Knockout of Na-glucose transporter SGLT2 attenuates hyperglycemia and glomerular hyperfiltration but not kidney growth or injury in diabetes mellitus

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Vallon V, Rose M, Gerasimova M, Satriano J, Platt KA, Koepsell H, Cunard R, Sharma K, Thomson SC, Rieg T. Knockout of Na-glucose transporter SGLT2 attenuates hyperglycemia and glomerular hyperfiltration but not kidney growth or injury in diabetes mellitus. Am J Physiol Renal Physiol 304: F156–F167, 2013. First published November 14, 2012; doi:10.1152/ajprenal.00409.2012.—The Na-glucose cotransporter SGLT2 mediates high-capacity glucose uptake in the early proximal tubule and SGLT2 inhibitors are developed as new antidiabetic drugs. We used gene-targeted Sglt2 knockout (Sglt2−/−) mice to elucidate the contribution of SGLT2 to blood glucose control, glomerular hyperfiltration, kidney growth, and markers of renal growth and injury at 5 wk and 4.5 mo after induction of low-dose streptozotocin (STZ) diabetes. The absence of SGLT2 did not affect renal mRNA expression of glucose transporters SGLT1, NaGLT1, GLUT1, or GLUT2 in response to STZ. Application of STZ not affect renal mRNA expression of glucose transporters SGLT1, GLUT1, or GLUT2 in response to STZ. The absence of SGLT2 did not affect renal mRNA expression of glucose transporters SGLT1, NaGLT1, GLUT1, or GLUT2 in response to STZ. Application of STZ increased blood glucose levels to a lesser extent in Sglt2−/− vs. wild-type (WT) mice (∼300 vs. 470 mg/dl) but increased glucosuria and food and fluid intake to similar levels in both genotypes. Lack of SGLT2 prevented STZ-induced glomerular hyperfiltration but not the increase in kidney weight. Knockout of SGLT2 attenuated the STZ-induced renal accumulation of p62/sequestosome, an indicator of impaired autophagy, but did not attenuate the rise in renal expression of markers of kidney growth (p27 and proliferating cell nuclear antigen), oxidative stress (NADPH oxidases 2 and 4 and heme oxygenase-1), inflammation (interleukin-6 and monocyte chemoattractant protein-1), fibrosis (fibronectin and Sirius red-sensitive tubulointerstitial collagen accumulation), or injury (renal/urinary neutrophil gelatinase-associated lipocalin). SGLT2 deficiency did not induce ascending urinary tract infection in nondiabetic or diabetic mice. The results indicate that SGLT2 is a determinant of hyperglycemia and glomerular hyperfiltration in STZ-induced diabetes mellitus but is not critical for the induction of renal growth and markers of renal injury, inflammation, and fibrosis.

diabetes; diabetic nephropathy; glomerular hyperfiltration; proximal tubule; renal growth; fibrosis; inflammation

The bulk of tubular glucose uptake across the apical membrane of the kidney occurs in the early proximal tubule and is mediated by the high-capacity SGLT2 whereas the low-capacity SGLT1 (SLC5A1) is thought to “cleanup” most of the remaining luminal glucose in further distal parts of the proximal tubule (for review see Refs. 28, 47). In accordance, recent studies directly localized SGLT2 and SGLT1 protein expression in the brush border membrane of the early and later sections of the proximal tubule, respectively (1, 41). Micropuncture studies in knockoumice directly showed that SGLT2 is responsible for all glucose reabsorption in the early proximal tubule and, overall, is the major pathway of glucose reabsorption in the kidney (41). In comparison, mice lacking SGLT1 have only a minor reduction in fractional renal glucose reabsorption (12). In accordance with these studies in mice, individuals with gene mutations in SGLT1 have little or no glucosuria, whereas those with mutations in SGLT2 have persistent renal glucosuria (28).

Thus the quantitative contribution of SGLT2 to renal glucose reabsorption has been well established; however, little is known about the consequences of its inhibition on early changes in the diabetic kidney. The kidney in general and the proximal tubules in particular grow large from the onset of diabetes (8, 26, 33), and diabetic kidney growth has been linked to the development of nephropathy (3, 5, 20, 39, 48). Whether glucose uptake via SGLT2 is a critical stimulus for diabetic kidney growth or the development of kidney injury in diabetes is not known. Moreover, tubular growth (35) and phlorizin-sensitive SGLTs (42) increase proximal tubular NaCl and fluid reabsorption in the early diabetic kidney. Increased proximal reabsorption contributes to diabetic glomerular hyperfiltration by lowering the Na-CI-K concentration at the macula densa and increasing the glomerular filtration rate (GFR) through the physiology of tubuloglomerular feedback (35, 42, 43). Evidence for primary hyperreabsorption upstream of the macula densa and a potential role in glomerular hyperfiltration was also proposed in diabetic patients (15, 46). Thus inhibition of SGLT2 may attenuate diabetic glomerular hyperfiltration and/or kidney damage. To further assess the role of SGLT2 in the early diabetic kidney, we used a mouse model that lacks SGLT2 (41), in which diabetes mellitus was induced using the low dose streptozotocin scheme.

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. The generation of
gene-targeted mice lacking Sglt2 has been described previously (41). Homozygous breeding of Sglt2 knockout (Sglt2−/−) or wild-type mice (WT; all on C57BL/6 background) was performed to yield experimental male mice of matched age (3–7.5 mo) that were housed in the same animal room with a 12:12-h light-dark cycle and free access to standard rodent chow (1% NaCl; Harlan Teklad, Madison, WI) and tap water.

To determine renal SGLT2 protein expression under diabetic conditions, additional mouse strains were used including homozygous C57BLKS/J db/db mice (db/db and littermate heterozygote db−/+) as an obese type 2 diabetic model and Akita mice (Ins2−/− congenic and littermate Ins2−/+ controls) as a nonobese insulin-dependent model of spontaneous type 1 diabetes (both from The Jackson Laboratories, Bar Harbor, ME). All studies were performed in male mice.

Induction of diabetes. C57BL/6 mice were made diabetic using the protocol recommended by the Animal Model of Diabetic Complications Consortium (http://www.DiaComp.org/shared/showProtocol.aspx?id=19). Briefly, mice were injected with streptozotocin (STZ; 50 mg/kg ip) on 5 consecutive days. Nondiabetic control mice received vehicle injections (Na+-citrate buffer with pH adjusted to 4.5).

Food and fluid intake and blood and urine collection. Food and fluid intake was determined over 3 days 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 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 isoflurane anesthesia.

Measurement of GFR in awake mice. Five days before final plasma/urine collection and kidney harvesting, GFR measurements were performed in conscious mice using the plasma kinetics of FITC-inulin following a single dose intravenous injection as described previously (27, 43). Briefly, FITC-inulin (5% in 0.85% NaCl) was dialedyzed 24 h against 0.85% NaCl (resulting in a 2.5–3% solution, which also served to establish the standard curve). The dialyzed FITC-inulin solution was sterile filtered and injected into the retro-orbital plexus (2 μl/g body wt) during brief isoflurane anesthesia. At 3, 7, 10, 15, 25, 35, and 75 min after injection, blood was collected from the end of the tail into a Na+-heparinized 10-μl microcap (Hirschmann Laborgeräte). After centrifugation, 1 μl of plasma was diluted 1:10 in 0.5 mol/l HEPES (pH 7.4) and fluorescence was determined in 2-μl samples using a Nanodrop ND-3300 fluorospec-trometer (Nanodrop Technologies, Wilmington, DE). GFR was calculated using a two-compartment model of two-phase exponential decay (GraphPad Prism, San Diego, CA).

Western blot analysis. Whole kidneys were harvested under terminal isoflurane anesthesia and prepared for Western blot analysis of SGLT2 in the membrane fraction as previously described (41). For analysis of other proteins [cyclin-dependent kinase inhibitor p27 (p27), proliferating cell nuclear antigen (PCNA), heme oxygenase 1 (HO-1), and fibronectin], 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). 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, PCNA, HO-1, and fibronectin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for p62, KIM-1, and NGAL were acquired from Sigma-Aldrich (St. Louis, MO), Abcam (Cambridge, MA), and R&D Systems (Minneapolis, MN), respectively. 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). Densitometric analysis was performed by ImageJ Software (National Institutes of Health). 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 (38). One-hundred microliters of cystolic fraction obtained from kidney homogenization were added to 1 ml of picro-Sirius red solution (NovoUltra Sirius Red stain kit; IHC World, Woodstock, MD) and agitated for 45 min followed by centrifugation at 10,000 g 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.

Sirius red staining. Kidney sections were stained with Sirius red as previously described (34). In brief, kidneys were snap-frozen on dry ice using OCT compound (Sakura Finetek, Torrance, CA), sliced at a thickness of 5 μm using a cryostat microtome, and placed on Super-frost Plus microscope slides (Thermo Fisher Scientific, San Diego, CA). After being air-dried, the section was fixed in 4% paraformaldehyde for 20 min at room temperature and immersed for 1 h in picro-Sirius red solution. Then, the section was rinsed for 2 min in 0.01 N HCl to remove any unbound dyes and mounted in Permount (Thermo Fisher Scientific, San Diego, CA).

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 a 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. Amplification efficiencies were normalized against the housekeeping gene rpl19 and relative fold increases were calculated using the Pfaffl technique of relative quantification, which accounts for real-time efficiencies (25). Each experiment was performed in triplicate.

Blood and urine analysis. Blood glucose was determined using the Ascensia Elite XL glucometer (Bayer, Mishawaka, IN). Urine glucose was determined by the hexokinase/glucose-6-phosphate dehydrogenase method (Infinity; Thermo Electron, Louisville, CO). Concentrations of albumin and creatinine in urine were measured using commercial assays (Exocell, Philadelphia, PA; Thermo Fisher Scientific, Waltham, MA). Plasma creatinine was determined by using the Ascensia ELX 800 spectrophotometer (Thermo Fisher Scientific, San Diego, CA). Plasma aldosterone was determined by radioimmunoassay (Beckman Coulter, Brea, CA).

Screening for urinary tract infection. Ten microliters of spontaneously voided urine (collected 48 h before death) and 50 μl bladder

Table 1. Real-time PCR primers used

<table>
<thead>
<tr>
<th>Target</th>
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<th>Reverse</th>
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<tr>
<td>Sglt1</td>
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<td>Sglt2</td>
<td>Mn00453831_m1 (Applied Biosystems)</td>
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urine (collected at the time of death) were 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) per milliliter of urine was determined. A density of \( >10^5 \) CFU/ml was considered to indicate infection, \( <10^4 \) CFU/ml indicated urethral contamination, and between \( 10^4 \) and \( 10^5 \) CFU/ml indicated one or the other (as recommended by manufacturer).

Statistical analysis. Data are shown as means \( \pm \) SE. ANOVA and unpaired Student’s t-test were performed to analyze for statistical differences between groups. \( P < 0.05 \) was considered statistically significant.

RESULTS

Two sets of studies were performed: STZ or vehicle was applied at an age of 3 mo. One set of mice was followed over 5 wk and is named “5 wk”; these mice had an age of \( \sim4 \) mo when studied. The other set of mice was followed over 4.5 mo after STZ or vehicle application and is named “4.5 mo”; these mice had an age of \( \sim7.5 \) mo when studied. These two mouse sets were used to gain insights on early and later responses to STZ diabetes in the absence of SGLT2 as well as to gain insights on the time-dependent effects of the SGLT2 knockout itself independent of STZ diabetes.

Lack of Sglt2 induces glucosuria in nondiabetic mice. Urinary glucose-to-creatinine ratios and absolute urinary glucose concentrations were significantly greater in nondiabetic Sglt2 \(-/-\) compared with WT mice, while simultaneously determined blood glucose levels were not different between genotypes (Fig. 1A; Table 2); the urinary glucose/caloric loss was associated with greater food (and fluid) intake in Sglt2 \(-/-\) mice (5 wk: Fig. 1D; 4.5 mo: Fig. 1E) confirming previously reported results (41).

Lack of Sglt2 attenuated STZ-induced hyperglycemia. The STZ-induced increase in blood glucose concentration was attenuated in Sglt2 \(-/-\) compared with WT mice as determined in nonfasted awake mice after 3 wk (279 \( \pm \) 13 vs. 434 \( \pm \) 20 mg/dl; \( P < 0.05 \)), 5 wk, and 4.5 mo following STZ application (Fig. 1A). STZ diabetes enhanced urinary glucose-to-creatinine ratios and absolute urinary glucose concentrations to similar levels in both genotypes (Fig. 1A; Table 2). For a given level of hyperglycemia, the urinary glucose-to-creatinine ratio was greater in STZ diabetic Sglt2 \(-/-\) compared with WT mice consistent with lower renal glucose reabsorption in the absence of SGLT2 (5 wk: Fig. 1B; 4.5 mo: Fig. 1C). STZ diabetes lowered body weight to a similar extent and increased food and fluid intake (measured in regular cages) to similar levels in Sglt2 \(-/-\) and WT mice (5 wk: Fig. 1D; 4.5 mo: Fig. 1E).
Lack of Sglt2 does not affect GFR but increases kidney weight in nondiabetic control mice. No evidence for altered GFR was observed in nondiabetic Sglt2−/− compared with WT mice based on measurement of plasma FITC-inulin kinetics in awake mice (5 wk: Fig. 2A; 4.5 mo: Fig. 3A). Determination of plasma concentrations of creatinine in the 7.5-mo-old set of mice revealed lower levels in Sglt2−/− vs. WT mice (0.08 ± 0.02 vs. 0.17 ± 0.04 mg/dl; n = 8/group; P < 0.05) while blood urea nitrogen concentrations were not different (40 ± 3 vs. 40 ± 1 mg/dl; n = 8/group; NS). In the nondiabetic mice, kidney weight was not different at 5 wk between genotypes (Fig. 2B); however, in the 4.5-mo groups (age of ~7.5 mo), kidney weight was greater in Sglt2−/− compared with WT mice (Fig. 3B).

Lack of Sglt2 dissociated STZ-induced changes in GFR and kidney weight. STZ diabetes increased GFR in WT mice with a greater increase being observed at 4.5 mo (Fig. 3A) vs. 5 wk after STZ (Fig. 2A). This increase in GFR was blunted in mice lacking SGLT2. In the 5-wk groups, the STZ-induced minor
increase in urinary albumin-to-creatinine ratio observed in WT mice was absent in Sglt2−/− mice (Fig. 2C). The linear relationship between urinary albumin-to-creatinine ratios and blood glucose levels in diabetic WT mice and the lack of enhanced albuminuria in diabetic WT mice with low blood glucose levels suggested that knockout of Sglt2 may have prevented albuminuria by lowering blood glucose concentration. After 4.5 mo of STZ diabetes, albuminuria in WT mice was again only modestly increased compared with nondiabetic mice and, at this time, a similar increase was observed in Sglt2−/− mice (Fig. 3C). In contrast to GFR, STZ increased the kidney weight to similar levels in both genotypes (5 wk: Fig. 2B; 4.5 mo: Fig. 3B).

Five weeks of STZ-induced diabetes resulted in similar plasma concentrations in Sglt2−/− and WT mice of Na+ and K+ and aldosterone and a modestly greater hematocrit in Sglt2−/− vs. WT mice (Table 2). The lower plasma aldosterone concentration of nondiabetic Sglt2−/− vs. WT mice had been noted before (41). In the 4.5-mo STZ-treated groups, hematocrit values were increased to similar values in both genotypes (Table 2).

STZ diabetes lowers renal expression of SGLT2. STZ diabetes for 5 wk reduced renal mRNA and protein expression of SGLT2 to ~40% of nondiabetic WT mice (Fig. 4, A and C). A difference in renal SGLT2 protein expression was maintained, although reduced, at 4.5 mo after STZ application (Fig. 4B). In comparison, both db/db mice and Akita Ins+/-C99Y mice (all hyperglycemic, 6–12 wk of age) had greater renal SGLT2 expression than their controls (Fig. 4D).

Lack of Sglt2 did not affect the renal mRNA expression of Sltl1, Naglt1, Glut1, or Glut2 in response to STZ. In nondiabetic mice, the renal mRNA expression of Sltl1 was lower in Sglt2−/− mice compared with WT mice, whereas the expression of Glut1 and Glut2 was similar (Fig. 4C), as previously reported (41). Renal mRNA expression of the Na-dependent glucose transporter Naglt1 previously detected in the kidney (16), was likewise not affected by the absence of Sglt2 (Fig. 4C). STZ diabetes for 5 wk did not change the renal mRNA expression of Sltl1 or Glut2 but lowered the expression of Naglt1 (like Sglt2) and increased the expression of Glut1 in both genotypes (Fig. 4C).

Effect of Sglt2 knockout on markers of renal growth, injury, inflammation, and fibrosis in nondiabetic mice. Independent of age, the renal protein or mRNA expression of the following tissue growth and stress markers were not significantly affected by the absence of Sglt2 in nondiabetic mice: PCNA (Fig. 5); the cytokines and chemokines interleukin IL-6, CCL2 (monocyte chemoattractant protein-1), CCL5 (regulated upon activation normal T-cell expressed and presumably secreted, RANTES), and transforming growth factor-β (TGF-β), and NADPH oxidases NOX2 and NOX4 (all Fig. 6). Expression of p27 and HO-1 was modestly increased in nondiabetic 4-mo-old Sglt2−/− compared with WT mice.
A greater renal expression of p62, fibronectin, and NGAL (Fig. A, although the rise in CCL2 was attenuated). STZ diabetes also raised mRNA levels of IL-6, CCL2, and NOX2 remained elevated 4.5 mo of STZ diabetes in WT mice (Fig. 5 A), and renal mRNA expression of NOX2 but not that of IL-6, CCL2, and CCL5 and tended to increase TGF-β-interstitial staining (Fig. 6 E). In comparison, CCL5 returned to control levels and TGF-β to subnormal levels in WT mice at 4.5 mo of STZ diabetes (Fig. 6 B), and renal KIM-1 protein expression was not affected by STZ diabetes in WT mice and KIM-1 was not detectable in urine of any group (not shown).

In response to STZ, renal protein expression of p27, PCNA, and HO-1 increased to similar levels in Sglt2−/− compared with WT mice (Fig. 5). The responses in renal mRNA expression of cytokines and NOX2/4 were likewise similar in the absence of Sglt2 with the exception that CCL5 remained modestly higher in Sglt2−/− compared with WT mice at 4.5 mo after STZ application (Fig. 6 B); the latter was associated with increased renal NGAL expression and urinary NGAL-to-creatinine ratios in mice lacking Sglt2 (Fig. 7, A–C). In contrast, the STZ-induced rise in p62 was blunted in Sglt2−/− mice (Fig. 8 A). Lack of Sglt2 did not affect the STZ-induced increase in renal fibronectin expression (Fig. 7 A) or the upregulation or tubulointerstitial localization of Sirius red-sensitive renal collagen content (Fig. 7 D) and tubulointerstitial staining (Fig. 7 E). Lfack of Sglt2 did not induce ascending urinary tract infection. Neither STZ diabetes nor the absence of Sglt2 induced a detectable bacteruria when urine was collected by direct bladder puncture under terminal anesthesia in the 4.5-mo groups (Fig. 8 A). In contrast, a previous study reported more urinary tract infections in mice with a loss of function mutation in SGLT2. In this previous study, STZ applications were repeated

(5-wk series). A trend persisted at 7.5 mo of age, but these differences were not statistically significant (Fig. 5).

As determined in 7.5-mo-old nondiabetic mice (4.5-mo series), the renal expressions of p62 (Fig. 7 A), a marker of autophagy; the kidney injury markers NGAL (Fig. 7 A) and KIM-1 (not shown); and the fibrosis marker fibronectin (Fig. 7 A) were not affected by the absence of Sglt2. Likewise no differences were observed in urinary NGAL-to-creatinine ratios (Fig. 7, B and C) or renal collagen content as determined by the Sirius red method (Fig. 7, D and E).

Lack of Sglt2 did not attenuate the effect of STZ on markers of renal growth, injury, inflammation, and fibrosis. Five weeks of STZ diabetes enhanced the renal protein expression of p27, PCNA, and HO-1 in WT mice (Fig. 5 A). This was associated with an increase in renal mRNA expression of the cytokines IL-6, CCL2, and CCL5 and tended to increase TGF-β (Fig. 6 A). The renal mRNA expression of NOX2 but not that of NOX4 was increased at this time point in STZ-diabetic vs. nondiabetic WT mice (Fig. 6 A).

The described effects on p27, PCNA, and HO-1 persisted at 4.5 mo of STZ diabetes in WT mice (Fig. 5 B), and renal mRNA levels of IL-6, CCL2, and NOX2 remained elevated (although the rise in CCL2 was attenuated). STZ diabetes also increased renal mRNA expression of NOX4 in WT mice at 4.5 mo of STZ diabetes. These changes were associated with greater renal expression of p62, fibronectin, and NGAL (Fig. 7 A), urinary NGAL-to-creatinine ratios (Fig. 7, B and C), and Sirius red-sensitive renal collagen content (Fig. 7 D) and tubulointerstitial staining (Fig. 7 E). In comparison, CCL5 returned to control levels and TGF-β to subnormal levels in WT mice at 4.5 mo of STZ diabetes (Fig. 6 B), and renal KIM-1 protein expression was not affected by STZ diabetes in WT mice and KIM-1 was not detectable in urine of any group (not shown).

In response to STZ, renal protein expression of p27, PCNA, and HO-1 increased to similar levels in Sglt2−/− compared with WT mice (Fig. 5). The responses in renal mRNA expression of cytokines and NOX2/4 were likewise similar in the absence of Sglt2 with the exception that CCL5 remained modestly higher in Sglt2−/− compared with WT mice at 4.5 mo after STZ application (Fig. 6 B); the latter was associated with increased renal NGAL expression and urinary NGAL-to-creatinine ratios in mice lacking Sglt2 (Fig. 7, A–C). In contrast, the STZ-induced rise in p62 was blunted in Sglt2−/− vs. WT mice (Fig. 7 A). Lack of Sglt2 did not affect the STZ-induced increase in renal fibronectin expression (Fig. 7 A) or the upregulation or tubulointerstitial localization of Sirius red-sensitive renal collagen (Fig. 7, D and E).

Lack of Sglt2 did not induce ascending urinary tract infection. Neither STZ diabetes nor the absence of Sglt2 induced a detectable bacteruria when urine was collected by direct bladder puncture under terminal anesthesia in the 4.5-mo groups (Fig. 8 A). In contrast, a previous study reported more urinary tract infections in mice with a loss of function mutation in SGLT2. In this previous study, STZ applications were repeated
to induce similar blood glucose levels in mutant and control mice (21). Therefore, to further enhance the glucosuric challenge in the absence of higher STZ doses, a separate group of Sglt2\(^{-/-}\) mice received 5% glucose in the drinking water for a period of 4.5 mo following STZ application. The maneuver increased blood glucose concentration, but mean levels remained below those in STZ-treated WT mice (Fig. 8B). The maneuver did not change absolute urine glucose concentrations (Fig. 8C) but significantly increased fluid intake (87 ± 6 ml/day; compare with Fig. 1E) and urinary glucose-to-creatinine ratios (Fig. 8C). Still, no bacteriuria was detectable by bladder puncture (Fig. 8A). Urine collected by direct bladder puncture from five nondiabetic 7.5-mo-old Sglt2\(^{-/-}\) mice also did not show any bacterial growth.

Providing 5% glucose in the drinking water did not enhance mean GFR in STZ-treated WT mice (Fig. 8D). Comparing animals with similar blood glucose concentrations in STZ-treated WT and Sglt2\(^{-/-}\) mice revealed lower levels of GFR in the knockout mice (circle symbols in Fig. 8D).

**DISCUSSION**

The main findings of the current study are that genetic knockout of SGLT2 in mice lowers blood glucose levels in STZ-induced diabetes mellitus and prevents glomerular hyperfiltration. Lack of SGLT2 did not induce ascending urinary tract infection in nondiabetic or diabetic mice. The absence of SGLT2 did not prevent the STZ-induced increase in kidney weight or in markers of renal growth, injury, inflammation, and fibrosis.

The results support the concept that inhibition of SGLT2 increases urinary glucose excretion thereby lowering blood glucose levels in diabetes mellitus (9, 22, 44). This is consistent with a recent study in mice carrying a loss of function mutation in SGLT2 (21). Food intake was enhanced in nondiabetic Sglt2\(^{-/-}\) mice compared with WT mice, possibly to match the urinary glucose and caloric loss. In comparison, food intake and probably urinary glucose excretion (as estimated from similar fluid intake and thus excretion and absolute urinary glucose concentrations) were not different between genotypes following STZ application. Thus the lack of SGLT2 reduced the glomerular filtration of glucose in STZ-diabetic mice such that glucosuria was similar to WT mice despite attenuated renal glucose reabsorption.

The reduced glomerular filtration of glucose in STZ-diabetic Sglt2\(^{-/-}\) vs. WT mice was due to lower blood glucose levels and blunted glomerular hyperfiltration. The latter is consistent with a role of SGLT-mediated proximal tubular hyperreabsorption in diabetes, which through the tubuloglomerular feedback mechanisms contributes to the rise in GFR (42). Selective pharmacological inhibition of SGLT2 has recently been proposed to lower GFR in diabetic rats through the same mechanism (36).

All diabetic patients experience episodes of hyperglycemia, and preventing the early proximal tubule from "seeing" these episodes of hyperglycemia through inhibition of SGLT2 may attenuate the negative effects of glucose on renal structure and function. Diabetic kidney growth and its unique molecular signature have been linked to the development of diabetic
nephropathy (for review see, Refs. 39, 45). The tubular growth mechanism in diabetes includes an early, growth factor-driven hyperplastic phase, which through the activation of cyclin-dependent kinase inhibitors, like p27, is switched to a hypertrophic phase that also shows aspects of senescence (29, 45). The current results indicate that the diabetes-induced increase in kidney weight (including the upregulation of p27 and HO-1) in younger mice (7.5 mo of age; Fig. 3B) occurred together with a modest upregulation of p27 and HO-1 in younger nondiabetic Sglt2−/− mice, which may reflect tissue responses to increased tubular glucose delivery. Further studies are needed to more precisely localize kidney growth and its molecular determinants in the absence of SGLT2 and in response to diabetes.

How can the lack of SGLT2 dissociate STZ-diabetes-induced kidney growth from the increase in GFR? The tubular glucose reabsorption rate is known to increase with kidney weight in early STZ-diabetic rats (32). Diabetic tubular growth is a major cause of proximal hyperreabsorption and glomerular hyperfiltration (35) and may do so in part by enhancing SGLT2 activity, consistent with the observed dissociation in the absence of SGLT2. Despite the observed early downregulation of renal SGLT2 expression in STZ-diabetic WT mice, the absolute tubular transport activity through that pathway was most likely increased compared with nondiabetic WT mice due to the enhanced filtered glucose load in diabetes and the fact that only part of the SGLT2 capacity is used under euglycemic conditions. This is reminiscent of glucose reabsorption via SGLT1 in nondiabetic Sglt2−/− mice, which appears greatly increased compared with nondiabetic WT mice due to enhanced proximal glucose delivery through that pathway (41). SGLT2 expression partly recovered after 4.5 mo of STZ diabetes, which was associated with a further rise in GFR in WT but not Sglt2−/− mice. In other words, early suppression of SGLT2 expression in STZ-diabetic WT mice may have attenuated the rise in proximal hyperreabsorption and thereby in GFR.

In WT mice, STZ diabetes increased renal mRNA expression of the proinflammatory cytokine IL-6 and the chemokine CCL2 (23) as well as NADPH oxidases NOX2 (24) and NOX4, the latter being implicated in glucose-induced renal
oxidative stress (31). STZ diabetes also enhanced renal expression of oxidative stress-induced HO-1, which decreases renal oxidative stress and inflammation in the diabetic kidney (11). These changes were associated with upregulation of renal and urinary NGAL, a marker of renal tubular injury (6), and fibrosis, as indicated by increased renlar fibronectin expression and in tubulointerstitial Sirius red staining, which has been shown to match with type I collagen immunostaining in normal and fibrotic kidneys (34). Despite lower GFR and blood glucose levels, lack of SGLT2 in STZ-diabetic mice did not attenuate these changes. How could that be explained assuming that we sufficiently mitigated nonspecific cytotoxicity of STZ by using the multiple low-dose injection scheme to induce diabetes as recommended by the Animal Model of Diabetic Complications Consortium (http://www.DiaComp.org/shared/showProtocol.aspx?id=19)? Considering similar urinary glucose concentrations and probably total glucose excretion (see above) and no differences in renal Sglt1/Naglt1/ Glut1/Glut2 mRNA expression in STZ-diabetic WT vs. Sglt2-/- mice, it seems possible that STZ-induced glucose delivery downstream of the SGLT2-expressing early proximal tubule was not affected by the absence of SGLT2 (the lack of early proximal glucose reabsorption via SGLT2 in Sglt2-/- mice was balanced by lower filtration of glucose). If luminal glucose delivery to further distal segments is important, then similar mid/late-proximal glucose delivery may explain why most of the STZ-induced renal growth and injury factors were independent of SGLT2. Alternatively, the levels of hyperglycemia induced in the absence of SGLT2 (~300 mg/dl) were sufficient to trigger these changes, i.e., lack of SGLT2 lowered blood glucose but not enough to affect kidney outcome, and/or glucose was taken up into proximal tubules across the basolateral membrane. Knockout of Sglt2 dissociated kidney growth from glomerular hyperfiltration but not from kidney injury. These results in Sglt2-/- mice are consistent with a link between diabetes-induced kidney growth and injury/fibrosis (39, 45). We observed that the renal mRNA expression of some of the inflammatory markers and TGF-β were lower at 4.5 mo vs. 5 wk after STZ-application while other markers, like NOX4, HO-1, and IL-6, appeared to increase with time. These findings indicate complex time-dependent renal processes in the STZ-

Fig. 7. Lack of Sglt2 did not attenuate the effect of STZ on markers of renal injury or fibrosis. At 4.5 mo after STZ, the renal protein expression of p62, neutrophil gelatinase-associated lipocalin (NGAL), and fibronectin was determined (A) as well as urinary NGAL-to-creatinine ratios (B and C) and Sirius red-sensitive renal collagen content (D) and cortical staining (E). Sglt2-/- mice showed increased STZ-induced renal NGAL expression and urinary NGAL-to-creatinine ratios but an attenuated rise in p62 vs. WT mice. In comparison, the fibrosis markers fibronectin and Sirius red-sensitive renal collagen content and tubulointerstitial staining increased to similar levels in both genotypes in response to STZ. *P < 0.05 vs. WT; #P < 0.05 vs nondiabetic control; n = 5–15 per group.
diabetes model that may include stronger responses to the initiation of hyperglycemia and STZ-application than to sustained high blood glucose levels, which may activate protective mechanisms (e.g., HO-1).

The NGAL response to STZ diabetes was enhanced in the absence of SGLT2. Renal NGAL and urinary NGAL were not increased in nondiabetic Sglt2−/− compared with WT mice despite high renal luminal glucose concentrations in the former. It remains to be determined whether the tonically enhanced tubular glucose load before diabetes induction in the knockout mice (which increased p27 and HO-1) sensitized the tubular system to STZ-diabetes-induced damage. In β-cells of the pancreas, STZ is taken up via GLUT2 (30, 37). Whether knockout of Sglt2, which may lower intracellular glucose and enhances the basolateral gradient for glucose uptake via GLUT2, enhanced or reduced proximal tubular STZ uptake and toxicity is not known.

Diabetes mellitus, including the STZ-model, has been shown to inhibit cellular autophagy in proximal and distal tubular cells (2, 14, 18). Autophagy involves the degradation of cellular components through the lysosomal machinery and contributes to cell growth, development, and homeostasis. The p62 is degraded through an autophagy-lysosome pathway (19), and renal p62 accumulation is an indicator of impaired autophagy (4, 17). In accordance, p62 is enhanced in the kidneys of diabetic Wistar fatty rats (18) as well as in STZ-diabetic mice (current study). In contrast to NGAL, the renal p62 accumulation was blunted in STZ-diabetic Sglt2−/− compared with WT mice. This may reflect lower blood glucose levels induced in Sglt2−/− mice or indicate a role of SGLT2-induced glucose uptake in STZ-diabetes-induced inhibition of autophagy.

Insights on the maximum effect of SGLT2-inhibition on the diabetic kidney may have been limited by the fact that STZ diabetes reduced renal SGLT2 expression. There is evidence for enhanced, unchanged, or increased SGLT2 expression in the diabetic kidney (39). In vitro studies in renal proximal tubule cells indicated that high glucose-induced oxidative stress may reduce SGLT expression (13), which may serve to limit renal glucose uptake and toxicity. In addition to SGLT2 and SGLT1, the low-affinity Na+/glucose cotransporter NaGLT1 has been cloned in the rat where it is located in the brush border membrane of proximal tubules (16). We found that lack of SGLT2 did not affect the renal mRNA expression of NaGLT1 (in contrast to SGLT1). However, like the response in SGLT2, the expression of NaGLT1 mRNA was reduced in STZ diabetes. In contrast to the STZ-diabetes model, we found that the Akita model of type 1 diabetes, as well as the db/db model of type 2 diabetes, has enhanced renal SGLT2 expression. Therefore, the evaluation of the maximum effect of SGLT2 inhibition in diabetes may require studies in the latter...
kind of models. Moreover, the response in SGLT2 expression may affect the susceptibility to diabetic nephropathy and contribute to observed mouse strain differences (7). Lower renal SGLT2 expression may contribute to the relative resistance of the C57BL/6 strain to STZ-induced diabetic kidney disease, which is a limitation associated with the use of that strain and the current study.

One concern with regard to the long-term safety of pharmacological SGLT2 inhibition is that increased urinary glucose excretion may enhance the risk of ascending urinary tract infections. A recent study reported urinary tract infections and enhanced mortality in mice with a loss of function mutation in SGLT2 when STZ applications were repeated to induce similar blood glucose levels in mutant and control mice, thereby inducing much greater glucosuria in mutant compared with control mice (21). Possible consequences and toxicity of greater STZ doses were not considered or discussed. The current study, following a similar time course as the previous study, demonstrated that a lack of SGLT2 alone (in the absence of greater STZ doses) did not cause greater urinary glucose concentrations than observed in WT mice given the same dose of STZ. No mortality or ascending urinary tract infections, as assessed by sterile bladder puncture, were detected in any of the STZ-treated groups. Moreover, application of 5% glucose to STZ-treated Sglt2−/− mice raised glucosuria above the levels observed in STZ diabetic Sglt2−/− and WT mice; yet the maneuver did not induce ascending urinary tract infections or mortality. This maneuver also allowed us to compare GFR at similar levels of hyperglycemia, which indicated that a lack of SGLT2 lowers GFR in diabetes independent of its effect on hyperglycemia, consistent with the tubular hypothesis of diabetic glomerular hyperfiltration (39).

In summary, genetic knockout of SGLT2 in mice lowers blood glucose levels in STZ-induced diabetes mellitus and prevents glomerular hyperfiltration. Lack of SGLT2 did not induce ascending urinary tract infection or increase mortality in nondiabetic or diabetic mice. The absence of SGLT2 attenuated the STZ-induced renal accumulation of p62, an indicator of impaired autophagy, but did not attenuate the increase in kidney weight or in markers of renal growth, injury, inflammation, and fibrosis.

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