats (King *et al.*, 1989). In the rodents, doses of ET-1 similar to those used in the present study produced a transient depressor response that preceded a sustained increase in blood pressure. The immediate fall in blood pressure has been attributed to stimulation of the endothelial ET_B receptors (Saeki *et al.*, 1991; Takayanagi *et al.*, 1991). In the present study, the lower doses of 0.03 to 0.3 nmol kg^{-1} i.v. of sarafotoxin S6c or ET-1 transiently increased blood pressure without an initial depressor response in conscious monkeys. This monophasic pressor activity was similar to previous reports that ET-1 infusions increased blood pressure in conscious dogs (Goetz *et al.*, 1988; Donckier *et al.*, 1991) and reduced cardiac contractility (Donckier *et al.*, 1991). This finding raises the possibility that the secondary fall in blood pressure produced by the highest dose of 1 nmol kg^{-1} i.v. of sarafotoxin S6c in the present study may have resulted from decreases in cardiac performance rather than activation of endothelial ET_B receptors.

In summary, we have shown that ET_A receptors occur throughout the vasculature but are concentrated primarily on the high pressure side of the circulation. Vasoconstrictor ET_B-like receptors are located on the low pressure side of the circulation in many species. These ET_B-like receptors can apparently mediate pressor responses elicited by sarafotoxin S6c in conscious cynomolgus monkeys.

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