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, ETA and ETB, underlying GPER1 natriuretic action remain unclear. In this study, we used genetic and pharmacologic methods to identify the contributions of ETA and ETB 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+ excretion and urine flow in anesthetized female ETB-deficient (ETB def) rats and littermate controls but did not affect blood pressure or urinary K+ excretion in either group. Pretreatment with the selective ETA inhibitor ABT-627 (5 mg/kg, intravenous) abolished G1-induced natriuresis and urine flow in SD rats. However, simultaneous antagonism of both receptors completely abolished these effects. These data suggest that ETA and ETB 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% (Ostchega 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 (Bairey 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; ETA, endothelin receptor subtype A; ETB, endothelin receptor subtype B; ETB def, ETB-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⁺ excretion via an endothelin-1 (ET-1)–dependent pathway (Gohar et al., 2020), but the contribution of endothelin receptors ET₁ and/or ET₂, 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⁺ channels and Na⁺/K⁺ 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⁺ homeostasis (Kohan, 2011; Speed et al., 2015). ET-1 elicits downstream actions via activation of the G protein–coupled receptors ET₁ and ET₂, which are expressed at high levels within the medulla of the kidney (Kohan et al., 2011). In particular, established evidence indicates that the ET₂ receptor has a central role in mediating the excretory effects of ET-1 (Ge et al., 2006), and loss of ET₂ receptor function results in a salt-sensitive phenotype (Hocher and Ehrenreich, 2002). However, natriuretic actions have also been ascribed to ET₁ receptors specifically in female rats under conditions of ET₂ receptor dysfunction (Nakano and Pollock, 2009). Additional evidence indicates that ET₁ and ET₂ receptors may act synergistically to facilitate Na⁺ and water excretion (Ge et al., 2008; Nakano and Pollock, 2009; Boesen and Pollock, 2010). Of note, ET receptor antagonists, combined ET₁ and ET₂ or selective ET₁ receptor–selective agents, are currently used in the treatment of pulmonary hypertension (Boesen, 2015). Despite limitations related to higher doses causing fluid retention, ET₁ 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 ET₂ receptor antagonists are not beneficial clinically, as they induce vasoconstriction and salt and water retention (Dhaun and Webb, 2010).

The current study was designed to use genetic and pharmacologic approaches to identify the relative contributions of ET₁ and ET₂ 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 ET₂-deficient (ET₂ def) rats and transgenic (TG) control littermates were obtained from our in-house colony. ET₂-deficient rats were originally produced from the spotting lethal (TG) control littermates were obtained from our in-house colony. Female ET₂-deficient (ET₂ def) rats and transgenic (TG) control littermates were obtained from our in-house colony. Of note, ET receptor antagonists, combined ET₁ and ET₂ or selective ET₁ receptor–selective agents, are currently used in the treatment of pulmonary hypertension (Boesen, 2015). Despite limitations related to higher doses causing fluid retention, ET₁ 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 ET₂ receptor antagonists are not beneficial clinically, as they induce vasoconstriction and salt and water retention (Dhaun and Webb, 2010; Meyers and Sethna, 2013; Moorhouse et al., 2013).

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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 −280°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 Na⁺ and K⁺ concentrations were determined using an atomic absorption spectrometer (iCE 3000 series paired with a CETAC ASX-520 AutoSampler; 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 ± S.E.M. P values less than 0.05 were considered statistically significant. Statistical analysis was performed using GraphPad Prism version 8.

Results

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

Pretreatment of ETB def rats with the ETₐ receptor antagonist ABT-627 prevented the G1-induced increase in urinary Na⁺ excretion and urine flow (Fig. 2, A and B) but did not affect urinary K⁺ excretion (Fig. 2C). Pretreatment of ETₐ 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 ETB def and TG controls.

Sprague-Dawley Rats. To examine the effects of blocking either ETₐ or ETₐ receptor alone, we conducted similar experiments in female SD rats. Intramedullary infusion of G1 in SD rats increased urinary Na⁺ excretion by 70% ± 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 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 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 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 ETₐ 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 ETₐ and ETₐ 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...
GPER1-induced natriuresis. These results are compatible with previous evidence showing that ETA and ETB receptors promote water and Na⁺ 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⁺ homeostasis was not defined. Our recently published study showed that renal

Fig. 2. Blockade of ET₄ receptor abolished the natriuretic response to renal medullary GPER1 activation in female ETB def rats. Urinary Na⁺ excretion (UNaV) (A), urine flow (UV) (B), and urinary K⁺ excretion (UKV) (C) were measured during two 20-minute urine collection periods in anesthetized female ETB 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. ETA 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 ETB 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 ETA and ETB 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 ETB receptors does not attenuate the G1-induced natriuresis observed in female SD rats, suggesting that the pronatriuretic actions of GPER1 can be mediated via ETA receptors. Hence, the inhibition of GPER1–induced natriuresis requires concomitant antagonism of ETA and ETB receptors in female rats. Nakano and Pollock (2009) demonstrated that ETA receptor contributes to ET-1–dependent natriuresis in female rats, and it has been shown that ETA and ETB receptors work synergistically to promote water and Na+ excretion (Ge et al., 2008; Boesen and Pollock, 2010). These latter findings are consistent with our present findings, suggesting that ETA and ETB receptors help efficient excretion of salt and water.

Our observation that ETA or ETB 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 ETA and ETB 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 ETA and ETB receptors contribute to ET-1–induced vasconstriction of afferent arterioles, highlighting a possible interaction between ETA and ETB receptors in the control of afferent arteriolar vascular tone. Similarly, ET-1 actions via ETB and possibly ETA receptors contribute to the blunted renal autoregulation in salt-loaded rats (Fellner et al., 2015). It has also been demonstrated that ETA receptor expression in the lung is diminished in ETB knockout mice (Kuc et al., 2006). Notably, fluorescence resonance energy transfer experiments demonstrated that ETA and ETB receptors exist as constitutive homodimers and heterodimers (Gregan et al., 2004a, b).

In addition, Kapsokalyvas et al. (2014) provided imaging evidence for ETA and ETB 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 ETA and ETB receptors.

We demonstrated that ETA receptor blockade alone does not mitigate G1-induced natriuresis in female SD rats. However, whether the GPER1/ETA receptor–mediated natriuretic effect is a compensatory mechanism that occurs only under conditions of ETB receptor dysfunction remains unclear. The observation that both ETA and ETB 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 ETA and ETB 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 ETA in the presence or absence of ETB 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 ETA antagonist. We also found that genetic deficiency or pharmacologic blockade of ETB receptors elevates blood pressure, similar to previous observations (Gariépy 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 ETB receptor function. Whether GPER1–induced natriuresis is evident in other experimental models of hypertension remains to be determined.

The localization of GPER1 and ETA and ETB 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 [3H]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
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/ETA/ETB pronatriuretic signaling system, supporting...
the importance of the intrarenal ET-1 system in renal Na⁺ 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⁺/K⁺ 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 vaso-dilatory 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 preconstricted 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 nitricergic nerves. GPER1 also appears to exert an inhibitory effect on neuronal apoptosis in the hippocampus (Han et al., 2019). Furthermore, GPER1 activation induces bradykinin 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 ovariec-tomized 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 ET₁α and ET₂β 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/ET₁α/ET₁β 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 Ca²⁺ influx and specific renal Na⁺ 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⁺ 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.

References