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

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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 [e.g. 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 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 et al., 2005), renal afferent but not efferent arterioles (Inscho et al., 2005), and pulmonary arteries (Sauvageau et al., 2005).

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 as agonist affinity, potency and efficacy, as well as receptor trafficking and internalization (Bulenger et al., 2005; Maggio et al., 2005; Milligan and Bouvier, 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-

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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 wild-type 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; $MgSO_4{\cdot}7H_2O$, 1.17; $CaCl_2{\cdot}2H_2O$, 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., 2004). 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 1 h 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 1 h 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 1 h 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 1 h, 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 BQ-788 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 BQ-788 (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 mm ring of aorta were cut into small pieces and equilibrated at room temperature for 10 min 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 min 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 min; vena cava: 45 min 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 5 min, 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 min (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 min, room temperature), permeabilized with Triton-X 100 (0.5%, 20 min) and incubated with ImageiT signal enhancer (30 min, 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 h (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 h (37 °C). Coverslips were mounted on slides with ProFound anti-fade mounting media (Invitrogen). Confocal images (stacks of 6 μm slices, image resolution=512 x 512 pixels) were captured at the Center for Advanced Microscopy at Michigan State University on a Zeiss confocal microscope.