

# Functional Interaction of Endothelin Receptors in Mediating Natriuresis Evoked by G Protein–Coupled Estrogen Receptor 1

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## ABSTRACT

The G protein–coupled estrogen receptor 1 (GPER1) mediates rapid estrogenic signaling. We recently reported that activation of GPER1 in the renal medulla evokes endothelin-1–dependent natriuresis in female, but not male, rats. However, the involvement of the ET receptors, ET<sub>A</sub> and ET<sub>B</sub>, underlying GPER1 natriuretic action remain unclear. In this study, we used genetic and pharmacologic methods to identify the contributions of ET<sub>A</sub> and ET<sub>B</sub> in mediating this female-specific natriuretic effect of renal medullary GPER1. Infusion of the GPER1-selective agonist G1 (5 pmol/kg per minute) into the renal medulla for 40 minutes increased Na<sup>+</sup> excretion and urine flow in anesthetized female ET<sub>B</sub>-deficient (ET<sub>B</sub> def) rats and littermate controls but did not affect blood pressure or urinary K<sup>+</sup> excretion in either group. Pretreatment with the selective ET<sub>A</sub> inhibitor ABT-627 (5 mg/kg, intravenous) abolished G1-induced natriuresis in ET<sub>B</sub> def rats. To further isolate the effects of inhibiting either receptor alone, we conducted the same experiments in anesthetized female

Sprague–Dawley (SD) rats pretreated or not with ABT-627 and/or the selective ET<sub>B</sub> inhibitor A-192621 (10 mg/kg, intravenous). Neither antagonism of ET<sub>A</sub> nor antagonism of ET<sub>B</sub> receptor alone affected the G1-induced increase in Na<sup>+</sup> excretion and urine flow in SD rats. However, simultaneous antagonism of both receptors completely abolished these effects. These data suggest that ET<sub>A</sub> and ET<sub>B</sub> receptors can mediate the natriuretic and diuretic response to renal medullary GPER1 activation in female rats.

## SIGNIFICANCE STATEMENT

Activation of G protein–coupled estrogen receptor 1 (GPER1) in the renal medulla of female rats evokes natriuresis via endothelin receptors A and/or B, suggesting that GPER1 and endothelin signaling pathways help efficient sodium excretion in females. Thus, GPER1 activation could be potentially useful to mitigate salt sensitivity in females.

## Introduction

Hypertension is the leading cause of cardiovascular morbidity and mortality among women (Benjamin et al., 2018). In the United States, the prevalence of hypertension among adult women from 2017 to 2018 was 39.7% (Osthega et al., 2020), and one in three deaths of women were attributed to cardiovascular disease (Benjamin et al., 2018). Hypertension control resulted in the largest reduction, 38%, in cardiovascular mortality in women, according to NHANES (National Health and Nutrition Examination Survey) data modeling (Patel et al., 2015). Yet despite the availability of multiple antihypertensive regimens, almost half of hypertensive individuals in the United States do not have their blood pressure under control, highlighting the need for personalized therapeutic treatment options for the management of high blood pressure. Impaired natriuresis is a fundamental mechanism

in the initiation of hypertension (Hall et al., 2012; Elijovich et al., 2016). Therefore, it is vital that we expand our knowledge of the varied mechanisms of natriuresis. As studies indicate that women often receive suboptimal care for cardiovascular disease (Bailey Merz et al., 2015; Leifheit-Limson et al., 2015), improved understanding of the female-specific mechanisms underlying hypertension will help narrow the gender-related gap in health care.

We recently provided evidence for the G protein–coupled estrogen receptor 1 (GPER1) as a novel pronatriuretic factor in female rats but not male rats (Gohar et al., 2020). GPER1 is a nonclassic estrogen receptor that elicits rapid activation of signaling pathways (Revankar et al., 2005; Thomas et al., 2005). In particular, this heptahelical membrane-associated receptor has been shown to elicit protective actions in the cardiovascular and renal systems of several animal models (Lindsey et al., 2009, 2011; Jessup et al., 2010; Kurt et al., 2016; Liu et al., 2016; Qiao et al., 2018; Chang et al., 2019; Gohar et al., 2020). Indeed, many studies have shown that GPER1 activation mitigates salt-induced cardiovascular and kidney disease (Jessup et al., 2010; Lindsey et al., 2011; Liu et al., 2016), although the underlying mechanism has not been completely defined. Within the kidney, GPER1 expression has

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**ABBREVIATIONS:** ET-1, endothelin-1; ET<sub>A</sub>, endothelin receptor subtype A; ET<sub>B</sub>, endothelin receptor subtype B; ET<sub>B</sub> def, ET<sub>B</sub>-deficient; GPER1, G protein–coupled estrogen receptor 1; MAP, mean arterial pressure; SD, Sprague–Dawley; TG, transgenic.

been detected in tubular and epithelial cells (Lindsey et al., 2011; Cheng et al., 2014). We found that activation of GPER1 within the renal medulla of female rats promotes an increase in urinary Na<sup>+</sup> excretion via an endothelin-1 (ET-1)-dependent pathway (Gohar et al., 2020), but the contribution of endothelin receptors ET<sub>A</sub> and/or ET<sub>B</sub>, involved in GPER1-induced natriuretic effect, have not been identified.

ET-1 is a well established pronatriuretic peptide that inhibits the activity of epithelial Na<sup>+</sup> channels and Na<sup>+</sup>/K<sup>+</sup> ATPase (Zeidel et al., 1989; Kohan, 2011; Speed et al., 2015). In fact, ET-1 has a fundamental role in the maintenance of blood pressure and Na<sup>+</sup> homeostasis (Kohan, 2011; Speed et al., 2015). ET-1 elicits downstream actions via activation of the G protein-coupled receptors ET<sub>A</sub> and ET<sub>B</sub>, which are expressed at high levels within the medulla of the kidney (Kohan et al., 2011). In particular, established evidence indicates that the ET<sub>B</sub> receptor has a central role in mediating the excretory effects of ET-1 (Ge et al., 2006), and loss of ET<sub>B</sub> receptor function results in a salt-sensitive phenotype (Hoher and Ehrenreich, 2002). However, natriuretic actions have also been ascribed to ET<sub>A</sub> receptors specifically in female rats under conditions of ET<sub>B</sub> receptor dysfunction (Nakano and Pollock, 2009). Additional evidence indicates that ET<sub>A</sub> and ET<sub>B</sub> receptors may act synergistically to facilitate Na<sup>+</sup> and water excretion (Ge et al., 2008; Nakano and Pollock, 2009; Boesen and Pollock, 2010). Of note, ET receptor antagonists, combined ET<sub>A</sub> and ET<sub>B</sub> or selective ET<sub>A</sub> receptor-selective agents, are currently used in the treatment of pulmonary hypertension (Boesen, 2015). Despite limitations related to higher doses causing fluid retention, ET<sub>A</sub> receptor-selective agents hold promise in the management of hypertension and renal disease (Meyers and Sethna, 2013; Boesen, 2015; Pollock and Pollock, 2019), whereas concomitant ET<sub>B</sub> receptor antagonists are not beneficial clinically, as they induce vasoconstriction and salt and water retention (Dhaun and Webb, 2008; Meyers and Sethna, 2013; Moorhouse et al., 2013).

The current study was designed to use genetic and pharmacologic approaches to identify the relative contributions of ET<sub>A</sub> and ET<sub>B</sub> receptors to renal medullary GPER1-induced natriuresis. Considering that the pronatriuretic effects of GPER1 are evident in female rats but absent in males (Gohar et al., 2020), the experiments in this study were conducted solely in female animals.

## Materials and Methods

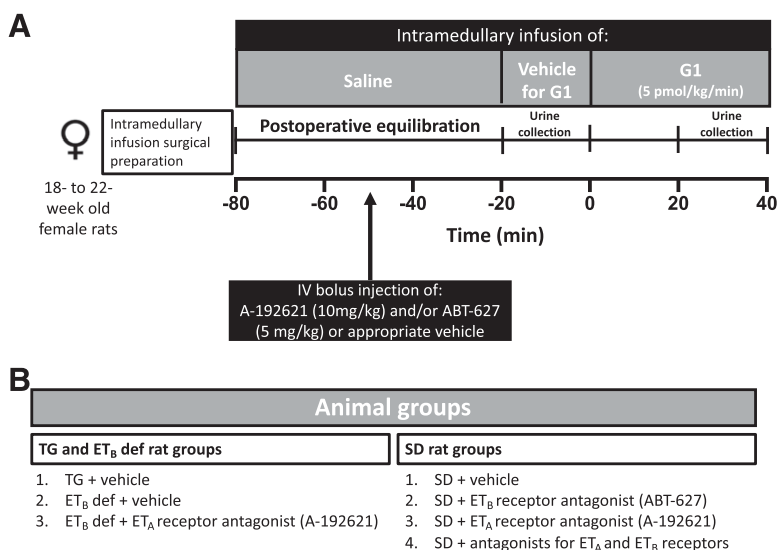
**Animals.** All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and the ARRIVE ((Animal Research: Reporting of In Vivo Experiments) guidelines (Kilkenny et al., 2010) and approved and monitored by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Female ET<sub>B</sub>-deficient (ET<sub>B</sub> def) rats and transgenic (TG) control littermates were obtained from our in-house colony. ET<sub>B</sub>-deficient rats were originally produced from the spotting lethal rat, which has a naturally occurring 301-bp deletion in the gene encoding ET<sub>B</sub> that results in a lethal phenotype of congenital intestinal megacolon (Garipey et al., 1998). This model was rescued by expressing a human ET<sub>B</sub> transgene only in sympathetic tissue using the human dopamine-β-hydroxylase promoter (Garipey et al., 1998). Thus, the ET<sub>B</sub> def rats express ET<sub>B</sub> receptors only in nervous tissue because of the transgene, whereas TG controls express the transgene and the normal ET<sub>B</sub> receptor. In total, 20 ET<sub>B</sub> def and TG rats were used. Separate experiments used an additional 38 Sprague-Dawley

(SD) rats purchased from Envigo (Indianapolis, IN). All rats were aged 18–22 weeks and weighed 200–300 g at time of the experimental protocol. Rats were housed at the institutional animal facilities at the University of Alabama at Birmingham in temperature- and humidity-controlled rooms with a 12-hour light/dark cycle. Experiments were conducted during the light period. Rats had free access to water and food (7917 Irradiated NIH-31 Mouse/Rat diet, 0.8% NaCl; Envigo).

**Surgical Procedure.** Female ET<sub>B</sub> def rats and TG littermates were surgically prepared for acute intramedullary infusion experiments as detailed in our previous studies (Gohar et al., 2016a, 2017). Briefly, rats were anesthetized by intraperitoneal injections of thiobutabarbitone (Inactin hydrate, 100 mg/kg per milliliter, catalog number T133; Sigma-Aldrich Co., St. Louis, MO). The trachea was cannulated with polyethylene tubing (PE-205) to facilitate free breathing. The left femoral vein was cannulated with a PE-50 catheter for intravenous supplementation of 3% bovine serum albumin (catalog number A7906; Sigma-Aldrich Co.) in phosphate-buffered saline (catalog number 2810305; MP Biomedicals, Irvine, CA) at a rate of 1.2 ml/h to compensate for fluid loss and maintain euolemia. Then, the left femoral artery was cannulated with a PE-50 catheter for blood pressure recording, after which a midline incision was made and the left ureter was cannulated by a PE-10 catheter to collect urine. Finally, 5 mm of stretched PE-10 catheter was inserted into the left kidney and positioned to deliver fluids at the outer-inner medullary junction at a rate of 0.5 ml/h. After surgery, the animals were allowed to equilibrate for 60 minutes before baseline urine collection. At the end of each experiment, the kidney was dissected to confirm the proper positioning of the catheter within the renal medullary interstitium. Surgeries were performed on a heated surgical table to avoid hypothermia. In separate experiments, the female SD rats underwent surgical procedures identical to those described above. Importantly, the intramedullary infusion technique we used has been shown to induce localized effects in the renal medulla, not the renal cortex (Stec et al., 1997; Speed and Hyndman, 2016).

**Blood Pressure Measurement.** Blood pressure was recorded in anesthetized animals via femoral artery catheterization. The catheter was connected to a blood pressure transducer (DPT-200, Deltran II; Utah Medical Products Ltd., Midvale, UT) with an output to PowerLab analog-to-digital converter (4/35; ADInstruments, Colorado Springs, CO) via a Quad Bridge Amplifier (FE224; ADInstruments). LabChart software version 7 was used to record and analyze blood pressure. Blood pressure data are presented as mean arterial pressure (MAP).

**Experimental Protocol.** Our experimental protocol is depicted in Fig. 1A. After the 60-minute postsurgical stabilization period, urine was collected over a 20-minute baseline period, and vehicle (0.02% DMSO in saline) for the selective GPER1 agonist G1 was infused into the renal medulla (DMSO, catalog number D8418; Sigma-Aldrich Co.). Then, G1 (5 pmol/kg per minute, catalog number 41004001; Sandia Biotech Inc. Albuquerque, NM) was infused into the renal medulla for 40 minutes (Fig. 1A). This intramedullary dose of G1 promotes natriuresis in female SD rats (Gohar et al., 2020). After 20 minutes of G1 infusion, a 20-minute urine sample was collected while G1 was still being infused to the renal medulla (Fig. 1A). Urine volume was determined gravimetrically. To pharmacologically inhibit the ET<sub>A</sub> receptor, ET<sub>B</sub> def rats were treated with an intravenous bolus injection of the ET<sub>A</sub> receptor antagonist ABT-627 (5 mg/kg; AbbVie Inc., Abbott Park, IL; *n* = 5) or vehicle (*n* = 6) 30 minutes before the end of the postsurgical stabilization period (Fig. 1A). Animal groups used are depicted in Fig. 1B. To pharmacologically inhibit ET receptors, SD rats were treated with an intravenous bolus injection of ABT-627 (5 mg/kg; *n* = 8) and/or the ET<sub>B</sub> receptor antagonist A-192621 (10 mg/kg; PepTech Corp., Bedford, MA; *n* = 10 and 9, respectively) 30 minutes before the end of the postsurgical stabilization period (Fig. 1A). These doses of ABT-627 and A-192621 have been shown to elicit efficient pharmacologic blockade of ET<sub>A</sub> and ET<sub>B</sub>, respectively, for this experimental duration (Gohar et al., 2016a), as ABT-627 and A-192621 have half-lives of 6 and 5 hours, respectively, in rats



**Fig. 1.** Schematic presentation of the experimental timeline (A) and animal groups (B) used in intramedullary infusion experiments.

(Wessale et al., 2002). A separate experimental group of SD ( $n = 11$ ) and TG rats ( $n = 9$ ) received an intravenous bolus injection of vehicle (0.5 ml/kg) and served as controls (Fig. 1B). The vehicle used to solubilize ABT-627 and A-192621 was composed of 60% polyethylene glycol 400 (catalog number 91893; Sigma-Aldrich Co.) and 25% ethanol (molecular-grade, catalog number E7023; Sigma-Aldrich Co.) in saline. Urine samples were stored at  $-80^{\circ}\text{C}$  until further analysis.

Of note, G1 is a highly selective agonist for GPER1 and displays no activity against classic estrogen receptors (Bologa et al., 2006; Albanito et al., 2007) or a panel of 25 other G protein-coupled receptors (Blasko et al., 2009). Moreover, the physiologic actions of G1 are not evident in GPER1-knockout mice (Haas et al., 2009; Liu et al., 2009; Wang et al., 2009).

**Measurement of Urinary Electrolytes.** Urine  $\text{Na}^+$  and  $\text{K}^+$  concentrations were determined using an atomic absorption spectrometer (ICE 3000 series paired with a CETAC ASX-520 Auto-Sampler; ThermoFisher Scientific, Waltham, MA) in the flame photometry mode.

**Statistics.** Comparisons between groups were analyzed by a repeated-measures two-way ANOVA followed by Dunnett's post hoc tests. Parameters measured during the first 20 minutes of G1 infusion were included in the statistical analysis. For clarity in data presentation, only responses during the second 20 minutes and not the first 20 minutes of G1 infusion are presented in the figures. Values are presented as means  $\pm$  S.E.M.  $P$  values less than 0.05 were considered statistically significant. Statistical analysis was performed using GraphPad Prism version 8.

## Results

**ET<sub>B</sub>-Deficient Rats.** Intramedullary infusion of G1, the selective GPER1 agonist, increased the urinary  $\text{Na}^+$  excretion by  $97\% \pm 19\%$  and  $126\% \pm 36\%$  relative to baseline in TG control rats and ET<sub>B</sub> def rats, respectively (Fig. 2A). Urine flow also increased in response to G1 infusion in female TG control rats and ET<sub>B</sub> def rats (Fig. 2B). In both groups, urinary  $\text{K}^+$  excretion remained relatively stable during the infusion (Fig. 2C). As previously reported, MAP was higher in ET<sub>B</sub> def rats than in TG littermates at baseline and did not change after G1 infusion in either group (Fig. 3).

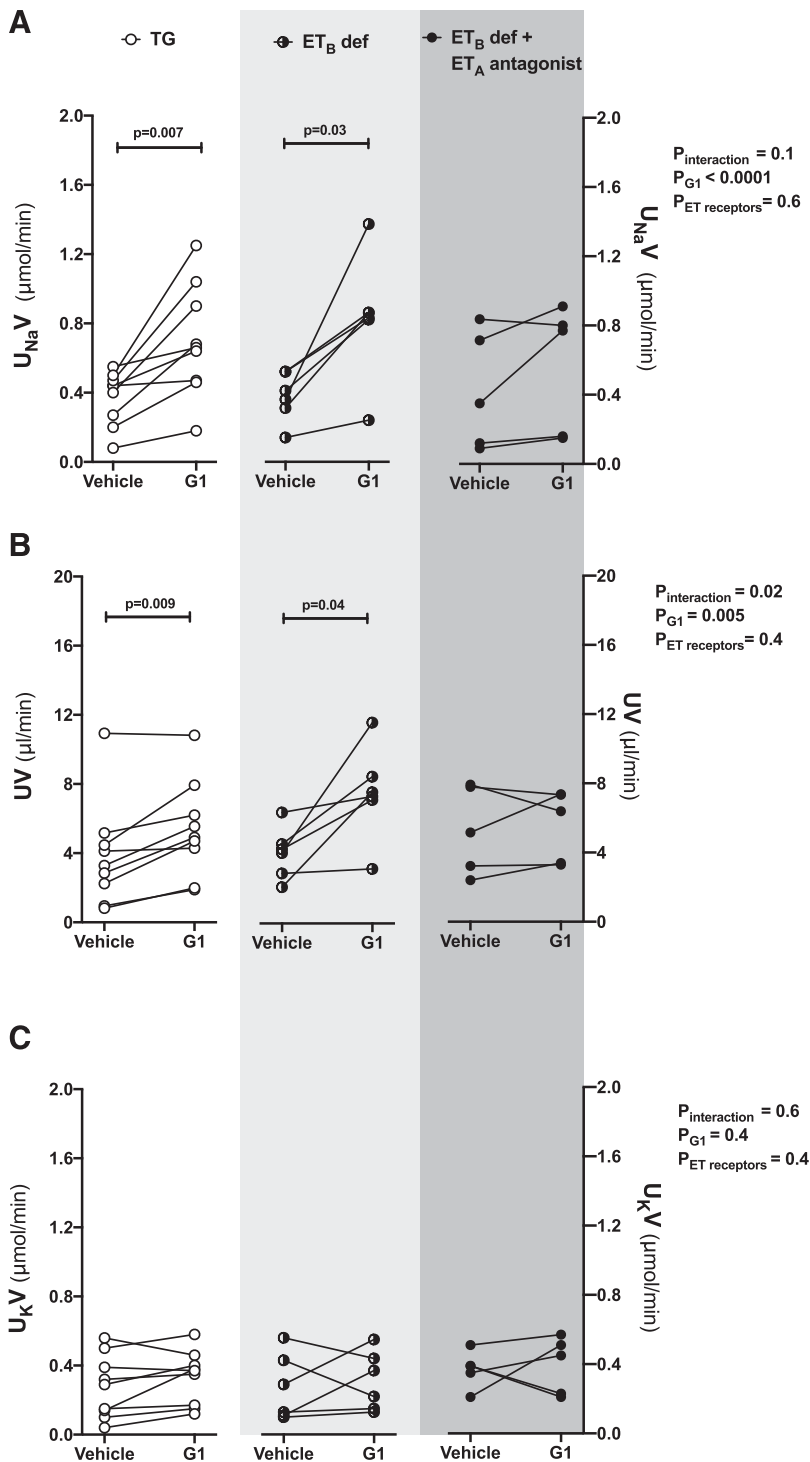
Pretreatment of ET<sub>B</sub> def rats with the ET<sub>A</sub> receptor antagonist ABT-627 prevented the G1-induced increase in urinary  $\text{Na}^+$  excretion and urine flow (Fig. 2, A and B) but did

not affect urinary  $\text{K}^+$  excretion (Fig. 2C). Pretreatment of ET<sub>B</sub> def rats with ABT-627 also caused a slight decrease in MAP that did not reach statistical significance (Fig. 3) but did eliminate the difference in MAP between ET<sub>B</sub> def and TG controls.

**Sprague-Dawley Rats.** To examine the effects of blocking either ET<sub>A</sub> or ET<sub>B</sub> receptor alone, we conducted similar experiments in female SD rats. Intramedullary infusion of G1 in SD rats increased urinary  $\text{Na}^+$  excretion by  $70\% \pm 24\%$  relative to baseline (Fig. 4A), which was similar to our recent study (Gohar et al., 2020). Urine flow was also increased in response to G1 infusion in SD rats (Fig. 4B). No significant differences were observed in urinary  $\text{K}^+$  excretion or MAP during G1 infusion in these rats when compared with corresponding baseline values (Fig. 4C; Fig. 5). Pretreatment of the SD rats with either ABT-627 or A-192621 did not alter the G1-induced increase in urinary  $\text{Na}^+$  excretion or urine flow (Fig. 4, A and B). However, pretreatment with ABT-627 and A-192621 simultaneously completely abolished the natriuretic and diuretic response to G1 infusion (Fig. 4, A and B). G1 elicited a significant overall effect on urinary  $\text{K}^+$  excretion in SD rats (Fig. 4C). However, there was no significant increase in comparison with corresponding baseline values in SD rats pretreated with either ET-1 receptor antagonist alone or in combination (Fig. 4C). Similar to the increase in MAP observed in ET<sub>B</sub> def rats, treatment with A-192621 alone resulted in an increase in MAP that was stable across the experimental period (Fig. 5). However, this increase in MAP did not occur after pretreatment with ABT-627 and A-192621 simultaneously (Fig. 5). In fact, pretreatment with ABT-627 alone or in combination with A-192621 resulted in a small but statistically significant decline in MAP during G1 infusion (Fig. 5).

## Discussion

In this study, we sought to elucidate the contribution of ET<sub>A</sub> and ET<sub>B</sub> receptors as downstream mediators of renal medullary GPER1-induced natriuresis. Overall, we provide genetic and pharmacologic evidence for a functional role of these receptors within the renal medulla in mediating

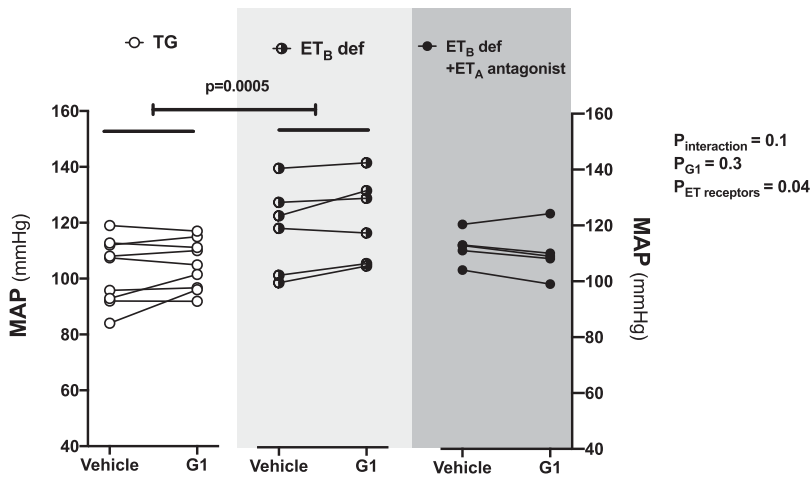


**Fig. 2.** Blockade of ET<sub>A</sub> receptor abolished the natriuretic response to renal medullary GPER1 activation in female ET<sub>B</sub> def rats. Urinary Na<sup>+</sup> excretion (U<sub>Na</sub>V) (A), urine flow (UV) (B), and urinary K<sup>+</sup> excretion (U<sub>K</sub>V) (C) were measured during two 20-minute urine collection periods in anesthetized female ET<sub>B</sub> def rats or TG control rats: at baseline during the intramedullary infusion of vehicle and 20 minutes after the infusion of the GPER1 agonist G1 (5 pmol/kg per minute) was initiated. ET<sub>A</sub> receptor blockade was achieved by intravenous bolus injections of ABT-627 (5 mg/kg) 30 minutes before the beginning of the baseline urine collection period; TG and ET<sub>B</sub> def controls received intravenous bolus injections of vehicle. Each line shows data for an individual animal. Statistical comparisons were performed by repeated-measures two-way ANOVA followed by Dunnett's post hoc tests.

GPER1-induced natriuresis. These results are compatible with previous evidence showing that ET<sub>A</sub> and ET<sub>B</sub> receptors promote water and Na<sup>+</sup> excretion (Ge et al., 2008; Boesen and Pollock, 2010).

In the present study, we demonstrated that activation of GPER1 within the medulla of the kidney of female SD rats evokes a natriuretic and a diuretic effect, consistent with our recent findings (Gohar et al., 2020). Notably, several studies have demonstrated that GPER1 activation ameliorates salt-induced cardiovascular and kidney damage (Jessup et al., 2010;

Lindsey et al., 2011; Liu et al., 2016). Specifically, GPER1 activation was shown to attenuate salt-induced cardiac remodeling (Jessup et al., 2010), vascular injury (Liu et al., 2016), and proteinuria (Lindsey et al., 2011) in mRen2.Lewis rats in a blood pressure-independent manner. The renoprotective actions of GPER1 activation were linked to decreased tubular oxidative stress and increased megalin-mediated protein reabsorption (Lindsey et al., 2011). However, the relationship between GPER1 activation and Na<sup>+</sup> homeostasis was not defined. Our recently published study showed that renal



**Fig. 3.** Effect of ET<sub>A</sub> receptor blockade on blood pressure during renal medullary GPER1 activation in female ET<sub>B</sub> def rats. MAP was measured during two 20-minute urine collection periods in anesthetized female ET<sub>B</sub> def rats or TG control rats: at baseline during the intramedullary infusion of vehicle and 20 minutes after the infusion of the GPER1 agonist G1 (5 pmol/kg per minute) was initiated. ET<sub>A</sub> receptor blockade was achieved by intravenous bolus injection of ABT-627 (5 mg/kg) 30 minutes before the beginning of the baseline urine collection period in female ET<sub>B</sub> def rats; TG and ET<sub>B</sub> def controls received intravenous bolus injections of vehicle. Each line shows data for an individual animal. Statistical comparisons were performed by repeated-measures two-way ANOVA followed by Dunnett's post hoc tests.

medullary GPER1 functions as a novel female-specific pronatriuretic factor via an ET-1-dependent signaling pathway. We also revealed that the mRNA expression of ET-1 and the ET<sub>A</sub> and ET<sub>B</sub> receptors is diminished in female GPER1-knockout mice compared with wild-type controls (Gohar et al., 2020). This effect was not evident in male mice (Gohar et al., 2020).

In the current study, we found that genetic deficiency or pharmacologic blockade of ET<sub>B</sub> receptors does not attenuate the G1-induced natriuresis observed in female SD rats, suggesting that the pronatriuretic actions of GPER1 can be mediated via ET<sub>A</sub> receptors. Hence, the inhibition of GPER1-induced natriuresis requires concomitant antagonism of ET<sub>A</sub> and ET<sub>B</sub> receptors in female rats. Nakano and Pollock (2009) demonstrated that ET<sub>A</sub> receptor contributes to ET-1-dependent natriuresis in female rats, and it has been shown that ET<sub>A</sub> and ET<sub>B</sub> receptors work synergistically to promote water and Na<sup>+</sup> excretion (Ge et al., 2008; Boesen and Pollock, 2010). These latter findings are consistent with our present findings, suggesting that ET<sub>A</sub> and ET<sub>B</sub> receptors help efficient excretion of salt and water.

Our observation that ET<sub>A</sub> or ET<sub>B</sub> receptor can mediate the G1 natriuretic effect alternatively under conditions of loss of the other receptor subtype is interesting, suggesting the capability of one ET receptor to compensate for the loss of function of the other receptor. However, this observation does not negate the possibility that one receptor may be solely or predominantly mediating GPER1 natriuretic action under normal physiologic conditions when both ET receptors are functional.

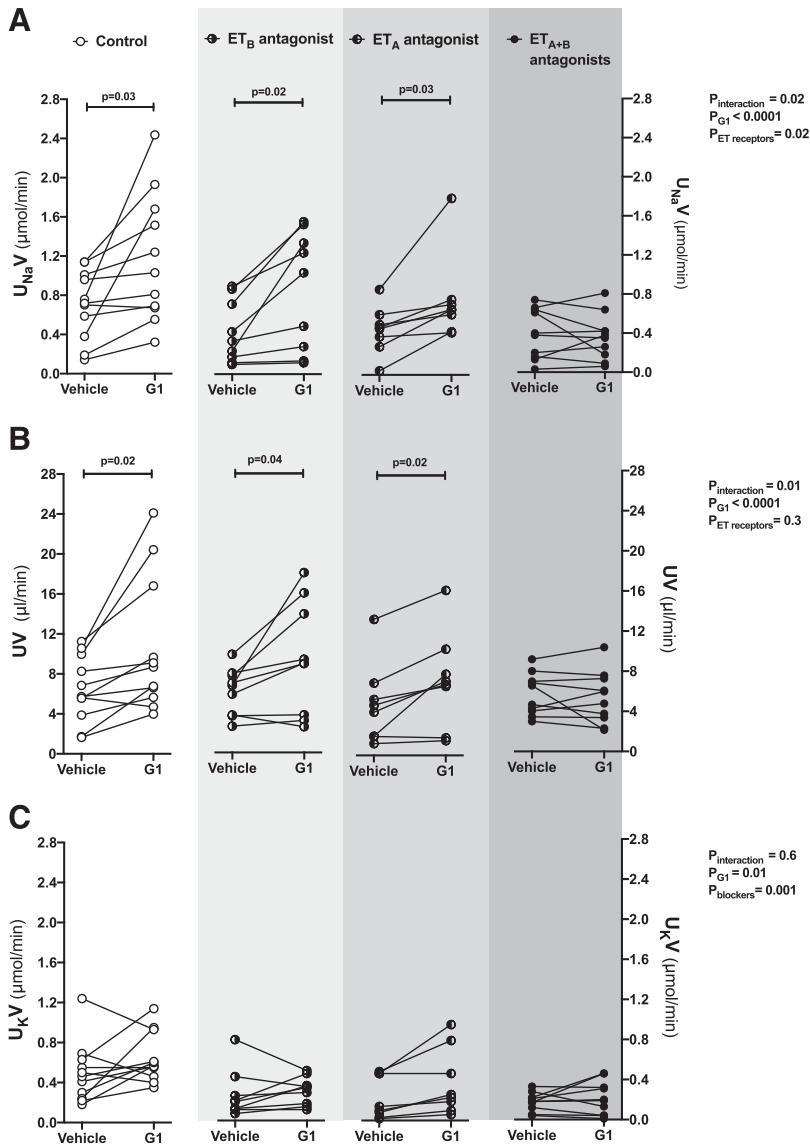
This interaction between ET<sub>A</sub> and ET<sub>B</sub> receptors has also been documented within the vasculature (White et al., 1993; Seo et al., 1994; Inscho et al., 2005). Inscho et al. (2005) showed that both ET<sub>A</sub> and ET<sub>B</sub> receptors contribute to ET-1-induced vasoconstriction of afferent arterioles, highlighting a possible interaction between ET<sub>A</sub> and ET<sub>B</sub> receptors in the control of afferent arteriolar vascular tone. Similarly, ET-1 actions via ET<sub>B</sub> and possibly ET<sub>A</sub> receptors contribute to the blunted renal autoregulation in salt-loaded rats (Fellner et al., 2015). It has also been demonstrated that ET<sub>A</sub> receptor expression in the lung is diminished in ET<sub>B</sub> knockout mice (Kuc et al., 2006). Notably, fluorescence resonance energy transfer experiments demonstrated that ET<sub>A</sub> and ET<sub>B</sub> receptors exist as constitutive homodimers and heterodimers (Gregan et al., 2004a,b).

In addition, Kapsokalyvas et al. (2014) provided imaging evidence for ET<sub>A</sub> and ET<sub>B</sub> receptor heterodimerization in rat mesenteric resistance arteries. Further experiments are required to identify whether heterodimers exist in epithelial cells and whether such dimers have relevance to the functional interaction between ET<sub>A</sub> and ET<sub>B</sub> receptors.

We demonstrated that ET<sub>A</sub> receptor blockade alone does not mitigate G1-induced natriuresis in female SD rats. However, whether the GPER1/ET<sub>A</sub> receptor-mediated natriuretic effect is a compensatory mechanism that occurs only under conditions of ET<sub>B</sub> receptor dysfunction remains unclear. The observation that both ET<sub>A</sub> and ET<sub>B</sub> receptors contribute to mediating the natriuretic response to GPER1, which is endogenously activated by estradiol in females, may have important implications for therapeutic use of dual ET<sub>A</sub> and ET<sub>B</sub> antagonists in female patients.

Changes in blood pressure and consequently pressure natriuresis do not appear to account for G1 natriuretic actions. We found that pharmacologic blockade of ET<sub>A</sub> in the presence or absence of ET<sub>B</sub> receptor blockade in SD rats decreases blood pressure before and after GPER1 activation. Importantly, GPER1-induced natriuresis was still evident despite the lower blood pressure produced by the ET<sub>A</sub> antagonist. We also found that genetic deficiency or pharmacologic blockade of ET<sub>B</sub> receptors elevates blood pressure, similar to previous observations (Garipey et al., 2000; Pollock and Pollock, 2001; Ge et al., 2006). Furthermore, G1 evoked a natriuretic action in animals with elevated blood pressure due to loss of ET<sub>B</sub> receptor function. Whether GPER1-induced natriuresis is evident in other experimental models of hypertension remains to be determined.

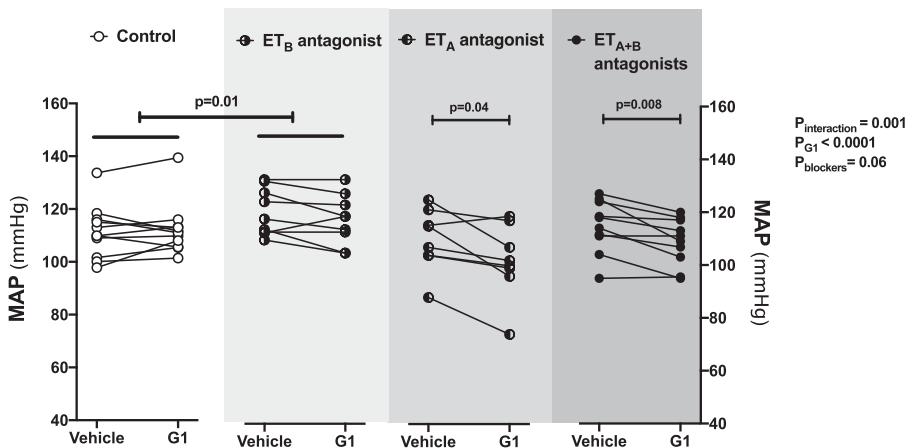
The localization of GPER1 and ET<sub>A</sub> and ET<sub>B</sub> receptors is also appropriate to support the crosstalk we observed between these receptors in the kidney. Multiple studies have shown that GPER1 is expressed in kidney cells (Hazell et al., 2009; Lindsey et al., 2011; Cheng et al., 2014; Li et al., 2014; Cheema et al., 2015). Earlier studies in which Davidoff et al. (1980) tested the binding capacity and affinity for [<sup>3</sup>H]estradiol in the cytosolic fraction of rat renal homogenates revealed radioactivity within the distal part of the tubule, specifically in the inner medullary collecting duct cells (Davidoff et al., 1980). In line with this finding, immunohistology revealed that GPER1 is expressed in the rat renal inner medulla (Hazell et al., 2009; Gohar et al., 2020). Similarly, studies with radioactively



**Fig. 4.** Combined blockade of ET<sub>A</sub> and ET<sub>B</sub> receptors abolished the natriuretic response to renal medullary GPER1 activation in female SD rats. Urinary Na<sup>+</sup> excretion ( $U_{NaV}$ ) (A), urine flow (UV) (B), and urinary K<sup>+</sup> excretion ( $U_{KV}$ ) (C) were measured during two 20-minute urine collection periods in anesthetized female SD rats: at baseline during the intramedullary infusion of vehicle and 20 minutes after intramedullary infusion of the GPER1 agonist G1 (5 pmol/kg per minute) was initiated. ET<sub>A</sub> and ET<sub>B</sub> receptor blockade was achieved by intravenous bolus injection of ABT-627 (5 mg/kg) and A-192621 (10 mg/kg), respectively, 30 minutes before the beginning of the baseline urine collection period; controls received intravenous bolus injections of vehicle. Each line shows data for an individual animal. Statistical comparisons were performed by repeated-measures two-way ANOVA followed by Dunnett's post hoc tests.

labeled ET-1 showed ET-1 binding primarily in the renal medulla (Davenport et al., 1989), and a high density of ET receptors was detected in inner medullary collecting duct cells

(Kohan et al., 1992). Overall, our study identifies a novel role for rapid estrogenic signaling as an upstream regulator of an ET-1/ET<sub>A</sub>/ET<sub>B</sub> pronatriuretic signaling system, supporting



**Fig. 5.** Effect of ET<sub>A</sub> and/or ET<sub>B</sub> receptor blockade on blood pressure during renal medullary GPER1 activation in female SD rats. MAP was measured during two 20-minute urine collection periods in anesthetized female SD rats: at baseline during the intramedullary infusion of vehicle and 20 minutes after intramedullary infusion of the GPER1 agonist G1 (5 pmol/kg per minute) was initiated. ET<sub>A</sub> and ET<sub>B</sub> receptor blockade was achieved by intravenous bolus injection of ABT-627 (5 mg/kg) and A-192621 (10 mg/kg), respectively, 30 minutes before the beginning of the baseline urine collection period; controls received intravenous bolus injections of vehicle. Each line shows data for an individual animal. Statistical comparisons were performed by repeated-measures two-way ANOVA followed by Dunnett's post hoc tests.

the importance of the intrarenal ET-1 system in renal Na<sup>+</sup> handling, particularly in females.

The regulation of natriuresis is highly integrated and involves many neural, vascular, and tubular signaling events within the kidney. We previously demonstrated that G1 downregulates Na<sup>+</sup>/K<sup>+</sup> ATPase activity in the renal outer medulla (Gohar et al., 2020). However, we do not know the contribution of the vascular and neuronal components to GPER1-induced natriuresis, if any. Evidence points to a vasodilatory response to GPER1 activation in multiple extrarenal vascular beds (Haynes et al., 2002; Broughton et al., 2010; Lindsey et al., 2014; Tropea et al., 2015; Peixoto et al., 2017; Fredette et al., 2018). Similarly, the GPER1 agonist, G1, induces a vasodilation in isolated perfused rat kidneys precontracted with phenylephrine (Kurt and Buyukafsar, 2013). In contrast, G1 evokes a vasoconstrictor response in the isolated perfused rat kidney under basal renal perfusion pressure (Kurt and Buyukafsar, 2013). Importantly, renal medullary GPER1 activation did not change blood flow in the medulla of the kidney (Gohar et al., 2020). Despite reports that GPER1 can modulate neuronal function, its role in regulating renal nerves is not clear. Li et al. (2016) provided evidence that GPER1 inhibits colonic motility by enhancing nitric oxide release from nitrergic nerves. GPER1 also appears to exert an inhibitory effect on neuronal apoptosis in the hippocampus (Han et al., 2019). Furthermore, GPER1 activation induces bradycardic effects via activation of cardiac parasympathetic neurons (Brailoiu et al., 2013). Additional studies are required to determine the contribution of renal nerves, if any, to GPER1-dependent natriuresis and how that relates to hypertension.

Previous studies have shown that systemic GPER1 activation lowers blood pressure in ovariectomized SD rats (Gohar et al., 2020) and mRen2.Lewis rats (Lindsey et al., 2009). Further, natriuresis evoked by renal medullary GPER1 activation is evident after ovariectomy (Gohar et al., 2020). Indeed, ovariectomy did not impact GPER1 protein expression in the renal medulla (Gohar et al., 2020). Of note, ovariectomy increased the mRNA expression of ET<sub>A</sub> and ET<sub>B</sub> in renal inner medulla (Gohar et al., 2016b). However, the contribution of ET receptors to GPER1-induced natriuresis under conditions of sex hormonal deficiency remains to be determined.

Collectively, we have demonstrated that G1 activates the GPER1/ET<sub>A</sub>/ET<sub>B</sub> axis, which in turn triggers natriuresis, suggesting that targeting GPER1 may be a useful approach to maintaining cardiovascular health in women. The question of whether GPER1 activation triggers an increase in ET-1 release and/or production remains to be answered. The effects of GPER1 activity on transport activity related to Ca<sup>2+</sup> influx and specific renal Na<sup>+</sup> transporters need to be investigated. Further research is also required to determine the functional significance of the interaction between GPER1 and ET receptors with regard to Na<sup>+</sup> balance and hypertension and to expand our understanding of GPER1 actions on renal electrolyte handling and salt sensitivity.

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#### Authorship Contributions

Participated in research design: Gohar, Pollock

Conducted experiments: Gohar.

Performed data analysis: Gohar.

Wrote or contributed to the writing of the manuscript: Gohar, Pollock.

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