SGLT2 Protein Expression Is Increased in Human Diabetic Nephropathy

SGLT2 PROTEIN INHIBITION DECREASES RENAL LIPID ACCUMULATION, INFLAMMATION, AND THE DEVELOPMENT OF NEPHROPATHY IN DIABETIC MICE*

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There is very limited human renal sodium gradient-dependent glucose transporter protein (SGLT2) mRNA and protein expression data reported in the literature. The first aim of this study was to determine SGLT2 mRNA and protein levels in human and animal models of diabetic nephropathy. We have found that the expression of SGLT2 mRNA and protein is increased in renal biopsies from human subjects with diabetic nephropathy. This is in contrast to db/db mice that had no changes in renal SGLT2 protein expression. Furthermore, the effect of SGLT2 inhibition on renal lipid content and inflammation is not known. The second aim of this study was to determine the potential mechanisms of beneficial effects of SGLT2 inhibition in the progression of diabetic renal disease. We treated db/db mice with a selective SGLT2 inhibitor JNJ 39933673. We found that SGLT2 inhibition caused marked decreases in systemic blood pressure, kidney weight/body weight ratio, urinary albumin, and urinary thiobarbituric acid-reacting substances. SGLT2 inhibition prevented renal lipid accumulation via inhibition of carbohydrate-responsive element-binding protein-β, pyruvate kinase L, SCD-1, and DGAT1, key transcriptional factors and enzymes that mediate fatty acid and triglyceride synthesis. SGLT2 inhibition also prevented inflammation via inhibition of CD68 macrophage accumulation and expression of p65, TLR4, MCP-1, and osteopontin. These effects were associated with reduced mesangial expansion, accumulation of the extracellular matrix proteins fibronectin and type IV collagen, and loss of podocyte markers WT1 and synaptopodin, as determined by immunofluorescence microscopy. In summary, our study showed that SGLT2 inhibition modulates renal lipid metabolism and inflammation and prevents the development of nephropathy in db/db mice.

Diabetes mellitus is the leading cause of renal and cardiovascular disease in the United States. This is of significant concern given the rising prevalence of diabetes. Current models predict that as many as 1 in 4 Americans are expected to have diabetes by the year 2030 (1–3). The pathogenesis of diabetes-associated renal disease is multifactorial. Different pathogenic mechanisms work in concert resulting in a progressive decline in renal function (4–12). Despite all the beneficial interventions available for patients with diabetes, including tight glucose control, tight blood pressure control, angiotensin-converting enzyme inhibition, angiotensin II receptor, or mineralocorticoid receptor antagonism, renal disease remains prevalent and is likely to progress in most of these patients (13, 14). Additional treatment modalities that modulate the injurious pathways involved in diabetic nephropathy are therefore urgently needed to slow the progression of renal disease in patients with diabetes.

There is increasing evidence that renal proximal tubular glucose absorption is increased in humans and animal models of diabetes mellitus (15–19). Renal proximal tubular glucose transport is mediated by sodium gradient-dependent glucose transporters SGLT2 and SGLT1, located on the apical membrane of the early and late segments of the proximal tubule, respectively (19, 20). In addition, GLUT2 and GLUT1 located on the basolateral membrane of the early and late segments of the proximal tubule, respectively, facilitate glucose transport across the basolateral membrane (19, 21). Studies in SGLT2 and SGLT1 KO mice have made it possible to determine the role and contribution of each transporter in regulation of reabsorption of the filtered glucose load (20, 22). It is estimated that 97%...
of proximal tubular glucose transport is mediated via SGLT2 and only 3% by SGLT1 (20, 22). However, during SGLT2 inhibition, glucose reabsorption via the SGLT1 transporter increases to ~40% of the filtered load (20). Similarly, the SGLT2 inhibitors in human clinical trials have good efficacy in increasing urine glucose excretion and decreasing serum glucose, but they do not inhibit more than 30–50% of the filtered glucose load.

Recent development of specific and selective SGLT2 inhibitors and SGLT2 knock-out mice has also made it possible to determine the role of SGLT2 in regulating the renal hemodynamic and biochemical alterations in diabetes and the consequences of SGLT2 inhibition on diabetic renal disease (17, 23–39). However, there is very limited human renal sodium gradient-dependent glucose transporter protein SGLT2 mRNA and protein expression data reported in the literature. Aim 1 of this study was to determine SGLT2 mRNA and protein levels in human and animal models of diabetic nephropathy.

The increase in proximal tubular glucose reabsorption has been shown to result in decreases in solute delivery to the macula densa, and adenosine, which deactivates tubuloglomerular feedback, decreases afferent arteriolar resistance and increases the glomerular filtration rate, which characterizes the glomerular hyperfiltration seen in early diabetes (19, 40–42). Alternatively, the increase in proximal tubular glucose absorption can also activate the renin-angiotensin-aldosterone system, with a resultant increase in efferent arteriolar resistance, an increase in intraglomerular pressure, and an increase in glomerular hyperfiltration (43, 44). In addition, the increase in proximal tubular reabsorption of glucose mediates the increase in kidney growth, proteinuria, and inflammation, which play an important role in eventual decline in renal function (19, 44).

Studies in SGLT2 knock-out mice with streptozotocin-induced hyperglycemia indicated that absence of SGLT2 attenuated hyperglycemia and glomerular hyperfiltration but did not prevent kidney growth, oxidative stress, inflammation, fibrosis, or injury (16). SGLT2 inhibition with empagliflozin in type 1 diabetic Akita mice had a more profound impact with decreased blood glucose, blood pressure, glomerular filtration rate, kidney weight, urine albumin/creatinine ratio, as well as gluconeogenesis and inflammatory genes but no changes in oxidative stress or fibrosis genes or renal histology (17). In contrast, in db/db mice empagliflozin attenuated markers of renal fibrosis without improving albuminuria (34). In BTBR (black and tan brachyury) ob/ob model of leptin deficiency and type 2 diabetes mellitus, SGLT2 inhibition with empagliflozin decreased blood glucose, albuminuria, glomerular hypertrophy, mesangial matrix expansion, and inflammation (29).

Our laboratory has found that in several models, including streptozotocin-induced diabetic mice and rats (45, 46), Akita mice (47), OVE26 mice (47), and db/db mice (48), that kidney disease is associated with lipid accumulation and increased activity of proinflammatory cytokines, resulting in albuminuria, glomerular mesangial expansion, and tubulointerstitial fibrosis. The lipid accumulation was caused by increased fatty acid synthesis, mediated by increased expression and activity of the sterol regulatory element-binding protein-1 (SREBP-1) and carbohydrate regulatory element-binding protein (ChREBP), and decreased fatty acid oxidation, mediated by decreased expression and activity of peroxisome proliferator-activated receptor α (49). We have seen similar regulation of renal lipid metabolism in kidney biopsies of humans with type 2 diabetes mellitus (50, 51). In addition, in glomerular mesangial cells, podocytes, and proximal tubular cells in culture, we have found that in cells grown in the presence of high glucose concentrations, similar to what is seen in diabetic rats and mice, there is significant accumulation of lipids and induction of inflammatory gene expression signature associated with increased expression of profibrotic growth factors and extracellular matrix protein production. These studies suggested that increased glucose entry into the renal cells may initiate cellular biochemical events that drive diabetic renal disease.

We hypothesized that inhibition of renal proximal tubular glucose absorption by SGLT2 inhibition would decrease renal lipid accumulation and inflammation, resulting in prevention or attenuation of diabetic nephropathy. Indeed, our study shows that SGLT2 inhibition decreases renal lipid accumulation and inflammation and prevents the development of nephropathy in db/db mice.

Results

SGLT2 mRNA and Protein Expression Are Increased in Kidney Biopsies from Human Subjects with Diabetic Nephropathy

SGLT2 mRNA abundance is increased in kidney biopsies obtained from human subjects with diabetic nephropathy. In contrast, there is no change in SGLT1 mRNA abundance (Fig. 1A). The characteristics of the human subjects are shown in Table 1. This finding was confirmed by immunohistochemical (IHC) analysis of SGLT2 protein expression, which showed increased apical border staining of proximal tubule epithelium of diabetics, in contrast to weaker staining in the same pattern in normal kidneys (Fig. 1B). In contrast, SGLT2 protein abundance as determined by IHC was not altered in db/db mice (Fig. 1C). We next explored the impact of SGLT2 inhibition on kidney disease in insulin-deficient and insulin-resistant diabetic db/db mice.

db/db Mice with Insulin-deficient Diabetes Mellitus

SGLT2 Inhibition Corrected Metabolic Parameters and Proteinuria—The db/db mice consumed more food than the db/m mice. The db/db mice treated with the SGLT2 inhibitor consumed even more food than the non-treated db/db mice. The db/db mice treated with the SGLT2 inhibitor continued to gain body weight throughout the study. In contrast, the non-treated db/db mice started to lose weight, and at the end of 12 weeks they were approximately the body weight of db/m mice (Table 2).

SGLT2 inhibition decreased and normalized blood glucose immediately in db/db mice. SGLT2 inhibition also increased urine glucose excretion and urine volume in db/db mice (data

2 The abbreviations used are: ChREBP, carbohydrate-responsive element-binding protein; IHC, immunohistochemistry; TBARS, thiobarbituric acid-reacting substance; LPK, pyruvate kinase L; FFPE, formalin-fixed paraffin-embedded; AMG, α-methyl D-glucopyranoside; TPE, two-photon excitation; SHG, second harmonic generation; QPCR, quantitative PCR; PAS, periodic acid-Schiff.
SGLT2 Inhibition and Diabetic Nephropathy

not shown). SGLT2 inhibition decreased and normalized hemoglobin A1C in db/db mice (Table 2); db/db mice had low serum insulin levels compatible with the db/db mice on the BLKS/J genetic background developing insulin deficiency due to beta cell failure induced by glucolipotoxicity (52). However, treatment with SGLT2 inhibitor prevented the decrease in serum insulin levels (Table 2). SGLT2 inhibition resulted in a significant decrease in systolic blood pressure in db/db mice (Table 2).

SGLT2 Inhibition Blocks Glomerular Changes in db/db Mice—SGLT2 inhibition prevented the increases in urinary albumin excretion in db/db mice (Fig. 2A). This was associated with the decrease in mesangial expansion in db/db mice as determined by PAS staining but no impact on glomerular size (Fig. 2B and Table 3).

SGLT2 inhibition prevented the increases in extracellular matrix accumulation in db/db mice as determined by fibronectin

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Clinical data on patient samples</th>
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<tr>
<td></td>
<td>Normal</td>
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<tr>
<td></td>
<td>Total numbers</td>
</tr>
<tr>
<td>Male numbers (%)</td>
<td>8 (53)</td>
</tr>
<tr>
<td>Age</td>
<td>50.3 ± 21.0</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>NA</td>
</tr>
<tr>
<td>Body mass index</td>
<td>NA</td>
</tr>
<tr>
<td>Blood pressure on admission</td>
<td>126/75 ± 26/12</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>Serum urea (mg/dl)</td>
<td>39 ± 15</td>
</tr>
<tr>
<td>Estimated glomerular filtration rate (eGFR) (ml/min/1.73 m²)</td>
<td>97.4 ± 29.5</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.6 ± 2.7</td>
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<tr>
<td>Serum albumin (mg/dl)</td>
<td>4.1 ± 0.5</td>
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<tr>
<td>Proteinuria</td>
<td>28.5 ± 66.3</td>
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</tbody>
</table>

* p < 0.05 versus normal is shown.
* p < 0.01 versus normal is shown.

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Metabolic parameters for db/db mice with insulin-deficient diabetes</th>
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<tbody>
<tr>
<td></td>
<td>db/m</td>
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<tr>
<td>Body weight (g)</td>
<td>30.9 ± 0.63</td>
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<td>Food intake (g/mouse/day)</td>
<td>4.5 ± 0.08</td>
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<td>Blood glucose (mg/dl)</td>
<td>143 ± 1.67</td>
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<tr>
<td>HbA1c (%)</td>
<td>4.0 ± 0.10</td>
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<td>Plasma insulin (ng/ml)</td>
<td>1.36 ± 0.20</td>
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<td>Kidney weight/body weight (×10^-3)</td>
<td>6.3 ± 0.12</td>
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<td>Systolic blood pressure (mm Hg)</td>
<td>130 ± 3</td>
</tr>
</tbody>
</table>

* p < 0.05 versus db/m is shown.
* p < 0.05 versus db/db is shown.

FIGURE 1. SGLT2 mRNA and protein expression in kidney biopsies from human subjects with diabetic nephropathy and SGLT2 protein abundance in kidneys from db/m and db/db mice. A, human SGLT2 mRNA levels as determined by RT-QPCR in RNA isolated from FFPE sections are increased in kidney biopsies from subjects with diabetic nephropathy, whereas the SGLT1 mRNA level is not changed. B, human SGLT2 protein level as determined by IHC is also increased in kidney biopsies from subjects with diabetic nephropathy. C, SGLT2 protein level as determined by IHC is not altered in db/db mouse kidneys.
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FIGURE 2. Effects of SGLT2 inhibition on diabetic kidney disease. A, urinary albumin level is decreased with the SGLT2 inhibition in db/db mice. B, periodic acid Schiff staining indicates marked mesangial expansion in db/db mice, and treatment with SGLT2 inhibitor results in a significant decrease in mesangial expansion. C, fibronectin immunofluorescence microscopy indicates marked accumulation of glomerular matrix in db/db mice, and SGLT2 inhibition results in a significant decrease in fibronectin immunostaining. D, type IV collagen immunofluorescence microscopy indicates marked accumulation of glomerular matrix in db/db mice, and SGLT2 inhibition results in a significant decrease in type IV collagen immunostaining. G, glomerulus. TPE and SHG microscopy shows increased fibrillary collagen expression in the tubulointerstitial (red signal) of db/db mice, which is decreased after SGLT2 inhibition.

(Fig. 2C and Table 3) and type IV collagen (Fig. 2D) immunofluorescence microscopy. Furthermore, two-photon excitation (TPE) and second harmonic generation (SHG) microscopy revealed a decrease in fibrillary collagen accumulation following SGLT2 inhibition (Fig. 2E).

SGLT2 inhibition prevented podocyte injury and loss of podocytes in db/db mice as determined by WT1 (Fig. 3A and Table 3) and synaptopodin (Fig. 3B and Table 3) immunofluorescence microscopy. However, SGLT2 inhibition was not able to prevent the decrease in nephrin immunostaining (Fig. 3C and Table 3).

SGLT2 Inhibition Blocks Inflammation in db/db Mice—SGLT2 inhibition prevented the increase in macrophage infiltration in db/db mice as determined by immunofluorescence microscopy for the macrophage marker CD68 (Fig. 4A and Table 3). SGLT inhibition similarly decreased the expression level of proinflammatory p65 subunit of NF-κB.
(Fig. 4B), toll-like receptor 4 (TLR4) (Fig. 4C), monocyte chemotactic protein 1 (MCP-1) (Fig. 4D), and osteopontin (Fig. 4E).

**TABLE 3**

Quantification of histology and immunofluorescence microscopy

Data are means ± S.E. (n = 12 mice in each group).

<table>
<thead>
<tr>
<th></th>
<th>db/m</th>
<th>db/db</th>
<th>db/db + SGLT-2 inhibition</th>
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<tr>
<td>Mesangial expansion index</td>
<td>1.53</td>
<td>2.16</td>
<td>1.85</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>550</td>
<td>941</td>
<td>739</td>
</tr>
<tr>
<td>WT1-positive cells/</td>
<td>12.18</td>
<td>5.14</td>
<td>10.44</td>
</tr>
<tr>
<td>glomerulus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synaptopodin</td>
<td>360</td>
<td>173</td>
<td>835</td>
</tr>
<tr>
<td>Nephrin</td>
<td>677</td>
<td>381</td>
<td>282</td>
</tr>
<tr>
<td>CD68</td>
<td>2.65</td>
<td>16.49</td>
<td>10.44</td>
</tr>
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</table>

*p < 0.05 versus db/m is shown.

SGLT2 Inhibition Blocks Lipid Accumulation and Alters Lipid Metabolism—SGLT2 inhibition also prevented the increase in neutral lipid (triglycerides and cholesterol ester) accumulation in db/db mice as determined by Oil Red O staining (Fig. 5A). Oil Red O staining revealed increased staining in the glomeruli and tubules of db/db mice, which was prevented following SGLT2 inhibition. The effects of SGLT2 inhibition on lipid accumulation was mediated in part through inhibition of the transcriptional factor ChREBP-β (Fig. 5B), and fatty acid and triglyceride synthesis genes pyruvate kinase L (LPK) (Fig. 5C), stearoyl-CoA desaturase-1 (SCD-1) (Fig. 5D), and diacylglycerol O-acyltransferase 1 (DGAT1) (Fig. 5E). In contrast, there were no...
effects on the transcription factors and their target enzymes that mediate fatty acid oxidation.

**SGLT2 Inhibition Modulates Intrarenal Renin Angiotensin System**—SGLT2 inhibition resulted in significant increases in renin, renin receptor, angiotensinogen, angiotensin-converting enzyme, and angiotensin 1 receptor mRNA levels (Table 4).

SGLT2 inhibition also resulted in significant increases in angiotensin 2 receptor and angiotensin-converting enzyme 2 mRNA levels, but there was no effect on Mas receptor mRNA level (Table 4).

**SGLT2 Inhibition Modulates Intrarenal Adenosine Receptors**—CD73 protein and mRNA levels are significantly decreased in db/db mice (Fig. 6A). CD73 also known as 5'-nu-
cleotidase, an enzyme that in humans is encoded by the NT5E gene. CD73 commonly serves to convert AMP to adenosine (53). SGLT2 inhibition resulted in normalization of CD73 protein and mRNA. The adenosine receptors Adora1a, Adora2a, and Adora2b mRNA levels were increased in db/db mice, and SGLT2 inhibition resulted in normalization of Adora2b mRNA levels and a downward trend in Adora1a and Adora2a mRNA levels (Fig. 6B).
SGLT2 Inhibition and Diabetic Nephropathy

**TABLE 4**
The effects of SGLT2 inhibition in regulation of the RAAS in db/db mice. Data are means ± S.E. (n = 12 mice in each group).

<table>
<thead>
<tr>
<th></th>
<th>db/m</th>
<th>db/db</th>
<th>db/db + SGLT2 inhibition</th>
</tr>
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<tbody>
<tr>
<td>Renin</td>
<td>10.75 ± 2.34</td>
<td>6.73 ± 2.35</td>
<td>27.68 ± 9.03</td>
</tr>
<tr>
<td>Renin receptor</td>
<td>1.21 ± 0.09</td>
<td>1.21 ± 0.08</td>
<td>1.67 ± 0.14</td>
</tr>
</tbody>
</table>

a p < 0.05 versus db/m is shown.
b p < 0.05 versus db/db is shown.

db/db Mice with Insulin-resistant Diabetes Mellitus

SGLT2 Inhibition Corrected Metabolic Parameters and Proteinuria—SGLT2 inhibition normalized blood glucose in db/db mice. In these experiments, db/db mice did not lose weight, and both untreated and treated db/db mice gained body weight throughout the experiment. This may have been due to the fact that this time the db/db mice had high insulin levels. The insulin level, however, did not change with SGLT2 inhibition. SGLT2 inhibition markedly decreased the increase in serum triglyceride levels, but it did not have a significant effect on serum cholesterol levels (Table 5). SGLT2 inhibition also markedly decreased urine albumin excretion in db/db mice (Table 5).

SGLT2 Inhibition Alters Brush-border Membrane Sodium Gradient Glucose Transport Activity and SGLT2 and SGLT1 Protein Abundance—SGLT2 inhibition resulted in a significant increase in apical brush-border membrane sodium gradient glucose transport activity in db/db mice (Fig. 7A). The increase in apical brush-border membrane sodium gradient glucose transport activity was associated with a significant increase in brush-border membrane SGLT2 but a decrease in SGLT1 protein abundance (Fig. 7B). However, the abundance of mRNA in both SGLT2 and SGLT1 was significantly decreased (Fig. 7, C and D). In addition, SGLT2 inhibition also cause a significant decrease of the abundance of mRNA in GLUT2 (Fig. 7E).

Effect of SGLT2 Inhibition on Kidney Gluconeogenesis—SGLT2 inhibition had no significant effects on phosphoenolpyruvate carboxykinase and glucose-6-phosphatase mRNA abundance (Fig. 7F).

Discussion

Although current clinical trials show that SGLT2 inhibitors are less effective on glycemic control in patients with renal impairment than in a population without renal impairment (54), experimental evidence in diabetic rodents and humans indicates that the renal SGLT2 threshold for glucose reabsorption is paradoxically increased in hyperglycemia (55). In cultured human renal proximal epithelial cells from the urine of patients with type 2 diabetes, SGLT2 mRNA and protein expression are also markedly increased compared with nondiabetics (18). We show here for the first time that SGLT2 mRNA and protein expression are increased in human biopsies of diabetic nephropathy even with advanced kidney disease (Table 1) indicating that SGLT2 can be an effective target in treatment of diabetic nephropathy.

Our hypothesis was that inhibition of renal proximal tubular glucose absorption by SGLT2 inhibition would result in prevention of renal lipid accumulation and inflammation, resulting in prevention or attenuation of diabetic nephropathy. Indeed, our study shows that SGLT2 inhibition decreases renal lipid accumulation and inflammation and prevents the development of nephropathy in db/db mice regardless whether they have insulin-deficient diabetes or insulin-resistant diabetes.

The beneficial effects of SGLT2 inhibition could be mediated by normalization of serum glucose that results in secondary beneficial effects in the kidney. The beneficial effects of SGLT2 inhibition could also be mediated, at least in part by the significant decrease in systolic blood pressure, which are mediated by osmotic diuresis following inhibition of renal tubular glucose absorption or increase in arterial compliance by decreasing glomerular hyperfiltration secondary to increase on distal sodium delivery to the macula densa, which regulate tubuloglomerular feedback (16, 17, 20, 24–26, 28, 30, 32, 56, 57).

In addition, beneficial effect of SGLT2 inhibition can also be mediated locally in the kidney tubular cells where glucose reabsorption causes glucotoxicity. Increased glucose uptake in the diabetic kidney is an early event of metabolic adaptation that leads to an increase in renal gluconeogenesis (58) and also abnormal fatty acid oxidation and lipid accumulation (59).

In direct support for an additional direct effect of SGLT inhibition in the kidney, in HK2 cells (human kidney proximal tubular cell line) the SGLT2 inhibitor empagliflozin prevented high glucose-induced increases in TLR4, NF-κB, IL-6, and type IV collagen (57). In another study in human proximal tubular cells in culture, SGLT2 inhibition via siRNA approach decreased high glucose-induced increase in reactive oxygen species generation and receptor of advanced glycation end product expression levels in tubular cells. In addition, high glucose was found to augment the advanced glycation end product-induced tubular cell apoptosis, which was also inhibited by SGLT2 siRNAs (60). Furthermore, another SGLT2 inhibitor tofogliflozin has been shown to block high glucose-induced oxidative stress, inflammation, and apoptosis (33). These studies in proximal tubular cells in culture therefore indicate that inhibition of glucose entry via SGLT2 inhibition has direct protective effects in the kidney, independent of or in addition to any systemic factors of SGLT2 inhibition in the intact animal.

The inhibition of renal tubular glucose transport that results in increased NaCl delivery to the macula densa has been shown to result in increased adenosine release (19). Adenosine and adenosine receptors play a role in regulation of renal hemodynamics and renal inflammation (61). Adenosine receptor mRNA and protein levels have been shown to be significantly altered in streptozotocin-induced diabetes in the rat. There were significant increases in A1-AR (Adora1) and A2a-AR (Adora2a), and a significant decrease in A2b-AR (Adora2b) protein (62). In direct cause and effect studies, in Akita and A1-AR (Adora1) double mutants there were increased glomerular hyperfiltration and increased glomerular injury and urinary albumin excretion (63). In contrast, in streptozotocin diabetic rats, activation of the A2a-AR (Adora2a) resulted in decrease in renal injury and attenuation of inflammation (64). In a recent study in streptozotocin diabetic mice, we found that CD73-dependent generation of adenosine attenuates diabetic nephropathy. CD73 mRNA and protein were elevated in dia-
betic mice, and CD73 knock-out mice had more severe diabetic nephropathy. In addition, A2b-AR (Adora2b) expression was increased in the mice and A2b-AR (Adora2b) knock-out mice had more severe diabetic nephropathy. Treatment of mice with a selective A2b-AR (Adora2b) agonist induced protection from diabetic nephropathy, and this beneficial effect was mediated by endothelial A2b-AR (Adora2b) signaling (53). In contrast, activation of A2b-AR (Adora2b) mediates TGF-β1 (65) and VEGF (66) release from glomeruli of streptozotocin diabetic rats, and in vivo treatment of A2b-AR (Adora2b) antagonist blocks overexpression of VEGF, glomerular injury, and proteinuria (66). In this study in db/db mice, however, we have found that CD73 protein and mRNA expression were decreased, and the adenosine receptors Adora1a, A2a-AR (Adora2a), and A2b-AR (Adora2b)mRNA were increased in diabetic db/db mice. Treatment with SGLT2 inhibitor normalized CD73 and A2b-AR (Adora2b) mRNA. These studies suggest that the regulation and the renal effects of adenosine receptors may be species-specific (rat versus mice), and the models of diabetes were investigated.

Our studies in both insulin-deficient and insulin-intact db/db animals are in agreement with other studies that have shown that SGLT2 inhibition results in decreases in proteinuria and inflammation in the kidney (17, 25, 28–30, 32, 67). In addition, we have also determined that these beneficial effects are associated with significant decreases in mesangial expansion, accumulation of extracellular matrix proteins, fibrillary collagens, and prevention of podocyte loss, which are the hallmarks of diabetic nephropathy (68).

Another new and novel finding of our study is that we have determined that SGLT2 inhibition prevents the accumulation of neutral lipids in the kidney. We have shown that this was mediated in part through inhibition of the fatty acid and triglyceride synthesis genes stearoyl-CoA desaturase-1 (Scd-1) and diacylglycerol O-acyltransferase 1 (Dgat1), which are direct downstream targets of SREBP-1 and Lpk, which is a direct downstream target of the ChREBP, which we have previously shown to be activated in the kidneys of diabetic mice and rats (45–48). Actually, SGLT2 inhibition resulted in inhibition of the novel transcription factor ChREBP-β. In previous studies we showed that increased expression of SREBP-1 per se in the

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**FIGURE 6. Effects of SGLT2 inhibition on kidney adenosine receptor regulation.** A, SGLT2 inhibition increases kidney CD73 protein expression and mRNA level in db/db mice. B, mRNA expression of kidney adenosine receptors Adora1a, Adora2a, and Adora2b are increased in db/db mice. Adora2a and Adora2b are increased in db/db mice. SGLT2 inhibition significantly decreases Adora2b mRNA level in db/db kidneys, whereas the mRNA expression of Adora1a and Adora2a is trending down with SGLT2 inhibition.

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**TABLE 5**

Metabolic parameters for db/db mice with insulin-resistant diabetes

Data are means ± S.E. (n = 12 mice in each group).

<table>
<thead>
<tr>
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<th>db/m</th>
<th>db/db</th>
<th>db/db + SGLT-2 inhibition</th>
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</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>30.9 ± 0.64</td>
<td>54.1 ± 1.92</td>
<td>56.4 ± 1.18</td>
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<td>Food intake (g/mouse/day)</td>
<td>4.0 ± 0.10</td>
<td>7.9 ± 0.20</td>
<td>7.0 ± 0.28</td>
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<tr>
<td>Blood glucose (mg/dl)</td>
<td>116 ± 6.8</td>
<td>376 ± 19.1</td>
<td>122 ± 8.7</td>
</tr>
<tr>
<td>Plasma insulin (ng/ml)</td>
<td>1.67 ± 0.24</td>
<td>3.94 ± 1.62</td>
<td>3.75 ± 1.21</td>
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<tr>
<td>Plasma triglycerides (mg/dl)</td>
<td>64.3 ± 8.5</td>
<td>215.3 ± 26.8</td>
<td>100.7 ± 12.6</td>
</tr>
<tr>
<td>Plasma cholesterol (mg/dl)</td>
<td>53.4 ± 3.1</td>
<td>128.8 ± 14.3</td>
<td>103.3 ± 9.4</td>
</tr>
<tr>
<td>Kidney weight/body weight (×10⁻³)</td>
<td>6.1 ± 0.14</td>
<td>4.6 ± 0.23</td>
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</tr>
<tr>
<td>24-h urine ACR (mg/mmol)</td>
<td>258.3 ± 123.4</td>
<td>973.4 ± 237.4</td>
<td>390.9 ± 115.3</td>
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*a p < 0.05 versus db/m is shown.

*b p < 0.05 versus db/db is shown.
kidneys of SREBP-1 transgenic mice reproduced manifestations of diabetic nephropathy, including proteinuria, mesangial expansion, lipid accumulation, and increased expression of proinflammatory cytokines and profibrotic growth factors (46). In contrast, the manifestations of obesity-related renal disease was prevented in SREBP-1 knock-out mice (69).

Our studies also determine a mode of action for the SGLT2 inhibitor used in this study. Although the administration of the SGLT2 inhibitor induces immediate glycosuria, in isolated proximal tubular brush-border membranes we have found a significant increase in sodium gradient glucose transport activity in db/db mice. This was associated with a significant increase in brush-border membrane SGLT2 protein but a decrease in SGLT1 protein abundance. However, both SGLT2 and SGLT1 mRNA abundance were significantly decreased. These results suggest that the SGLT2 inhibitor used in this study is a competitive inhibitor of apical membrane glucose uptake with compensatory increases in SGLT2 protein abundance. In a recent study SGLT2 inhibition with empagliflozin was shown to increase SGLT2 protein abundance in cortical membranes in wild type mice but not in Akita mice, which had a baseline increase in SGLT2 protein abundance (17). In contrast, SGLT1 protein abundance was decreased in wild type mice following SGLT2 inhibition, but there were no effects in Akita mice that had a baseline decrease in SGLT1 protein abundance (17). This study, however, did not measure sodium glucose transport activity or SGLT2 or SGLT1 mRNA abundance.

In summary, our study shows new and novel effects of SGLT2 inhibition in decreasing renal lipid accumulation and preventing the development of nephropathy in db/db mice by preventing podocyte loss, accumulation of extracellular matrix protein accumulation, and inhibition of inflammation. These beneficial effects are associated with a significant decrease in systolic blood pressure and occur despite stimulation of the intrarenal renin angiotensin system.

**Experimental Procedures**

**Human Kidney Samples**—Formalin-fixed paraffin-embedded tissue specimens (FFPE) of renal biopsy material were obtained from the archives of the Columbia Renal Pathology Laboratory, Department of Pathology at Rabin Medical Center, and Department of Pathology at the University of Colorado. These studies were approved for research by the IRB from Columbia University, Rabin Medical Center, and the University of Colorado. Kidney samples were obtained from left over portions of diagnostic kidney biopsies of patients with diabetic nephropathy (n = 34) or normal kidneys (n = 29). Control tissues were deemed unremarkable on routine histological, immunofluorescence, and electron microscopic examination.

RNA was extracted from archives of FFPE specimens. Total RNA was isolated using RNeasy FFPE Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. RNA quantity and quality were determined by optical density 260/280 nm ratio on a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA was converted to cDNA using RevertAid First Strand cDNA synthesis kit (Fermentas); cDNA was then amplified using TaqMan PreAmp Master Mix (Applied Biosystems) for 14 cycles of preamplification according to the manufacturer’s protocol using target gene assays (51).

Candidate gene expressions were analyzed by real time RT-PCR, performed as described previously (51), using the TaqMan and SYBR system based on real-time detection of accumulated fluorescence (ABI Prism Step One; PerkinElmer Life Sciences). Fluorescence for each cycle was quantitatively analyzed by an ABI Step One sequence detection system (PerkinElmer Life Sciences). To control for variation in the amount of DNA that was available for PCR in the different samples, gene expression of the target sequence was normalized in relation to the expression of an endogenous control, 18S rRNA, or RPLPO (large ribosomal protein).

**Immunohistochemistry**—Deidentified renal biopsy sections (University of Colorado, Columbia University, The John Hopkins University, and Rabin Medical Center) were used per each institution’s IRB guidelines. Staining was performed on 5-μm formalin-fixed paraffin-embedded sections using the Daco EnVision + HRP Mouse (DAB+) staining kit (Dako, product number K400711-2). Paraffin was removed from sections using xylene and dehydrated with a series of graded ethanol. Heat-induced antigen retrieval (pH 9) was performed, and samples were prepared with peroxidase block (Dako, product number S200380-2) and protein block (2% skim milk). Antibody staining of human tissue for hSGLT2 was performed with a polyclonal antibody against a peptide of human raised in rabbit that has been described (56) and detection with DAB.

**Animal Studies**—8-Week-old male db/m and db/db mice (BLK/lj genetic background) were obtained from The Jackson Laboratory (Bar Harbor, ME). They were maintained on a 12-h light/12-h dark cycle. They were acclimated for 2 weeks. Baseline blood glucose and body weight were measured, and the mice were assigned to (a) control diet with no drugs (Research Diets) or (b) control diet supplemented with the SGLT2 inhibitor JNJ-3993673 at a concentration of 0.07 g/kg of diet (Research Diets).

JNJ-3993673 is a potent and selective SGLT2 inhibitor. The IC<sub>90</sub> values of JNJ-3993673 for human SGLT1 and SGLT2 were determined in CHO-K1-hSGLT1 and CHO-K1-hSGLT2 cells treated with 300–500 μM α-methyl D-glucopyranoside (AMG, Sigma) mixed with [<sup>14</sup>C]AMG (PerkinElmer Life Sciences or Amersham Biosciences). The radioactive counts in the cells were assessed in a TopCount (Packard Instrument, Meriden, CT). For pharmacokinetics study, db/db mice were fed with regular diet containing JNJ-3993673 at 0.07 g/kg (Research Diets) for 7 days, and compound concentrations in the plasma were determined at different time points in day 8.

**FIGURE 7. Effects of SGLT2 inhibition on SGLT activity and SGLT2 and SGLT1 protein and mRNA.** A. SGLT2 inhibition increases sodium gradient-dependent glucose transport activity in apical (BBM) in db/db mice. B. Brush-border membrane SGLT2 protein abundance is increased, but SGLT1 protein abundance is decreased with SGLT2 inhibition. Equal amounts of total brush-border membrane were loaded into the protein gels, and the signals were normalized to β-actin. Both SGLT2 (C) and SGLT1 (D) mRNA abundance are significantly decreased with SGLT2 inhibition in db/db mice. E. Glut2 mRNA but not Glut1 mRNA are significantly decreased with SGLT2 inhibition. F. Glucconeogenesis genes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pc) mRNA levels are not significantly changed with SGLT2 inhibition.
**SGLT2 Inhibition and Diabetic Nephropathy**

JNJ39933673 inhibited the [14C]AMG uptake with IC50 values of 3 nm for human SGLT2 and 468 nm for human SGLT1. When db mice were fed with JNJ-39933673 at a concentration of 0.07 g/kg of diet for 8 days, the Cmax values and areas under the curve of JNJ 39933673 were 343 ng/ml and 50,247 hmg/ml.

All control nondiabetic db/m mice and diabetic db/db mice were matched according to their body weight and blood glucose. We studied n = 12 mice in each experimental and treatment group. The mice were treated with these diets for 12 weeks.

We studied two different cohorts of mice as follows: (i) in the first cohort, the db/db mice became insulin-deficient, lost weight, and behaved like type I diabetic mice; (ii) in the second cohort, the db/db mice were the typical insulin-resistant mice and developed hyperglycemia and obesity as a function of time.

Food intake, body weight, and tail vein blood glucose were measured every 2 weeks. Mice were placed in metabolic cages for 24-h urine collection for measurement of volume, creatinine, albumin, and TBARS. Blood pressure was measured using tail artery technique with the BP-2000 Blood Pressure Analysis System (Visitech Systems, Inc., Apex, NC).

At the end of the study period, the mice were anesthetized, and blood was drawn for measurement of glucose, hemoglobin A1C, insulin, triglyceride, and cholesterol.

The kidneys were then rapidly removed for biochemical studies. The kidneys were processed for the following: (a) histology and immunofluorescence microscopy; (b) RNA extraction and real time QPCR; (c) protein extraction and Western blotting; and (d) isolation of apical brush-border membranes from the kidney for the measurement of sodium gradient-dependent glucose transport activity and SGLT2 and SGLT1 protein abundance.

Animal studies and relative protocols were approved by the Animal Care and Use Committee at the University of Colorado Denver. All animal experimentation was conducted in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD).

**Urine Chemistry**—Urine albumin and creatinine concentrations were determined using kits from Exocell (Philadelphia, PA). Urinary TBARS level was measured using the kit from Bioassay Systems (Hayward, CA).

**RNA Extraction and Quantitative Real Time PCR**—RNA extraction and quantitative real time PCR was performed as we have previously described in our publications (45, 50, 72–74).

**Western Blotting**—Brush-border membrane protein content was measured by BCA assay (Thermo Fisher Scientific, Waltham, MA). An equal amount of total protein was separated by SDS-polyacrylamide gels and transferred onto PVDF membranes. The primary antibodies used include anti-SGLT2 (22), anti-SGLT1 (56), and anti-β-actin (Sigma). After HRP-conjugated secondary antibodies, the immune complexes were detected by chemiluminescence captured on UVP Biospectrum (Upland, CA). An equal amount of total protein was separated by SDS-polyacrylamide gels and transferred onto PVDF membranes. The primary antibodies used include anti-SGLT2 (22), anti-SGLT1 (56), and anti-β-actin (Sigma). After HRP-conjugated secondary antibodies, the immune complexes were detected by chemiluminescence captured on UVP Biospectrum (Upland, CA). An equal amount of total protein was separated by SDS-polyacrylamide gels and transferred onto PVDF membranes. The primary antibodies used include anti-SGLT2 (22), anti-SGLT1 (56), and anti-β-actin (Sigma). After HRP-conjugated secondary antibodies, the immune complexes were detected by chemiluminescence captured on UVP Biospectrum (Upland, CA). An equal amount of total protein was separated by SDS-polyacrylamide gels and transferred onto PVDF membranes. The primary antibodies used include anti-SGLT2 (22), anti-SGLT1 (56), and anti-β-actin (Sigma). After HRP-conjugated secondary antibodies, the immune complexes were detected by chemiluminescence captured on UVP Biospectrum (Upland, CA).

**Histology Staining and Immunofluorescence Microscopy**—Sections (4 μm thick) cut from 10% formalin-fixed, paraffin-embedded kidney samples were used for periodic acid-Schiff (PAS) staining. Frozen sections were used for Oil Red O staining of neutral lipid (cholesterol esters and triglycerides) deposits or for immunostaining for fibronectin (Sigma), type IV collagen (Sigma), nephrin (a gift from Dr. Larry Holzman, University of Pennsylvania, Philadelphia, PA), synaptophysin (Sigma), WT1 (Sigma), and CD68 (AbD Serotec, Raleigh, NC) and imaged with a laser scanning confocal microscope (LSM 510, Zeiss, Jena, Germany).

**Quantification of Morphology**—All quantifications were performed in a masked manner. Using coronal sections of the kidney, 30 consecutive glomeruli per mouse, six mice per group, were examined for evaluation of glomerular mesangial expansion. The index of the mesangial expansion was defined as ratio of mesangial area/glomerular tuft area. The mesangial area was determined by assessment of the PAS-positive and nucleus-free area in the mesangium using ScanScope image analyzer (Aperio Technologies, Vista, CA).

**TP-EHG Microscopy**—We performed SHG and TPAF microscopy for label-free imaging collagen and related structures. These images were acquired using a Zeiss 780 microscope (Carl Zeiss, Jena, Germany) equipped with a Coherent Chameleon Ultra II laser (Coherent, Santa Clara, CA). The laser produces a maximum average power of ~4.3 watts of average power at 775 nm at the laser aperture with a pulse duration and repetition rate of 140 fs and 80 MHz, respectively. After passing through the microscope optics, the pulse duration is ~300 fs. For SHG, the laser was tuned to 800 nm. For a ×10 0.3 NA objective, maximum average power at the objective at this wavelength is 45.2 milliwatts. A filter cube containing a narrow band 390–410-nm emission filter (h400/20m-2p, Chroma Technology, Bellows Falls, VT) was used to detect the SHG signal on a non-descanned detector. A dichroic mirror at 425 nm allows detection of the TPAF signal at 450–700 nm (h575/250m-2p, Chroma Technology, Bellows Falls, VT), also with a non-descanned detector.

**Statistical Analysis**—Results are presented as the means ± S.E. for at least three independent experiments. Data were analyzed by analysis of variance and Student-Newman-Keuls tests for multiple comparisons or by Student’s t test for unpaired data between two groups. Statistical significance was accepted at the p < 0.05 level.

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**References**


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