Introduction:
Sodium-glucose cotransporter 2 inhibitors such as empagliflozin (EMPA) protect against diabetic kidney disease. Prostaglandin E2 (PGE2), the main renal product of cyclooxygenase-2, inhibits vasopressin (AVP)-water reabsorption in the collecting duct (CD). The novelty of this study is that for the first time, we examined if EMPA affects the renal PGE2/EP receptor system and determined if CD responses to EMPA prevent water loss.

Methods:
Four groups of adult male mice were studied after 6 weeks of treatment: control (db/m), db/m+EMPA (10 mg/kg/day in chow), type 2 diabetic (db/db), and db/db+EMPA. Tubules were microdissected for quantitative polymerase chain reaction (qPCR) and CD water transport was measured in response to AVP, with or without PGE2.

Results:
Hyperglycemia and albuminuria were attenuated by EMPA. Renal mRNA expression for COX, PGE synthase, PGE2 (EP) receptor subtypes, CD AVP V2 receptors and aquaporin-2 was elevated in db/db mice, but unchanged by EMPA. Urine PGE2 levels increased in db/db but were unchanged by EMPA. AVP-water reabsorption was comparable in db/m and db/m+EMPA, and equally attenuated by 50% by PGE2. In db/db mice, AVP-water reabsorption was reduced by 50% compared to non-diabetic mice, and this reduction was unaffected by EMPA. In db/db mice, AVP-stimulated water transport was more significantly attenuated with PGE2 (62%), compared to non-diabetic mice, but this attenuation was reduced in response to EMPA, to 28%.

Conclusion:
In summary, expression of renal PGE2/EP receptors is increased in db/db mice, and this expression is unaffected by EMPA. However, in diabetic CD, PGE2 caused a greater attenuation in AVP-stimulated water reabsorption, and this attenuation is reduced by EMPA. This suggests that EMPA attenuates diabetes-induced excess CD water loss.
of type 1 diabetes, we showed that renal EP₁ promotes glomerular filtration barrier and tubular injury, contributing to hyperfiltration and proteinuria, while gene deletion of EP₁ deletion is beneficial [12]. We also showed that renal EP₂ contributes to polyuria by impacting urine concentrating function in diabetic mice [13]. However, the role of renal PGE₂/EP receptors in kidney disease associated with type 2 diabetes is unclear.

The purpose of this study was to examine the effect of EMPA on CD responses to PGE₂ in type 2 diabetic (db/db) mice. Though EMPA is nephroprotective in type 2 diabetes, the implications of increasing glucose and fluid delivery along the nephron has not been studied in detail. Therefore, we also examined how EMPA affects the renal PGE₂/EP receptor system, and how CD hormonal systems respond to EMPA to prevent excessive water loss.

Methods

Type 2 diabetic mice and EMPA treatment

Male db/db mice (BKS.Cg-Dock7m/ Leprdb/J; Jackson Labs) and their control heterozygous (db/m) littermates mice, 12 weeks-old of age, were studied. We randomly assigned mice into 4 groups: control (db/m), db/m+EMPA, type 2 diabetic (db/db), db/db+EMPA, and administered empagliflozin (EMPA; 10 mg/kg/day; Boehringer-Ingelheim, Germany) in standard chow (prepared by Envigo Labs). The dose of 10 mg/kg/day for 6 weeks was chosen according to previous rodent studies [14]. Mice were housed on a 12-hr light-dark cycle, with free access to standard chow and water. Mice were sacrificed by exsanguination under isoflurane anesthesia. All animal procedures were undertaken at the University of Ottawa's Animal Care and Veterinary Services vivarium and were approved by the University of Ottawa Animal Care Committee.

Mouse characteristics, blood pressure, and urine analysis

The effects of EMPA on various metabolic and renal parameters, body weight and blood glucose were measured. Kidney weights were normalized to body weight. Spot urine was collected at the same time daily. Food and water consumption, and urine volume were monitored daily for 3 consecutive days with one day of acclimation from 24 hrs metabolic cages. Urine parameters analyzed included albumin by ELISA (Bethyl Labs, TX, USA), PGE₂ levels by ELISA (Cayman Chemicals), and osmolality by freezing point depression (Advanced Model 3MO Plus Plus Osmometer, Advanced Instruments Inc, MA, USA). Values were normalized to creatinine (Exocell Inc).

Determination of glomerular filtration rate (GFR)

GFR was measured in conscious mice by FITC-inulin clearance. Briefly, 5% FITC-inulin (Sigma-Aldrich, St.Louis, MO, USA) dissolved in 0.9% saline was dialyzed overnight and filtered. Mice were injected with 3.74 μl/g BW of FITC-inulin via tail-vein. Blood was collected from the saphenous vein into heparinized capillary tubes after 3, 7, 10, 15, 35, 55 and 75 min, and centrifuged. Samples were buffered in HEPES (500 mM, pH 7.4) and fluorescence was measured (excitation 488 nm/emission 538 nm). GFR was calculated using a two-compartment clearance model as previously reported [15].

Quantitative PCR analysis

To examine the expression of renal PGE₂/EP receptor systems and water and solute transporters along the nephron, we performed quantitative PCR (qPCR). The left kidneys were dissected to separate cortex and medulla and microdissected tubules (proximal tubule, thick ascending limb, cortical and inner medullary CD), then snap-frozen in liquid nitrogen. Tissue was homogenized using the TP-103 Agalmarator COE Capmixer (GC America Inc, IL, USA). RNA from microdissected tubules was obtained by following the RNase Free Mini Kit (Invitrogen, MA, USA) according to the manufacturer’s instructions. We measured the expression of PGE₂ synthesis enzymes (microsomal PGE₂ synthase: mPGES-1 and mPGES-2, cytosolic PGE₂ synthase: cPGES, COX1, COX2) and EP₁-4 receptors by qPCR. Expression was normalized to 18S RNA and the 2⁻ΔΔCT method was used for analysis as done previously [15].

Ex vivo microperfusion of collecting ducts and water transport

Empa is already a well-known anti-diabetic drug to treat type 2 diabetes, but nothing is known in regards of how Empa affects the renal membrane transport in db/db mice. Mice

<table>
<thead>
<tr>
<th>Product name</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>β2-MG</td>
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<td>5'-CGGGTGCCACCTGACTTCACT-3'</td>
</tr>
<tr>
<td>COX-1</td>
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<td>5'-CAAGCTGCTGATTACCACT-3'</td>
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<tr>
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<td>5'-CAGGGAGTTCAGACACTTC-3'</td>
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<tr>
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<td>5'-ACGTGCCGCTGCGCTGACCC-3'</td>
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<tr>
<td>mPGES2</td>
<td>5'-CATCCTCCATCCGGTCCATCA-3'</td>
<td>5'-ACGTGGGCTGCTCCACCA-3'</td>
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<td>PGE₂</td>
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<td>5'-ACGTGACGCTACGCTGACAC-3'</td>
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<tr>
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<td>5'-CTGACGCTGCACTGACTTC-3'</td>
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<tr>
<td>EP₂ receptor</td>
<td>5'-GCTGCTTGCTGCTCTCAACC-3'</td>
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<td>EP₄ receptor</td>
<td>5'-GGCCCAATACACGCTGACAC-3'</td>
<td>5'-CTGACGCTGCACTGACTTC-3'</td>
</tr>
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</table>

| Primer sequence for qPCR. |
were euthanized after 6 wks EMPA, and IMCD from right kidneys were microdissected for in vitro microperfusions and measurement of net fluid reabsorption ($J_v$). $^3$H-inulin (75 μCi/ml) was used as a volume marker. In control periods, two collections were made for calculation of basal $J_v$ following 30 min equilibration. Tubules with a negative basal $J_v$ were discarded. AVP (10^{-12} mM, Sigma-Aldrich, St. Louis, MO, USA) was added to the bath and four 10-min collections were made. PGE$_2$ (10^{-7} mM, Sigma-Aldrich, St. Louis, MO, USA) was then added with five additional 10-min collections. Mean $J_v$ was calculated as previously described (15). Compositions of bath, dissecting, and perfusion solutions have been published [16-18]. For diabetic perfusions, glucose was increased from normal bath of 8.3 to 25 mM. To control for osmotic responses the control group was perfused with 16.7 mannitol + 8.3 mM glucose. The perfusate was hypotonic at 290 vs. 440 mOsm for the bath.

Statistical analysis

Graphpad Prism (San Diego, CA, USA) was used for data analysis. Values are expressed as means ± SEM. Statistical analysis was done using One-way ANOVA followed by Tukey’s post-test. The significance was indicated for $p < 0.05$. Additionally, a one sample t-test with a hypothetical value of 1 was performed for qPCR.

Results

Physiological parameters, blood pressure, and GFR

To study the effect of type 2 diabetes and EMPA on mouse physiology and renal function, we first measured food and water intake, body weight and blood glucose. As shown in figure 1, food and water intake, body weights, and blood glucose were elevated in db/db mice compared to db/dm. EMPA had no effect on food and water intake or body weight, but significantly lowered blood glucose levels compared to db/db mice.

In figure 2A, FITC-inulin clearance was increased up to 500 ul/min in db/db mice compared to 300 ul/min in db/m, but only slightly lower in db/db+EMPA compared to db/db. Urine PGE$_2$ levels were 10-fold higher in db/db mice compared to db/m, but unchanged by EMPA (Figure 2B). Urine osmolalities were reduced by 50% in db/db mice compared to db/m, but also unaffected by EMPA (Figure 2C). However, urine albumin levels were increased 3-fold in db/db mice and lowered to near control levels by EMPA (Figure 2D).

Expression of renal cyclooxygenases, PGE$_2$ synthases, and EP receptors

To determine whether the renal PGE$_2$/EP receptor system is altered in type 2 diabetes or in response to EMPA, we characterized the regional and segmental mRNA expression profile of the PGE$_2$ pathway, including the synthetic enzymes: COX-1 and COX-2 and PGE$_2$ synthases (microsomal mPGES1, mPGES2, and cytosolic cPGES), as well as the four EP receptors (EP$_{1-4}$).

As shown in figure 3A, cortical COX-1 mRNA was reduced by 50% in all groups compared to db/m, including db/m+EMPA. EMPA did not further lower COX1 in db/db mice. Medullary COX1 mRNA was significantly increased in db/db mice, but unchanged by EMPA (Figure 3B). In comparison, COX2 mRNA was increased in the cortex and medulla of db/db mice by 2.6-fold and 3.5-fold, respectively, compared to db/m; although this was not significant in the medulla ($p = 0.06$). Levels were similar in db/db and db/db+EMPA (Figure 3C and 3D). Medullary mPGES1 mRNA was also increased in db/db mice by 2.6-fold and 3.5-fold, respectively, compared to db/m; although this was not significant in the medulla ($p = 0.06$). Levels were similar in db/db and db/db+EMPA (Figure 3C and 3D). Medullary mPGES1 mRNA was also increased in db/db mice compared to db/m, and unchanged by EMPA (Figure 4A,B). However, in the medulla mPGES1 was also increased in db/m+EMPA (Figure 4B) compared to db/m. Renal expression of mPGES2 (Figure 4C,D) and cPGES was similar in all groups (data not shown).

EP receptor expression was measured in microdissected proximal tubules (PT), thick ascending limb (TAL), and cortical and inner medullary CCD (CCD and IMCD respectively). As shown in figures 5C+D, EP$_1$ mRNA was increased 2-fold in the CCD ($p < 0.05$) in db/db mice compared to db/m, but unaffected by EMPA. EP$_1$ mRNA was reduced in db/db PT and db/m+EMPA.
Collecting duct PGE2 responses reduce water loss with empagliflozin in mice with type 2 diabetes mellitus

TAL and unaffected by EMPA, but EP1 was also reduced by 75% in the PT of db/m+EMPA like db/db (Figures 5A,B). EP3 expression was significantly reduced by 50% in the TAL of db/db+EMPA compared to db/m (figure 5F), but otherwise unchanged. EP4 was not detectable in microdissected TAL and CCD. In the PT, EP4 mRNA was reduced over 75% in all groups, and almost undetectable in the db/m+EMPA and db/db compared to db/m (Figure 5I). EP2 was also reduced by 50% in db/m+EMPA compared to db/m in the IMCD, but unchanged in db/db and db/db+EMPA (Figure 5J). EP2 was unchanged in the cortex and medulla in diabetic mice, unaffected by EMPA, and undetectable in micro dissected tubules (data not shown).

Expression of renal sodium transporters

We examined the mRNA expression of major sodium transporters in the renal cortex and microdissected tubule segments. As shown in figures 6A,B, cortical expression of sodium-hydrogen exchanger (NHE)-3 and epithelial sodium channel (αENaC) were increased 2-fold and 1.8-fold respectively in db/db mice compared to db/m, but unaffected by EMPA. In contrast, cortical sodium-potassium-ATPase was reduced by 40% - 50% in all groups compared to db/m, though the reduction was not significant in db/db+EMPA (Figure 6C).

Next, we showed in microdissected PT that sodium glucose cotransporter SGLT1 mRNA was reduced by 50% in db/m+EMPA compared to db/m, but unaltered in diabetic tubules (Figure 6D). SGLT2 mRNA however was comparable in all groups (Figure 6E). Interestingly, TAL sodium-potassium-2-chloride cotransporter (NKCC2) mRNA was increased 2-fold in both db/m+EMPA and db/db tubules compared to db/m, but the expression returned to control levels in db/db+EMPA (Figure 6F).

Expression of CD aquaporins, AVP V2 receptor, and urea transporter

We next examined the mRNA expression of cortical and medullary aquaporins (AQP)-1 and AQP2, the two major renal water channels. As shown in figure 7, cortical AQP1 was increased 2.5-fold in db/db+EMPA compared to db/m, and unchanged in the other two groups (Figure 7A). AQP1 was unchanged in both regions in db/db mice. In contrast, AQP2 was increased 4-fold and 2-fold respectively, in the cortex and medulla of db/db mice compared to db/m (Figure 7C,D), however this increase was unaltered by EMPA. AQP2 was unchanged in cortex and medulla of db/m+EMPA.

We also examined the expression of AVP V2 receptors (V2R) in microdissected cortical and inner medullary CD (CCD and IMCD). As shown in figure 8A, CCD V2R mRNA was increased 3-fold in db/db mice compared to db/m. Moreover, medullary V2R mRNA expression was increase 2.5 fold in db/db and db/db+EMPA compared to db/m (Figure 8B). We also
examined the mRNA expression of the urea transporter A1 (UTA1) at the collecting ducts. As shown in figure 8C, UTA1 expression increased by 8-fold in db/db+EMPA compared to db/m, and had little to no expression in the other two groups.

Figure 6: Renal sodium transporters gene expression. RNA was isolated from the renal cortex (A-C) and from microdissected proximal tubules (D-E) and thick ascending limb (F) from 4 mouse groups: control (db/m), db/m+EMPA, diabetic (db/db), and db/db+EMPA. Expression of sodium-hydrogen exchanger (NHE)-3, epithelial sodium channel (ENaC), sodium-potassium-ATPase (NaK-ATPase), sodium-glucose transporter (SGLT)-1 and SGLT-2, and sodium-potassium-2-chloride cotransporter (NKCC2) were measured by qPCR. Data was normalized to 18S and is presented as fold db/m of mean +/- SEM. *p < 0.05 vs. db/m, n = 3-6.

Collecting duct water transport

Functional studies in isolated perfused IMCD were conducted to determine whether water reabsorption is increased to compensate for the osmotic diuresis induced by EMPA. Our work first determined whether basal water flux is altered in db/db mice, and then how EMPA alters basal water transport. Next, we assessed water flux in response to 10^{-8} M AVP and AVP+10^{-7} M PGE_2. As shown in figure 9, AVP-stimulated water reabsorption was comparable in db/m and db/m+EMPA at around 5 nl/mm/min, and equally attenuated by 45 and 46% respectively by PGE_2. In db/db mice, the AVP response was reduced by 50%, and this reduction was unaffected by EMPA. There was a greater attenuation of AVP-mediated water transport in db/db in response to PGE_2 by 62%, but this attenuation was reduced in response to EMPA, to 28% in db/db+EMPA.

Discussion

Four groups of adult male mice were studied after 6 weeks of treatment: control (db/m), db/m+EMPA (10 mg/kg/day in chow), type 2 diabetic (db/db), and db/db+EMPA. Tubules were microdissected for qPCR and CD water transport was measured in response to AVP, with or without PGE_2. Hyperglycemia and albuminuria were attenuated by EMPA. Renal mRNA expression for COX, PGE synthase, EP receptors, CD AVP V2 receptors and aquaporins was elevated in db/db mice, but unchanged by EMPA. Urine PGE_2 levels increased in db/db but were unchanged by EMPA. AVP-water reabsorption was comparable in db/m and db/m+EMPA, and equally attenuated to 50% by PGE_2. In db/db mice, AVP-water reabsorption was reduced by 50% compared to non-diabetic mice, and this reduction was unaffected by EMPA.
In db/db mice, AVP-stimulated water transport was more significantly attenuated with PGE$_2$ (62%), compared to non-diabetic mice, but this attenuation was reduced in response to EMPA, to 28%.

In addition to the classical disturbances implicated in the pathogenesis of DKD, namely altered hemodynamics and filtration barrier dysfunction, proximal tubule transport and injury are also involved in the initiation and progression of kidney disease [19]. SGLT2 transporters reabsorb over 90% of filtered glucose and are elevated in the diabetic proximal tubule, contributing to hyperglycemia [20]. SGLT2 is now considered a key target for anti-hyperglycemic therapy [21]. In fact, Jurczak, [22] examined the effects of SGLT2 gene (SLC5A2) deletion on db/db mouse pancreas and other organs, and metabolic and renal outcomes were improved, but the transport properties of the diabetic nephron were not studied. The major novel finding of our study is that the collecting duct compensates for diuresis in response to EMPA, by increasing water reabsorption in the terminal IMCD. PGE$_2$ is implicated in this response, since the attenuation of AVP-mediated water reabsorption was significantly reduced in db/db mice treated with EMPA compared to the non-diabetic mice.

Following SGLT2 inhibition, the tubules in the distal nephron will be exposed to increased solute and water delivery. To prevent excessive water losses, the CD must adapt and increase water reabsorption. We recently reported a key role for PGE$_2$/EP1 in attenuating mouse CD AVP-mediated water transport [15]. Our data suggest that reductions in EP1-mediated attenuation of AVP transport may underlie the reductions we observed. Another possibility is increased AVP-independent water reabsorption by the CD. Gao, et al. [24] reported a major role for CD PGE$_2$/EP$_2$ in AVP-independent water reabsorption. In our mice, following 6 wks of EMPA, volume status is maintained despite reduced proximal tubule reabsorption, suggesting that compensation occurs downstream of the proximal tubule. It is quite possible that a combination of the above-mentioned mechanisms occurs to ensure optimal compensation.

SGLT2 inhibitors reduce glomerular hyperfiltration in human DKD and reduce proteinuria [2,3]. While hyperfiltration may be attenuated in diabetic patients, GFR ultimately normalizes, which is promising in terms of therapeutic usefulness of these drugs for CKD patients already at risk for reduced renal filtration function [2,22]. In our study, db/db mice were hyperfiltering, but EMPA had no effect on GFR. However, we did observe a significant decrease in proteinuria concomitant with reductions in hyperglycemia, but independent of changes in GFR. The mechanisms underlying the reduced proteinuria were not explored and warrant further investigation. The most recent work in rodents highlights the positive renal outcomes associated with SGLT2 inhibition in type 2 diabetes, up to 16 wks treatment [1,25,26]. Renal benefits were not observed following SGLT2 inhibition with canagliflozin in type 1 diabetic eNOS knockout mice [28], however in type 1 Akita mice hyperfiltration and albuminuria were both improved by EMPA [29]. However, the beneficial outcomes are inconsistent even in type 2 diabetic db/db mice. For example, in a study by Gallo, et al. [27], molecular markers of renal fibrosis were ameliorated in db/db mice following 10 wks of EMPA, without reductions in albuminuria or protection against kidney injury. Perhaps following longer treatment, improvement in renal hyperfiltration is more likely in our study, but we observed significant amelioration of albuminuria without changes in GFR. The reason for the discrepancy in all these rodent studies is unclear, but drug class, timing, and dose may be important factors, and in previous reports EMPA treatment was prolonged up to 16 wks. More work is needed to fully understand the mechanisms underlying the benefits of EMPA, since in our study they are clearly not associated with improvements in hyperglycemia or hyperfiltration.

Overall, PGE$_2$ promotes diuresis and natriuresis, acting on EP$_3$ and EP$_4$ and stimulates water reabsorption via EP$_1$. All three subtypes have been localized to the proximal tubule, TAL, and CD by our group, and their expression compared in normotensive and hypertensive mice [15]. Here we show the pattern of expression of EP receptors in the db/db mouse kidney and its heterozygous control. To the best of our knowledge, this is the first characterization of renal EP receptor expression in type 2 diabetes. The most noteworthy findings of this study are that renal EP$\_3$ and EP$\_4$ are mostly increased in type 2, as they are in type 1 diabetes [6,8], and EP$\_4$ is diminished. The increase in mRNA may occur as a feedback response to lower EP receptor protein expression. The expression profile depends on the nephron segment, and as in our previous study we found that PT EP receptor expressions are diminished in diabetes. The significance of this decrease has yet to be studied. We previously reported that PGE$_2$ plays a stimulatory role in PT water transport [30], but very little is known about other PT transport processes linked to each EP receptor subtype. Of interest, we also observed molecular changes in the expression of sodium transporters and aquaporins in EMPA treated mice, independent of the diabetic state, namely PT SGLT1, TAL NKCC2, and CD aquaporins 1 and 2. It is not clear whether the mRNA expression patterns result from feedback responses or are a result of compensatory responses to conserve sodium and water and prevent excessive losses.

Altogether our work emphasizes the need to fully investigate the transport responses of the nephron following proximal tubule SGLT2 inhibition, especially since these inhibitors have become a central part of the treatment arsenal to reduce hyperglycemia and prevent cardiovascular and renal complications associated with type 2 diabetes. It is not surprising that the PGE$_2$/EP receptor system is responsive...
to EMPA treatment to prevent excessive water losses. After all it has long been recognized that both PGE_{2} and AVP are key hormones in the fine-tuning of water balance, especially in diabetes, but even more so when the nephron is further challenged with increased solute and water delivery. The long-term consequences of EMPA treatment on distal nephron transport warrant further investigation.

**Data availability**

The data used to support the findings of this study are included within the article.

**Financial support**

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**Author contributions**

RN wrote the initial manuscript. RN, JZ, VC and JFT performed experimental procedures. JFT, KDB and RLH revised the manuscript.

**References**

Collecting duct PGE2 responses reduce water loss with empagliflozin in mice with type 2 diabetes mellitus


