Long-term treatment with the SGLT2 inhibitor, dapagliflozin, ameliorates glucose homeostasis and diabetic nephropathy in *db/db* mice

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Abstract

Inhibition of sodium glucose cotransporter 2 (SGLT2) has been reported as a novel therapeutic approach for treating diabetes. However, the effect of SGLT2 inhibitors on the kidney is unknown. In addition, whether SGLT2 inhibitors have an anti-inflammatory or antioxidative stress effect is still unclear. In this study, to resolve these issues, we investigated the effects of the SGLT2 inhibitor, dapagliflozin, using a mouse model of type 2 diabetes and murine proximal tubular epithelial (mProx24) cells. Eight-week-old male *db/db* mice were treated with 0.1 or 1.0 mg/kg of dapagliflozin for 12 weeks. Body weight, blood glucose, hemoglobin A1c, urinary albumin excretion, creatinine clearance and blood pressure were measured. Mesangial matrix accumulation and interstitial fibrosis in the kidney and pancreatic β-cell mass were evaluated by histological analysis. Furthermore, gene expression of inflammatory mediators, such as monocyte chemoattractant protein-1, transforming growth factor-β and osteopontin, was evaluated by quantitative reverse transcriptase-polymerase chain reaction. In addition, oxidative stress was evaluated by dihydroethidium and NADPH oxidase 4 staining. Administration of 0.1 or 1.0 mg/kg of dapagliflozin ameliorated hyperglycemia, β-cell damage and albuminuria in db/db mice. Serum creatinine, creatinine clearance and blood pressure were not affected by the administration of dapagliflozin, but glomerular mesangial expansion and interstitial fibrosis were suppressed in a dose-dependent manner. Dapagliflozin treatment markedly decreased macrophage infiltration and gene expressions of inflammatory cytokines and oxidative stress in the diabetic kidney. Moreover, dapagliflozin suppressed high-glucose-induced gene expressions of inflammatory cytokines and oxidative stress in cultured mProx24 cells. These data suggest that dapagliflozin ameliorates diabetic nephropathy by improving hyperglycemia along with inhibiting inflammation and oxidative stress in *db/db* mice.

Introduction

Diabetic nephropathy is the most common cause of end-stage renal disease in developed countries [1]. In the past, several mechanisms have been suggested to contribute to the onset and progression of diabetic nephropathy, including genetic and hemodynamic factors, intracellular metabolic anomalies, and advanced glycation end products [2]. Emerging evidence suggests that inflammation is crucially involved in the pathogenesis of diabetic nephropathy [3]. Recently, numerous studies have also suggested that hyperglycemia is associated with enhanced generation of reactive oxygen species (ROS), and oxidative stress has been implicated in the development of diabetic nephropathy [4]. Therefore, the regulation of oxidative stress and inflammation could be a potential therapeutic target in diabetic nephropathy.

Sodium glucose cotransporter 2 (SGLT2), which is located on the apical side of the proximal tubules, can transport sodium and glucose concurrently within the proximal tubular cells [5]. Under normoglycemic conditions, SGLT2 can reabsorb about 90% of filtered glucose in the early segments of the proximal tubules [6]. In recent years, SGLT2 inhibitors, which can block reabsorption of filtered glucose by inhibiting SGLT2, have been developed and proposed as novel hypoglycemic agents for treating patients with diabetes mellitus [7]. Growing numbers of SGLT2 inhibitors are being developed and hundreds of preclinical and clinical studies have been carried out in the last decade [8]. Although SGLT2 inhibitors are novel and promising drugs for treating patients with type 2 diabetes, the effect of SGLT2 inhibition on diabetic nephropathy is unknown.

Dapagliflozin is a very potent and selective SGLT2 inhibitor [9], and is the first-in-class SGLT2 inhibitor launched on the market in 2012 [10]. Various clinical trials have shown improvements in postprandial blood glucose with dapagliflozin monotherapy and combination therapy [11]. In addition, dapagliflozin was associated with additional non-glycemic benefits

including reduction in body weight and blood pressure in most clinical trials [12]. Although several studies with animal models suggest that long-term administration of SGLT2 inhibitors, including dapagliflozin, preserves pancreatic β-cell function with improved glucose homeostasis [9,13,14,15], the effects of SGLT2 inhibition on diabetic nephropathy and renal function have not been reported.

The purpose of this study was to investigate the hypothesis that inhibition of SGLT2 by dapagliflozin ameliorates glucose homeostasis with preserving β-cell mass, and prevents the development of diabetic nephropathy with inhibiting inflammation and oxidative stress in a mouse model of obesity and type 2 diabetes.

Materials and Methods

Experimental Protocol

Six-week-old male diabetic *db/db* mice (BKS.Cg-*lepr*^{db}/*lepr*^{db}) and non-diabetic *db/m* mice (BKS.Cg-*lepr*^{db}/+) were purchased from CLEA Japan (Tokyo, Japan). All mice were maintained under a 12-h light/12-h dark cycle with free access to food and tap water. Dapagliflozin was kindly supplied by Bristol-Myers Squibb (Pennington, NJ, USA). Dapagliflozin (0.1 or 1.0 mg/kg/day) was administrated to *db/db* mice (n = 6) by gavage for 12 weeks starting at the age of eight weeks. Control *db/db* mice (n = 5) and control *db/m* mice (n = 5) received saline for 12 weeks. The mice were euthanized at 20 weeks of age, and the kidneys were removed and weighed. The kidneys were fixed in 10% formalin for periodic acid-methenamine silver (PAM) staining, and some of the other tissues were embedded in optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan) and immediately frozen in acetone cooled on dry ice. Other tissues were snap-frozen in liquid nitrogen and stored at −80°C. Animal care and procedures were performed according to the Guidelines for Animal Experimentation at Okayama University, the Japanese Government Animal Protection and Management Law, and the Japanese Government Notification on Feeding and Safekeeping of Animals. The experimental protocol was approved by the Animal Ethics Review Committee of Okayama University (OKU-2012356). All surgery was performed under sodium pentobarbital anesthesia, and every effort was made to minimize suffering.

Metabolic Data

Body weight was measured weekly. Blood pressure, plasma glucose, urinary glucose and 24-h urinary albumin excretion (UAE) were measured every four weeks. Plasma glucose and blood pressure were measured after an overnight fast. Hemoglobin A1c (HbA1c), water intake, food

intake, kidney weight, blood urea nitrogen (BUN), creatinine and creatinine clearance (Ccr) were measured at the age of 20 weeks. Blood pressure was measured by the tail-cuff method (Softron, Tokyo, Japan). HbA1c was measured using high-pressure liquid chromatography, and serum creatinine was measured using an enzymatic method. Urine was collected for 24 h with each mouse housed individually in a metabolic cage and provided with food and water *ad libitum*. UAE was measured as previously described [16].

Light Microscopy

Sections (4 μm thick) were cut from the 10% formalin-fixed, paraffin-embedded kidney samples taken at 20 weeks of age and subjected to PAM and Masson trichrome staining. All tissue sections were examined using a BZ-9000 microscope (Keyence, Osaka, Japan). The PAM-positive area and the tuft area were calculated using BIOZERO software (Keyence). The mesangial matrix index (MMI) was defined as the PAM-positive area in the tuft area, and calculated using the following formula: MMI = (PAM-positive area) / (tuft area). To determine the MMI, we examined 10 randomly selected glomeruli in the cortex per animal under high magnification (×400). The results are expressed as mean ± SEM (per μm^2 for tuft area; arbitrary units for MMI).

Immunofluorescent Staining

Immunofluorescent staining was performed as described previously [17]. Renal expression of type IV collagen was detected using rabbit anti-type IV collagen (Millipore, Temecula, CA) followed by Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA). Similarly, pancreatic β-cells were detected using guinea pig anti-insulin (Abcam, Cambridge, UK) followed by Alexa Fluor 488 goat anti-guinea pig IgG (Invitrogen). Type IV collagen-positive area in

glomerulus was calculated same as MMI. The proportion of the area of pancreatic tissue occupied by β-cells was calculated using BIOZERO software (Keyence). The insulin-positive area relative to the area of the whole pancreatic tissues was analyzed for more that 100 islets per group.

Immunoperoxidase Staining

Immunoperoxidase staining was performed as described previously [16]. Macrophage infiltration was evaluated using a rat anti-mouse monocyte/macrophage (F4/80) monoclonal antibody (Abcam), followed by HRP-conjugated goat anti-rat IgG antibody (Millipore). We counted the number of F4/80-positive cells in 10 glomeruli per animal. The mean number of positive cells per glomerulus and interstitial tissue (number per mm^2) were used for the estimation.

NADPH oxidase 4 (Nox4) immunoperoxidase staining was performed as previously described [18]. Tissue sections were stained with Nox4 rabbit antibody (Novus Biologicals, Littleton, CO, USA) for 12 h at 4°C followed by biotin-labeled anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories). To quantify the proportional area of staining, 10 views of the renal cortex were randomly selected in each slide.

Quantitative Analysis of Renal Cortex Gene Expression

RNA from the renal cortex was isolated from 20-week-old mice using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Single-strand cDNA was synthesized from the extracted RNA using a reverse transcriptase-polymerase chain reaction (RT-PCR) kit (Perkin Elmer, Foster City, CA, USA). To determine the mRNA expression of *CD14*, *CD11c*, *CD206*, *transforming growth factor* (*TGF*)*-β*, *monocyte chemoattractant protein* (*MCP*)*-1*, *osteopontin*, *intercellular adhesion molecule* (*ICAM*)*-1*, *caspase 12* and *Bax* in the renal cortex, quantitative RT-PCR (qRT-PCR) was performed using StepOnePlus (Applied Biosystems, Tokyo, Japan) and FastStart SYBR

Premix Ex Taq II (Takara Bio, Otsu, Japan). All primers were purchased from Takara Bio. Each sample was analyzed in triplicate and normalized against *Atp5f1* mRNA expression.

Expression of ROS

To evaluate the effect of dapagliflozin on ROS production, superoxide anion radicals were detected by dihydroethidium (DHE) staining (Molecular Probes, Eugene, OR, USA). Briefly, the kidney sections were incubated with 2 μmol/l DHE at 37°C for 45 min in a humidified chamber protected from light. Fluorescence pictures were obtained using BIOZERO software (Keyence). The mean DHE fluorescence intensity was calculated by dividing the combined fluorescence value of the pixels by the total number of pixels in 10 randomly selected fields observed under identical laser and photomultiplier settings.

Terminal Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Assay

To evaluate the effect of dapagliflozin on apoptosis, kidney sections were incubated with *in situ* apoptosis detection kit (Takara Bio) according to the manufacturer's protocol. The mean number of positive cells per interstitial tissue (number per mm^2) was determined by observing more than 10 interstitia from each section.

Cell Culture and Treatment

Murine proximal tubular epithelial (mProx24) cells were generously provided by Dr. Takeshi Sugaya (CMIC Co., Japan), and cultured as reported previously [17]. mProx24 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5.5 mM D-glucose (low glucose), 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine. DHE staining and qRT-PCR were performed as described above.

Statistical Analysis

All values are given as mean ± SEM. Statistically significant differences between groups were examined using one-way ANOVA followed by Scheffe's test. A P value < 0.05 was considered statistically significant.

Results

Effect of Dapagliflozin on Body Weight, Hyperglycemia and Renal Function

Body weight was higher in the *db/db* groups than in the *db/m* group during the study, and body weight in the *db/db* group treated with 0.1 or 1.0 mg/kg/day of dapagliflozin (*db/db*+0.1 dapa group and *db/db*+1.0 dapa group, respectively) was higher than in the *db/db* group from 10 to 20 weeks of age (Fig. 1A). Plasma and urinary glucose excretion progressively increased in the *db/db* groups during the study. However, dapagliflozin significantly reduced plasma and urinary glucose, and HbA1c compared with those of the *db/db* group at 20 weeks of age (Fig. 1B, 1C and Table 1). There were no significant differences in systolic and diastolic blood pressure between the four groups at 20 weeks of age. In addition, there were no significant differences in water and food intake between the *db/db*, the *db/db*+0.1 dapa and the *db/db*+1.0 dapa groups (Table 1).

UAE, a characteristic feature of diabetic nephropathy, progressively increased in the *db/db* group during the study. However, dapagliflozin significantly reduced the mean UAE compared with that of the *db/db* group from 12 to 20 weeks of age (Fig. 1D). The other parameters are summarized in Table 2. There were no significant differences in BUN and serum creatinine between the four groups at 20 weeks of age. Kidney weight and relative kidney weight were significantly lower in the *db/db* groups than in the *db/m* group, but there were no significant differences between the *db/db*, the *db/db*+0.1 dapa and the *db/db*+1.0 dapa group. Ccr was higher in the *db/db* group and the *db/db*+0.1 dapa group than in the *db/m* group, but there were no significant differences between the *db/db*, the *db/db*+0.1 dapa, and the *db/db*+1.0 dapa group.

Dapagliflozin Suppresses Mesangial Matrix Accumulation and Interstitial Fibrosis

Kidneys were isolated and processed for pathological analysis using PAM staining, Masson's trichrome staining, and immunofluorescence for type IV collagen. As revealed by PAM and type

IV collagen staining (Fig. 2A), mesangial matrix expansion was observed in the *db/db* group at 20 weeks of age. However, this outcome was ameliorated in the *db/db*+1.0 dapa group compared with the *db/db* group, as demonstrated by a reduction in the MMI from 4.9 ± 0.1% in the *db/db* group to 2.1 ± 0.6% in the *db/db*+1.0 dapa group ($P < 0.05$; Fig. 2B). Immunofluorescent staining for type IV collagen also showed the same tendency (Fig. 2C). Similarly, representative interstitia in the Masson's trichrome-stained sections are shown in Fig. 2D. Interstitial fibrosis was significantly higher in the *db/db* group compared with that in the *db/m* group, and was suppressed in the *db/db*+0.1 dapa group and the *db/db*+1.0 dapa group (Fig. 2E). Collectively, these results demonstrate that administration of dapagliflozin ameliorates mesangial matrix accumulation and interstitial fibrosis in *db/db* mice.

Proinflammatory Macrophage Infiltration in the Kidney

We performed qRT-PCR analysis to evaluate the macrophage infiltration into the kidney. Gene expression of *CD14*, a macrophage marker, was lower in the *db/db*+1.0 dapa group than in the *db/db* group (Fig. 3A). To distinguish which proinflammatory or anti-inflammatory macrophages are dominant in the kidney, we used the primers for *CD11c* and *CD206*. CD11c is a marker for the proinflammatory (M1) subtype of macrophages, while CD206 is specific for the anti-inflammatory (M2) subtype of macrophages. The renal expression of *CD11c* was lower in *db/db*+1.0 dapa group than in the *db/db* group (Fig. 3B); however, there were no significant differences in *CD206* between the *db/db*, the *db/db*+0.1 dapa and the *db/db*+1.0 dapa group (Fig. 3C). To confirm these findings, we performed immunoperoxidase staining for F4/80, a marker for M1 macrophages. The number of macrophages in both the glomeruli and interstitium were remarkably higher in the *db/db* group than in the *db/m* group (Fig. 3D). The macrophage infiltration into the glomeruli was significantly reduced in the *db/db*+0.1 dapa and the *db/db*+1.0

dapa group compared with the *db/db* group (Fig. 3D and 3E). Similarly, the macrophage infiltration into the interstitium was increased in the *db/db* group but suppressed in the *db/db*+1.0 dapa group (Fig. 3D and 3F). These findings indicate that dapagliflozin suppresses proinflammatory macrophage infiltration into the diabetic kidney.

Inflammatory Gene Expression in the Renal Cortex

qRT-PCR analysis of kidney tissue demonstrated that the expression of several proinflammatory genes, including *TGF-β, MCP-1*, *osteopontin* and *ICAM-1*, was significantly suppressed by dapagliflozin in the *db/db* group (Fig. 4A–D).

Oxidative Stress and Apoptosis in the Kidney

To investigate the role of oxidative stress and apoptosis, and the effects of dapagliflozin on the pathogenesis of diabetic nephropathy, we conducted DHE staining, Nox4 immunostaining and the TUNEL assay on the kidney. ROS production, which was detected by DHE, was higher in the cortex of the *db/db* group than in the *db/m* group, but it was reduced in the *db/db*+0.1 and *db/db*+1.0 dapa groups (Fig. 5A and B). Similarly, Nox4, a subunit of NADPH oxidase, was upregulated in the cortex of the *db/db* group, but its expression was attenuated in the *db/db*+1.0 dapa group (Fig. 5C and D). TUNEL staining confirmed that apoptosis was increased in the *db/db* group, and that dapagliflozin markedly reduced the number of apoptotic cells (Fig. 6A and B). Furthermore, dapagliflozin markedly reduced the high gene expression of the proapoptotic factors, *Caspase-12* and *Bax*, in the *db/db* group (Fig. 6C and D). These data indicate that diabetes increases oxidative stress and apoptosis, and that oxidative stress and apoptosis are suppressed by dapagliflozin.

Oxidative Stress and Inflammatory Gene Expression in Cultured Proximal Tubular Epithelial Cells

To evaluate high-glucose-induced ROS production in cultured proximal tubular epithelial cells, we performed DHE staining. High-glucose medium increased ROS production in mProx24 cells, and that dapagliflozin treatment signigicantly attenuated this increase in mProx24 cells (Fig. 6A and B). qRT-PCR analyses of mProx24 cells demonstrated that high-glucose-induced Nox4 mRNA expression was also attenuated by dapagliflozin (Fig. 7C). Similarly, expression levels of inflammatory genes including MCP-1 and OPN were increased by exposure to high glucose and suppressed by dapagliflozin (Fig. 7D and 7E). These findings suggest that dapagliflozin ameliorates high-glucose-induced oxidative stress and inflammation in renal proximal tubular epithelial cells.

Effect of Dapagliflozin on β-cell mass in db/db mice

We evaluated the effect of dapagliflozin on β-cell morphology by immunoperoxidase staining for insulin (Fig. 8A). β-cell mass was significantly decreased in the *db/db* group compared with the *db/m* group at 20 weeks of age. However, treatment of dapagliflozin significantly prevented the decrease in β-cell mass in a dose dependent manner (Fig. 8B)

Discussion

In the present study, we demonstrated that dapagliflozin, a novel SGLT2 inhibitor, suppressed hyperglycemia and restored β-cell mass in diabetic *db/db* mice. Administration of dapagliflozin reduced macrophage infiltration and the gene expression of inflammatory cytokines, including *MCP-1*, *TGF-β* and *OPN* in the kidney of diabetic *db/db* mice. Furthermore, oxidative stress and apoptosis were lower in dapagliflozin-treated *db/db* mice than in the untreated mice. Our findings revealed that dapagliflozin exhibits potent antihyperglycemic effects and prevents the development of diabetic nephropathy.

SGLT2 inhibitors are a novel class of antihyperglycemic drugs that target the process of renal glucose reabsorption and induce glycuresis independently of insulin secretion or action. To date, data on dapagliflozin, a selective SGLT2 inhibitor in development, have demonstrated that the kidney is an efficacious and safe target for therapy, and that SGLT2 inhibition may benefit patients with type 2 diabetes mellitus beyond glycemic control [19]. Although many studies in animals and humans have demonstrated that SGLT2 inhibitors reduce hyperglycemia measurements, including HbA1c, fasting plasma glucose and postprandial glucose, the effects of SGLT2 inhibitors on the organs are not well known. Several studies have demonstrated that genetic and pharmacological inhibition of SGLT2 preserve pancreatic β-cell function [15,20,21]; however, the effects of SGLT2 inhibitors on renal structures and function are not understood. Therefore, we investigated how dapagliflozin influences the progression of diabetic nephropathy using a mouse model of type 2 diabetes.

Inflammation is associated with the development of diabetic nephropathy, and targeting inflammation could be a therapeutic approach for the treatment of diabetic nephropathy [3,22]. We have demonstrated that activation of nuclear hormone receptors, including peroxisome proliferator-activated receptor (PPAR) γ, PPARδ and liver x receptor, inhibits macrophage

infiltration and inflammation, and ameliorates diabetic nephropathy in animal models [23,24,25]. In the present study, dapagliflozin suppressed the gene expression of the proinflammatory M1 macrophage marker, *CD11c*, but not the anti-inflammatory M2 macrophage marker, *CD206*, and decreased macrophage infiltration into the kidney in a dose-dependent manner. Similarly, dapagliflozin suppressed the expression levels of the chemokine *MCP-1*, the adhesion molecule *ICAM-1*, and the cytokines *TGF-β* and *OPN*. Moreover, in vitro study suggests that dapagliflozin inhibit MCP-1 and OPN expression in cultured proximal tubular epithelial cells. These results indicate that dapagliflozin inhibits proinflammatory macrophage infiltration and inflammation in diabetic nephropathy.

Many studies have also proposed an important role for oxidative stress and apoptosis in the pathogenesis of diabetic nephropathy [2,26]. To investigate the role of oxidative stress and the effects of dapagliflozin on the pathogenesis of diabetic nephropathy, we evaluated oxidative stress in the kidney by assessing ROS generation in this study. DHE staining revealed that ROS were increased in the interstitia of diabetic *db/db* mice compared with non-diabetic *db/m* mice. The intensity of DHE staining was lower in dapagliflozin-treated *db/db* mice than in control *db/db* mice. We also performed immunohistochemistry of Nox4, as a promoter of ROS generation in the diabetic kidney. The fact that Nox4 expression was increased in diabetic *db/db* mice and decreased by the administration of dapagliflozin suggests that dapagliflozin may reduce oxidative stress by suppressing Nox4-derived ROS generation in the kidney of *db/db* mice. Furthermore, we evaluated apoptosis in the kidney by TUNEL staining and quantitative analysis of gene expression of proapoptotic factors. Diabetes-induced apoptotic cells were decreased in dapagliflozin-treated *db/db* mice compared with control *db/db* mice. Similarly, the expression levels of *Caspase-12* and *Bax* were also suppressed by the administration of dapagliflozin. Finally, we performed in vitro study and revealed that dapagliflozin suppresses

high-glucose-induced ROS generation and Nox4 expression in cultured proximal tubular epithelial cells. Overall, these results indicate that diabetes increases oxidative stress and apoptosis in the kidney, and dapagliflozin suppresses diabetes-induced oxidative stress and apoptosis in the kidney.

To date, no studies have explored the effect of SGLT2 inhibitors on the progression of diabetic nephropathy in detail, and only two studies reported renoprotective effects of SGLT2 inhibitors. First is that the SGLT2 inhibitor, tofogliflozin, is reported to reduce albuminuria and glomerular hypertrophy in *db/db* mice [21]. Second is that luseogliflozin is also reported to slow the progression of diabetic nephropathy in rat model of type 2 diabetes [27]. However, neither inflammation nor oxidative stress in renal tissue or in cultured renal cells was examined in these studies. To the best of our knowledge, this is the first study to investigate the protective effect of an SGLT2 inhibitor on the development of diabetic nephropathy by inhibiting inflammation and oxidative stress by both in vivo and in vitro studies.

Vallon *et al.* showed that SGLT2 knockout mice attenuated hyperglycemia and glomerular hyperfiltration, but not of renal injury, oxidative stress and inflammation in streptozotocin (STZ)-induced type 1 diabetes model [28]. There are two speculations for the discrepancy between their and our studies. First, it is well known that STZ has toxicity and STZ itself might affect to the kidney and induce renal injury, oxidative stress and inflammation. Second, the glucose level was lower in STZ-induced diabetic SGLT2 knockout mice than in diabetic wild-type mice (~300 vs. 470 mg/dl), however, it was still extremely higher than normal level. The glucose level of their diabetic SGLT2 knockout mice is almost similar to that of our untreated *db/db* mice. Therefore, hyperglycemia per se might induce oxidative stress, inflammation and renal injury. Recent clinical study reported that empagliflozin ameliorated hyperfiltration but not urine albumin/creatinine ratio in patients with type 1 diabetes [29]. The

treatment period was only 8 weeks in this study and it is too short to expect the effect of SGLT2 inhibitor to reduce albuminuria. Furthermore, we should be careful not to administer SGLT2 inhibitors to type 1 diabetic patients since the indication of SGLT2 inhibitors is to type 2 diabetic patients.

Tahara *et al*. have reported that the SGLT2 inhibitor, ipragliflozin, reduced plasma and liver levels of oxidative stress biomarkers and inflammatory markers, and ameliorated hyperglycemia in a mouse model of diabetes [30]. Chen *et al*. have shown that the SGLT2 inhibitor, BI-38335, suppressed the gene expression of inflammatory cytokines in pancreas, and improved glycemic control in *db/db* mice [15]. However, the effects of SGLT2 inhibitors on kidney were not investigated in these studies. To elucidate the precise mechanisms by which dapagliflozin prevents diabetes-induced oxidative stress and inflammation, and thus protects against diabetic nephropathy, further investigations are needed.

In conclusion, we demonstrated that the SGLT2 inhibitor, dapagliflozin, ameliorates the primary features of diabetic nephropathy and reduces albuminuria as well as hyperglycemia and β-cell damage in *db/db* mice. Dapagliflozin shows renoprotective effects through its glucose lowering effect and at least in part by anti-inflammatory/oxidative stress effects in the diabetic kidney. Our findings suggest that dapagliflozin may thus be a therapeutic option for the treatment of diabetic nephropathy.

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Figure Legends

Figure 1. Effect of dapagliflozin on body weight, hyperglycemia and urinary albumin excretion (UAE). (A) Body weight was higher in the *db/db* group than in the *db/m* group during the study. Body weight in the *db/db* with 1.0 mg/kg dapagliflozin group (*db/db*+0.1 dapa group) was higher than in the *db/db* group from 10 to 20 weeks of age. Data are mean ± SEM. **P* < 0.05. (B-D) Plasma and urinary glucose, and UAE progressively increased in the *db/db* group during the 12-week observation period. These parameters were significantly lower in the *db/db*+1.0 dapa group than in the *db/db* group. Data are mean ± SEM. **P* < 0.05.

Figure 2. Dapagliflozin suppresses mesangial matrix accumulation and interstitial fibrosis. (A) Periodic acid-methenamine silver (PAM) staining of kidney sections. Mesangial matrix expansion was evident in the *db/db* group. Dapagliflozin suppressed the increase in mesangial matrix accumulation compared with that in the *db/db* group. Original magnification, ×400. (B) Mesangial matrix index of the glomeruli. Data are mean ± SEM. **P* < 0.05. (C) Type IV collagen positive area in the glomeruli. Data are mean ± SEM. **P* < 0.05. (D) Masson's trichrome staining of kidney sections. Interstitial fibrosis was significantly higher in the *db/db* group than in the *db/m* group, and significantly lower in the *db/db*+1.0 dapa group than in the *db/db* group. Original magnification, ×100. (E) Percentages of fibrosis in interstitia. Data are mean ± SEM. **P* < 0.05.

Figure 3. Dapagliflozin inhibits proinflammatory macrophage infiltration in the renal cortex. Quantitative RT-PCR analysis of the expression of *CD14* (A), *CD11c* (B) and *CD206* (C) showed that dapagliflozin suppressed gene expression in proinflammatory macrophages in the kidney. mRNA levels were normalized against *Atp5f1* expression. Data are mean ± SEM. **P* <

0.05. (D) Macrophage infiltration into the glomeruli and the interstitium was clearly evident in the *db/db* group compared with that in the *db/m* group, and was suppressed in the *db/db*+dapa groups compared with that in the *db/db* group. Original magnifications: ×400 for glomeruli and ×100 for interstitium. (E) Number of intraglomerular macrophages. Data are mean ± SEM. **P* < 0.05. (F) Number of macrophages in the interstitium. Data are mean ± SEM. **P* < 0.05.

Figure 4. Dapagliflozin suppresses inflammatory gene expression in the renal cortex. Quantitative RT-PCR analysis of the expression of *TGF-β* (A), *MCP-1* (B), *osteopontin* (C) and *ICAM-1* (D) showed that dapagliflozin inhibited diabetes-induced inflammatory gene expression in the kidney. mRNA levels were normalized against *Atp5f1* expression. Data are mean ± SEM. **P* < 0.05.

Figure 5. Dapagliflozin inhibits oxidative stress in the kidney. (A, B) ROS production was detected by fluorescence microscopy using dihydroethidium (DHE). ROS was predominantly localized in the interstitia of *db/db* mice, and was suppressed in the *db/db*+dapa groups compared with that in the *db/db* group. Original magnification, ×100. Data are mean ± SEM. **P* < 0.05. (C, D) Localization of renal Nox4 expression by immunohistochemistry. The expression of Nox4 was predominantly localized in the interstitia of *db/db* mice, and was suppressed in the *db/db*+dapa groups compared with that in in the *db/db* group. Original magnification, ×100. Data are mean ± SEM. **P* < 0.05.

Figure 6. Dapagliflozin inhibits apoptosis in the kidney. (A, B) Apoptosis was detected by TUNEL staining. Arrow heads indicate the apoptotic nuclei. The number of apoptotic cells was higher in the interstitia of *db/db* mice than in *db/m* mice, and was lower in the *db/db*+dapa groups

than in the *db/db* group. Original magnification, ×400. Data are mean ± SEM. *$P < 0.05$. (C, D) Dapagliflozin reduced the mRNA levels of *Caspase-12* and *Bax* in the kidney. mRNA levels were normalized against *Atp5f1* expression. Data are mean ± SEM. *$P < 0.05$.

Figure 7. Dapagliflozin suppresses oxidative stress and inflammatory gene expression in cultured proximal tubular epithelial cells. (A) ROS production was detected by fluorescence microscopy using dihydroethidium. ROS production was not increased by mannitol (b) compared with normal glucose (a), but was increased by high glucose (c). High-glucose-induced ROS production was attenuated by dapagliflozin pretreatment in a dose-dependent manner (d: 0.2 nM; e: 2.0 nM; f: 20.0 nM). The cells depicted are representative of three independent experiments. (B) Densitometric quantification of ROS production. Data are mean ± SEM of three independent experiments. *$P < 0.05$ versus high glucose; NG: normal glucose; Man: mannitol; HG: high glucose; dapa: dapagliflozin. Quantitative RT-PCR analysis of the expression of *Nox4* (C), *MCP-1* (D) and *osteopontin* (E) showed that dapagliflozin inhibited diabetes-induced inflammatory gene expression in the kidney. mRNA levels were normalized against *Atp5f1* expression. Data are mean ± SEM. *$P < 0.05$.

Figure 8. Treatment with dapagliflozin increases β-cell mass in db/db mice. (A) Representative immunofluorescent staining for insulin performed with pancreatic tissue sections derived from *db/m*, *db/db*, *db/db* with 0.1 and 1.0 mg/kg dapagliflozin mice. Original magnification, ×400. (B) The β-cell area is shown as a proportion of the area of the entire pancreas. Data are mean ± SEM. *$P < 0.05$.

525 **Table 1.** Influence of dapagliflozin on metabolic and physiologic parameters in *db/db* and *db/m*

526 mice at 20 weeks

	db/m	*db/db*	*db/db* + 0.1 dapa	*db/db* + 1.0 dapa
Systolic blood pressure (mmHg)	120.0 ± 5.2	116.6 ± 4.5	121.2 ± 2.3	115.2 ± 4.5
Diastolic blood pressure (mmHg)	79.4 ± 3.2	78.8 ± 2.3	86.3 ± 1.6	84.3 ± 3.0
HbA1c (%)	4.0 ± 0.1	9.2 ± 0.2 [a]	8.5 ± 0.3 [a]	6.6 ± 0.2 [abc]
Water intake (ml/day)	4.8 ± 0.4	31.1 ± 4.1 [a]	22.3 ± 2.9 [a]	19.8 ± 1.9 [a]
Food intake (g/day)	3.2 ± 0.1	4.5 ± 0.7	4.8 ± 0.3	6.1 ± 0.3 [a]

527 *db/m*, nondiabetic control mice; *db/db*, untreated diabetic mice; *db/db*+0.1 dapa, dapagliflozin

528 (0.1 mg/kg)-treated diabetic mice; *db/db*+1.0 dapa, dapagliflozin (1.0 mg/kg)-treated diabetic

529 mice; HbA1c, hemoglobin A1c. Data are presented as mean ± SEM; [a]$P < 0.05$ *vs*. *db/m*, [b]$P < 0.05$

530 *vs*. *db/db*, [c]$P < 0.05$ *vs*. *db/db*+0.1 dapa.

531

532 **Table 2.** Effects of dapagliflozin on renal functional and structural parameters at 20 weeks

	db/m	*db/db*	*db/db* + 0.1 dapa	*db/db* + 1.0 dapa
Kidney weight (mg)	379.0 ± 39.6	247.0 ± 6.8 [a]	239 ± 9.4 [a]	252.5 ± 9.1 [a]
Relative kidney weight (mg/g body weight)	11.5 ± 1.0	6.0 ± 0.3 [a]	5.2 ± 0.4 [a]	4.5 ± 0.1 [a]
BUN (mg/dl)	20.3 ± 0.7	29.1 ± 2.8	24.6 ± 2.5	25.9 ± 0.6
Serum creatinine (mg/dl)	0.10 ± 0.01	0.12 ± 0.02	010 ± 0.01	0.12 ± 0.02
Urine volume (ml/day)	1.0 ± 0.1	23.4 ± 3.1 [a]	19.2 ± 2.4 [a]	16.3 ± 1.9 [a]
Ccr (ml/min)	4.80 ± 0.54	9.42 ± 0.96 [a]	9.81 ± 0.78 [a]	6.40 ± 0.65 [c]

533 *db/m*, nondiabetic control mice; *db/db*, untreated diabetic mice; *db/db*+0.1 dapa, dapagliflozin
534 (0.1 mg/kg)-treated diabetic mice; *db/db*+1.0 dapa, dapagliflozin (1.0 mg/kg)-treated diabetic
535 mice; BUN, blood urea nitrogen; Ccr, creatinine clearance. Data are presented as mean ± SEM;
536 [a]$P < 0.05$ *vs*. *db/m*, [b]$P < 0.05$ *vs*. *db/db*, [c]$P < 0.05$ *vs*. *db/db*+0.1 dapa.