Role for ovarian hormones in purinoceptor-dependent natriuresis

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Short Title: Sex differences in P2-mediated natriuresis
Abstract

**Background:** Premenopausal women have a lower risk of hypertension compared to age-matched men and postmenopausal women. P2Y₂ and P2Y₄ purinoceptor can be considered potential contributors to hypertension due to their emerging roles in regulating renal tubular Na⁺ transport. Activation of these receptors inhibits epithelial Na⁺ channel activity (ENaC) via a phospholipase C (PLC)-dependent pathway resulting in natriuresis. We recently reported that activation of P2Y₂ and P2Y₄ receptors in the renal medulla by UTP promotes natriuresis in male and ovariectomized (OVX) rats, but not in ovary-intact females. This led us to hypothesize that ovary-intact females have greater basal renal medullary activity of P2 (P2Y₂ and P2Y₄) receptors regulating Na⁺ excretion compared to male and OVX rats.

**Methods:** To test our hypothesis, we determined (i) the effect of inhibiting medullary P2 receptors by suramin (750 μg/kg/min) on urinary Na⁺ excretion in anesthetized male, ovary-intact female and OVX Sprague Dawley rats, (ii) mRNA expression and protein abundance of P2Y₂ and P2Y₄ receptors and (iii) mRNA expression of their downstream effectors (PLC-1δ and ENaCα) in renal inner medullary tissues obtained from these three groups. We also subjected cultured mouse inner medullary collecting duct cells (segment 3, mIMCD3) to different concentrations of 17β-estradiol (E₂, 0, 10, 100 and 1000 nM) to test whether E₂ increases mRNA expression of P2Y₂ and P2Y₄ receptors.

**Results:** Acute P2 inhibition attenuated urinary Na⁺ excretion in ovary-intact females, but not in male or OVX rats. We found that P2Y₂ and P2Y₄ mRNA expression was higher in the inner medulla from females compared to males or OVX. Inner medullary lysates showed that ovary-intact females have higher P2Y₂ receptor protein abundance, compared to males, however, OVX
did not eliminate this sex difference. We also found that E<sub>2</sub> dose-dependently upregulated P2Y<sub>2</sub> and P2Y<sub>4</sub> mRNA expression in mIMCD3.

**Conclusion:** These data suggest that females have enhanced P2Y<sub>2</sub> and P2Y<sub>4</sub>-dependent regulation of Na<sup+</sup> handling in the renal medulla, compared to male and OVX rats. We speculate that the P2 pathway contributes to facilitated renal Na<sup+</sup> handling in premenopausal females.
Introduction

Women are largely protected from hypertension during their premenopausal age, compared to age-matched men [1]. The risk of hypertension is increased after menopause which is a state characterized by the cessation of ovarian production of the female sex steroid, estradiol (E$_2$) [2, 3]. Data suggest that E$_2$ exerts protective effects on cardiovascular and renal health in premenopausal females [4-7].

Maintenance of Na$^+$ balance and efficient renal Na$^+$ excretion are fundamental aspects in the regulation of blood pressure [8, 9]. Multiple overlapping systems contribute to regulation of renal tubular reabsorption of Na$^+$. Data implicate an important role for purinoceptors (P2 receptors) in controlling urinary Na$^+$ excretion [10, 11]. P2 receptors are classified into ligand-gated ion channels, P2X$_{1-7}$, and G protein-coupled receptors, P2Y$_{1,2,4,6,11-14}$ receptors [11-13].

Recently, it has been shown that P2Y$_2$ and P2Y$_4$ purinoceptor subtypes play a central role in promoting urinary Na$^+$ excretion and influencing blood pressure [10, 11]. Genetic deletion of P2Y$_2$ receptors results in elevated renal Na$^+$ reabsorption and hypertension [14]. Increased dietary salt increases ATP release from collecting duct cells. Activation of P2Y$_2$ and P2Y$_4$ purinoceptors by ATP increases intracellular Ca$^{2+}$ and reduces epithelial Na$^+$ channel (ENaC) activity in collecting ducts via phospholipase C (PLC), resulting in a natriuretic effect [10, 11, 15, 16].

Regulation of Na$^+$ excretion via the P2-mediated signaling cascade has been studied almost exclusively in males. Despite ample evidence for sex differences in P2-mediated signaling outside the kidney [17-19], sex differences in the renal P2 system are poorly understood. We recently reported that activation of renal medullary P2Y$_2$ and P2Y$_4$ receptors by UTP infusion for 1 hour promotes natriuresis in male rats [20]. In addition, we showed that 1 hour of UTP infusion
to the renal medulla did not stimulate natriuresis in ovary-intact female rats while ovariectomy unmasked UTP-induced natriuretic actions [21], pointing to sex-related differences in P2-mediated inhibitory tone on tubular Na\(^+\) reabsorption.

The goal of the current study was to test whether renal medullary P2 (P2\(_Y2\) and P2\(_Y4\)) receptors exert a greater role in regulating Na\(^+\) excretion in ovary-intact female rats, compared to males, and whether ovariectomy blunts P2-dependent natriuretic pathway. We also determined the expression of P2\(_Y2\) and P2\(_Y4\) receptors and their downstream effectors in the renal inner medulla. Of note, the inner medullary collecting duct plays an integral role in fine-tuning Na\(^+\) reabsorption [22]. Given that the female sex steroid, E\(_2\), has an established role in mediating sex-differences in cardiovascular and renal disease females [4-7], we also investigated the impact of E\(_2\) treatment of mouse inner medullary collecting duct segment 3 (mIMCD3) cells on P2\(_Y2\) and P2\(_Y4\) receptor mRNA expression.

Methods

Animals. Male and female (16-20 weeks of age) Sprague Dawley (SD) rats from Envigo (Indianapolis, IN) were used. All animal protocols were in accordance with the ARRIVE guidelines and the Guide for the Care and Use of Laboratory Animals and were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Animals were housed in a temperature (18-23°C) controlled room with a 12:12-h light-dark cycle with free access to food and water. Animals were maintained on 7917 Irradiated NIH-31 Mouse/Rat diet (0.8% NaCl, Envigo).
Ovariectomy. Rats (13-17 weeks of age) were subjected to bilateral ovariectomy, as detailed in our previous studies [23]. Three weeks later, acute intramedullary infusion experiments were performed. Briefly, female rats were anesthetized using isoflurane (2%, 502017, Vetone). Bilateral incisions were made on both sides of the back. Ovaries were then exteriorized, tied off and removed. Then, the muscle layer was sewed and the incision was closed using wound clips.

Acute intramedullary infusion. Male, ovary-intact female and OVX rats were anesthetized using thiobutabarbitone (Inactin, hydrate, 100 mg/kg, ip, T133, Sigma-Aldrich Co.) and surgically prepared similar to our previous studies [20]. Briefly, animals were maintained on a heated surgical table to maintain body temperature at 37°C. Tracheotomy was performed using PE-205 to facilitate breathing. The femoral vein was catheterized (PE-50) to allow fluid resuscitation with 3% bovine serum albumin in phosphate-buffered saline at a rate of 1.2 ml/h to maintain euvoelemia. The femoral artery was catheterized (PE-50) to measure mean arterial pressure (MAP). A 5-6 mm catheter (PE-10) was inserted into the renal medulla of the left kidney to deliver fluids to the renal medullary interstitium at a rate of 0.5 ml/h. Positioning of the catheter tip was confirmed by kidney dissection at the end of each experiment. Urine was collected from the infused kidney by ureter catheterization (PE-10). Animals were allowed to equilibrate for 80 min during which saline was infused into the renal medulla. This was followed by intramedullary infusion of the P2 antagonist, suramin (750 μg/kg/min [20], S2671, Sigma-Aldrich Co., dissolved in saline) or vehicle for a 30 min urine collection period (Fig. 1). Urinary electrolyte levels were measured using an atomic absorption spectrometer (iCE 3000 series...
paired with a CETAC ASX-520 AutoSampler, ThermoFisher Scientific) in the flame photometry mode.

**Gene expression assessment by RT-PCR.** RNA was isolated from tissues or cultured cells using a Purelink Mini extraction kit (12183018A, ThermoFisher Scientific) or a Purelink miRNA extraction kit (K157001, ThermoFisher Scientific), respectively, according to manufacturer’s instructions. The isolated RNA was reverse transcribed using a QuantiTect Reverse Transcription kit (205311, Qiagen). mRNA was quantified by RT-PCR (CFX96 Real-Time System, BIORAD) using TaqMan primer gene expression assays with rat P2Y<sub>2</sub> receptor (Rn02070661_s1), rat P2Y<sub>4</sub> (Rn02133903_s1), rat β-Actin (Rn00667869_m1), mouse P2Y<sub>2</sub> receptor (Mm02619978_s1), mouse P2Y<sub>4</sub> receptor (Mm00445136_s1) and mouse β-Actin (Mm02619580_g1) primers. mRNA expression was quantified relative to β-Actin using 2<sup>-ΔΔCt</sup> method. Gene expression data are expressed as the fold change from the mean mRNA expression values in ovary-intact female rats.

**Western blotting.** Renal inner medullary tissues were processed as previously described [24]. Briefly, inner medulla protein lysates were transferred and incubated with rabbit anti-P2Y<sub>2</sub> or anti-P2Y<sub>4</sub> receptor primary antibody (APR-010, APR-006, respectively, Alomone Labs) at 1:1500 dilution at 4°C overnight. The blots were then incubated for 1 hour at room temperature with anti-rabbit IgG, HRP-Linked secondary antibody (7074, Cell Signaling Technology) at 1:7000 dilution. Images were developed after exposure to X-ray film. The blots were then re-probed with anti-β-actin (A2228, Sigma-Aldrich Co.) at 1:10000 dilution as a loading control. Relative band densities were quantified using AlphaEaseFC™ software version 3.1.2 (Genetic Technologies Inc.).
Densitometry results are expressed as the fold change from the mean values in ovary-intact female rats.

**Cell culture.** mIMCD-3 cells (ATYCC CRL-2123, American Type Culture Collection) were cultured as previously described [25] in Dulbecco’s Modified Eagle Medium (F12, ThermoFisher Scientific) containing 10% fetal bovine serum (ThermoFisher Scientific) and 1% penicillin-streptomycin (ThermoFisher Scientific). Cells were incubated at 37°C in 5% CO₂-95% air. Passages 4–6 were used. Cells were grown in 12-well plates and allowed to reach 100% confluency. Cells were serum starved for 3 hours, then they were treated with 17β-estradiol (E₂, E2758, Sigma-Aldrich Co.) or vehicle for 24 hours at final concentrations of 10, 100 or 1000 nM. E₂ was dissolved in 0.1% ethanol (molecular grade, E7023, Sigma-Aldrich Co.). Values reported are means ± SE and represent results of cells from three experiments with cell lysates assayed in triplicate.

**Statistical analysis.** Statistical tests used for each data set are specified in each figure legend. This includes analysis by one-way ANOVA followed by assessment of differences between the means of the groups using Bonferroni’s or Dunnett’s multiple comparison tests. Two-way ANOVA followed by assessment of differences between the means of the groups using Sidak’s post-hoc tests was used for analysis of figure 2 data (data are presented in different panels for clarity). Data are presented as means ± SEM, with a probability of p<0.05 considered significant. Statistical analysis was performed using GraphPad Prism version 8.

**RESULTS**

**Natriuretic role for P2 receptors in the renal medulla.** To determine the contribution of renal medullary P2 receptors, we infused the P2 antagonist, suramin, into the renal medulla of
male, ovary-intact female and OVX rats (Fig. 2). Suramin significantly decreased urinary Na\(^+\) excretion and urine flow relative to vehicle-infused values (Fig 2B, E) only in ovary-intact females. Urine flow and Na\(^+\) excretion did not significantly change during medullary blockade of P2 receptors in male or OVX rats (Fig. 2A, C, D, F). Urinary K\(^+\) excretion (Fig. 2G-I) and MAP (Fig. 2J-L) were not significantly altered by suramin in males, ovary-intact females or OVX.

**Renal inner medullary P2Y\(_2\) and P2Y\(_4\) receptor mRNA expression.** We determined P2Y\(_2\) and P2Y\(_4\) receptor expression in inner medullary tissues from kidneys obtained from male, ovary-intact female and OVX rats. We found that P2Y\(_2\) receptor mRNA expression is higher in the inner medulla of ovary-intact female rats compared to males (Fig. 3A). This sex difference was eliminated by ovariectomy (Fig. 3A). Renal inner medullary P2Y\(_4\) receptor mRNA expression followed the same pattern as P2Y\(_2\) receptor mRNA expression (Fig. 3B).

**Renal inner medullary P2Y\(_2\) and P2Y\(_4\) receptor protein abundance.** As expected, based on the molecular weight of P2Y\(_2\) receptor, Western blots demonstrated an intense band at approximately 42-kD (Fig. 3C), that was completely ablated by preincubation with the blocking peptide (Supplemental Fig. 1A). No differences were evident in the intensity of this band between groups. Western blots for P2Y\(_2\) receptor consistently demonstrated another slightly lower molecular weight band (approximately 36-kD), that also underwent complete ablation when incubated with the blocking peptide (Supplemental Fig. 1A). This band may represent a posttranslational modified form of the P2Y\(_2\) receptor, but this will require further investigation. This 36-kD band was significantly higher in ovary-intact females, in comparison with males (p=0.03), however, ovariectomy did not impact this 36-kD band. Overall, the combined mean densities of the two bands for P2Y\(_2\) receptor were higher in inner medulla from kidneys obtained
from ovary-intact females, compared to males, consistent with the mRNA data (Fig. 3C).

Ovariectomy did not change the combined mean densities of the two bands for P2Y<sub>2</sub> receptor (Fig. 3C).

Western blots of renal inner medullary lysates for P2Y<sub>4</sub> receptor also showed two apparently distinct bands (Fig. 3D). The lower molecular weight band is consistent with the expected molecular weight for P2Y<sub>4</sub> receptor protein (approximately 42-kD). The other more intense band had a slightly higher molecular weight, which may represent the glycosylated form of P2Y<sub>4</sub> receptor [26]. Importantly, preincubation with P2Y<sub>4</sub> blocking peptide resulted in complete ablation of these two bands (Supplemental Fig. 1B). The 42-kD band for P2Y<sub>4</sub> receptor had a slightly higher abundance in ovary-intact females relative to males, however, this trend did not reach statistical significance. Notably, the relative abundance of the 42-kD band for P2Y<sub>4</sub> receptor in OVX rats was significantly lower, compared to ovary intact females (p=0.02), consistent with the mRNA data. As quantified in figure 3D, the combined relative intensity of the both bands was not different between male, ovary-intact or OVX female rats. The higher prevalence of the higher molecular weight band for P2Y<sub>4</sub> receptors completely masked the effect of OVX observed in the 42-kD band.

**Renal inner medullary PLC-1δ and ENaC<sub>α</sub> mRNA expression.** Downstream of purinoceptor activation, PLC-1δ-dependent inhibition of ENaC activity was shown to promote natriuresis [10, 11, 15, 16]. We determined the mRNA expression levels of PLC-1δ and ENaC<sub>α</sub> (SCNN1A) in kidneys from male, ovary-intact female and OVX rats. We found that mRNA expression of PLC-1δ was higher in the renal inner medulla of ovary-intact female rats compared to males (Fig. 4A). Ovariectomy abolished this male-female difference in PLC-1δ mRNA
expression (Fig. 4A). In contrast, no significant differences were detected in the mRNA expression of inner medullary ENaCα between groups (Fig. 4B).

**E₂ increases P2Y₂ and P2Y₄ receptor mRNA.** To identify the impact of the female sex steroid, E₂, on P2Y₂ and P2Y₄ receptor mRNA expression in the inner medullary collecting ducts, we treated mIMCD3 cells with different doses of E₂ (10, 100, 1000 nM) or vehicle (0.1% ethanol) for 24 hours. We observed that E₂ dose-dependently increases the mRNA expression of P2Y₂ and P2Y₄ receptors in mIMCD3 cells (Fig. 5).

**Discussion**

The current report establishes an important role for sex and sex steroids in regulating P2-mediated Na⁺ excretion. Our results showed that (i) infusion of the P2 antagonist, suramin, to the renal medulla attenuated urinary Na⁺ excretion in ovary-intact female rats, but not in male or OVX rats, (ii) OVX abolished the male-female difference in the mRNA expression of P2Y₂, P2Y₄ receptors and PLC-1δ in the inner medulla of the kidney and (iii) the protein abundance of the P2Y₂ receptor is higher in renal inner medulla from ovary-intact female rats, compared to males. (iv) We also provide *in vitro* evidence that E₂ upregulates the mRNA expression of P2Y₂ and P2Y₄ receptors in mIMCD3. All together, these findings suggest an interaction between E₂ and P2 signaling in the inner medulla to promote renal Na⁺ excretory function under basal physiological conditions in ovary-intact females.

Studies suggest that females have a more advanced capacity to excrete salt, compared to age-matched males [27, 28]. This female advantage appears in both experimental animals and humans [27-29]. Renal Na⁺ handling is a complex and highly regulated physiological process that
involves multiple mechanistic pathways. The renal tubular P2 signaling plays important roles in regulating urinary Na⁺ excretion \cite{10, 11}, however, whether P2-dependent renal signaling is differentially regulated based on sex and sex hormones is not clear. We recently reported that infusion of the P2Y₂ and P2Y₄ agonist, UTP \cite{30}, into the renal medulla evokes natriuresis in male and OVX rats, but not ovary-intact females \cite{20, 21}. This observation directed us to focus on the P2Y₂/P2Y₄ signaling cascade as a potential contributor to sex-related physiological differences in renal salt handling. This is particularly relevant to evidence demonstrating that sex hormones regulate extrarenal purinoceptor signaling \cite{31-34}.

To elucidate the role of endogenous activation of P2 receptors on urinary Na⁺ excretion under basal physiological conditions, we determined the effect of intramedullary infusion of the non-selective P2 antagonist, suramin, on basal urinary Na⁺ excretion in male rats and female rats with and without ovaries. We found that suramin attenuates urinary Na⁺ excretion in ovary-intact females, but not in males or OVX rats (Fig. 5), indicating that endogenous activation of P2 receptors inhibits tubular Na⁺ reabsorption in ovary-intact females, but not males or OVX females. Given that suramin is a non-selective blocker for P2 receptors, this experiment does not provide us with definite clues regarding which P2 receptor subtype(s) enhance(s) urinary Na⁺ excretion in ovary-intact females. Evidence primarily points to P2Y₂ and P2Y₄ as important players in evoking natriuresis and regulating blood pressure \cite{10, 11}. Thus, in the present study, we focused on studying aspect of the P2Y₂ and P2Y₄-mediated signaling cascades as potential natriuretic pathways that may contribute to sex-related differences in Na⁺ excretion. Additional studies are needed to fully understand the impact of sex and sex steroids on the control of Na⁺ excretory function by P2Y and P2X receptors.
The signaling mechanisms by which P2 receptor activation evokes natriuresis involves inhibition of ENaC activity via a PLC-dependent pathway [10, 11, 15, 16]. It has been shown that inhibiting P2 receptors rapidly enhances ENaC activity [16]. Experimental evidence documents the expression and functionality of P2Y_2 and P2Y_4 receptors in the collecting duct [11, 13, 16, 35]. P2Y_2 knockout mice are hypertensive, possibly due to ENaC hyperactivity leading to enhanced renal Na^+ reabsorption [14, 16]. The current study demonstrates that the renal inner medulla from ovary-intact female rats have higher mRNA expression of P2Y_2 and P2Y_4 receptors and PLC, compared to males. Importantly, this male-female difference is eliminated by ovariectomy. We did not observe sex or OVX-related differences in inner medullary ENaCα mRNA expression; however, it is possible that there are sex-related differences in the activity, rather than the expression, of this ion channel. It has been demonstrated that the distal nephrons of female rats have a higher abundance of cleaved forms of ENaCα and γ, compared to males [27]. Given that PLC signaling couples P2 receptor to ENaC [16], sex-dependent regulation of PLC may present an indirect regulatory role for sex on ENaC activity. Further functional studies are necessary to identify sex and sex hormonal-dependent modulation of ENaC activity.

Similar to our mRNA data, the protein abundance of P2Y_2 receptor was higher in ovary-intact females, compared to males. This sex difference at the protein level was not abolished by OVX. Despite our observation that ovary-intact female rats exhibit higher P2Y_4 receptor mRNA expression in their renal inner medulla compared to males or OVX rats, no differences were observed at the total protein level. The disconnect between the level of mRNA expression and the receptor protein levels for P2Y_4 receptor may reflect differences in programmed receptor destruction or post-translational modifications, rather than differences in transcription. Further
studies are needed to address potential sex and sex hormone-dependent differences in the processing of mRNA to translation, modification, localization and protein degradation.

E2 is pivotal for maintenance of cardiovascular and renal health in females [4, 5]. We provide in vitro evidence that E2 dose-dependently increases mRNA expression of P2Y2 and P2Y4 receptor in mIMCD3 cells. These data are consistent with our finding that ovary-intact female rats have an enhanced renal P2Y2/P2Y4 signaling system, compared to males. Thus, we propose that E2 regulates the renal medullary P2 system. Notably, renal estrogen receptor, ER, expression has been shown in multiple studies [36-38]. Binding studies using radiolabeled E2 revealed that radioactivity localizes to the proximal tubule and the inner medullary collecting duct [39], which is relevant to our findings in mIMCD3 cells. Data showed that classical ER, ERα and ERβ, and membrane-associated ER, G protein-coupled ER, are expressed in the collecting ducts [40]. However, the exact relationship between the ER and P2 signaling systems in the kidney is not clear.

Overall, studies in recent years generally reinforce the importance of ovarian hormones in determining quality of life and prognosis of cardiovascular and renal diseases in female patients. When results of needed studies of the modulatory role of sex hormones on critically important systems involved in the control of Na+ homeostasis and blood pressure are available, developing new clinical practice guidelines will be applicable.

Study limitations

Despite that it is established that OVX is the standard approach for studying the impact of ovarian hormones on female health in preclinical research [41], it is important to note that
there are limitations for OVX as a model for the study of postmenopausal females. Aging is a confounding factor that contributes to postmenopausal physiological changes, however OVX surgery was conducted in the current study in relatively young animals. In addition, OVX results in an abrupt decline in the plasma concentration of ovarian hormones, which is different from the slow nature of the human menopause transition, which typically spans over few (4-6) years [41]. Due to the sudden nature combined with the age of the animals employed in the current study, OVX accurately models surgical, rather than natural, menopause in women.

In addition to the sex-related differences in the signaling pathway downstream to P2Y$_2$ and P2Y$_4$ activation in the renal medulla that we identified in the current study, it is possible that there are differences in P2Y$_2$ and P2Y$_4$ receptor upstream signaling that may contribute to sex differences in P2-mediated natriuresis. Future studies are needed to determine whether there are discrepancies between males and females in renal medullary ATP levels, ecto-ATPases and Connexin 30 channel expression and function.

**Perspective and significance**

To our knowledge, this is the first study showing sex and sex-hormonal related differences in P2-dependent regulation of urinary Na$^+$ excretion. This finding may contribute to the lower prevalence of hypertension and enhanced ability to handle salt challenges evident in premenopausal females. Additional studies are necessary to identify the contribution of ATP/P2Y$_2$/P2Y$_4$/PLC/ENaC signaling cascade to salt sensitivity in postmenopausal female population.
Declarations

Ethics approval and consent to participate

All experimental procedures were executed in accordance with National Institutes of Health guidelines for the care and use of laboratory animals, ARRIVE guidelines and were approved by the Institutional Animal Care and Use Committee at University of Alabama at Birmingham.

Consent for publication

Not applicable

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that there are no conflicts of interest. Dr. Gohar is also affiliated with the Department of Pharmacology and Toxicology, Faculty of Pharmacy, Alexandria University, Egypt.

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Authors’ contributions
EYG, MK, EWI and DMP contributed to the conception and design of the research. EYG and SZ performed the experiments and analyzed the data. EYG prepared the figures and drafted the manuscript. MK, EWI and DMP edited and revised the manuscript. All authors approved the final manuscript.

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FIGURE LEGENDS

Fig. 1. Schematic presentation of the experimental timeline employed in intramedullary infusion experiments.

Fig. 2. Anti-natriuretic response to renal medullary P2 blockade in ovary-intact female rats only. Urinary Na⁺ excretion ($U_{Na}V$, $P_{interaction}=0.04$) (A-C), urine flow ($UV$, $P_{interaction}=0.07$) (D-F), urinary K⁺ excretion ($U_{K}V$, $P_{interaction}=0.9$) (G-I) and mean arterial blood pressure (MAP, $P_{interaction}=0.9$) (J-L) in anesthetized male, ovary-intact female and OVX Sprague Dawley rats receiving renal medullary interstitial infusions of suramin (P2 antagonist, 750 μg/kg/min) or vehicle. n=6 in each group. Statistical comparisons performed by two-way ANOVA followed by Sidak’s post-hoc tests.

Fig. 3. Inner medullary P2Y₂ and P2Y₄ receptor mRNA expression and protein abundance. Relative mRNA expression and total protein abundance of P2Y₂ (A, C) and P2Y₄ (B, D) receptor in the inner medulla from male, ovary-intact female and OVX Sprague Dawley rats (representative Western blots are presented). Gene expression and protein abundance values represent fold change from ovary-intact female levels. n= 4-8 rats in each group. Statistical comparisons performed using one-way ANOVA followed by Bonferroni’s post-hoc tests.

Fig. 4. Inner medullary PLC-1δ and SCNN1A mRNA expression. Relative mRNA expression of PLC-1δ (A) and SCNN1A (ENaCα) (B) in the inner medulla from male, ovary-intact female and OVX Sprague Dawley rats. Gene expression values represent fold change from ovary-intact female levels. n= 5 rats in each group. Statistical comparisons performed using one-way ANOVA followed by Bonferroni’s post-hoc tests.

Fig. 5. E₂ promotes P2Y₂ and P2Y₄ receptor mRNA expression in the inner medullary collecting duct cells (IMCD-3). P2Y₂ (A) and P2Y₄ (B) receptor mRNA expression in IMCD-3 cells incubated
with E₂ (1, 10, 100, 1000 nM) or vehicle for 24h. n=3-6 in each group. Statistical comparisons performed using one-way ANOVA followed by Dunnett’s post-hoc tests.
Experimental Timeline

- Male
- Ovary-intact female
- OVX

16-20 wks old

Surgery

80 min Equilibration

Intramedullary infusion of saline

30 min

urine collection period

IM infusion of saline or suramin
Fig. 2.

### Males

- **A**: $U_{Na V}$ (μmol/min) over Cont and Suramin
- **D**: Urine Flow (μmol/min) over Cont and Suramin
- **G**: $U_{K V}$ (μmol/min) over Cont and Suramin
- **J**: MAP (mmHg) over Cont and Suramin

### Females

- **B**: $U_{Na V}$ (μmol/min) over Cont and Suramin
- **E**: Urine Flow (μmol/min) over Cont and Suramin
- **H**: $U_{K V}$ (μmol/min) over Cont and Suramin
- **K**: MAP (mmHg) over Cont and Suramin

### OVX

- **C**: $U_{Na V}$ (μmol/min) over Cont and Suramin
- **F**: Urine Flow (μmol/min) over Cont and Suramin
- **I**: $U_{K V}$ (μmol/min) over Cont and Suramin
- **L**: MAP (mmHg) over Cont and Suramin
Fig. 3.

A) Relative P2Y2 receptor mRNA (Fold change) for Males, Females, and OVX groups, with significance levels p=0.02 and p=0.008.

B) Relative P2Y4 receptor mRNA (Fold change) for Males, Females, and OVX groups, with significance levels p=0.009 and p=0.01.

C) Western blot showing relative P2Y2 receptor protein abundance for Males, Females, and OVX groups, with p-value p=0.03.

D) Western blot showing relative P2Y4 receptor protein abundance for Males, Females, and OVX groups.
Fig. 4.

A. Relative PLC-1 mRNA (Fold change) between Males, Females, and OVX.

B. Relative SCNN1A mRNA (Fold change) between Males, Females, and OVX.

Significance: p=0.006, p=0.0007
Fig. 5.

**A**

Relative P2Y$_2$ receptor mRNA (Fold change)

- $E_2$ [nM]

- Vertical axis: 0.0 to 2.4

- Horizontal axis: 0, 10, 100, 1000

- $p=0.04$

**B**

Relative P2Y$_4$ receptor mRNA (Fold change)

- $E_2$ [nM]

- Vertical axis: 0 to 140

- Horizontal axis: 0, 10, 100, 1000

- $p=0.01$
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