Ipragliflozin improves mitochondrial abnormalities in renal tubules induced by a high-fat diet

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INTRODUCTION
Metabolic syndrome and type 2 diabetes are pandemic. However, effective treatments for the organ failure associated with these health problems have not been found. Sodium–glucose cotransporter 2 (SGLT2) inhibitors are a new class of antidiabetic drugs that have been confirmed to reduce the risk of cardiovascular events and show renoprotective effects in patients with type 2 diabetes.

Empagliflozin Cardiovascular Outcome Event Trial in Type 2 Diabetes Mellitus Patients—Removing Excess Glucose was used to show that empagliflozin suppressed the incidence or worsening of diabetic nephropathy and deterioration of renal function. Indeed, renal replacement therapy was reduced by 55% in the empagliflozin group. In addition, the Canagliflozin Cardiovascular Assessment Study trial showed that canagliflozin significantly reduced cardiovascular risk, reduced the urinary albumin excretion rate, resulted in a 40% reduction in the composite renal replacement therapy and reduced renal death.
SGLT2 inhibitor, empagliflozin, has been shown to attenuate renal hyperfiltration in individuals with type 1 diabetes, likely by affecting tubuloglomerular feedback mechanisms. Such attenuation of hyperfiltration with SGLT2 inhibitors is likely a class effect of all SGLT2 inhibitors, and is the most sophisticated explanation for the renoprotective effects of SGLT2 inhibitors. However, various potential renoprotective effects of SGLT2 inhibitors have been proposed in experimental animal models. The kidney is an organ with a high energy demand and is rich in mitochondria. In addition, persistent mitochondrial dysfunction can also lead to chronic deficiencies in cell and organ function similar to disorders of the heart, brain and kidney, which are organs known to be associated with mitochondrial disease. Mitochondrial dysfunction causes podocyte injury, tubular epithelial cell damage and endothelial dysfunction. Mitochondrial fusion and fission are necessary not only for mitochondrial morphology maintenance, but also for mitochondrial deoxyribonucleic acid (DNA) integrity, regulating cellular survival and death, transmitting redox-sensitive signals, and participating in metabolic processes. Mitochondrial fusion depends on mitofusion (Mfn1) or Mfn2 and the dynamin family GTPase optic atrophy factor 1 (Opal). Dynamin-related protein-1 (Drp-1) is required for mitochondrial fission in mammalian cells. The proximal tubules in the kidney have many mitochondria that are in a more oxidized state than those in the distal tubule segments in which tubules can utilize glycolysis as their main energy source. Mitochondrial dysfunction was shown to occur in insulin resistance associated with high-fat diet (HFD)-induced oxidative stress in mice.

Here, we show that in mice fed a HFD, the SGLT2 inhibitor improves proximal tubular cell integrity through modulation of mitochondrial damage.

METHODS
Animal experimental model
Eight-week-old male C57/BL6 mice (Sankyo Lab Service, Tokyo, Japan) were utilized in all experiments. The mice were first fed a control diet or HFD (60% fat; Research Diets Inc., New Brunswick, NJ, USA) for 8 weeks. After 8 weeks of being on the HFD, the HFD-induced obese mice were divided into two groups: HFD with vehicle (0.5% methylcellulose) or HFD with oral 1-1gloflazin administration (HFD ipra; 10 mg/kg; Figure 1a). All mice were euthanized 16 hours after HFD feeding. Ipragliflozin was provided by Astellas (Astellas Pharma Inc., Tokyo, Japan) with a co-research agreement. The bodyweight and blood glucose levels (Antsense III; Fukuoka Densi Co., Tokyo, Japan) were monitored every week. The amount of food intake was measured every 2 days. Urinary 8-hydroxydeoxyguanosine (8-OHdG) levels were measured according to the manufacturer’s protocol (Japan Institute for the Control of Aging [JalICA], Shizuoka, Japan). The animal experiment was approved by the Institutional Animal Care and Use Committee of Kanazawa Medical University (protocol number. 2014-86).

Electron microscopy
Kidney tissues were fixed in 0.1 mol/L cacodylate acid with 2% glutaraldehyde at 4°C and dehydrated at room temperature. Electron microscopy and transmission electron microscopy were carried out by the Hanaichi Ultrastructure Research Institute (Aichi, Japan). The mitochondrial morphology was imaged and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Western blot analysis
Proteins were harvested using radioimmunoprecipitation assay lysis buffer (lysis buffer, phenylmethylsulfonyl fluoride, protease inhibitor cocktail and sodium orthovanadate, which were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Protein lysates in sample buffer were boiled at 94°C for 5 min, and the lysates were separated on sodium dodecylsulfate-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Pall Corporation, Pensacola, FL, USA) by using the semidy method. After blocking with Tris-buffered saline with Tween 20 containing 5% non-fat dry milk or 5% bovine serum albumin, the membranes were incubated with primary antibodies (SGLT2:1:50, Opal1:1:1000, Mfn2:1:1000, Drp-1:1:800 dilution) at 4°C overnight. The membranes were washed with Tris-buffered saline with Tween 20 three times and then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. After washing with Tris-buffered saline with Tween 20 three times, the blots were developed with an enhanced chemiluminescence detection system (Pierce Biotechnology, Rockford, IL, USA) and visualized using an Image-Quant LAS 400 camera system (GE Healthcare Life Sciences, Uppsala, Sweden). Antibodies were purchased from following (SGLT2:GTX59872 (GeneTex, Inc. Irvine, CA, USA), Opal1:ab119685 and Mfn2:ab56889 (Abcam, Cambridge, UK), Drp-1:8570 (Cel Signaling Technology, Danvers, MA, USA)).

Cell culture
HK-2 cells (human proximal endothelial cells) were cultured in medium (Opti-MEM, Manassa, VA, USA) with epidermal growth factor human recombinant and bovine pituitary extract. Palmitate (PO500; Sigma, St. Louis, MO, USA) was dissolved in 0.1% dimethyl sulfoxide into final stock concentration 10 mmol/L1,12, and dimethyl sulfoxide was used as the control. In the presence or absence of SGLT2 inhibitor or small interfering ribonucleic acid (siRNA)-mediated knockdown of SGLT2, palmitate (adjusted to 150 μmol/L) or high glucose (30 mmol/L) were incubated in media for 48 h.

Transfection experiment
HK-2 cells were transfected with siRNA (100 nmol/L) targeting SGLT2 (Invitrogen, Carlsbad, CA, USA; SGLT2: 5’-GUCAUUGCGAUAAUUCG). When the cells were 70% confluent, Lipofectamine 2000 (following the manufacturer’s instructions; Invitrogen, Carlsbad, CA, USA) and the target RNA were added to the keratinocyte-SFM (1X; Life
Technologies, Green Island, NY, USA) and incubated in cell culture for 6 h. The medium was replaced with fresh medium with or without high glucose (30 mmol/L) or palmitate (150 μmol/L) treatment for 48 h.

Mito-tracker green staining
The treated HK-2 cells were cultured in eight-well culture slides. When the cells reached 80–90% confluence, we removed the medium and added pre-warmed MitoTracker® Green (100 nmol/L; Invitrogen) for 30 min at 37°C. After being washed twice with phosphate-buffered saline, the cells were fixed by 4% formaldehyde and permeated by TritonX-100. Slides were washed twice by phosphate-buffered saline and then mounted with mounting medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA).

Statistical analysis
The data are expressed as the mean ± standard error of the mean. One-way ANOVA followed by Tukey’s multiple comparison test (statistical significance was defined as \( P < 0.05 \)) was used for statistical analysis using the GraphPad Prism software (Ver 5.0f; La Jolla, CA, USA).

RESULTS
To elucidate whether SGLT2 inhibitors induced renoprotective effects regardless of improvements in metabolic status or body-weight, we analyzed ad libitum HFD-fed mice, the model of metabolic syndrome and type 2 diabetes with insulin resistance. In HFD-fed mice, caloric intake and bodyweight increased compared with those of control-fed mice (Figure 1b–d). Kidney weights and blood glucose levels appeared to be unchanged (Figure 1e,f). Interestingly, ipragliflozin-
treated HFD-fed mice had increased dietary food intake compared with the HFD group (Figure 1b). Consistent with the enhanced food intake in ipragliflozin-treated mice, there was no reduction in bodyweight by ipragliflozin treatment in the HFD group (Figure 1c). Kidney weights and blood glucose levels were not significantly altered by ipragliflozin in HFD-fed mice (Figure 1d–f).

Compared with control diet-fed mice, the mice in the HFD group showed tubular vacuolation, tubular dilatation and tubular epithelial cell detachment. Ipragli flozin ameliorated these alterations (Figure 2a). The ultrastructural analysis of the kidney by transmission electron microscopy showed rounded and fragmented mitochondria in HFD-fed mice when compared with control-fed mice (Figure 2a). The mitochondrial inner membrane was also broken in the HFD group (Figure 2a). The mitochondrial damage was ameliorated by ipragliflozin treatment (Figure 2a). Mitochondrial dysfunction can accelerate glycolysis and oxidative stress, and mitochondrial abnormalities are induced by oxidative stress in metabolically abnormal mice fed an HFD13. Oxidative stress reduced Opa1 levels and disrupted mitochondrial morphological dynamic alterations14.
Therefore, we analyzed mitochondrial membrane GTPase protein expression by western blot analysis. In the HFD group, the levels of Opa1 and Mfn2, the GTPases related to mitochondrial fusion, were decreased (Figure 2b). Drp-1, the GTPase related to mitochondrial fission, was increased in the HFD group (Figure 2b). In the ipragliflozin group, Opa1 and Mfn2 were restored (Figure 2b). HFD-induced Drp-1 levels were not significantly altered by ipragliflozin (Figure 2b).

Mitochondrial damage and deficiency in either Opa1 or Mfn2 induced oxidative stress. In regard to this, urinary 8-OHdG, an oxidative stress marker, was increased in the HFD group, and ipragliflozin treatment restored the levels to those seen in control mice (Figure 3). 8-OHdG is produced when DNA is damaged by oxidative stress. Because mitochondrial DNA has a single-stranded circular structure, mitochondrial DNA is less protected against oxidative stress15,16.

To validate the effects of SGLT2 inhibition on tubular mitochondria, we carried out an in vitro analysis utilizing tubular epithelial cell line HK-2 cells. We first examined the effect of SGLT2 inhibition on the expression of the glucose-regulated mitochondrial membrane GTPase protein in proximal tubular cells. Incubation in a high-glucose (HG) medium (30 mmol/L) significantly suppressed Mfn2 and Opa1 protein levels (Figure 4a–c) in HK-2 cells. SGLT2 knockdown using a specific siRNA in HK-2 cells restored HG-suppressed Mfn2 and Opa1 levels (Figure 4a–c). Furthermore, the incubation with palmitate remarkably decreased the expression of Mfn2 and Opa1 in

Figure 4 | Sodium–glucose cotransporter 2 (SGLT2) knockdown or SGLT2 inhibition improved the expression levels of mitochondrial GTPase under the high glucose or palmitate condition. Western blot analysis for (a–c) mitochondrial GTPase expression and SGLT2 suppression by small interfering SGLT2 (siSGLT2) or (d–f) inhibition by ipragliflozin (10 nmol/L) incubated in (a–c) high-glucose (HG) medium (30 mmol/L) or (d–f) palmitate (150 μmol/L) for 48 h. (a–c) HK-2 cells were transfected with small interfering ribonucleic acid (siRNA; 100 nmol/L) targeting SGLT2 or control scramble. For (d–f), dimethyl sulfoxide was used as the control. The harvested proteins were analyzed by western blot analysis. Densitometric analysis of the western blot was normalized to β-actin (n = 3 in each group). The values are expressed as the mean ± standard error of the mean for animals in each group. *P < 0.05; **P < 0.01. Con, control group; DMSO, dimethyl sulfoxide; Ipra, ipragliflozin; Mfn2, mitofusion 2.
HK-2 cells (Figure 4d–f); in the presence of the SGLT2 inhibitor ipragliflozin, palmitate lost its effect on Mfn2 and Opa1 levels in HK-2 cells (Figure 4d–f), suggesting that the free fatty acid-altered mitochondrial membrane GTPase protein in proximal tubular cells relies on the glucose uptake mediated by SGLT2. HG and palmitate combination media significantly disrupted mitochondria in HK-2 cells analyzed by mitotracker green; either ipragliflozin or SGLT2 siRNA restored the expression of mitochondria (Figure 5a,b).

**DISCUSSION**

In the present study, we found that ipragliflozin affected kidney tubular mitochondrial homeostasis independently from either bodyweight reduction or glucose-lowering effect. Here, we showed the following: (i) in HFD-fed mice, mitochondria kidney proximal tubular cells were damaged and ipragliflozin restored normal mitochondrial morphology; (ii) small GTPases – Opa1 and Mfn2 – that are important for mitochondrial fusion, were reduced in HFD-fed mice kineys, but ipragliflozin restored them; (iii) ipragliflozin suppressed oxidative stress evaluated by urinary 8-OHdG in HFD-fed mice; and (iv) in *in vitro* culture proximal tubular cell line HK-2 cells, suppression of SGLT2 restored mitochondrial biogenesis in HG or/and high-palmitate media associated with normalization of Opa1 and Mfn2 levels. These data shed light on novel insights into the molecular mechanisms of the renoprotective effects of SGLT2 inhibitors in diabetes patients.

To understand the renoprotective effects of SGLT2 inhibition, we might require separate interpretation of the *in vivo* and *in vitro* models in our study. HFD has been shown to induce a metabolic abnormality that suppressed both Opa1 and Mfn2 levels. According to a recent report, Opa1, which plays a key role in mitochondrial fusion and participates in cristae remodeling, was increased by insulin signals. Interestingly, Mfn2 deficiency led to oxidative stress that contributed to the onset of insulin resistance; overexpression of Mfn2 suppressed the insulin resistance induced by palmitate in rat skeletal muscle cells. Interestingly, these data suggested that Mfn2 could restore Opa1 levels *in vivo* through amelioration of insulin signaling; another report stated Mfn2 itself contributes in the degradation process of Opa1. Molecular deficiencies related to mitochondrial biogenesis subsequently induced mitochondrial defects. Mitochondrial defects or the HFD-diet itself caused oxidative stress, a key inducer of mitochondrial damage. Ipragliflozin intervention inhibited such a vicious cycle by restoring Opa1 and Mfn2 levels to normal values *in vivo* without altering blood glucose levels or reducing bodyweight. In contrast, HFD-induced Drp-1 overexpression was not altered by ipragliflozin *in vivo*. Although the mechanisms by which Drp-1 expression could not be restored by the inhibition of SGLT2 are still unclear, systemic metabolic alterations, such as improvement of insulin resistance and lipid metabolism, could be responsible for such altered regulation between Opa1, Mfn2 and Drp-1 *in vivo*.

*In vitro*, our data showed the presence of metabolic insults with either HG or high palmitate, and mitochondrial biogenesis is significantly diminished by the loss of mitochondrial fusion proteins. Inhibition of SGLT2 can inhibit the effect of these metabolic insults by preventing the loss of these molecules and restoring mitochondrial homeostasis. Even though we utilized HFD-fed mice as a model for metabolic syndrome or type 2 diabetes that showed insulin resistance with metabolic defects, fundamentally, ipragliflozin directly acted on the proximal tubule of the kidney. Therefore, the effects of ipragliflozin on Opa1 and Mfn2 in kidney tubules should be based on the inhibition of glucose influx into the tubular cells. In regard to this, the *in vitro* experiment using HG or/and high-palmitate media...
experiments supported such local effects of ipraglirozin or SGLT2 suppression.

There was a significant limitation to the present study. In regard to in vivo models, we believed some of the effects of ipraglirozin could be associated with the amelioration of insulin resistance in HFD-fed animals. However, we did not see direct evidence of such alterations, as initially we did not thing such an amelioration of insulin resistance could affect the mitochondrial phenotype of our experimental set. For complete understanding, we should utilize some interventions, such as metformin, pioglitazone or exercise, to adjust the levels of insulin resistance together with the ipraglirozin group as a reference. However, this could be beyond of our the of the present study. Second, our in vitro study clearly showed metabolic insults, HG or/and high-palmitate media, and induced mitochondrial defects with deficiencies in Opa1 and Mfn2. However, the mechanisms by which glucose influx into tubular cells affected such alterations in these GTPase were not entirely clear. Son et al. reported that higher glycolysis showed a close association with suppressed levels of Opa1 and Mfn2; therefore, high metabolic stress induced by HG or/and high-palmitate conditions could induce higher demand for glycolysis in the kidney tubule. However, the presence of glycolysis in the proximal tubule has been under intense debate, even though recent analysis showed a pathological significance of glycolysis in the kidney proximal tubule. To answer these questions, further studies are obviously required.

In conclusion, we showed that SGLT2-mediated uptake of glucose diminished mitochondrial homeostasis and found a novel, potentially renoprotective effect of ipraglirozin.

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DISCLOSURE

The funding source collaborated with the authors in data interpretation and writing of the report. TT is an employee of Astellas Pharma Inc. KK and DK received lecture honoraria from Astellas Pharma Inc.

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