Purinergic signaling is enhanced in the absence of UT-A1 and UT-A3

Nathaniel J. Himmel, *Emory University School of Medicine*
Richard T. Rogers, *Emory University School of Medicine*
Sara K. Redd, *Emory University School of Medicine*
Yirong Wang, *Emory University School of Medicine*
Mitsi Blount, *Emory University*

Journal Title: Physiological Reports

Volume: Volume 9, Number 1

Publisher: Wiley Periodicals LLC | 2021-01-01, Pages e14636-e14636

Type of Work: Article | Final Publisher PDF

Publisher DOI: 10.14814/phy2.14636

Permanent URL: https://pid.emory.edu/ark:/25593/vrq3z

Final published version: http://dx.doi.org/10.14814/phy2.14636

Copyright information:

Received: 26 February 2020 | Revised: 30 June 2020 | Accepted: 1 July 2020

DOI: 10.14814/phy2.14636

Physiological Reports

ORIGINAL RESEARCH

Purinergic signaling is enhanced in the absence of UT-A1 and UT-A3

Nathaniel J. Himmel[1] | **Richard T. Rogers**[1] | **Sara K. Redd**[1] | **Yirong Wang**[1] | **Mitsi A. Blount**[1,2]

[1]Renal Division, Department of Medicine, Emory University School of Medicine, Atlanta, GA, USA

[2]Department of Physiology, Emory University School of Medicine, Atlanta, GA, USA

Correspondence
Mitsi A. Blount, 338D Woodruff Memorial Building, 1639 Pierce Drive, Atlanta, GA 30322+, USA.
Email: mabloun@emory.edu

Funding Information
Author RTR received a research grant from the American Society of Nephrology (ASN Foundation for Kidney Research Student Scholar Grant). Author MAB has received research grants from the NIH/NIDDK (K01-DK082733, R03-DK091501, K01-DK082733-S1, and P30-DK079312).

Abstract

ATP is an important paracrine regulator of renal tubular water and urea transport. The activity of $P2Y_2$, the predominant P2Y receptor of the medullary collecting duct, is mediated by ATP, and modulates urinary concentration. To investigate the role of purinergic signaling in the absence of urea transport in the collecting duct, we studied wild-type (WT) and UT-A1/A3 null (UT-A1/A3 KO) mice in metabolic cages to monitor urine output, and collected tissue samples for analysis. We confirmed that UT-A1/A3 KO mice are polyuric, and concurrently observed lower levels of urinary cAMP as compared to WT, despite elevated serum vasopressin (AVP) levels. Because $P2Y_2$ inhibits AVP-stimulated transport by dampening cAMP synthesis, we suspected that, similar to other models of AVP-resistant polyuria, purinergic signaling is increased in UT-A1/A3 KO mice. In fact, we observed that both urinary ATP and purinergic-mediated prostanoid (PGE_2) levels were elevated. Collectively, our data suggest that the reduction of medullary osmolality due to the lack of UT-A1 and UT-A3 induces an AVP-resistant polyuria that is possibly exacerbated by, or at least correlated with, enhanced purinergic signaling.

KEYWORDS

ATP, P2Y receptors, polyuria, Urea transporters, urine concentration

1 | INTRODUCTION

A hypertonic inner medulla is necessary for concentrating urine during antidiuresis, and urea is essential to this mechanism (Sands et al., 2011). Along with sodium and chloride, urea is a major contributor to the increased osmolality of the inner medullary interstitium (Sands & Layton, 2009). High interstitial osmolality is sustained by urea reabsorption in the inner medullary collecting duct (IMCD), which in turn drives water reabsorption through the aquaporin channel AQP2 (Klein et al., 2010; Pannabecker et al., 2008; Sands & Layton, 2009). While urea is freely membrane permeable and, with sufficient time, will equalize across a lipid bilayer, maintenance of a hypertonic inner medulla requires rapid urea reabsorption in the IMCD (Sands, 2003). This is facilitated by the urea transport proteins UT-A1 and UT-A3 (Klein et al., 2011).

Arginine vasopressin (AVP) is the chief regulator of water reabsorption in the collecting duct. Antidiuresis is initiated by AVP binding to the G protein-coupled receptor vasopressin receptor 2 (V2R), which leads to increased intracellular levels of cAMP (Rieg et al., (2010); Vallon & Rieg, 2011).

Elevated cAMP levels promote phosphorylation of UT-A1 and AQP2, increasing UT-A1-mediated urea reabsorption, and thereby AQP2-mediated water entry (Blount et al., 2008; Hoffert et al., 2006). In the presence of a transepithelial osmolality gradient, AVP induces IMCD cell swelling (Chou et al., 2008; Ganote et al., 1968), which releases local factors such as the purine nucleotide adenosine-5'-triphosphate (ATP), augmenting the AVP response (Vallon & Rieg, 2011).

Renal epithelial cells release ATP in response to multiple stimuli, including increased luminal flow rate and elevated cell volume (Schwiebert, 2001; Vallon & Rieg, 2011). Once released into the extracellular environment of the inner medulla, ATP acts locally on membrane-bound P2 purinergic receptors. Several receptor subtypes—including both ionotropic P2X receptors and metabotropic P2Y receptors—are localized to the rat collecting duct (Wildman et al., 2009). In humans, of the seven known P2X receptor subunits, only $P2X_4$ has been detected in significant amounts in the collecting duct Chabardes-Garonne et al., 2003. Studies have shown that broad activation of P2X receptors increases water excretion; however, these effects were largely attributed to the proximal tubule (Jankowski et al., 2011). Although evidence suggests that P2X receptors are important to various aspects of renal physiology, the specific role P2X receptors play in the IMCD has not yet been elucidated (Burnstock et al., 2014; Unwin et al., 2003).

Several studies have demonstrated that extracellular purines modulate water handling in the IMCD through P2Y receptors (Boone & Deen, 2008; Burnstock et al., 2014). These G-protein-coupled receptors are subdivided based on the targets of their associated G-protein subunits; of the eight P2Y receptors identified, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ stimulate phospholipase C through the G-protein Gαq (Erb et al., 2006; Vallon & Rieg, 2011). Although many P2Y receptors have been reported in renal epithelia and cultured cell lines of renal origin, the $P2Y_2$ subtype is apparently the most predominant P2Y receptor expressed in native collecting duct tissue (Insel et al. 2001; Kishore et al., 2000; Schwiebert & Kishore, 2001; Turner et al., 2003).

Following the identification of the receptor's location in the collecting duct, studies revealed that ATP-driven $P2Y_2$ receptor activation is an important regulator of water reabsorption (Ecelbarger et al., 1994; Edwards, 2002; Kishore et al., 1995; Rouse et al., 1994; Sun et al., 2005a; Vallon & Rieg, 2011). As discussed above, elevated cAMP levels promote AQP2-mediated water entry, increasing cell volume (Blount et al., 2008; Hoffert et al., 2006). Increases in cell volume lead to the release of ATP at both the basolateral and apical sides of the IMCD (Hovater et al., 2008), and subsequent ATP driven $P2Y_2$ receptor activation inhibits AVP-mediated water permeability via phospholipase C inhibition of cAMP, thereby normalizing cell volume and accelerating the excretion of free water (Ecelbarger et al., 1994; Edwards, 2002; Kishore et al., 1995; Rouse et al., 1994; Sun, et al., 2005; Vallon & Rieg, 2011). Experiments in isolated IMCD indicate that ATP can stimulate the release of prostaglandin E_2 (PGE_2) by activation of $P2Y_2$ receptors (Sun, et al., 2005; Welch et al., 2003). PGE_2 is known to affect the transport of water and urea in the IMCD (Rouch & Kudo, 2000), presumably through interactions of a purinergic system, by tonically inhibiting the stimulating effect of AVP in the renal medulla.

While not fully understood, evidence does suggest that urea transport in the IMCD is regulated through purinergic signaling. In mice lacking $P2Y_2$ receptors, urea transporter expression is elevated, which likely contributes to the observed increased urinary concentration capacity of these animals (Zhang et al., 2008). Furthermore, in animals with elevated inner medullary purinergic signaling, UT-A1 protein abundance is reduced (Blount et al., 2010; Zhang et al., 2009), and functional data demonstrate that urea transport can be modulated by PGE_2 post-cAMP-dependent events (Rouch & Kudo, 2000). However, how urea transport might affect the purinergic-prostanoid system remains to be fully elucidated. In this study, we aimed to elucidate the importance of urea transport to purinergic signaling in the IMCD using transgenic mice lacking UT-A1 and UT-A3. We show that, in the absence of UT-A1 and UT-A3, there are unexpectedly low levels of luminal cAMP, despite elevated vasopressin levels. Levels of ATP and PGE2 are increased, collectively suggesting an increase in purinergic receptor activation in the absence of urea transport.

2 | MATERIALS AND METHODS

2.1 | Animals

All animal protocols were approved by the Emory University Institutional Animal Care and Use Committee. All studies were performed using male mice between 8 and 10 weeks of age that either (1) did not express UT-A1 or UT-A3 (UT-A1/A3$^{-/-}$; UT-A1/A3 KO) or (2) were control, wild-type littermates (UT-A1/A3$^{+/+}$; WT). UT-A1/A3 KO mice were a generous gift from Dr. Jeff M. Sands (Emory University, Atlanta, GA) who regenerated the line on a C56BL/6J background from the original NIH source, as previously described (Fenton et al., 2004; Ilori et al., 2013).

2.2 | Analysis of urine and serum samples

Mice were individually studied in Tecniplast Single Metabolic Cages (Tecniplast USA Inc) for 24 hr of acclimation, followed by 24 hr of urine collection. Food and water were provided ad libitum except where indicated. Urine osmolality was measured using a Wescor Vapro 5,520 Vapor Pressure Osmometer (Wescor, Logan, UT). Urine creatinine

was measured by the Jaffe reaction with a colorimetric kit (BioVision, Inc.). Urinary levels of cAMP, prostaglandin, and ATP were measured using a Cyclic AMP EIA kit (Caymen Chemical, Ann Arbor, MI), a Prostaglandin E2 Express EIA kit (Caymen Chemical, Ann Arbor, MI), and a colorimetric ATP assay kit (BioVision, Inc.), respectively. Blood was collected via intracardiac bleed, at the time of sacrifice. Serum vasopressin levels were measured using an Arg^8-Vasopressin ELISA Kit (Enzo Life Sciences,). All assay kits were used according to manufacturer specifications.

2.3 | Western blot analysis

Whole kidneys were collected, dissected into inner medulla (IM) and outer medulla (OM), and lysates prepared using an SDS lysis buffer. Lysates were spun at 5,000 RPM for 10 min at 4°C and protein content was quantified using the Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Proteins (20 μg/lane) were resolved on 12.5% SDS-PAGE gels, and then, electroblotted to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). After blotting, membranes were blocked for 1 hr in Odyssey Blocking Buffer (Li-COR, Lincoln, NE) and then, probed overnight at 4ºC with a primary antibody specific against UT-A1 (1:1,000; previously characterized (Naruse et al., 1997)), UT-A3 (1:100; previously characterized (Blount et al., 2007) or AQP2 (1:2000; SPC-503; StressMarq, Victoria, BC, Canada). Membranes were washed in TBS-tween solution, then, probed with Alexa680-conjugated anti-rabbit secondary antibody (1:4,000; Life Technologies, Grand Island, NY). Probed membranes were again washed, and then, visualized using infrared detection via the LI-COR Odyssey protein analysis system. To ensure equal loading and to normalize densitometric scanning, membranes were stained with coomassie blue to visualize total protein load. All densitometries were collected using ImageJ software (National Institutes of Health, Bethesda, MD).

2.4 | Immunofluorescence

Tissues were prepared as described previously (Sim et al., 2014). Whole kidney sections were hydrated with ethanol, and endogenous peroxides were quenched with 3% H_2O_2. After antigen retrieval using TEG buffer and quenching free aldehyde groups with 50 mM NH_4Cl/PBS, sections were blocked in 1% BSA/PBS for 30 min at room temperature. Sections were incubated overnight at 4ºC with an antibody specific to AQP2 (1:300; sc-9882; Santa Cruz Biotechnology, Dallas, Texas). After several PBS washes, sections were incubated for 2 hr at room temperature with Alexa Fluor 456 conjugated donkey anti-goat secondary antibody (1:200, Invitrogen, Carlsbad, CX). After several PBS washes, sections were mounted with ProLong Gold Antifade with DAPI (Cell Signaling), and observed under an Olympus IX71 inverted microscope.

2.5 | Real Time qRT-PCR

IM tissue was collected and stored in RNAlater solution (Life Technologies) until homogenization. Purified RNA was extracted using PureLink RNA Mini Kit (Ambion - Life Technologies), and quantified via UV spectrophotometry. Taqman RT-PCR reagents (Applied Biosystems) were used to generate all cDNA. Using predeveloped Taqman assays, real-time quantitative PCR was performed for $P2Y_2$ and the housekeeping transcript 18S (Applied Biosystems, Carlsbad, CA). PCR was performed with 20 ng of cDNA and the corresponding primer pairs using an iCycler Real-Time Detection System (BioRad Laboratories). The relative quantity of mRNA was determined using the comparative Ct method and expression levels of $P2Y_2$ receptor mRNA were normalized to ribosomal 18S expression.

2.6 | Statistical analysis

Data are expressed as the mean ± standard error of the mean, and n is the number of animals. Differences were determined by Student's *t* test using GraphPad Instat Software (La Jolla, CA). p values < 0.05 were considered statistically significant.

3 | RESULTS

3.1 | UT-A1/A3 KO mice are polyuric and have a reduced ability to reabsorb urea

Western blot analysis of inner medullary tissues collected from the UT-A1/A3 null strain developed by Ilori et al. confirms that UT-A1/A3 KO mice do not express either UT-A1 or UT-A3 proteins (Figure 1a) (Ilori et al., 2013). Inner medullary AQP2 protein expression was unaffected by the loss of urea transporters under basal conditions (Figure 1b), and immunofluorescence staining confirms that AQP2 was present at the apical membrane of IMCD cells in both WT and UT-A1/A3 KO animals (Figure 2).

Compared to WT mice, UT-A1/A3 KO mice had an elevated 24-hr urine volume (Figure 3a) and a much lower urine osmolality (Figure 3b), demonstrating that they were polyuric. Additionally, water intake was increased in the UT-A1/A3 KO mice compared to WT mice; however, a significant weight loss or difference in food intake was not observed between the two groups (Table 1). When corrected for urine dilution, urinary urea concentrations were roughly 7.5-fold

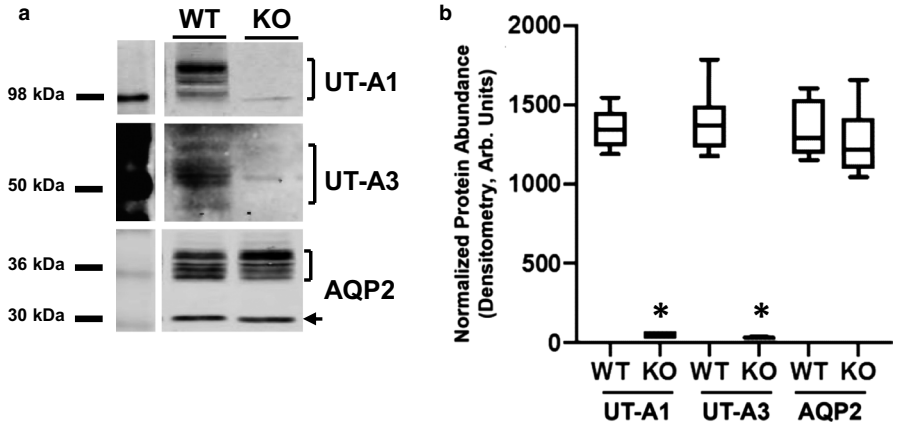

FIGURE 1 AQP2 is unchanged in the inner medulla of UT-A1/A3 KO mice. Inner medullary tissue was dissected from control, wild-type mice (UT-A1/A3+/+; WT) and UT-A1/UT-A3 null mice (UT-A1/A3-/-; KO). (a) Representative western blot images from tissues probed for UT-A1, UT-A3, and AQP2. Brackets indicate the positive identification of the glycosylated proteins. The arrow indicates the un-glycosylated form of AQP2. (b) Cumulative protein abundance quantification by densitometry presented as a box and whisker plot where whiskers mark minimum and maximum values. Differences in abundances were determined using a Student's *t* test where $p < .05$ was significant (*); $n = 6$

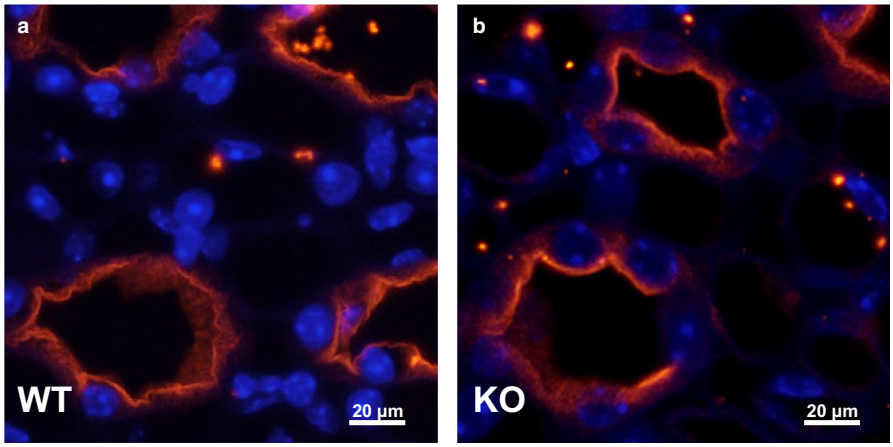

FIGURE 2 AQP2 localization in the IMCD is similar in WT and UT-A1/A3 KO mice. Perfusion-fixed kidneys were collected from wild-type mice (WT) and UT-A1/A3 KO mice (KO). Following paraffin embedding, 4 µm-thick sections were stained for AQP2 (red) and DAPI (blue). Shown are representative images
taken fromthe inner medulla of WT (a) and KO (b) mice. Scale bar = 20 µm; $n = 3$

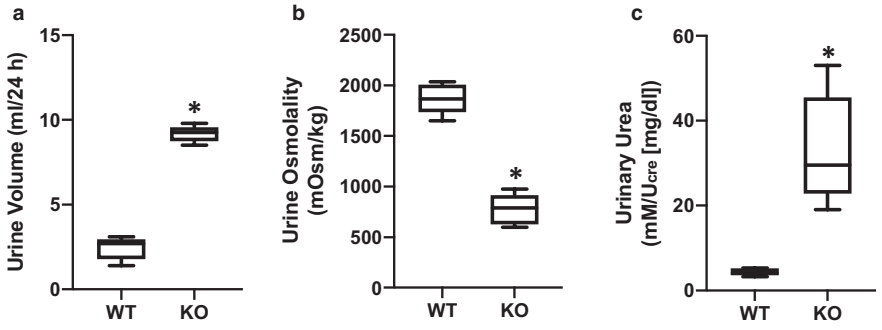

FIGURE 3 UT-A1/A3 KO mice are polyuric. Control, wild-type mice (WT) and UT-A1/A3 KO mice (KO) were studied in metabolic cages and urine was collected over 24 hr. (a) Total volume of urine collected under oil during the timed 24-hr period. (b) Urine osmolality of the collected urine. (c) Urinary urea measured from collected urine normalized to creatinine (U_{cre}) to correct for differences in urine dilution. All data presented as box and whisker plots where whiskers mark minimum and maximum values. Differences were determined using a Student's *t* test where $p < .05$ was significant (*); $n = 6$

TABLE 1 UT-A1/A3 KO mice have increased water intake compared to WT mice

	WT	UT-A1/A3 KO
Food Intake (g/g BW/24 hr)	0.14 ± 0.01	0.15 ± 0.01
Water Intake (ml/g BW/24 hr)	0.18 ± 0.01	0.28 ± 0.02*
Body Weight (g)	28.2 ± 1.1	27.5 ± 0.9

After 24 hr of acclimation, food and water intake were measured in male age-matched mice for 24-hr using timed metabolic caging. Body weight was measured at the beginning and end of the 24-hr timed collection which followed a 24-hr acclimation period. Data are ± SE. Differences were determined using a Student's *t* test where $p < .05$ was significant (*); $n = 3$

higher in UT-A1/A3 KO mice compared to WT animals (Figure 3c), demonstrating a decreased ability to reabsorb urea in the absence of UT-A1 and UT-A3. This metabolic characterization of UT-A1/A3 KO mice confirms previous reports establishing the importance of UT-1 and UT-A3 in urine concentration (Fenton et al., 2004; Ilori et al., 2013).

3.2 | UT-A1/A3 KO mice have low urinary cAMP excretion despite elevated AVP levels

Serum analysis indicated that circulating AVP levels were significantly elevated in UT-A1/A3 KO mice (Figure 4a). As previously discussed, AVP activates V2R, increasing intracellular cAMP levels (Hoffert et al., 2006; Pearce et al., 2014). With this mechanism in mind, we investigated urinary levels of cAMP. Interestingly, urinary cAMP levels were significantly lower in UT-A1/A3 KO mice as compared to WT animals, despite elevated AVP levels (Figure 4b).

AVP-mediated water reabsorption is inhibited by ATP-mediated activation of $P2Y_2$, thereby dampening cAMP synthesis (Kishore et al., 1995). Given the decreased urinary cAMP levels observed in UT-A1/A3 KO mice, we suspected that there was an increase in ATP-mediated $P2Y_2$ activation. Analysis confirms that urinary ATP levels were higher in UT-A1/A3 KO mice as compared to WT mice (Figure 4c).

This elevation in urinary ATP led us to speculate that, similar to other animal models of AVP-resistant polyuria (Sun, et al., 2005), purinergic signaling was increased in the UT-A1/A3 KO mice. Although the IMCD releases moderate amounts of PGE_2 under basal conditions, specific activation of the $P2Y_2$ receptor results in an enhanced release of PGE_2 from IMCD cells in a time- and concentration-dependent manner (Welch et al., 2003). Therefore, to indirectly analyze the ATP-activation of the $P2Y_2$ receptor in the IMCD, we measured urinary excretion of PGE_2. Immunoassays revealed that PGE_2 levels were higher in UT-A1/A3 KO mice as compared to WT mice (Figure 4d).

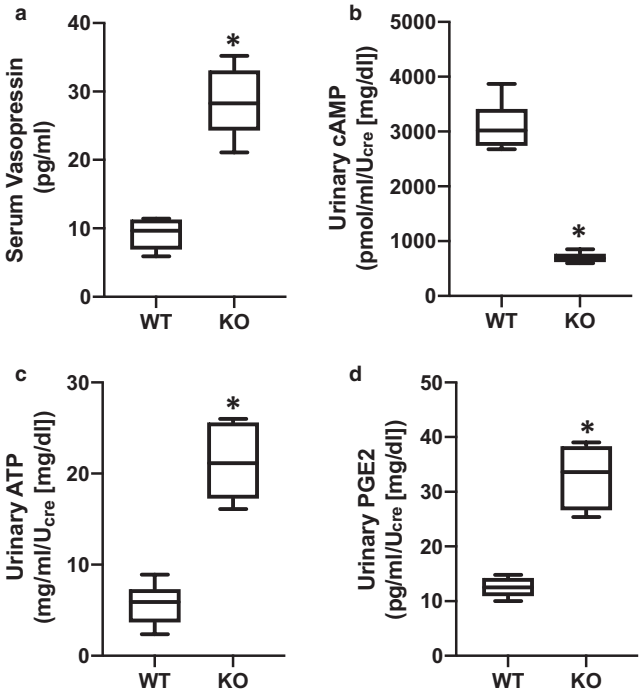

FIGURE 4 UT-A1/A3 KO mice excrete low amounts of cAMP, ATP, and PGE_2 despite high circulating AVP. (a) Vasopressin was measured in serum samples collected from wild-type mice (WT) and UT-A1/A3 KO mice (KO). Mice were studied in metabolic cages and urine was collected over 24 hr. Fresh urine was then analyzed for (b) cAMP, (c) ATP, and (d) PGE_2. All concentrations were normalized to creatinine (U_{cre}) to correct for differences in urine dilution. All data presented as box and whisker plots where whiskers mark minimum and maximum values. Differences were determined using a Student's *t* test where $p < .05$ was significant (*); $n = 4$

3.3 | UT-A1/A3 KO mice have unchanged $P2Y_2$ mRNA expression in the inner medulla

$P2Y_2$ activation in response to ATP release in the collecting duct is critical for modulating water transport (Vallon et al., 2012). To determine if an increase in $P2Y_2$ receptor abundance contributed to the observed increase in purinergic activity in UT-A1/A3 KO mice, we measured $P2Y_2$ mRNA expression in the medullary tissues of these animals using quantitative real-time RT-PCR. There was no significant difference in $P2Y_2$ mRNA expression in the medullary tissues of WT and UT-A1/A3 KO mice (fold change in mRNA expression: WT, 1.00; KO 0.90).

4 | DISCUSSION

Many have shown that local factors, including ATP, may modulate the actions of AVP (Pearce et al., 2014). In the IMCD, ATP activates $P2Y_2$ receptor, thereby inhibiting AVP-mediated water permeability via phospholipase C inhibition of

cAMP. The activation of the $P2Y_2$ receptor ultimately leads to PGE_2 release (Sun, et al., 2005; Welch et al., 2003). ATP modulation of AVP-stimulated water transport in the IMCD has been studied extensively employing both pharmacological approaches (Ecelbarger et al., 1994; Kishore et al., 1995; Sun, et al., 2005; Sun, et al., 2005), and metabolic examination of mice lacking $P2Y_2$ receptors (Rieg et al., 2007; Zhang et al., 2008); however, the role that urea transport plays in the purinergic-prostanoid system has not been extensively studied.

In the studies described herein, we found that mice simultaneously lacking both the apical urea transporter, UT-A1, and the basolateral urea transporter, UT-A3, have extensive polyuria despite unchanged levels of AQP2 expression and elevated circulating AVP. We observed lower levels of urinary cAMP in UT-A1/A3 KO mice as compared to wild-type litter mates. Furthermore, we observed that UT-A1/A3 KO mice have increased urinary PGE_2 levels as compared to wild-type litter mates, as well as elevated expression of the $P2Y_2$ receptor in inner medullary tissues. These data collectively indicate an increase in $P2Y_2$ activity in UT-A1/A3 KO mice.

Increased levels of ATP might drive increased $P2Y_2$ activation, and we observed an increase of urinary ATP in UT-A1/A3 KO mice. As previously discussed, rapid changes to the tonicity of the inner medullary interstitium is responsible for increased IMCD cell size, and the subsequent release of ATP is essential to normalizing cell volume by driving $P2Y_2$ activation. Previous studies indicate that acute water loading increases the release of ATP due to the reduction of extracellular tonicity and a resultant increase in cell volume (Rieg et al., 2007). It has been previously demonstrated, and we have confirmed, that UT-A1/A3 KO mice have a reduced ability to reabsorb urea, and thus, have higher levels of urinary urea when compared to wild-type animals. Given that urea is a major osmotic player, its presence in luminal fluid could be building osmotic potential toward the lumen of the collecting duct. Water moving from the interstitium toward the collecting duct, through the basolateral aquaporins AQP3 and AQP4, could increase cell volume, releasing ATP at both the basolateral and apical membranes, contributing to the observed increase in ATP, and ultimately to an increase in $P2Y_2$ activation.

Among previously mentioned stimulators, increased luminal flow increases luminal ATP expression in principal cells of the collecting duct (Jensen et al., 2007; Praetorius & Leipziger, 2010). UT-A1/A3 KO mice are polyuric, and as such, luminal flow rates in the collecting duct are likely increased as compared to wild-type mice. It is worth noting, however, that urinary flow rate cannot be simply inferred based on ATP excretion; for example, water loading and V2R blocking both increase flow rate, but differentially affect ATP excretion (Rieg & Vallon, 2009). At this time, it is unclear what other factors may be involved. As $P2Y_2$ receptors are also activated by UTP (Soltoff et al., 1998), this constitutes one possible area for future investigation.

We observed that AQP2 protein abundance remains unchanged in UT-A1/A3 KO mice, and as such the potential for water movement is likely unaffected. Granted that urea recycling in the inner medulla is ablated in the UT-A1/A3 KO mice, there are no reported differences in the expression of sodium transporters in the distal nephron and collecting ducts of these animals (Fenton et al., 2005), suggesting that sodium reabsorption can still contribute to generating a weakened osmotic gradient in the medullary interstitium. However, because of possible elevated purinergic-prostanoid activity in the UT-A1/A3 KO mice, we predict that any possible water reabsorption by AQP2 would be inhibited despite the presence of a dampened osmotic gradient. Therefore, the polyuria seen in the UT-A1/A3 KO mice is likely compounded by the inability of these mice to reabsorb luminal water in the IMCD.

While many studies agree that PGE_2 plays an important role in regulating water excretion by modulating the effects of AVP, PGE_2-diuretic effects are extremely variable. For instance, PGE_2 stimulates water transport in the absence of AVP, but decreases water and urea permeability in the presence of AVP (Hebert et al., 1993; Rouch & Kudo, 2000). Although the understanding of the underlying molecular mechanisms behind the PGE_2-mediated inhibition of urea and water transport remains incomplete, the differences in PGE2-mediated actions are likely explained by the diverse range of biologic actions elicited by the four E-prostanoid receptor subtypes, EP1, EP2, EP3, and EP4 (Breyer & Breyer, 2001; Olesen & Fenton, 2013). While not investigated herein, we speculate that the elevated PGE_2 levels observed in UT-A1/A3 KO mice may inhibit AVP-induced water reabsorption through the EP3 receptor. EP3 receptor stimulation, which is coupled to G_i, would explain the decrease levels of urinary cAMP in these mice despite elevated AVP. EP3-deficient mice can concentrate urine similar to WT mice after both water loading and water deprivation; however, pharmacological inhibition of PGE_2 production only increases urine osmolality in WT mice. These studies suggest that PGE_2 action through the EP3 receptor is not essential for urine concentration under normal conditions, but when PGE_2 is elevated, as observed in the UT-A1/A3 KO mice, the EP3 receptor likely plays a crucial role (Fleming et al., 1998). Further support comes from a recent study demonstrating that lithium-induced polyuria is attenuated in $P2Y_2$ KO mice (Zhang et al., 2013). EP3 receptor expression is downregulated in the collecting ducts of lithium-treated $P2Y_2$ KO mice, and PGE_2-stimulation of isolated IMCD tubules from these mice stimulates cAMP production at higher rates than lithium-treated WT mice. Not only do these findings suggest that the IMCD can become more sensitive to PGE_2 in response to altered physiological settings like elevated urine flow, but these studies also demonstrate that $P2Y_2$ is a prominent regulator of EP3 abundance.

Another explanation could be due to an increase in $P2Y_2$ receptor protein abundance. An increase in $P2Y_2$ protein abundance in the inner medulla of UT-A1/A3 KO mice would lead to an increase in total $P2Y_2$ activity. Previous studies have linked UT-A1 and $P2Y_2$ abundance in a backward fashion, demonstrating that in the absence of $P2Y_2$, UT-A1 protein abundance is increased (Zhang et al., 2008). Additionally, in models of heightened purinergic activity, UT-A1 protein abundance is reduced (Blount et al., 2010; Zhang et al., 2009). A likely explanation is that in the absence of urea transport in the IMCD, medullary tonicity is decreased, resulting in enhanced $P2Y_2$ abundance. In vivo studies suggest that alterations in $P2Y_2$ abundance are regulated by medullary tonicity (Kishore et al., 2005), which was later supported by in vitro studies in mpkCCD cells (Wildman et al., 2009). In fact, inner medullary tissue osmolality is significantly higher in mice lacking $P2Y_2$ receptors, suggesting that the elevated UT-A1 abundance observed in these same mice increases their urea recycling capability (Zhang et al., 2008, 2013).

Although we did not observe changes in mRNA expression of $P2Y_2$ in UT-A1/A3 KO mice, others investigating the expression profiles of $P2Y_2$ in rodent IMCD found that mRNA and protein levels of the receptor can be discordant (Kishore et al., 2005). It is not uncommon for G protein-coupled receptors like $P2Y_2$ to be sequestered into the cytoplasm to either be recycled back to the plasma membrane or sorted for degradation into lysosomes. Biochemical studies examining the cellular trafficking of a tagged-$P2Y_2$ demonstrate that the receptor can be internalized by a clathrin-mediated pathway and degraded through a proteasome pathway (Sromek & Harden, 1998; Tulapurkar et al., 2005). Increased AVP levels induce a cAMP-dependent translocation of $P2Y_2$ receptors into the basolateral membrane (Wildman et al., 2009) suggesting that the observed receptor recycling and possibly degradation may be mediated by AVP. This and other possible explanations will need to be explored in more extensive investigations to elucidate the reason for the observed differences between mRNA and protein expression; other explanations may involve posttranslational modification or other aspects of $P2Y_2$ protein stability and turnover. Future studies and improved tools are needed to clarify this relationship between the urea transporters and $P2Y_2$ in the IMCD.

In conclusion, using UT-A1/A3 KO mice we have demonstrated that urea transport in the inner medulla is modulated in some part by the purinergic-prostanoid system. Although most previous studies have focused on local regulation of water reabsorption in the IMCD by ATP, the effect of this nucleotide on urea handling in the inner medulla is largely unknown. This work represents the first step in understanding the connection between $P2Y_2$-regulation of the urea transporters UT-A1 and UT-A3.

ACKNOWLEDGMENTS

The authors would like to thank Jessie Kuo for technical assistance, and Dr. Jeff M. Sands for generously providing UT-A1/A3 KO mice. Present addresses: (NJH) Neuroscience Institute, Georgia State University, Atlanta, GA; (RTR) Department of Surgery, Mayo Clinic, Rochester, MN; (SKR) Department of Behavioral Sciences and Health Education, Rollins School of Public Health, Emory University, Atlanta, GA; and (YW) Department of Nutritional Sciences, University of Wisconsin-Madison, Madison, WI.

CONFLICT OF INTEREST

This study was funded by a Norman S. Coplon Extramural Grant provided by Satellite Healthcare (MAB). The remaining authors declare that they have no conflict of interest.

ORCID

Nathaniel J. Himmel https://orcid.org/0000-0001-7876-6960
Mitsi A. Blount https://orcid.org/0000-0002-1637-3341

REFERENCES

Blount, M. A., Klein, J. D., Martin, C. F., Tchapyjnikov, D., & Sands, J. M. (2007). Forskolin stimulates phosphorylation and membrane accumulation of UT-A3. *American Journal of Physiology Renal Physiology*, *293*, F1308–F1313. https://doi.org/10.1152/ajprenal.00197.2007

Blount, M. A., Mistry, A. C., Fröhlich, O., Price, S. R., Chen, G., Sands, J. M., & Klein, J. D. (2008). Phosphorylation of UT-A1 urea transporter at serines 486 and 499 is important for vasopressin-regulated activity and membrane accumulation. *American Journal of Physiology. Renal Physiology*, *295*, F295–F299. https://doi.org/10.1152/ajprenal.00102.2008

Blount, M. A., Sim, J. H., Zhou, R., Martin, C. F., Lu, W., Sands, J. M., & Klein, J. D. (2010). Expression of transporters involved in urine concentration recovers differently after cessation of lithium treatment. *American Journal of Physiology-Renal Physiology*, *298*, F601–608. https://doi.org/10.1152/ajprenal.00424.2009

Boone, M., & Deen, P. M. T. (2008). Physiology and pathophysiology of the vasopressin-regulated renal water reabsorption. *Pflugers Archiv*, *456*, 1005–1024. https://doi.org/10.1007/s00424-008-0498-1

Breyer, M. D., & Breyer, R. M. (2001). G protein-coupled prostanoid receptors and the kidney. *Annual Review of Physiology*, *63*, 579–605. https://doi.org/10.1146/annurev.physiol.63.1.579

Burnstock, G., Evans, L. C., & Bailey, M. A. (2014). Purinergic signalling in the kidney in health and disease. *Purinergic Signalling*, *10*, 71–101. https://doi.org/10.1007/s11302-013-9400-5

Chabardes-Garonne, D., Mejean, A., Aude, J. C., Cheval, L., Di Stefano, A., Gaillard, M. C., Imbert-Teboul, M., Wittner, M., Balian, C., Anthouard, V., Robert, C., Segurens, B., Wincker, P., Weissenbach, J., Doucet, A., & Elalouf, J. M. (2003). A panoramic view of gene expression in the human kidney. *Proceedings of the National Academy of Sciences of the United States of America*, *100*, 13710–13715. https://doi.org/10.1073/pnas.2234604100

Chou, C.-L., Yu, M.-J., Kassai, E. M., Morris, R. G., Hoffert, J. D., Wall, S. M., & Knepper, M. A. (2008). Roles of basolateral solute uptake via NKCC1 and of myosin II in vasopressin-induced

cell swelling in inner medullary collecting duct. *American Journal of Physiology Renal Physiology*, *295*, F192–F201. https://doi.org/10.1152/ajprenal.00011.2008

Ecelbarger, C. A., Maeda, Y., Gibson, C. C., & Knepper, M. A. (1994). Extracellular ATP increases intracellular calcium in rat terminal collecting duct via a nucleotide receptor. *American Journal of Physiology. Renal Physiology*, *267*, F998–F1006. https://doi.org/10.1152/ajprenal.1994.267.6.F998

Edwards, R. M. (2002). Basolateral, but not apical, ATP inhibits vasopressin action in rat inner medullary collecting duct. *European Journal of Pharmacology*, *438*, 179–181. https://doi.org/10.1016/S0014-2999(02)01293-1

Insel, P. A., Ostrom, R. S., Zambon, A. C., Hughes, R. J., Balboa, M. A., Shehnaz, D., Gregorian, C., Torres, B., Firestein, B. L., Xing, M., & Post¥, S. R. (2001). Extracellular ATP and cAMP as paracrine and interorgan regulators of renal function P2Y Receptors of MDCK Cells: Epithelial Cell Regulation by Extracellular Nucleotides. *Clinical and Experimental Pharmacology and Physiology*, *28*, 351–354.

Erb, L., Liao, Z., Seye, C., & Weisman, G. (2006). P2 receptors: Intracellular signaling. *Pflügers Archiv*, *452*, 552–562.

Fenton, R. A., Chou, C.-L., Stewart, G. S., Smith, C. P., & Knepper, M. A. (2004). Urinary concentrating defect in mice with selective deletion of phloretin-sensitive urea transporters in the renal collecting duct. *Proceedings of the National Academy of Sciences of the United States of America*, *101*, 7469–7474. https://doi.org/10.1073/pnas.0401704101

Fenton, R. A., Flynn, A., Shodeinde, A., Smith, C. P., Schnermann, J., & Knepper, M. A. (2005). Renal phenotype of UT-A urea transporter knockout mice. *Journal of the American Society of Nephrology : JASN*, *16*, 1583–1592. https://doi.org/10.1681/ASN.2005010031

Fleming, E. F., Athirakul, K., Oliverio, M. I., Key, M., Goulet, J., Koller, B. H., & Coffman, T. M. (1998). Urinary concentrating function in mice lacking EP3 receptors for prostaglandin E2. *The American Journal of Physiology*, *275*, F955–961.

Ganote, C. E., Grantham, J. J., Moses, H. L., Burg, M. B., & Orloff, J. (1968). Ultrastructural studies of vasopressin effect on isolated perfused renal collecting tubules of the rabbit. *The Journal of Cell Biology*, *36*, 355–367. https://doi.org/10.1083/jcb.36.2.355

Hebert, R. L., Jacobson, H. R., Fredin, D., & Breyer, M. D. (1993). Evidence that separate PGE2 receptors modulate water and sodium transport in rabbit cortical collecting duct. *The American Journal of Physiology*, *265*, F643–650. https://doi.org/10.1152/ajprenal.1993.265.5.F643

Hoffert, J. D., Pisitkun, T., Wang, G., Shen, R.-F., & Knepper, M. A. (2006). Quantitative phosphoproteomics of vasopressin-sensitive renal cells: Regulation of aquaporin-2 phosphorylation at two sites. *Proceedings of the National Academy of Sciences*, *103*, 7159–7164.

Hovater, M., Olteanu, D., Hanson, E., Cheng, N.-L., Siroky, B., Fintha, A., Komlosi, P., Liu, W., Satlin, L., Bell, P. D., Yoder, B., & Schwiebert, E. (2008). Loss of apical monocilia on collecting duct principal cells impairs ATP secretion across the apical cell surface and ATP-dependent and flow-induced calcium signals. *Purinergic Signalling*, *4*, 155–170. https://doi.org/10.1007/s11302-007-9072-0

Ilori, T. O., Blount, M. A., Martin, C. F., Sands, J. M., & Klein, J. D. (2013). Urine concentration in the diabetic mouse requires both urea and water transporters. *American Journal of Physiology. Renal Physiology*, *304*, F103–111. https://doi.org/10.1152/ajprenal.00385.2012

Jankowski, M., Szamocka, E., Kowalski, R., Angielski, S., & Szczepanska-Konkel, M. (2011). The effects of P2X receptor agonists on renal sodium and water excretion in anaesthetized rats. *Acta Physiologica (Oxford, England)*, *202*, 193–201. https://doi.org/10.1111/j.1748-1716.2011.02276.x

Jensen, M. E. J., Odgaard, E., Christensen, M. H., Praetorius, H. A., & Leipziger, J. (2007). Flow-Induced [Ca2+]i increase depends on nucleotide release and subsequent purinergic signaling in the intact nephron. *Journal of the American Society of Nephrology*, *18*, 2062–2070.

Kishore, B. K., Chou, C. L., & Knepper, M. A. (1995). Extracellular nucleotide receptor inhibits AVP-stimulated water permeability in inner medullary collecting duct. *The American Journal of Physiology*, *269*, F863–869. https://doi.org/10.1152/ajprenal.1995.269.6.F863

Kishore, B. K., Ginns, S. M., Krane, C. M., Nielsen, S., & Knepper, M. A. (2000). Cellular localization of P2Y(2) purinoceptor in rat renal inner medulla and lung. *American Journal of Physiology Renal Physiology*, *278*, F43–51.

Kishore, B. K., Krane, C. M., Miller, R. L., Shi, H., Zhang, P., Hemmert, A., Sun, R., & Nelson, R. D. (2005). P2Y2 receptor mRNA and protein expression is altered in inner medullas of hydrated and dehydrated rats: Relevance to AVP-independent regulation of IMCD function. *American Journal of Physiology Renal Physiology*, *288*, F1164–1172.

Klein, J. D., Blount, M. A., Fröhlich, O., Denson, C. E., Tan, X., Sim, J. H., Martin, C. F., & Sands, J. M. (2010). Phosphorylation of UT-A1 on serine 486 correlates with membrane accumulation and urea transport activity in both rat IMCDs and cultured cells. *American Journal of Physiology Renal Physiology*, *298*, F935–F940. https://doi.org/10.1152/ajprenal.00682.2009

Klein, J. D., Blount, M. A., & Sands, J. M. (2011). Urea Transport in the Kidney. *Comprehensive Physiology*. John Wiley & Sons Inc.

Naruse, M., Klein, J. D., Ashkar, Z. M., Jacobs, J. D., & Sands, J. M. (1997). Glucocorticoids downregulate the vasopressin-regulated urea transporter in rat terminal inner medullary collecting ducts. *Journal of the American Society of Nephrology*, *8*, 517–523.

Olesen, E. T. B., & Fenton, R. A. (2013). Is There a Role for PGE2 in Urinary Concentration? *Journal of the American Society of Nephrology*, *24*, 169–178. https://doi.org/10.1681/ASN.2012020217

Pannabecker, T. L., Dantzler, W. H., Layton, H. E., & Layton, A. T. (2008). Role of three-dimensional architecture in the urine concentrating mechanism of the rat renal inner medulla. *American Journal of Physiology. Renal Physiology*, *295*, F1271–F1285. https://doi.org/10.1152/ajprenal.90252.2008

Pearce, D., Soundararajan, R., Trimpert, C., Kashlan, O. B., Deen, P. M., & Kohan, D. E. (2014). *Collecting Duct Principal Cell Transport Processes and Their Regulation*. Clinical journal of the American Society of Nephrology.

Praetorius, H. A., & Leipziger, J. (2010). Intrarenal purinergic signaling in the control of renal tubular transport. *Annual Review of Physiology*, *72*, 377–393. https://doi.org/10.1146/annurev-physiol-021909-135825

Rieg, T., Bundey, R. A., Chen, Y., Deschenes, G., Junger, W., Insel, P. A., & Vallon, V. (2007). Mice lacking P2Y2 receptors have salt-resistant hypertension and facilitated renal Na+ and water reabsorption. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *21*, 3717–3726.

Rieg, T., Tang, T., Murray, F., Schroth, J., Insel, P. A., Fenton, R. A., Hammond, H. K., & Vallon, V. (2010). Adenylate Cyclase 6 Determines cAMP Formation and Aquaporin-2 Phosphorylation and Trafficking in Inner Medulla. *Journal of the American Society of Nephrology*, *21*, 2059–2068. https://doi.org/10.1681/ASN.2010040409

Rieg, T., & Vallon, V. (2009). ATP and adenosine in the local regulation of water transport and homeostasis by the kidney. *American Journal of Physiology Regulatory, Integrative and Comparative Physiology*, *296*, R419–427. https://doi.org/10.1152/ajpregu.90784.2008

Rouch, A. J., & Kudo, L. H. (2000). Role of PGE(2) in alpha(2)-induced inhibition of AVP- and cAMP-stimulated H(2)O, Na(+), and urea transport in rat IMCD. *American Journal of Physiology Renal Physiology*, *279*, F294–301.

Rouse, D., Leite, M., & Suki, W. N. (1994). ATP inhibits the hydrosmotic effect of AVP in rabbit CCT: Evidence for a nucleotide P2u receptor. *American Journal of Physiology Renal Physiology*, *267*, F289–F295.

Sands, J. M. (2003). Molecular mechanisms of urea transport. *Journal of Membrane Biology*, *191*, 149–163. https://doi.org/10.1007/s00232-002-1053-1

Sands, J. M., Blount, M. A., & Klein, J. D. (2011). Regulation of renal urea transport by vasopressin. *Transactions of the American Clinical and Climatological Association*, *122*, 82–92.

Sands, J. M., & Layton, H. E. (2009). The physiology of urinary concentration: An update. *Seminars in Nephrology*, *29*, 178–195.

Schwiebert, E. M. (2001). ATP release mechanisms, ATP receptors and purinergic signalling along the nephron. *Clinical and Experimental Pharmacology & Physiology*, *28*, 340–350.

Schwiebert, E. M., & Kishore, B. K. (2001). Extracellular nucleotide signaling along the renal epithelium. *American Journal of Physiology Renal Physiology*, *280*, F945–F963. https://doi.org/10.1152/ajprenal.2001.280.6.F945

Sim, J. H., Himmel, N. J., Redd, S. K., Pulous, F. E., Rogers, R. T., Black, L. N., Hong, S. M., von Bergen, T. N., & Blount, M. A. (2014). Absence of PKC-alpha attenuates lithium-induced nephrogenic diabetes insipidus. *PLoS One*, *9*, e101753. https://doi.org/10.1371/journal.pone.0101753

Soltoff, S. P., Avraham, H., Avraham, S., & Cantley, L. C. (1998). Activation of P2Y2 Receptors by UTP and ATP stimulates mitogen-activated kinase activity through a pathway that involves related adhesion focal tyrosine kinase and protein kinase C. *Journal of Biological Chemistry*, *273*, 2653–2660.

Sromek, S. M., & Harden, T. K. (1998). Agonist-induced internalization of the P2Y2 receptor. *Molecular Pharmacology*, *54*, 485–494.

Sun, R., Carlson, N. G., Hemmert, A. C., & Kishore, B. K. (2005). P2Y2 receptor-mediated release of prostaglandin E2 by IMCD is altered in hydrated and dehydrated rats: Relevance to AVP-independent regulation of IMCD function. *American Journal of Physiology. Renal Physiology*, *289*, F585–592.

Sun, R., Miller, R. L., Hemmert, A. C., Zhang, P., Shi, H., Nelson, R. D., & Kishore, B. K. (2005). Chronic dDAVP infusion in rats decreases the expression of P2Y2 receptor in inner medulla and P2Y2 receptor-mediated PGE2 release by IMCD. *American Journal of Physiology. Renal Physiology*, *289*, F768–776.

Tulapurkar, M. E., Schafer, R., Hanck, T., Flores, R. V., Weisman, G. A., Gonzalez, F. A., & Reiser, G. (2005). Endocytosis mechanism of P2Y2 nucleotide receptor tagged with green fluorescent protein: Clathrin and actin cytoskeleton dependence. *Cellular and Molecular Life Sciences : CMLS*, *62*, 1388–1399.

Turner, C. M., Vonend, O., Chan, C., Burnstock, G., & Unwin, R. J. (2003). The pattern of distribution of selected ATP-sensitive P2 receptor subtypes in normal rat kidney: An immunohistological study. *Cells, Tissues, Organs*, *175*, 105–117.

Unwin, R. J., Bailey, M. A., & Burnstock, G. (2003). Purinergic signaling along the renal tubule: The current state of play. *News in Physiological Sciences : An International Journal of Physiology Produced Jointly by the International Union of Physiological Sciences and the American Physiological Society*, *18*, 237–241.

Vallon, V., & Rieg, T. (2011). Regulation of renal NaCl and water transport by the ATP/UTP/P2Y2 receptor system. *American Journal of Physiology Renal Physiology*, *301*, F463–475.

Vallon, V., Stockand, J., & Rieg, T. (2012). P2Y receptors and kidney function. *Wiley Interdisciplinary Reviews Membrane Transport and Signaling*, *1*, 731–742. https://doi.org/10.1002/wmts.61

Welch, B. D., Carlson, N. G., Shi, H., Myatt, L., & Kishore, B. K. (2003). P2Y2 receptor-stimulated release of prostaglandin E2 by rat inner medullary collecting duct preparations. *American Journal of Physiology Renal Physiology*, *285*, F711–721.

Wildman, S. S., Boone, M., Peppiatt-Wildman, C. M., Contreras-Sanz, A., King, B. F., Shirley, D. G., Deen, P. M., & Unwin, R. J. (2009). Nucleotides downregulate aquaporin 2 via activation of apical P2 receptors. *Journal of the American Society of Nephrology : JASN*, *20*, 1480–1490. https://doi.org/10.1681/ASN.2008070686

Zhang, Y., Li, L., Kohan, D. E., Ecelbarger, C. M., & Kishore, B. K. (2013). Attenuation of lithium-induced natriuresis and kaliuresis in P2Y2 receptor knockout mice. *American Journal of Physiology. Renal Physiology*, *305*, F407–416.

Zhang, Y., Nelson, R. D., Carlson, N. G., Kamerath, C. D., Kohan, D. E., & Kishore, B. K. (2009). Potential role of purinergic signaling in lithium-induced nephrogenic diabetes insipidus. *American Journal of Physiology Renal Physiology*, *296*, F1194–1201. https://doi.org/10.1152/ajprenal.90774.2008

Zhang, Y., Sands, J. M., Kohan, D. E., Nelson, R. D., Martin, C. F., Carlson, N. G., Kamerath, C. D., Ge, Y., Klein, J. D., & Kishore, B. K. (2008). Potential role of purinergic signaling in urinary concentration in inner medulla: Insights from P2Y2 receptor gene knockout mice. *American Journal of Physiology Renal Physiology*, *295*, F1715–1724.

How to cite this article: Himmel NJ, Rogers RT, Redd SK, Wang Y, Blount MA. Purinergic signaling is enhanced in the absence of UT-A1 and UT-A3. *Physiol Rep*. 2021;9:e14636. https://doi.org/10.14814/phy2.14636