Published in final edited form as:
Vascul Pharmacol. 2008 July ; 49(1): 6–13. doi:10.1016/j.vph.2008.03.005.

Pharmacological endothelin receptor interaction does not occur in veins from ET_B receptor deficient rats

Keshari Thakali[a], **James J. Galligan**[a], **Gregory D. Fink**[a], **Cheryl E. Gariepy**[b], and **Stephanie W. Watts**[a]

a *Department of Pharmacology and Toxicology, Michigan State University, B445 Life Sciences Bldg, East Lansing, MI, 48823 USA*

b *Department of Pediatrics and Communicable Disease, University of Michigan, Rm A520B, 1150 W. Medical Center Drive, Medical Science Research Building I, Ann Arbor, MI 48108 USA*

Abstract

Heterodimerization of G-protein coupled receptors can alter receptor pharmacology. ET_A and ET_B receptors heterodimerize when co-expressed in heterologous expression lines. We hypothesized that ET_A and ET_B receptors heterodimerize and pharmacologically interact in vena cava from wild-type (WT) but not ET_B receptor deficient (sl/sl) rats. Pharmacological endothelin receptor interaction was assessed by comparing ET-1-induced contraction in rings of rat thoracic aorta and thoracic vena cava from male Sprague Dawley rats under control conditions, ET_A receptor blockade (atrasentan, 10 nM), ET_B receptor blockade (BQ-788, 100 nM) or ET_B receptor desensitization (Sarafotoxin 6c, 100 nM) and ET_A plus ET_B receptor blockade or ET_A receptor blockade plus ET_B receptor desensitization. In addition, similar pharmacological ET receptor antagonism experiments were performed in rat thoracic aorta and vena cava from WT and sl/sl rats. ET_A but not ET_B receptor blockade or ET_B receptor desensitization inhibited aortic and venous ET-1-induced contraction. In vena cava but not aorta, when ET_B receptors were blocked (BQ-788, 100 nM) or desensitized (S6c, 100 nM), atrasentan caused a greater inhibition of ET-1-induced contraction. Vena cava from WT but not sl/sl rats exhibited similar pharmacological ET receptor interaction. Immunocytochemistry was performed on freshly dissociated aortic and venous vascular smooth muscle cells to determine localization of ET_A and ET_B receptors. ET_A and ET_B receptors qualitatively co-localized more strongly to the plasma membrane of aortic compared to venous vascular smooth muscle cells. Our data suggest that pharmacological ET_A and ET_B receptor interaction may be dependent on the presence of functional ET_B receptors and independent of receptor location.

1. Introduction

Veins maintain responsiveness while arteries lose responsiveness to the vasoactive hormone endothelin-1 (ET-1) in situations of exposure to ET-1 [eg. hypertension (Watts et al. 2002) and experimental protocols (Thakali et al. 2004)]. Also, many veins have contractile ET_B receptors while most arterial beds do not (Watts et al. 2002; Thakali et al. 2004; Perez-Rivera et al. 2005). Thus, receptors for ET-1 – the G-protein coupled ET_A and ET_B receptors – may function

Corresponding author (and present address): Keshari Thakali, PhD Dept. of Pharmacology and Toxicology University of Arkansas for Medical Sciences 4301 W. Markham St., Slot 611 Little Rock, AR 72205 Phone: (501) 686-7146 Fax: (501) 686-5521 Email: KMThakali@uams.edu.

differently in arteries and veins. Several reports suggest "cross-talk" occurs between ET_A and ET_B receptors, meaning that activation of one receptor subtype alters the function of the other receptor subtype. For example, in rabbit jugular and saphenous veins and hamster aorta (vessels with contractile ET_B receptors), ET_A receptor blockade alone did not inhibit ET-1-induced contraction. Only when ET_B receptors in these vessels were selectively desensitized with sarafotoxin 6c (S6c), an ET_B selective agonist, was ET-1-induced contraction sensitive to ET_A receptor blockade. In vessels lacking contractile ET_B receptors, like the rat aorta and rabbit carotid artery, ET_B receptor desensitization did not alter ET_A receptor blockade of ET-1-induced contraction (Lodge et al. 1995). Functional endothelin receptor "cross-talk" or interaction has also been observed in mouse mesenteric veins but not arteries (Perez-Rivera and Galligan 2005), renal afferent but not efferent arterioles (Inscho et al. 2006), and pulmonary arteries (Sauvageau et al. 2006).

While heptahelical receptors canonically interact with G-proteins in a 1:1 ratio, G-protein coupled receptor (GPCR) dimerization (hetero- or homo-) also occurs, potentially affecting pharmacological receptor properties such agonist affinity, potency and efficacy, as well as receptor trafficking and internalization (Bulenger et al. 2005; Maggio et al. 2005; Milligan et al. 2005; Prinster et al. 2005). Human ET_A and ET_B receptors constitutively heterodimerize when over-expressed in HEK-293 cells (Gregan et al. 2004) and ET_A and ET_B receptor co-expression in HEK-293 cells is required for trafficking and membrane expression of ET_B receptors (Dai and Galligan 2006). Evidence for GPCR dimerization has been well characterized in over-expression systems but there is a paucity of data examining GPCR dimerization in physiologically relevant systems, such as the vasculature. ET_B receptor deficient rats were derived from the spotting lethal rat, which carries a natural 301 base pair deletion of the ET_B receptor gene encoding the first and second transmembrane domains of the receptor. Since homozygous spotting lethal rats develop aganglionic megacolon and die shortly after birth, the human dopamine-β-hydroxylase promoter was introduced to drive ET_B receptor expression primarily in the neonatal enteric nervous system, but also in other catecholaminergic nerves (Gariepy et al, 1996; Gariepy et al, 1998). We hypothesized that ET_A and ET_B receptors physically interact *via* receptor heterodimerization in vena cava from wild-type (WT) but not ET_B receptor deficient rats and this heterodimerization functionally affects venous ET_A and ET_B receptor pharmacology.

2. Methods

2.1 Isolated tissue bath protocol

All animal studies were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* at Michigan State University. Thoracic aorta and vena cava were removed from deeply anesthetized male Sprague-Dawley rats (SD), male homozygous ET_B receptor deficient rats (sl/sl) and their male wildtype litter mates (WT) (200-250 g) [pentobarbital (50 mg/kg, *i.p.*)] and placed in physiological salt solution (PSS) containing (in mM): NaCl, 130; KCl, 4.7; KH_2PO_4, 1.18; $MgSO_4$ $7H_2O$, 1.17; $CaCl_2$ $2H_2O$, 1.6; $NaHCO_3$, 14.9; dextrose, 5.5; and $CaNa_2EDTA$, 0.03 (pH 7.2). Vessels were cleaned of fat, and rings (3-4 mm) of aorta and vena cava were prepared for measurement of isometric tension as described previously (Thakali et al. 2006). Briefly, rings of aorta and vena cava were placed between two wire hooks; one hook was attached to a stationary glass rod, the other was connected to a force transducer for measurement of isometric contraction. Passive tension was pulled (aorta: 4000 mg; vena cava: 1000 mg) and vessels were equilibrated for one hour in warmed (37°C), aerated (95% O_2, 5% CO_2) PSS, with frequent buffer changes. Tissue viability was assessed by contraction to an adrenergic agonist (aorta: phenylephrine, 10 μM; vena cava: norepinephrine, 10 μM). Norepinephrine was used to contract vena cava because phenylephrine did not reproducibly contract vena cava and phenylephrine was used to contract aorta such that comparisons to past

experiments could be made. Endothelial integrity was confirmed by greater than 80% relaxation to acetylcholine (1 μM) in aorta contracted with phenylephrine (10 nM) and vena cava contraction with norepinephrine (10 μM).

2.1.1—In receptor desensitization studies, vessels were incubated with vehicle (water), ET_A receptor antagonist (atrasentan, 10 nM) plus vehicle, ET_B receptor agonist (S6c, 100 nM) or ET_A receptor antagonist plus ET_B receptor agonist (atrasentan + S6c) for one hour and then cumulative concentration response curves to ET-1 (10 pM – 100 nM) were performed. To confirm that the ET_B receptor desensitization protocol actually desensitized ET_B receptors, vena cava were incubated with S6c (100 nM) for one hour without washing and then challenged again with S6c (100 nM) or norepinephrine (10 μM).

2.1.2—In receptor antagonism studies, vessels were incubated with vehicle (0.0001% DMSO), ET_A receptor antagonist (atrasentan, 10 nM) plus vehicle, BQ-788 (100 nM, ET_B receptor antagonist, solubilized in vehicle), or ET_A plus ET_B receptor antagonists [atrasentan (10 nM) + BQ-788 (100 nM)] for one hour, and cumulative concentration response curves to ET-1 (10 pM - 100 nM) were performed. The selective ET_A receptor antagonist atrasentan, also known as ABT627, binds ET_A and ET_B receptors with an IC_{50} of 0.055 nM and 84.8 nM, respectively (Wu-Wong *et al*, 2002), while the ET_B selective antagonist BQ788 binds ET_A and ET_B receptors with an IC_{50} of 1300 nM and 1.2 nM, respectively (Ishikawa *et al*, 1994). The concentrations of atrasentan (10 nM) and BQ788 (100 nM) were chosen to selectively block ET_A and ET_B receptors, respectively.

2.2 Western blot analysis

Rat thoracic aorta and vena cava were isolated, dissected, cleaned and then snap-frozen in liquid nitrogen. Vessels were homogenized and protein isolated as previously described (Watts et al. 2002). Fifty micrograms of total protein were loaded on 10% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to PVDF, membranes blocked in 5% milk overnight and incubated with anti-ET_B receptor antibody (1:200 in 5% milk + 0.025% sodium azide, Alomone Labs) overnight. After rinsing blots in tris-buffered saline (+0.5% Tween-20), blots were incubated with an anti-rabbit secondary antibody (1:1000) and developed using standard chemiluminescence protocols.

2.3 Dissociation of vascular smooth muscle cells

Rat thoracic aorta and vena cava were isolated, dissected and cleaned in chilled dissociation solution containing (in mM): NaCl, 136; $MgCl_2$, 1; Na_2HPO_4, 0.42; NaH_2PO_4, 0.43; $NaHCO_3$, 4.2; HEPES, 10; sodium nitroprusside, 8.72 and bovine serum albumin, 1 mg/mL (pH 7.4 with NaOH). The entire vena cava and a 4-5 millimeter ring of aorta were cut into small pieces and equilibrated at room temperature for 10 minutes in fresh dissociation solution. Vessels were then incubated in an enzymatic solution (dissolved in dissociation solution) containing papain (26 U/mL) and dithiothreitol (1 mg/mL) (45 minutes with shaking at 37°C). Then vessels were incubated in a second enzymatic solution (dissolved in dissociation solution) containing collagenase (1.95 U/mL), elastase (0.15 mg/mL) and soybean trypsin inhibitor (1 mg/mL) (aorta: 35 minutes; vena cava: 45 minutes with shaking at 37°C). The digestion solution was carefully pulled off (leaving the tissue and cells in the tube) and fresh, cold dissociation solution was added. Cells were placed on ice for five minutes, the dissociation solution was discarded and cells were rinsed again with fresh, cold dissociation solution. The second wash of dissociation solution was gently pipetted off and cells were suspended and triturated (forcefully pipetted approximately 10 times) in OptiMEM (Invitrogen) (plus sodium nitroprusside, 872 nM) to dissociate vascular smooth muscle cells from the blood vessel matrix.

2.4 Immunocytochemistry in freshly dissociated vascular smooth muscle cells

Two hundred microliters of freshly dissociated vascular smooth muscle cells (in OptiMEM) were placed on poly-lysine (50 μg/mL) coated coverslips (12 mm) and allowed to adhere for 45 minutes (37°C, 4% CO_2). Some cells were stimulated with ET-1 (100 nM), which was added while cells were adhering to coverslips. Cells were fixed in Zamboni's fixative (20 minutes, room temperature), permeabilized with Triton-X 100 (0.5%, 20 minutes) and incubated with ImageiT signal enhancer (30 minutes, 37°C, Invitrogen). Coverslips were incubated with primary antibodies (ET_A: anti-sheep, Fitzgerald Industries; ET_B: anti-rabbit, Alomone Laboratories; pan-cadherin: anti-mouse, Sigma; 1:200 dilution in phosphate buffered saline, 0.5% Triton-X 100) for 2 hours (37°C). Coverslips were then incubated with secondary antibodies (Alexa555 anti-rabbit, 1:200; Alexa488 anti-sheep, 1:200; Alexa633 anti-mouse, 1:200; Invitrogen) for 1 hour (37°C). Coverslips were mounted on slides with ProFound anti-fade mounting media (Invitrogen). Confocal images (stacks of 6 micron slices, image resolution = 512×512 pixels) were captured at the Center for Advanced Microscopy at Michigan State University on a Zeiss confocal microscope.

2.5 Data analysis and statistical procedures

Contractility data are presented as mean ± standard error of the mean as a percentage of the initial response to PE/NE (10 μM) for the number of vessels (N) in parentheses. Agonist EC_{50} values (representing the logarithm of agonist concentration required to cause half-maximal contraction) were calculated using a nonlinear regression analysis (GraphPad Prism, San Diego, CA). When clear maximal responses were not obtained, EC_{50} values were considered estimates with the true EC_{50} value being equal to or greater than the calculated value. When comparing two groups, the appropriate Student's *t*-test was used and when comparing three or more groups, one-way ANOVA and Bonferroni's post-hoc test was performed. In all cases, a *P* value less than or equal to 0.05 was considered statistically significant. Immunocytochemical analysis of receptor co-localization was qualitatively determined because though confocal imaging parameters were kept constant, experiments were performed on separate days, giving an inherent variability in fluorescence intensity.

2.6 Chemicals

Phenylephrine and norepinephrine were solubilized in water and purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ET-1 and S6c were obtained from Peninsula Laboratories (Belmont, CA, U.S.A.) and solubilized in deionized water. BQ-788 was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.) and was solubilized in dimethylsulfoxide (DMSO). Atrasentan (DMSO) was a gift from Abbott Laboratories.

3. Results

3.1 Functional endothelin receptor interaction occurs in vena cava but not aorta

To establish an experimental protocol for examining functional endothelin receptor interaction, ET_A receptor blockade (atrasentan, 10 nM) was compared when ET_B receptors were unbound (control) or agonist-bound (S6c-desensitized) (results summarized in Table 1) in thoracic aorta and vena cava from male Sprague Dawley rats, as negative and positive examples of functional receptor interaction, respectively. In vena cava, a 1-hour S6c (100 nM) incubation prevented constriction to an additional S6c (100 nM) challenge, but not constriction to norepinephrine (10 μM) (Fig 1), confirming that this protocol desensitized ET_B receptors without reducing vascular reactivity to other agonists. Aorta did not contract to S6c (100 nM). In aorta with unbound ET_B receptors (control) compared to aorta with agonist bound ET_B receptors (S6c-desensitized), ET_A receptor antagonism (atrasentan, 10 nM) of ET-1-induced contraction was not different from aorta with agonist-bound ET_B receptors (S6c-desensitized) (Fig 2a, Table

1). In vena cava with agonist-bound ET_B receptors (S6c-desensitized) compared to vena cava with unbound ET_B receptors (control), ET_A receptor blockade caused a significantly greater rightward shift in ET-1-induced contraction (control vena cava: no rightward shift in estimated EC_{50} values; S6c-desensitized vena cava: 3.4-fold rightward shift) (Fig 2b, Table 1).

As another way of examining functional ET receptor interaction, cumulative ET-1 concentration response curves were performed in thoracic aorta and vena cava from male Sprague Dawley rats in the presence of vehicle (0.001% DMSO), ET_A receptor blockade (atrasentan, 10 nM), ET_B receptor blockade (BQ-788, 100 nM) or ET_A plus ET_B receptor blockade (atrasentan plus BQ-788) (results summarized in Table 2). In aorta with and without ET_B receptor blockade, atrasentan caused a similar inhibition of ET-1-induced contraction (Fig 3a). In vena cava without ET_B receptor blockade, atrasentan caused a 1.7-fold rightward shift in estimated EC_{50} values that was significantly less than the 11.1-fold rightward shift induced by atrasentan in the presence of ET_B blockade (Fig 3b).

To further demonstrate a critical role for functional ET_B receptors in pharmacological endothelin receptor interaction, we compared ET_A receptor inhibition of ET-1-induced contraction in aorta and vena cava from male homozygous ET_B receptor deficient rats (sl/sl) and their male wild-type littermates (WT) (results summarized in Tables 3 and 4, respectively). Western blot analysis of ET_B receptor protein expression demonstrates that aorta and vena cava from both WT and sl/sl rats express ET_B receptors (Fig 4a). We confirmed that vena cava from sl/sl rats lacked functional ET_B receptors by lack of contraction to the ET_B receptor agonist S6c (100 nM) (Figure 4b), while venous norepinephrine (10 μM)-induced contraction was not different from WT and sl/sl rats (WT: 142 ± 22 mg; sl/sl: 103 ± 4 mg). In vena cava (Fig 5a) but not aorta (Fig 6a) from WT rats, ET_A receptors were more sensitive to receptor blockade when ET_B receptors were blocked (BQ788, 100 nM) (rightward shift in ET-induced contraction - WT vena cava: atrasentan, 1.1-fold; atrasentan plus BQ788, 2.4 fold). In both vena cava and aorta from sl/sl rats (Fig 5b and 6b, respectively), the presence or absence of ET_B receptor blockade had no effect on efficiency of ET_A receptor blockade (rightward shift in ET-induced contraction – sl/sl vena cava: atrasentan, 2.1-fold; atrasentan plus BQ788, 1.1-fold; sl/sl aorta: atrasentan, 3.7-fold; atrasentan plus BQ788, 4.4-fold).

3.2 Immunocytochemical localization of ET_A and ET_B receptors

We hypothesized that functional venous ET receptor interaction occurred because ET_A and ET_B receptors heterodimerize at the plasma membrane of venous vascular smooth muscle cells (VSMCs). We used immunocytochemical analysis to localize ET_A and ET_B receptors on freshly dissociated aortic and venous VSMCs. Surprisingly, ET_A and ET_B receptors qualitatively co-localized more strongly with cadherin, a plasma membrane marker, in aortic compared to venous VSMCs under basal conditions (Fig 7a and 8a, respectively). ET_B receptors on aortic and venous VSMCs were also present in intracellular compartments, though we did not specifically identify these compartments. ET-1 (100 nM) stimulation (applied when cells were adhering to coverslips) did not induce internalization of either ET_A or ET_B receptors on the plasma membrane of aortic and venous VSMCs and actually appeared to intensify aortic ET_A and ET_B receptor co-localization (Fig 7b and 8b, respectively).

Another technique commonly used to evaluate receptor dimerization is receptor co-immunoprecipitation. Due to technical limitations, we were unable to co-immunoprecipitate ET_A and ET_B receptors from arterial or venous lysates.

4. Discussion and Conclusions

4.1 Pharmacological ET_A and ET_B receptor interaction

We hypothesized that ET_A and ET_B receptors heterodimerize in rat thoracic vena cava from wild-type but not ET_B receptor deficient rats, and that this receptor heterodimerization would alter ET_A and ET_B receptor pharmacology. ET_A and ET_B receptor heterodimerization provides one reasonable explanation for why veins have a continued response to ET-1 in hypertension, as opposed to arteries, and do not desensitize to the magnitude arteries do when exposed acutely to ET-1. Contractility data comparing ET_A receptor function when ET_B receptors were desensitized, blocked or non-functional demonstrated that in rat thoracic vena cava, functional ET_B receptors were capable of altering ET_A receptor pharmacology. Venous ET_A receptor blockade inhibited ET-1-induced contraction to a larger degree when ET_B receptors were blocked, desensitized or non-functional (because of genetic disruption). Functional ET_A and ET_B receptor cross-talk occurs in other vessels including rat mesenteric veins (Claing et al. 2002), rat isolated small mesenteric resistance arteries (Mickley et al. 1997), mouse mesenteric veins (Perez-Rivera et al 2005), hamster saphenous and jugular veins (Lodge et al. 1995), pulmonary arteries (Sauvageau et al. 2006), and renal afferent arteries (Inscho et al. 2006). All of these vessels, like the vena cava from wild-type rats, possess contractile ET_A and ET_B receptors, suggesting that pharmacological ET_A and ET_B receptor interaction may require the presence of contractile ET_B receptors.

We found it surprising that aorta and vena cava from homozygous ET_B receptor deficient (sl/sl) rats expressed ET_B receptor protein as detected by Western blot analysis. Spotting lethal rats, have a natural deletion of the first exon of the ET_B receptor, which encodes for transmembrane domains 1 and 2. Because these rats lack functional ET_B receptors during development, they fail to develop an enteric nervous system and die at birth. Gariepy *et al* (1996 and 1998) created a line of transgenic rats in which a transgene containing the ET_B receptor driven by a dopamine β hydroxylase (DβH) promoter was inserted into the DNA of spotting lethal rats. Thus, tissues that express DβH – primarily nerves – also express ET_B receptors and the rats develop a normal enteric nervous system. It is possible that the protein detected in our Western blots could represent DβH-driven ET_B receptor expression in sympathetic nerves innervating the aorta and vena cava. It is also possible that the mutant ET_B receptor, though it lacks 301 base pairs of exon 1, is still transcribed into mRNA and translated into (nonfunctional) protein. The Alomone ET_B receptor antibody used in our Western blot experiments targets amino acids 298-314 in the third intracellular loop of the receptor, a region that is not deleted in the mutant ET_B receptor. It is also possible that the mutant ET_B receptor expressed in sl/sl rats could still physically interact with ET_A receptors, as the mutant ET_B receptor lacks only the first and second transmembrane domains (Gariepy et al. 1996; Gariepy et al. 1998). However, our pharmacological data demonstrates that though both aorta and vena cava from ET_B receptor deficient rats express ET_B receptors, these ET_B receptors do not couple to contraction, raising the possibility that if ET_A and ET_B receptors do physically interact, the 1^{st} and 2^{nd} transmembrane domains of the ET_B receptor might be important for this interaction.

It is important to note that ET_B receptors located on endothelial cells couple to nitric oxide release and vascular relaxation (Hirata et al. 1993). When ET-1 is administered in vivo, endothelial ET_B receptors mediate a transient decrease in blood pressure followed by smooth muscle ET_A receptor-mediated increase in blood pressure (Kedzierski and Yanagisawa 2001). While we have not observed a biphasic response (transient relaxation followed by a prolonged contraction) to ET-1 in aorta or vena cava when performing cumulative concentration response curves (Watts et al. 2002) or challenge with a bolus (Thakali et al 2004), we cannot exclude the possibility that endothelial ET_B receptor blockade alters ET-1-induced contraction. To our knowledge there are no endothelial ET_B receptor specific

antagonists. We observed that ET_A plus ET_B receptor blockade caused a greater inhibition or no difference in ET-1-induced contraction compared to ET_A receptor blockade alone in vena cava and aorta, respectively, suggesting that in these vessels, endothelial ET_B receptors have little effect in modulating ET-1-induced contraction.

4.2 ET_A and ET_B receptor co-localization in aortic and venous VSMCs

Gregan et al. elegantly demonstrated that ET_A and ET_B receptors heterodimerize when co-expressed in HEK293 cells, providing the first report that endothelin receptors are included among the myriad of other GPCRs that heterodimerize. Dai et al. (2006) observed that transiently transfected ET_A and ET_B receptors co-localize at the plasma membrane of HEK cells and that proper plasma membrane expression of ET_B receptors requires ET_A receptor co-expression. These data support the popular theory that for many pairs of receptor heterodimers, receptor co-expression is a necessary step in receptor processing, maturation and targeting to the plasma membrane (Prinster et al. 2005). Our immunocytochemical experiments demonstrate that both aortic and venous VSMCs express ET_A and ET_B receptors and that these receptors are present at the plasma membrane. We chose to examine ET_A and ET_B receptor co-localization in freshly dissociated VSMCs because of the significant autofluorescence in whole vessels sections and because of increased ET_B receptor expression in cultured VSMCs (Adner et al. 1998; Moller et al. 2000). Our data suggest that ET_A and ET_B receptor heterodimerization in vena cava may not account for pharmacological ET_A and ET_B receptor interaction because ET_A and ET_B receptors co-localize in both aortic and venous vascular smooth muscle cells and suggest that downstream signaling events mediated by contractile ET_B receptors are important in determining pharmacological ET_A and ET_B receptor interaction.

4.3 Limitations

Receptor dimerization is typically assessed using three methods: 1) fluorescence resonance energy transfer (FRET) to determine receptor proximity, 2) co-immunoprecipitation to determine if the receptors physically interact with each other and, 3) some measure of functional receptor interaction (Angers et al. 2002; Maggio et al. 2005; Milligan and Bouvier 2005; Prinster et al. 2005). One significant limitation of traditional confocal microscopy is that the limit of resolution is approximately 3,000 – 4,000 Å, while FRET allows resolution down to 50 – 100 Å (Wallrabe and Periasamy, 2005). However, the available tools for immunocytochemical analysis of ET receptors in freshly dissociated VSMCs are not powerful enough to make accurate FRET measurements.

We attempted to co-immunoprecipitate ET_A and ET_B receptors in the lysates of rat thoracic aorta and vena cava to determine if there was a physical interaction between ET_A and ET_B receptors. Our co-immunoprecipitation experiments were inconclusive because the heavy chain Ig band of the immunoprecipitating antibody resolved at 50 kDa, masking detection immunoprecipitated ET receptors that resolved at the same molecular weight. We tried several commercial kits from Pierce, Santa Cruz and eBioscience designed to eliminate the contaminating Ig bands, but none these kits effectively removed the contaminating Ig band (data not shown). Sauvageau et al. (2006) successfully co-immunoprecipitated ET_A and ET_B receptors from small pulmonary arteries, but in our hands (and in our samples), these same antibodies used by Sauvageau et al did not specifically bind ET_A or ET_B receptors and thus were not useful for co-immunoprecipitation. We conclude that like the FRET experiments, the available tools are inadequate for determining if ET_A and ET_B receptors co-immunoprecipitate in rat thoracic aorta and vena cava.

Most accounts of receptor dimerization have been reported in cell lines over-expressing tagged receptors. These over-expression systems are excellent tools to probe how receptor

dimerization occurs, but do not answer the fundamental question: is receptor dimerization a physiological process? A few studies have examined GPCR dimerization in blood vessels and other types of smooth muscle cells. For example, angiotension (AT_1) receptors heterodimerize with bradykinin (B_2) receptors on human omental vessels and rat renal mesangial cells and increased AT_1/B_2 receptor heterodimer formation contribute to enhanced angiotensin II reactivity in human preeclampsia and rodent models of hypertension (AbdAlla et al. 2000; AbdAlla et al. 2001; AbdAlla et al. 2005). In cultured airway smooth muscle cells and mouse tracheal rings, prostaglandin EP_1 receptors heterodimerize with β_2 adrenergic receptors (McGraw et al. 2006) and in aortic vascular smooth muscle cells, prostacyclin receptor and thromboxane A_2 receptor heterodimerization determines receptor trafficking (Wilson et al. 2007). However, in most of these studies while functional receptor interaction was observed in smooth muscle cells or blood vessels, direct evidence for receptor dimerization (FRET or co-immunoprecipitation experiments) was determined using over-expression systems. While it is possible that high receptor expression levels in heterologous expression systems compared to natively expressed receptors may artificially induce receptor dimerization, it also possible that the use of epitope-tagged receptors in heterologous expression systems provides a more sensitive method for detection of receptor heterodimers. In vena cava, pharmacological ET_A and ET_B receptor interaction occurs independently of receptor co-localization, suggesting that interaction between ET_A and ET_B receptor-activated signal transduction and not receptor heterodimerization mediate functional ET_A and ET_B receptor interaction.

4.4 Conclusions

Receptor dimerization is a nascent field in receptor characterization and has more recently been recognized as a phenomenon with potential clinical ramifications as many therapeutic drugs target G-protein coupled receptors. Contractility experiments using ET_A and ET_B receptor antagonists, ET_B receptor desensitization and ET_B receptor deficient rats demonstrated that pharmacological endothelin receptor requires functional ET_B receptors. Our data suggest that functional ET_A and ET_B receptor interaction in vena cava does not appear to be dependent on receptor co-localization and thus receptor heterodimerization, and may be due to interactions between downstream signaling events. Only with improved tools to assess receptor dimerization, like better ET_A and ET_B receptor antibodies and novel co-immunoprecipitation approaches, can we actually determine if ET_A and ET_B receptors heterodimerize when natively expressed in tissues.

Acknowledgements

This work was supported by National Heart, Lung, and Blood Institute Grant PO1 HL-70687 (to SWW, JJG and GDF) and a Ford Foundation Diversity Fellowship (to KT).

5. References

AbdAlla S, Lother H, Quitterer U. AT_1-receptor heterodimers show enhanced G-protein activation and altered receptor sequestration. Nature 2000;407:94–98. [PubMed: 10993080]

AbdAlla S, Lother H, el Massiery A, Quitterer U. Increased AT(1) receptor heterodimers in preeclampsia mediate enhanced angiotensin II responsiveness. Nat Med 2001;7:1003–1009. [PubMed: 11533702]

AbdAlla S, Abdel-Baset A, Lother H, el Massiery A, Quitterer U. Mesangial AT_1/B_2 receptor heterodimers contribute to angiotensin II hyperresponsiveness in experimental hypertension. J Mol Neurosci 2005;26:185–192. [PubMed: 16012192]

Adner M, Uddman E, Cardell LO, Edvinsson L. Regional variation in appearance of vascular contractile endothelin-B receptors following organ culture. Cardiovasc Res 1998;37:254–262. [PubMed: 9539881]

Adner M, Shankley N, Edvinsson L. Evidence that ET-1, but not ET-3 and S6b, ET_A-receptor mediated contractions in isolated rat mesenteric arteries are modulated by co-activation of ET_B receptors. Br J Pharmacol 2001;133:927–935. [PubMed: 11454667]

Angers S, Salahpour A, Joly E, Hilairet S, Chelsky D, Dennis M, Bouvier M. Detection of beta 2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). Proc Natl Acad Sci U S A 2000;97:3684–3689. [PubMed: 10725388]

Bulenger S, Marullo S, Bouvier M. Emerging role of homo- and heterodimerization in G-protein-coupled receptor biosynthesis and maturation. Trends Pharmacol Sci 2005;26:131–137. [PubMed: 15749158]

Claing A, Shbaklo H, Plante M, Bkaily G, D'Orleans-Juste P. Comparison of the contractile and calcium-increasing properties of platelet-activating factor and endothelin-1 in the rat mesenteric artery and vein. Br J Pharmacol 2002;135:433–443. [PubMed: 11815379]

Dai X, Galligan JJ. Differential trafficking and desensitization of human ET_A and ET_B receptors expressed in HEK 293 cells. Exp Biol Med (Maywood) 2006;231:746–751. [PubMed: 16740992]

Gariepy CE, Cass DT, Yanagisawa M. Null mutation of endothelin receptor type B gene in spotting lethal rats causes aganglionic megacolon and white coat color. Proc Natl Acad Sci U S A 1996;93:867–872. [PubMed: 8570650]

Gariepy CE, Williams SC, Richardson JA, Hammer RE, Yanagisawa M. Transgenic expression of the endothelin-B receptor prevents congenital intestinal aganglionosis in a rat model of Hirschsprung disease. J Clin Invest 1998;102:1092–1101. [PubMed: 9739043]

Gregan B, Jurgensen J, Papsdorf G, Furkert J, Schaefer M, Beyermann M, Rosenthal W, Oksche A. Ligand-dependent differences in the internalization of endothelin A and endothelin B receptor heterodimers. J Biol Chem 2004;279:27679–27687. [PubMed: 15075338]

Hirata Y, Emori T, Eguchi S, Kanno K, Imai T, Ohta K, Marumo F. Endothelin receptor subtype B mediates synthesis of nitric oxide by cultured bovine endothelial cells. J Clin Invest 1993;91:1367–1373. [PubMed: 7682570]

Inscho EW, Imig JD, Cook AK, Pollock DM. ET_A and ET_B receptors differentially modulate afferent and efferent arteriolar responses to endothelin. Br J Pharmacol 2005;146:1019–1026. [PubMed: 16231007]

Ishikawa K, Ihara M, Noguchi K, Mase T, Mino N, Saeki T, Fukuroda T, Fukami T, Ozaki S, Nagase T, Nishikibe M, Yano M. Biochemical and pharmacological profile of a potent and selective endothelin B-receptor antagonist, BQ-788. Proc Natl Acad Sci U S A 1994;91:4892–4896. [PubMed: 8197152]

Kedzierski RM, Yanagisawa M. Endothelin system: the double-edged sword in health and disease. Annu Rev Pharmacol Toxicol 2001;41:851–876. [PubMed: 11264479]

Lodge NJ, Zhang R, Halaka NN, Moreland S. Functional role of endothelin ET_A and ET_B receptors in venous and arterial smooth muscle. Eur J Pharmacol 1995;287:279–285. [PubMed: 8991802]

Maggio R, Novi F, Scarselli M, Corsini GU. The impact of G-protein-coupled receptor hetero-oligomerization on function and pharmacology. FEBS J 2005;272:2939–2946. [PubMed: 15955054]

McGraw DW, Mihlbachler KA, Schwarb MR, Rahman FF, Small KM, Almoosa KF, Liggett SB. Airway smooth muscle prostaglandin-EP_1 receptors directly modulate $beta_2$-adrenergic receptors within a unique heterodimeric complex. J Clin Invest 2006;116:1400–1409. [PubMed: 16670773]

Mickley EJ, Gray GA, Webb DJ. Activation of endothelin ET_A receptors masks the constrictor role of endothelin ET_B receptors in rat isolated small mesenteric arteries. Br J Pharmacol 1997;120:1376–1382. [PubMed: 9105715]

Milligan G, Bouvier M. Methods to monitor the quaternary structure of G protein-coupled receptors. FEBS J 2005;272:2914–2925. [PubMed: 15955052]

Moller S, Adner M, Edvinsson L. Increased levels of endothelin ET_B receptor mRNA in human omental arteries after organ culture: quantification by competitive reverse transcription-polymerase chain reaction. Clin Exp Pharmacol Physiol 2000;25:788–794. [PubMed: 9784917]

Perez-Rivera AA, Fink GD, Galligan JJ. Vascular reactivity of mesenteric arteries and veins to endothelin-1 in a murine model of high blood pressure. Vascul Pharmacol 2005;43:1–10. [PubMed: 15975530]

Prinster SC, Hague C, Hall RA. Heterodimerization of g protein-coupled receptors: specificity and functional significance. Pharmacol Rev 2005;57:289–298. [PubMed: 16109836]

Sauvageau S, Thorin E, Caron A, Dupuis J. Evaluation of endothelin-1-induced pulmonary vasoconstriction following myocardial infarction. Exp Biol Med (Maywood) 2005;231:840–846. [PubMed: 16741009]

Thakali K, Fink GD, Watts SW. Arteries and veins desensitize differently to endothelin. J Cardiovasc Pharmacol 2004;43:387–393. [PubMed: 15076222]

Wallrabe H, Periasamy A. Imaging protein molecules using FRET and FLIM microscopy. Curr Opin Biotechnol 2005;16:19–27. [PubMed: 15722011]

Watts SW, Fink GD, Northcott CA, Galligan JJ. Endothelin-1-induced venous contraction is maintained in DOCA-salt hypertension; studies with receptor agonists. Br J Pharmacol 2002;137:69–79. [PubMed: 12183332]

Wilson SJ, Dowling JK, Zhao L, Carnish E, Smyth EM. Regulation of thromboxane receptor trafficking through the prostacyclin receptor in vascular smooth muscle cells: role of receptor heterodimerization. Arterioscler Thromb Vasc Biol 2007;27:290–296. [PubMed: 17110599]

Wu-Wong JR, Dixon DB, Chiou WJ, Sorensen BK, Liu G, Jae HS, Tasker A, von Geldern TW, Winn M, Opgenorth TJ. Pharmacology of endothelin receptor antagonists ABT-627, ABT-546, A-182086 and A-192621: in vitro studies. Clin Sci (Lond) 2002;103:107S–111S. [PubMed: 12193066]

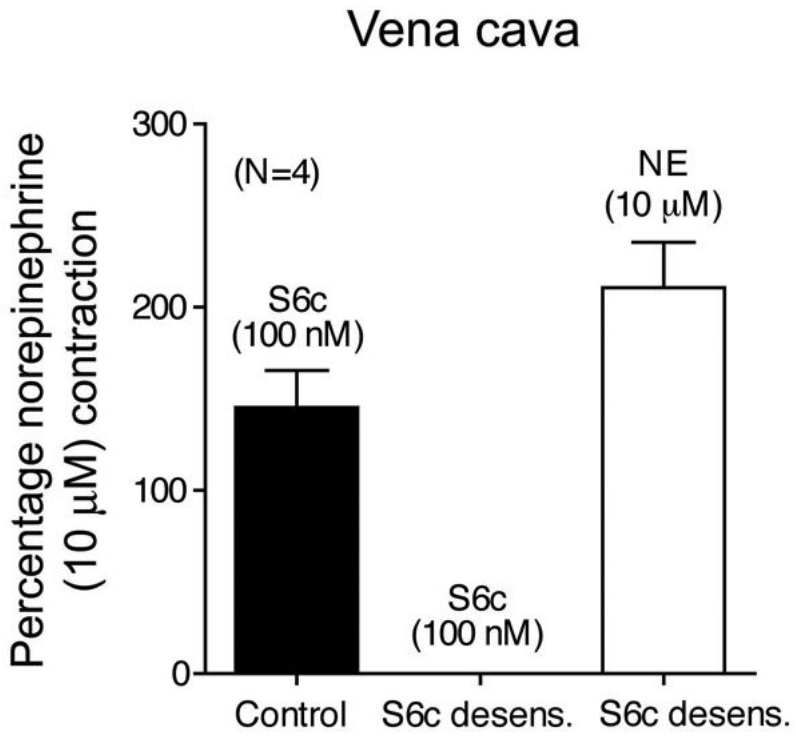

Figure 1.

S6c (100 nM) incubation for one hour desensitizes venous ET_B receptors, but does not alter the response to NE (10 μM). Vena cava were challenged with S6c (100 nM) and then one hour later were challenged with NE (10 μM) or S6c (100 nM). Data are represented as means ± S.E.M. for the number (N) of animals in parentheses. NE, norepinephrine; S6c, sarafotoxin 6c.

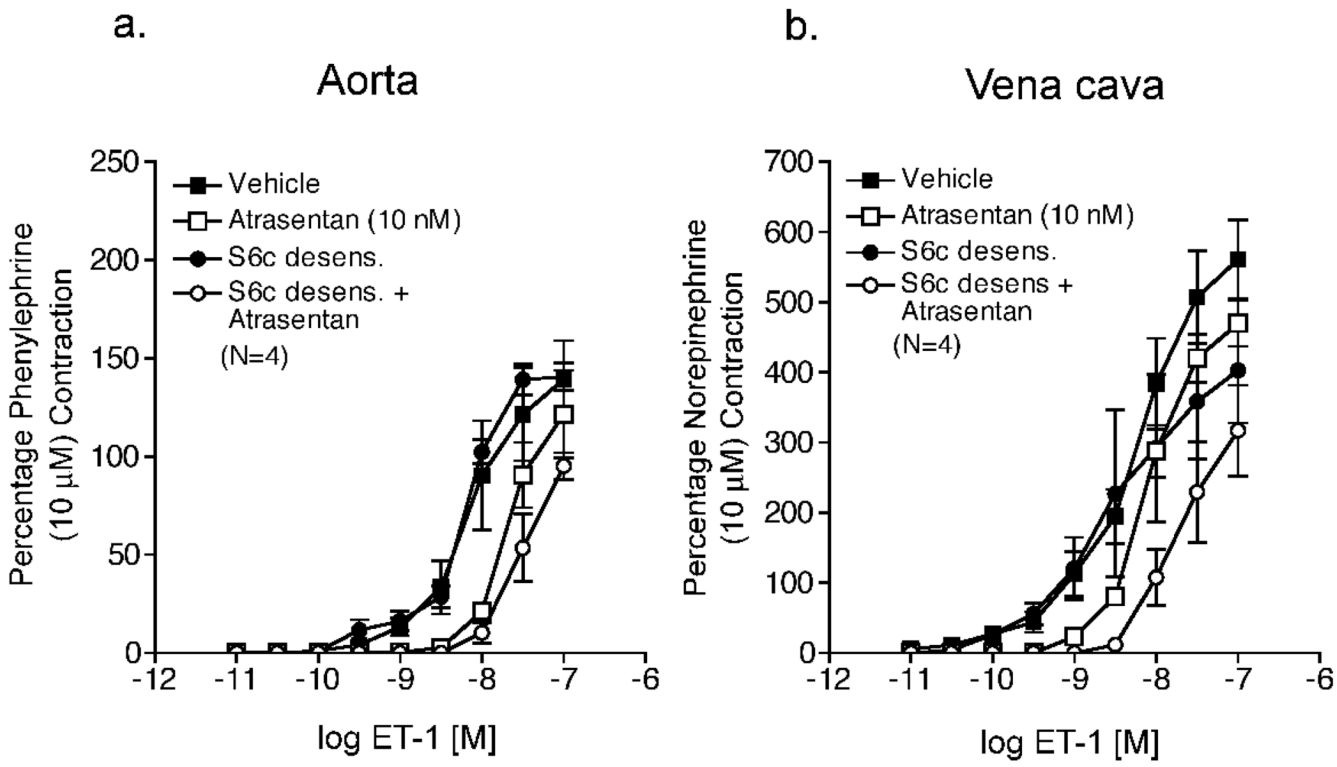

Figure 2.

S6c (100 nM) desensitization augments ET_A receptor blockade of ET-1-induced contraction in veins but not arteries. ET-1-induced contraction of aorta (a) and vena cava (b) incubated with vehicle, atrasentan (10 nM), S6c (100 nM), or S6c (100 nM) plus atrasentan (10 nM). Data are represented as means ± S.E.M. for the number (N) of animals in parentheses. S6c, sarafotoxin 6c.

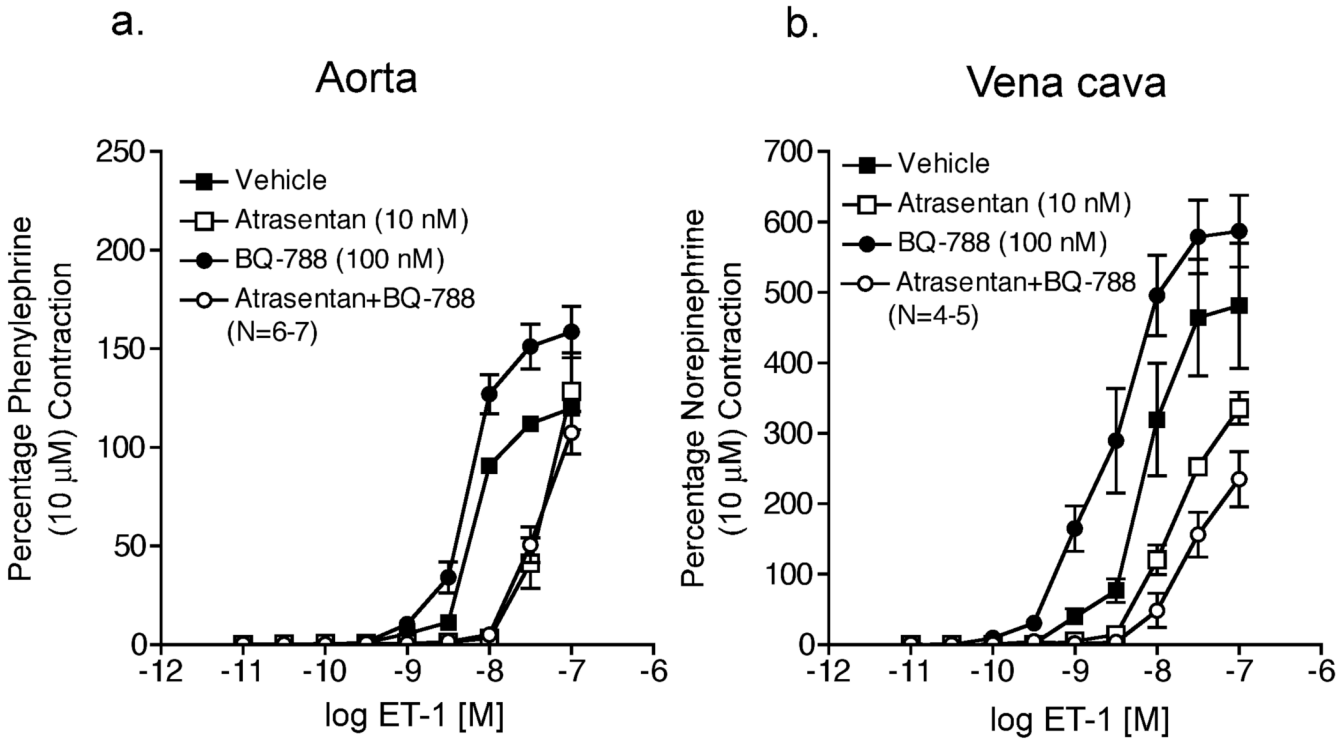

Figure 3.

ET_B receptor blockade enhances ET_A receptor blockade of ET-1-induced contraction in vena cava but not aorta. ET-1-induced contraction of aorta (a) and vena cava (b) incubated with vehicle, atrasentan (10 nM), BQ788 (100 nM), or atrasentan (10 nM) plus BQ788 (100 nM). Data are represented as means ± S.E.M. for the number (N) of animals in parentheses.

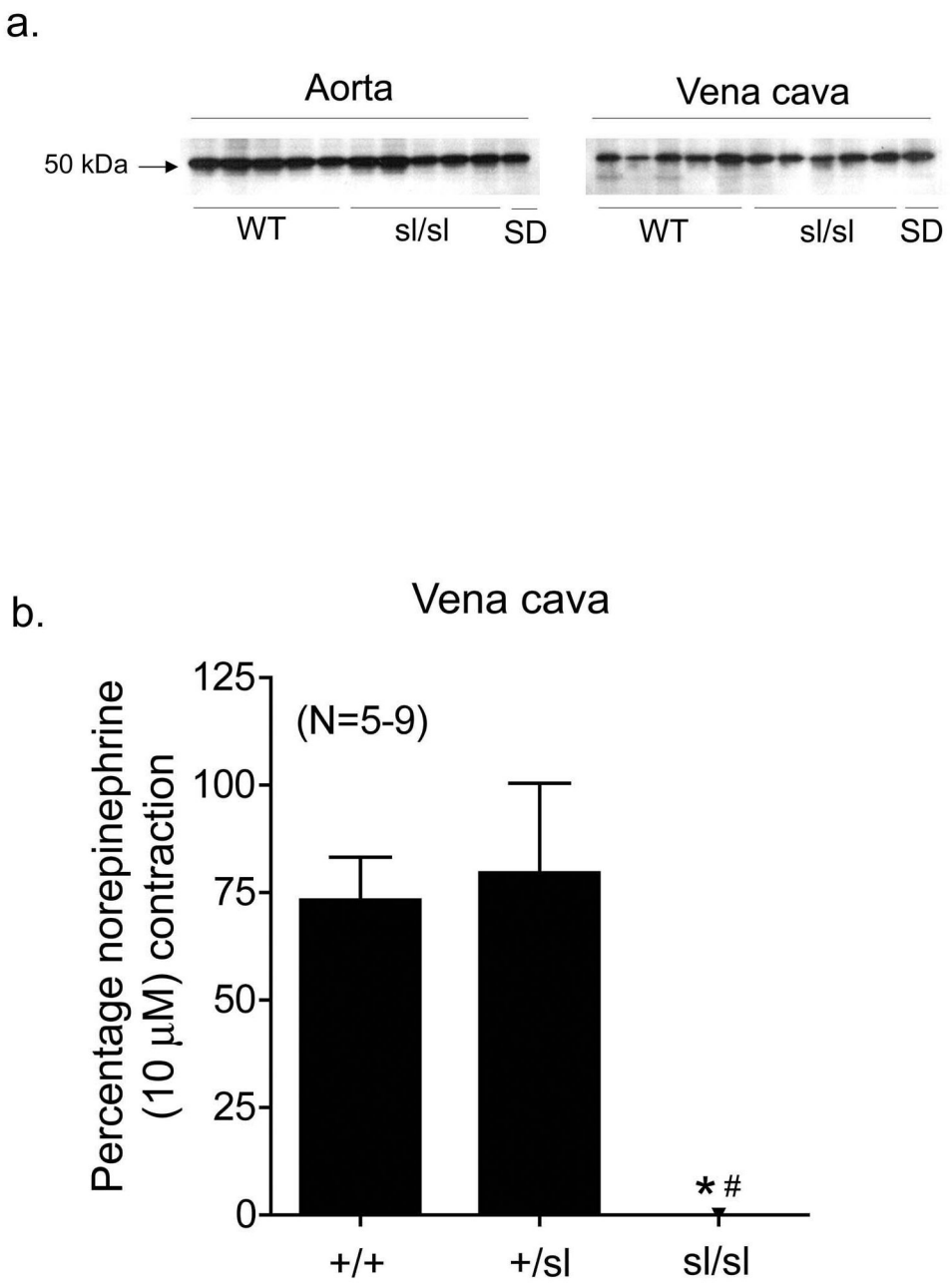

Figure 4.

Aorta and vena cava from WT and sl/sl rats express ET_B receptor protein (a). Each lane represents aortic or venous lysates from a different animal. The ET_B receptor agonist S6c (100 nM) constricts vena cava from WT and heterozygous (+/sl) rats but not vena cava from sl/sl rats (b). Data are represented as means ± S.E.M. for the number (N) of animals in parentheses. S6c, sarafotoxin 6c; sl/sl, homozygous ET_B receptor deficient; SD, Sprague Dawley; WT, wild-type.

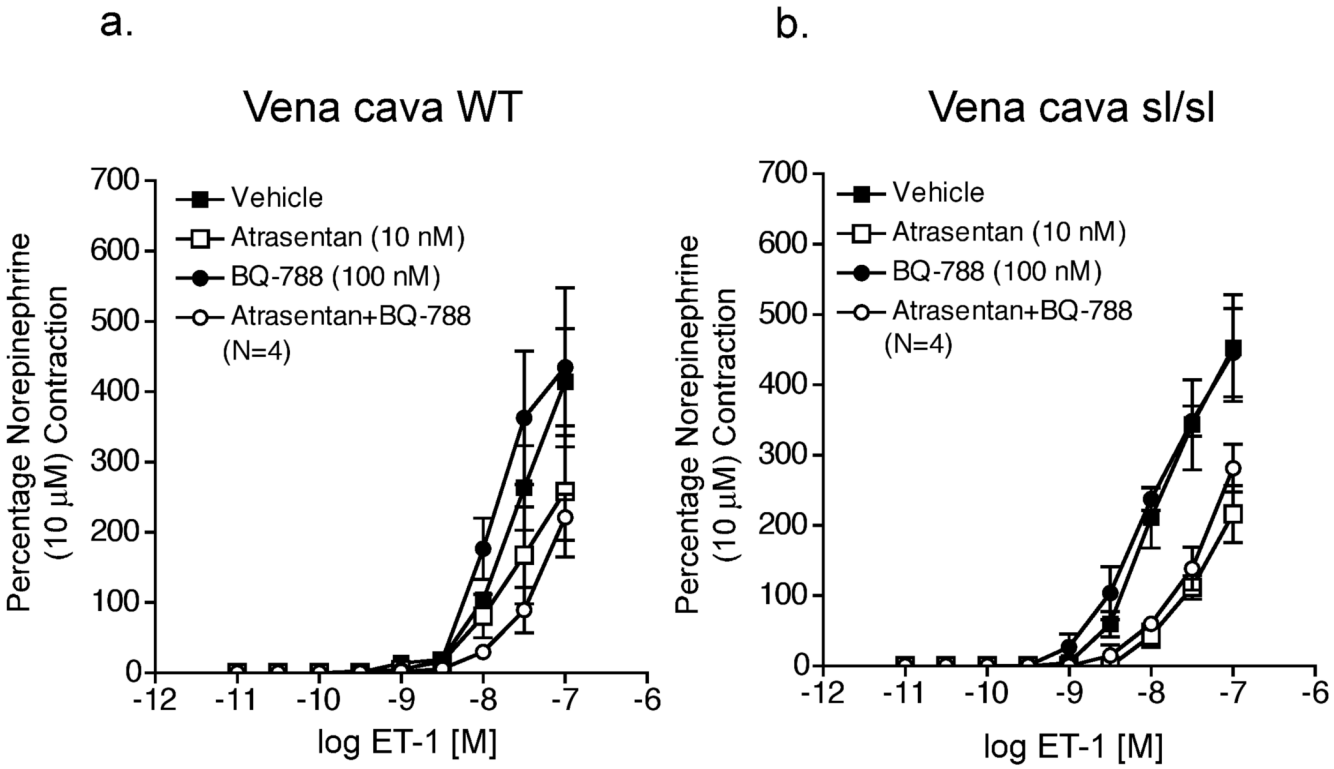

Figure 5.

ET_B receptor blockade enhanced ET_A receptor blockade of ET-1-induced contraction of vena cava from WT but not sl/sl rats. ET-1-induced contraction of vena cava from WT rats (a) and sl/sl rats (b) incubated with vehicle, atrasentan, BQ788 (100 nM), or atrasentan (10 nM) plus BQ788 (100 nM). Data are represented as means ± S.E.M. for the number (N) of animals in parentheses. sl/sl, homozygous ET_B receptor deficient; WT, wild-type.

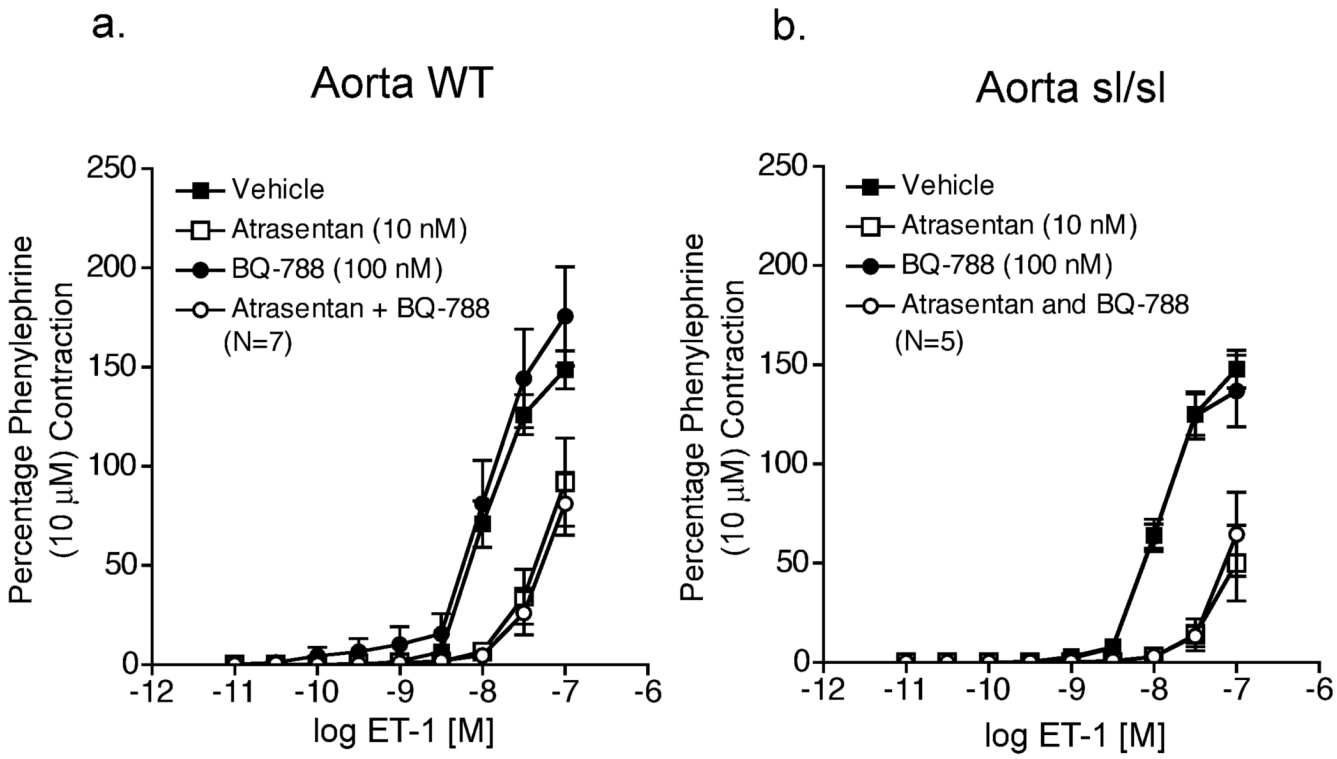

Figure 6.

ET_B receptor blockade has no effect on ET_A receptor blockade of ET-1-induced contraction of aorta from WT and sl/sl rats. ET-1-induced contraction of aorta from WT rats (a) and sl/sl rats (b) incubated with vehicle, atrasentan, BQ788 (100 nM), or atrasentan (10 nM) plus BQ788 (100 nM). Data are represented as means ± S.E.M. for the number (N) of animals in parentheses. sl/sl, homozygous ET_B receptor deficient; WT, wild-type.

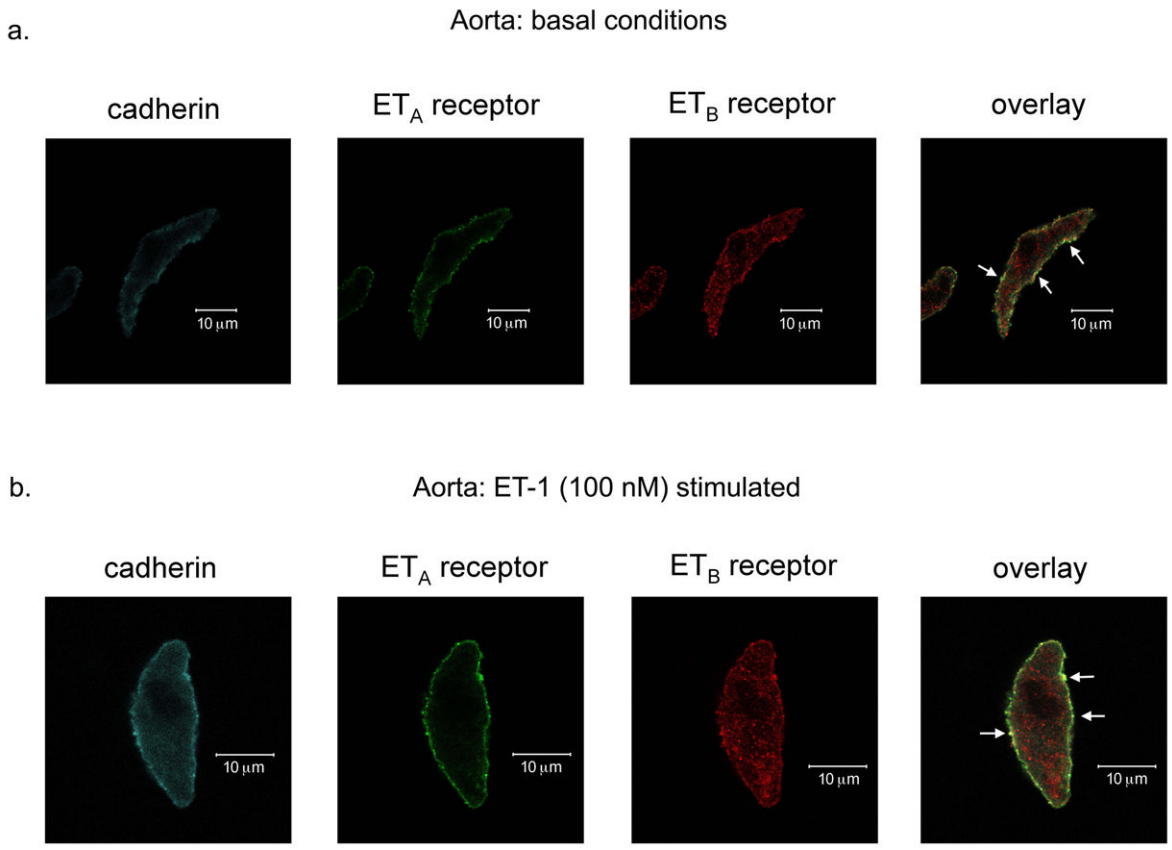

Figure 7.

ET_A and ET_B receptors co-localize to the membrane of aortic vascular smooth muscle cells. Confocal images (6 μm) of ET_A receptor, ET_B receptor and cadherin (a membrane marker) expression in freshly dissociated aortic vascular smooth muscle cells under basal conditions (a) and after ET-1 (100 nM) stimulation (b). White arrows pointing to yellow on the overlay images represent areas of co-localization of cadherin, ET_A receptors and ET_B receptors. Images are representative of aortic vascular smooth muscle cells from 5 different rats.

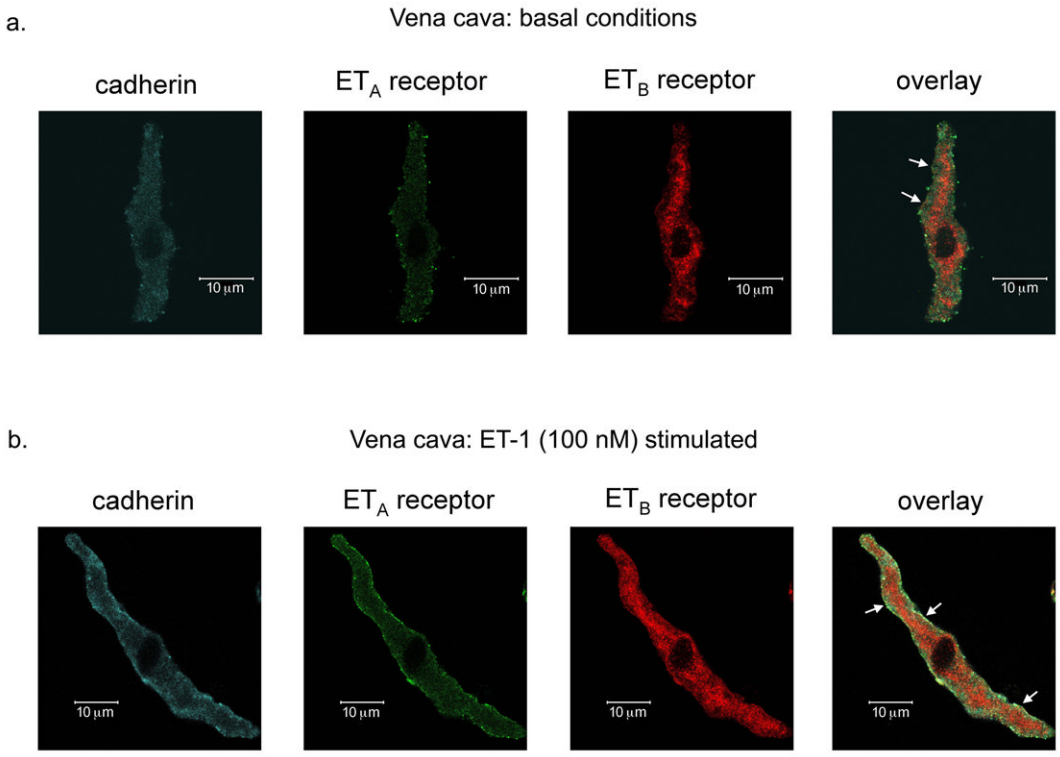

Figure 8.

ET_A and ET_B receptors co-localize to the membrane of venous vascular smooth muscle cells. Confocal images (6 μm) of ET_A receptor, ET_B receptor and cadherin (a membrane marker) expression in freshly dissociated venous vascular smooth muscle cells under basal conditions (a) and after ET-1 (100 nM) stimulation (b). White arrows pointing to yellow on the overlay images represent areas of co-localization of cadherin, ET_A receptors and ET_B receptors. Images are representative of venous vascular smooth muscle cells from 4 different rats.

Table 1

Maximum contraction and estimated EC_{50} values for ET-1 (10 pM – 100 nM) - induced contraction in rat thoracic aorta and vena cava under control conditions, ET_A receptor blockade, ET_B receptor desensitization and ET_A blockade plus ET_B receptor desensitization.

	Aorta		Vena cava	
Treatment	**Max contraction [% PE (10 μM)]**	**EC_{50} (nM)**	**Max contraction [% NE (10 μM)]**	**EC50 (nM)**
Vehicle	139±20	6.95±2.13†	561±56	10.58±4.84†
Atrasentan (10 nM)	122±22	19.90±1.12 *†	470±33	8.15±0.60†
S6c (100 nM)	141±7	7.74±1.60*	403±75*	6.68±1.90
Atrasentan (10 nM) + S6c (100 nM)	102±8 *†	32.15±13.25 *†	319±63*	22.45±4.70 *†

Data are represented as mean ± S.E.M. Atrasentan, ETA receptor antagonist; NE, norepinephrine; PE, phenylephrine; S6c, (sarafotoxin 6c) ETB receptor agonist.

* represents a statistically significant difference from control ($p<0.05$)

† represents a statistically significant difference from S6c ($p<0.05$).

Table 2

Maximum contraction and estimated EC_{50} values for ET-1 (10 pM – 100 nM) - induced contraction in rat thoracic aorta and vena cava under control conditions, ET_A receptor blockade, ET_B receptor blockade and ET_A plus ET_B receptor blockade.

	Aorta		Vena cava	
Treatment	**Max contraction [% PE (10 μM)]**	**EC_{50} (nM)**	**Max contraction [% NE (10 μM)]**	**EC_{50} (nM)**
Vehicle	120±7	7.32±0.70	481±89	9.70±2.66†
Atrasentan (10 nM)	128±19†	53.12±20.19 *†	336±28 *†	16.84±3.02 *†
BQ-788 (100 nM)	158±13*	4.97±0.78*	587±51	3.76±1.17*
Atrasentan (10 nM) + BQ-788 (100 nM)	107±11†	52.84±12.03 *†	235±39 *†	41.71±15.12 *†

Data are represented as mean ± S.E.M. Atrasentan, ETA receptor antagonist; BQ-788, ETB receptor antagonist; NE, norepinephrine; PE, phenylephrine.

*represents a statistically significant difference from control ($p<0.05$)

†represents a statistically significant difference from BQ-788 ($p<0.05$).

Table 3

Maximum contraction and estimated EC_{50} values for ET-1 (10 pM – 100 nM) - induced contraction in thoracic aorta from WT and sl/sl rats under control conditions, ET_A receptor blockade, ET_B receptor blockade and ET_A plus ET_B receptor blockade.

	Aorta WT		Aorta sl/sl	
Treatment	**Max contraction [% PE (10 μM)]**	**EC_{50} (nM)**	**Max contraction [% PE (10 μM)]**	**EC_{50} (nM)**
Vehicle	149±10	11.70±1.31	148±9	12.36±1.23
Atrasentan (10 nM)	92±22 *†	44.00±0.5 *†	50±19 *†	45.73±2.27 *†
BQ-788 (100 nM)	176±25	13.51±2.26	137±18	10.97±1.00
Atrasentan (10 nM) + BQ-788 (100 nM)	81±16 *†	45.68±3.72 *†	65±21 *†	48.56±0.83 *†

Data are represented as mean ± S.E.M. Atrasentan, ET_A receptor antagonist; BQ-788, ET_B receptor antagonist; PE, phenylephrine; WT, homozygous wildtype rats; sl/sl, homozygous ET_B receptor deficient rats.

* represents a statistically significant difference from control ($p<0.05$)

† represents a statistically significant difference from BQ-788 ($p<0.05$).

Table 4

Maximum contraction and estimated EC_{50} values for ET-1 (10 pM – 100 nM) - induced contraction in thoracic vena cava from WT and sl/sl rats under control conditions, ET_A receptor blockade, ET_B receptor blockade and ET_A plus ET_B receptor blockade.

	Vena cava WT		Vena cava sl/sl	
Treatment	**Max contraction [% PE (10 μM)]**	**EC_{50} (nM)**	**Max contraction [% NE (10 μM)]**	**EC_{50} (nM)**
Vehicle	414±76	16.98±4.24	452±76	10.82±2.93
Atrasentan (10 nM)	258±93†	18.62±2.87†	216±41 *†	22.30±5.48*
BQ-788 (100 nM)	443±88	12.39±1.55	357±46	16.10±10.21
Atrasentan (10 nM) + BQ-788 (100 nM)	221±33 *†	29.12±4.83 *†	206±27 *†	18.20±3.08*

Data are represented as mean ± S.E.M. Atrasentan, ET_A receptor antagonist; BQ-788, ET_B receptor antagonist; NE, norepinephrine; WT, homozygous wildtype rats; sl/sl, homozygous ET_B receptor deficient rats.

* represents a statistically significant difference from control ($p<0.05$)

† represents a statistically significant difference from BQ-788 ($p<0.05$).