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Original Paper

Dapagliflozin Aggravates Renal Injury via Promoting Gluconeogenesis in db/db Mice

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Key Words
SGLT2 inhibitor • Type 2 diabetes mellitus • Diabetic nephropathy • Glucose homeostasis • Gluconeogenic key enzymes

Abstract
Background/Aims: A sodium-glucose co-transporter-2 inhibitor dapagliflozin is widely used for lowering blood glucose and its usage is limited in type 2 diabetes mellitus patients with moderate renal impairment. As its effect on kidney function is discrepant and complicated, the aim of this study is to determine the effect of dapagliflozin on the progression of diabetic nephropathy and related mechanisms. ***Methods:*** Twelve-week-old male C_{57}BL/6 wild-type and db/db mice were treated with vehicle or 1 mg/kg dapagliflozin for 12 weeks. Body weight, blood glucose, insulin tolerance, glucose tolerance, pyruvate tolerance and 24-hour urine were measured every 4 weeks. At 24 weeks of age, renal function was evaluated by blood urea nitrogen level, creatinine clearance, urine output, urinary albumin excretion, Periodic Acid-Schiff staining, Masson's trichrome staining and electron microscopy. Changes in insulin signaling and gluconeogenic key regulatory enzymes were detected using Western blot analysis. ***Results:*** Dapagliflozin did not alleviate but instead aggravated diabetic nephropathy manifesting as increased levels of microalbuminuria, blood urea nitrogen, and glomerular and tubular damage in db/db mice. Despite adequate glycemic control by dapagliflozin, urinary glucose excretion increased after administration before 24 weeks of age and was likely associated with renal impairment. Increased urinary glucose excretion was mainly derived from the disturbance of glucose homeostasis with elevated hepatic and renal gluconeogenesis induced by dapagliflozin. Although it had no effect on insulin sensitivity and glucose tolerance, dapagliflozin further induced the expression of gluconeogenic key rate-limiting enzymes through increasing the expression levels of FoxO1 in the kidney and liver. ***Conclusion:*** These experimental results indicate that dapagliflozin aggravates diabetes mellitus-induced kidney injury, mostly through increasing gluconeogenesis.

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Introduction

According to the International Diabetes Federation, the prevalence of diabetes mellitus (DM) was almost 8.8% in 2017, and by 2045, approximately 629 million people will have DM worldwide. In high-income countries up to 91% of adult DM belongs to type 2, T2DM. Long-term poor glycemic control in patients with DM can lead to cardiovascular diseases, nephropathy, neuropathy and retinopathy, which are responsible for high morbidity and mortality in patients with T2DM [1, 2]. It is reported that diabetic nephropathy (DN) occurs in 20~30% of diabetic patients [3]. Normalizing hyperglycemia is necessary to slow the progression of the diabetic process [1] and to reduce the risk of complications including cardiovascular disease and nephropathy [2].

Although the glycemic control in patients with T2DM is improving with the use of common clinical drugs, it is still difficult for most cases to achieve adequate glycemic control without dose-limiting side effects such as the potential for hypoglycemia, weight gain and sustained insulin resistance [1, 4]. Moreover, current interventions are almost insulin-dependent, mainly through stimulating insulin secretion or addressing peripheral insulin resistance. Therefore, the researches and development of novel anti-hyperglycemic agents for T2DM with an insulin-independent mechanism are of interest in the drug discovery field.

More recent research has focused on the kidney as a therapeutic target, especially because maximal renal glucose reabsorption is increased in T2DM. Sodium-glucose co-transporter-2 (SGLT2) is responsible for 97% of glucose reabsorption in the renal proximal tubule, and its expression and activities are increased in T2DM [5]. SGLT2 inhibitors are new medications that improve cardiovascular and renal complications in patients with T2DM. Dapagliflozin is the first highly selective SGLT2 inhibitor approved by the European Medicine Agency and the U.S. Food and Drug Administration. A clinical trial of dapagliflozin is promising because it significantly reduces hemoglobin A1c without increasing risk of hypoglycemia [6, 7]. Dapagliflozin also improves insulin sensitivity and β-cell functions [8, 9], decreases body weight and lowers systolic blood pressure independently from insulin secretion or action [10]. Clinical data show that dapagliflozin lowers the estimated glomerular filtration rate [11] and then slows the progression of DN. It is also reported that dapagliflozin improves DN not only by lowering blood glucose but also by inhibiting inflammation and oxidative stress in db/db mice [12]. However, the albuminuria-lowering action of dapagliflozin is variable and dependent on renal function [13]. The U.S. Food and Drug Administration has issued alerts regarding increased acute kidney injury risk with canagliflozin or dapagliflozin. Consistent with this, the effectiveness of SGLT2 inhibitors is decreased with the increasing severity of renal impairment, requiring dosage adjustments or restrictions with moderate-to-severe renal dysfunction. Therefore, further evaluation regarding the long-term effect of dapagliflozin on kidney with impaired function is needed. The purpose of this study is to determine the effects of dapagliflozin on the progression of DN and related mechanism in db/db mice with renal impairment.

Materials and Methods

Animals

Male C_{57}BL/6 wild-type mice and male C_{57}BL/6 db/db mice at 12 weeks of age were acquired from the Animal Center of Peking University Health Science Center. The mice were maintained on a standard diet and had free access to water. For the experiment, mice were housed in cages in a light-, temperature- and humidity- controlled environment. The wild-type mice and db/db mice were randomly divided into groups: control group and dapagliflozin treated group. Vehicle or 1 mg/kg dapagliflozin (product code: HY-10450, brand: MCE) was orally administered once daily for 12 weeks. The mice were placed in metabolic cages adapted for one day, and 24-hour urine was collected every 4 weeks. The body weight was measured every 4 weeks. The blood glucose was measured after 8 h of fasting every 4 weeks. The mice at 24 weeks of age were killed to collect blood and tissues for the subsequent experiments. Data were collected from 9-10

animals in each group. All animal experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, eighth Edition, 2011) and were approved by the Peking University Health Science Center Animal Experimentation Ethics Committee (Laboratory animal use license No. XYSK (JING) 2011-0039, Laboratory animal production license No. SCXK (JING) 2011-0012).

Insulin, glucose, and pyruvate tolerance testing

An insulin tolerance test (1 IU/kg insulin i.p.) and glucose tolerance test (1.5 g/kg glucose i.p.) were performed in mice (every 4 weeks after vehicle or dapagliflozin treatment) after 6 h of fasting. A pyruvate tolerance test (2 g/kg pyruvate i.p.) was performed in mice (every 4 weeks after vehicle or dapagliflozin treatment) after 16 h of fasting. Blood (5 μl) was collected from the tail vein at 0, 15, 30, 60, 90, and 120 min after injection and then mixed with 45 μl saline. The samples were centrifuged at 1100×g, at 4 °C for 15 min and the supernatant was immediately collected to determine glucose concentrations using the glucose oxidase-peroxidase method (NJJC Bio) according to the manufacturer's instructions. Data were expressed as a blood glucose change relative to 0 min.

Urine and blood chemistry

Urea concentrations in urine and blood was measured using a QuantiChrom Urea Assay kit (BioAssay Systems Q20). Creatinine and glucose concentration in urine and blood were measured with commercial kits (NJJC Bio) according to the manufacturer's instructions. Albumin was measured with mouse microalbuminuria ELISA kits (EIA06044m, Wuhan Xinqidi Biological technology) according to the manufacturer's instructions. Urinary osmolality was measured using freezing point depression (Micro-osmometer, FISKER ASSOCIATES, Norwood, Massachusetts, USA).

Renal histological assessment and ultrastructural examination

The kidneys were fixed with 4% paraformaldehyde overnight, dehydrated in graded alcohol and then embedded in paraffin for staining with Periodic Acid-Schiff (PAS) to assess renal injury and Masson staining to assess the level of collagen deposition. Renal tubular damages were assessed using a tubular damage score, as previously described [14, 15]. In brief, tubular injury was scored in a blinded manner according to the percentage of damage including atrophy and flattening of proximal tubule epithelial cell, and tubular dilation: 0 = normal; 1 = < 20%; 2 = 20 to 40%; 3 = 40 to 60%; 4 = 60 to 80%; and 5 = > 80%. Ten random pictures per kidney section were quantified. Glomerular diameter was measured using Image-Pro Plus 3.0 (Media Cybernetics, Silver Spring, MD).

For ultrastructural evaluation, kidneys that were 1 mm^3 in size, were fixed in 2.5% glutaraldehyde, postfixed in osmium tetroxide, and stained with uranyl acetate and lead citrate. The specimen was thin-sectioned and examined under a transmission electron microscope. Electron microscopic pictures were randomly taken in each group.

Western blot analysis

Renal cortex or liver were lysed in RIPA lysis buffer containing protease inhibitor cocktail (Roche). Total protein was measured by BCA (Pierce). The lysates were electrophoresed on polyacrylamide gels and electrotransferred to polyvinylidene difluoride membranes (Amersham Biosciences). After blocking, the membranes were incubated with antibodies against β-actin (Santa Cruz, sc-47778, 1:5000 dilution), thymoma viral proto-oncogene 1 (Akt1, Santa Cruz, sc-5298, 1:500 dilution), p-Akt1 Ser473 (Abclonal, AP0140, 1:2000 dilution), p-Akt2 Ser474 (Abcam, ab38513, 1:1000 dilution), p-S6 Ser235/236 (CST, 2211, 1:1000 dilution), S6 (CST, 2217, 1:1000 dilution), cytosolic phosphoenolpyruvate carboxykinase 1 (PEPCK1, Bioworld, BS6870, 1:5000 dilution), glucose-6-phosphatase (G6Pase, Santa Cruz, sc-25840, 1:500 dilution), hepatic nuclear factor 4, alpha (HNF-4α, Abcam, ab181604, 1:1000 dilution), PPARγ coactivator-1α (PGC-1α, Abcam, ab54481, 1:1000 dilution), forkhead box O1 (FoxO1, CST, 2880, 1:1000 dilution), p-FoxO1 Ser 256 (CST, 9461, 1:1000 dilution), glycogen synthase kinase 3 beta (Gsk3β, CST, 9315, 1:1000 dilution), p-Gsk3β Ser9 (CST, 9336, 1:1000 dilution), insulin receptor substrate 1 (IRS1, CST, 2382, 1:1000 dilution), p-IRS1 Ser302 (CST, 2384, 1:1000 dilution), IRS2 (CST, 4502, 1:1000 dilution), p-IRS2 Ser731 (Abcam, ab3690, 1:1000 dilution), insulin receptor beta (InRβ, CST, 3025, 1:1000 dilution), and anti-p-InRβ (Santa Cruz, sc-81500, 1:500 dilution). Goat anti-rabbit IgG or goat anti-mouse IgG (Santa Cruz) were added and the blots

were developed with ECL plus kit (Amersham Biosciences). Quantitation was performed by scanning and analyzing the intensity of the hybridization band.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). The data were expressed as the means ± SEM, and each experiment was performed at least three times. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparison test. A "P value" < 0.05 was considered statistically significant.

Results

Dapagliflozin promoted urinary glucose excretion and lowered fasting blood glucose in db/db mice

Db/db mice are usually used as the typical T2DM animal model and show significant metabolic syndromes, including obesity, hyperglycemia and lipid metabolism disorders [16]. In our study, db/db mice showed increased body weight (Fig. 1A), fasting blood glucose (Fig. 1B), urine output (Fig. 1C) and urinary glucose excretion (Fig. 1D) compared with wild-type mice from 12 to 24 weeks. Dapagliflozin treatment did not affect body weight, but significantly decreased fasting blood glucose level in db/db mice from 12 to 24 weeks (Fig. 1). Additionally, it increased urine output and urinary glucose excretion at 16 and 20 weeks and had no effect in db/db mice at 24 weeks (Fig. 1). Body weight and fasting blood glucose were unchanged, but urine output and urinary glucose excretion increased significantly after dapagliflozin treatment in wild-type mice (Fig. 1).

Dapagliflozin aggravated DM-induced nephropathy in db/db mice

To determine the functional and structural changes in kidneys, blood and kidney samples were collected after 12 weeks of vehicle or dapagliflozin administration. The ratio of kidney weight to body weight (Fig. 2A) significantly decreased and urinary albumin excretion (Fig. 2B), urinary albumin/creatinine ratio (Fig. 2C), and creatinine clearance (Fig. 2D) increased evidently in db/db mice compared with wild-type mice. However, Fig. 2E shows that there was no difference in BUN between db/db mice and wild-type mice. The increased urinary albumin excretion and urinary albumin/creatinine ratio implied that db/db mice had progressive mild renal impairment from 12 weeks to 24 weeks (Fig. 2B and Fig. 2C). The results showed that dapagliflozin treatment increased the ratio of kidney weight to body weight, urinary albumin excretion, urinary albumin/creatinine ratio and BUN but had no effect on creatinine clearance (Fig. 2). Urinary creatinine excretion, urinary urea excretion, and urinary osmolality

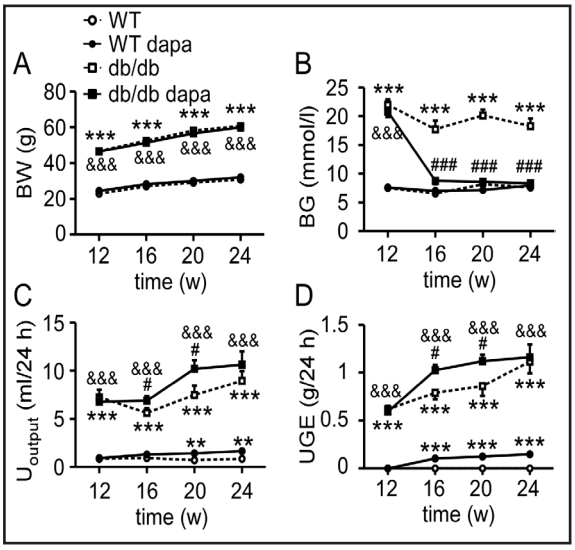

Fig. 1. Dapagliflozin lowered blood glucose through promoting urinary glucose excretion in db/db mice. Wild-type mice and db/db mice were administrated with dapagliflozin or saline once daily for 12 weeks. (A) Body weight (BW). (B) Blood glucose (BG). (C) Urine output (U_{output}). (D) Urinary glucose excretion (UGE). Means ± SEM, n = 9. *P<0.05, **P<0.01 and ***P<0.001 vs. wild-type (WT) mice. #P<0.05, ##P<0.01 and ###P<0.001 vs. db/db mice. &P<0.05, &&P<0.01 and &&&P<0.001 vs. dapagliflozin treated wild-type mice. One-way ANOVA, Bonferroni's multiple comparison test.

significantly increased in db/db mice and were not affected by dapagliflozin (Fig. 2F-H).

To determine the effect of dapagliflozin treatment on renal morphology and ultrastructure, PAS staining, Masson's trichrome staining, and transmission electron microscopy were performed. The renal morphological changes were found in db/db mice, including tubular dilatation, loss of proximal tubule brush border (Fig. 3A), and collagen deposition (Fig. 3B). Transmission electron microscopy showed ultrastructures of normal glomerulus (Fig. 3C) and tubular epithelial cell damage including mitochondrial swelling and necrosis (Fig. 3D) in db/db mice. Dapagliflozin treatment induced glomerular injury, including collagen deposition (Fig. 3B), glomerular basement membrane thickening, fusion of endothelial cells, foot process fusion (Fig. 3C), and increased glomerular diameter (Fig. 3E). It also further accelerated serious tubular injury (Fig. 3F) and tubular epithelial cell damage including mitochondrial swelling and necrosis (Fig. 3D). No obvious change in renal morphology was observed in wild-type and dapagliflozin treated wild-type kidneys. These data indicated that dapagliflozin treatment aggravated the progression of nephropathy in db/db mice with renal damage.

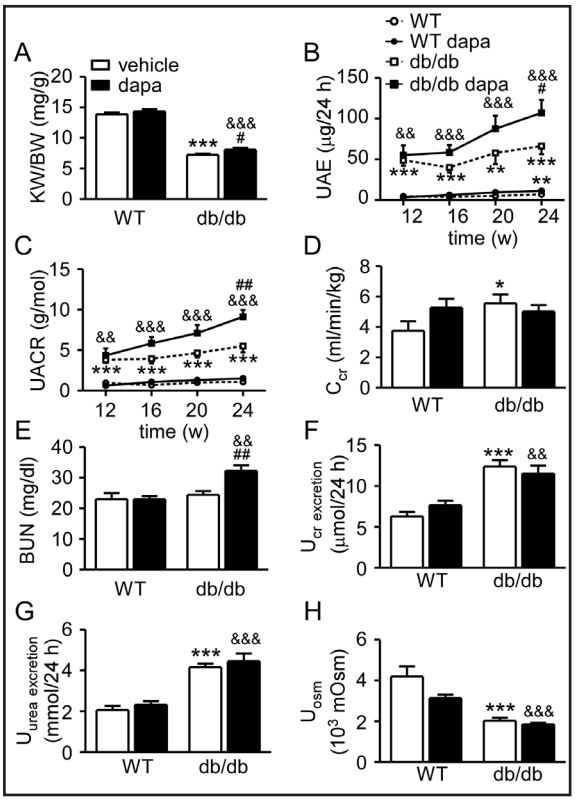

Fig. 2. Dapagliflozin aggravated impaired renal function induced by DM in db/db mice. (A) The ratio of kidney weight to body weight (KW/BW). (B) Urinary albumin excretion (UAE). (C) The ratio of urinary albumin and creatinine (UACR). (D) Creatinine clearance (C_{cr}). (E) Blood urea nitrogen (BUN). (F) Urinary creatinine excretion ($U_{cr\ excretion}$). (G) Urinary urea excretion ($U_{urea\ excretion}$). (H) Urinary osmolality (U_{osm}). Means ± SEM, n = 9. *P<0.05, **P<0.01 and ***P<0.001 vs. wild-type mice (WT). #P<0.05, ##P<0.01 vs. db/db mice. &P<0.05, &&P<0.01 and &&&P<0.001 vs. dapagliflozin treated wild-type mice. One-way ANOVA, Bonferroni's multiple comparison test.

Dapagliflozin promoted the disorder of glucose homeostasis in db/db mice

To investigate the consequences of dapagliflozin treatment on glucose homeostasis, key metabolic parameters were measured. The intraperitoneal insulin tolerance test showed that insulin sensitivity was significantly reduced in db/db mice as evidenced by an increase in the area under the curve (AUC) by 70.4% at 24 week, treatment with dapagliflozin for 12 weeks had no effect on insulin sensitivity (Fig. 4A and Fig. 4D). A glucose tolerance test showed that glucose clearance was significantly reduced in db/db mice as demonstrated by the upregulation of AUC by 31.6% at 24 week and slight downregulation after dapagliflozin administration (Fig. 4B and Fig. 4E). Surprisingly, gluconeogenesis was enhanced in db/db mice evidenced by a 32.1% increase in the AUC and was further elevated after dapagliflozin administration (increased AUC by 40.5%) at 24 week (Fig. 4C and Fig. 4F). Increased gluconeogenesis by dapagliflozin treatment occurred from 20 weeks in db/db mice (Fig. 4C and Fig. 4F). It was also found that dapagliflozin had no effect on glucose homeostasis in wild-type mice (Fig. 4).

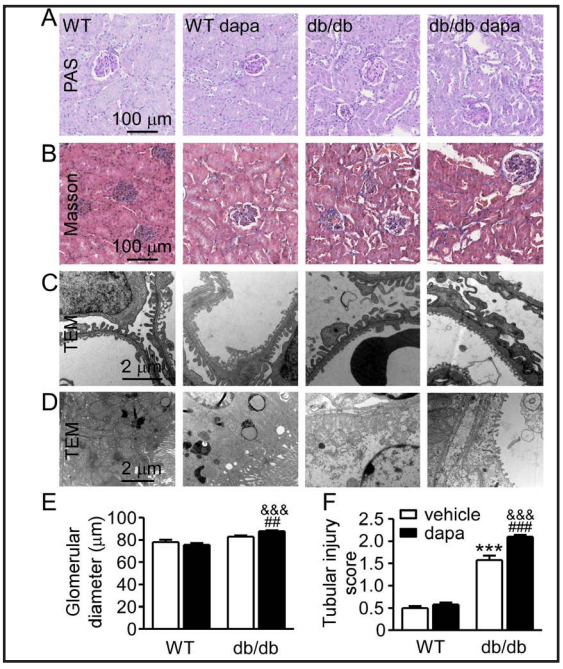

Fig. 3. Dapagliflozin aggravated tubular injury and induced glomerular damage in db/db mice. (A) PAS staining of kidney sections. (B) Masson's trichrome staining of kidney sections. (C) Electron micrograph of glomerulus. (D) Electron micrograph of proximal tubules. (E) Glomerular diameter. (F) Tubular injury score. Values were means ± SEM, n = 4. *P<0.05, **P<0.01 and ***P<0.001 vs. wild-type mice (WT). #P<0.05, ##P<0.01 and ###P<0.001 vs. db/db mice. &P<0.05, &&P<0.01 and &&&P<0.001 vs. dapagliflozin treated wild-type mice. One-way ANOVA, Bonferroni's multiple comparison test for E and F.

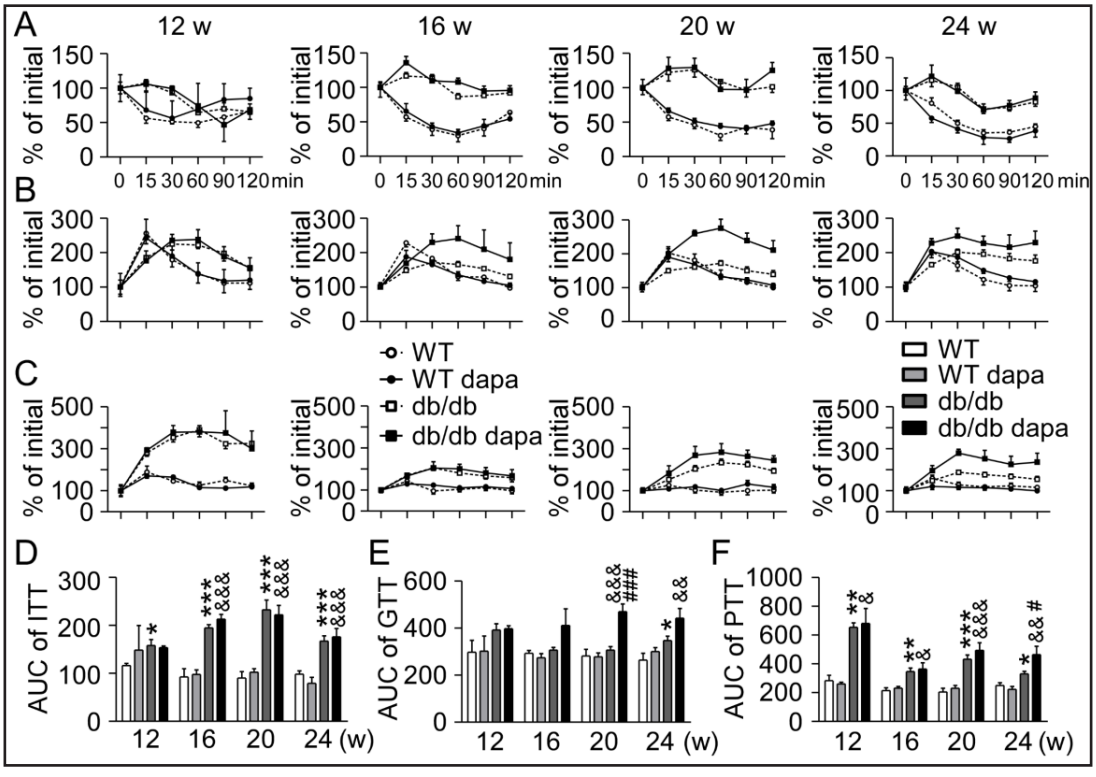

Fig. 4. Dapagliflozin affected glucose homeostasis in db/db mice. Blood was collected at 0, 15, 30, 60, 90, and 120 min after insulin, glucose or sodium pyruvate injection (A) Insulin tolerance test at 12, 16, 20, and 24 week. (B) Glucose tolerance test at 12, 16, 20, and 24 week. (C) Pyruvate tolerance test at 12, 16, 20, and 24 week. (D) Area under the curve (AUC) of insulin tolerance test (ITT). (E) AUC of glucose tolerance test (GTT). (F) AUC of pyruvate tolerance test (PTT). Values were means ± SEM, n = 6. *P<0.05, **P<0.01 and ***P<0.001 vs. wild-type (WT) mice. #P<0.05, ##P<0.01 and ###P<0.001 vs. db/db mice. &P<0.05, &&P<0.01 and &&&P<0.001 vs. dapagliflozin treated wild-type mice. One-way ANOVA, Bonferroni's multiple comparison test.

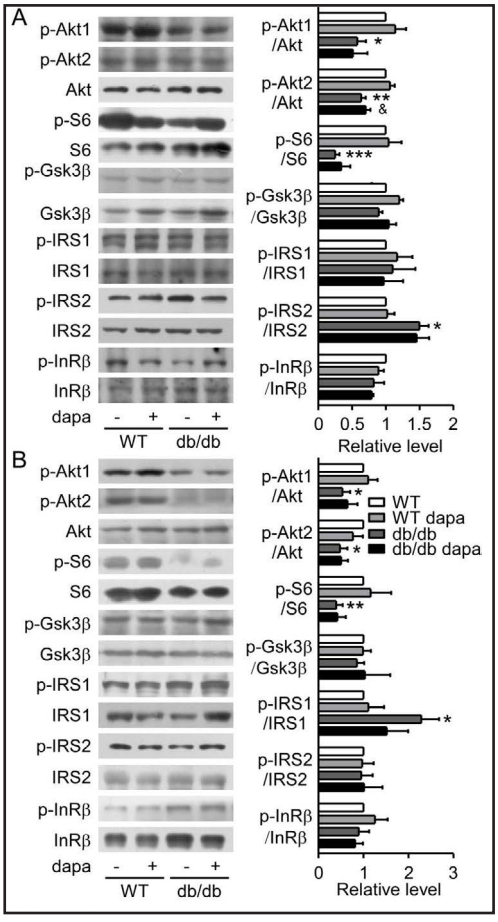

Fig. 5. Dapagliflozin had no effect on insulin signaling in kidney and liver in db/db mice. (A) Expression of proteins associated with insulin signaling in renal cortex. Representative blotting (left) and quantification of protein levels (right) are shown. (B) Expression of proteins associated with insulin signaling in liver. Representative blotting (left) and quantification of protein levels (right) are shown. Values were means ± SEM, n = 6. *P<0.05, **P<0.01 and ***P<0.001 vs. wild-type mice (WT). &P<0.05 vs. dapagliflozin treated wild-type mice. One-way ANOVA, Bonferroni's multiple comparison test.

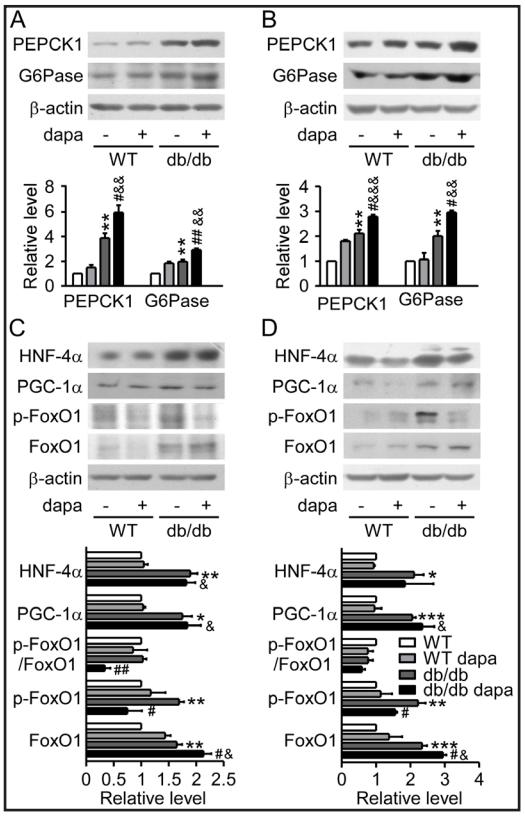

Fig. 6. Dapagliflozin increased expression of gluconeogenic key regulatory enzymes in renal cortex and liver in db/db mice. (A) Expression of gluconeogenic key regulatory enzymes in renal cortex. Representative blotting (up) and quantification of protein levels (down) are shown. (B) Expression of gluconeogenic key regulatory enzymes in liver. Representative blotting (up) and quantification of protein levels (down) are shown. (C) Expression of transcriptional activators in renal cortex. Representative blotting (up) and quantification of protein levels (down) are shown. (D) Expression of transcriptional activators in liver. Representative blotting (up) and quantification of protein levels (down) are shown. Values were means ± SEM, n = 6. *P<0.05, **P<0.01 and ***P<0.001 vs. wild-type mice (WT). #P<0.05, ##P<0.01vs. db/db mice. &P<0.05, &&P<0.01 and &&&P<0.001 vs. dapagliflozin treated wild-type mice. One-way ANOVA, Bonferroni's multiple comparison test.

Dapagliflozin had no effect on insulin signaling in db/db mice

Because glucose homeostasis is closely related to insulin signaling, the effect of dapagliflozin on hepatic and renal insulin signaling is further studied. The expression and phosphorylation levels of InRβ and IRS1 did not significantly change in the renal cortex of db/db mice (Fig. 5A). Although the phosphorylation levels of IRS2 significantly increased (Fig. 5A), the phosphorylation levels of Akt1, Akt2, and S6 significantly decreased (Fig. 5A). There was also no change in the expression and phosphorylation levels of Gsk3β (Fig. 5A).

Analogously, the expression and phosphorylation levels of InRβ and IRS2 did not significantly change in livers of db/db mice (Fig. 5B). However, the expression of IRS1 significantly decreased (Fig. 5B). Likewise, the phosphorylation levels of Akt1, Akt2, and S6 were significantly reduced in livers of db/db mice (Fig. 5B). There was no change in either the expression or thephosphorylation of Gsk3β either (Fig. 5B). These results indicate that there was impaired insulin signaling in the renal cortex and liver in db/db mice. Dapagliflozin only increased expression of IRS2 in renal cortex and IRS1 in liver in db/db mice (Fig. 5). Beyond that, dapagliflozin did not affect the phosphorylation and expression levels of other proteins in renal cortex and liver of db/db mice (Fig.5), showing that treatment with dapagliflozin for 12 weeks had no effect on insulin signaling in db/db mice.

Dapagliflozin increased renal and hepatic gluconeogenesis in db/db mice

Increased gluconeogenesis in DM is the main cause of fasting hyperglycemia and contributes greatly to endogenous glucose production. A pyruvate tolerance test indicated that gluconeogenesis significantly increased in dapagliflozin-treated db/db mice. Therefore, the expression levels of gluconeogenic key rate-limiting enzymes PEPCK1 and G6Pase in the renal cortex and liver were detected. As shown in Fig. 6A and B, the expression levels of PEPCK1 and G6Pase were increased in the renal cortex and livers of db/db mice and were further markedly promoted by dapagliflozin treatment. To obtain mechanistic insights into increased gluconeogenesis, the expression of transcriptional activators was investigated. The expression of FoxO1, HNF-4α, PGC-1α and phosphorylation of FoxO1 increased observably in the renal cortex and liver of db/db mice (Fig. 6C and D). Moreover, only increased expression of FoxO1 and decreased phosphorylation levels of FoxO1 were found after dapagliflozin treatment. In dapagliflozin treated wild-type mice, there was no alteration in the expression of key rate-limiting enzymes and transcriptional activators (Fig. 6).

Discussion

The motivation of this study was to determine whether SGLT2 inhibitors may prevent diabetic nephropathy in an animal model of T2DM with obesity. SGLT2 inhibitors, such as dapagliflozin, have been shown to improve glomerular hyperfiltration and blood pressure [10, 11], as add-ons to their action on blood glucose and glycated hemoglobin levels. The renoprotective effect of dapagliflozin in diabetic patients is still discrepant [13, 17]. Therefore, we chose diabetic db/db mice at an age of 12 to 24 weeks to investigate the effect of dapagliflozin on renal function. Db/db mice at 12 weeks of age showed serious hyperglycemia, glucosuria and polyuria, and mildly impaired renal function with microalbuminuria. Moreover, higher levels of BUN and urinary albumin excretion were observed in dapagliflozin treated db/db mice at the age of 24 weeks, indicating that dapagliflozin could further aggravate DN.

Observation of histomorphology and electron microscopy also provided sufficient evidence that glomerular damages was present, including hypertrophy and impairment of the glomerular filtration barrier in dapagliflozin-treated db/db mice. More serious tubular and tubulointerstitial injuries, such as interstitial fibrosis, epithelial cell swelling, necrosis and tubular dilatation, were also found in dapagliflozin-treated db/db mice compared to db/db mice. Despite adequate glycemic control with dapagliflozin, urinary glucose excretion did not decline but increased after administration before 24 weeks of age. The amounts of glucose filtered through the glomerulus and urinary glucose excretion have been shown to be correlated with renal function [5, 18].

Unexpectedly, in this study, we found kidney injury induced by dapagliflozin, contrary to previous reports in db/db mice with diabetic nephropathy [12]. Of note, db/db mice treated with dapagliflozin have lower urinary glucose excretion than db/db mice; this finding contradicts our results. Furthermore, glucosuria has typically been considered to be a “bad thing” and is associated with untreated DM or generalized disorders of proximal

tubule function [19-22]. Therefore, increased urinary glucose excretion itself may pose a heavy burden on the kidneys. In another study, dapagliflozin slowed renal fibrosis in db/db mice with right uninephrectomy [23]. Consistent with our findings, several pharmacokinetic or pharmacodynamics studies have identified that the effects of SGLT2 inhibitors on urinary glucose excretion and glycemic levels are attenuated by reduced renal function [18, 24, 25]. When uninephrectomy is performed, compensatory changes may occur in the kidney of db/db mice and may affect the role of dapagliflozin in glucose metabolism.

The kidney contributes to glucose homeostasis through four major processes, glucose filtration, glucose consumption, gluconeogenesis and glucose reabsorption in the proximal tubule [26]. These processes can be adaptively altered in patients with T2DM, likely contributing to hyperglycemia and providing potential targets for novel therapies. The most important process among these is the paradoxical increase in glucose reabsorption because of the increased expression and/or activity of SGLT2 in the kidneys that occurs in T2DM.

The abnormal change in SGLT2 is akin to increased hepatic gluconeogenesis that also occurs in T2DM. Endogenous glucose production is mainly derived from two pathways: gluconeogenesis and glycogenolysis, both of these are enhanced in T2DM and contribute to fasting and postprandial hyperglycemia [27, 28]. In addition to the liver, the renal cortex also has the highest expression of enzymes related to gluconeogenesis and is therefore capable of generating considerable amounts of glucose (by gluconeogenesis) to be released into the circulation [29]. The human liver and kidneys release approximately equal amounts of glucose via gluconeogenesis in the post-absorptive state [29]. In patients with T2DM, hepatic glucose release is increased by approximately 30%, and renal glucose release is increased nearly 300% [30]. A key point of gluconeogenesis control is transcriptional regulation of PEPCK1and G6Pase, the first and last key rate-limiting enzymes for gluconeogenesis respectively.

To identify if inhibiting SGLT2 will induce subsequent alterations of gluconeogenesis, we examined the levels of PEPCK1 and G6Pase. We found that dapagliflozin can significantly induce gluconeogenesis, as manifested by increased expression of PEPCK1 and G6Pase and the AUC of the glucose tolerance test. Although there is conflicting evidence on the role of SGLT2 inhibitors in reflecting renal pharmacodynamic effects in patients with DN and diabetic animal models, our results at least suggest that increased renal and hepatic gluconeogenesis induced by dapagliflozin is an important contributor to the progression of DN.

To date, the significance of the kidney in glucose homeostasis both under physiological and pathological conditions has been the focus of attention; however, the precise mechanism has not been fully clarified. Our data show that expression levels of transcriptional activators FoxO1, HNF-4α, and PGC-1α were increased both in the liver and renal cortex in db/db mice. Accumulating evidences demonstrates that the expression or the promoter-binding activity of FoxO1, HNF4, PGC-1α or cAMP responsive element binding protein (CREB), which bind to the G6Pase and PEPCK1promoters, regulate gluconeogenesis in the liver [27, 31-35]. Recently, the transcriptional regulators of hepatic gluconeogenesis are also considered to be potential therapeutic targets for the treatment of T2DM [27, 35, 36]. However, dapagliflozin only further increased the expression of FoxO1, suggesting that FoxO1 might play an important role in dapagliflozin-induced renal and hepatic gluconeogenesis.

Mammalian FoxO proteins are found throughout the body [37]. It is well established that FoxOs can be phosphorylated and that their transcriptional activity is then repressed by insulin-Akt signaling [38]. As phosphorylation of FoxO1 by PI3K/Akt signaling leads to its nuclear exclusion and eventual ubiquitylation-dependent proteasomal degradation [39], a lowered level of p-FoxO1 by dapagliflozin treatment may contribute to persistent activation of FoxO1. In this study, there was impaired insulin sensitivity with down-regulation of p-Akt and p-S6 and an increased AUC for the insulin tolerance test and glucose tolerance test curve in db/db mice that were not improved by dapagliflozin treatment. Even though our data revealed that subsequent persistent activation of FoxO1 was an important factor for the activation of gluconeogenic key enzymes induced by dapagliflozin, we failed to draw a conclusion that dapagliflozin treatment mainly promotes gluconeogenesis by insulin-Akt-

FoxO1 signals. Although elevated gluconeogenesis is involved in dapagliflozin-induced renal damage, the exact mechanisms are not yet known and still need further research.

Conclusion

We suggest that dapagliflozin may further aggravate diabetic renal impairment in db/db mice and is most likely associated with increased renal and hepatic gluconeogenesis and glucose homeostasis disturbance. Forthcoming data on the long-term efficacy and safety profile of SGLT2 inhibitors should help to solidify the role of these agents in the management of DM and DN.

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Disclosure Statement

The authors declare no duality of interest associated with this manuscript.

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