The Effect Sodium Glucose Co-Transporter-2 inhibitor on Renal Proximal Tubular Gluconeogenesis

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The Effect Sodium Glucose Co-Transporter-2 inhibitor on Renal Proximal Tubular Gluconeogenesis

Directed by Professor Eun Seok Kang

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ABSTRACT

The Effect Sodium Glucose Co-Transporter-2 inhibitor on Renal Proximal Tubular Gluconeogenesis

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(Directed by Professor Eun Seok Kang)

Dapagliflozin, a sodium glucose cotransporter-2 (SGLT-2) inhibitors, is an anti-diabetic medication which improved hyperglycemia via inhibition of renal tubular glucose reabsorption. SGLT-2 is almost exclusively expressed in renal proximal tubule and its physiological roles other than glucose reabsorption were not widely studied. Here, we explore the effects of SGLT-2 inhibitor on renal gluconeogenesis in vitro, ex vivo and in vivo. We administered dapagliflozin to BL6 mice in short-term (7 days) and long term (6 weeks). Although dapagliflozin treatment improved glycemia, it enhanced renal gluconeogenesis both directly and indirectly. Increased gluconeogenic enzymes (PEPCK and G6Pase) mRNA and

protein expression was coupled with increased expression of CREB phosphorylation and decreased FoxO1 phosphorylation. In addition, renal proximal tubular epithelial cell-specific knockdown of SGLT-2 showed same effects on renal gluconeogenesis as dapagliflozin. Therefore, we found that dapagliflozin induced SGLT-2 mediated enhancement in gluconeogenesis directly. However, increase in glucose output in kidney via dapagliflozin did not affect whole body glucose metabolism as dapagliflozin reduced fasting glucose significantly. We suggest another role of dapagliflozin in kidney glucose metabolism and the direct effect of dapagliflozin in kidney glucose metabolism should be studied further.

Key words: SGLT-2, Dapagliflozin, Gluconeogenesis, Renal proximal tubule

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I. INTRODUCTION

Sodium-glucose cotransporter-2 (SGLT-2) accounts for approximately 90% of the glomerular filtered glucose reabsorption in the proximal tubule, and SGLT-1 mediates the rest. SGLT-2 inhibition causes not only glycosuria but also natriuresis due to co-inhibition of sodium reabsorption, both leading to osmotic diuresis.

SGLT-2 inhibitors decrease the blood glucose level in an insulin-independent manner by inhibiting glucose reabsorption in the proximal renal tubule. Pharmacological inhibition of SGLT-2 showed alleviated glycemia without weight gain and minimal risk of

hypoglycemia.[1,2] This class of medications do not stimulate insulin secretion and may preserve pancreatic beta cell function. These, anti-diabetic drugs now are used widely, following metformin, especially in patients with diabetes and metabolic syndrome. Dapagliflozin is a highly selective and potent SGLT-2 inhibitor and has a protective effect on diabetic nephropathy.[3]

Gluconeogenesis is an essential metabolic pathway that maintains normal blood glucose levels and prevents hypoglycemia. In the fasting state, levels of phosphoenolpyruvate carboxykinase (PEPCK), the rate-limiting enzyme in gluconeogenesis, and glucose-6-phosphatase (G6Pase) are increased and suppress glycolysis. The multifaceted kidney function in glucose homeostasis includes such functions as glucose reabsorption and gluconeogenesis, the former being mediated by active and passive transporters.[4] Because glycogen stores are reduced during long-term fasting, glucose homeostasis is vital to convert non-carbohydrate precursors to glucose. During long-term fasting, level of the stored glycogen in the liver decreases gradually leading to a decrease in gluconeogenesis, and its contribution to glucose metabolism by the kidney is further enhanced.[5] Insulin suppresses the hormonal regulation of renal gluconeogenesis, whereas glucagon is known to in enhancing renal gluconeogenesis.[6] The renal cortex contains many enzymes associated with gluconeogenesis and produces glucose under various physiological conditions.[7] Thus, the primary function of SGLT-2 inhibitors is to inhibit glucose reabsorption in the kidney; it is important to investigate whether the effect of SGLT-2 inhibitors on renal glucose metabolism is direct or indirect.

SGLT-2 inhibitor treatment leads to a reduction in the insulin:glucagon ratio and

increases hepatic glucose production by gluconeogenesis.[8,9] In a recent study, administration of SGLT-2 inhibitors alleviated glucose toxicity and improved peripheral insulin sensitivity but increased glucose production in the liver by increasing glucagon levels.[10] Recent studies also showed that NAD-dependent deacetylase sirtuin-1 (SIRT1) interacts with and deacetylates peroxisome proliferative activated receptor-g co-activator 1 (PGC-1α or PPARGC1) leading to induction of gluconeogenic gene expression in the liver.[11] In addition, SGLT-2 inhibitors increase SIRT1 expression.[12] Therefore, we postulate that SGLT-2 inhibitors might affect renal gluconeogenesis by direct interaction with SGLT-2 on the renal cortex. To test the hypothesis that intrinsic renal gluconeogenesis is stimulated by dapagliflozin treatment, we conducted studies in both healthy mice and mice with hyperglycemia induced by high-fat diet (HFD). We studied the effect of the SGLT-2 inhibitor dapagliflozin on the mechanism of renal gluconeogenesis via inhibition of glucose reabsorption.

II. MATERIALS AND METHODS

1. Animals

Six-week-old C57BL/6J male mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). The mice were maintained at a temperature of 23ºC ± 2ºC and a humidity level of 60 ± 10% under a 12 hours light/dark cycle. Mice had free access to standard or high-fat diet (60% of calories derived from fat, D12492; Research Diets, Inc., New Brunswick, NJ, USA) for 20 weeks. Body weight and food intake are measured once a week, and random glucose measured three times a week using a blood sample collected from a tail vein to confirm hyperglycemia. After normal and hyperglycemia mice were sacrificed, blood collected via heart puncture and tissues harvested. Specimens were snap-frozen in liquid nitrogen and maintained at −80ºC until analyzed. All animal studies were approved by the Animal Care and Use Committee of the Yonsei University College of Medicine.

2. Experimental Treatments

To evaluate the short-term effect of SGLT-2 inhibitors on the normoglycemic condition, we administered 10 mg/kg of dapagliflozin for 7 days to 12-week-old BL6 mice. For prolonged fasting experiments, vehicle group and dapagliflozin group were fasted for 36 hours and then sacrificed. To investigate the effect of SGLT-2 inhibitor on the hyperglycemic condition, the mice were fed with 60% HFD for 20 weeks and then randomly assigned to either vehicle (saline) group or dapagliflozin (2 mg/kg/day) group. All HFD fed mice were fasted for 16 hours before sacrifice.

3. Metabolic measurements

For the oral glucose tolerance test (OGTT), 25% glucose (2 g/kg body weight) was administered via oral gavage after 16 hours fast. Blood was collected from the tail vein at 0, 30, 60, 90, and 120 minutes after glucose administration. Effect of dapagliflozin on insulin resistance before and after drug treatment was by an insulin tolerance test (ITT) conducted after drug administration. After fasting for 4 h, human insulin (0.75 units/kg) was injected intraperitoneally, and blood glucose measured at 0, 15, 30, 45, 60 and 120 minutes after insulin injection. For estimation of renal gluconeogenesis, 2 g/kg glutamine (Sigma-Aldrich, G3126, St Louis, MO, USA) dissolved in phosphate-buffered saline (PBS) was injected intraperitoneally after 16 hours fasting. Blood collected from the tail vein before glutamine injection (0 h) and at 15, 30, 60, 90, and 120 minutes after injection. Glucose levels were assessed using an Accu-Chek Performa glucometer (Boehringer-Mannheim, Indianapolis, IN, USA).

4. Isolation of mouse renal proximal tubular epithelial cells (mRPTECs)

Primary mouse renal proximal tubule epithelial cells (mRPTECs) were isolated from the kidney of mice. Briefly, kidneys were removed from sacrificed mice, weighed, and washed twice with ice-cold PBS. The kidney was de-capsulated and bisected to remove the medulla. Cortex and medulla separated with a scalpel and the cortex chopped into ~1 mm^3 pieces. Cortical tissues were transferred to ice-cold Dulbecco's modified Eagle's medium: Ham's medium F12 (DMEM-F12) containing 50 μg/ml gentamycin. The cortical tissue chopped by

fine scissors and transferred to DMEM-F12 containing 0.5 mg/ml collagenase type-II and digested by incubating at 37ºC for 10 minutes and vortexed for 1 minute. Next, we filtered the kidney digests through a series of brass sieves with increasingly smaller mesh sizes. Kidney digests were washed with the buffer for primary cells and filtered by a 40 μm nylon mesh. The collected cell fragments from the 40 μm nylon mesh were centrifuged at 150 x g for 10 minutes. The cell pellet was suspended in medium selective for epithelial cell growth, seeded onto 1% collagen-coated tissue culture plates and incubated at 37ºC with 5% CO_2. Cells were grown for 1 week in (DMEM: F12 (1:1), pH 7.4, with 20 mM HEPES-$NaHCO_3$, 2.5 mM L-glutamine, 1% penicillin and streptomycin, supplemented with 5 pg/ml of insulin, 35 pg/ml of transferrin, 50 nM dexamethasone, 5 nM triiodothyronine, 40 ng/ml hydrocortisone, 10 ng/ml EGF, and 1% FBS and medium was changed every 2-3 days until cell confluence (5-7 days).

5. Cell cultures and siRNA transfections

The primary cells described above were sub-cultured one week after isolation. Human renal proximal tubular epithelial cells (HK-2) were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA; SH30243.01). Media were supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA; SH30071.03), 1% penicillin, and 1% streptomycin (Thermo Fisher Scientific, Waltham, MA, USA; SV30010) in a 5% CO^2 incubator at 37ºC. For cell experiments, HK-2 cells and primary mRPTECs were maintained in a serum-free, high-glucose medium for 24 hours, then switched to low-glucose medium and treated with or without 10 μM dapagliflozin for 24 hours.

For siRNA-transfection experiments, HK-2 cells were transfected with 6 or 8 nM of control or human SGLT-2 siRNA (Santa Cruz Biotechnology Inc., Santa cruz, CA, USA) for 24 hours. Each experiment was conducted in triplicate in 6-well plates.

6. Western blot analysis

Total cell lysates were prepared from mouse kidney, liver, HK-2 cells, and primary mRPTECs with radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate; Cell Signaling Technology, Beverly, MA, USA; 9806) and the protein content was measured by Bradford assay (Bio-Rad, Hercules, CA, USA; 500–0006). Equivalent amounts of each protein extract were heat denatured in 5× sample buffer (2% sodium dodecyl sulfate (Tech & Innovation, Chuncheon, Korea; BSS-9005), 62.5 mM Tris, pH 6.8, 0.01% bromophenol blue, 1.43 mM mercaptoethanol, and 0.1% glycerol), separated on 10% polyacrylamide gels, and transferred electrophoretically onto a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA; 1620175). After blocking, membranes were treated with the following antibodies: anti-FOXO1 (Cell Signaling Technology, Beverly, MA, USA; 2880), anti-phospho-FOXO1 (Cell Signaling Technology, Beverly, MA, USA; 9461), anti-CREB (Abcam, Cambridge, UK; ab31387), anti-phospho-CREB (Cell Signaling Technology, Beverly, MA, USA; 9198), anti-PEPCK (Abcam, Cambridge, UK; ab28455), anti-G6Pase (Abcam, Cambridge, UK; Ab83690), anti-SGLT2 (Abcam, Cambridge, UK; ab37296), anti-PGC1α (NOVUS, Littleton, CO, USA; NBP1-04676), and anti-ACTB (Sigma-Aldrich, St Louis, MO, USA; A1978). Immunostaining was performed using chemiluminescent reagent (SuperSignal West Pico

Luminol/Enhancer solution; Thermo Fisher Scientific, Waltham, MA, USA; 34080) and Agfa medical X-ray film (Mortsel, CURIX 60). ACTB protein levels were used as loading control.

7. RNA extraction and Quantitative Real-Time PCR Analysis

Total RNA was isolated from tissues and cells with TRIzol reagent (15596–018, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions, and 2 mg of total RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (4368814, Applied Biosystems, Foster City, CA, USA). The cDNA was amplified in the ABI 7500 sequence detection system (4350584, Applied Biosystems, Foster City, CA, USA) using Power SYBR Green PCR Master Mix (4367659, Applied Biosystems, Foster City, CA, USA) with the following cycling conditions: 40 cycles of 95°C for 5 sec, 58°C for 10 sec, and 72°C for 20 sec. Target gene expression was normalized to that of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or ACTB, and quantitative analyses were conducted using the ΔΔcycle threshold method and StepOne Software version 2.2.2. The primer sets used for quantitative polymerase chain reaction (qPCR) are as follows:

Mouse Gapdh forward, 5'–GGCATTGCTCTCAATGACAA–3'

Mouse Gapdh reverse, 5'–ATGTAGGCCATGAGGTCCAC–3'

Mouse Pepck forward, 5'–TGACAGACTCGCCCTATGTG–3'

Mouse Pepck reverse, 5'–TGCAGGCACTTGATGAACTC–3'

Mouse G6pase forward, 5'–ACTCCAGCATGTACCGGAAG–3'

Mouse G6pase reverse, 5'–AAGAGATGCAGGAGGACCAA–3'

Mouse Sglt-2 forward, 5'–TGTGTGGCTACCTGAAGCTG–3'

Mouse Sglt-2 reverse, 5'–TTAGAGCAGCCCACCTCAGT–3'

Human ACTIN forward, 5'– GGACTTCGAGCAAGAGATGG–3'

Human ACTIN reverse, 5'– AGCACTGTGTTGGCGTACAG–3'

Human PEPCK forward, 5'–GAGAGAACTCCAGGGTGCTG–3'

Human PEPCK reverse, 5'–TTGCTTCAAGGCAAGGATCT–3'

Human G6pase forward, 5'–TTTGGGATCCAGTCAACACA–3'

Human G6pase reverse, 5'–CAGATGGGGAAGAGGACGTA–3'

Human SGLT-2 forward, 5'–TCTACTTCGCCATTGTGCTG–3'

Human SGLT-2 reverse, 5'–TATGCCGGAGACTGAAGACC–3'

8. Glucose output assay

For glucose output studies, HK-2 cells were cultured in serum-free media with or without dapagliflozin for 24 hours. After washing, the medium was replaced by glucose-free medium containing 2 mM sodium pyruvate, 20 mM sodium lactate, 5 mM L-glutamine, 15 mM HEPES, and without phenol red (GIBCO, GrandIsland, NY, USA; A14430-01). Glucose production was determined by the measurement of glucose in the media over a 5-hour peroid using a glucose assay kit (BioVision Inc., Milpitas, CA, USA, K606-100). Cells were lysed in modified RIPA buffer as described above and subjected to western blot analysis or protein normalization for glucose output studies. Alternatively, cells were harvested for RNA isolation and subsequent gene expression analysis as described above.

9. Statistical analysis

The data are presented as the mean ± SEM from multiple samples (n = 3–12 for each group in the animal study). All the in vitro experiments were replicated at least three independent experiments. Differences between groups were determined by the two-tailed, unpaired Student's t-test or one-way ANOVA with Bonferroni's post hoc test. Statistical analysis was performed using GraphPad PRISM 5.0 software (GraphPad Software, La Jolla, CA). p values less than 0.05 were considered statistically significant.

II. RESULTS

1. Study design

For normoglycemic condition, male C57BL/6J mice were orally treated with dapagliflozin at a dose of 10 mg/kg body weight for 7 days once a day. At seven days following dapagliflozin treatment, the fasting blood glucose was measured after 36 hours fasting as shown in Fig. 1A. To investigate the effect of dapagliflozin on hyperglycemia, the subjects who developed hyperglycemia after 14 weeks of high fat diet (HFD) were administrated daily to oral gavage of dapagliflozin (2 mg/kg body weight) for 6 weeks maintaining HFD (Fig. 1B).

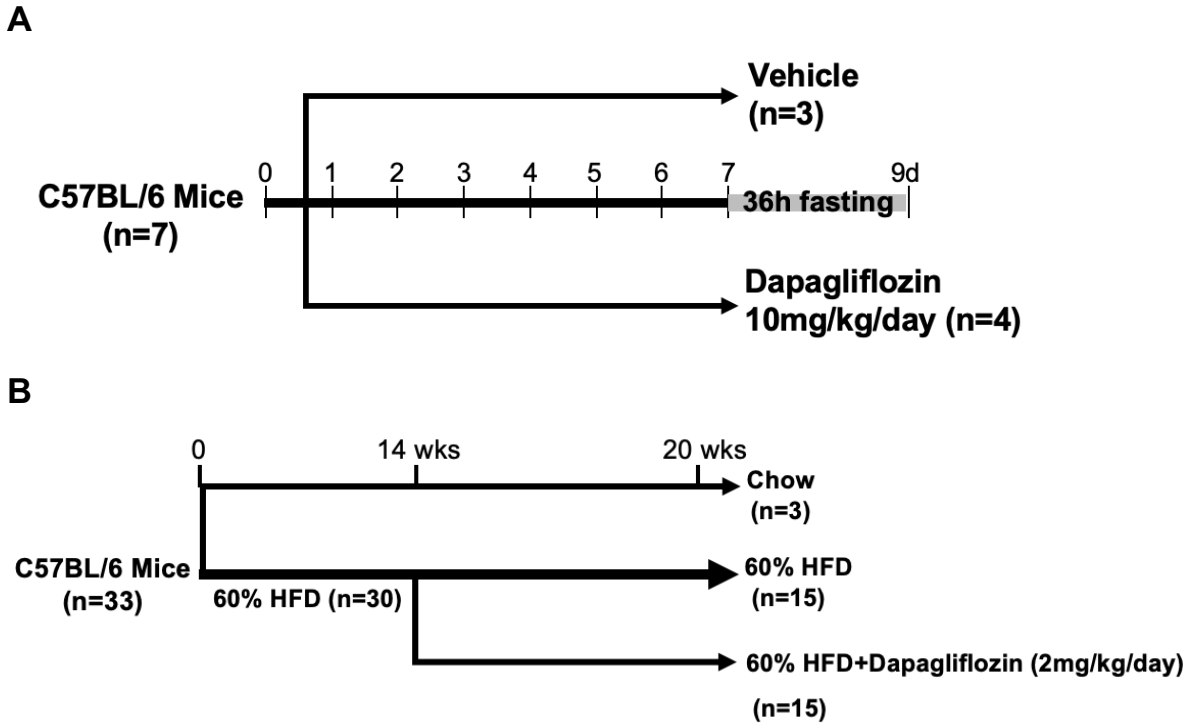


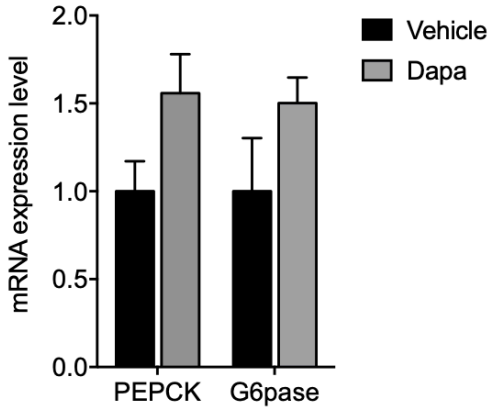
Figure 1. Outline of study design. (A) C57BL/6 mice were placed on a standard fat diet for 7 days, followed by a week treatment period with either vehicle or dapagliflozin (10 mg/kg) once a day. (B) Hyperglycemia was induced by placing C57BL/6 mice on a 60 % high fat diet for 14 weeks, followed by a six-week treatment period with either vehicle or dapagliflozin (2 mg/kg) once a day.

2. Dapagliflozin induces gluconeogenesis in human renal proximal tubular epithelial cells (HK-2)

To investigate the effect of dapagliflozin, a SGLT-2 inhibitor on renal gluconeogenesis, cells were treated, and related mRNA and protein changes were evaluated. We first studied the expression of gluconeogenic enzymes after 24 hours dapagliflozin treatment in HK-2 cells. HK-2 cells were exposed to different concentration of glucose in the presence or absence of dapagliflozin, and changes in the expression of PEPCK and related signaling pathways was studied. We studied the effect of both high (25 mM) and physiological (5.5 mM) glucose conditions (Fig. 2A and 2B). Under both conditions, dapagliflozin treatment increased PEPCK and G6Pase; however, the effect of dapagliflozin was more prominent under physiological glucose concentration. All of the following experiments were conducted with physiological glucose concentration after maintaining cells in high glucose for 24 hours. Under these conditions, qRT-PCR analysis revealed a decrease in the expression level of glucokinase (GCK) mRNA and pyruvate kinase (PKLR) in HK-2 cells treated with dapagliflozin, while dapagliflozin did not significantly affect the expression of GLUT2 (Fig. 2C). HK-2 cells also expressed significantly higher PEPCK and G6Pase proteins, the key enzymes of the glucose synthesis pathway, in the presence of dapagliflozin (Fig. 2D). We next examined whether changes in the gluconeogenic enzyme are regulated at the transcription level in HK-2 cells. We examined changes in phosphorylation of Forkhead Box O1 (FOXO1) and cyclic AMP response element binding protein (CREB), which play an important role in hepatic gluconeogenesis. Dapagliflozin-treated cells showed increased transcription factors and cofactors related to gluconeogenesis as shown in Fig 2. We observed a significant increase

in CREB activation as determined by the ratio of phosphorylated CREB to total CREB. In addition, dapagliflozin enhanced expression of proteins involved in the gluconeogenic pathway via the dephosphorylation and activation of the FOXO1 protein. When dephosphorylated-FOXO1 translocates from the cytoplasm to the nucleus, it upregulates the expression of gluconeogenesis-related genes; however, PGC-1α increased slightly (Fig. 2E). We also confirmed that glucagon treatment did not affect renal gluconeogenesis (Fig. 2F).

A



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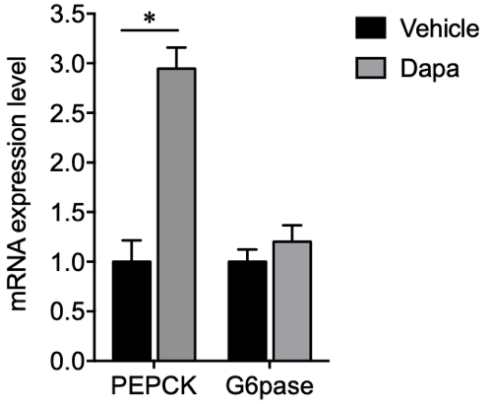

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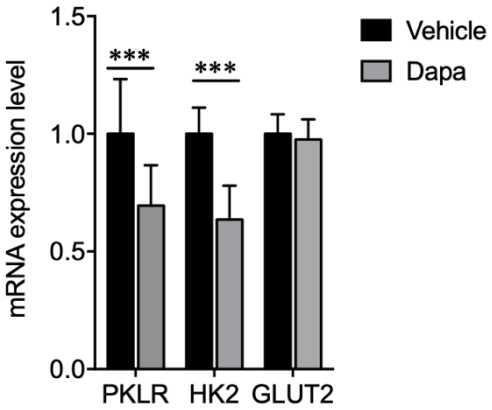

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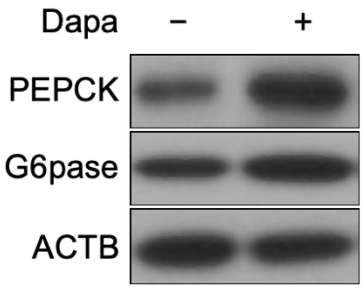

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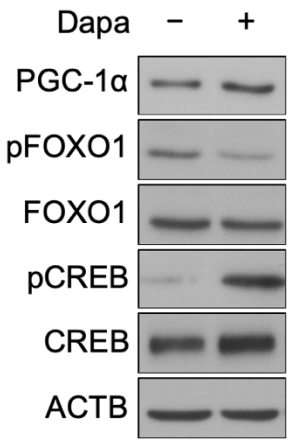

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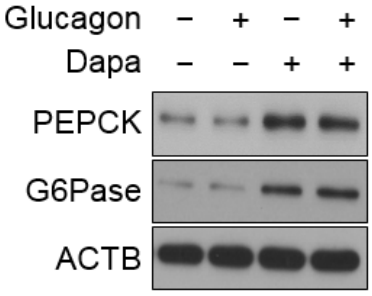


Figure 2. Induction of gluconeogenic enzymes by dapagliflozin in HK-2 cells. mRNA levels of PEPCK and G6pase in HK-2 cells on high (A) or high to low concentration (B) of glucose. mRNA expression of target genes was normalized to that of GAPDH. (C) mRNA levels of GLUT2 and glycolysis-related gene (PKLR and HK2) in HK-2 cells. (D) Representative western blot showing the levels of PEPCK (Pepck) and G6pase in HK-2 cells. (E) Representative western blot showing the levels of total protein and of phosphorylated protein for the indicated molecules in HK-2 cells. (F) Western blot for PEPCK and G6pase in HK-2 cells in dapagliflozin conditions (10 μM) with or without glucagon (10 nM). Protein levels were normalized to those of ACTB. Data were expressed as the mean ± SEM (n=3). *$p<0.05$ vs. untreated cells (Vehicle). Abbreviations: Dapa, dapagliflozin.

3. Increased glucose output in HK-2 cells with dapagliflozin and gluconeogenesis substrate

To confirm the above results, we performed glucose output assay with glutamine, the preferred precursors of gluconeogenesis in kidney. This assay aims to evaluate the ability of renal proximal tubular epithelial cells to release glucose, newly synthesized using glutamine as the substrate under basal and dapagliflozin-treated conditions. We measured glutamine driven glucose production in HK-2 cells (Fig. 3A). Moreover, we compared glutamine and alanine as gluconeogenic precursors. In the presence of dapagliflozin, glutamine increased glucose output, whereas alanine had not increased glucose production significantly (Fig. 3B).

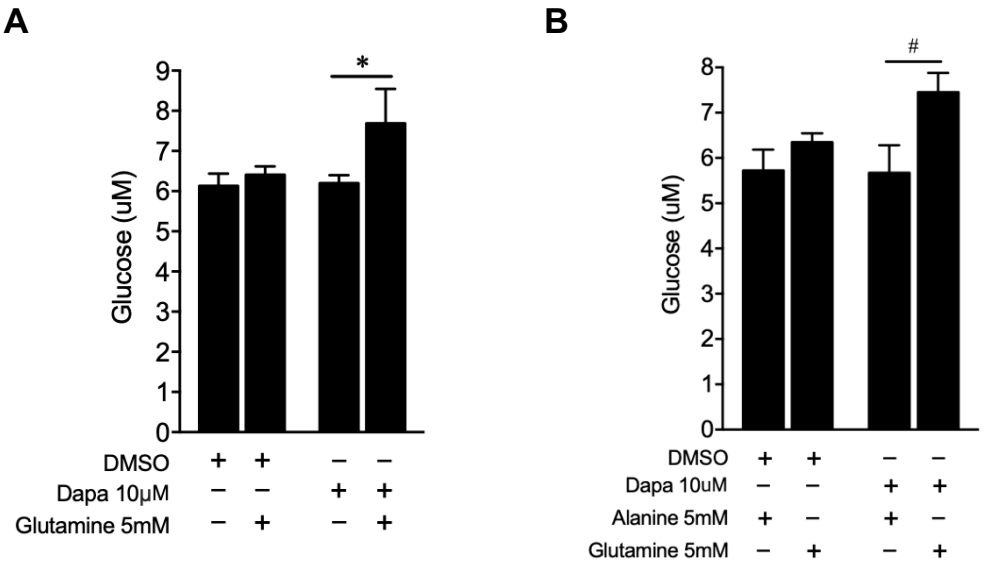


Figure 3. Renal glucose production from glutamine in HK-2 cells. (A) Glucose production in HK-2 cells when glutamine is abundant in the state of increased gluconeogenesis by dapagliflozin. (B) Comparison of alanine and glutamine. Data are presented as mean ± SEM of three independent experiments. Significance level at *$p < 0.05$ vs. non-amino acid group and #$p<0.05$ vs. alanine-treatment. Abbreviations: Dapa, dapagliflozin.

4. SGLT-2 knockdown in HK-2 cells regulates gluconeogenic enzyme and associated transcription factors

We used small interfering RNA (siRNA) technology to knock down SGLT-2 in HK-2 cells, to determine whether the enhanced renal gluconeogenesis could be observed by direct inhibition of SGLT-2. HK-2 cells were treated with SGLT-2 siRNA for 24 hours. We used siRNA to reduce expression of the SGLT-2 in HK-2 cells (Fig. 4A). Compared with that in the scrambled siRNA group, the knockdown of SGLT-2 significantly promoted gluconeogenic enzyme expression, and the result is similar to that of dapagliflozin treatment. siRNA treated-cells showed significant increase in PEPCK and G6pase expression (Fig. 4B). The transcription factor was also altered to activate gluconeogenesis.

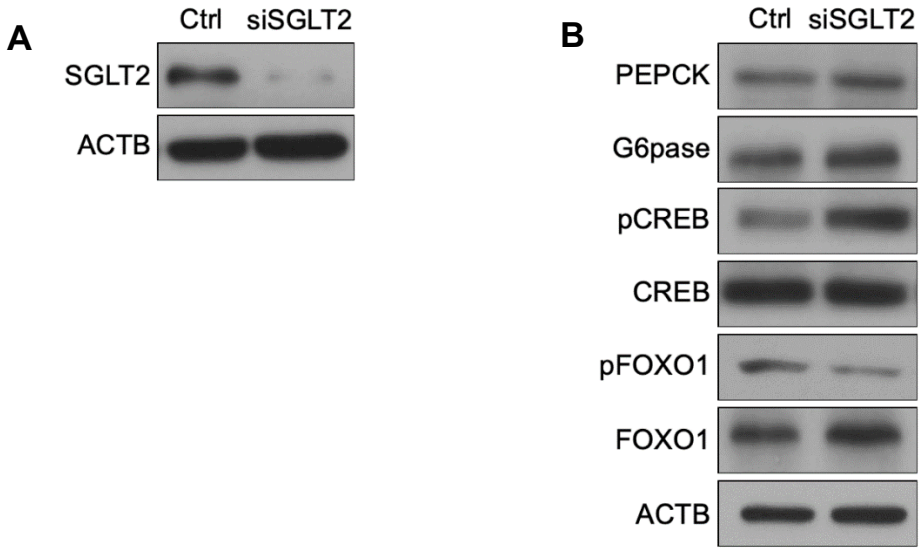


Figure 4. Effect of SGLT-2 knockdown in HK-2 cells. (A) SGLT-2 siRNA treatment decreased SGLT-2 protein expression (A) and increased gluconeogenesis related protein in HK-2 cells (B). Overexpression of siRNA for 24 hours promoted the endogenous expression of PEPCK, G6pase, phosphorylated-CREB, and dephosphorylated-FOXO1.

5. Ex-vivo analysis of renal proximal tubular cells

Cells isolated from HFD-fed animals were incubated for 24 hours with high glucose (25 mM). Then we switched the cells to low glucose (5.5 mM) as physiological conditions with and without dapagliflozin treatment for 24 hours. Exposure of cells to dapagliflozin enhanced the upregulation of PEPCK and G6Pase, known targets for FOXO1 and CREB (Fig. 5A). In addition, PGC-1a, FOXO1, and CREB were more active in dapagliflozin-treated cells compared to that in untreated-cells (Fig. 5B).

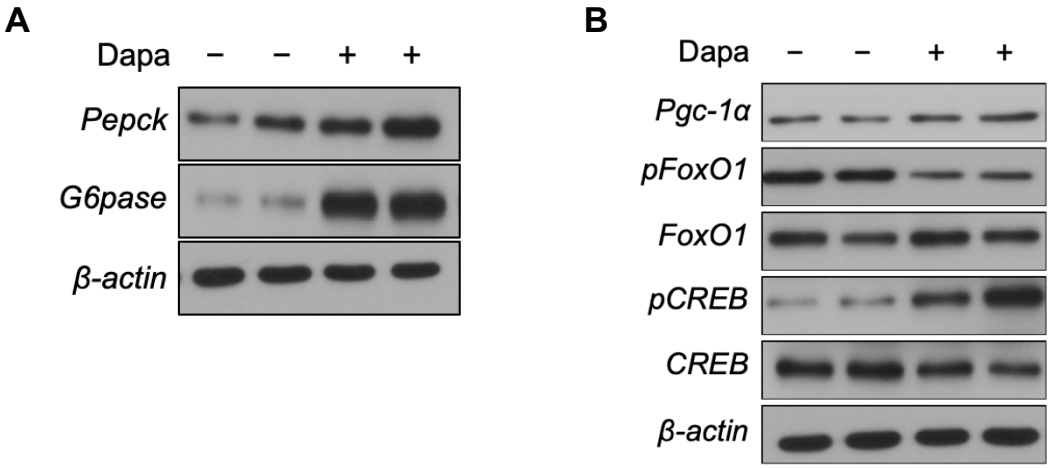


Figure 5. Western blot analysis of ex vivo-treated renal proximal tubular cells. Western blot showing effects of dapagliflozin on mRPTECs expression of Pepck, G6pase (A), Pgc-1α and transcription factors protein expression (B). Abbreviations: Dapa, dapagliflozin.

6. Effects of short-term treatment with dapagliflozin in normoglycemic mice

For evaluation of the short-term effect of SGLT-2 inhibitors under normoglycemic condition, 12-week old male C57BL/6J mice were treated with dapagliflozin orally at a dose of 10 mg/kg/day for 7 days. After seven days following dapagliflozin treatment, mice were fasted for 36 hours and then sacrificed. (n = 3-4 in each group). In the dapagliflozin-treated mice, blood glucose level was lower than that of vehicle-treated mice without a statistical difference (Fig. 6A). qPCR analysis of the effect on renal expression of gluconeogenic genes in mice showed increased expression of PEPCK and G6Pase (Fig. 6B), and significantly increased the expression of phosphorylated CREB protein levels. Moreover, PGC-1α was enhanced (Fig. 6C).

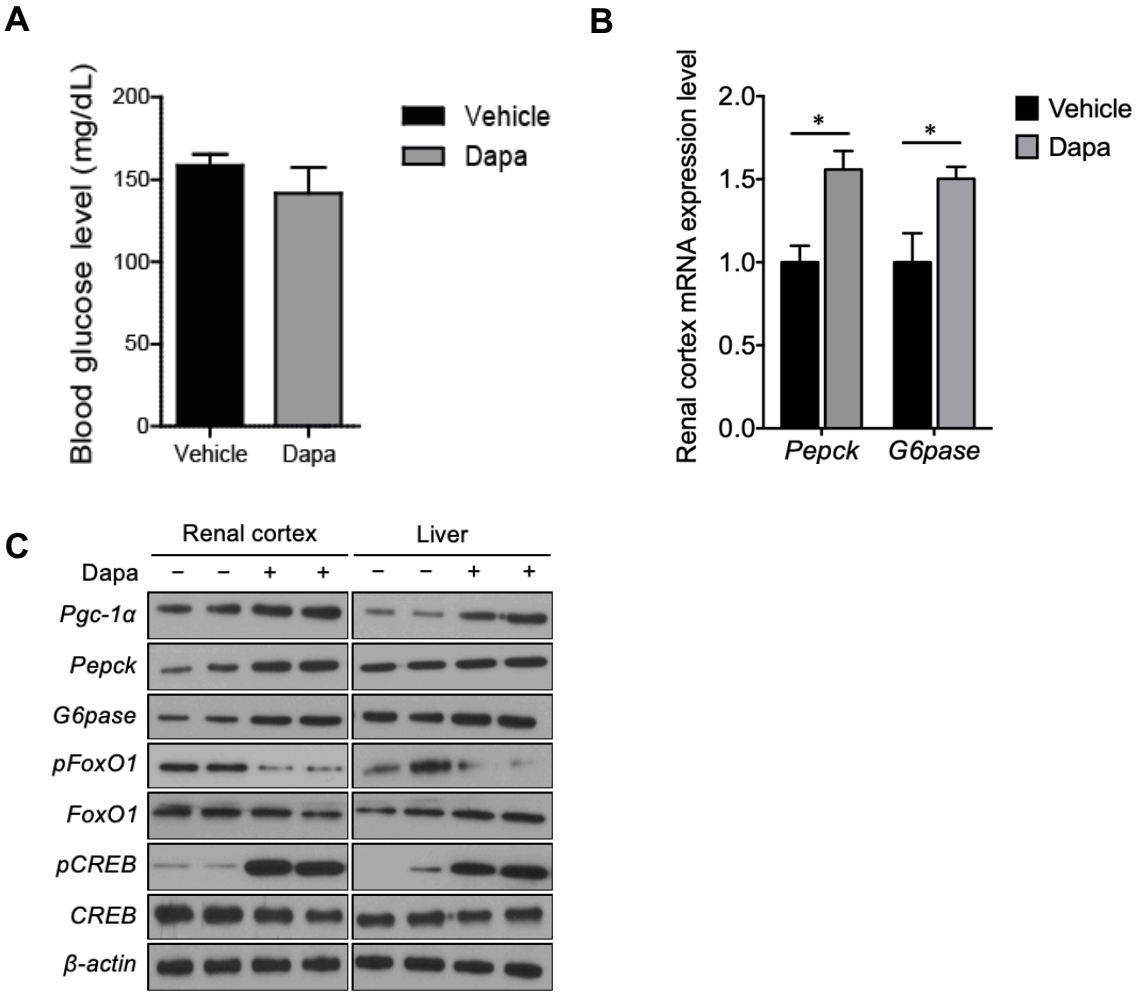
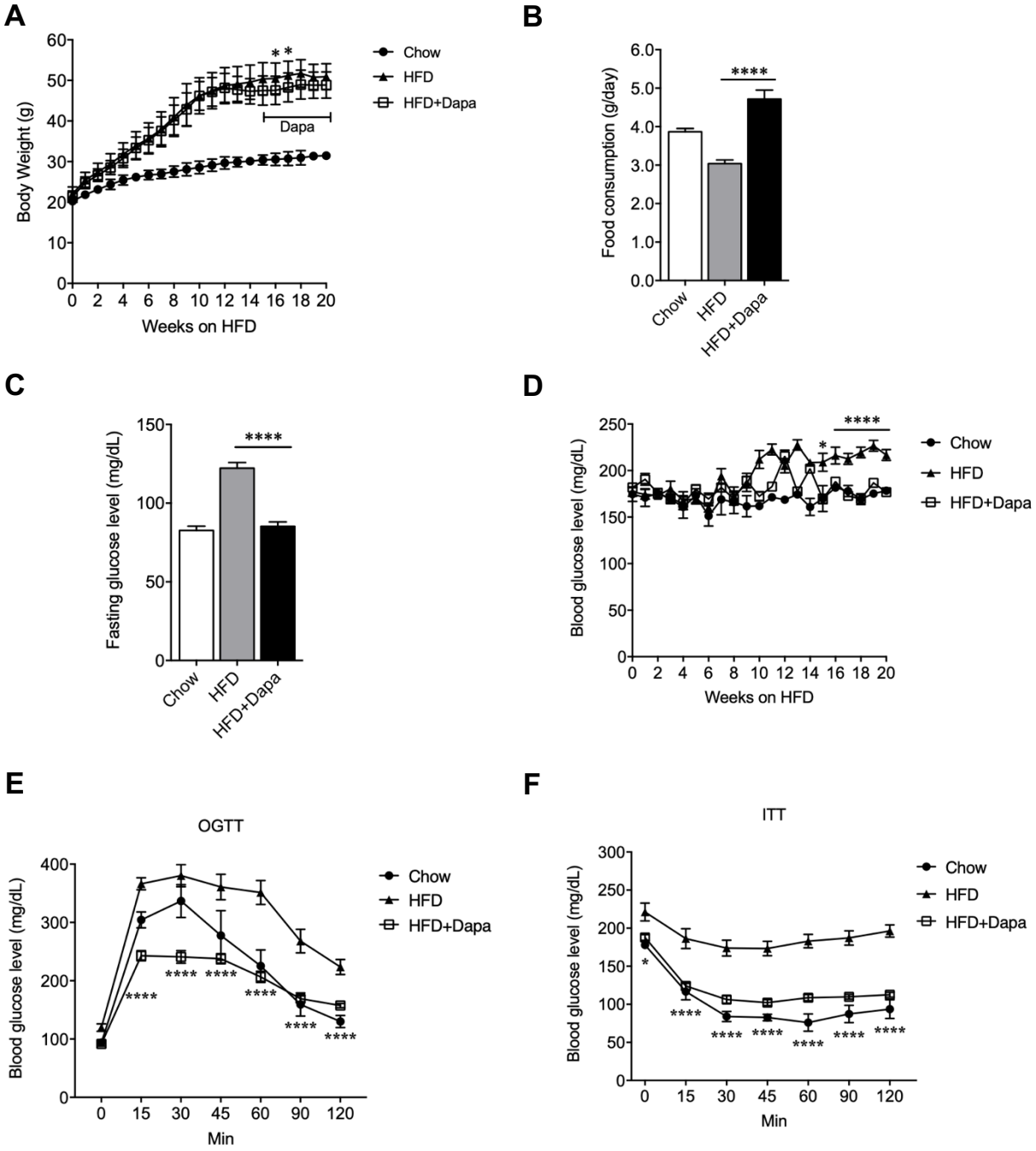


Figure 6. Effect of short-term administration of dapagliflozin in normoglycemic mice. (A) Blood glucose levels (B) mRNA levels of gluconeogenic genes (Pepck and G6pase) in the renal cortex of male C57BL/6J mice (n = 3-4 mice per group). mRNA expression of target genes was normalized to that of Gapdh. (C) Representative western blot showing levels of total and phosphorylated CREB and FoxO1 in the renal cortex and liver of male C57BL/6J mice. Results shown are representative of three blots. Protein levels were normalized to those

of ACTB (β-actin). Data presenting as mean (± SEM) values. *p<0.05 and ****p<0.01 using Student's t-test against HFD group. Abbreviations: Dapa, dapagliflozin.

7. Long-term dapagliflozin treatment improves glucose homeostasis in HFD-fed hyperglycemic mice

To investigate the long effect of dapagliflozin on the HFD fed mice, mice that developed hyperglycemia after 14 weeks of HFD feeding were administrated daily dapagliflozin (2 mg/kg body weight) or vehicle by oral gavage for 6 weeks while maintaining HFD. All HFD-fed mice fasted for 16 hours before sacrifice. Dapagliflozin treatment led to a slight reduction in the body weight during the study period (Fig. 7A), but the amount of food intake was significantly higher in HFD + Dapa groups than in the HFD group (Fig. 7B). In the HFD + Dapa group, the fasting blood glucose levels (Fig. 7C) and random glucose levels (Fig. 7D) were significantly lower than those in the HFD group. To evaluate glucose homeostasis, we measured physiological and metabolic parameters in the three groups of mice. HFD + Dapa group showed significant improvement in oral glucose tolerance (Fig. 7E) and insulin resistance (Fig. 7F) than the HFD group. We performed a glutamine tolerance test (QTT) to assess renal gluconeogenesis. QTT result showed increased glutamine tolerance in dapagliflozin treated hyperglycemic mice (Fig. 7G). Kidney size was not different among the groups (Fig. 7H). Serum alanine transaminase (ALT) level decreased with dapagliflozin administration (Fig. 7I).



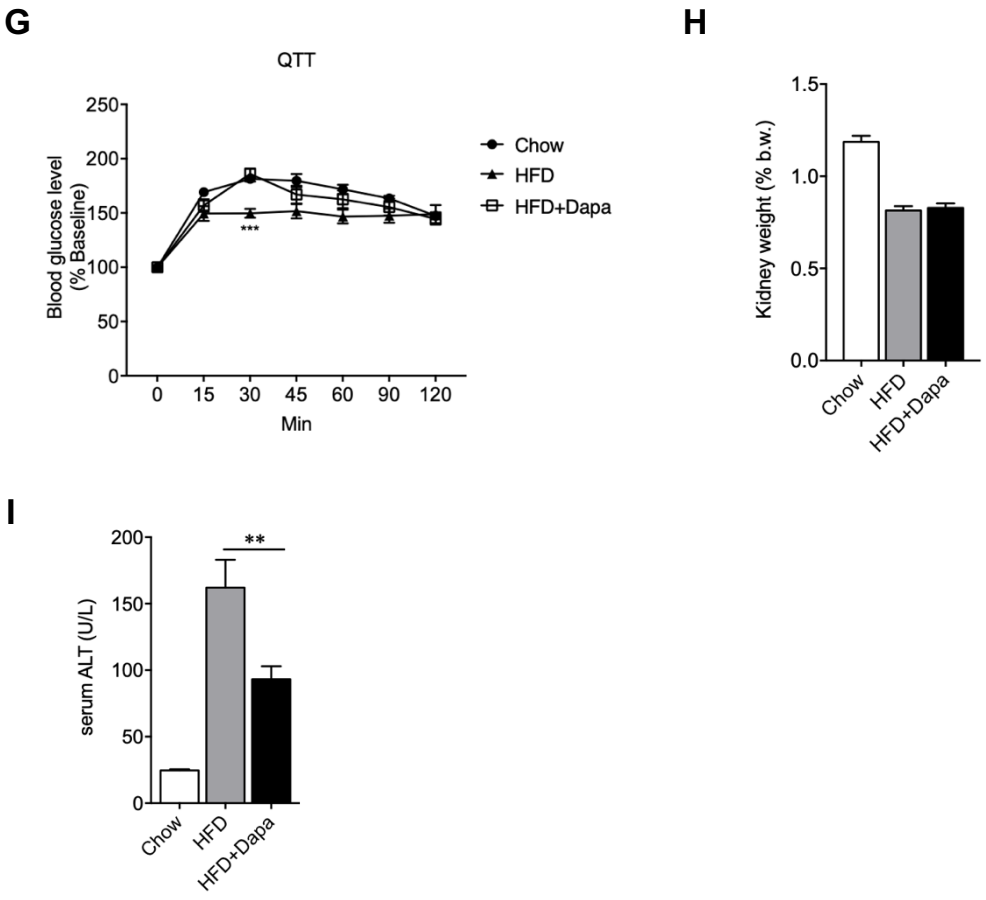


Figure 7. Improved metabolic profile of HFD-fed hyperglycemic mice by administration of dapagliflozin. Metabolic parameters. (A) Body weight, (B) food consumption, (C) fasting blood glucose levels (D) blood glucose levels, (E) Blood glucose levels during OGTT (F), blood glucose levels after an insulin challenge (G) blood glucose levels after glutamine challenge are shown for BL6 mice on a chow diet, high-fat diet (HFD) and HFD+Dapa. (H) The ratio of parametric kidney weight and (I) levels of serum ALT (n = 3 for chow groups; n = 15 for HFD groups; n = 15 for HFD+Dapa groups) *p<0.05, **p<0.01, ***p<0.005 and ****p<0.001 using Student's t-test against HFD group. Data presenting as mean (± SEM) values.

8. Long-term treatment of dapagliflozin induces gluconeogenesis in the renal cortex of HFD-fed hyperglycemic mice

On the 6th week of dapagliflozin administration, mice were killed and their kidneys were analyzed. As shown in Fig 8A, the mRNA expression of PEPCK and G6Pase was enhanced with dapagliflozin treatment. In addition, protein expression of PEPCK and G6Pase was higher in dapagliflozin treated HFD group than in vehicle treated HFD group (Fig. 8B) Dapagliflozin also increased the levels of phosphorylated CREB and PGC-1α. FOXO1 phosphorylation decreased in the dapagliflozin treated group (Fig. 8C)

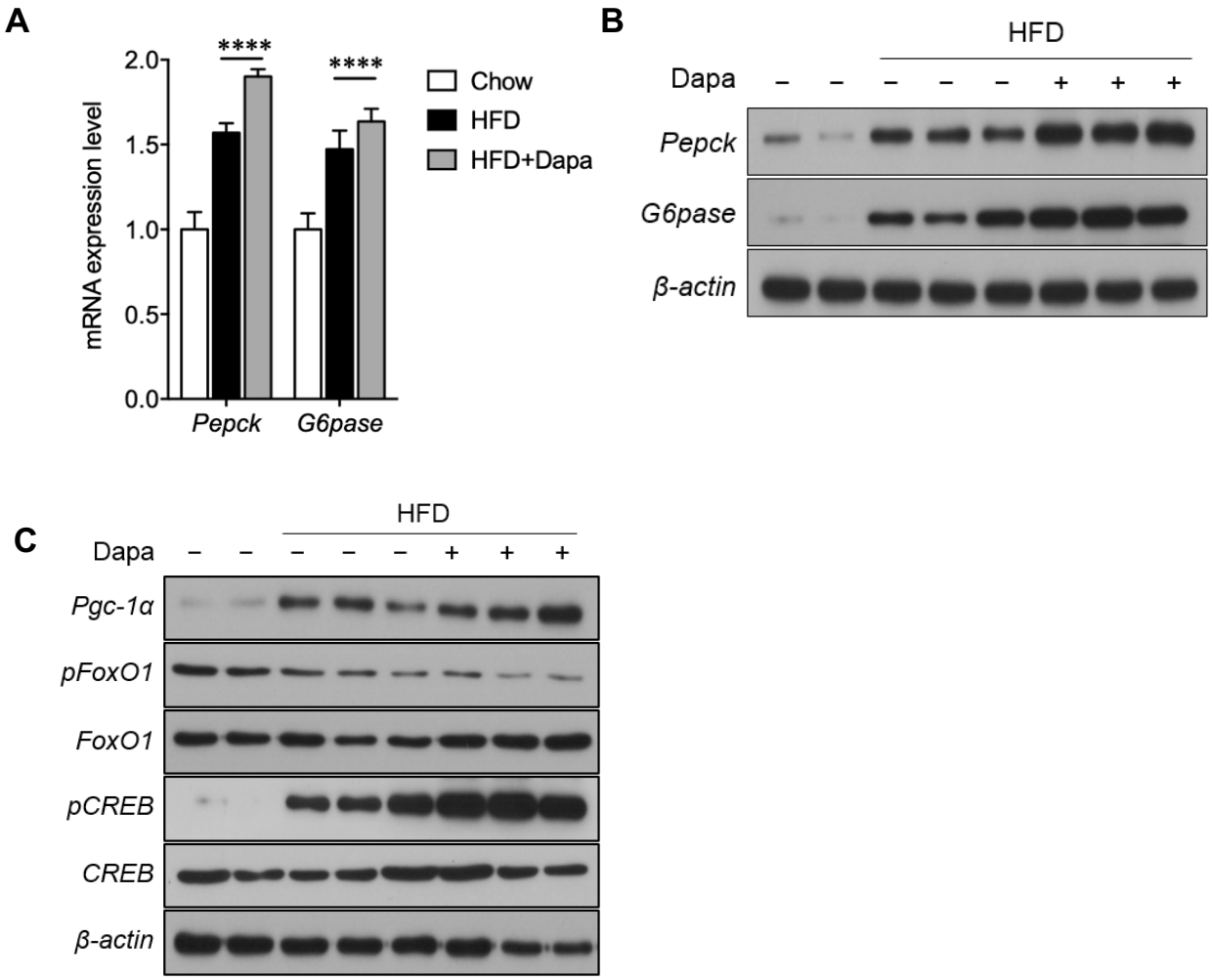


Figure 8. Enhancement of gluconeogenesis in HFD-fed hyperglycemic mice by administration of dapagliflozin. (A) Real-time PCR for selected genes involved gluconeogenesis (Pepck and G6pase) performed on mRNA isolated from the renal cortex of male C57BL/6J mice fed 60% HFD for 20 weeks with or without dapagliflozin (n = 12 mice per group). mRNA expression of target genes was normalized to that of Gapdh. (B) Protein levels of PEPCK and G6PC in the renal cortex. (C) Representative western blot showing

levels of total and phosphorylated CREB and FoxO1 and protein level of cofactor PGC-1α in the renal cortex. Results shown are representative of three blots. Expression of target protein was normalized to that of β-actin. Data presenting as mean (± SEM) values. ****$p<0.001$ vs. HFD group.

IV. DISCUSSION

Although the effect of dapagliflozin on the improvement of whole-body glucose metabolism has been characterized, its effect on glucose metabolism in the kidney is not well documented. Specifically, studies on the influence of the SGLT-2 inhibitor on mechanisms linking gluconeogenesis activation in the renal cortex and control of circulating glucose are lacking. Here, we identified an unknown role for dapagliflozin in renal gluconeogenesis. Our results differ from other studies in that dapagliflozin was shown to regulate glucose levels by direct interaction with SGLT-2.

SGLT-2 inhibitor is an anti-diabetic drug that lowers blood glucose by inducing glycosuria. SGLT-2 plays a vital role in glucose uptake in proximal tubule cells. Under hyperglycemic conditions, proximal renal tubules increase SGLT-2 activity and expression to increase glucose reabsorption in the proximal tubules.[13,14] Inhibition of SGLT-2, therefore, has the potential to not only acutely lower hyperglycemia but also improve glucose homeostasis by reducing glucose toxicity and preventing β-cell failure. The primary function of SGLT-2 inhibitors is to prevent kidney glucose reabsorption; however, the role of the kidneys in glucose metabolism requires attention, just as liver altering glucose output indirectly. A previous study showed a significantly higher level of hepatic PEPCK after dapagliflozin administration compared to untreated mice.[15] However, in that study, changes in renal PEPCK expression by dapagliflozin administration is not examined. We evaluated the effect of dapagliflozin on two key gluconeogenesis enzymes, PEPCK, and G6Pase, in the renal cortex.

Gene transcription of PEPCK and G6Pase is regulated not only by transcription factors but also by a complex interaction between cofactors.[16] CREB and FOXO1 are important transcription factors in hepatic gluconeogenesis pathway. However, because the functions of CREB and FOXO1 are not clear in the kidney, we investigated changes in levels of the CREB and FOXO1 proteins and phosphorylation status, involved in the regulation of the gluconeogenic genes. We showed that dapagliflozin effectively induces the expression of gluconeogenic enzymes through activation of these transcription factors in the renal cortex, thereby increasing gluconeogenesis. In addition, similar results were shown in ex-vivo experiments.

We showed upregulated expression of PEPCK and G6Pase and dephosphorylated-FOXO1 and PGC-1α regulated when HK-2 cells were exposed to dapagliflozin. Of note, the phosphorylation of CREB increased significantly. This result shows increased gluconeogenesis under *in-vitro* conditions where physiological conditions were not met. However, glucagon did not affect the dapagliflozin-induced increased renal gluconeogenesis. Western blot analysis showed increased levels of PEPCK, G6Pase, PGC-1α, and phosphorylated CREB in dapagliflozin-treated primary cells compared to that in untreated cells. In glucose production assays, changes in gluconeogenesis related-genes and proteins eventually led to increased production of glucose. For evaluation of effect of the SGLT-2–mediated glucose synthesis pathway on the enhancement of renal gluconeogenesis, we tested glucose production in HK-2 cells, a major target of dapagliflozin action. In the glutamine-rich state, glucose production increased significantly, while alanine levels did not show a significant difference. In addition to the above results, it is essential to confirm whether

dapagliflozin has a direct or indirect effect. Thus, HK-2 cells were treated with human SGLT-2 siRNA. Knockdown of SGLT-2 showed the same effect as its inhibitor dapagliflozin, thus showing that increased renal glucose production induced by dapagliflozin was due to its direct inhibition of SGLT-2.

We showed that dapagliflozin enhances gluconeogenesis by regulating gluconeogenic enzymes and transcription factors under in vitro conditions According to our results of in vivo experiments, there was no difference in blood glucose between vehicle group and dapagliflozin-treated group after fasting for 36 hours. These results show that even if the liver and kidney contribute to the whole-body glucose level, the degree of contribution of the kidney to whole-body glucose level is not large when liver function is normal. In addition, the expression of the gluconeogenic genes PEPCK and G6Pase in the renal cortex was increased further in normoglycemic mice treated acutely with dapagliflozin for 7 days. Activation of CREB and FOXO1 was also shown at the protein level. Dapagliflozin had no effect on plasma blood glucose levels in 36 hours fasted mice, but it regulated gluconeogenic enzymes and transcription factors related gluconeogenesis compared to vehicle-treated mice. In a hyperglycemic model with obesity and diabetes induced by HFD, gluconeogenesis is higher than in normoglycemic conditions.[17] In several studies with animal models, a trend towards a significant increase in caloric intake without body weight gain when SGLT-2 inhibitors were administered, and the similar trend was observed in our study. As expected, our data showed a significant reduction in both random non-fasting and fasted blood glucose levels when administered with dapagliflozin. In addition, glucose tolerance and insulin resistance in HFD-fed mice aggravated but was mitigated by dapagliflozin. Intraperitoneal glutamine tolerance

test used to examine renal gluconeogenesis showed a slight increase in the dapagliflozin treated HFD group compared to the HFD group with statistical significance. Our results showing the increased glucose response to exogenous glutamine loading in mice provide additional evidence for elevated renal gluconeogenesis with dapagliflozin treatment. Moreover, the phosphorylated-CREB protein levels increased and phosphorylated-FOXO1 protein levels decreased after treatment with dapagliflozin. Increased renal tubular transcription factor activity also results in a higher induction of PEPCK and G6Pase. In addition, PGC-1α, a cofactor of FOXO1 regulates glucose metabolism and increases protein and mRNA expression, indicating that dapagliflozin has an effect not only on inhibition of glucose reabsorption in the kidney but also on enhancing gluconeogenesis in the renal cortex. After excretion of excess glucose by the kidneys, the body will use the *de novo* synthesis of glucose for gluconeogenesis. Therefore, gluconeogenesis is an important process when inhibiting SGLT-2.

It is well recognized that dapagliflozin, because of its insulin independent mechanism, has an advantage with a low risk for induction of hypoglycemia. Therefore, our results cautiously suggest the possibility of a partial reduction in the risk of hypoglycemia by increasing glucose synthesis in the kidney as well as in the liver. Finally, further studies are needed when the treatment of SGLT-2 inhibitors such as dapagliflozin is combined with other hypoglycemic drugs.

V. CONCLUSION

In this study, we investigated the effects of SGLT-2 inhibitor on glucose metabolism in renal cortex. We found that the representative SGLT-2 inhibitor, dapagliflozin, significantly increased the expression of enzymes and transcription factors involved in gluconeogenesis and also increased final product glucose output. Moreover, we also confirmed by siRNA experiments that these alterations are the SGLT-2-mediated pathway. In summary, the data in this study clearly show that dapagliflozin increased the gluconeogenic enzyme in the renal cortex and consequently increased gluconeogenesis. Our findings provide insights into the mechanism of dapagliflozin in the regulation of glucose homeostasis in the renal cortex.

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ABSTRACT (IN KOREAN)

나트륨-포도당 공동수송체 2 (SGLT-2) 억제제가 신장 근위 세뇨관의 Gluconeogenesis 에 미치는 영향

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김진희

Dapagliflozin 은 SGLT-2 억제제로서 신장 관 포도당 재 흡수를 억제하여 고혈당을 개선 한 항 당뇨병 치료제이다. SGLT-2 는 신장 근위 세뇨관에서 거의 독점적으로 발현되며 포도당 재흡수 이외의 생리학적 역할은 널리 연구되지 않았다. 본 연구에서는 우리는 시험 관내, 생체 내 및 생체 내 신장 gluconeogenesis 에 SGLT - 2 억제제의 효과를 탐구하였다. BL6 마우스에 단기간 (7 일 투여) 또는 장기간 (6 주 투여) dapagliflozin 을 투여했다. Dapagliflozin 투여에 의해 혈당이 개선되었지만, 직접적으로나 간접적으로 신장의 gluconeogenesis 를 향상시켰다. CREB 인산화의 발현 증가와 FoxO1 인산화를 감소는 포도당 생성 효소(PEPCK 와 G6Pase)의 mRNA 와 단백질 발현을 증가시켰다. 또한 신장 근위 세뇨관 상피 세포 특이적인 SGLT-2 의 knockdown 은 포도당신생합성에 대하여 dapagliflozin 과 동일한 효과를 나타냈다. 따라서, dapagliflozin 은 SGLT-2 를 매개하여 포도당신생합성의 증가를 직접적으로

유도한다는 것을 발견했다. 그러나 dapagliflozin 을 통한 신장의 포도당 생산량의 증가는 dapagliflozin 이 공복 혈당을 유의하게 감소 시킴에 따라 전신 포도당 대사에 영향을 미치지 않았다. 신장의 당 대사에서 dapagliflozin 의 또 다른 역할 제안 및 직접적인 효과에 대한 향후 추가적인 연구가 필요해 보인다.

핵심되는 말: SGLT-2, Dapagliflozin, 포도당신생합성, 신장근위세뇨관