Dapagliflozin Prevents Kidney Glycogen Accumulation and Improves Renal Proximal Tubule Cell Functions in a Mouse Model of Glycogen Storage Disease Type 1b

Mariavittoria D’Acierno, Roberta Resaz, Anna Iervolino, Rikke Nielsen, Donato Sardella, Sabrina Siccardi, Vincenzo Costanzo, Luciano D’Apolito, Yoko Suzumoto, Daniela Segalerba, Simonetta Astigiano, Alessandra F. Perna, Giovambattista Capasso, Alessandra Eva, and Francesco Trepiccione

1 Biogem Institute of Molecular Biology and Genetics, Ariano Irpino, Italy
2 Laboratory of Molecular Biology, IRCCS Istituto Giannina Gaslini, Genoa, Italy
3 Department of Translational Medical Sciences, University of Campania "L. Vanvitelli," Naples, Italy
4 Department of Biomedicine, Aarhus University, Aarhus, Denmark
5 IRCCS Ospedale Policlinico San Martino, Genoa, Italy

ABSTRACT

Background Mutations in SLC37A4, which encodes the intracellular glucose transporter G6PT, cause the rare glycogen storage disease type 1b (GSD1b). A long-term consequence of GSD1b is kidney failure, which requires KRT. The main protein markers of proximal tubule function, including NaPi2A, NHE3, SGLT2, GLUT2, and AQP1, are downregulated as part of the disease phenotype.

Methods We utilized an inducible mouse model of GSD1b, TM-G6PT−/−, to show that glycogen accumulation plays a crucial role in altering proximal tubule morphology and function. To limit glucose entry into proximal tubule cells and thus to prevent glycogen accumulation, we administered an SGLT2-inhibitor, dapagliflozin, to TM-G6PT−/− mice.

Results In proximal tubule cells, G6PT suppression stimulates the upregulation and activity of hexokinase-I, which increases availability of the reabsorbed glucose for intracellular metabolism. Dapagliflozin prevented glycogen accumulation and improved kidney morphology by promoting a metabolic switch from glycogen synthesis toward lysis and by restoring expression levels of the main proximal tubule functional markers.

Conclusion We provide proof of concept for the efficacy of dapagliflozin in preserving kidney function in GSD1b mice. Our findings could represent the basis for repurposing this drug to treat patients with GSD1b.

JASN 33: ***-***, 2022. doi: https://doi.org/10.1681/ASN.2021070935

Glycogen storage disease type 1b (GSD1b) is a rare autosomal recessive disease characterized by hypoglycemia, growth retardation, hepatomegaly, nephromegaly, lactic acidemia, hyperlipidemia, and hyperuricemia. Moreover, patients with GSD1b suffer from neutropenia and neutrophil dysfunction, which expose them to life-threatening infections and predispose them to development of autoimmune disorders, such as chronic inflammatory bowel disease.
No causative therapy is available for patients with GSD1b except for a dietary approach, to control glyceremia and metabolic dysfunctions, and a treatment with GM-CSF, to increase neutrophil counts and to reduce the frequency and severity of infections.3 Strict compliance to diet increased patients’ life expectancy, but long-term complications consequently emerge, such as the development of hepatic adenomas and kidney failure. Thus, new therapies are required.

GSD1b is caused by a deficiency of glucose-6-phosphate transporter (G6PT),4 which transports G6P from the cytoplasm to the endoplasmic reticulum where glucose-6-phosphatase (G6Pase) catalyzes the hydrolysis of G6P in glucose and phosphate.5 Disruption of the activity of the G6PT/G6Pase complex in liver and kidney affects glucose homoeostasis, impairing glucose release in the bloodstream, and leading to severe glycogen accumulation in these tissues.3

By CRE-lox recombination strategy, we generated a tamoxifen (TM) inducible G6PT-deficient mouse, TM-G6PTfl/fl, that recapitulates the hallmarks of human GSD1b.6 This inducible G6PT-knockout (KO) model was conceived to overcome the limitations related to early lethality of a constitutive G6PT-KO mouse,7 thus allowing the study of the long-term pathophysiology of the disease.

The working hypothesis we wanted to address was whether, by limiting new glucose entry into the proximal tubule (PT) cells, we could reduce glycogen accumulation and, thus, restore PT function. To address this hypothesis, we used dapagliflozin, a sodium-glucose cotransporter 2 (SGLT2) inhibitor. Gliflozins constitute a new class of drugs used for treating type 2 diabetes mellitus, because they reduce the renal threshold for glucose reabsorption and consequently facilitate glucose clearance. Empagliflozin, a SGLT2 inhibitor, improved neutrophils’ function in mice8 and patients with GSD1b,9 but the lack of data on its effect on the kidney (the target organ) imposed cautions on its usage.

Here, we provide proof of concept that treatment with dapagliflozin, by reducing intracellular glycogen accumulation, improves renal function in TM-G6PTfl/fl mice.

MATERIALS AND METHODS

TM-G6PTfl/fl Mice
All animals were maintained in a conventional animal facility in 12-hour light/dark cycles, fed ad libitum, and monitored for their lifespan. All experiments were reviewed and approved by the internal Review Board and authorized by the Italian Ministry of Health, accordingly with the current national and European regulations and guidelines for the care and use of laboratory animals (D.L. 26/2014; 86/609/EEC Directive). The 6- to 8-week-old G6PTloxlox;c-reER/wt mice,8 from now on named G6PT+/+, were injected intraperitoneally with 1 mg/10 g body weight per day of TM for 5 consecutive days to obtain TM-G6PTfl/fl mice. Genotyping and confirmation of G6PT excision was performed by PCR analysis using genomic DNA isolated from tail tip and renal tissue. The following primers were used: 1S: 5'-GAGATCGCCTGAGGACAGGA; 2AS: 5'-AACTTGCTGATGGCGTAGG; CRE-S: 5'-TTTGGGCCCAGCTAAACATGC; CRE-AS: 5'-TTACGTATATCCTGGCAGGC.

Dapagliflozin Treatment
Dapagliflozin has been extensively studied in rodents.10 Dapagliflozin was obtained from AstraZeneca (NCR-16-12171) and was administered to adult mice in drinking water for 30 days, starting 3 days before TM induction. Dapagliflozin concentration in drinking water was calculated according to water intake to guarantee an equal dose of 1.2 mg/kg per day to all mice.

Urine and Blood Analysis
Mice were housed individually in metabolic cages. After 4 days of adjustment, physiologic parameters were evaluated as previously described.11 Urinary protein concentration was evaluated by Bradford assay (BioRad Protein Assay; BioRad). Urinary Clara cell protein (CC16) concentration was evaluated by BioRad. Urinary albumin was evaluated by mouse albumin ELISA (Santa Cruz). Urine and blood samples were collected as previously described.12

Histology and Immunohistochemistry
Mice were anesthetized with isoflurane and euthanized. The left kidney was harvested for protein and mRNA evaluation, whereas the right kidney was fixed in 10% buffered formalin for 24 hours and paraffin embedded. For histologic analysis, sections 4-μm thick were randomly collected and stained with hematoxylin and eosin (Sigma-Aldrich). Serial 2-μm-thick sections were treated with or without amylase solution (04-130803; Bio-Optica) before periodic acid–Schiff staining.

Immunofluorescence and immunohistochemistry were carried out according to a standard protocol as detailed previously.13 For immunofluorescence, sections were treated overnight with anti-SGLT2 (1:100, 85626; Abcam). Anti-rabbit IgG was used to detect SGLT2. Immunofluorescence was detected using a Bio-Rad FluorCam. Sections were counterstained with anti-SGLT2 (1:100, 85626; Abcam). Anti-rabbit IgG was used to detect SGLT2. Immunofluorescence was detected using a Bio-Rad FluorCam. Sections were counterstained with anti-SGLT2 (1:100, 85626; Abcam). Anti-rabbit IgG was used to detect SGLT2. Immunofluorescence was detected using a Bio-Rad FluorCam.
conjugated Alexa Fluor 488 (1:800, A-11094; Invitrogen) was used as secondary antibody for 1 hour at room temperature.

For double labeling, sections were incubated with anti-NaPi2A (1:300, NPT27-A; Alpha Diagnostic) and Lotus Tetragonolobus Lectin, Fluorescein (1:400, FL-1321–2; Vector Lab). Goat anti-rabbit Cyanine3-conjugated (1:600; A-10520; Invitrogen) was used as secondary antibody for 1 hour at room temperature.

Stained sections were mounted with fluorescent mounting medium (Dako). Zeiss spinning disk Axio Observer Z1 confocal microscope (Zeiss) was used for image acquisition. For immunohistochemistry, sections were incubated with anti-NaPi2A antibody (1:200, NPT27-A; Alpha Diagnostic), followed by an anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (1:1000, 2016–09; Dako). Coverslips were mounted with Eukitt (09–00250; Bio-Optica).

Electron Microscopy

Male mice (three control and four TM-G6PT−/−) were perfused and fixed with Karnovsky solution (2% paraformaldehyde and 2.5% glutaraldehyde in 0.01 M pH 7.2 sodium phosphate buffer). Tissue slices 1 mm thick were further fixed overnight by immersion in the same fixative, before treatment with 1% OsO4 for 2 hours, and processed according to Storm et al.14

In Vitro Experiments

TKPTS, a mouse PT cell line, was a gift from Dr. Ivonne Loeffler.13 TKPTS cells were maintained in DMEM low glucose medium (5.5 mM d-glucose) (AL006; Microgen) containing FBS (10%), insulin (3.4 mg/500 ml medium), and penicillin/streptomycin (0.5%). For high glucose medium, DMEM high-glucose 24.9 mM (D1145; Sigma) was used. TKPTS cells were transfected using Lipofectamine 3000 reagent (Invitrogen). Cells (3×105), suspended in low glucose serum-free medium, were transfected with siRNA A, B, or C (100 nM). To increase transfection efficiency, two consecutive transfections were performed 48 hours apart. Cells were harvested after additional 72 hours from the second transfection. The target sequence of the siRNAs (ON-Target Plus Set; Dharmacon) was: siRNA-A: CCUC-GUGGUCUUCGGAUA, siRNA-B: GCACCAAGAUGG GCCGAGU, siRNA-C: UGUACUAAUUCAACCGCAA and siCONTROL was non-Targeting siRNA (Dharmacon).

Hexokinase-I Activity Assay

Hexokinase (HK) activity was measured in TKPTS cells using an HK-1 Colorimetric Assay Kit (K789; Biovision). The assay for HK is on the basis of spectrophotometric detection of NADH produced from HK activity and was performed according to the manufacturer's instructions. Protein concentrations were determined using Bradford reagent (Bio-Rad). An equal amount of protein per sample (20 μg) was loaded.

Computer-Assisted Image Analysis

Images from hematoxylin and eosin–stained sections were acquired on Slide Scanner (Nanoloozer 2.0RS; Hamamatsu) with a pixel size of 226 nm/pixel. Images were fused together to compose the entire cortex and analyzed using the Fiji software,16 as previously described.17

A region of interest (ROI) in the external cortex was manually traced over the entire perimeter, moving from the renal capsule up to 300 pixels inside the parenchyma (545 μm). The area occupied by Bowman’s capsules and glomeruli was manually segmented out and excluded by the computational analysis. The parenchyma was segmented using the Color Threshold option. The setting was manually adjusted to compensate for sample variance (excluding areas of sections damage), and, finally, a ROI of the parenchyma of interest was drawn. In this way parenchyma containing large vacuoles was less represented in the counting. Finally, the area of the segmented parenchyma over the total area of interest was quantified with the ROI manager option.

Immunoblotting

Whole kidney was used for sample preparation. Immunoblotting was carried out as previously described.11 Briefly, kidneys were homogenized with a TissueLyser (Qiagen) in lysis buffer (Sucrose 0.3 M, TrisHepes 100 mM pH 7.4, pepstatin A) supplemented with protease inhibitor cocktail (Complete Protease Inhibitor Cocktail, Sc-29130; Santa Cruz Biotechnology) and phosphatase inhibitors (PhosSTOP, 4906837001; Roche). The membrane-enriched fractions were prepared as previously described.18 Equal amounts of proteins were loaded into 8%, 10%, or 12.5% SDS-PAGE and transferred to nitrocellulose membranes (106000; Amersham Protran). Samples from cell lysates were prepared as previously described.19

The following primary antibodies were used: anti-GLUT2 (1:1000; kindly provided by Prof. Thorens),20 anti-SGT2L (1:2000, ab85626; Abcam), anti-NaPi2A (1:1000, NPT27-A; Alpha Diagnostic), anti-AQP1 (1:500, sc-25287; Santa Cruz Biotechnology), anti-AQP2 (1:500, sc-9882; Santa Cruz Biotechnology), anti-GYS (1:1000, 3886; Cell Signaling), anti-pS641 GYS (1:500, 3891; Cell Signaling), anti-PYGL (1:1000, E-AB-11510; Elab Science), anti-pS15 PYGL (1:500, ab227043; Abcam), anti-NHE3 (1:500, AB3085; Millipore), anti-HK1 (1:1000, 2024; Cell Signaling Technology), and anti–β–ACTIN (1:40000, 76793; Sigma). Donkey anti-rabbit HRP (1:2000, NA934–1ML; GE Healthcare Lifescience), rabbit anti-goat HRP (1:1000, 172–1034; Bio-Rad), and sheep anti-mouse IgG (1:2000, NA931; GE Healthcare Lifescience) were used as secondary antibodies. Protein signals were visualized with ECL Western Blotting Detection Reagent (GE Healthcare) using a HRP-conjugated goat antimouse antibody (Thermo Fisher Scientific) and normalized to loading control. Densitometry was
performed using ImageJ (National Institutes of Health) software.

Glycogen Quantification in Tissues
Glycogen extraction and quantification in renal cortex samples was performed as previously described. Briefly, glycogen precipitation was induced with 30% KOH and then with a solution of 0.25M Na2SO4 and ethanol. Glycogen was digested by amyloglucosidase (A7095; Sigma) (6 mg/ml in 0.2 M sodium acetate, pH 4.8) for 3 hours at 37°C into glucose and then measured with the Glucose Hexokinase kit (Greiner Diagnostic), according to the manufacturer’s instructions.

RNA Extraction and Quantitative PCR
Total RNA was purified from tissues using TRIzol reagent (BIO-38033; BIOLINE). Then 1 μg of RNA was reverse transcribed by Quantitect Reverse Transcription Kit (205311; Qiagen) according to the manufacturer’s instructions. The quantitative RT-PCR was performed on Quantity Studio 7 (Applied Biosystems), using Fast SYBR Green Master Mix (4385612; Applied Biosystems) according to the manufacturer’s instructions. Expression data were normalized on the values obtained in parallel for the reference gene (GAPDH).

The following primers were used: G6PT Fw: CCTATCTCTGGGTGCTGTCC; G6PT Rev: TCGAGGGCACTGATGGCTGGGTGCTGTCC; GAPDH Rev: GGGCCCTCAAGGACGCGG.

RESULTS

6

BASIC RESEARCH www.jasn.org

The results are reported as mean±SEM. Comparison between two groups was made by unpaired t test or one-way ANOVA, as indicated in the figure legend. A value of P<0.05 was considered statistically significant.

RESULTS

TM-G6PT−/− Mice Present with Proximal and Distal Nephron Dysfunction
G6PT expression was evaluated in all dissected kidney zones in 6- to 8-week old TM-G6PT−/− and their littermate G6PT+/+ control mice. As shown in Figure 1A, G6PT mRNA is mostly expressed in the cortex/outer stripe of outer medulla (CTX/OSOM), whereas expression in the inner stripe of outer medulla (ISOM) and inner medulla (IM) is lower. TM administration resulted in the suppression of G6PT mRNA expression by 90% (Figure 1B).

Then, 1 month after TM administration, TM-G6PT−/− mice presented with a lower body weight compared with control mice, whereas no difference was observed in serum BUN, urinary protein/creatinine ratio (Table 1), and urinary albumin/creatinine ratio (Figure 1C), suggesting no major glomerular dysfunction, as expected in the early stages of the disease. However, TM-G6PT−/− mice showed glycosuria (Figure 1C), phosphaturia, and low-molecular-weight proteinuria (Figure 1D). This was associated with a severe morphologic alteration of the PT cells, which appeared swollen with the nuclei mislocated at the apical domain of the cell, assuming the typical Armanni–Ebstein phenotype (Figure 1E). All these elements together suggest a Fanconi-like phenotype, as in patients with GSD1b.

As shown before, the kidney damage is progressive as proven by an overt albuminuria manifested from 5 months of age. To investigate the functional mechanisms behind this phenotype, we analyzed the abundance of NaPi2A, the main phosphate transporter, and NHE3, the main sodium transporter expressed along the PT. NaPi2A and NHE3 were severely downregulated in TM-G6PT−/− compared with control mice (Figure 1F). In addition, residual NaPi2A expression in TM-G6PT−/− mice was limited to the intracellular domain (Figure 1E).

Ablation of G6PT Alters the Main Glucose Transport Machinery in PTs
PT cells are the main site of the nephron devoted to glucose metabolism and glucose release in the bloodstream. To dissect the overall glucose metabolism along PT cells, we investigated the glycogen content in the CTX/OSOM in relation to expression levels of the PT main glucose transporters, SGLT2 and GLUT2, located on the apical and basolateral membrane of PT cells, respectively. One month after TM administration, TM-G6PT−/− mice presented with almost six-fold increase in glycogen abundance in CTX/OSOM, compared with control mice (Figure 2A). Periodic acid–Schiff staining of kidney sections revealed an eosinophilic signal located in the enlarged PT cell cytoplasm. This signal disappeared by pretreatment with amylase, a glycogen lytic enzyme, suggesting that the cytosolic swelling is, at least in part, due to intracellular glycogen trapping (Supplemental Figure 1). Electron microscopy examination of renal cortex indicated the presence of cytosolic sparse granules assuming the aspect of beta-particles specifically in TM-G6PT−/− mice (Figure 2B). This material contributes to cell swelling and alteration of intracellular ultrastructure, by promoting apical repositioning of the nuclei. These alterations were associated with a significant downregulation of SGLT2 and GLUT2 transporters, the glucose-reabsorbing machinery of the PT cells (Figure 2C).
Figure 1. Renal phenotype in TM-G6PT−/− mice. (A) Quantitative RT-PCR (RT-qPCR) expression of G6PT mRNA along the renal zone in wild-type C57BL/6J mice. Data are expressed as percentage of CTX/OSOM expression (n=3). ISOM (n=4) and IM (n=4). Statistics was performed by one-way ANOVA followed by Bonferroni post-test. (B) RT-qPCR expression of G6PT mRNA in control (ctr, gray dots) or TM-G6PT−/− mice (black dots) along the three renal zones (CTX/OSOM n=3+4; ISOM n=4+3; IM n=4+3, unpaired t test). (C) Urinary excretion of glucose and albumin over creatinine (ACR) (for glucose/creatinine n=4+8 and for ACR n=3+4; unpaired t test). (D) Urinary phosphate (Pi)/creatinine excretion and CC16/creatinine, as marker of low-molecular-weight proteinuria (Pi/creatinine n=5+14 and CC16/creatinine n=5+5; unpaired t test). I Representative images from renal cortex of Ctr and TM-G6PT−/− mice stained with anti-NaPi2A antibody (upper panels, scale bar: 50 μm; lower panels, scale bar: 20 μm). (F) Immunoblotting from membrane fractions of whole kidney samples from Ctr and TM-G6PT−/− mice reveals a downregulation of NaPi2A, NHE3, AQP1, and AQP2 in TM-G6PT−/− mice (n=5+5, unpaired t test). All data are expressed as mean±SEM; ***P<0.001, **P<0.01, *P<0.05.
Immunofluorescence staining of renal cortical sections confirmed a severe reduction, but not disappearance of the SGLT2 transporter in TM-G6PT^{−/−} mice (Figure 2D).

**G6PT Suppression in PT Cells Upregulates HK1 Expression and Activity**

Under physiologic conditions, the glucose reabsorbed by PT cells is transcellularly transported and released into peritubular capillaries.\(^{25}\) This mainly occurs because the metabolism of PT cells does not rely on glucose oxidation, but mainly on fatty acids and amino acids. Here, to check whether HK1, the key enzyme of glucose metabolism, is regulated by G6PT, we acutely suppressed G6PT expression in a mouse PT cell line, namely TKPTS.

In vitro, HK1 expression is sensitive to glucose concentration of the cultured media (Supplemental Figure 2), decreasing at glucose concentration similar to PT lumen (5.5 mM). Here, in low glucose cultured cells, we silenced G6PT mRNA transcription by transfecting three different siRNAs A, B, and C. Table 1.

### Table 1. Physiologic parameters at baseline

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ctr</th>
<th>n</th>
<th>TM-G6PT^{−/−}</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>19.0±1.1</td>
<td>(6)</td>
<td>15.32±0.5</td>
<td>(6)*</td>
</tr>
<tr>
<td>Water intake, μl/h per g BW</td>
<td>8.7±1.3</td>
<td>(10)</td>
<td>12.34±1.1</td>
<td>(11)*</td>
</tr>
<tr>
<td>Food intake, mg/h per g BW</td>
<td>7.4±0.6</td>
<td>(8)</td>
<td>6.795±1.3</td>
<td>(12)</td>
</tr>
<tr>
<td>Osmolality, mOsm/kg H₂O</td>
<td>1532±188.7</td>
<td>(6)</td>
<td>870.9±153.9</td>
<td>(10)*</td>
</tr>
<tr>
<td>Urine volume, μl/h per g BW</td>
<td>2.01±0.3</td>
<td>(10)</td>
<td>10.8±1.4</td>
<td>(14)***</td>
</tr>
<tr>
<td>UPCR mg/mg</td>
<td>5.0±0.7</td>
<td>(5)</td>
<td>4.80±0.9</td>
<td>(9)</td>
</tr>
<tr>
<td>BUN, mg/dl</td>
<td>10.62±0.5</td>
<td>(5)</td>
<td>9.05±1.2</td>
<td>(5)</td>
</tr>
</tbody>
</table>

Physiologic parameters from 3-month-old mice. The number of replicates is reported in brackets. For all, the statistics were calculated with unpaired t test. All data are expressed as mean±SEM; ***P < 0.001, **P < 0.01, *P < 0.05. BW, body weight; UPCR, urinary protein/creatinine ratio.

![Figure 2. PTs of TM-G6PT^{−/−} mice have large glycogen deposit and alteration in glucose transporters.](image)

Figure 2. PTs of TM-G6PT^{−/−} mice have large glycogen deposit and alteration in glucose transporters. (A) Cortical glycogen content is about six-fold larger in TM-G6PT^{−/−} (black dots) compared with control mice (Ctr, gray dots) (n=4+5, unpaired t test). (B) Representative electron microscopy images from renal cortex of control and TM-G6PT^{−/−} mice at low (upper panels, scale bar: 10 μm) and high (lower panels, scale bar: 1 μm) magnification. (C) Immunoblotting of membrane fraction of whole renal samples from control and TM-G6PT^{−/−} mice reveals a severe downregulation of both luminal glucose transporter, SGLT2 and basolateral glucose transporter, GLUT2 (n=5+5, unpaired t test). (D) Representative pictures from renal cortex of Ctr and TM-G6PT^{−/−} mice stained with anti-SGLT2 antibody (1:100 dilution; scale bar: 20 μm). All data are expressed as mean±SEM; **P<0.01, *P<0.05.
C. After 3 days, G6PT mRNA was significantly downregulated by 50%–70% compared with siRNA control transfected cells (Figure 3A). This was associated with a significant upregulation of HK1 mRNA (Figure 3B) and protein abundance (Figure 3C). In addition, HK1 activity resulted increased in siRNA-treated cells (Figure 3D). All together, these results suggest that acute suppression of G6PT leads to an increase in the abundance and the activity of HK1 and could represent the first step for glycogen synthesis.

Dapagliflozin Prevents Glycogen Accumulation in TM-G6PT<sup>−/−</sup> Mice Facilitating Glycogen Phosphorylase Activity

To evaluate whether reducing the glucose uptake along the PT cells would prevent the intracellular glycogen accumulation, for 30 days we administered the selective inhibitor of SGLT2 transporter, dapagliflozin, to TM-G6PT<sup>−/−</sup> mice, starting 2 days before TM-induced G6PT ablation (Figure 4A). Weekly dipstick evaluation of glycosuria was used to control that all of the mice received dapagliflozin at effective glycosuria-induced dose. No major alteration of the physiologic parameters was induced by dapagliflozin, neither elevation in serum BUN compared with nontreated mice (Table 2). Figure 4B shows that dapagliflozin significantly prevented cortical glycogen accumulation in TM-G6PT<sup>−/−</sup> mice, keeping glycogen content at the level of TM-untreated mice (Figure 2A). To strengthen these results, we analyzed the expression level of two crucial kidney enzymatic checkpoints of glycogen metabolism, namely glycogen synthase, GYS, and glycogen phosphorylase, PYGL. In TM-G6PT<sup>−/−</sup> mice both p-S641 GYS, the inhibitory form of GYS, and p-S15 PYGL, the active form of PYGL were severely downregulated compared with the control mice. However, a small residual expression of GYS can be detected, suggesting active glycogen formation. Dapagliflozin treatment blocked the synthesis of new glycogen by promoting the expression of the p-S641 inhibitory form of GYS. At the same time, dapagliflozin promoted glycogenolysis by stimulating PYGL and its active phosphorylated isoform, p-S15 PYGL (Figure 4C). These results suggested that dapagliflozin can directly or indirectly modulate glycogen metabolism in PT cells.

![Figure 3. In vitro silencing of G6PT leads to upregulation and increase activity of HK-1.](image-url)

(A) RT-qPCR expression of G6PT mRNA in TKPTS cells transfected with control siRNA (Ctr; gray dots) or three different siRNA interfering with the G6PT mRNA, namely A, B, and C. (A) RT-qPCR expression of G6PT mRNA and (B) HK-I (mRNA G6PT/Gapdh n=9+8+8+9 and mRNA HK1/Gapdh n=9+9+9+8). (C) Immunoblotting of cell lysate probed with an anti-HK-I antibody (n=3 per group). (D) HK-I activity from cell lysate (n=3 per group). For all of the panels statistics was performed with one-way ANOVA followed by Bonferroni post-test. All data are expressed as mean±SEM; ***P<0.001, **P<0.01, *P<0.05.
Figure 4. Dapagliflozin prevents glycogen accumulation by facilitating its catabolism. (A) Protocol for dapagliflozin and TM administration. (B) Dapagliflozin prevents glycogen accumulation in renal cortex of TM-G6PT−/− mice (n=4+6; unpaired t test). (C) Immunoblotting from total fraction of whole renal samples from control (Ctr) and TM-G6PT−/− mice treated and untreated with dapagliflozin (n=3+4+4; one-way ANOVA). Dapagliflozin inhibits GYS by favoring its inactive S641-phosphorylated form and stimulates the activity of PYGL by favoring the expression of its active S15-phosphorylated form. All data are expressed as mean±SEM; ***P<0.001, **P<0.01, *P<0.05. The single asterisk (*) is for comparison Ctr versus TM-G6PT−/− mice; the pound symbol (#) is for comparison versus untreated TM-G6PT−/− mice.

Dapagliflozin Treatment Ameliorates the Morphology and the Expression Level of PT-Specific Transporters

Along with reduction of cortical glycogen, we observed a marked improvement in kidney morphology. As evaluated by a computer-based analysis of renal histology, in dapagliflozin-treated mice the amount of nonvacuolized parenchyma significantly increased (Figure 5, A and B). Along with the changes in morphology, dapagliflozin reduced urinary phosphate excretion, but not low-molecular-weight proteinuria, compared with nontreated TM-G6PT−/− mice (Figure 5C). Concomitantly, we observed an increased expression in the membrane fraction of the main functional markers of PT, including NaPi2A, NHE3, SGLT2, GLUT2, and AQP1 (Figure 6A). In addition, after dapagliflozin treatment, the sorting to the apical membrane of NaPi2A significantly improved and likely is responsible of a reduction in phosphaturia (Figure 6B). As an effect of dapagliflozin, SGLT2 expression to the apical membrane domain increased as well (Figure 6C). Finally, we observed an increase of AQP2 expression (Figure 6A) along with an amelioration of the urine concentrating ability (lower urinary volume and increased in urinary osmolality) (Table 2), suggesting dapagliflozin effect was not limited to the PT, but somehow influenced the function of distal tubule as well. All together, these results indicate dapagliflozin is effective in restoring PT cells’ functions and suggest it may represent a new therapeutic drug for kidney disease in GSD1b.

DISCUSSION

Here, we report that ablation of G6PT in the kidney affects mainly the PT cells and, to a lesser extent, the principal cells of the collecting duct. We show data for a new mechanism in the pathogenesis of the Fanconi-like phenotype occurring in GSD1b that involves cellular metabolism. In addition, we provide proof of concept that dapagliflozin, an SGLT2 inhibitor, is effective in preventing glycogen accumulation and in recovering kidney morphology and function.

In TKPTS cells, a mouse PT cell line, G6PT suppression triggers the expression and the activity of the HK1. This is a major event in PT metabolism, because it potentially makes available a huge amount of glucose for the intracellular metabolism and it is likely to be the crucial event for the glycogen accumulation that we observe in TM-G6PT−/− mice and patients with GSD1b.

Table 2. Physiologic parameters during dapagliflozin treatment

<table>
<thead>
<tr>
<th>TM-G6PT−/−</th>
<th>− Dapa</th>
<th>n</th>
<th>+ Dapa</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>14.59±1.14</td>
<td>(6)</td>
<td>16.43±1.15</td>
<td>(8)*</td>
</tr>
<tr>
<td>Water intake, µl/h per g BW</td>
<td>11.71±1.3</td>
<td>(5)</td>
<td>14.26±0.8</td>
<td>(8)</td>
</tr>
<tr>
<td>Food intake, mg/h per g BW</td>
<td>7.49±0.6</td>
<td>(5)</td>
<td>10.59±0.4</td>
<td>(8)**</td>
</tr>
<tr>
<td>Osmolality, mOsm/kg H2O</td>
<td>510.6±112.2</td>
<td>(5)</td>
<td>1126±110.4</td>
<td>(5)**</td>
</tr>
<tr>
<td>Urine volume, µl/h per g BW</td>
<td>9.67±1.82</td>
<td>(5)</td>
<td>6.51±1.0</td>
<td>(6)**</td>
</tr>
<tr>
<td>UPCR mg/mg</td>
<td>5.3±0.6</td>
<td>(5)</td>
<td>5.3±0.6</td>
<td>(6)</td>
</tr>
<tr>
<td>BUN, mg/dl</td>
<td>9.41±0.8</td>
<td>(5)</td>
<td>10.29±0.6</td>
<td>(5)</td>
</tr>
</tbody>
</table>

*Physiologic parameters after 1 month of dapagliflozin treatment. The number of replicates is reported in brackets. For all, the statistics were performed with unpaired t test. All data are expressed as mean±SEM; **P<0.01 and *P<0.05. BW, body weight; UPCR, urinary protein/creatinine ratio.
HK1 is usually not expressed in PT cells, and this guarantees a continuous reabsorption of glucose from the apical membrane into the bloodstream; indeed, it precludes its utilization for intracellular metabolism and likely glucotoxicity. Neoglucogenesis and glycogen synthesis in the PT cells derive mainly from the catabolism of tricarboxylic acids. However, we showed that G6PT suppression triggers the expression and the activity of the HK1. This could be the main trigger for an uncontrolled glycogen synthesis and aberrant accumulation of glycogen that we observed in our mice model.

As a result of glycogen accumulation, severe cellular swelling, and ultrastructure reorganization lead to overall kidney enlargement. In this scenario, as determinants of the Fanconi-like phenotype, crucial proteins for PT function, namely NHE3, SGLT2, GLUT2, NaPi2A, and AQP1, are downregulated. This latter event could be due to a feedback mechanism to further limit sodium, glucose, phosphate, and water uptake in the attempt to limit further PT cells swelling. However, we cannot exclude that the cytoskeleton alteration secondary to cell swelling affect the sorting to the plasma membrane of these proteins.

Dapagliflozin treatment seems to effect glycogen metabolism by stimulating the expression of p-S15 PYGL, the active phosphorylated isoform of PYGL. This enzyme is mainly involved in glycogen catabolism not only in the kidney, but also the liver. At the same time, dapagliflozin treatment arrests new glycogen synthesis by promoting the expression of p-Ser 641 GYS, the inactive isoform of GYS. This enzymatic shift from GYS toward PYGL activation facilitates glycogen catabolism and, likely, contributes to PT cell function amelioration, causing an energetic switch from a glycogen-accumulation to a glycogen-degradation state.

As additional mechanism, the SGLT2 inhibitors play a crucial role on renal reabsorption of the 1,5-anhydroglucitol-6-phosphate (1,5AG6P), a noncanonical metabolite that results from the phosphorylation of 1,5AG. 1,5AG6P is responsible of neutropenia in G6PT and G6Pase-β KO mice. Indeed, 1,5AG6P accumulation in neutrophil cytosol inhibits glucose phosphorylation by hexokinases.
Figure 6. Dapagliflozin ameliorates the expression of proteins markers of PT function. (A) Immunoblotting from membrane fraction of whole kidney samples from Ctr or TM-G6PT−/− mice treated with or without dapagliflozin (n=3+4+4 respectively; one-way ANOVA). Dapagliflozin improves the expression of functional markers of PT such as NaPi2A, NHE3, SGLT2, GLUT2, and AQP1, but also of distal tubule as AQP2. Representative pictures of renal cortex from Ctr or TM-G6PT−/− mice treated with or without Dapa stained with (B) anti-NaPi2A (red) (1:300 dilution) and Lotus Tetragonolobus Lectin (LTL) (green) (1:400 dilution); scale bar: 10 μm; white asterisk indicates intracellular retention of NaPi2A or (C) anti-SGLT2 antibody (green) (1:100 dilution). All data are expressed as mean±SEM; ***P<0.001, **P<0.01, *P<0.05. The single asterisk (*) is for comparison Ctr versus TM-G6PT−/− mice; the pound symbol (#) is for comparison versus untreated TM-G6PT−/− mice.
and, thus, glycolysis. The glycosuria induced by SGLT2 inhibition is expected to compete with 1,5AG at the site of the SGLT4 transporter and favor its urinary excretion. This, in turn, reduces the systemic level of 1,5AG and is beneficial to neutrophils’ function as demonstrated in the experimental models and in a small cohort of patients with GSD1b. Because there is no evidence that SGLT2 can transport 1,5AG and the activity of HK-1 in PT cells is negligible in basal conditions, a pathogenetic mechanism that interferes with glycolysis seems unlikely to be crucial for the PT cells.

Renal involvement in GSD1b shares common features with diabetic kidney disease (DKD). Indeed, patients with GSD1b present with hyperfiltration in the early disease state and, progressively, develop chronic kidney disease with proteinuria and glomerulosclerosis. In addition, large intracellular glycogen accumulation, leading to Armanni-Ebstein lesions in PT cells, is reported in patients with DKD and animal models as well, pointing out that glycogen metabolism in PT may be crucial in the pathogenesis of both diseases. Similar histologic findings were reported in a kidney-specific model of GSD1a lacking the functional partner gene of G6PT, namely G6P-ase in PT cells.

Because the molecular mechanisms driving the SGLT2 inhibitor–dependent kidney protection in DKD are not yet completely elucidated, our results could inspire new mechanisms of disease to explore in experimental models of DKD.

Finally, TM-G6PT−/− mice present with hypoosmotic polyuria that involves a general dysfunction in renal water handling, characterized by a downregulation in both AQP1 and AQP2 expression. G6PT ablation could be directly involved in water homeostasis, because the expression level of G6PT in IM is reduced by 90% in TM-G6PT−/− mice. However, we cannot exclude that AQP2 downregulation is a result of PT dysfunction. Indeed, AQP2 expression and urine concentration ability improved after treatment with dapagliflozin in parallel with PT function rescuing.

In conclusion, for the first time we provide evidence that dapagliflozin is effective in reducing glycogen accumulation in PT cells and thus, by ameliorating the expression profile of the main PT functional markers, in improving renal function in a mouse model of GSD1b. These results, together with our recent evidence on the amelioration of neutrophils’ dysfunction, could represent the basis for a substantial repositioning of the SGLT2 inhibitors, dapagliflozin, for the treatment of patients GSD1b.

M. D’Acierno reports employment with Biogem scarl. All remaining authors have nothing to disclose.

FUNDING

This work was supported by Telethon Foundation (GSP20002_PasGlic1b008), A. Eva was supported by grants from Associazione Italiana Glicogenosi and Italian Ministry of Health (Ricerca Corrente).

ACKNOWLEDGMENTS

The authors thank AstraZeneca for providing the dapagliflozin. The authors also thank the Associazione Italiana Glicogenosi, whose continuous support and encouragement has been essential for the execution of this work.

AUTHOR CONTRIBUTION

V. Costanzo, M. D’Acierno, A. Iervolino, R. Resaz, D. Sardella, S. Siccardi, and F. Trepiccione were responsible for the data curation; V. Costanzo, D. Sardella, and F. Trepiccione were responsible for the formal analysis; F. Trepiccione was responsible for the funding acquisition; L. D’Apolito, M. D’Acierno, A. Iervolino, R. Nielsen, R. Resaz, D. Sardella, S. Siccardi, and Y. Suzumoto were responsible for the investigation; D. Segalerba and F. Trepiccione were responsible for the methodology; A. Iervolino was responsible for the project administration; V. Costanzo was responsible for the software; S. Astigiano, G. Capasso, A. Eva, A. Perna, D. Segalerba, and F. Trepiccione provided supervision; G. Capasso, V. Costanzo, M. D’Acierno, R. Resaz, S. Siccardi, and F. Trepiccione were responsible for the validation; S. Astigiano, G. Capasso, A. Eva, A. Perna, and F. Trepiccione were responsible for the visualization; and M. D’Acierno, A. Eva, and F. Trepiccione wrote the original draft.

DATA SHARING STATEMENT

All data used in this study are available in this article.

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2021070935/-/DC Supplemental.

Supplemental Figure 1. Periodic acid–Schiff positivity and amylase sensitivity reveal glycogen deposition in kidney from TM-G6PT−/− mice.

Supplemental Figure 2. HK1 expression in TKPTS cells cultured at different glucose and insulin concentration.

REFERENCES