SGLT2 Inhibitor Empagliflozin Reduces Renal Growth and Albuminuria

In Proportion to Hyperglycemia and Prevents Glomerular Hyperfiltration

in Diabetic Akita Mice

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Running title: SGLT2 inhibition in the diabetic kidney

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ABSTRACT

Our previous work has shown that gene-knockout of the sodium-glucose cotransporter SGLT2 modestly lowered blood glucose in streptozotocin-diabetic mice (BG; from 470 to 300mg/dl) and prevented glomerular hyperfiltration, but did not attenuate albuminuria or renal growth and inflammation. Here we determined effects of the SGLT2 inhibitor empagliflozin (300mg/kg of diet for 15 weeks; corresponding to 60-80mg/kg/d) in type 1 diabetic Akita mice that, opposite to streptozotocin-diabetes, upregulate renal SGLT2 expression. Akita-diabetes, empagliflozin, and Akita+empagliflozin similarly increased renal membrane SGLT2 expression (by 38-56%) and reduced the expression of SGLT1 (by 33-37%) vs. vehicle-treated wild-type controls (WT). The diabetes-induced changes in SGLT2/SGLT1 protein expression are expected to enhance the BG lowering potential of SGLT2 inhibition, and empagliflozin strongly lowered BG in Akita (means of 187-237 vs. 517-535mg/dl in vehicle group; 100-140mg/dl in WT). Empagliflozin modestly reduced GFR in WT (250 vs. 306µl/min), and completely prevented the diabetes-induced increase in GFR (255 vs. 397µl/min). Empagliflozin attenuated increases in kidney weight and urinary albumin/creatinine ratio in Akita in proportion to hyperglycemia. Empagliflozin did not increase urinary glucose/creatinine ratios in Akita, indicating the reduction in filtered glucose balanced the inhibition of glucose reabsorption. Empagliflozin attenuated/prevented the increase in systolic blood pressure, glomerular size, and molecular markers of kidney growth, inflammation, and gluconeogenesis in Akita. We propose that SGLT2 inhibition can lower GFR independent of reducing BG (consistent with the tubular hypothesis of diabetic glomerular hyperfiltration), while attenuation of albuminuria, kidney growth, and inflammation in the early diabetic kidney may mostly be secondary to lower BG.
Introduction

Diabetes-associated kidney disease is the commonest cause of end stage renal disease in most countries of the world. The pathophysiology, however, is still incompletely understood.

Control of blood glucose levels is important in diabetic patients but often associated with hypoglycemia and weight gain. Pharmacological inhibition of the renal sodium-glucose cotransporter SGLT2 (SLC5A2) is a new approach that inhibits the renal reabsorption of filtered glucose thereby lowering blood glucose levels without increasing body weight (6; 17; 40).

The concept to inhibit SGLT2 in diabetes is based on the fact that SGLT2, which is localized in the early proximal tubule, mediates the bulk of tubular glucose uptake across the apical membrane of the kidney (27; 36; 43). In comparison, SGLT1 is thought to have a lower capacity for glucose reabsorption, which, in euglycemia, “cleans up” most of the remaining luminal glucose in further distal parts of the proximal tubule (1; 11; 27; 43). Besides their role in glucose reabsorption, SGLTs contribute to the increase in Na⁺ and fluid reabsorption in the proximal tubule of the early diabetic kidney (37). Increased proximal reabsorption contributes to diabetic glomerular hyperfiltration by lowering the Na-Cl-K concentrations at the macula densa and increasing GFR through the physiology of tubuloglomerular feedback (32; 37; 39). Direct luminal application of the SGLT-inhibitor, phlorizin, into the free-flowing early proximal tubule, just downstream of Bowman space, acutely normalized the Na-Cl-K concentrations at the macula densa as well as single nephron GFR in streptozotocin (STZ)-diabetic rats (37). These studies demonstrated the contribution of SGLTs to the diabetic hyperreabsorption and that inhibition of SGLTs can lower glomerular hyperfiltration without altering blood glucose levels.

Selective pharmacological inhibition of SGLT2 with dapagliflozin has recently been proposed to lower GFR in STZ-diabetic rats through the same macula densa mechanism (33), and gene knockout of Sglt2 prevented glomerular hyperfiltration in STZ-diabetic mice (38). Both studies provided additional evidence that this response did not require effects on blood glucose levels.

Similarly, preliminary studies in patients with type 1 diabetes showed that the SGLT2 inhibitor, empagliflozin, lowers GFR independent of effects on blood glucose levels (4).
Besides its role in GFR control, renal glucose uptake via SGLT2 may contribute to kidney growth in the early diabetic kidney. The latter involves diabetes-induced growth of the proximal tubule (5; 23; 30), and diabetic kidney growth has been linked to the development of nephropathy (2; 3; 13; 26; 35; 44). Using the model of low-dose STZ-induced type 1 diabetes in mice lacking Sglt2, we recently concluded that glucose uptake via SGLT2 is not a critical stimulus for diabetic kidney growth or the development of kidney inflammation and injury (38). The study further indicated that, in steady state, urinary glucose excretion was not affected by the absence of Sglt2 in STZ-diabetic mice, i.e., the lack of early proximal glucose reabsorption via SGLT2 was balanced by the resulting lowering of glomerular glucose filtration (lower blood glucose x lower GFR). As a consequence, STZ-induced glucose delivery downstream of the SGLT2-expressing early proximal tubule was likely not different between WT and Sglt2-/- mice. If luminal glucose delivery downstream of the early proximal tubule is an important stimulus for kidney growth, inflammation, and injury in diabetes, then this may explain why these changes were independent of SGLT2. Alternatively, growth and other tubulointerstitial changes may be triggered by sensing of hyperglycemia via the basolateral tubular membrane and/or glomerular filtration of specific factors, and the absence of SGLT2, which lowered blood glucose in this model from ~470 mg/dl in wild-type mice to ~300 mg/dl in Sglt2-/- mice, just did not lower blood glucose enough to limit kidney growth and inflammation (38).

The previous experiments, testing for a role of SGLT2 in STZ-diabetic mice, were also confounded by a 60% decline in renal SGLT2 expression following STZ administration in wild-type mice, which may have masked some effects of knocking out the Sglt2 gene (38). This problem of reduced SGLT2 expression probably owes to a direct effect of STZ on the mouse proximal tubule, since SGLT2 expression is actually increased in both the db/db model of type 2 diabetes and the Akita model of type 1 diabetes (38). Hence, to more fully assess the potential of SGLT2 inhibition in the early diabetic kidney, we tested in diabetic Akita mice the effect of the selective SGLT2 inhibitor, empagliflozin (12), on blood glucose control, GFR, kidney growth, and markers of renal inflammation.
Methods

Animals. All animal experimentation was conducted in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD) and was approved by the local Institutional Animal Care and Use Committee. Akita mice (Ins2^+/C96Y; Akita/+) (and littermate Ins2^{+/+} controls (WT)) were used as a non-obese insulin-dependent model of spontaneous type 1 diabetes (The Jackson Laboratories, Bar Harbor, ME). All studies were performed in male mice, which were housed in the same animal room with a 12:12 hour light-dark cycle and free access to standard rodent chow and tap water. At 4 weeks of age, mice received a diet containing empagliflozin (300 mg/kg of diet; in regular rodent diet [in %: 19 protein, 3.3 fat, 4.9 fiber, 6.4 ash, 36.5 starch, 4.7 sugar, 12.8 MJ ME/kg]; synthesized at ssniff Spezialdiäten GmbH, Soest, Germany) or repelleted control diet (vehicle) for 15 weeks (n=10-13/group). Empagliflozin was provided by Boehringer Ingelheim Pharma GmbH & Co.KG, Biberach, Germany.

Food and fluid intake, blood and urine collection. Food and fluid intake was determined while the mice were maintained in their regular cages. Urine was obtained by picking up the mice to elicit reflex urination and holding them over a clean Petri dish for sample collection. For paired glucose measurements, blood was collected by tail snip immediately after urine collection in awake mice. For other blood/plasma analyses, blood was collected from the retrobulbar plexus under brief isoflurane anesthesia.

Systolic blood pressure and heart rate in awake mice. Systolic blood pressure and heart rate were determined using an automatic tail-cuff system (Visitech-Systems, Apex, NC, USA) after appropriate training as described (36).

Measurement of GFR in awake mice. GFR measurements were performed in conscious mice (at 14 weeks after starting treatment with empagliflozin or vehicle) using the plasma kinetics of FITC-Sinistrin (Fresenius-Kabi, Linz, Austria)(19) following a single dose i.v. injection of this GFR marker as described (24; 38; 39). Briefly, FITC-Sinistrin (2% in 0.85% NaCl, which
also served to establish the standard curve), was injected into the retro-orbital plexus (2 µl/g/body weight) during brief isoflurane anesthesia. At 3, 5, 7, 10, 15, 35, 56, and 75 min after injection, blood was collected from the end of the tail into a Na+-heparinized 10 µl microcap (Hirschmann Laborgeräte, Eberstadt, Germany). After centrifugation plasma was diluted 1:10 in 0.5 mol/l HEPES (pH 7.4) and fluorescence determined using a Nanodrop ND-3300 fluorospectrometer (Nanodrop Technologies, Wilmington, DE) by pipetting 2 µl of samples onto the pedestal. GFR was calculated using a two-compartment model of two-phase exponential decay (GraphPad Prism, San Diego, CA).

**Blood and urine analysis.** Blood glucose was determined using the Ascensia Elite XL glucometer (Bayer Corporation, Mishawaka, IN, USA). Urine glucose was determined by the hexokinase / glucose-6-phosphate dehydrogenase method (Infinity, Thermo Electron, Louisville, CO). Concentrations of plasma aldosterone (Beckman Coulter, Brea, CA) and urinary albumin (Exocell, Philadelphia, PA, USA) and creatinine (Thermo Fisher Scientific, Waltham, MA, USA) were measured using commercial assays.

**Glomerular size.** Kidneys were harvested under terminal isoflurane anesthesia and fixed in 4% paraformaldehyde (PFA), embedded in O.C.T. compound (Sakura Finetek, Torrance, CA), sliced at a thickness of 10 µm using a cryostat microtome, and placed on Superfrost Plus microscope slides (Thermo Fisher Scientific, San Diego, CA). After being air-dried, sections were blocked in 5% dry milk (in PBST), and incubated with Alexa Fluor® 555 Phalloidin (Invitrogen, Carlsbad, CA) for 30 minutes at room temperature. Sections were washed 3X for 5 minutes in PBST and mounted with Prolong (Invitrogen) and imaged on an Olympus DSU confocal microscope. The area (in pixel units) of twenty random glomeruli was measured per mouse kidney using Adobe Photoshop 7.0 software.

**Western blot analysis.** Whole kidneys were harvested under terminal isoflurane anesthesia and prepared for Western blot analysis of SGLT2 and SGLT1 in the membrane fraction as previously described (36). For the former and the analysis of other proteins (cyclin
dependent kinase inhibitors p27 and p21, and heme oxygenase 1 (HO-1)), kidneys were
harvested and homogenized in a buffer containing 250 mM sucrose, 10 mM triethanolamine,
and protease inhibitors (Complete protease cocktail, Roche Molecular Biochemicals, Mannheim,
Germany) with a tissue homogenizer (Tissumizer; Tekmar, Cincinnati, OH). Cell fractionation
was achieved by differential centrifugation at 4000xg for 10 minutes (nuclear pellet for p27 and
p21) followed by 16000xg for 1 hour (membrane pellet for SGLT2/SGLT1 and cytosolic
supernatant for HO-1). Protein concentration was determined with a DC Protein Assay (Bio-
Rad). Lysates at 40 µg/lane were resolved on NuPAGE gels in MOPS buffer (Invitrogen,
Carlsbad, CA). Gel proteins were transferred to nitrocellulose membranes and immunoblotted
with the appropriate primary antibody. The secondary antibody was horseradish peroxidase-
conjugated for autoradiographic detection by ECL Plus (Amersham Pharmacia, Piscataway,
NJ). Antibodies for p27 and HO-1 were purchased from Santa Cruz Biotechnology (Dallas, TX).
The antibody for p21 was acquired from Abcam (Cambridge, MA). To verify equal protein
loading, membranes were stripped (0.2 M NaOH for 5 min) and reprobed with monoclonal anti-
β-actin antibody (Sigma-Aldrich, St. Louis, MO) for cytosolic and membrane proteins and with
anti-lamin-B antibody (Santa Cruz) for nuclear proteins. Densitometric analysis was performed
by ImageJ Software (National Institutes of Health, Bethesda, MD). For the indirect assessment
of renal collagen content, a modification of a previously described method was used involving
precipitation of collagen with picro-Sirius red (34; 38). Briefly, 100 µl of cytosolic fraction
obtained from kidney homogenization was added to 1 ml of picro-Sirius red solution (NovaUltra
Sirius Red Stain Kit, IHC World, Woodstock, MD) and agitated for 45 min followed by
centrifugation at 10,000g for 10 min. The Sirius red dye was released from the pellet with alkali
reagent (1 N NaOH) and spectrophotometric readings were taken at 540 nm on a microplate
reader (Molecular Devices, Sunnyvale, CA). Results were expressed relative to WT controls.

Reverse transcription and real-time PCR. Whole kidney RNA was prepared with the
RNeasy® Plus Mini Kit and cDNA was prepared with the Superscript II® First Strand Synthesis
System. For quantification, specific primers were used with Power SYBR® Green PCR Master Mix (10 min at 95°C with 50 cycles of 15 sec at 95°C and 1 min at 60°C) in an AB7300 Real Time PCR System (Applied Biosystems, Foster City, CA). For some genes, we used Taqman® PCR Universal Mastermix and primers to improve specificity and sensitivity of these reactions (Applied Biosystems). Please see Table 1 for primer details. Relative fold increases were calculated using the Pfaffl technique of relative quantification, which accounts for real-time efficiencies (18). Each experiment was performed in triplicate.

**Determination of adipocyte size.** Epididymal white adipose tissue (WAT) was excised during terminal anesthesia, immediately fixed with PFA for 24 h, and embedded in paraffin. Paraffin embedded fat tissue was cut into 5 μm sections and stained with PAS. Pictures were taken on an Olympus IX81 microscope and adipocyte cross-sectional area was determined using ImageJ software. At least 300 adipocytes were analyzed per mouse.

**Screening for urinary tract infection.** Twenty to fifty μl bladder urine (collected at the time of sacrifice) was streaked onto cystine-lactose-electrolyte-deficient medium plates (Thermo Scientific Remel, Lenexa, KS) which were incubated overnight at 37°C. The number of colony-forming units (CFU's) per ml of urine was determined. A density of >10⁵ CFU/ml was considered to indicate ascending urinary tract infection (as recommended by manufacturer).

**Statistical analysis.** Data are shown as means ± SEM. ANOVA and unpaired Student's t-test were performed to analyze for statistical differences between groups. Regression analysis was performed to test for a linear relationship between blood glucose concentrations and various parameters. P<0.05 was considered statistically significant.
**Results**

*Empagliflozin-treatment of non-diabetic mice and Akita-diabetes increased renal membrane expression of SGLT2 and reduced SGLT1 expression.* Renal membrane SGLT2 expression was increased in Akita/+ versus WT mice by 38%, associated with a trend for higher SGLT2 mRNA expression (P=0.087) (Figure 1A-C). Empagliflozin enhanced the renal protein expression of SGLT2 in WT mice by 47% without upregulation of mRNA (Figure 1A-C). Renal SGLT2 expression in empagliflozin-treated Akita/+ was similar to vehicle-treated Akita/+ or empagliflozin-treated WT.

Renal membrane protein expression of SGLT1 provided a mirror image of the changes in SGLT2. Akita-diabetes, empagliflozin, and their combination reduced the renal membrane protein expression of SGLT1 by 33-37% (Figure 1A,B). In comparison, renal mRNA expression of SGLT1 was increased in Akita/+ compared with WT and not affected by empagliflozin-treatment (Figure 1C), indicating divergent effects of Akita-diabetes on renal SGLT1 mRNA and membrane protein expression. The specificity of the analyses for SGLT2 and SGLT1 protein and mRNA were confirmed by the use of kidneys from gene knockout animals.

In WT mice, empagliflozin did not affect renal mRNA expression of GLUT1 or GLUT2 (Figure 1C). Akita/+ increased renal mRNA expression of both transporters, and these responses were attenuated by empagliflozin.

*Effects of empagliflozin on glucose excretion, plasma glucose, food intake, and body weight.* In WT mice, empagliflozin induced sustained increases in urinary glucose/creatinine ratios (Figure 2B) and absolute urinary glucose concentrations (470±34 vs. 4±1 mM, P<0.05), and modestly lowered blood glucose levels (Figure 2A). The increased loss of glucose calories into the urine of empagliflozin-treated WT mice was partially offset by increased food (and fluid) intake (Figure 2D,E), consistent with previous reports in non-diabetic mice lacking Sglt2 (36). The compensatory increase in food intake is deemed to be "partial" because the body weight of the glucosuric WT mice was lower than in their non-glucosuric counterparts (Figure 2F).
In Akita/+ mice, mean plasma insulin concentration was less by 90% while blood glucose concentration was elevated 5-fold relative to WT (Figures 2A and 3A). Empagliflozin reduced blood glucose concentration by 60% in hyperglycemic Akita/+ while further lowering plasma insulin levels, indicating presence of ~10% residual beta islet function in Akita/+ that remains sensitive to changes in plasma glucose within the range of 200-600 mg/dl. Inhibition of glucose reabsorption by empagliflozin in Akita/+ was well-matched to the resulting decline in the filtered load of glucose, as indicated by the finding that treated and untreated animals wound up excreting similar amounts of glucose in their urine after coming into balance with disparate blood glucose concentrations (Figure 2A, 2B, and 2C). This was associated with similar food intake (measured in regular cages)(Figure 2E). Similar food intake and urinary glucose/creatinine ratios and absolute urinary glucose concentrations (urinary calorie loss) were previously reported between STZ-diabetic Sglt2/-/- and control mice (36). Up to 10 weeks of treatment did not significantly alter body weight in Akita/+ mice, but empagliflozin modestly increased body weight following 15 weeks of treatment (Figure 2F), i.e. over the course of the study the SGLT2 inhibitor induced opposite effects on body weight in WT versus Akita/+ mice. The observed changes in body weight in response to diabetes and empagliflozin were accompanied by parallel changes in epididymal fat adipocyte size, i.e. empagliflozin reduced mean adipocyte size in WT and increased adipocyte size in Akita/+ mice (Figure 2G).

Renal gluconeogenesis is upregulated in diabetic animal models and in humans with type 1 or type 2 diabetes, and may significantly contribute to hyperglycemia (for review see (10)). Phosphoenolpyruvate carboxykinase (PEPCK) is a principal gluconeogenic enzyme, whose activity is directly and proportionally modulated by the rate of gene transcription and thus the amount of mRNA (21). We found PEPCK mRNA expression to be 60% elevated in Akita/+ versus WT mice and normalized by empagliflozin (Figure 3B). Empagliflozin did not affect the expression of PEPCK in WT.
Empagliflozin prevented the diabetes-induced modest increase in systolic blood pressure and hematocrit. Systolic blood pressure (determined in trained and awake mice by an automatic tail cuff system) and hematocrit were modestly increased in Akita/+ versus WT mice (Figure 3D, F). These increases in systolic blood pressure or hematocrit in Akita/+ were prevent with empagliflozin (Figure 3D, F). Heart rate and plasma concentrations of aldosterone were not affected by empagliflozin (Figure 3C, E). Note that aldosterone concentrations were elevated in Akita/+ compared with WT mice. Plasma Na⁺ concentration (not shown) was not modified by the treatment and similar between WT and Akita/+ mice.

Empagliflozin prevented the diabetes-induced increase in GFR and attenuated the increase in kidney weight and albuminuria in proportion to hyperglycemia. In WT mice, empagliflozin modestly reduced GFR (Figure 4A) while tending to increase kidney weight (Figure 4B; \(P=0.085\)) and significantly increasing the kidney to body weight ratio compared with vehicle-treatment (12.0±0.1 vs. 10.9±0.2 mg/g bw; \(P<0.001\)).

GFR was 30% greater among Akita/+ versus WT mice, commensurate with diabetic glomerular hyperfiltration. Hyperfiltration was prevented by empagliflozin, which reduced GFR to the levels observed in WT (Figure 4A). Empagliflozin attenuated increases in kidney weight and urinary albumin/creatinine ratio observed in Akita/+ versus WT mice in proportion to lowering hyperglycemia (Figure 4B, C), and normalized glomerular size (Figure 4D).

Empagliflozin attenuated diabetes-induced rise in renal expression of markers of kidney growth and inflammation. Overexpression of the cyclin-dependent kinase inhibitors p27 and p21 has been implicated in the renal growth and G₁ cell cycle arrest in the early diabetic kidney (35). Expression of p27 and p21 proteins was greater in renal nuclei from Akita/+ mice compared with WT (Figure 5A). Renal content of the stress response protein, hemoxygenase-1 (HO-1), was also modestly increased in Akita/+ versus WT. p21, p27, and HO-1 levels in Akita/+ mice were reduced by empagliflozin, though remaining slightly elevated. Empagliflozin did not suppress p21, p27, or HO-1 expression in WT.
NFkB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a transcriptional regulator responsible for cytokine production, including inflammatory cytokines. Renal content of NFkB mRNA was elevated in Akita/+ as were mRNA for CCL2 (a cytokine that recruits proinflammatory cells), CD14 (a cell surface marker of macrophages), IL-6 (a dual pro- and anti-inflammatory cytokine), and TIMP2 (a tissue inhibitor of matrix metallo-proteinases that arises in kidney injury). Empagliflozin reduced or normalized the renal mRNA content of all these inflammatory markers in Akita/+, without major effects on their expression in WT mice (Figure 5B).

Several markers of oxidative stress and fibrosis were examined and found not to be different between the hyperfiltering Akita/+ mice with early diabetes and WT controls. Nor was there evidence that empagliflozin affected these markers. Markers included renal mRNA content of NADPH oxidases, NOX2 and NOX4, and the cytokine CCL5. Markers of fibrosis included TGFβ and renal collagen expression, as determined by Sirius red binding (data not shown). These results indicated that diabetes-induced renal changes in the current study were restricted to the early phase of renal hyperfunction (GFR), albuminuria, growth, and inflammation, rather than extensive oxidative stress or fibrosis.

*Empagliflozin did not induce ascending urinary tract infection.* Glucosuria predisposes to urinary tract infection, but no animal in the present study contracted urinary tract infection as defined by >10^5 bacterial colony-forming units (CFU's) per ml of urine collected by direct bladder puncture under terminal anesthesia following 15 weeks of treatment. Most samples from all groups produced no bacterial growth and no sample in any mouse produced more than >10^3 CFU/ml; n=11-13). These findings are consistent with previous studies using non-diabetic and diabetic Sglt2-/- mice (36).
The main findings of the current study are that the selective SGLT2 inhibitor, empagliflozin, lowered blood glucose levels in diabetic Akita mice by ~60% from >500 mg/dl to ~200 mg/dl and prevented glomerular hyperfiltration. Empagliflozin also attenuated, without completely eliminating, diabetes-associated increases in albuminuria, kidney weight, markers of renal growth, and several markers of inflammation. Finally, empagliflozin prevented the diabetes-induced overexpression of the gluconeogenic enzyme, PEPCK. Empagliflozin appeared to mitigate the effects of diabetes on kidney growth, albuminuria, and inflammation to the same extent that it mitigated hyperglycemia, whereas the mitigation of hyperfiltration by empagliflozin was complete. Together with previous findings discussed below, including studies in STZ-treated Sglt2/-/- mice, we propose that inhibition of SGLT2 can suppress diabetic glomerular hyperfiltration independent of its blood glucose effect, consistent with the tubular hypothesis of diabetic glomerular hyperfiltration (35; 41). In comparison, the beneficial effect of SGLT2 inhibition on albuminuria, kidney growth, and inflammation in the early diabetic kidney may mostly be secondary to lower blood glucose.

The current results confirm that pharmacological inhibition of SGLT2 reduces renal glucose reabsorption thereby lowering blood glucose levels in diabetes mellitus. This is consistent with prior studies in STZ-diabetic mice lacking Sglt2 (38) or carrying a loss of function mutation in SGLT2 (15), and with studies using empagliflozin or other SGLT2 inhibitors in diabetic rats and humans (6; 7; 14; 17; 31; 40). Uptake experiments with a non-metabolizable glucose analogue in stable cell lines over-expressing human and mouse glucose transporters showed that empagliflozin inhibits SGLT2-mediated glucose uptake in a dose-dependent manner with an IC50 of 3.1 nM for human SGLT2 and 1.9 nM for mouse SGLT2, and is highly selective for SGLT-2 over SGLT-1 in humans (>2500-fold) and mice (approximately 5800-fold), respectively (12). Comparing multiple SGLT2 inhibitors, empagliflozin had the highest selectivity for SGLT-2 over SGLT-1 (12). Empagliflozin was given at a dose of 300 mg/kg of diet, which
corresponded to 60-80 mg/[kg body wt and day]. Empagliflozin given in the diet at a dose of 30-
35 mg/[kg body wt and day] induced a free plasma concentration of 1-2 nM in clearance studies
performed during daytime in non-diabetic mice (25), which is close to the IC_{50} for SGLT2 in the
mouse (12). Therefore, the dose of ~60-80 mg/[kg body wt and day] applied in the current study
was expected to have inhibited SGLT2 function by more than 50% during daytime and possibly
even stronger during nighttime when food and thus drug intake occurred, taking into
consideration that the plasma half life of empagliflozin in the male mouse is about 5.6 hours.

Empagliflozin lowered blood glucose (measured during daytime) more potently in Akita/+ mice (by ~300 mg/dl: from >500 to ~200 mg/dl) than did knocking out the Sglt2 gene in STZ-
diabetic mice (by ~170 mg/dl: from ~470 to ~300 mg/dl) (38). One possible reason is the
difference in renal SGLT2 expression. SGLT2 expression was upregulated in Akita/+ mice
(similar to mice (38) and humans (22) with type 2 diabetes) and, therefore, the amount of
transporter that can be inhibited by empagliflozin was increased. In contrast, STZ-diabetes
reduced the renal expression of SGLT2 and, thereby, the glucose reabsorption available to be
suppressed by the knockout (38). SGLT2 expression was also increased by empagliflozin in WT
mice, probably a compensatory response. Divergent effects on mRNA expression indicate that
Akita-diabetes and chronic SGLT2 inhibition may increase SGLT2 expression via different
mechanisms. While the increase in SGLT2 expression in Akita/+ can contribute to a greater
reduction or change in blood glucose levels in response to empagliflozin compared with the
effect of the Sglt2 gene knockout in STZ-diabetes, the differences in SGLT2 expression can not
explain why blood glucose was reduced to lower absolute levels in the current study.

Renal membrane SGLT1 protein expression provided a mirror image of the changes in
SGLT2, i.e. Akita-diabetes, empagliflozin, and their combination reduced renal membrane
protein expression of SGLT1. Previous studies provided evidence for increased, unchanged or
reduced renal SGLT1 expression and/or activity in diabetes or under high glucose conditions
(41). The effect of the SGLT2 inhibitor empagliflozin in non-diabetic mice mimicked the
suppression of renal membrane SGLT1 protein expression reported in mice lacking Sglt2 (36).

Glucose transport through SGLT1 is strongly increased when SGLT2 is deleted by gene targeting or inhibited by empagliflozin (25) or when luminal glucose delivery is increased in diabetes. Therefore, lowering renal membrane SGLT1 protein expression may serve to limit glucose reabsorption and possibly toxicity in the S2 and S3 segments of the proximal tubule when glucose delivery and uptake are increased. In vitro studies in renal proximal tubule cells indicated that high glucose-induced oxidative stress may reduce SGLT expression and Na+/glucose cotransport activity (53). The observed divergent effects of Akita/+ and empagliflozin on mRNA and membrane protein expression of SGLT1 indicate a complex translational and posttranslation regulation. With regard to glucose homeostasis, downregulation of SGLT1 reduces its glucose transport and compensatory capacity and is expected to enhance the glucosuric and blood glucose lowering effect of SGLT2 inhibition (20; 25).

In addition to inhibiting renal glucose reabsorption, empagliflozin attenuated, in proportion to hyperglycemia, the diabetes-associated increase in renal mRNA expression of PEPCK. This enzyme is a principal regulator of gluconeogenesis in the proximal tubule that is regulated at the mRNA level and upregulated in the diabetic kidney of rats and humans (9; 10; 21). This observed effect was specific for the diabetic kidney since empagliflozin did not affect the expression of PEPCK in WT kidneys. PEPCK expression by proximal tubules is mainly down-regulated by insulin and up-regulated by glucagon, though its expression also increases during chronic acidosis as part of the pathway for generating ammonium and bicarbonate (10; 21). The increase in PEPCK in Akita/+ was expected based on prior reports and known pathways (9; 10; 21). The mechanism whereby empagliflozin prevented this is not obvious. Effects of empagliflozin on plasma insulin levels cannot explain the effect on renal PEPCK expression in Akita/+ mice since insulin levels were modestly lower compared with vehicle-treated Akita/+ mice, consistent with lower blood glucose levels reducing the stimulus for insulin
secretion. Further studies will examine the basis for the novel relationship of PEPCK expression to SGLT2 and will determine the extent to which a reduction in renal PEPCK overexpression contributed to the blood glucose lowering effect of empagliflozin.

Similar to the phenotype of knocking out the Sglt2 gene in non-diabetic mice (38), SGLT2 inhibition by empagliflozin increased food intake in WT mice, possibly to match the sustained urinary glucose and calorie loss. This was associated with lower body weight and smaller epididymal adipocytes, corroborating the notion that increased food intake among SGLT2-blocked mice is a compensatory response to a decrease in energy stores. The glucosuria in empagliflozin-treated WT mice and in non-diabetic Sglt2/-/- mice (38) was sustained in steady-state indicating the reduction in glomerular filtration of glucose (due to lowering blood glucose levels and GFR (see below)) was too small to match the reduction in proximal tubular glucose reabsorption. In comparison, and as observed in STZ-diabetic Sglt2/-/- mice (38), pharmacological inhibition of SGLT2 by empagliflozin in diabetic Akita/+ mice did not increase glucosuria after they reached steady-state. Glucosuria did not increase because the reduction in glomerular filtration of glucose (due to lowering blood glucose and glomerular hyperfiltration) was large enough to nullify the impact of reduced tubular reabsorption. As a consequence of similar glucosuria, empagliflozin did not reduce body weight or fat stores (even small increases) or stimulate increased food intake in Akita/+ mice. This simple paradigm for the effects of SGLT2 blockade on body weight and food intake applies universally to mice, rats, and humans. Whatever differences may appear among various studies in humans, rats, and mice with respect to the effect of SGLT2 blockade on body weight can be accounted for by quantitative differences in the effect on glucosuria and the effect of glucosuria on blood glucose and the sensitivity of appetite to weight loss.

Hyperglycemia causes a primary increase in proximal tubular Na⁺ and fluid reabsorption, in part due to enhanced Na⁺-glucose cotransport. Enhanced reabsorption reduces the luminal Na-Cl-K concentration at the macula densa, and via the physiology of
tubuloglomerular feedback and a possible reduction in the hydrostatic pressure in Bowman space increases GFR (35; 37). Empagliflozin slightly reduced GFR in WT mice, but normalized GFR in Akita/+ mice. This is consistent with attenuation of hyperreabsorption by SGLT2 inhibition, which through the described mechanisms lowers GFR, in particular in diabetic conditions (33; 35; 37). The stronger GFR response in diabetes is due to the greater tubular glucose delivery and, therefore, greater empagliflozin-inhibitable Na⁺-glucose cotransport in the proximal tubule. The described GFR lowering mechanism of SGLT2 inhibition does not require a blood glucose lowering effect (4; 33; 37; 38). Correcting hyperglycemia may, however, have additional effects on GFR, e.g., via inhibition of glomerular growth, assuming filtration pressure disequilibrium allows for an influence of glomerular capillary growth on GFR, which may be more relevant in humans than in rodents. If glomerular hyperfiltration is not only associated with but actually contributes to the development and progression of diabetic nephropathy, then lowering GFR by inhibiting SGLT2 has long-term nephroprotective potential (which may not become immediately obvious in short-term studies in rodents).

Diabetic kidney growth and its unique molecular signature have been linked to the development of diabetic nephropathy (35; 41). The tubular growth mechanism in diabetes includes an early, growth factor-driven hyperplastic phase which through the activation of cyclin-dependent kinase inhibitors, including p27 and p21, is switched to a hypertrophic phase that shows aspects of senescence (28; 41). We reported previously that knockout of Sglt2 in STZ-diabetic mice, which lowered blood glucose levels from ~470 to ~300 mg/dl, did not limit the rise in albuminuria, kidney weight or markers of kidney growth, inflammation, and injury (38), indicating that SGLT2-mediated glucose-reabsorption itself is not critical for these changes. Here we observed that empagliflozin given to Akita/+ mice induced a stronger blood glucose lowering effect, from >500 to ~200 mg/dl, and did not prevent but attenuated the rise in albuminuria and kidney growth, including the upregulation of p27 and p21, and reduced markers of renal inflammation in proportion to hyperglycemia. Together these studies indicate that
inhibition of SGLT2 can have beneficial effects on the kidney beyond lowering GFR. The
observed linear correlations between blood glucose levels and albuminuria, kidney weight or
markers of kidney inflammation in the current study would be expected if blood glucose was the
only determinant, but the correlations can not prove a causal relationship. Considering that the
absence of SGLT2 itself or the associated reduction in BG to 300 mg/dl was not
nephroprotective in STZ-diabetic rats (38), the current results are consistent with the notion that
beneficial effects of SGLT2 inhibition on these parameters require and are mostly secondary to
a strong blood glucose lowering effect.

In non-diabetic mice, empagliflozin tended to increase kidney weight and a small,
numerical upregulation of the renal expression of p27 and HO-1 was observed. The changes
did not reach statistical significance, but are reminiscent of the reported modest increase in
kidney weight and renal expression of p27 and HO-1 observed in non-diabetic Sglt2/- versus
control mice (38). These changes may reflect tissue responses of growth and upregulation of
protective mechanisms, respectively, when tubular glucose delivery downstream of the early
proximal tubule is increased in the absence of hyperglycemia. Available data in humans with
mutations in SGLT2 (SLC5A2), a rare and not well studied genetic disorder, indicate that no
other complications, including chronic nephrologic complications, are known to be consistently
associated with mutations of this gene (8; 16; 27; 29; 42). In this regard, the observed changes
with SGLT2 inhibition in non-diabetic mice may reflect adaptive and minor changes, but further
studies are necessary. In diabetic mice, empagliflozin attenuated but did not completely prevent
kidney growth or p27 upregulation, which may reflect the fact that the SGLT2 inhibitor did not
normalize blood glucose levels and/or did not reduce glucose delivery downstream of the early
proximal tubule (see above). The finding that empagliflozin reduced kidney growth in Akita/+ mice without lowering urinary glucose excretion indicated that an increase in luminal glucose
delivery downstream of the early proximal tubule alone is not sufficient to induce full diabetic
kidney growth, arguing that additional factors (e.g. from glomerular filtrate and/or peritubular sites) also contribute.

Empagliflozin lowered blood glucose and diabetes-induced increases in GFR, kidney weight, albuminuria, markers of kidney inflammation, hematocrit, and blood pressure, yet the increase in plasma levels of aldosterone in Akita/+ mice was not affected by the SGLT2 inhibitor. This observation argues against a major pathophysiological role of enhanced aldosterone levels in these diabetes-induced changes. The results may also provide some clues to the mechanism(s) that enhance(s) plasma aldosterone levels in diabetes: a minor isotonic volume constriction, as indicated by higher hematocrit values in Akita/+ mice, may not be crucial since it was normalized by SGLT2 inhibition; in comparison and as discussed above, the enhanced glucose delivery downstream of the early proximal tubule, including the macula densa segment and the distal nephron, was not affected by empagliflozin in Akita/+ mice and can provide interactions with the renin-angiotensin-aldosterone system. Further studies are needed to better understand the nuances of enhanced luminal glucose concentrations along the nephron.

In summary, inhibition of SGLT2 by empagliflozin in diabetic Akita/+ mice strongly lowered blood glucose levels. This was the consequence of lowering renal glucose reabsorption, but empagliflozin also prevented the diabetes-induced upregulation of renal PEPCK mRNA expression, a principal regulator of gluconeogenesis. Downregulation of renal membrane protein expression of SGLT1 in Akita/+ reduces SGLT1-mediated compensation and is expected to enhance the blood glucose lowering effect of empagliflozin. Empagliflozin attenuated diabetes-induced albuminuria, kidney growth, and renal expression of molecular markers of kidney growth and inflammation. These modifications appear to depend on blood glucose reduction. In contrast, SGLT2 inhibition prevented glomerular hyperfiltration and this effect can occur independent of lowering blood glucose (due to tubular Na and fluid transport inhibition). In addition, empagliflozin prevented the increase in systolic blood pressure observed
in Akita/+ mice, similar to findings described in diabetic hypertensive patients. These results suggest that inhibition of SGLT2, with empagliflozin, could affect the development or progression of diabetic nephropathy by blood glucose-dependent and independent mechanisms. Further studies are needed to confirm the putative nephroprotective effects of SGLT2 inhibitors in clinical practice.
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Table 1: Real-time PCR Primers Used

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Figure legends

Figure 1: Empagliflozin-treatment of nondiabetic mice and Akita-diabetes increased renal membrane protein expression of SGLT2 and reduced SGLT1 protein expression. Empagliflozin attenuated the diabetes-induced increase in renal mRNA expression of GLUT1 and GLUT2 but not SGLT1. Divergent effects on mRNA and membrane protein expression of SGLT2 and SGLT1 indicate complex translational and posttranslational regulation. Empagliflozin (300 mg/kg of diet) or repelleted diet (vehicle) were given to Akita/+ and WT mice. A,B: Renal membrane SGLT2 and SGLT1 protein expression. Kidneys from knockout mice served as negative controls (Sglt2-/-, Sglt1-/-). C: Renal mRNA expression of glucose transporters. For Sglt2 and Sglt1 mRNA analysis, kidneys from knockout mice confirmed specificity of primers (data not shown). * \( P<0.05 \) vs. vehicle-treatment in same genotype; # \( P<0.05 \) vs. WT; ANOVA and unpaired Student's t-test and linear regression analysis. Linear regression lines were included when statistical significance was achieved. N=10-13 per group.

Figure 2: Empagliflozin strongly lowered hyperglycemia in diabetic Akita/+ mice. Empagliflozin (300 mg/kg of diet) or repelleted diet (vehicle) were given to Akita/+ and WT mice. A) Empagliflozin lowered blood glucose levels in WT and much stronger in Akita/+ mice. B) Empagliflozin increased urinary glucose/creatinine ratios in WT but not in Akita/+ mice. C) For a given level of hyperglycemia, urinary glucose/creatinine ratios were greater in empagliflozin-treated compared with vehicle-treated Akita/+ mice. D-G) In WT, empagliflozin lowered body weight and adipocyte size and increased food and fluid intake. In Akita/+ mice, empagliflozin did not affect food intake and modestly increased body weight and adipocyte size, respectively. * \( P<0.05 \) vs. vehicle-treatment in same genotype; # \( P<0.05 \) vs. WT. ANOVA and unpaired Student's t-test. A), D) E), F) statistical analysis refers to last time point shown. N=10-13 per group for A-F); n=3-5 for G).
Figure 3: Empagliflozin further lowered plasma insulin in diabetic Akita/+ mice and prevented diabetes-induced increase in renal mRNA expression of phosphoenolpyruvate carboxykinase (PEPCK), systolic blood pressure, and hematocrit. Empagliflozin (300 mg/kg of diet) or repelleted diet (vehicle) were given to Akita/+ and WT mice. Depicted are results for plasma insulin in non-fasted mice (A), renal PEPCK mRNA (B), plasma aldosterone (C), systolic blood pressure (D), heart rate (E), and hematocrit (F). * P<0.05 vs. vehicle-treatment in same genotype; # P<0.05 vs. WT. ANOVA and unpaired Student's t-test. N=10-13 per group.

Figure 4: Empagliflozin prevented the diabetes-induced increase in GFR and attenuated the increase in kidney weight, glomerular size, and albuminuria in proportion to hyperglycemia. Empagliflozin (300 mg/kg of diet) or repelleted diet (vehicle) were given to Akita/+ and WT mice. Depicted are results for GFR (A), kidney weight (B), urinary albumin/creatinine ratios (C), and glomerular size (D). * P<0.05 vs. vehicle-treatment in same genotype; # P<0.05 vs. WT. ANOVA and unpaired Student's t-test and linear regression analysis. Linear regression lines were included when statistical significance was achieved. N=10-13 per group for A-C) and n=6-7 for D).

Figure 5: Empagliflozin attenuated diabetes-induced rise in renal expression of markers of kidney growth and inflammation. Empagliflozin (300 mg/kg of diet) or repelleted diet (vehicle) were given to Akita/+ and WT mice and kidneys harvested after 15 weeks. Depicted are results for renal nuclear expression of p27 and p21 and renal cytosolic expression of HO-1 (A), and renal mRNA expression of NFkB, CCL2, CD14, TIMP2, and IL6 (B). * P<0.05 vs. vehicle-treatment in same genotype; # P<0.05 vs. WT. ANOVA and unpaired Student's t-test and linear regression analysis. Linear regression lines were included when statistical significance was achieved. N=10-13 per group.
Figure 1
Figure 2
Figure 4
Figure 5

A) Renal nuclear protein expression (related to lamin B) for p27 and p21, as well as HO-1 expression, in WT and Akita/+ mice with and without empagliflozin treatment.

B) Renal mRNA expression (related to total mRNA) for NFkB, CCL2, CD14, TIMP2, and IL6 in WT and Akita/+ mice with and without empagliflozin treatment.

Statistical significance:
- * indicates p < 0.05
- # indicates p < 0.01
- significant correlation coefficients (r²) and p-values for each treatment group are provided.