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ET_A AND ET_B RECEPTORS COEXIST ON RABBIT PULMONARY ARTERY VASCULAR SMOOTH MUSCLE MEDIATING CONTRACTION

Donnelle M. LaDouceur, Michael A. Flynn, Joan A. Keiser, Elwood Reynolds, and Stephen J. Haleen[1]

Parke-Davis Pharmaceutical Research Division, Warner Lambert Company, 2800 Plymouth Road, Ann Arbor, Michigan 48105

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Summary: The possibility that both ET_A and ET_B endothelin receptor subtypes could mediate contractile activity in the same tissue was investigated in isolated, endothelium denuded rabbit pulmonary arteries. The ET_B selective agonist, sarafotoxin 6c (S6c), produced potent contractile activity, equal to the non-selective ET_A and ET_B receptor agonist endothelin-1 (ET-1), indicating a contractile role for ETB receptors in this tissue. In addition BQ-123 (10.0 μM), the ET_A selective antagonist, was only partially effective in blocking ET-1 induced contractions further indicating a contractile role for ET_B receptors. However, the partial blockade by BQ-123 suggested a possible contractile role for ET_A receptors. To address this possibility, ET_B receptors were desensitized with a 30 minute pretreatment of S6c (0.01 μM). Under these conditions, we were able to demonstrate full ET-1 contractile activity that was now sensitive to blockade by BQ-123. The coexistence of both ET_A and ET_B receptors was confirmed through receptor binding experiments indicating 40/60 ratio, respectively. We conclude that 1) both ET_A and ET_B receptors coexist on vascular smooth muscle of rabbit pulmonary artery, 2) activation of either receptors subtype results in contraction, and 3) prolong activation of the ET_B receptor subtype produces tachyphylaxis preventing further activation by S6c or ET-1.

Until recently, the endothelin receptor mediating vasoconstriction has been characterized as the ET_A receptor (1). New reports, however, indicate endothelin ET_B receptors can mediate vasoconstriction in some vascular tissues including rabbit pulmonary artery (2), rabbit vein (3,4), and the rat kidney (5-8). Functional evidence includes potent vasoconstrictor activity by ET_B selective agonists (ET-3, [$Ala^{1,3,11,15}$]ET-1, and S6c) and the inability of BQ-123 (9), an ET_A selective antagonist, to block vasoconstrictor activity by ET-1, which activates both ET_A and ET_B receptors non-selectively. Although these results indicate a vasoconstrictor role for ET_B receptors in these tissues, they do not exclude the possibility of coexistent ET_A receptors within these same tissues that are also capable of mediating vasoconstriction.

[1]To whom correspondence and reprint requests should be addressed.

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The purpose of this study was to investigate possible ET_A receptor-mediated vasoconstrictor activity in isolated, endothelium denuded rabbit pulmonary arteries. Since there are no highly selective ET_B receptor antagonist available for this purpose, a new method of desensitizing ET_B receptors with S6c was employed thereby permitting independent study of ET_A activity.

MATERIALS AND METHODS

Materials: Norepinephrine and carbachol were purchased from Sigma Chemical Co., (St. Louis, MO), ET-1, and S6c from American Peptide (Sunnyvale, CA). BQ-123 was synthesized by our Medicinal Chemistry Department (Parke-Davis Pharmaceutical Research Division); purity (99.9%) was verified by HPLC. ET-1 and S6c were dissolved in 0.1% acetic acid in distilled water. BQ-123 was dissolved in DMSO. The maximum concentration of DMSO in the bath was 0.1% which did not significantly affect developed tension in response to either ET-1 or S6c.

Isolated Pulmonary Artery Preparation: New Zealand white male rabbits 2.0-2.4 kg (Hazelton) were euthanized by CO_2 gas and exsanguination. The lungs were removed and pulmonary arteries (secondary segments) were isolated, cleaned of connective tissue, and cut into 4 mm rings. The endothelium was partially denuded by first placing the rings over a wooden skewer and gently rolling, this was repeated with hypodermic tubing (28 gauge) to complete denudation. Denuded rings were mounted in 20 mL baths containing Krebs-bicarbonate buffer (2). Resting tension was adjusted to 4 g, left for 90 minutes to equilibrate, and then **readjusted to 4 g of** tension.

Vascular rings were tested for a lack of an endothelium-dependent relaxation response to carbachol (1.0 μM) in norepinephrine (0.3 μM) contracted rings. Agonist peptides were cumulatively added at 10-minute intervals. In separate experiments, the endothelin antagonist BQ-123 was added 30 minutes prior to adding the agonist. In experiments where pretreatment included S6c and BQ-123, they were added at 30 minute intervals, respectively, followed by ET-1.

Radioligand Binding Assay: Pulmonary arteries (secondary branches) were purchased from Pel-Freeze and stored at -80C until use. Vessels were thawed, cleaned of connective endothelium removed by gentle rubbing and vigorous rinsing in buffer. The tissue was frozen in liquid nitrogen and pulverized in a freezer/mill (Spex). The tissue was resuspended in 10 ml binding buffer (BB) containing (mM) Tris (20), EDTA (2), PMSF (0.1), bacitracin (0.1), leupeptin (0.001), pepstatin A (0.0001), pH 7.4, then homogenized in a Dounce homogenizer. The suspension was centrifuged for 5 minutes at 500 x g (4°C) and the supernatant filtered through cheesecloth. The supernatant was then centrifuged for 30 minutes at 40,000 xg (4°C) and the pellet resuspended in binding buffer at a concentration of 66 μg protein/ml.

Binding assays were carried out by the addition of 50 μl [^{125}I] ET-1 (30,000; NEP), 50 μl drug, and 150 μl pulmonary artery membranes to polypropylene tubes. Drug and [^{125}I] ET-1 were prepared in BB = 0.2% BSA. The assay mixture was incubated for 2 hours at 37°C, and the incubation terminated by filtration over Wattman GF/B filters presoaked in 50 mM Tris, 0. 2% BSA. Radioactivity retained on filters was measured in a gamma counter. Non-specific binding was defined as the binding in the presence of 100 nM ET-1 and specific binding was defined as total binding minus non-specific binding.

Statistics: An F test for parallelism was used to evaluate the effects of pretreatment (BQ-123 or S6c + BQ-123) on the contractile activity of ET-1. Statistical differences between parallel curves were determined using a t-test on EC_{50} values. Binding data was analyzed by nonlinear least-square curve fitting using InPlot (GraphPad Software), and IC_{50} values calculated using a one-site fit.

RESULTS

Evidence for contractile activity via ET_B receptors. Figure 1 summarizes the contractile activity of ET-1 and S6c in rabbit pulmonary arteries. S6c was significantly more potent than ET-1 (EC_{50} 0.7 versus 2.6 nM), whereas maximal activity (6.4 versus 6.9 g) was not significantly different. Pretreatment of the rabbit pulmonary arteries with 10.0 μM BQ-123 (Figure 2) resulted in a biphasic ET-1 response curve which was slightly shifted to the right with a significantly smaller maximal response (Table 1).

Desensitization of the ET_B receptor. Figure 3 (panel a) illustrates the effects of prolonged exposure of S6c on rabbit pulmonary arteries. Initially, 0.01 uM S6c produced a strong contraction lasting approximately 10 minutes. This was followed by a slow recovery over 40 minutes to near baseline levels. Subsequently increasing S6c up to a concentration of 1.0 μM had no effect. Pretreatment with S6c (0.01 μM) did not, however, affect the contractile potency of ET-1 (Figure 3, panel b). The EC_{50} values for ET-1 in S6c pretreated and control rings were not significantly different (Table 1). In addition, maximal activity to ET-1 was comparable between S6c pretreated and control rings (Table 1).

Evidence for contractile activity via ET_A receptors. In S6c pretreated rings (Figure 4), BQ-123 (1.0 and 10.0 μM) produced concentration-dependent rightward shifts in ET-1 response curves. The calculated pA_2 value for BQ-123 was 6.6 in S6c pretreated rings. Maximal activity of ET-1 was comparable among control, S6c pretreated, and S6c + BQ-123 rings (Table 1).

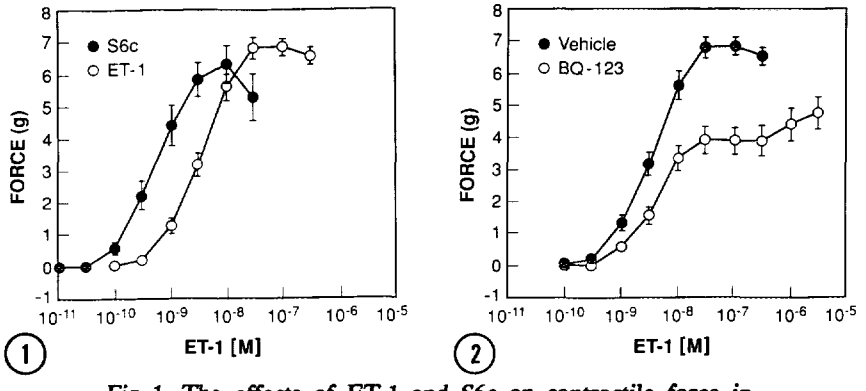


Fig. 1. The effects of ET-1 and S6c on contractile force in denuded pulmonary arteries isolated from rabbits. Each point represents the mean ± S.E.M of 8 to 10 rings isolated from four to six animals.

Fig. 2. The effects of BQ-123 (10.0 μM) pretreatment on contractile responses to ET-1 in denuded pulmonary arteries isolated from rabbits. Each point represents the mean ± S.E.M of 10 to 12 rings isolated from four to six animals.

Table 1. The effects of BQ-123, S6c, and BQ-123 + S6c pretreatment on the potency and maximal activity of ET-1 in denuded rabbit pulmonary arteries

Pretreatment	ET-1 Activity	
	EC_{50} (nM)	Maximal (g)
Vehicle	2.6	6.9±0.3
BQ-123 (10.0 μM)	6.2*	4.8±0.5*
S6c	1.4	6.2±0.4
S6c + BQ-123 (1.0 μM)	12.3∞	6.0±0.7
S6c + BQ-123 (10.0 μM)	108.8∞	5.8±1.0

Values are mean ± S.E.M. (N=4-10). *Indicates a significant difference from vehicle, ∞ indicates a significant difference from S6c (alone) pretreatment group and indicates a significant difference from BQ-123 (10.0 μM) (alone) treatment group, $p<0.05$.

ET_A and ET_B receptor binding. In competition binding experiments with [^{125}I]ET-1 in pulmonary artery membranes, ET-1 was able to completely inhibit [^{125}I]ET-1 binding with an IC_{50} value of 0.13 nM. The subtype selective ligands BQ-123 (ET_A) and S6c (ET_B) only

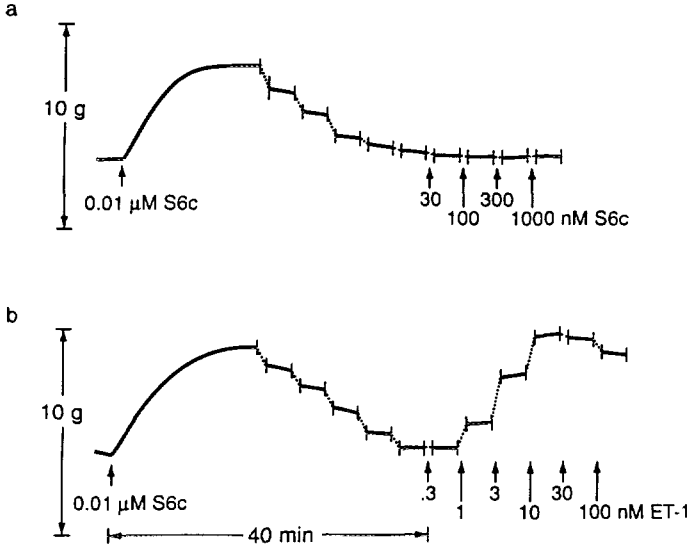
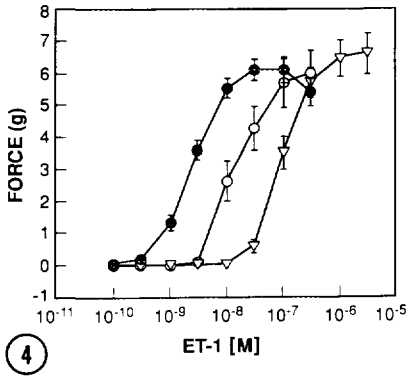


Fig.3. Representative traces of the transient effects of S6c (0.01 μM) on contractile force over 30 minutes, followed by further increases in S6c concentration (panel a), or ET-1 concentration (panel b) in denuded pulmonary arteries isolated from rabbits.



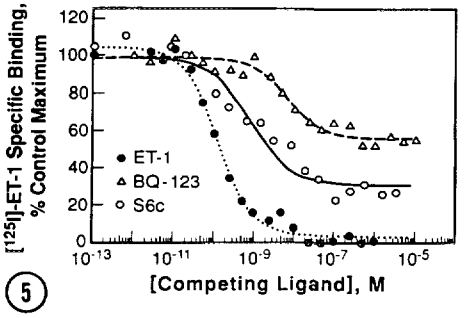


Fig.4. The effects of S6c pretreatment (●), S6c + BQ-123, 1.0 μM (○), and S6c + BQ-123, 10.0 μM (▽) on contractile force in denuded rabbit pulmonary arteries isolated from rabbits. Each point represents the mean ± S.E.M. of 8 to 10 rings from four to six animals.

Fig.5. Inhibition of specific [^{125}I]ET-1 binding to membranes prepared from endothelium denuded rabbit pulmonary arteries.

partially inhibited [^{125}I]ET-1 binding, demonstrating that [^{125}I]ET-1 was binding to both ET_A and ET_B receptors in this tissue. BQ-123 only inhibited 44% of [^{125}I]ET-1 binding with an IC_{50} of 7.0 nM, whereas S6c only inhibited 74% of [^{125}I]ET-1 binding with an IC_{50} of 0.32 nM. These results suggest that 35-45% of the ET receptors in denuded rabbit pulmonary artery are ET_A (BQ-123-sensitive, S6c-insensitive), and 55-65% are ET_B (BQ-123-insensitive, S6c-sensitive(Fig. 5).

DISCUSSION

The aim of this study was to demonstrate that ET_A and ET_B endothelin receptor subtypes coexisted on vascular smooth muscle and that both could mediate contraction independently. To do this a method of eliminating ET_B mediated contractile activity was developed so that ET_A receptor activity could be studied independently.

In denuded rabbit pulmonary arteries, S6c was slightly more potent than ET-1 with comparable maximal activity. A previous study produced similar results with S6c in rabbit pulmonary arteries and also demonstrated nanomolar affinity by S6c for (^{125}I)ET-1 binding sites in this same tissue (2). Since S6c has been characterized as a highly selective ligand for the ET_B receptor (IC_{50} ET_B:ET_A, >100,000) (10), it seems probable that the contractile activity by S6c in rabbit pulmonary artery occurs via ET_B receptors. Both studies used denuded rabbit pulmonary arteries making the vascular smooth muscle, as opposed to the endothelium, the likely site for the receptors mediating the ET_B contractile activity.

Further evidence for a role of ET_B receptors in the rabbit pulmonary artery was provided by our results with the ET_A selective antagonist, BQ-123. Pretreatment with BQ-123 at 10.0 μM significantly attenuated the maximal activity of ET-1, but did not produce a parallel shift in the ET-1 response curve. In contrast, BQ-123 produced parallel shifts in ET-1 response curves at 1.0 μM in rat aorta (3) and rabbit carotid arteries (4), and at 10.0 μM BQ-123 completely blocked the contractile activity of ET-1 in rabbit carotid arteries (4). The contractile activity by ET-1 in rabbit pulmonary artery, despite ET_A blockade, is consistent with the presence of non-ET_A receptors in this tissue.

The biphasic effect BQ-123 had on ET-1 contractile activity, along with the reduction in maximal activity, suggested a contractile role for ET_A receptors in rabbit pulmonary artery. To specifically study this, we desensitized the ET_B receptor with prolonged exposure to S6c. S6c at 0.01 μM produced a transient increase in tension that gradually returned to near baseline levels in approximately 40 minutes. Thereafter, the vessels were insensitive (no contractile activity) to further increases in S6c (0.3 to 1.0 μM). This desensitization of the ET_B receptor in the rabbit pulmonary artery (coupled to contraction) may be similar to the tachyphylaxis reported for ET_B receptor on the vascular endothelium (coupled to relaxation) following repeated exposure to ET-1 (11).

Although desensitized rabbit pulmonary arteries would no longer respond to S6c, they would respond to ET-1 with full contractile activity. The EC_{50} values for ET-1 in S6c pretreated rings were not significantly different than the EC_{50} values from control rings. Furthermore, the maximal activity to ET-1 was comparable between S6c pretreated and control rings. If the ET_B receptor was desensitized by S6c pretreatment, then the activity by ET-1 was mediated through yet another receptor. With the use of BQ-123, we tested the likely possibility that the other receptor was ET_A. In S6c desensitized arteries, BQ-123 (1.0 and 10.0 μM) produced concentration-dependent rightward shifts in ET-1 response curves demonstrating functional antagonism. These results indicate that ET_A receptors can mediate ET-1 contractile activity in denuded rabbit pulmonary arteries.

To confirm the presence of both ET_A and ET_B receptors in rabbit pulmonary arteries denuded of endothelium, competition receptor binding experiments were performed with [^{125}I]ET-1. We found that 35-45% of the ET receptors are ET_A (BQ-123 sensitive, S6c-insensitive), and 55-65% are ET_B (BQ-123-insensitive, S6c-sensitive). Pervious studies using endothelium-intact rabbit pulmonary arteries demonstrated little or no presence of ET_A receptor activity (2). In addition to using endothelium-intact arteries, differences in binding reaction conditions (e.g., absence of BSA, presence of 1.0 μM ET-1 to define non-specific binding versus. 0.1 μM ET-1) may have lessened the apparent presence of ET_A receptors.

Several recent papers have shown that ET_B receptors may be responsible for mediating ET-1 vasoconstrictor/contractile activity in certain tissues (2-8). Typically, the results come from experiments with selective ET_B agonists and/or ET_A selective antagonists, much like the initial experiments in this study. However, the implications of our results in ET_B desensitized vessels raises the possibility that ET_A receptors - in addition to ET_B receptors, mediate the vasoconstrictor/contractile activity by ET-1 within these tissues. In addition, desensitization of ET_B receptors activity, if not regarded, may confound the results of those studies involving prolonged exposure to ET_B agonist.

We conclude that ET_A and ET_B receptors coexist on the vascular smooth muscle of rabbit pulmonary artery and that both receptor subtypes mediate contractile activity. In addition, the contractile activity mediated by the ET_B receptor develops tachyphylaxis with prolonged exposure to agonist.

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