

G protein- and β -arrestin Signaling Profiles of Endothelin Derivatives at the Type A Endothelin Receptor

Xinyu Xiong,^{1,2} Nour Nazo,^{1,2} Ritika Revoori,³ Sudarshan Rajagopal,^{1,2} and Matthew A. Sparks^{3,4}

Key Points

- Endothelins activated a wide range of G proteins at the ET_AR
- Endothelin derivatives did display structure-activity relationships in their degrees of agonism
- β -arrestin 1 and 2 does not augment the diurnal blood pressure, acute, or chronic pressor response to endothelin 1

Abstract

Background Endothelin-1 (ET-1) is a potent vasoconstrictor in the cardiovascular system, an effect mediated through the type A endothelin receptor (ET_AR), a G protein-coupled receptor (GPCR). Antagonists of the ET_AR have shown promising results in randomized clinical trials. However, side effects limit widespread use. Biased agonists have been developed to mitigate the untoward effects of a number of GPCR antagonists. These agents block deleterious G-coupled pathways while stimulating protective β -arrestin pathways. The goal of this study was to test whether there was any significant ligand bias between endothelin derivatives, and whether this could have any physiologic effects in the cardiovascular system.

Methods A panel of endothelin derivatives were tested in assays of G protein signaling and β -arrestin 2 recruitment at the ET_AR. We then tested the effects of ET-1 on the vasopressor response in wild-type and β -arrestin 1 and 2 KO mice.

Results We found the endothelins activated a wide range of G proteins at the ET_AR, but none of the endothelin derivatives demonstrated significant biased agonism. Endothelin derivatives did display structure-activity relationships with regards to their degrees of agonism. β -arrestin 1 and 2 knockout mice did not display any differences to wild-type mice in the acute pressor response to ET-1, and β -arrestin 2 knockout mice did not display any blood pressure differences to wild-type mice in the chronic responses to ET-1.

Conclusions Our findings are consistent with vasoconstriction being mediated by G proteins with a lack of significant desensitization by β -arrestins at the ET_AR. These findings suggest that G protein- and β -arrestin-biased ET_AR agonists could have distinct physiologic effects from balanced agonists, although the endothelin peptide scaffold does not appear suitable for designing such ligands.

KIDNEY360 2: 1124–1131, 2021. doi: <https://doi.org/10.34067/KID.0005462020>

Introduction

Endothelins (ET) are 21-residue peptides that bind to two G protein-coupled receptors (GPCRs), type A (ET_A) and B (ET_B) receptors (ET_AR and ET_BR). ET-1 is the most potent vasoconstrictor in the human cardiovascular system, an effect that is mediated through the ET_AR (1). A number of highly selective peptide ET_AR and ET_BR antagonists and agonists have been developed at these receptors (2), along with small molecule antagonists that are used in the treatment of

pulmonary arterial hypertension (3). Recently, atrasentan, a selective ET_AR antagonist, demonstrated impressive reductions the composite primary outcome of ESKD, doubling of serum creatinine, and death from kidney failure in patients with diabetic nephropathy (4). However, the use of atrasentan is known to cause fluid retention, and necessitated the use of a unique trial design, called an enrichment period, to ensure tolerability and select those individuals with albuminuria reductions, who were randomized in the study. Thus,

¹Department of Biochemistry, Duke University, Durham, North Carolina

²Division of Cardiology, Duke University School of Medicine, Durham, North Carolina

³Division of Nephrology, Duke University School of Medicine, Durham, North Carolina

⁴Renal Section, Durham Veterans Affairs Health Care System, Durham, North Carolina

Correspondence: Dr. Matthew A. Sparks, Department of Medicine, Division of Nephrology, Duke University School of Medicine, Room 1013 MSRB2, 2 Genome Court, Durham, NC 27710. Email: matthew.sparks@duke.edu

selective ET antagonists with a diminished side effect profile are needed. GPCRs, such as the ET_AR, can signal through multiple transducers, including heterotrimeric G proteins, G protein receptor kinases, and β -arrestins. Although all of these signaling pathways downstream of a GPCR can be activated or blocked by conventional “balanced” agonists or antagonists, they can also be selectively activated by “biased agonists” (5). A biased agonist binding to a GPCR promotes a response that can result in preferential signaling through one of its downstream pathways, such as G proteins (by a G protein–biased agonist) or β -arrestins (by a β -arrestin–biased agonist), while blocking signaling through the other pathways.

At many GPCRs, G protein– and β -arrestin–mediated signaling has been shown to have biochemical and physiologic actions distinct from one another. For example, at the type 1 angiotensin II receptor (AT₁R), β -arrestin–biased agonists have been shown to promote signaling through mitogen-activated protein kinases (6), which results in antiapoptotic effects (7). In physiologic studies, an AT₁R β -arrestin–biased agonist increased cardiac performance and preserved cardiac stroke volume (8). This was associated with increased cardiac unloading, likely related to its vasodilatory properties (9), which is the opposite of what was observed with the balanced agonist angiotensin II. Much like the AT₁R, the ET_AR is a GPCR with a peptide agonist that couples primarily through G_q and G_i and promotes vasoconstriction. β -arrestin–biased ET_AR agonists would be predicted to decrease vasoconstriction while maintaining other important physiologic signaling through the receptor. Conversely, a G protein–biased ET_AR agonist would be predicted to promote increased vasoconstriction with decreased receptor desensitization. Therefore, ET_AR–biased agonists may have interesting physiologic profiles that could allow them to serve as tool compounds or novel therapeutics.

The goal of this study was to test for bias between the endogenous ET peptides and whether any derivatives of the ET peptides display significant bias between G protein and β -arrestin signaling. We also tested the effects of selective β -arrestin 1 or 2 knockout (KO) on the acute and chronic pressor response in mice.

Materials and Methods

Materials

ET-1, 2, and 3 and ET derivatives were purchased from Sigma-Aldrich (St. Louis, MO) and BACHEM (Torrance, CA). D-Luciferin and coelenterazine-h was obtained from Cayman Chemicals (Ann Arbor, MI). HBSS was obtained from Sigma-Aldrich (St. Louis, MO).

Mice

All animal experiments were conducted in compliance with institutional guidelines and were approved by Duke University and the Durham Veterans Affairs (VA) Institutional Animal Care and Use Committee (Protocol: A17516–08). β -arrestin 1 and 2 KO mice were provided by Dr Robert J. Lefkowitz and have been described previously (10,11). β -arrestin 1 KO mice were maintained on a C57BL/6J background and the β -arrestin 2 on a 129/SvEv. All mice studied were littermate males (KO and wild-type

[WT]) from same parents, aged 12–20 weeks of age. Mice were housed at the Durham VA Health Care Center animal research facility (ARF). Animals were housed on substrate bedding in individually ventilated polycarbonate cages. *Ad libitum* water was chlorinated and triple filtered, and provided *via* automatic watering with back-up water-bottles as needed. *Ad libitum* feed was provided in conventional pellet form *via* a feed hopper. Rooms were maintained on a 12:12 light cycle, with temperatures set at 72°F and relative humidity at 50%. Lighting, temperature, and humidity were continuously monitored using an alarmed TempTrak system. In addition, each occupied animal room in the ARF was equipped with a manual thermohygrometer that was monitored daily by ARF staff. Daily maximum and minimum temperature and humidity ranges were recorded and maintained on file in the ARF (Supplemental Methods).

Cell Culture

HEK293T cells were used in G protein and β -arrestin signaling assays. The cells were maintained in MEM (Corning) containing 1% penicillin/streptomycin and 10% FBS (Corning). Cells were grown at 37°C in a humidified 5% CO₂ incubator in poly-d-lysine–coated tissue culture plates. Media was changed every 48–72 hours.

Detection of Plasma Endothelin-1 Levels

ET-1 levels were undetectable in WT, β -arrestin 1 KO and β -arrestin 2 KO mice. Plasma samples from six biologic replicates were tested using the Mouse Endothelin-1 ELISA Kit (Thermo Fisher Scientific, #EM26RB). ET-1 was undetectable in all samples.

TGF- α Shedding Assay

GPCR G _{α} activity was assessed by the TGF- α shedding assay as previously described (12). Briefly, HEK 293 cells lacking G_q, G₁₁, G_{s/olf}, and G_{12/13} (“ Δ Gsix” HEK 293 cells) were transiently transfected with receptor, modified TGF- α containing alkaline phosphatase (AP-TGF- α), and the indicated G _{α} subunit. Cells were reseeded 24 hours later in HBSS (Gibco, Gaithersburg, MD) supplemented with 5mM HEPES in a Costar 96-well plate (Corning Inc., Corning, NY). Cells were then stimulated with the indicated concentration of ligand for 1 hour. Conditioned media (CM) containing the shed AP-TGF- α was transferred to a new 96-well plate. Both the cell and CM plates were treated with paranitrophenylphosphate (100 mM) (Sigma-Aldrich, St. Louis, MO) substrate for 1 hour, which is converted to paranitrophenol by AP-TGF- α . This activity was measured at OD₄₀₅ in a Synergy Neo2 Hybrid Multi-Mode (BioTek, Winooski, VT) plate reader immediately after paranitrophenylphosphate addition and after 1 hour incubation. G _{α} activity was calculated by first determining paranitrophenol amounts by absorbance through the following equation:

$$100 * \left(\frac{\Delta OD_{405} \text{ CM}}{\Delta OD_{405} \text{ CM} + \Delta OD_{405} \text{ cell}} \right),$$

where ΔOD_{405} = OD₄₀₅ 1 hour – OD₄₀₅ 0 hour and ΔOD_{405} cell and ΔOD_{405} CM represent the changes in absorbance after 1 hour in the cell and CM plates, respectively. Data were normalized to a single well that produced the maximal signal.

β -arrestin 2 Recruitment by Bioluminescence Resonance Energy Transfer

A 10 cm dish of HEK293T cells was transfected with 2 μ g each of constructs encoding the ET_AR with a C-terminal *Renilla* luciferase II and β -arrestin 2, with a C-terminal yellow fluorescent protein construct by calcium chloride transfection, as previously described for other receptors (13). Then, 24 hours after transfection, cells were plated onto a 96-well plate at 50,000–100,000 cells/well. Approximately 44 hours after transfection, media was changed to MEM (Corning) supplemented with 0.1% bovine serum albumin and 1% penicillin/streptomycin. After approximately 3 to 4 hours of serum starvation, cells were washed with room temperature PBS. Next, 80 μ l of a coelenterazine-h/HBSS solution (3 μ M coelenterazine-h) was added. Ligands were prepared at 5 \times concentration, and read by a Mithras LB940 instrument (Berthold) with 485 nm and 530 nm emission filters. The Bioluminescence Resonance Energy Transfer (BRET) ratio was calculated using Equation 1.

$$\text{Net BRET ratio} = \left[\frac{\text{YFP emission at 530nm}}{\text{Rluc emission at 485 nm}} \right] - cf \quad (1)$$

In Equation 1, *cf* represents BRET ratio in the vehicle control group. The net BRET ratio was quantified 5 minutes after ligand addition.

Bias Analysis

All concentration-response data was fit in GraphPad Prism 8.0 (GraphPad, San Diego, CA) with three-parameter fits (Baseline, Span, EC₅₀) after baseline correction and normalization. Intrinsic relative activities (E_{max}/EC₅₀) and bias factors were calculated as previously described (14). Bias plots to assess potential bias on the basis of E_{max} alone was performed as previously described (15).

Acute Pressor Studies

We examined acute vasoconstrictor responses to ET-1 (reconstituted in sterile saline) in mice anesthetized with 2% isoflurane, as described previously. A catheter (PE-50) was inserted into the left jugular vein for administration of basal fluids and vasoconstrictors. A second catheter (pulled-down PE-50) attached to a pressure transducer (model MLT844, ADInstruments, Colorado Springs, CO) was placed in the left carotid artery. Intra-arterial BP was recorded continuously through the carotid catheter using the PowerLab data acquisition system and LabChart software (ADInstruments). At 5 minute intervals (or until baseline BP was re-established, average 8 minutes), increasing doses (1 and 2 nmol/kg) of ET-1 were injected intravenously into the internal jugular vein at a volume of 1 μ l/g body wt (20–25 μ l total volume), followed immediately by a 25 μ l bolus of saline. Before injection of vasoactive agents, each mouse received an equivalent volume (20–25 μ l) of saline (1 μ l/g body WT) as a vehicle control. Intra-arterial pressures were continuously monitored.

Telemetry Studies

Mice were housed at the Durham VA Health Care Center ARF. Animals are housed on substrate bedding in individually ventilated polycarbonate cages. *Ad libitum* water was chlorinated and triple filtered and provided *via* automatic

watering with back-up water-bottles as needed. *Ad libitum* feed was provided in conventional pellet form *via* a feed hopper. Rooms were maintained on a 12:12 light cycle, with temperatures set at 72°F and relative humidity at 50%. Lighting, temperature, and humidity were continuously monitored using an alarmed TempTrak system. In addition, each occupied animal room in the ARF was equipped with a manual thermohygrometer that is monitored daily by ARF staff. Daily maximum and minimum temperature and humidity ranges were recorded and maintained on file in the ARF. Radiotelemetry (model PA-C10, Data Science International, St. Paul, MN) was used to measure BP in conscious unrestrained SMKO and control mice, as described previously (16). Arterial BP data were collected, stored, and analyzed using Dataquest ART software (version 4.0, Data Sciences International). Mice were allowed to recover for 7 days after placement of the telemetry implant to reestablish normal circadian rhythms. After recovery, telemetry data were collected continuously, with sampling every 5 minutes at 10 second intervals. ET-1 (2 pmol kg⁻¹, min⁻¹; Sigma-Aldrich) was infused *via* subcutaneously implanted osmotic minipump (Alzet, Cupertino, CA), and BP measurements continued after minipump implantation.

Results

Endothelins Activate A Wide Range of G Proteins

To compare signaling by ETs at the ET_AR across multiple G proteins, we used a previously described TGF- α shedding assay that provides a similar readout (AP-TGF- α release promoted by matrix metalloproteinase cleavage by a recombinant G α subunit) (12). We found the ETs could promote ET_AR signaling through a wide range of G proteins (Figure 1), consistent with a previously published study (17). Assessing these concentration-response data (Figure 1, data normalized to ET-1 signaling *via* G₁₁ in Supplemental Table 1), ET-1 and ET-2 demonstrated similar efficacies and potencies in all G protein assays, whereas ET-3 demonstrated significantly lower potency in all of the assays. Notably, in the β -arrestin 2 recruitment assay by BRET, which has significantly less amplification than the TGF- α release assay, ET-2 was a log-fold more potent than ET-1, suggesting the potential for relative G protein bias of ET-1. Although bias factors calculated from intrinsic relative activities suggested the potential for bias between these compounds (Supplemental Table 2 and Supplemental Figure 1), a bias plot of the raw data did not suggest significant bias between compounds due to the large difference in amplification between the β -arrestin and G protein assays (Supplemental Figure 2). Thus, it is most likely there is not significant G protein or β -arrestin bias between the ET peptides at ET_AR because an analysis on the basis of bias factors can be prone to errors when there are large differences in amplification between assays (15).

Structure-activity Relationships of Endothelin Derivatives

To determine whether any ET derivatives displayed significant G protein or β -arrestin bias at the ET_AR, we characterized a number of ET_AR peptide agonists and antagonists that were commercially available (Figure 2A). These peptides displayed a range of structures, including modifications

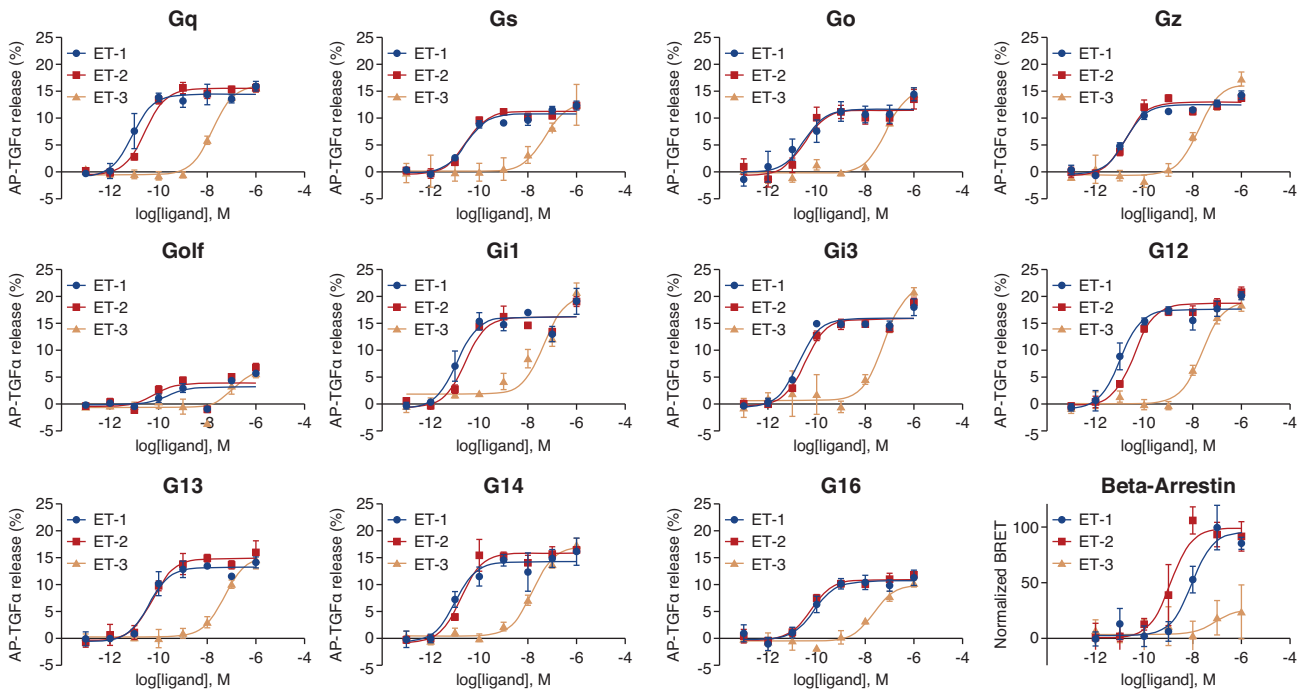


Figure 1. | Concentration response of endothelin-1 (ET-1), ET-2, and ET-3 in assays of G protein signaling and β -arrestin recruitment. The slight reversal of potencies of ET-1 and ET-2 in the β -arrestin recruitment assay are consistent with mild ligand bias. G protein signaling data are shown as percent of alkaline phosphatase TGF- α (AP-TGF- α) release as previously described (normalized G protein signaling data is shown in Supplemental Table 1). β -arrestin recruitment data are displayed as net BRET signal normalized to ET-1.

in the residues of the loop between the disulfide between Cys3 and Cys11, and more significant changes such as deletion of the N-terminal or C-terminal residues (an image of the crystal structure of ET-1 with the ET_BR with residues that differ between ET-1, 2, and 3 is shown in Figure 2B for orientation). Due to the large difference in amplification in assays, we focused on an assessment of efficacies at saturating concentrations from ligand, followed by a model-independent assessment of bias (15). Although we did not observe any evidence of significant bias between these compounds on the basis of their E_{max} across multiple assays (Figure 3 and Supplemental Table 3), we could make a number of

conclusions regarding structure-activity between these compounds. Removing either the C-terminus of the peptide (in ET-1[1–15] and ET-1[1–15] amide), which interacts with the hydrophobic pocket of the receptor, or the disulfides ([Ala1,3,11,15]-ET-1, BQ-3020, IRL-1620, IRL-1038, AC-ET-1[16–21]) that are critical for the conformation of the peptide that interact with the extracellular loops, resulted in a loss of agonism. Of note, sarafotoxin S6c demonstrated a significant loss of agonism, which is likely due to replacement of a positively charged residue (Lys9) with a negatively charged residue (Glu9) that is not capable of the same interactions with extracellular loop 2 (Figure 2B).

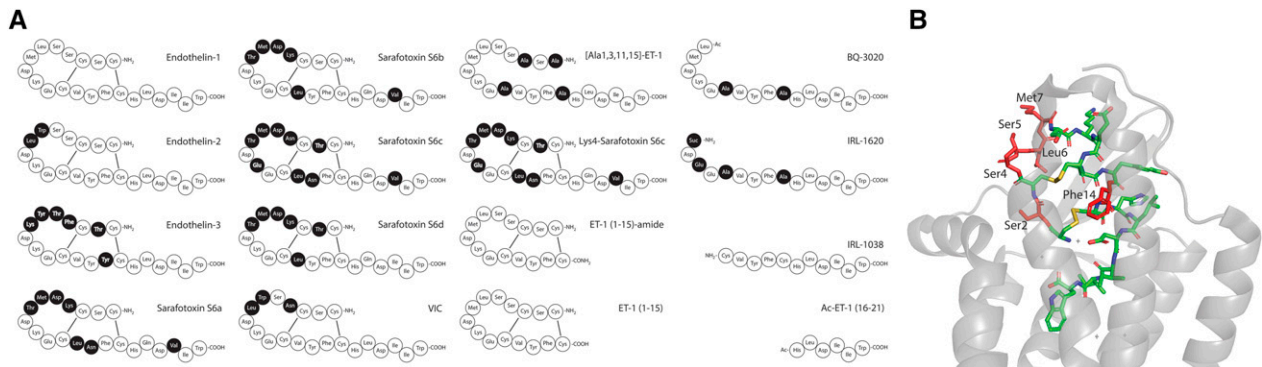


Figure 2. | ET derivatives tested for activity. (A) Derivatives largely involved modifications of residues between positions 2 and 13. Residues modified from ET-1 are shown as filled circles; those that are unchanged are open circles. (B) Residues of ET-1 modified in derivatives (shown in red) in the crystal structure of ET-1 with the type B ET receptor (ET_BR).

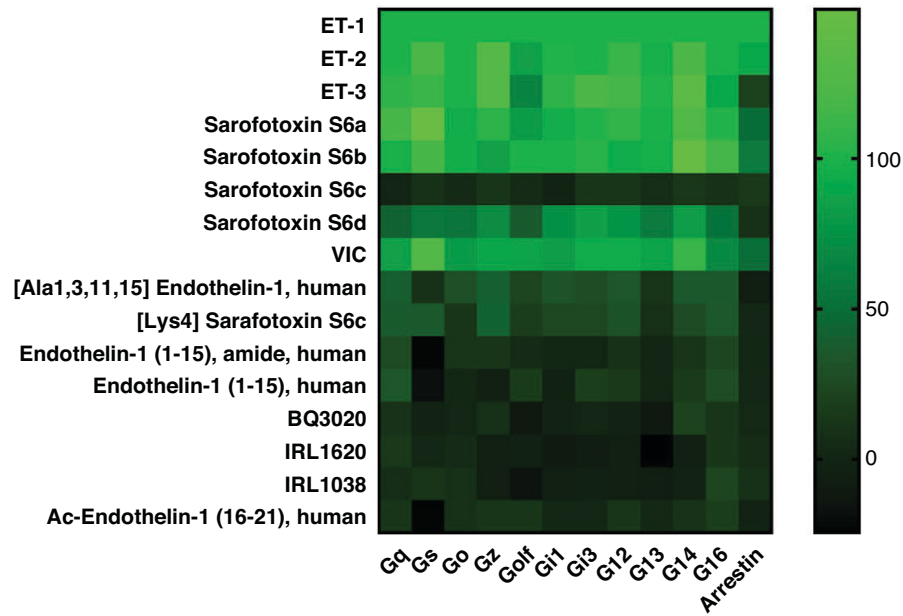


Figure 3. | Heatmap of efficacies (E_{max}) of different derivatives compared with ET-1 (100 on the scale). None of the derivatives demonstrated significant bias, because the differences in efficacies observed could be explained by differences in amplification between different G proteins (see Supplemental Figure 2).

β -arrestins Are Not Required for Acute Pressor Response to ET-1

With the evidence that both G protein and β -arrestin signaling was relatively balanced across the peptide ligands tested at the ET_A R, we next tested whether G protein-biased signaling would have any effect on ET-1 signaling *in vivo*. First, we assessed the relative expression of the ET_A R and ET_B R mRNA in mouse lung, and found that β -arrestin 2 KO mice had increased expression of both receptors compared with WT and β -arrestin 1 KO mice (Supplemental Figure 3). However, we were unable to detect ET-1 in the plasma using a commercially available ELISA kit in all genotypes. We tested the effects of ET-1 infusion on the acute pressor response and chronic exposure with radiotelemetry in WT and β -arrestin 1 and 2 KO mice (Figure 4, Table 1). We did not find any significant difference in the acute pressor response to ET-1 in WT or β -arrestin 1 in the B6 background (Figure 4A) and no effect on β -arrestin 2 KO mice in the 129

background (Figure 4B). The doses of ET-1 used in these acute studies were at the top end of the dose-response curve and therefore may not represent physiologic ET_A R and ET_B R activation. As β -arrestin 2 has been shown to play a significant role in the pressor response of angiotensin II (8), we then tested the effects of chronic ET-1 infusion by minipump in WT and β -arrestin 2 KO mice. We did not observe a significant difference in the blood pressure, heart rate, or diurnal blood pressure between WT and β -arrestin 2 KO mice in response to ET-1 infusion (Figure 5). However, it should be noted that the mice in the chronic ET-1 infusion (2 pmol/kg per min) *via* osmotic minipump had minimal elevation in blood pressure. This could be secondary to rapid metabolism of ET-1 by endothelial ET_B Rs resulting in diminished ET-1 interaction with the ET_A R, thus tempering the results of this experiment. This suggests that β -arrestins do not play a significant role in modifying the acute pressor responses to ET-1.

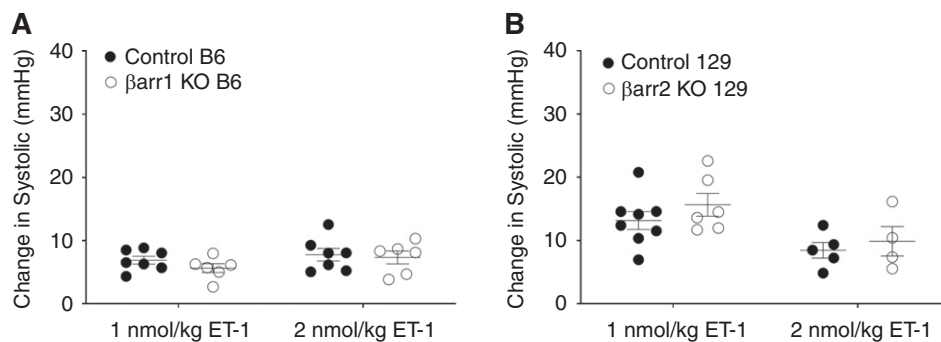


Figure 4. | Acute pressor effects of ET-1 in wild-type (WT) and β -arrestin 1 and 2 knockout (KO) mice. No difference in acute pressor response to ET-1 in (A) WT and β -arrestin 1 KO mice in a B6 background; (B) WT and β -arrestin 2 KO mice in a 129 background.

Table 1. Baseline hemodynamic parameters in acute blood pressure studies

Parameter	Control B6	β -arrestin 1 KO B6	Control 129	β -arrestin 2 KO 129
HR, bpm	488 \pm 14	472 \pm 9	429 \pm 11	427 \pm 23
SBP, mm Hg	91 \pm 2	92 \pm 2	110 \pm 4	107 \pm 4
Mean BP, mm Hg	75 \pm 3	75 \pm 2	93 \pm 3	91 \pm 2
DBP, mm Hg	67 \pm 3	67 \pm 2	81 \pm 2	79 \pm 2

HR, heart rate; bpm, beats per minute; SBP, systolic BP; DBP, diastolic BP.

Discussion

In this study, we characterized the G protein and β -arrestin signaling profiles of a number of ET derivatives at the ET_AR. For the ETs (ET-1, ET-2, ET-3), we found there was no significant bias between G protein and β -arrestin signaling at the ET_AR, although the analysis was limited by the significant difference in amplification between

the G protein signaling (amplification with AP generation) and β -arrestin recruitment (no amplification in resonance energy transfer) assays. None of the ET derivatives demonstrated significant biased agonism on the basis of a comparison of their maximal efficacies, although they did display structure-activity relationships with regards to their degrees of agonism.

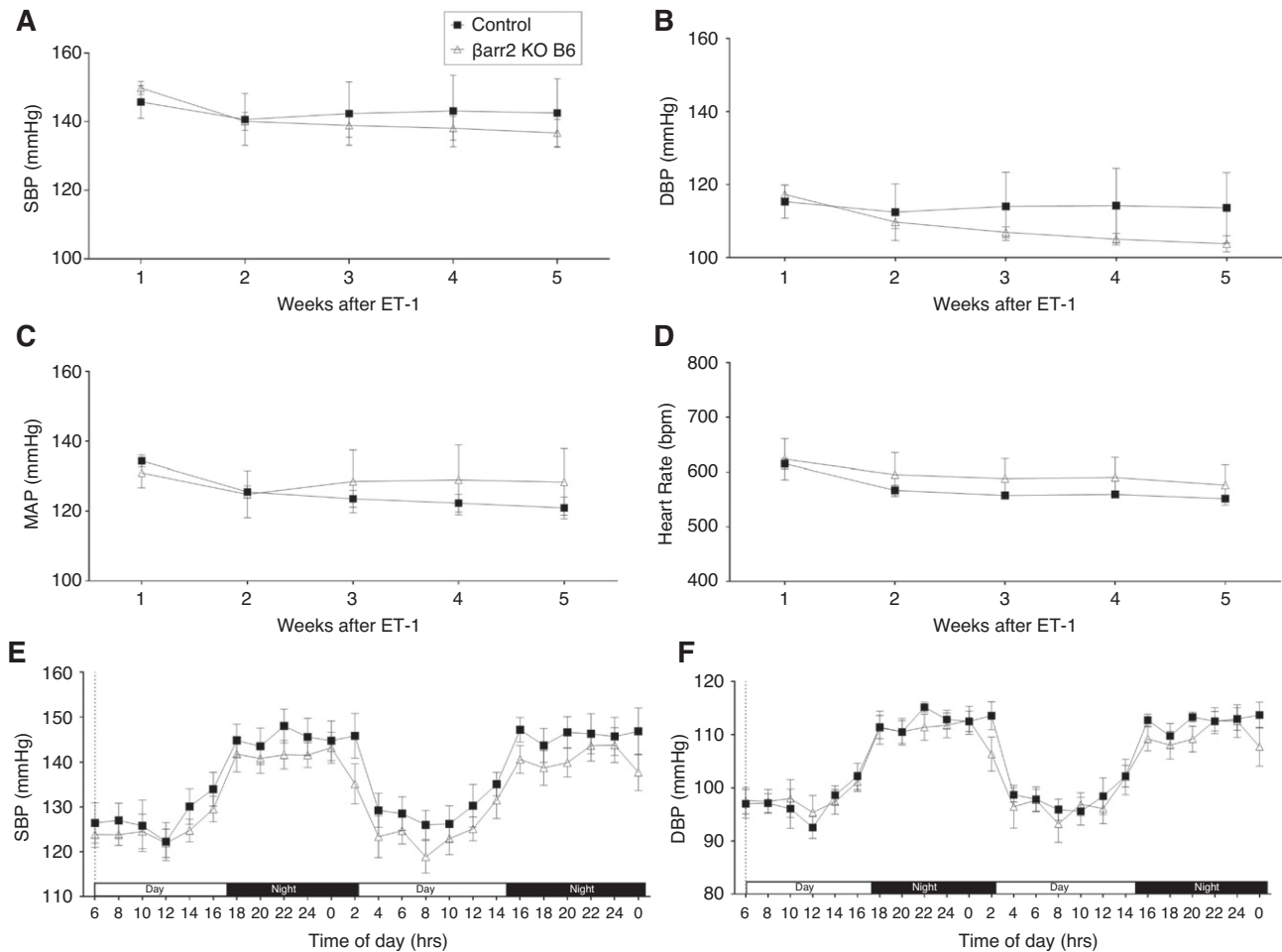


Figure 5. | Chronic pressor effects of ET-1 in WT and β -arrestin 2 KO mice. No difference in chronic pressor response to ET-1 on (A) systolic BP (SBP), (B) diastolic BP (DBP), (C) mean arterial pressure (MAP), and (D) heart rate between WT and β -arrestin 2 KO with ET-1 (2 pmol/kg per day) infusion *via* osmotic minipump. (E) Diurnal variation of systolic blood pressure during ET-1 shows no difference between WT and β -arrestin 2 KO. (F) Diurnal variation of DBP during ET-1 shows no difference between WT and β -arrestin 2 KO. Statistics for chronic blood pressure studies were performed with a mixed-effects model, multiple comparisons were made using Sidak's multiple comparisons test. For SBP, $P=0.86$ for each week, 1 $P=1.0$, 2 $P>1.0$, 3 $P=1.0$, 4 $P=1.0$, and 5 $P=1.0$. For DBP, $P=0.62$, for each week 1 $P>1.0$, 2 $P>1.0$, 3 $P=1.0$, 4 $P=0.91$, 5 $P=1.0$. For MAP $P=0.8$ for each week 1 $P=1.0$, 2 $P=1.0$, 3 $P=1.0$, 4 $P=1.0$, 5 $P=1.0$. For heart rate (HR) $P=0.6$, for each week, 1 $P>1.0$, 2 $P=1.0$, 3 $P=1.0$, 4 $P=1.0$, 5 $P=1.0$.

We found that β -arrestin 1 and 2 KO did not have an effect on the acute pressor response to ET-1 and β -arrestin 2 KO did not have an effect on mitogen-activated proteins with a chronic infusion of ET-1 compared to control mice. However, it should be noted the mice in the chronic ET-1 infusion (2 pmol/kg per min) *via* osmotic minipump had minimal elevation in blood pressure. This could be secondary to rapid metabolism of ET-1 resulting in diminished ET-1 interaction with the ET_AR, thus tempering the results of this experiment. Nonetheless, the lack of vasoconstrictor effect in the acute experiments is consistent with the AT₁R, where G proteins and not β -arrestins are responsible for mediating vasoconstriction (18). We did not observe an increase in the acute pressor response to ET-1 in β -arrestin KO mice compared with WT mice, suggesting the β -arrestins may not play a central role in receptor desensitization in the vasculature. An alternative explanation is that increased receptor expression in the β -arrestin 2 KO lung results in relative preservation of acute and chronic pressor responses. Importantly, ET-1 receptors are also expressed on multitude of other organ systems that control blood pressure, such as tubular epithelial cells of the kidney, neurons of the central nervous system, and cardiomyocytes among others (1). The results of the chronic ET-1 administration show that β -arrestin 2 signaling may not play a role in mediating ET-1 receptor desensitization in a variety of these organ systems that control blood pressure. If the β -arrestins were playing a significant physiologic role in receptor desensitization, we would expect to have observed that β -arrestin KO mice would have an exaggerated blood pressure responses to ET-1. β -arrestins have been shown to promote signaling through a wide range of pathways downstream of the ET_AR, promoting cell invasion through β -catenin (19), proliferation through Akt (20), and regulating transcription through multiple mechanisms (21,22). These complementary pathways likely contribute to smooth muscle cell proliferation and the longer-term effects on ET-1 on the vasculature (1). Alternatively, it is possible that compensatory β -arrestin signaling is occurring in β -arrestin 1 or β -arrestin 2 KO mice. However, β -arrestin 1 signaling would occur in β -arrestin 2 KO mice and *vice versa*.

Our findings suggest the native ET peptide backbone is likely a poor starting point for the development of biased ET_AR agonists. This is surprising because other peptide and protein GPCRs, such as the AT₁R (23,24), the parathyroid hormone receptor (25), and chemokine receptors (13,26,27), display significant bias between their endogenous ligands and of derivatives of their endogenous agonists. Notably at the μ opioid receptor, the identification of a distinct agonist scaffold resulted in compounds with significant G protein bias (28). This suggests that alternative scaffolds that bind to the ET_AR, such as sulfamides and their derivatives (29), should be tested to see whether they display any ligand bias. Such compounds could be useful as tool compounds in delineating the roles of G protein- and β -arrestin-mediated signaling in the vasculature and potentially as novel therapies. Identifying biased ligands for the ET_AR could represent a novel way to block many of the deleterious actions of ETs, while potentially alleviating important side effects. Results from this study suggest that agonist and antagonists targeting the ET_AR to result in biased signaling should use nonpeptide scaffolds.

Disclosures

S. Rajagopal reports receiving grants and personal fees from Actelion, Altavant, Bayer, Insmad, and United Therapeutics outside the submitted work. M. Sparks reports receiving research funding from the Renal Research Institute; reports receiving honoraria from Elsevier Nephrology Secrets; reports being a scientific advisor or member of the American Board of Internal Medicine, Nephrology Board, Board of Directors, NephJC, Editorial Board of the *American Journal of Kidney Diseases*, *ASN Kidney News*, *Kidney360*, and *Kidney Medicine*; reports Kidney in Cardiovascular Disease Council Membership & Communications Committee American Heart Association; Kidney in Cardiovascular Disease Council Scientific & Clinical Education Lifelong Learning Committee of the American Heart Association; and the National Kidney Foundation of North Carolina Medical Advisory Board. All remaining authors have nothing to disclose.

Funding

This work was supported by National Institutes of Health grants HL114643 and GM122798 to S. Rajagopal and a Duke O'Brien Center for Kidney Research pilot grant DK038108 to M. Sparks and S. Rajagopal.

Acknowledgments

We thank Robert Griffiths for technical assistance.

Author Contributions

M. Sparks and S. Rajagopal conceptualized the study and were responsible for funding acquisition, project administration and resources, and visualization, and provided supervision; M. Sparks, N. Nazo, R. Revoori, S. Rajagopal, and X. Xiong were responsible for data curation; M. Sparks, S. Rajagopal, and X. Xiong were responsible for formal analysis, software, and validation; M. Sparks, N. Nazo, S. Rajagopal, and X. Xiong were responsible for the investigation and methodology; M. Sparks, S. Rajagopal, and R. Revoori reviewed and edited the manuscript; and S. Rajagopal wrote the original draft.

Supplemental Material

This article contains the following supplemental material online at <http://kidney360.asnjournals.org/lookup/suppl/doi:10.34067/KID.0005462020/-/DCSupplemental>.

Supplemental Methods.

Supplemental Figure 1. No significant bias between ET peptides.

Supplemental Figure 2. Bias plots of ET derivatives compared with equimolar response-response data.

Supplemental Figure 3. qPCR of EDNRA and EDNRB from lung tissue of wild type, β -arrestin 1 KO, and β -arrestin 2 KO mice from six biological replicates.

Supplemental Table 1. G protein and β -arrestin signaling by ET peptides.

Supplemental Table 2. Intrinsic relative activities and bias factors for ET peptides relative to ET-1.

Supplemental Table 3. Efficacies of ET derivatives in G protein- and β -arrestin-mediated signaling.

References

1. Davenport AP, Hyndman KA, Dhaun N, Southan C, Kohan DE, Pollock JS, Pollock DM, Webb DJ, Maguire JJ: Endothelin. *Pharmacol Rev* 68: 357–418, 2016 <https://doi.org/10.1124/pr.115.011833>

2. Maguire JJ, Davenport AP: Endothelin@25: New agonists, antagonists, inhibitors and emerging research frontiers: IUPHAR Review 12. *Br J Pharmacol* 171: 5555–5572, 2014 <https://doi.org/10.1111/bph.12874>
3. Viswanathan G, Mamazhakypov A, Schermuly RT, Rajagopal S: The role of G protein-coupled receptors in the right ventricle in pulmonary hypertension. *Front Cardiovasc Med* 5: 179, 2018 <https://doi.org/10.3389/fcvm.2018.00179>
4. Heerspink HJL, Parving HH, Andress DL, Bakris G, Correa-Rotter R, Hou FF, Kitzman DW, Kohan D, Makino H, McMurray JJV, Melnick JZ, Miller MG, Pergola PE, Perkovic V, Tobe S, Yi T, Wiggerson M, de Zeeuw D; SONAR Committees and Investigators: Atrasentan and renal events in patients with type 2 diabetes and chronic kidney disease (SONAR): A double-blind, randomised, placebo-controlled trial. *Lancet* 393: 1937–1947, 2019 [https://doi.org/10.1016/S0140-6736\(19\)30772-X](https://doi.org/10.1016/S0140-6736(19)30772-X)
5. Smith JS, Lefkowitz RJ, Rajagopal S: Biased signalling: From simple switches to allosteric microprocessors. *Nat Rev Drug Discov* 17: 243–260, 2018 <https://doi.org/10.1038/nrd.2017.229>
6. Ahn S, Shenoy SK, Wei H, Lefkowitz RJ: Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. *J Biol Chem* 279: 35518–35525, 2004 <https://doi.org/10.1074/jbc.M405878200>
7. Ahn S, Kim J, Hara MR, Ren XR, Lefkowitz RJ: Beta-arrestin-2 mediates anti-apoptotic signaling through regulation of BAD phosphorylation. *J Biol Chem* 284: 8855–8865, 2009 <https://doi.org/10.1074/jbc.M808463200>
8. Violin JD, Dewire SM, Yamashita D, Rominger DH, Nguyen L, Schiller K, Whalen EJ, Gowen M, Lark MW: Selectively engaging beta-arrestins at the AT1R reduces blood pressure and increases cardiac performance. *J Pharmacol Exp Ther* 377: 572–579, 2010 <https://doi.org/10.1124/jpet.110.173005>
9. Boerrigter G, Soergel DG, Violin JD, Lark MW, Burnett JC Jr: TRV120027, a novel β -arrestin biased ligand at the angiotensin II type I receptor, unloads the heart and maintains renal function when added to furosemide in experimental heart failure. *Circ Heart Fail* 5: 627–634, 2012 <https://doi.org/10.1161/CIRCHEARTFAILURE.112.969220>
10. Conner DA, Mathier MA, Mortensen RM, Christe M, Vatner SF, Seidman CE, Seidman JG: Beta-arrestin 1 knockout mice appear normal but demonstrate altered cardiac responses to beta-adrenergic stimulation. *Circ Res* 81: 1021–1026, 1997 <https://doi.org/10.1161/01.RES.81.6.1021>
11. Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG, Lin FT: Enhanced morphine analgesia in mice lacking beta-arrestin 2. *Science* 286: 2495–2498, 1999 <https://doi.org/10.1126/science.286.5449.2495>
12. Inoue A, Ishiguro J, Kitamura H, Arima N, Okutani M, Shuto A, Higashiyama S, Ohwada T, Arai H, Makide K, Aoki J: TGF α shedding assay: An accurate and versatile method for detecting GPCR activation. *Nat Methods* 9: 1021–1029, 2012 <https://doi.org/10.1038/nmeth.2172>
13. Smith JS, Nicholson LT, Suwanpradid J, Glenn RA, Knape NM, Alagesan P, Gundry JN, Wehrman TS, Atwater AR, Gunn MD, MacLeod AS, Rajagopal S: Biased agonists of the chemokine receptor CXCR3 differentially control chemotaxis and inflammation. *Sci Signal* 11: eaaq1075, 2018 <https://doi.org/10.1126/scisignal.aaq1075>
14. Rajagopal S, Ahn S, Rominger DH, Gowen-MacDonald W, Lam CM, Dewire SM, Violin JD, Lefkowitz RJ: Quantifying ligand bias at seven-transmembrane receptors. *Mol Pharmacol* 80: 367–377, 2011 <https://doi.org/10.1124/mol.111.072801>
15. Onaran HO, Ambrosio C, Uğur Ö, Madaras Koncz E, Grò MC, Vezzi V, Rajagopal S, Costa T: Systematic errors in detecting biased agonism: Analysis of current methods and development of a new model-free approach. *Sci Rep* 7: 44247, 2017 <https://doi.org/10.1038/srep44247>
16. Sparks MA, Stegbauer J, Chen D, Gomez JA, Griffiths RC, Azad HA, Herrera M, Gurley SB, Coffman TM: Vascular type 1A angiotensin II receptors control BP by regulating renal blood flow and urinary sodium excretion. *J Am Soc Nephrol* 26: 2953–2962, 2015 <https://doi.org/10.1681/ASN.2014080816>
17. Inoue A, Raimondi F, Kadji FMN, Singh G, Kishi T, Uwamizu A, Ono Y, Shinjo Y, Ishida S, Arang N, Kawakami K, Gutkind JS, Aoki J, Russell RB: Illuminating G-protein-coupling selectivity of GPCRs. *Cell* 177: 1933–1947, 2019 <https://doi.org/10.1016/j.cell.2019.04.044>
18. Boerrigter G, Lark MW, Whalen EJ, Soergel DG, Violin JD, Burnett JC Jr: Cardiorenal actions of TRV120027, a novel β -arrestin-biased ligand at the angiotensin II type I receptor, in healthy and heart failure canines: A novel therapeutic strategy for acute heart failure. *Circ Heart Fail* 4: 770–778, 2011 <https://doi.org/10.1161/CIRCHEARTFAILURE.111.962571>
19. Rosanò L, Cianfrocca R, Masi S, Spinella F, Di Castro V, Biroccio A, Salvati E, Nicotra MR, Natali PG, Bagnato A: Beta-arrestin links endothelin A receptor to beta-catenin signaling to induce ovarian cancer cell invasion and metastasis. *Proc Natl Acad Sci U S A* 106: 2806–2811, 2009 <https://doi.org/10.1073/pnas.0807158106>
20. Cianfrocca R, Rosanò L, Spinella F, Di Castro V, Natali PG, Bagnato A: Beta-arrestin-1 mediates the endothelin-1-induced activation of Akt and integrin-linked kinase. *Can J Physiol Pharmacol* 88: 796–801, 2010 <https://doi.org/10.1139/Y10-052>
21. Cianfrocca R, Tocci P, Semprucci E, Spinella F, Di Castro V, Bagnato A, Rosanò L: β -Arrestin 1 is required for endothelin-1-induced NF- κ B activation in ovarian cancer cells. *Life Sci* 118: 179–184, 2014 <https://doi.org/10.1016/j.lfs.2014.01.078>
22. Rosanò L, Cianfrocca R, Tocci P, Spinella F, Di Castro V, Spadaro F, Salvati E, Biroccio AM, Natali PG, Bagnato A: β -arrestin-1 is a nuclear transcriptional regulator of endothelin-1-induced β -catenin signaling. *Oncogene* 32: 5066–5077, 2013 <https://doi.org/10.1038/onc.2012.527>
23. Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, Lefkowitz RJ: Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. *Proc Natl Acad Sci U S A* 100: 10782–10787, 2003 <https://doi.org/10.1073/pnas.1834556100>
24. Holloway AC, Qian H, Pipolo L, Ziogas J, Miura S, Karnik S, Southwell BR, Lew MJ, Thomas WG: Side-chain substitutions within angiotensin II reveal different requirements for signaling, internalization, and phosphorylation of type 1A angiotensin receptors. *Mol Pharmacol* 61: 768–777, 2002 <https://doi.org/10.1124/mol.61.4.768>
25. Gesty-Palmer D, Flannery P, Yuan L, Corsino L, Spurney R, Lefkowitz RJ, Luttrell LM: A beta-arrestin-biased agonist of the parathyroid hormone receptor (PTH1R) promotes bone formation independent of G protein activation. *Sci Transl Med* 1: 1ra1, 2009 <https://doi.org/10.1126/scitranslmed.3000071>
26. Corbisier J, Galès C, Huszagh A, Parmentier M, Springael JY: Biased signaling at chemokine receptors. *J Biol Chem* 290: 9542–9554, 2015 <https://doi.org/10.1074/jbc.M114.596098>
27. Rajagopal S, Bassoni DL, Campbell JJ, Gerard NP, Gerard C, Wehrman TS: Biased agonism as a mechanism for differential signaling by chemokine receptors. *J Biol Chem* 288: 35039–35048, 2013 <https://doi.org/10.1074/jbc.M113.479113>
28. Manglik A, Lin H, Aryal DK, McCorvy JD, Dengler D, Corder G, Levit A, Kling RC, Bernat V, Hübner H, Huang XP, Sassano MF, Giguère PM, Löber S, Da Duan, Scherrer G, Kobilka BK, Gmeiner P, Roth BL, Shoichet BK: Structure-based discovery of opioid analgesics with reduced side effects. *Nature* 537: 185–190, 2016 <https://doi.org/10.1038/nature19112>
29. Maguire JJ, Davenport AP: Endothelin receptors and their antagonists. *Semin Nephrol* 35: 125–136, 2015 <https://doi.org/10.1016/j.semnephrol.2015.02.002>

Received: September 14, 2020 Accepted: May 4, 2021