The SGLT2 inhibitor empagliflozin ameliorates early features of diabetic nephropathy in BTBR ob/ob type 2 diabetic mice with and without hypertension

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The transport of glucose across cell membranes is accomplished by two gene families: facilitative glucose transporters (GLUTs) and Na+-glucose cotransporters (SGLTs). In the kidney, high-capacity SGLT2 (SLC5A2) is responsible for the majority of glucose reabsorption in the early proximal tubule, while low-capacity SGLT1 (SLC5A1) reabsors the remaining glucose further distal in the proximal tubule (26, 27). Investigations of transgenic mice have shown that the lack of SGLT1 only has minor effects on glucose reabsorption (15), whereas SGLT2 deficiency results in pronounced glucosuria (33). These experimental findings correspond with clinical data, in which patients with mutations in the Sglt2 gene have sustained renal glucosuria, whereas mutations in the Sglt1 gene have little or no impact on glucosuria (28). Recently, the development of specific and potent SGLT2 inhibitors offers a new antidiabetic therapy via enhanced glucose excretion (23).

Mice carrying the ob/ob mutation, an inactivating mutation in the leptin gene, develop hyperglycemia, hypercholesterolemia, elevated triglycerides, insulin resistance, and subsequently diabetes mellitus type 2, depending on the genetic background (9, 10). In contrast to other background strains, BTBR.Cg-Lep(ob)/H11022 mice have been recently described as an excellent animal model for diabetic nephropathy (18, 24). BTBR ob/ob mice develop progressive proteinuria and a renal histomorphological picture that is quite similar to that seen in human patients with advanced diabetic nephropathy (18). In contrast to the majority of patients with diabetic nephropathy, BTBR ob/ob mice do not develop hypertension but are rather slightly hypotensive (18). Therefore, besides investigation of usual diabetic BTBR ob/ob mice, we tried to further aggravate this diabetic nephropathy model by the induction of hypertension via chronic ANG II infusion in these mice.

To elucidate whether SGLT2 inhibition with empagliflozin (BI-10773, Boehringer Ingelheim) has beneficial effects on the development and progression of diabetic nephropathy in type 2 diabetes, we investigated the effects of empagliflozin compared with placebo in diabetic BTBR ob/ob mice without or with ANG II-mediated hypertension.

**MATERIALS AND METHODS**

**Animals.** Eight-week-old female BTBR ob/ob (18, 24) mice and sex- and age-matched wild-type (WT) control mice were used in the experiments. Animals of both genotypes were littermates from heterozygous parents. Genotypes were verified with PCR using the following primers: LEPPOB, forward 5'-ATTGAACCTGGAG-AATCTCC-3'; LEPOB, reverse 5'-GCCAGATGGGAGGTCTCA-3'; RFLP, forward 5'-TGGATTTGCACAGTGACC-3' and reverse 5'-GCGATCCAGGCTCTGGA-3' (13). Animals were housed at constant humidity (60 ± 5%) and temperature (24 ± 1°C) and with a 12:12-h light-dark cycle (6 AM to 6 PM light). Mice had access to water ad libitum.

All animal experiments were performed in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of...
Table 1. Primer pairs used for quantitative PCR analysis

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>SGLT1</td>
<td>5′-GGAGAGCCATATGACCTGTTCAG-3′</td>
<td>5′-GAGGACATGTTGACAAGACACAC-3′</td>
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<tr>
<td>SGLT2</td>
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<td>5′-ACACAAAGCCCTTGGGAAAGT-3′</td>
</tr>
<tr>
<td>GLUT1</td>
<td>5′-AACATGGAAACGCCACGTC-3′</td>
<td>5′-GTGCGAAGAGGCCACAAG-3′</td>
</tr>
<tr>
<td>GLUT2</td>
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<td>5′-GTGCGAAGAGGCCACAAG-3′</td>
</tr>
<tr>
<td>MCP-1</td>
<td>5′-GTGCGAAGAGGCCACAAG-3′</td>
<td>5′-GTGCGAAGAGGCCACAAG-3′</td>
</tr>
<tr>
<td>RANTES</td>
<td>5′-AACAGGCCCTTGGGACAG-3′</td>
<td>5′-GCAAGAGGACATGTTG-3′</td>
</tr>
<tr>
<td>IL-6</td>
<td>5′-AACAGGCCCTTGGGACAG-3′</td>
<td>5′-GCAAGAGGACATGTTG-3′</td>
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<tr>
<td>L32</td>
<td>5′-TGTGGGATGACGATATACGG-3′</td>
<td>5′-TGAAAGGAAATGGGCGACG-3′</td>
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SGLT, Na\(^+\) glucose cotransporter; GLUT, glucose transporter; MCP-1, monocyte chemoattractant protein-1; RANTES, regulated on activation normal T cell expressed and secreted.
Empagliflozin (EMPA) differentially regulates renal Na\(^+\)-glucose cotransporter (SGLT)1 and SGLT2 protein and mRNA expression, whereas renal glucose transporter (GLUT)1 and GLUT2 mRNA expression were not affected. A 300 ppm EMPA diet or an equicaloric control diet [placebo (P)] were fed to BTBR.Cg-Lep\(^{-}\)/WiscJ (BTBR ob/ob) mice. A: staining with SGLT1-specific antibody revealed SGLT1 protein expression in the brush-border membrane of late proximal convoluted tubules. B: staining with SGLT2-specific antibody revealed SGLT2 protein expression in early proximal convoluted tubules. C: representative Western blots showing renal membrane SGLT1 and SGLT2 protein expression. D: quantification of SGLT1 and E SGLT2 protein expression. Protein expression was normalized to β-actin. Relative protein expression is shown as fold changes of the wild-type (WT) group. F–I: renal mRNA expression of SGLT1 (F), SGLT2 (G), GLUT1 (H), and GLUT2 (I) were quantified. mRNA levels were normalized to ribosomal L32. Relative gene expression is shown as fold changes of the WT group. Data were analyzed using Student’s t-test; n ≥ 6. *P < 0.05 and **P < 0.01 vs. WT mice.

(STZ)-induced diabetic mice (8, 34), renal mRNA expression of SGLT2 was unchanged in BTBR ob/ob mice (Fig. 1G). Empagliflozin treatment did not regulate renal mRNA expression. To investigate the expression of other glucose transporters in kidneys of BTBR ob/ob mice, we quantified GLUT1 (Fig. 1H) and GLUT2 mRNA (Fig. 1I), but neither diabetic state in BTBR ob/ob mice compared with WT mice nor treatment with empagliflozin influenced the expression levels of the two transport proteins.

Basal characteristics of empagliflozin-treated BTBR ob/ob mice. WT mice at the age of 8 wk weighed 26 ± 0.5 g and gained 6 ± 0.6 g over the 12 wk of the experiment (Table 2).
Empagliflozin lowers blood glucose in diabetic BTBR ob/ob mice

Wild-Type Mice  Placebo-Treated BTBR ob/ob Mice  Empagliflozin-Treated BTBR ob/ob Mice

<table>
<thead>
<tr>
<th>Age, wk</th>
<th>Body weight, g</th>
<th>Glucose:creatinine ratio, mmol/mg creatinine</th>
<th>Systolic arterial pressure, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>26.2 ± 0.5</td>
<td>2.7 ± 0.6</td>
<td>116 ± 2.2</td>
</tr>
<tr>
<td>14</td>
<td>30.3 ± 0.3</td>
<td>3.4 ± 1.5</td>
<td>110 ± 2.0</td>
</tr>
<tr>
<td>16</td>
<td>33.2 ± 0.7</td>
<td>3.2 ± 0.8</td>
<td>120 ± 1.4</td>
</tr>
<tr>
<td>18</td>
<td>36.1 ± 1.2</td>
<td>2.9 ± 2.0</td>
<td>120 ± 1.4</td>
</tr>
<tr>
<td>20</td>
<td>39.8 ± 2.9</td>
<td>2.9 ± 2.1</td>
<td>120 ± 1.4</td>
</tr>
<tr>
<td>22</td>
<td>42.3 ± 3.0</td>
<td>2.9 ± 2.1</td>
<td>120 ± 1.4</td>
</tr>
<tr>
<td>24</td>
<td>45.0 ± 3.1</td>
<td>2.9 ± 2.1</td>
<td>120 ± 1.4</td>
</tr>
</tbody>
</table>

Note: Data are means ± SE; 8 mice/group. A 300 ppm empagliflozin diet or an equicaloric control diet (placebo) were fed to BTBR.Cg-Lep ob/ob mice. Empagliflozin treatment had no impact on body weight gain, induced strong and persisting glucosuria (glucose-to-creatinine ratio), significantly lowered blood glucose, had no impact on systolic arterial pressure, and attenuated the renin-angiotensin-aldosterone system.

Glomerular matrix expansion was quantified by the mesangial index fraction of the glomerular tuft. Diabetic BTBR ob/ob showed a significantly increased mesangial index fraction compared with WT mice (Fig. 3A). Similar to the effects on albuminuria and glomerular hypertrophy, empagliflozin treatment was able to ameliorate the increased glomerular matrix expansion. Furthermore, we analyzed the mRNA expression of the inflammatory markers monocye chemoattractant protein (MCP)-1 [chemokine (C-C motif) ligand 2 (Ccl2)], regulated on activation normal T cell expressed and secreted (RANTES) (Ccl5), and IL-6 in kidney samples. In 14-wk-old diabetic BTBR ob/ob mice (6 wk of placebo diet), MCP-1 (∼1.4-fold, P < 0.05 vs. WT mice; Fig. 3B), RANTES (∼2.6-fold, P < 0.05 vs. WT mice; Fig. 3C), and IL-6 (∼2.4-fold, P < 0.05 vs.
WT mice; Fig. 3D) mRNA expression were significantly increased. None of these inflammatory markers was significantly affected after 6 wk of empagliflozin treatment. However, 12 wk of empagliflozin treatment reduced renal mRNA expression of MCP-1 by ~75% ($P < 0.05$ vs. placebo; Fig. 3B, inset), RANTES by ~55% ($P < 0.05$ vs. placebo; Fig. 3C, inset), and IL-6 by 52% ($P < 0.05$ vs. placebo; Fig. 3D, inset).

**Characterization of hypertensive diabetic mice.** Chronic ANG II infusion using Alzet minipumps over 6 wk induced profound and lasting hypertension in diabetic BTBR ob/ob mice (Table 3), reaching blood pressure values of almost 150 mmHg. Similar to nonhypertensive BTBR ob/ob mice, ANG II-infused mice on both diets were obese (placebo: 52 ± 1.3 g and empagliflozin: 52 ± 1.6 g; Table 3) and, independently of the drug therapy, gained weight fast (placebo: 9 ± 1.4 g and empagliflozin: 12 ± 3.5 g). Chronic ANG II infusion led to more pronounced glucosuria in BTBR ob/ob mice before the treatment started (hypertensive 4,814 ± 630 μmol/mg creatinine and normotensive: 2,182 ± 972 μmol/mg creatinine, $P < 0.001$; Tables 2 and 3). In BTBR ob/ob mice, ANG II infusion itself led to a further increase of glomerular hypertrophy (3,877 ± 88.6 vs. 3,646 ± 59.8 μm², $P < 0.05$; Figs. 2A and 4A) but

Fig. 3. EMPA treatment reduces glomerular matrix expansion and renal expression of proinflammatory cytokines. A 300 ppm EMPA diet or an equicaloric control diet (P) were fed to BTBR ob/ob mice. A: glomerular matrix expansion was quantified using the mesangial index fraction of glomerular tufts. More than 30 glomeruli were analyzed per mouse. B–D: Renal mRNA expression of monocyte chemoattractant protein (MCP)-1 [chemokine (C-C motif) ligand 2 (Ccl2); B], regulated on activation normal T cell expressed and secreted (RANTES) (Ccl5; C), and IL-6 (D), were quantified, demonstrating a significant reduction after 12 wk (insets) but not 6 wk of empagliflozin treatment. mRNA levels were normalized to ribosomal L32. Relative gene expression is shown as fold changes of the control group. Data were analyzed using Student’s t-test; $n \geq 8$. *$P < 0.05$ vs. WT mice; **$P < 0.05$ vs. placebo-treated BTBR ob/ob mice.
Increased glucose excretion already 3 wk after the start of treatment (empagliflozin: 9,716 ± 3,820 μg/mg creatinine; placebo: 5,303 ± 2,291 μg/mg creatinine; P < 0.05). As seen in Table 3, empagliflozin treatment had no significant impact on creatinine clearance in hypertensive BTBR ob/ob mice. Data were analyzed using Student’s t-test; *P < 0.05 and †P < 0.001 vs. placebo treatment.

Effect of empagliflozin treatment in hypertensive BTBR ob/ob mice. Similar to normotensive mice, empagliflozin treatment in these hypertensive mice reduced blood pressure not significantly by 6 mmHg (Table 3) but was still able to further increase glucose excretion already 3 wk after the start of treatment (empagliflozin: 9,716 ± 658 μg/mg creatinine and placebo: 5,303 ± 947 μg/mg creatinine, P < 0.05), which was sustained for the full experimental time. Empagliflozin successfully reduced blood glucose levels also in ANG II-infused mice after 3 wk by 140 and 240 mg/dl after 6 wk, respectively (Table 3). In contrast to normotensive BTBR ob/ob mice, empagliflozin had no significant impact on creatinine clearance (Table 3) and subsequently no effect on glomerular hypertrophy in hypertensive BTBR ob/ob mice (Fig. 4A). Nevertheless, empagliflozin still ameliorated albuminuria by 70% in hypertensive BTBR ob/ob mice (1,470 ± 226 vs. 4,869 ± 1,776 μg/mg creatinine, P < 0.05; Fig. 4B). However, empagliflozin treatment had no effect on glomerular matrix expansion (Fig. 4C) in hypertensive BTBR ob/ob mice.

In hypertensive BTBR ob/ob mice, empagliflozin treatment did not influence renal mRNA expression of SGLT1 (data not shown) or SGLT2 (data not shown). Similar to normotensive diabetic mice, 6 wk of empagliflozin treatment was not able to reduce renal mRNA expression of MCP-1 (data not shown), RANTES (data not shown), or IL-6 (data not shown) in ANG II-induced hypertensive mice.

DISCUSSION

To our knowledge, this is the first study investigating the impact of long-term SGLT2 inhibitor treatment on the development of diabetic nephropathy.
empagliflozin also in ANG II-infused hypertensive mice. Increased glucosuria also in ANG II-infused hypertensive mice. The key findings of the present study are that the selective SGLT2 inhibitor empagliflozin, via increasing urinary glucose excretion, lowers blood glucose levels in type 2 diabetic BTBR ob/ob mice independently of accompanying hypertension. Empagliflozin markedly decreased albuminuria in both experimental settings, whereas empagliflozin ameliorated early diabetes-related features in the kidney, such as glomerular hypertrophy/hyperfiltration, markers of inflammation, and mesangial matrix expansion only in normotensive but not hypertensive BTBR ob/ob mice.

In contrast to STZ-induced (34) and Akita/+ (32) type 1 diabetes models, SGLT2 inhibition induced a profound glucosuria in BTBR ob/ob mice, as expected. Tubular glucose reabsorption is predominantly mediated by the two Na+-glucose transporters SGLT1 and SGLT2 and glucose transporters of the GLUT family (17). We hypothesized that the effectiveness of the SGLT2 inhibitor empagliflozin depends on the expression level of the renal target SGLT2 during the diabetic disease process. To verify this, we analyzed mRNA expression of SGLT1, SGLT2, GLUT1, and GLUT2. In contrast to the STZ-induced diabetes type 1 model, in which SGLT2 expression is downregulated (8), SGLT2 mRNA and protein expression are unaltered in the BTBR ob/ob model of type 2 diabetes. This may explain why the SGLT2 inhibitor empagliflozin more efficiently (by ~50%) lowered blood glucose levels compared with STZ-induced diabetic mice with a complete lack of SGLT2 (34). In addition, BTBR ob/ob mice compared with BTBR WT mice demonstrated decreased renal SGLT1 mRNA expression but increased renal SGLT1 protein levels, both of which were not further altered under complete SGLT2 blockade. While this result was surprising and cannot be explained, a previous study (4) has also demonstrated differential expression of SGLT1 mRNA and protein. From these results, we interpret that diabetic BTBR ob/ob mice do not show a mechanism of compensatory increased SGLT1-mediated glucose reabsorption under complete SGLT2 blockade, as has been demonstrated by Vallon and coworkers in C57Bl/6 mice (25).

The most common early pathophysiological changes seen in diabetic nephropathy in both human and animal models are glomerular hyperfiltration/hypertrophy and albuminuria. In the “tubulocentric model,” the glomerular hyperfiltration and hypertrophy in diabetes are caused by an enhanced tubuloglomerular feedback mechanism (31, 35). Recently, it has been shown that SGLT2 inhibition activates the tubuloglomerular feedback mechanism (35) and thereby normalizes glomerular hyperfiltration under diabetic conditions (30). These results are consistent with our study, in which empagliflozin treatment attenuated the glomerular hypertrophy seen in normotensive BTBR ob/ob mice, which was associated with a numerical reduction of the increased creatinine clearance.

Empagliflozin treatment reduced blood glucose levels and increased glucosuria also in ANG II-infused hypertensive BTBR ob/ob mice, whereas it had no effect on both creatinine clearance and glomerular hypertrophy. The results in our diabetic mice suggest that ANG II infusion markedly overrides the milder effects that may be achieved by the use of empagliflozin in regard to the tubuloglomerular feedback mechanism. Consistent with this assumption, a study by Moniwa and coworkers (22) implicated that ANG II inhibits the tubuloglomerular feedback, which seems to be necessary for the empagliflozin effect independent of successful reduction of the blood glucose levels.

An increase in urinary albumin excretion is one of the earliest clinical symptoms of microvascular damage (2). In diabetic conditions, albuminuria is the result of an increased amount of filtered albumin in the injured glomeruli and a disturbed tubular function, which leads to reduced albumin reabsorption in the proximal tubules by endocytosis (6). Diabetic BTBR ob/ob mice reveal a profound albuminuria. Treatment of normotensive and hypertensive BTBR ob/ob mice with empagliflozin led to a significant reduction of albuminuria. In accordance with a previous study in Akita/+ mice (32), our data suggest that the amelioration of albuminuria by empagliflozin is a result of a mechanism secondary to the substantial blood glucose reduction and apparently independent of the tubuloglomerular feedback loop. These early changes in diabetic nephropathy are subsequently followed by gradual and progressive accumulation of the mesangial extracellular matrix, which appeared to be reduced via empagliflozin treatment. Although this effect was only mild and restricted to BTBR ob/ob mice without hypertension, it further supports the potential of empagliflozin as a treatment for diabetic nephropathy. In recent years, data from experimental models (38) and clinical trials (16) have established inhibition of the renin-angiotensin system (RAS) as the major preventive strategy for patients with type 2 diabetes at risk to develop diabetic nephropathy. While we did not compare the effects of empagliflozin treatment with effects of RAS inhibition, the lower efficacy of empagliflozin in our hypertensive disease model, taken together with experimental data from diabetic endothelial nitric oxide synthase-deficient db/db mice (38), suggest that angiotensin-converting enzyme inhibition is more potent in hypertensive diabetic nephropathy. However, further studies are needed to dissect the individual effects of RAS and SGLT2 inhibitors in the same disease model and to elucidate the potential of a combined therapeutic approach.

Under diabetic conditions, hyperglycemia and the resulting glucotoxicity are the driving force for inflammatory processes in the kidney. Treatment of BTBR ob/ob mice with empagliflozin for 12 wk led to reduced renal inflammation, as demonstrated by the significant reduction in mRNA expression of the proinflammatory cytokines MCP-1, RANTES, and IL-6. Despite the significant reduction of blood glucose in ANG II-infused BTBR ob/ob mice, empagliflozin treatment for 6 wk did not influence the expression of those chemokines in hypertensive BTBR ob/ob mice. Again, ANG II elicits direct effect on the expression of such proinflammatory cytokines (21, 37), which could mask the positive empagliflozin effects seen in the normotensive group of animals. However, treatment of hypertensive mice was performed only for 6 wk. Such a short treatment period was also not sufficient in normotensive mice to reduce the expression of these cytokines.
Beside these “on target” effects, SGLT2 inhibition is also associated with additional favorable effects for type 2 diabetic patients, such as weight loss (7) and decreased blood pressure (3). In animal (20, 32) and clinical studies with type 2 diabetic patients (3, 36), it has been shown that SGLT2 inhibition leads to weight loss. Increased urinary Na+ and glucose excretion under the SGLT2 inhibitor therapy induces osmotic diuresis, which contributes to weight loss. In BTBR ob/ob mice, empagliflozin treatment did not influence body weight, which also could be a consequence of the manifested hyperphagia. Furthermore, polyuria in empagliflozin-treated diabetic mice was not further increased in our experiments, and, thus, osmotic diuresis does not contribute to the reduction of body weight. In type 2 diabetic patients, empagliflozin treatment slightly reduces blood pressure by 3–7 mmHg (14). In Akita/+ mice, empagliflozin treatment prevented the rise in blood pressure seen in these mice on a placebo diet (32). In diabetic BTBR ob/ob mice without or with hypertension, empagliflozin slightly reduced systolic blood pressure to a comparable extent as seen in humans, but the detected blood pressure effect did not reach significance, which was most likely due to the limited number of animals as well as some variability caused by the tail-cuff system used for blood pressure measurements.

Taken together, our present results demonstrate that SGLT2 inhibition by empagliflozin is a good therapeutic option to lower blood glucose levels in type 2 diabetes. Moreover, empagliflozin treatment differentially ameliorates markers of renal injury, such as albuminuria, hyperfiltration/hypertrophy, inflammation, and mesangial matrix expansion in murine diabetic nephropathy without or with hypertension, supporting the concept of SGLT2 inhibition for the prevention of diabetic nephropathy.

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AUTHOR CONTRIBUTIONS

REFERENCES


