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Research Article **Nephrology**

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RESEARCH ARTICLE

Metformin improves urine concentration in rodents with nephrogenic diabetes insipidus

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Urine concentration is regulated by vasopressin. Congenital nephrogenic diabetes insipidus (NDI) is caused by vasopressin type 2 receptor (V2R) mutations. We studied whether metformin could improve urine concentration in rodent models of congenital NDI by stimulating AMPK. To block the V2R in rats, tolvaptan (10 mg/kg/d) was given by oral gavage with or without metformin (800 mg/kg/d). Control rats received vehicle with or without metformin. Tamoxifen-induced V2R KO mice were given metformin (600 mg/kg) or vehicle twice daily. Urine osmolality in tolvaptan-treated rats (1,303 ± 126 mOsM) was restored to control levels by metformin (2,335 ± 273 mOsM) within 3 days and was sustained for up to 10 days. Metformin increased protein abundance of inner medullary urea transporter UT-A1 by 61% and aquaporin 2 (AQP2) by 44% in tolvaptan-treated rats, and immunohistochemistry showed increased membrane accumulation of AQP2 with acute and chronic AMPK stimulation. Outer medullary Na^+-K^+-$2Cl^-$ cotransporter 2 (NKCC2) abundance increased (117%) with AMPK stimulation in control rats but not in V2R-blocked rats. Metformin increased V2R KO mouse urine osmolality within 3 hours, and the increase persisted for up to 12 hours. Metformin increased AQP2 in the V2R KO mice similar to the tolvaptan-treated rats. These results indicate that AMPK activators, such as metformin, might provide a promising treatment for congenital NDI.

Introduction

Urine concentration in mammals requires (a) an osmotic gradient between the medullary interstitium and the collecting duct lumen and (b) that the collecting duct be permeable to water. A hypertonic interstitium is mainly generated by Na^+-K^+-$2Cl^-$ cotransporter 2 (NKCC2) in the outer medulla (OM) and urea transporters (UT) in the inner medulla (IM). Water is absorbed from the collecting duct lumen through aquaporin 2 (AQP2). In healthy subjects, vasopressin (AVP) activates these transporters by stimulating vasopressin type 2 receptors (V2Rs). V2R stimulates production of cAMP, which in turn, activates PKA to phosphorylate serines 486 and 499 of UT-A1 and serines 256, 264, and 269 of AQP2 (1–4). Phosphorylation of these transporters results in their insertion into the apical plasma membrane. Alternatively, phosphorylation of AQP2 Ser261 is downregulated by AVP (5).

Nephrogenic diabetes insipidus (NDI) is a consequence of resistance of the kidney to AVP. Congenital NDI is caused by mutation of V2Rs in 90% of cases (6). Present treatment options, such as indomethacin, thiazides, and salt restriction, are only partially effective. NDI patients urinate up to 10–20 liters in a day. In very young patients, this can cause mental retardation due to dehydration-rehydration cycles, and, at any age, it can result in chronic kidney disease due to urinary reflux. Although the disease is better understood and complications are less often seen currently due to our increased understanding of their causes, the necessity for frequent and high-volume urination is still a problem for survival and quality of life. There are several chemicals, including erlotinib, sildenafil, and simvastatin, that have been reported to activate urine-concentrating ability through nonvasopressin pathways in vivo and in vitro (7–10), but none of these studies showed complete restoration of urine-concentrating ability.

AMPK is an energy-sensing serine/threonine kinase with two types of catalytic subunits (α1 and α2) (11). Although present throughout the body, it has only recently been shown to be present in the kidney medulla (12). Metformin is an oral antidiabetic drug that stimulates both AMPK catalytic subunits (13). Our previous data have shown that metformin can stimulate AQP2 membrane accumulation, and water

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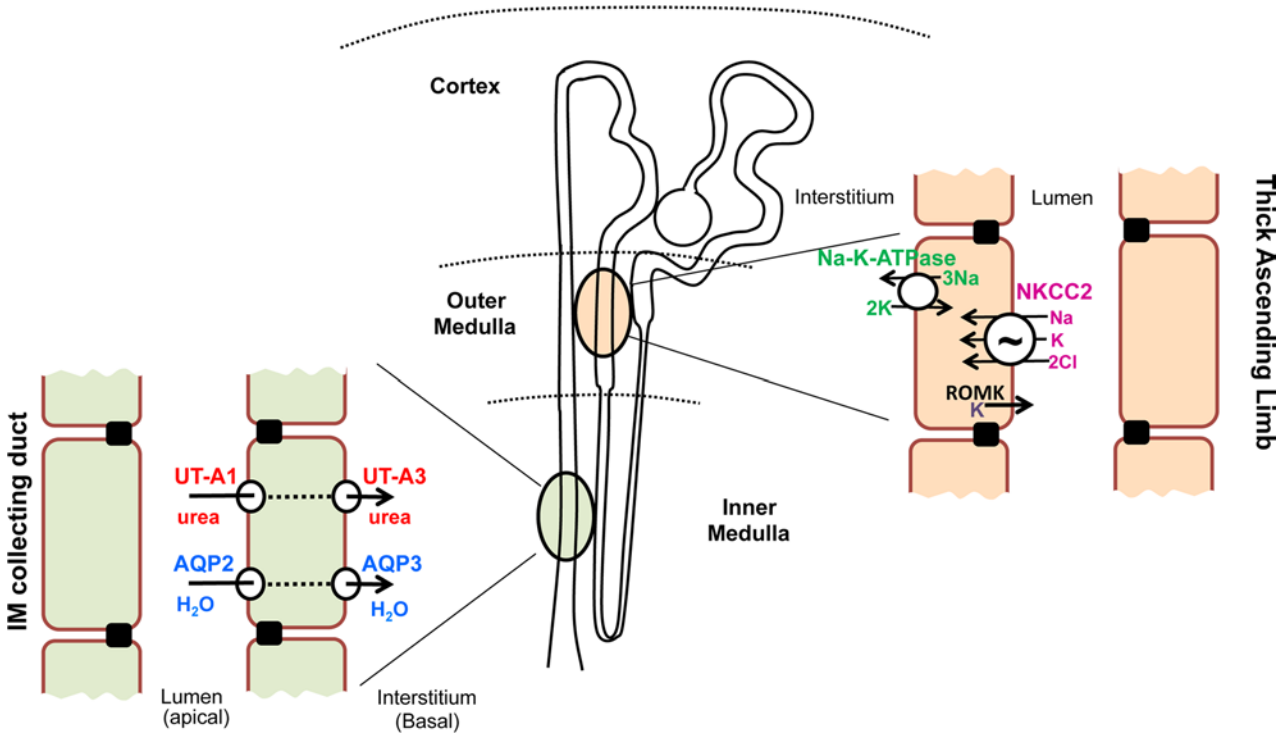

Figure 1. Influence of metformin on urine-concentrating transporters. The nephron is shown with 2 sections highlighted: the thick ascending limb and the inner medullary collecting duct (IMCD). In the thick ascending limb, metformin increases phosphorylation of the Na^+-K^+-$2Cl^-$ cotransporter 2 (NKCC2), activating it's reabsorption of Na, K, and Cl into the cell. The Na-K-ATPase moves sodium into the interstitium at the basolateral membrane, ROMK returns K to the lumen. The net result is a movement of Na to the interstitium to increase interstitial hyperosmolality. In the IMCD, aquaporin 2 (AQP2) and urea transporter A1 (UT-A1) are phosphorylated and activated by AMPK and stimulated by metformin. They move water and urea into the IMCD cells, and AQP3 (and AQP4, not shown) and UT-A3 complete the transfer out of the cell into the interstitium.

and urea permeability, in rat inner medullary collecting duct cells (12). In addition, AMPK was found to increase the phosphorylation of NKCC2 at Ser126 in vivo and in vitro (14) (Figure 1). In this study, we hypothesized that metformin, as a nonvasopressin activator of water and urea transport, could improve urine-concentrating ability in NDI rodent models. We used tolvaptan, a selective V2R antagonist, to produce a rat model of NDI. We also used V2R KO mice to verify our results. We observed the effects of metformin on both control and tolvaptan-treated rat and V2R KO mouse kidneys by assessing urine-concentrating ability and transporter abundance.

Results

Metformin improves urine-concentrating ability in rodent models of NDI. We produced an NDI model using tolvaptan to block the V2R in rats. We used metformin to stimulate AMPK. Tolvaptan significantly decreased urine osmolalities of treated rats within 24 hours (mean ± SEM for basal osmolality: 2,108 ± 134 mOsM vs. after tolvaptan: 1,303 ± 127 mOsM, $n = 32$, which includes all tolvaptan-treated rats prior to initiation of metformin treatment, $P < 0.01$). Urine osmolalities remained low in the tolvaptan group ($n = 15$) until the end of the experiment. Metformin was given along with tolvaptan (tol+met group) ($n = 17$) starting from day 3 and increased urine osmolalities back to control levels in 3 days. Average urine osmolalities of the tol+met group on days 3, 4, and 5 were 1,700 ± 189, 2,022 ± 168, and 2,335 ± 273 mOsM, respectively (Figure 2). Tolvaptan increased 24-hour urine volumes from 10 ± 2 ml to 22 ± 4 ml. Adding metformin reduced urine volume to 19 ± 4, 12 ± 1, and 10 ± 1 ml on days 3, 4, and 5, respectively, and the decrease on days 4 and 5 was significantly different from tolvaptan alone ($P < 0.05$).

In control rats, metformin increased urine osmolality, but the change did not reach statistical significance (2,097 ± 213 vs. 2,740 ± 88 mOsM, $P = 0.077$, $n = 5$). The 24-hour urine volumes in the control rats were significantly reduced after 3 days of oral metformin (15 ± 2 vs. 10 ± 1 ml, $P = 0.05$). Creatinine clearance, serum sodium, and vasopressin levels for both control and V2R-blocked rats are compared in Table 1.

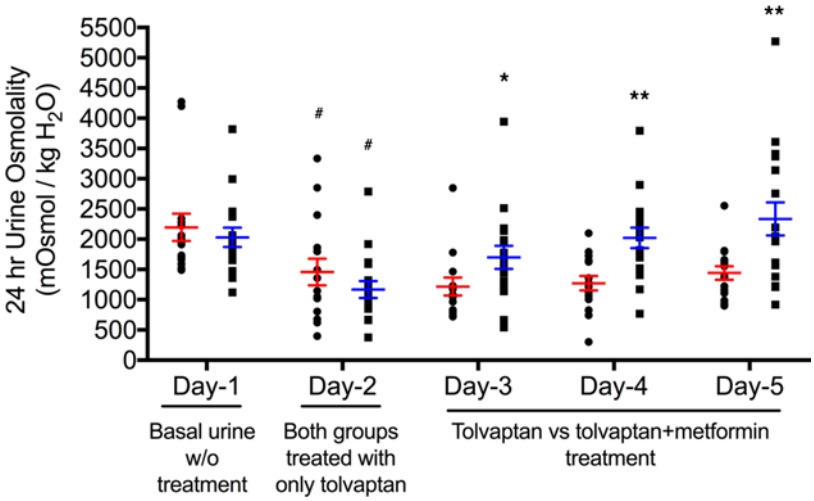

Figure 2. Daily urine osmolalities of rats given tolvaptan with or without metformin. Twenty-four–hour urine samples were assessed for osmolality before tolvaptan treatment, after initiation of tolvaptan, and after metformin treatment for the group that received this treatment. Each animal sample is indicated by a single dot. Average of each group is designated by a line bordered by lines showing the SEM. Red = tolvaptan alone group (n = 15). Blue = group receiving metformin from day 3 (n = 17). Student's t test was used to determine statistical significance, $^{\#}P < 0.05$ compared with untreated controls; $^{*}P < 0.05$, $^{**}P < 0.01$ for metformin-treated group compared with the tolvaptan only group on the same day.

Tamoxifen treatment induced V2R KO in Cre (+) mice (untreated urine osmolality: 1,796 ± 159 vs. urine osmolality after tamoxifen: 136 ± 16 mOsM) but not in Cre (–) mice. Metformin (given by gavage feeding) increased urine osmolalities of V2R KO mice. The increase was apparent at hour 3 but became statistically significant by the fifth hour and peaked at around hour 7 (Figure 3). Metformin also improved daily (24-hour) urine osmolalities significantly and reduced urine volumes of V2R KO mice. An increase of 25% in urine osmolality after treatment with metformin was used to indicate that the mice had responded. There were no mice that did not meet this minimal requirement (Figure 3).

Metformin increases protein abundances of transport proteins involved in urine concentration in rodent models of NDI. Metformin treatment of tolvaptan-treated (V2R-blocked) rats increased the expression of UT-A1 and AQP2 in the IM tip but not of NKCC2 in the OM. UT-A1 abundance in the IM tip was increased by 62% in tol+met rats relative to tolvaptan rats ($P < 0.05$). Similarly, the IM tip AQP2 expression in tol+met rats was 44% higher than the tolvaptan group ($P = 0.05$). There was no statistically significant difference in the levels of NKCC2 in the OM of tolvaptan-treated rats with or without metformin (Figure 4). There was no change in the level of AQP2 in the OM (data not shown). In V2R KO mice, metformin appeared to increase UT-A1 abundance, but the increase was not statistically significant (Figure 5). AQP2 abundance in the IM of the V2R KO mice was significantly increased by 78% by metformin treatment (Figure 5). NKCC2 in the OM of the V2R KO mice also showed a tendency to be increased with metformin, but this did not reach statistical significance (Figure 5).

Metformin treatment of tolvaptan rats increased the phosphorylation of AQP2 in the IM tip. Phospho-serine256–AQP2 (pSer256-AQP2) abundance was significantly increased by 105% with metformin treatment of tolvaptan rats ($P < 0.05$). The ratio of pSer256-AQP2 to total AQP2 tended to be higher in the tol+met group, but the difference was not statistically significant (1.0 ± 0.1 vs. 1.5 ± 0.3, $P = 0.183$). Metformin treatment of tolvaptan rats did not change the pSer261-AQP2 amount (100±7% tolvaptan vs 88±10% tol+met) in the IM tip (100 ± 7% vs. 88 ± 10%) but reduced the pSer261-AQP2/total AQP2 ratio markedly (1.0 ± 0.1 tolvaptan vs. 0.6 ± 0.1, tol+met, $P < 0.01$) (Figure 6).

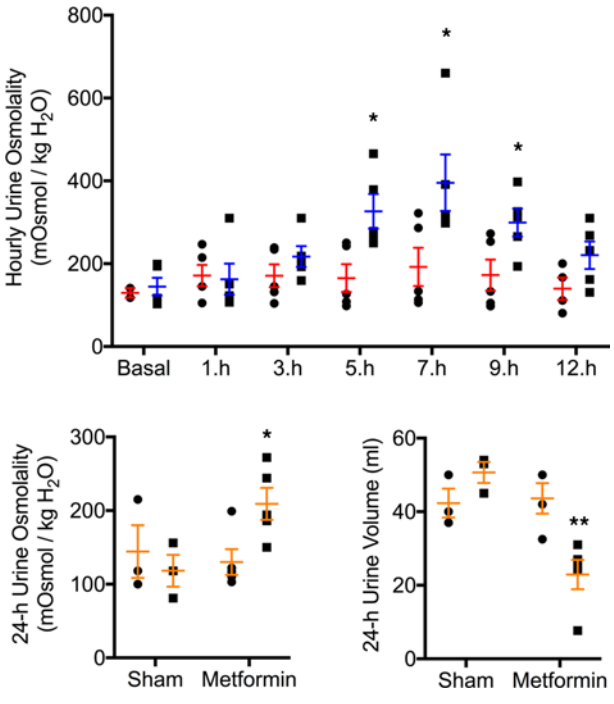

Figure 3. Urine osmolality of V2R KO mice. Top: Linear dot plot comparing hourly urine osmolality of V2R KO mice after sham (red, n = 5) versus metformin treatment (blue, n = 5). Each animal sample is indicated by a single dot. Average of each group is designated by a line bordered by lines showing the SEM. Bottom: Twenty-four–hour urine osmolality (left) and urine volume (right) of V2R KO mice before (circles) and after (squares) sham (n = 3) or metformin treatment (n = 5). Average values ± SEM are designated by orange lines. Student's t test was used to determine statistical significance, $^{*}P < 0.05$, $^{**}P < 0.01$ for metformin-treated compared with the sham controls.

Table 1. Serum parameters

	Control		Tolvaptan	
	(–) Metformin (+)	Metformin	(–) Metformin (+)	Metformin
Creatinine clearance (ml/min) (n = 5)	2.7 ± 0.5	2.7 ± 0.6	1.6 ± 0.3	1.4 ± 0.2
Serum Na (eMq/l) (n = 5)	140 ± 3.5	133 ± 3.2	141 ± 2.9	134 ± 4.7
Serum vasopressin (pg/ml) (n = 4)	0.93 ± 0.08	0.76 ± 0.1	0.85 ± 0.04	0.93±0.01

Kidney sections were subjected to immunohistochemical analysis to determine cellular localization of the transporters. Metformin treatment increased the membrane accumulation of AQP2 and pSer256-AQP2 but not pSer261-AQP2 in V2R-blocked rats (Figure 7). The tolvaptan group showed decreased membrane accumulation of AQP2 and pSer256-AQP2 compared with control rats. Simultaneous administration of metformin with tolvaptan for 4 days increased membrane association of both AQP2 and pSer256-AQP2 compared with rats receiving tolvaptan alone. Similarly, a single injection of metformin increased AQP2 and pSer256-AQP2 membrane accumulation in tolvaptan-treated rats. pSer261-AQP2 membrane association of tolvaptan-treated rats appears to be similar with or without metformin (Figure 7). Both metformin and tolvaptan caused a subtle enhancement of NKCC2 membrane accumulation in the OM. Four days of metformin treatment slightly increased membrane accumulation of NKCC2 in control rats (data not shown). Tolvaptan-treated rats with or without metformin also appeared to have slightly enhanced membrane staining relative to control-fed rats (data not shown).

Effect of metformin in control rats. Outer medullary expression of NKCC2 was dramatically increased by 117% by metformin (100 ± 32 control vs. 217 ± 20 metformin, n = 5, P < 0.01). Although slightly increased, UT-A1 expression in the IM tip of control rats was not significantly changed by metformin (100 ± 12 vs. 131 ± 19, n = 5, P = 0.9) (Figure 8).

Metformin increased IM tip AQP2 and pSer256-AQP2 abundance but not pSer261-AQP2 in control rats (Figure 9). Inner medullary tip AQP2 (100 ± 12 vs. 148 ± 17, P = 0.08) and pSer256-AQP2 (100 ± 38 vs. 250 ± 27, P < 0.01) expression was increased by 3 days of metformin treatment. The ratio of pSer256-AQP2 to total AQP2 was increased 90% by metformin treatment, although this increase was not statistically significant (n = 5, P = 0.11). IM tip pSer261-AQP2 abundance was not changed by AMPK stimulation (100 ± 7 vs. 131 ± 16, n = 5, P < 0.442).

Immunohistochemical staining of sham gavage fed, 4-day oral metformin, and single injection metformin-treated rats is shown in Figure 10. These results suggest that AQP2 and pSer256-AQP2 at the api-

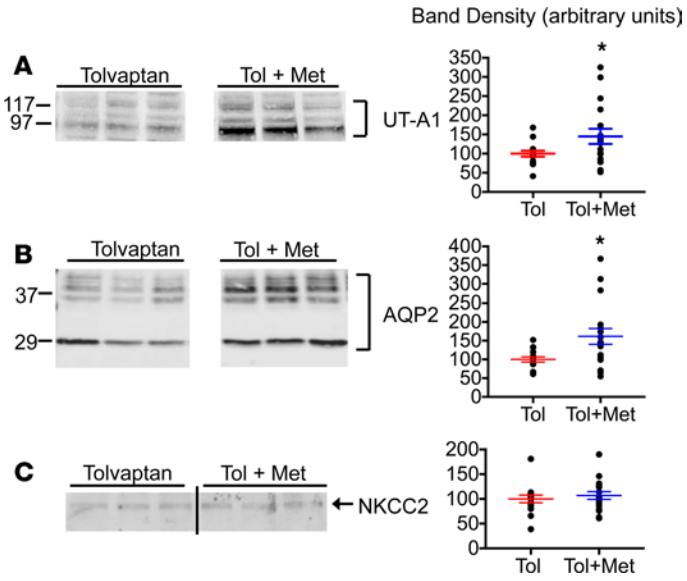

Figure 4. The effect of metformin on the IM tip AQP2, UT-A1, and OM NKCC2 abundances in tolvaptan-treated rats. Western blots for (**A**) UT-A1, (**B**) AQP2, and (**C**) NKCC2 (arrow at 160 kDa) and the densitometry of 3 combined experiments. Brackets/arrows show bands that were analyzed for densitometry. Linear dot plots compare band densities from animals receiving tolvaptan alone (Tol) or tolvaptan and metformin (Tol+Met). Each animal sample is indicated by a single dot (n = 15 tolvaptan only rats; n = 17 metformin-treated tolvaptan rats). Average of each group is designated by a line bordered by lines showing the SEM. Student's t test was used to determine statistical significance, *P < 0.05.

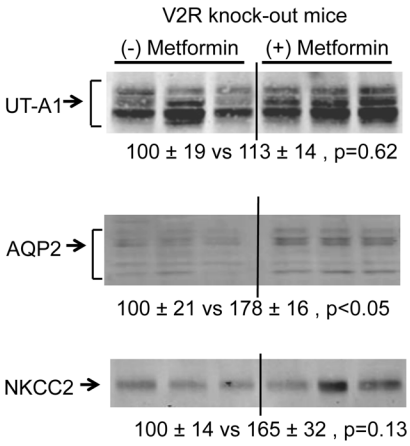

Figure 5. The effect of metformin on the IM AQP2, UT-A1, and OM NKCC2 abundances in V2R KO mice. Western blots show 3 control V2R KO mice and 3 V2R KO mice receiving metformin probed for UT-A1 (bracket indicates molecular weight range encompassing 117 and 97 kDa used for densitometry), AQP2 (bracket indicates molecular weight range encompassing 55 to 29 kDa), and NKCC2 (arrow indicates 160 kDa). Beneath each blot is the average densitometry ± SEM for each group of samples (n = 3).

cal membrane relative to the cytoplasmic aquaporin localization are increased by metformin treatment. Interestingly, apical staining for pSer261-AQP2 was denser in the 4-day metformin group relative to the control group, whereas the metformin injection appeared to have no significant effect on pSer261-AQP2 distribution in control rats (Figure 10).

The effect of metformin is sustained for 10 days. To determine whether the benefits of metformin persisted over time, rats were treated with tolvaptan for 4 days to achieve a stable NDI model, and then metformin was given for an additional 10 days. The increase in urine osmolality in the tolvaptan NDI rats was apparent at 2 days after initiation of metformin treatment (day 7) and remained elevated up to 10 additional days (day 14) (Figure 11).

Discussion

We investigated the possibility that activation of AMPK by metformin would improve urine concentration in congenital NDI. The data reported in this study revealed that metformin increases urine-concentrating ability in a rat model of NDI that was generated in response to tolvaptan treatment. This response to metformin was verified using V2R KO mice treated with metformin. Hypoosmolar urine outputs from animals that were given tolvaptan were restored to normal osmolality by 3 days of treatment with metformin. The beneficial effect of metformin was sustained for up to 10 days. Metformin, given by gavage, also enhanced urine osmolality in V2R KO mice, starting from the third hour and peaking at the seventh hour, consistent with our previous study (12). These results indicate that metformin, a well-known and safe oral antidiabetic drug, might be a novel treatment option for congenital NDI and potentially for other types of NDI as well.

Congenital NDI is mostly caused by mutations in the V2R. In about 10% of the cases, the condition is caused by mutations in the AQP2 gene. When AQP2 gene mutation is the cause, an alternate pathway to phosphorylate AQP2 will not be corrective. However, in the cases in which the V2R is mutated, the

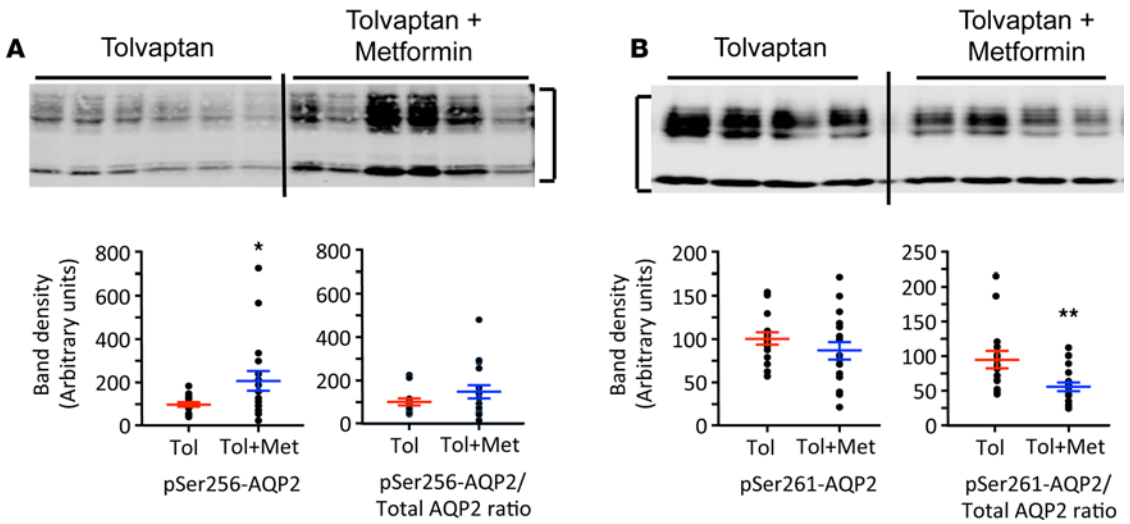

Figure 6. The effect of metformin on the IM tip pSer256-AQP2 and pSer261-AQP2 abundances in tolvaptan rats. (**A**) Representative Western blot of tolvaptan vs. tolvaptan plus metformin rats probed for pSer256-AQP2. Combined data from 3 experiments are also shown. (**B**) Representative Western blot probed for pSer261-AQP2. Combined data from 3 experiments are also shown. Brackets denote bands included in densitometry analysis between 55 and 29 kDa. Each animal sample is indicated by a single dot. Average of each group is designated by a line bordered by lines showing the SEM. Red = tolvaptan alone group (n = 15). Blue = group receiving both tolvaptan and metformin (n = 17). Under each blot, the left graph shows pSer AQP2 band densities, and the right graph shows the ratio of phospho-to-total band densities. Student's t test was used to determine statistical significance, $^*P < 0.05$, $^{**}P < 0.01$ for metformin-treated (n = 17) compared with the tolvaptan only (n = 15) group.

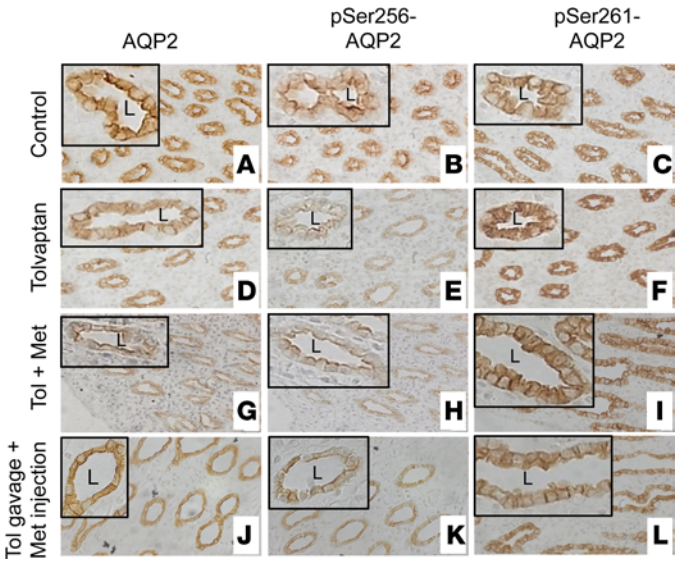

Figure 7. Immunohistochemical staining for AQP2, pSer256-AQP2, and pSer261-AQP2 in kidneys of rats treated with tolvaptan with or without metformin. Shown are inner medullary collecting ducts stained for AQP2 and pAQP2. Micrographs were collected at an original magnification of ×400; insets are magnified a further ×2.5. (**A**–**C**) Images are from 4-day sham gavage-fed rats. (**D**–**F**) Images are from the 4-days tolvaptan gavage-fed rat. (**G**–**I**) Images are from the rats given tolvaptan and metformin (Tol+Met) by gavage for 4 days. (**J**–**L**) Images are from the rats receiving tolvaptan gavage for 4 days and then single dose i.p. injection of metformin 1 hour before kidney perfusion. Brown indicates positive identification of the protein. Lumens are marked with an "L."

transport proteins are intact, so an alternate means of stimulating the activities of the urea and water transporters could improve or correct the concentrating defect that results from the lack of V2R-mediated PKA phosphorylations. To determine if AMPK activation could be effective in relieving NDI, we chemically blocked rat kidney V2Rs with tolvaptan to produce the rat model. A schematic of the proposed mechanism for AMPK-mediated improvement of urine concentration is shown in Figure 12. Miranda et al. reported that tolvaptan is an effective tool to study vasopressin-null systems (15). We used polyuria and reduced urine osmolality as bioassays to confirm the disease model. Tolvaptan decreased the urine osmolalities of Sprague-Dawley rats to 1,000 mOsM. After 4 days, AQP expression was reduced by 60% and membrane-associated AQP2 and pSer256-AQP2 was markedly decreased by tolvaptan. Surprisingly, UT-A1 in IM tip and NKCC2 in OM were not decreased in tolvaptan-treated rats compared with untreated controls (data not shown). These results are consistent with those reported by Miranda et al. using a rat model in which the animals were given tolvaptan by 4-day minipump (15). Similarly, UT-A1 and NKCC2 abundances were not different when comparing V2R KO and Cre (–) control mice. Li et al. also reported that conditional deletion of V2R in mice caused a 70% reduction in AQP2 expression but no significant change in NKCC2 levels (16). In a normal animal, increased vasopressin upregulates AQP2, UT-A1, and NKCC2 expression. One might expect that in the absence of vasopressin these transporters might be decreased, as observed for AQP2 with tolvaptan treatment, or remain unchanged. UT-A1 and NKCC2 did not decrease. In fact, they showed a tendency to increase, although the changes were not statistically significant, upon V2R blockade with tolvaptan. This suggests that there may be compensatory pathways for regulation of these transporters that are independent of V2R. The data presented here suggest that, in a normal animal, vasopressin represses AMPK, and, when this tonic repression is removed, AMPK might offer such a compensatory stimulatory pathway.

In previous studies, we showed that metformin treatment of isolated perfused inner medullary collecting ducts increased urea and water permeability (12). We also showed that AMPK directly phosphorylates

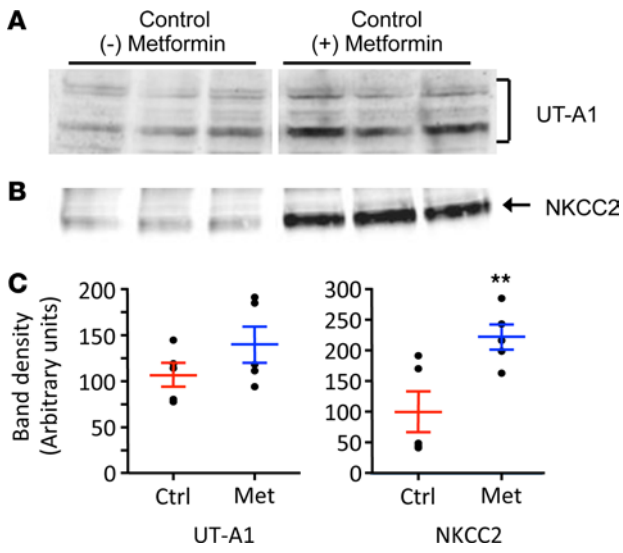

Figure 8. UT-A1 and NKCC2 abundance in control rats treated with or without metformin. (**A**) Representative Western blot from IM tip samples probed for UT-A1 between 97 and 117 kDa (bracket). (**B**) Representative Western blot showing outer medullary NKCC2 at 160 kDa (arrow). (**C**) Linear dot plot comparing metformin-treated (Met, blue) and sham-treated controls (Ctrl, red). Each dot is a separate sample. Averages for the group are presented as lines ± SEM. Student's 2-tailed *t* test was used to determine significance, $^{**}P < 0.01$ (n = 5/group).

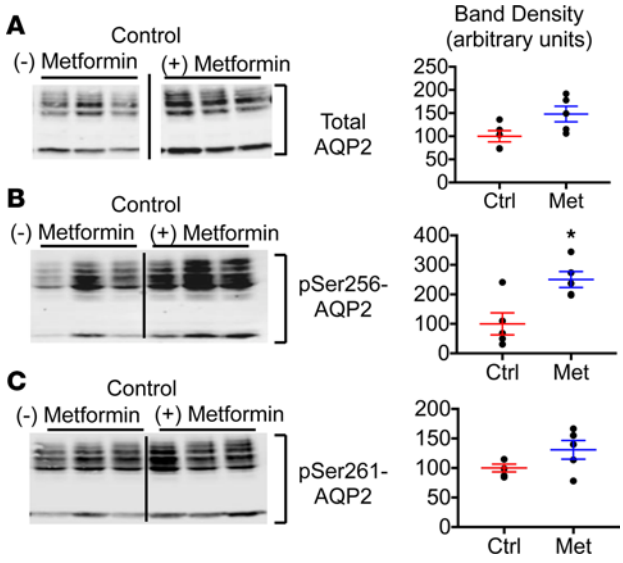

Figure 9. The effect of 4 days of metformin treatment on control rat IM tip AQP2, pSer256-AQP2, and pSer261-AQP2 abundances. Representative Western blots from IM tip tissue lysates probed for (**A**) AQP2, (**B**) pSer256-AQP2, and (**C**) pSer261-AQP2 (brackets denote gel area between 55 and 29 kDa used for densitometry) and linear dot plots comparing band densities from sham-treated rats (Ctrl, red) and rats receiving metformin (Met, blue). Each animal sample is indicated by a single dot. Average of each group is designated by a line bordered by lines showing the SEM. Student's *t* test was used to determine statistical significance, **A**: $P = 0.085$; **B**: $^*P < 0.05$; **C**: P = not significant; $n = 5$ rats per group.

AQP2 and UT-A1 in vitro (12). In inner medullary suspensions from control animals, metformin increased AQP2 membrane association but did not increase UT-A1 in the membrane (12). These experiments suggested regulation of transporter function by acute AMPK stimulation. In the current study we investigated the results of chronic metformin treatment in NDI rodent models. After longer treatment with metformin, AQP2, pSer256-AQP2, and UT-A1 were all upregulated compared with the levels in untreated V2R-blocked rats and V2R KO mice. There was also a decrease in the level of AQP2 phosphorylation at Ser261 relative to total AQP2 in response to AMPK activation. These data show that, in addition to regulating transporter function in control tissue, AMPK also upregulates AQP2 and UT-A1 at a protein level in the absence of V2R. It is unclear whether the increase in transporter protein is due to enhanced production or decreased degradation. Our results show that AMPK treatment results in phosphorylation of Ser256-AQP2 and dephosphorylation of Ser261-AQP2, which is similar to the changes observed upon treatment of Brattleboro rats, which have central diabetes insipidus, with vasopressin (5). Since phosphorylation of Ser261 in AQP2 has been linked to retrieval of AQP2 from the membrane, and phosphorylation of Ser256 has been shown to promote membrane insertion, the combination would result in increased AQP2-mediated water reabsorption, analogous to what was observed in the vasopressin-treated Brattleboro rat model.

The current study showed that metformin increases urine-concentrating ability in V2R-blocked rats, but it also revealed that metformin increases both expression and membrane accumulation of NKCC2, AQP2, and pSer256-AQP2 in control rats. We previously showed that rats with streptozotocin-induced diabetes mellitus have increased AQP2, UT-A1, and NKCC2 expression and hypothesized that these increases are a protective compensatory response for volume depletion in diabetes (17). In the current study, metformin treatment promoted similar chances, suggesting that this may be part of the mechanism by which metformin exerts its antidiabetic effects. Knowing that metformin may be promoting water reabsorption as part of its mechanism might influence decisions about using metformin (or not using it) in diabetic patients with other comorbidities that include aspects of water retention, such as congestive heart failure.

Calculated glomerular filtration rate values revealed no significant difference between any treatment condition in rats ($n = 5$ for each group), indicating that tolvaptan and metformin doses were safe for the animals and the

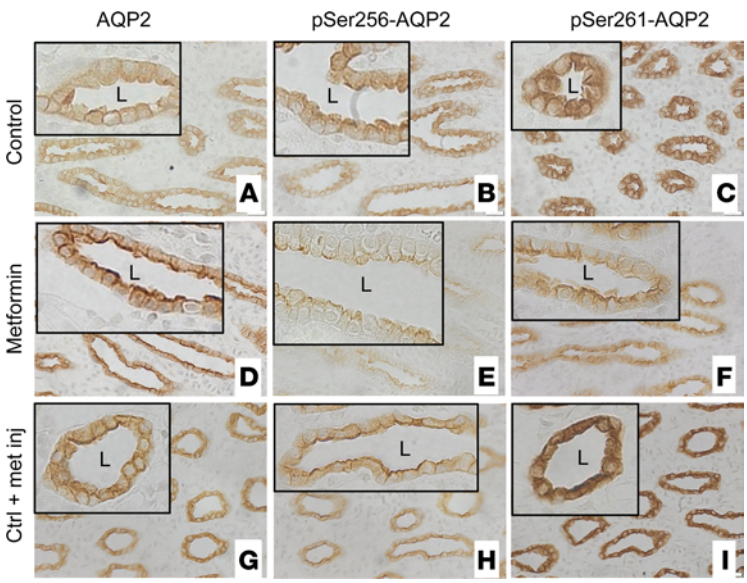

Figure 10. Immunohistochemical staining shows inner medullary tip collecting duct AQP2, pSer256-AQP2, and pSer261-AQP2 cell membrane distribution in control rats treated with or without metformin. (**A**–**C**) Images show rats that were sham gavage-fed for 4 days. (**D**–**F**) Images show staining in rats receiving metformin gavage-fed for 4 days. (**G**–**I**) Images show rats that had sham gavage feeding for 4 days and a single injection of metformin 1 hour before perfusion. Micrographs were collected at an original magnification of ×400; insets were magnified a further ×2.5 to visualize the membrane distribution more clearly. L, lumen of the tubule.

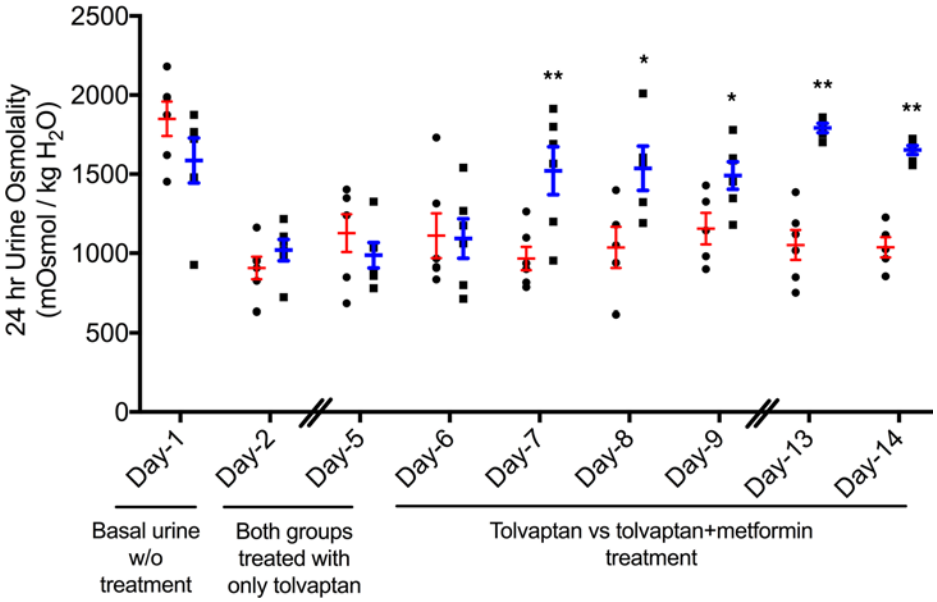

Figure 11. Urine osmolality result from longer-term metformin treatment of tolvaptan-treated rats. Twenty-four–hour urine samples were assessed for osmolality before tolvaptan treatment, after initiation of tolvaptan, and after metformin treatment for the group that received this treatment from day 6 to day 14. Each animal sample is indicated by a single dot. Average of each group is designated by a line bordered by lines showing the SEM. Red = tolvaptan alone group (n = 6). Blue = group receiving metformin from day 6 (n = 6). Student's t test was used to determine statistical significance, $^*P < 0.05$, $^{**}P < 0.01$ for metformin-treated compared with the tolvaptan only group on the same day.

decreased urine volumes were not due to kidney injury. Vasopressin serum values did not change with tolvaptan or metformin treatment, agreeing with the results of another published account that reported unchanged vasopressin levels in tolvaptan-treated animals (18). Serum Na^+ levels in the rats did not change with tolvaptan treatment; however, metformin treatment had a tendency to decrease Na^+ levels in both control and tolvaptan rats, consistent with enhanced water reabsorption from collecting ducts.

There were also some limitations of this study. We used tolvaptan, which is a competitive antagonist of vasopressin, to generate NDI. Therefore, our animals were not under complete V2R blockade. However, we verified our results with our inducible V2R KO mice. There is some question as to whether these results would be reiterated in a different model of NDI, for example Li-induced NDI. There are contradictory data about the mechanism of Li-induced NDI in the literature about whether this NDI results from interruption of the cAMP pathway or results from renal fibrosis, or both. To elucidate the efficacy of metformin for this type of NDI future studies with Li-treated rats will be required. In the current study, the metformin dose given to the rats was higher than doses prescribed for diabetic patients, which might explain why diabetic patients do not complain of symptoms of water retention. This dose was chosen to ensure maximal bioavailability of the drug. Creatinine measurements in control and tolvaptan-treated rats were not different when metformin was administered at this dose, so we concluded that it was not having a nephrotoxic effect.

Congenital NDI is usually treated with a thiazide, amiloride, and/or indomethacin combination, along with reduced salt intake. However, the treatment is only partially effective. There are some reports in the literature of other compounds stimulating urine-concentrating ability by bypassing the V2R. The EP4 PGE2 agonist, ONO, increased the urine osmolalities of the V2R KO mouse (16). Fluvastatin administration along with secretin stimulated urine-concentrating ability in the V2R KO mouse (19). Simvastatin was reported to decrease AQP2 endocytosis and increase urine-concentrating ability in Brattleboro rats (9). Sildenafil was also shown to increase AQP2 membrane accumulation in vitro and in vivo by enhancing cGMP levels in

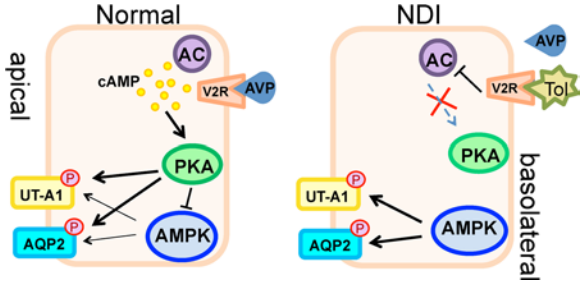

Figure 12. Proposed mechanism of AQP2 and UT-A1 activation in normal and nephrogenic diabetes insipidus conditions. Normally, vasopressin (AVP) binds to the vasopressin receptor type 2 (V2R) activating adenylyl cyclase (AC) to catalyze cAMP production. PKA is activated by cAMP to phosphorylate UT-A1 and AQP2, which results in their insertion into the membrane, in which they facilitate reabsorption of water and urea, respectively. PKA suppresses AMPK, so any phosphorylation/activation of AQP2 or UT-A1 by AMPK is minimal. In NDI, the V2R is unresponsive to AVP. In these studies, tolvaptan (Tol) was used to block the V2R, resulting in decreased cAMP, decreased PKA activation, and derepression of AMPK. The result is phosphorylation of AQP2 and UT-A1 by AMPK instead of PKA.

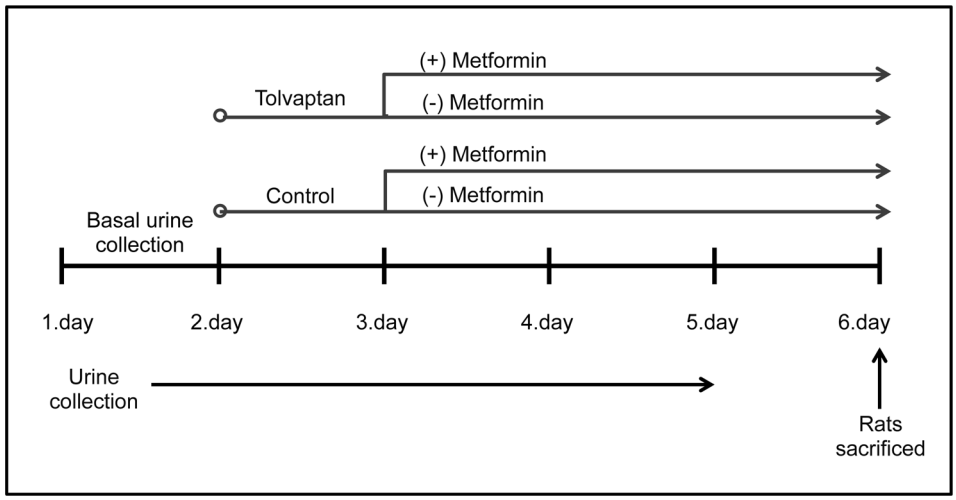

Figure 13. Schematic diagram of experiment time line.

Brattleboro rats (20). Assadi and Sharbaf reported a case of X-linked NDI treated with sildenafil; treatment decreased urine volume and enhanced urine osmolality compared with the combination of HTCZ, amiloride, and indomethacin (21). Rosiglitazone was shown to upregulate AQP2 and NKCC2 in MCD4 cells by a Ca-dependent/cAMP-independent pathway (22). Here, we presented the effects of AMPK activation as a novel approach. Sildenafil and simvastatin were shown to improve urine concentration for several hours in other rat and mouse models of NDI, but the beneficial effect was transient (23, 24). In the present study, metformin lead to a sustained restoration of urine-concentrating ability for 10 days.

In conclusion, we have shown that AMPK stimulation by metformin increases urine-concentrating ability in V2R-blocked rats and V2R KO mice by upregulating AQP2 and UT-A1 in the IM. AMPK stimulators including metformin might be a novel treatment option for patients with congenital NDI due to V2R mutations.

Methods

Animals and treatments. Male Sprague-Dawley rats, weighing between 100 and 350 g, were purchased from Charles River Labs. Cre (+) and Cre (–) V2R floxed mice, between 20 and 40 g, were originally obtained from Jurgen Wess (National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, Maryland, USA) and were bred and housed in the Emory Animal Care facility. Treatment of rats with metformin is outlined in Figure 13. Rats were fed with water and standard rat chow (Purina Lab Diet 5001) ad libitum. The rat studies included 3 independent cohorts. Tolvaptan-treated rats received tolvaptan (Otsuka Pharmaceutical Co. Ltd.) (10 mg/kg/d) with or without metformin (LKT Laboratories Inc.) (800 mg/kg/d) once a day by oral gavage for 4 days. Both drugs were administered in a suspension of 1% HPMC (Sigma-Aldrich) in water. Control rats were also given suspension media alone or with metformin (800 mg/kg/d) by oral gavage for 4 days. All animals were housed in metabolic cages during the experimental period to facilitate collection of 24-hour urine samples. Basal urine samples were collected on the first day without any treatment. To assess the V2R blockade, tolvaptan alone was given to both tolvaptan and tol+met groups on the second day prior to beginning the metformin treatment. Beginning on day 3, tolvaptan alone or tol+met was given daily for 4 subsequent days. Following 4 days of drug administration and daily urine collection, animals were decapitated. Trunk blood samples were collected and sera were obtained by centrifugation (3,000 *g* for 10 minute at 4°C). Rat kidneys were removed and dissected into IM tip, IM base, and OM.

Long-term treatment with metformin. To assess whether metformin's effect persists for a more extended time without development of a tolerance, metformin was started after 4 days of tolvaptan treatment and continued for 10 additional days. Control rats were treated with tolvaptan alone.

Metformin in V2R KO mice. Conditional V2R KO was induced in genetically modified mice by feeding tamoxifen (TD.130859, Envigo) as described previously (16). Mice were determined to have successful KO of V2R when their urine osmolality fell below 250 mOsmol/kg H_2O. Mice were given metformin (600 mg/kg prepared in water) or sham fed with water twice daily and housed in metabolic cages. Hourly and

24-hour urine samples were collected. When the 24-hour urine osmolality increased by more than 25%, mice were sacrificed and kidneys were dissected into IM and OM.

Western blot analysis. Dissected tissues were homogenized and protein content was determined by modified Lowry assay using the Bio-Rad DC protein assay reagent as previously described (25). Samples were diluted with Laemmli sample buffer to a final protein concentration of 1 mg/ml and boiled for 1–3 minutes. Proteins were separated by PAGE as follows: UT-A1, 25 μg/lane, 10% PAGE; AQP2, 10 μg/lane for rat tissue lysates and 30 μg/lane for mouse tissue lysates, 12.5% PAGE; pSer256-AQP2 and pSer261-AQP2, 25 μg/lane, 12.5% PAGE; and NKCC2, 30 μg/lane, 7.5% PAGE. Proteins were electroblotted to polyvinylidene difluoride membranes (PVDF-Immobilon, Millipore) as described previously (26). Blots were blocked with 5% nonfat dry milk in Tris-buffered saline (20 mM Tris·HCl, 0.5 M NaCl, pH 7.5) for 1 hour and then incubated overnight with our antibodies to UT-A1 (26), NKCC2 (27, 28), or AQP2 (29, 30) or with the following commercial antibodies: pSer256-AQP2 (Biorbyt, catalog orb317557) or pSer261-AQP2 (Avivasysbio, catalog OAPC00158). Alexa Fluor 680–linked anti-rabbit IgG (Molecular Probes, catalog A21076) was used as the secondary antibody. Then, the secondary antibody was visualized with the LICOR Odyssee protein analysis system and densities were analyzed with the LICOR densitometry software. Blots were stained with Ponceau S for total protein. Blots were scanned and ImageJ (NIH) was used to quantitate lane protein density. We normalized each protein of interest to the loading control (31).

Immunohistochemistry. Rats treated with or without tolvaptan with or without metformin, as described above, or receiving an acute 1-hour metformin treatment (single subcutaneous injection of 400 mg/kg metformin) were deeply anesthetized with isoflurane. The kidneys were perfused and fixed with 4% paraformaldehyde and paraffin embedded as previously described (32). Paraffin sections (4-μm thickness) were sliced, dewaxed, and hydrated for immunostaining as previously described (32). Sections were incubated overnight at 4°C with AQP2, pSer256-AQP2, pSer261-AQP2, or NKCC2 antibodies. Slices were then washed and incubated in horseradish peroxidase–conjugated secondary antibody (donkey anti-rabbit IgG) for 2 hours. Diaminobenzidine with 35% H_2O_2 was used to detect peroxidase activity. Then, slides were counterstained with Mayer's hematoxylin for nuclear visualization. Stained sections were examined with an Olympus inverted microscope IX71 at ×400 magnification. All results were confirmed in a second animal.

Urine and serum analysis. Twenty-four–hour urines were collected from the rats daily. Hourly and daily urines were collected from the mice. Urine osmolalities were measured using a Wescor 5520 Vapor Pressure Osmometer (Wescor). Urine and serum samples were also analyzed for Na^+ (EasyLyte, Medica), urea (using the colorimetric Infinity reagent according to manufacturer's instructions, Thermo Fisher Scientific), and creatinine (QuantiChrom Creatinine Assay kit according to the manufacturer's instructions, BioAssay Systems). Creatinine clearance was calculated using the following equation to assess kidney function in the rats: (urine creatinine concentration × urine volume/plasma creatinine concentration). Serum vasopressin was measured by ELISA according to manufacturer's instructions (Enzo Life Sciences).

Statistics. All data are presented as mean ± SEM. A 2-tailed Student's *t* test was used for comparisons of 2 groups. Statistical differences with $P < 0.05$ were considered significant. *N* represents the number of animals per condition in an experiment.

Study approval. All procedures involving animals were approved by the Emory Institutional Animal Care and Use Committee and adhere to NIH standards for animal use.

Author contributions

OE planned and executed experiments, collected and analyzed data, and wrote the manuscript. JDK planned experiments, prepared samples, analyzed data, and edited the manuscript. LML prepared samples and collected data. HR prepared samples and collected data. JMS planned the project, analyzed data, and edited the manuscript.

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