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ORIGINAL ARTICLE

# Inhibition of SGLT2 alleviates diabetic nephropathy by suppressing high glucose-induced oxidative stress in type 1 diabetic mice

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Diabetic nephropathy, hyperglycemia, oxidative stress, SGLT2 inhibitor, urinary albumin excretion.

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## Abstract

It is unclear whether the improvement in diabetic nephropathy by sodium glucose cotransporter 2 (SGLT2) inhibitors is caused by a direct effect on SGLT2 or by the improvement in hyperglycemia. Here, we investigated the effect of dapagliflozin on early-stage diabetic nephropathy using a mouse model of type 1 diabetes and murine proximal tubular epithelial cells. Eight-week-old Akita mice were treated with dapagliflozin or insulin for 12 weeks. Body weight, urinary albumin excretion, blood pressure, as well as levels of blood glucose and hemoglobin A1c were measured. Expansion of the mesangial matrix, interstitial fibrosis, and macrophage infiltration in kidneys were evaluated by histology. Oxidative stress and apoptosis were evaluated in kidneys and cultured proximal tubular epithelial cells. Compared with nontreated mice, dapagliflozin and insulin decreased blood glucose and hemoglobin A1c levels equally. Urine volume and water intake increased significantly in the dapagliflozin-treated group compared with those in the insulin-treated group, but there were no differences in body weight or blood pressure between the two groups. Macrophage infiltration and fibrosis in renal interstitium improved significantly in the dapagliflozin group compared with the insulin group. Oxidative stress was attenuated by dapagliflozin, and suppression occurred in a dose-dependent manner. RNAi knockdown of SGLT2 resulted in reduced oxidative stress. Dapagliflozin ameliorates diabetic nephropathy by suppressing hyperglycemia-induced oxidative stress in a manner independent of hyperglycemia improvement in Akita mice. Our findings suggest that dapagliflozin may be a novel therapeutic approach for the treatment of diabetic nephropathy.

## Abbreviations

BUN, blood urea nitrogen; DHE, dihydroethidium; DM, diabetes mellitus; ESRD, end-stage renal disease; $Hb_{A1c}$, hemoglobin A1c; Nox4, NADPH oxidase 4; PAS, periodic acid–Schiff; ROS, reactive oxygen species; SGLT2, sodium glucose cotransporter 2; SOD2, superoxide dismutase 2; STZ, streptozotocin; UAE, urinary albumin excretion.

## Introduction

Diabetic nephropathy is a leading cause of morbidity and mortality, and frequently leads to end-stage renal disease (ESRD) (Collins et al. 2012). Several treatment options for diabetes mellitus (DM) have become available in recent years, but the incidence of ESRD related to diabetic nephropathy has not changed (Gregg et al. 2014).

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Consequently, agents that improve diabetic nephropathy are needed.

Sodium glucose cotransporters (SGLTs) of the *Slc5* gene family play an important part in glucose reabsorption in the kidney. It has been reported that SGLT1 and SGLT2 are expressed on the apical side of proximal tubular cells, and that the renal threshold for glucose excretion is increased in DM patients, possibly because of upregulation of expression of SGLTs (Wilding 2014). SGLT2 (*Slc5a2*) is located in the S1 segment of the proximal tubule and accounts for ~90% of glucose reabsorption in the kidney (Mather and Pollock 2010).

Dapagliflozin was the first SGLT2 inhibitor to be approved, and was launched in the market in 2012. It is prescribed mainly in developed countries as an effective treatment against type 2 diabetes mellitus (T2DM) (Hasan et al. 2014). The efficacy and safety of dapagliflozin for DM patients have been shown by various robust studies (Ptaszynska et al. 2014). Recently, it was reported that dapagliflozin also reduces glucose levels, glycemic variability, and insulin doses in type 1 diabetes mellitus (T1DM) patients (Henry et al. 2015). SGLT2 inhibitors are promising drugs for DM treatment, but the effect of SGLT2 inhibition on diabetic nephropathy is not yet known.

Previously, we demonstrated that the SGLT2 inhibitor dapagliflozin prevents the development of diabetic nephropathy by inhibiting oxidative stress and inflammation in *db/db* mice (a model of T2DM) (Terami et al. 2014). In addition, Vallon et al. (2014) reported that another SGLT2 inhibitor, empagliflozin, attenuated DM-induced albuminuria and renal growth as well as hyperglycemia in Akita mice with DM. Dapagliflozin and empagliflozin improved hyperglycemia in those studies, so it is not yet known whether the improvement in diabetic nephropathy by SGLT inhibitors is caused by a direct effect on SGLT or by the improvement in hyperglycemia. Therefore, it is necessary to reduce the plasma glucose level with other agents to the level reduced by SGLT2 inhibitors, and to compare the effects on diabetic nephropathy.

We designed a study to test our hypothesis that SGLT2 inhibition by dapagliflozin ameliorates the progression of diabetic nephropathy directly by inhibiting high glucose-induced oxidative stress in Akita mice (a model of T1DM).

## Materials and Methods

### Ethical approval of the study protocol

The care of and procedures related to animals were carried out according to the Guidelines for Animal Experimentation at Okayama University (Okayama, Japan), Animal Protection and Management Law set by the Japanese Government, and the Notification on Feeding and Safekeeping of Animals set by the Japanese Government. The experimental protocol was approved by the Animal Ethics Review Committee of Okayama University (OKU-2013156). All surgeries were performed under sodium pentobarbital anesthesia, and every effort was made to minimize suffering.

### Experimental protocol

Seven-week-old male DM Akita mice (AKITA/Slc) and non-DM C57BL/6 mice (C57BL/6JJmsSlc) were purchased from Japan SLC (Hamamatsu, Japan). All mice were maintained under a 12-h light–dark cycle with free access to food and tap water. We gave standard forage, MF (Oriental Yeast, Tokyo, Japan) to all mice. Forage 100 g is equivalent to 359 kcal, and it contain 7.9% water, 23.1% protein, 5.1% fat, 5.8% mineral, 2.8% fiber, and 55.3% nitrogen-free extract. Dapagliflozin was kindly supplied by Bristol–Myers Squibb (Pennington, NJ).

Dapagliflozin (1.0 mg/kg per day) was administered to Akita mice ($n$ = 8) by gavage for 12 weeks starting at the age of 8 weeks. Insulin glargine (Sanofi, Tokyo, Japan) (10–20 unit/kg per day) was administered to Akita mice ($n$ = 6) by subcutaneous injection for 12 weeks starting at the age of 8 weeks. Insulin doses were titrated to maintain comparable blood glucose levels as those of the dapagliflozin group and to avoid hypoglycemia. Dapagliflozin was dissolved and diluted with pure water. Control C57BL/6 mice ($n$ = 8) and control Akita mice ($n$ = 8) were given pure water for 12 weeks. Mice were killed as described previously (Matsushita et al. 2011).

### Metabolic data

Body weight and blood glucose were measured every week after an overnight fast. Blood was drawn from tail vein, and blood glucose was measured by simple blood glucose measuring instrument, Glutest Neo Alpha (Sanwa Kagaku Kenkyusho, Nagoya, Japan). Blood pressure, urinary volume, urinary excretion of glucose, water intake, and food intake were measured every 4 weeks as described previously (Terami et al. 2014). Kidney weight, 24-h urinary albumin excretion (UAE), hemoglobin A1c ($Hb_{A1c}$), blood urea nitrogen (BUN), creatinine, immunoreactive insulin (IRI), and free fatty acid (FFA) were measured at the age of 20 weeks. Urinary levels of sodium ions and chloride ions were measured using the ion-selective electrode method. Total cholesterol (TC), low-density lipoprotein (LDL-C), high-density lipoprotein (HDL-C), and triglyceride (TG) were measured using gel filtration high-performance liquid chromatography.


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## Histological analyses

Sections (thickness 4 $\mu$m) were cut from 10% formalin-fixed, paraffin-embedded kidney samples taken at 20 weeks of age and subjected to periodic acid–Schiff (PAS) staining to evaluate expansion of the mesangial matrix. PAS-positive area was calculated as described previously (Terami et al. 2014). Kidney sections were also stained with Picrosirius Red F3BA Liquid (Polysciences, Warrington, PA) for 60 min to evaluate interstitial fibrosis. To quantify percentage renal fibrosis, 10 views of the renal cortex were selected randomly in each slide. Renal expression of type IV collagen, macrophages, and NADPH oxidase 4 (Nox4) was also analyzed, as described previously (Terami et al. 2014).

## Quantitative analyses of gene expression in the renal cortex

RNA isolation from the renal cortex and quantitative RT-PCR (qRT-PCR) were performed as described previously (Terami et al. 2014). All primers for transforming growth factor-$\beta$ (*Tgfb*), monocyte chemoattractant protein-1 (*Ccl2)*, *osteopontin*, intercellular adhesion molecule-1 (*Icam-1*), *Nox4*, and *Slc5a2* were purchased from Takara Bio (Otsu, Japan). Each sample was analyzed in triplicate and normalized against expression of glyceraldehyde 3-phosphate dehydrogenase mRNA.

## Expression of reactive oxygen species

To evaluate the effect of dapagliflozin on reactive oxygen species (ROS) production, superoxide anion radicals were detected by dihydroethidium (DHE) staining (Molecular Probes, Eugene, OR) as described previously (Terami et al. 2014). The mean fluorescence intensity of DHE was calculated by dividing the combined fluorescence value of pixels by the total number of pixels in 10 randomly selected fields observed under identical laser and photomultiplier settings.

## Western blotting of the renal cortex

Western blotting of SGLT2, Nox4, TGF-$\beta$, superoxide dismutase 2 (SOD2), and $\beta$-actin was undertaken as described previously (Matsushita et al. 2011; Terami et al. 2014). Antibodies for SGLT2 were purchased from Abcam (Cambridge, UK).

## Terminal transferase-mediated dUTP nick-end labeling assay

To evaluate the effect of dapagliflozin on apoptosis in the kidney, sections were incubated with an in situ apoptosis detection kit (Takara Bio) as described previously (Terami et al. 2014).

## Culture and treatment of cells

Murine proximal tubular epithelial (mProx24) cells were generously provided by Dr. Takeshi Sugaya (CMIC, Tokyo, Japan) and cultured, as reported previously (Terami et al. 2014). RNA interference (RNAi) experiments were performed using *Slc5a2* small interfering RNA (siRNA) (MSS217391; Invitrogen, Carlsbad, CA) and scrambled siRNA (Silencer Select siRNA Controls; Invitrogen). mProx24 cells were transfected with 10.0 nmol/L *Slc5a2* siRNA or scrambled siRNA in the presence of Lipofectamine RNAiMAX™ (Invitrogen). After siRNA transfection for 48 h, cells were stimulated with 25 mmol/L D-glucose (high glucose) for 24 h.

## Flow cytometry

Cellular ROS levels were assessed using a DCF-DA Cellular Reactive Oxygen Species Detection Assay kit (Abcam) and FACSCalibur (Becton Dickinson, Tokyo, Japan). All experiments were repeated at least three times with different cell preparations.

## Apoptosis assay

To evaluate the effect of dapagliflozin on apoptosis in mProx24 cells, apoptotic nuclei were detected by Hoechst staining. Briefly, cells were incubated with 1 mg/mL Hoechst 33342 solution (Dojindo, Kumamoto, Japan) at 37°C for 5 min in a humidified chamber protected from light. Fluorescence images were obtained using a BZ-9000 Microscope (Keyence, Osaka, Japan) and evaluated using BIOZERO software (Keyence). Mean number of apoptotic nuclei (number per mm$^2$) was calculated in 10 randomly selected fields observed under identical laser and photomultiplier settings.

## Glucose uptake into cultured proximal tubular epithelial cells

Cellular glucose uptake levels were assessed using a Glucose Cellular Uptake Measurement Kit (Cosmo Bio, Tokyo, Japan). All experiments were repeated at least three times with different cell preparations.

## Statistical analyses

Data are the mean $\pm$ SEM. Significant differences between groups were examined using one-way ANOVA followed by Scheffe's test. $P < 0.05$ was considered significant.

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## Results

### Effect of dapagliflozin on body weight, hyperglycemia, renal function, and lipid

Body weight was higher in the C57BL/6 group than in the Akita group. There was no difference in body weight between the Akita group, the Akita with 10–20 unit/kg insulin glargine group ("insulin group"), and the Akita with 1.0 mg/kg dapagliflozin group ("dapagliflozin group") throughout the 12-week observation period except for at 10, 12, 13, and 15 weeks of age (Fig. 1A). Blood glucose level, which was significantly higher in the Akita group compared with that in the C57BL/6 group, was significantly lower in the insulin group and dapagliflozin group than in the Akita group throughout the 12-week observation period ($P < 0.05$). There were no differences in glycemic control between the insulin group and dapagliflozin group and no hypoglycemic episodes during the study (Fig. 1B). Similarly, there was no significant difference in levels of $Hb_{A1c}$ between the insulin group and dapagliflozin group (Fig. 1C). UAE was higher in the Akita group compared with that in the C57BL/6 group, and was significantly lower in the dapagliflozin and insulin groups ($P < 0.05$). Moreover, UAE was significantly lower in the dapagliflozin group compared with that in the insulin group at 20 weeks of age ($P < 0.05$, Fig. 1D). There were no significant differences in systolic blood pressure or diastolic blood pressure between the four groups at 20 weeks of age (Table 1). Urinary excretion of glucose, water intake, and food intake were higher in the Akita group compared with the C57BL/6, dapagliflozin, and insulin groups (Table 1). Urinary excretion of glucose and water intake were higher in the dapagliflozin group than in the insulin group (Table 1). Other renal parameters are summarized in Table 2. Kidney weight and relative kidney weight were considerably higher in the Akita group than in the C57BL/6 group, and kidney weight and relative kidney weight were significantly lower in the dapagliflozin group compared with that in the Akita group at 20 weeks of age ($P < 0.05$). There was no significant difference in levels of urinary sodium and chloride concentrations between the insulin group and dapagliflozin group (Table 2). There were no significant differences in serum creatinine among the four groups at 20 weeks of age. Insulin levels and lipid parameters are summarized in Table 3. Of course, insulin levels were markedly higher in the insulin group, but there was no significant difference in levels of insulin in dapagliflozin group compared with Akita group. There were no significant differences in serum lipid parameters including free fatty acid, triglyceride, and cholesterol among the four groups at 20 weeks of age. This result indicates that neither triglycerides nor cholesterol affect renal injury in this model.

### Dapagliflozin suppresses expansion of the mesangial matrix and interstitial fibrosis

To evaluate the effect of dapagliflozin and insulin on renal injuries, kidneys at 20 weeks of age were processed for pathological analysis. As revealed by PAS staining and immunofluorescence of type IV collagen, the mesangial matrix expanded considerably in the Akita group, but expansion was ameliorated by dapagliflozin and insulin (Fig. 2A–C). Staining with picrosirius red revealed that interstitial fibrosis was more severe in the Akita group compared with that in the C57BL/6 group, and that it was suppressed in dapagliflozin and insulin groups (Fig. 2D [upper panels] and E). Similarly, expression of type IV collagen was significantly higher in the interstitium in the Akita group than in the C57BL/6 group as shown by immunofluorescence (Fig. 2D, lower panels). Dapagliflozin and insulin both suppressed interstitial fibrosis, but dapagliflozin was more effective at suppressing interstitial fibrosis than insulin (Fig. 2F). Segments of the late proximal tubule and other cortical segments from the distal tubule were not altered in dapagliflozin and insulin groups. Collectively, these results suggested that dapagliflozin administration reduced interstitial fibrosis by acting directly on the interstitium of Akita mice.

**Figure 1.** Effect of dapagliflozin on body weight and hyperglycemia. (A) Body weight in Akita with 1.0 mg/kg dapagliflozin group ("dapagliflozin group") was lower than that in the Akita group at 10, 12, 13, and 15 weeks of age. Data are the mean ± SEM. *$P < 0.05$ versus Akita. (B) Blood glucose level was higher in the Akita group than that in the C57BL/6 group during the 12-week observation period. Blood glucose levels in the dapagliflozin group and insulin-treated group ("insulin group") were similar to each other and significantly lower than that in the Akita group. Data are the mean ± SEM. *$P < 0.05$ versus Akita. (C) $Hb_{A1c}$ level was higher in the Akita group than that in the C57BL/6 group during the 12-week observation period. $Hb_{A1c}$ levels in the dapagliflozin and the insulin groups were similar to each other and significantly lower than that in the Akita group. Data are the mean ± SEM. **$P < 0.05$ versus Akita. (D) UAE was higher in the Akita group than in the C57BL/6 group, and was significantly lower in the dapagliflozin and insulin groups at 20 weeks of age. Urinary albumin excretion (UAE) was reduced significantly in the dapagliflozin group compared with that in the insulin group at 20 weeks of age. Data are the mean ± SEM. *$P < 0.05$; **$P < 0.01$ versus 8 weeks.


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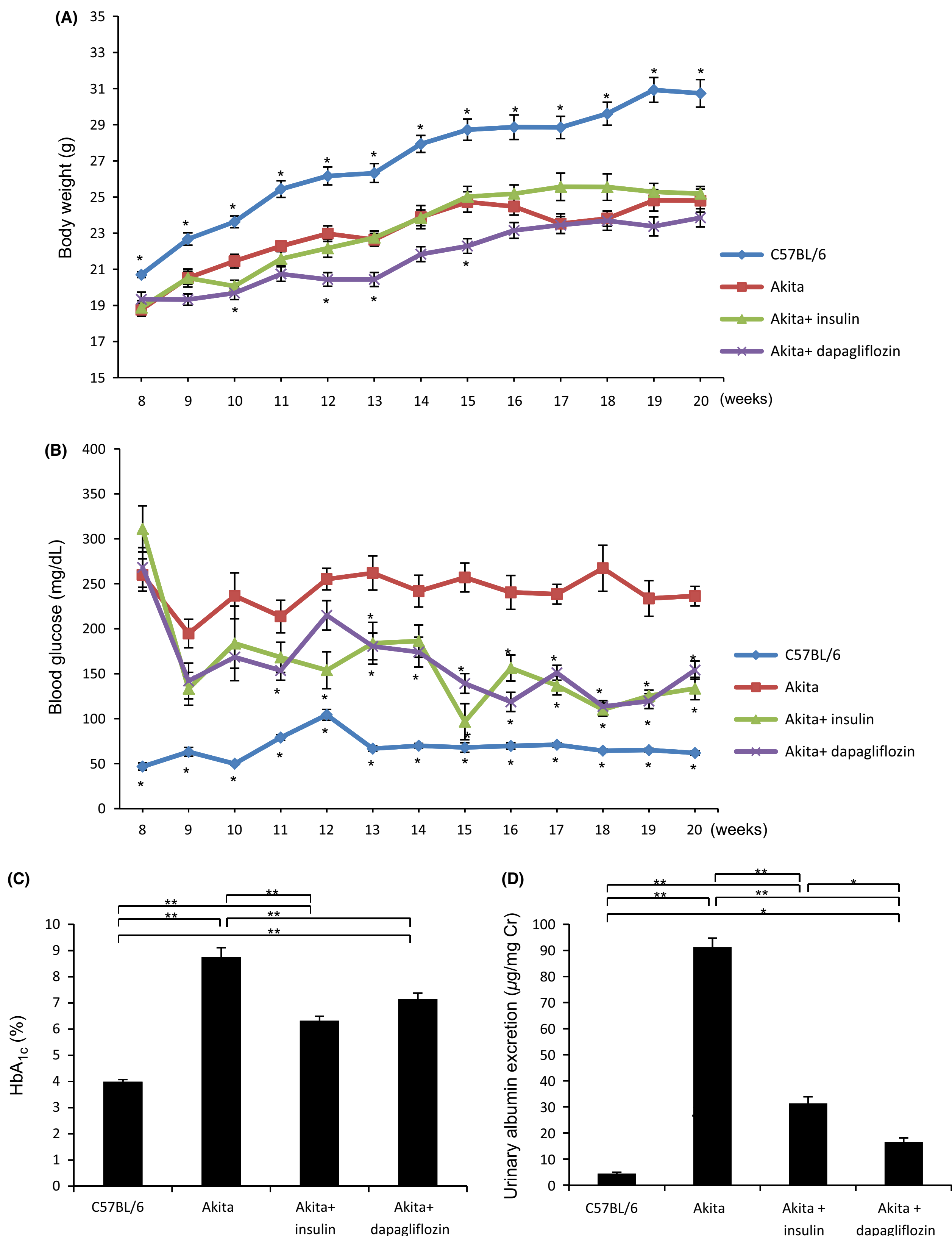
(A)
Body weight (g)
(weeks)
C57BL/6
Akita
Akita+ insulin
Akita+ dapagliflozin
(B)
Blood glucose (mg/dL)
(weeks)
C57BL/6
Akita
Akita+ insulin
Akita+ dapagliflozin
(C)
HbA1c (%)
C57BL/6
Akita
Akita+ insulin
Akita+ dapagliflozin
(D)
Urinary albumin excretion (μg/mg Cr)
C57BL/6
Akita
Akita + insulin
Akita + dapagliflozin

**Table 1.** Influence of dapagliflozin on metabolic and physiological parameters in Akita mice and C57BL/6 mice at 20 weeks.

| | C57BL/6 (*n* = 8) | Akita (*n* = 8) | Akita + Insulin (*n* = 6) | Akita + Dapagliflozin (*n* = 8) |
|---|---|---|---|---|
| Systolic blood pressure (mmHg) | 99.0 ± 2.6 | 104.0 ± 3.8 | 103.2 ± 2.1 | 102.4 ± 1.7 |
| Diastolic blood pressure (mmHg) | 63.8 ± 3.0 | 66.6 ± 3.9 | 63.8 ± 2.8 | 60.3 ± 4.2 |
| Urine volume (mL/day) | 1.2 ± 0.2 | 34.4 ± 2.8[1] | 11.1 ± 1.8[1,2] | 24.8 ± 1.3[1,2,3] |
| Urinary glucose excretion (mg/day) | 0.4 ± 0.1 | 3737.2 ± 286.9[1] | 1371.4 ± 158.3[1,2] | 2951.9 ± 156.8[1,2,3] |
| Water intake (mL/day) | 4.1 ± 0.7 | 44.1 ± 2.5[1] | 17.3 ± 2.0[1,2] | 33.3 ± 1.9[1,2,3] |
| Food intake (g/day) | 3.8 ± 0.3 | 5.5 ± 0.2[1] | 4.3 ± 0.2[2] | 4.8 ± 0.1 |

C57BL/6, non-DM control mice; Akita, untreated DM mice; Akita + Insulin, insulin (10–20 unit/kg)-treated DM mice; Akita + Dapagliflozin, dapagliflozin (1.0 mg/kg)-treated DM mice. Data are the mean ± SEM; [1]$P < 0.05$ versus C57BL/6, [2]$P < 0.05$ versus Akita, [3]$P < 0.05$ versus Akita + Insulin. DM, diabetes mellitus.

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

| | C57BL/6 (*n* = 8) | Akita (*n* = 8) | Akita + Insulin (*n* = 6) | Akita + Dapagliflozin (*n* = 8) |
|---|---|---|---|---|
| Kidney weight (mg) | 292.5 ± 16.6 | 445.0 ± 18.6[1] | 410.0 ± 17.3[1] | 320.0 ± 7.1[2,3] |
| Relative kidney weight (mg/g body weight) | 9.6 ± 0.2 | 18.5 ± 1.4[1] | 16.7 ± 0.8[1] | 13.3 ± 0.2[1,2,3] |
| BUN (mg/dL) | 19.4 ± 1.2 | 32.2 ± 1.6[1] | 18.8 ± 0.8[2] | 25.3 ± 1.1[1,2,3] |
| Serum creatinine (mg/dL) | 0.15 ± 0.02 | 0.24 ± 0.04 | 0.18 ± 0.04 | 0.15 ± 0.03 |
| Urinary sodium (mEq/L) | 132.5 ± 4.9 | 14.5 ± 0.8[1] | 22.3 ± 3.1[1] | 15.6 ± 0.8[1] |
| Urinary chloride (mEq/L) | 200.0 ± 12.0 | 21.3 ± 1.1[1] | 33.3 ± 5.2[1] | 21.6 ± 0.8[1] |

C57BL/6, non-DM control mice; Akita, untreated DM mice; Akita + Insulin, insulin (10–20 unit/kg)-treated DM mice; Akita + Dapagliflozin, dapagliflozin (1.0 mg/kg)-treated DM mice; BUN, blood urea nitrogen. Data are the mean ± SEM; [1]$P < 0.05$ versus C57BL/6, [2]$P < 0.05$ versus Akita, [3]$P < 0.05$ versus Akita + Insulin. DM, diabetes mellitus.

**Table 3.** Influence of dapagliflozin on insulin levels and lipid parameters in Akita mice and C57BL/6 mice at 20 weeks.

| | C57BL/6 (*n* = 8) | Akita (*n* = 8) | Akita + Insulin (*n* = 6) | Akita + Dapagliflozin (*n* = 8) |
|---|---|---|---|---|
| IRI (ng/mL) | 2.58 ± 0.80 | 0.10 ± 0.00[1] | 53.73 ± 6.49[1,2] | 0.23 ± 0.03[3] |
| TC (mg/dL) | 69.3 ± 3.6 | 84.9 ± 7.3 | 90.3 ± 10.1 | 89.8 ± 5.2 |
| LDL-C (mg/dL) | 11.9 ± 0.2 | 14.5 ± 0.7 | 16.0 ± 2.1 | 15.9 ± 1.0 |
| HDL-C (mg/dL) | 54.2 ± 3.6 | 62.3 ± 7.0 | 65.3 ± 3.4 | 64.9 ± 7.9 |
| TG (mg/dL) | 17.5 ± 2.2 | 14.6 ± 1.8 | 16.9 ± 1.9 | 16.1 ± 1.7 |
| FFA (mEq/L) | 0.93 ± 0.06 | 1.01 ± 0.08 | 0.88 ± 0.13 | 1.03 ± 0.09 |

C57BL/6, non-DM control mice; Akita, untreated DM mice; Akita + Insulin, insulin (10–20 unit/kg)-treated DM mice; Akita + Dapagliflozin, dapagliflozin (1.0 mg/kg)-treated DM mice; IRI, immunoreactive insulin; TC, total cholesterol; LDL-C, low-density lipoprotein; HDL-C, high-density lipoprotein; TG, triglyceride; FFA, free fatty acid. Data are the mean ± SEM; [1]$P < 0.05$ versus C57BL/6, [2]$P < 0.05$ versus Akita, [3]$P < 0.05$ versus Akita + Insulin. DM, diabetes mellitus.

### Infiltration of proinflammatory macrophages and expression of inflammatory genes in the renal cortex

To evaluate macrophage infiltration into the kidney, we performed immunoperoxidase staining of F4/80, a marker for proinflammatory macrophages. The number of macrophages in the glomeruli and interstitium was remarkably higher in the Akita group than in the C57BL/6 group (Fig. 3A). Macrophage infiltration into the glomeruli and interstitium was significantly lower in dapagliflozin and insulin groups compared with the Akita group (Fig. 3B and C). Interestingly, macrophage infiltration into the interstitium was suppressed to a greater extent in the dapagliflozin group than in the insulin group (Fig. 3C). These findings suggested that dapagliflozin suppresses infiltration of proinflammatory macrophages into the interstitium of DM kidneys.

qRT-PCR analyses of kidney tissue demonstrated that expression of several proinflammatory genes, including


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*Tgfb*, *Ccl2*, *osteopontin*, and *Icam-1*, was upregulated significantly in the Akita group compared with that in the C57BL/6 group, and was suppressed by dapagliflozin and insulin (Fig. 3D–G). Interestingly, expression of *Tgfb* and *Ccl2* was suppressed to a greater extent in the dapagliflozin group than in the insulin group (Fig. 3D and E). These results suggested that dapagliflozin has an anti-inflammatory effect independent of blood glucose levels, and contributes to suppression of macrophage infiltration and fibrosis in the interstitium of Akita mice.

## Oxidative stress and apoptosis in the kidney

To investigate the role of oxidative stress and apoptosis, as well as the effects of dapagliflozin on the pathogenesis of diabetic nephropathy, we conducted DHE staining, Nox4 immunostaining, and the terminal transferase-mediated dUTP nick-end labeling (TUNEL) assay in kidneys. ROS production, as detected by DHE, was higher in the renal cortices of mice in the Akita group than in the renal cortices of mice in the C57BL/6 group, and was suppressed by insulin and, more efficiently, dapagliflozin (Fig. 4A [upper panels] and B). Similarly, expression of Nox4, which is a subunit of NADPH oxidase, was upregulated in the renal cortices of mice in the Akita group compared with that of renal cortices of mice in the C57BL/6 group, but was attenuated in the dapagliflozin group and insulin group, though less effectively by the latter (Fig. 4A [lower panels] and C). As demonstrated by qRT-PCR analyses of kidney tissue, *Nox4* expression was suppressed significantly by dapagliflozin but not by insulin (Fig. 4D). Moreover, western blotting showed that dapagliflozin but not insulin suppressed expression of Nox4 protein (Fig. 4E). TUNEL staining revealed that apoptosis was higher in the Akita group compared with the C57BL/6 group, and that the number of apoptotic cells was slightly lower in the dapagliflozin group compared with that in the insulin group (Fig. 4F and G). Collectively, oxidative stress and apoptosis induced by DM were suppressed by glycemic control using insulin, and were more strongly suppressed by dapagliflozin. These data suggested that dapagliflozin reduced oxidative stress and apoptosis directly in DM kidneys.

## Oxidative stress and expression of inflammatory genes in cultured proximal tubular epithelial cells

To further investigate the effect of dapagliflozin on high glucose-induced ROS production in kidneys, we performed DCF-DA staining and FACS analyses using cultured mProx24 cells (a proximal tubular epithelial cell line). High-glucose medium increased ROS production in mProx24 cells, which was attenuated significantly by dapagliflozin treatment in a dose-dependent manner (Fig. 5A). To ascertain whether the suppressive effect of dapagliflozin is dependent on SGLT2 inhibition, we performed RNAi against *Slc5a2* (the gene encoding SGLT2) in mProx24 cells. In untransfected mProx24 cells, *Slc5a2* was similarly expressed in normal glucose, high glucose, and high glucose with dapagliflozin. However, transfection with *Slc5a2* siRNA, but not scrambled siRNA, resulted in potent inhibition of *Slc5a2* (Fig. 5B). Levels of SGLT2 protein were also similar in normal glucose, high glucose, and high glucose with dapagliflozin, and were suppressed significantly by *Slc5a2* knockdown in mProx24 cells (Fig. 5C). qRT-PCR analyses showed that expression of the high glucose-induced inflammatory genes *Ccl2* and *osteopontin* in mProx24 cells was inhibited by dapagliflozin. *Slc5a2* knockdown also suppressed expression of these inflammatory genes to similar levels as those observed with 20 $\mu$mol/L dapagliflozin (Fig. 5D and E). These results showed that dapagliflozin inhibited oxidative stress and subsequent inflammation by inhibiting SGLT2 in proximal tubular epithelial cells.

## Oxidative stress and apoptosis in cultured proximal tubular epithelial cells

We assessed high glucose-induced ROS production and apoptosis in mProx24 cells by DHE staining and Hoechst staining, respectively. High-glucose medium increased ROS production and the number of apoptotic nuclei in mProx24 cells, but dapagliflozin treatment significantly attenuated this increase in a dose-dependent manner (Fig. 6A–D). Western blotting demonstrated that expression of Nox4 and TGF-$\beta$ in mProx24 cells was induced by high-glucose stimulation, and was suppressed significantly by dapagliflozin or *Slc5a2* knockdown (Fig. 6E–G). Expression of the antioxidant enzyme SOD2 was unchanged by dapagliflozin or *Slc5a2* knockdown (Fig. 6E and H). These findings suggested that dapagliflozin suppresses expression of Nox4 and TGF-$\beta$ by inhibiting SGLT2 in mProx24 cells. These observations support the hypothesis that dapagliflozin ameliorates high glucose-induced oxidative stress and apoptosis in proximal tubular epithelial cells under high-glucose conditions.

## Glucose uptake into cultured proximal tubular epithelial cells

The amount of glucose uptake into the proximal tubular epithelial cells was suppressed significantly by dapagliflozin or *Slc5a2* knockdown (Fig. 7). This result indicates that both pharmacological and genetic inhibition of SGLT2 reduce glucose uptake into the cells.

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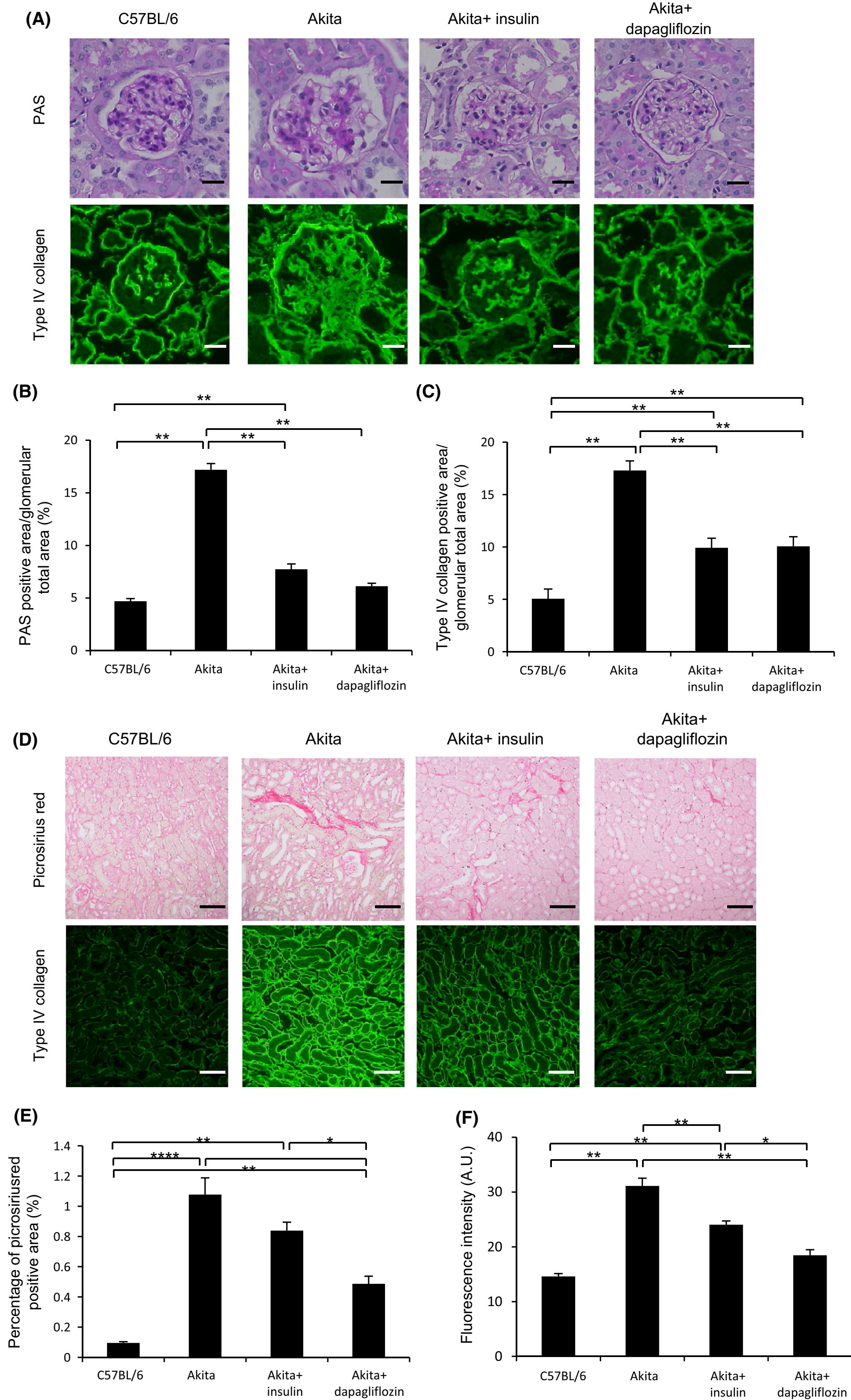



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**Figure 2.** Dapagliflozin suppresses expansion of the mesangial matrix and interstitial fibrosis. (A) Periodic acid–Schiff (PAS) staining and staining of type IV collagen of kidney sections. Scale bar, 25 $\mu$m. (B) PAS-positive area in glomeruli. (C) Type IV collagen-positive area in glomeruli. (D) Picrosirius red staining and staining of type IV collagen in kidney sections. Scale bar, 100 $\mu$m. (E) Percentages of picrosirius red-positive areas in the interstitium. (F) Type IV collagen-positive area in the interstitium. Data are the mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$.

## Discussion

The present study demonstrated that the selective SGLT2 inhibitor dapagliflozin improves hyperglycemia and delays development of diabetic nephropathy in T1DM Akita mice. By maintaining blood glucose at the same level in the dapagliflozin group and control insulin group, we detected suppressive effects specific to the SGLT2 inhibitor (Fig. 1B and C). Dapagliflozin suppressed fibrosis, inflammation, oxidative stress, and apoptosis in the interstitium of the kidney to a greater extent than insulin (Fig. 2D–F, 3C–E, 4). Our in vitro study demonstrated that dapagliflozin suppresses oxidative stress by inhibiting SGLT2 in cultured proximal tubular epithelial cells (Fig. 5, 6 and 7). These findings suggest that dapagliflozin reduces the progression of diabetic nephropathy instead of merely improving hyperglycemia. We assume that this renoprotective effect is owing to inhibition of SGLT2 expression.

Despite the progress that has been made in developing efficacious treatments for DM, diabetic nephropathy has remained the leading cause of ESRD worldwide in recent decades (de Boer et al. 2011). This fact indicates that the mechanisms involved in ESRD initiation are not completely understood. Glomerular lesions are major triggers for the development of diabetic nephropathy. However, several recent studies have demonstrated that tubular lesions and tubulointerstitial fibrosis are also key factors in the pathogenesis of diabetic nephropathy (Morii et al. 2003; Bagby 2007; Ninichuk et al. 2007; Tonolo and Cherchi 2014). It has also been shown that the progression of diabetic nephropathy is correlated with the degree of tubulointerstitial disease (Tonolo and Cherchi 2014). Therefore, strategies targeting tubules and the interstitium could help to retard the progression of diabetic nephropathy.

Exposure of proximal tubular epithelial cells to increased levels of glucose results in an increase in growth factors, inflammatory cytokines, and profibrotic mediators as well as the formation of advanced glycation end products and ROS (Kanwar et al. 2011). All of these factors have been implicated in the onset and progression of diabetic nephropathy. Induction of these pathways is initiated by glucose entry, which is mediated mainly by SGLT2. Thus, inhibition of SGLT2 should reduce intracellular glucose levels and their subsequent adverse effects in proximal tubular cells (Thomas 2014). In support of this notion, an in vitro study showed that SGLT2 inhibition antagonizes many of the deleterious actions of high-glucose exposure (Panchapakesan et al. 2013). However, it is not known whether this phenomenon is also applicable to in vivo events in rodents and human.

Previously, we demonstrated that dapagliflozin has protective effects on diabetic nephropathy in *db/db* mice (Terami et al. 2014). Investigators have reported that other SGLT2 inhibitors also have renoprotective effects (Arakawa et al. 2001; Nagata et al. 2013; Vallon et al. 2014). However, the effects of SGLT2 inhibitors were not compared with other glucose-lowering drugs, and the mechanisms were not investigated fully in those studies. Similar to the present study, Kojima et al. (2013) evaluated the renoprotective effects of SGLT2 inhibitors independent of the glucose-lowering effect of insulin. In accordance with our findings, they demonstrated that the SGLT2 inhibitor luseogliflozin reduced the degree of glomerular injury, renal fibrosis, and tubular necrosis in T2DM rats and control of hyperglycemia by insulin had no beneficial effects on the progression of diabetic nephropathy (Kojima et al. 2013), suggesting the renoprotective effects of luseogliflozin. The study by Kojima et al. (2013) did not evaluate the direct effects of SGLT2 inhibitors on cultured proximal tubular epithelial cells, so the possible mechanisms remain unexplored.

Komala et al. reported that the SGLT2 inhibitor empagliflozin had no beneficial effects on streptozotocin (STZ)-induced diabetic nephropathy in endothelial nitric oxide synthase knockout mice (Gangadharan Komala et al. 2014). There are two possibilities for the discrepancy between their results and our data. First, it is well known that STZ is toxic and that STZ itself may affect the kidney and induce renal injury, oxidative stress, and inflammation. Second, the glucose level in empagliflozin-treated mice was much higher than normal (~360 mg/dL). Komala et al. administered 10 mg/kg/day of empagliflozin, but this dose might have been too small to evaluate its effects. Indeed, Vallon et al. (2013) used 60–80 mg/kg per day of empagliflozin, and hyperglycemia was suppressed (~180 mg/dL) and albuminuria was improved. Therefore, hyperglycemia per se may induce oxidative stress, inflammation, and renal injury. Similar to our study, Vallon et al. (2014) reported that the SGLT2 knockout itself appears not to be nephroprotective in DM mice. However, they also used an STZ-induced model of T1DM, and glucose levels in STZ-induced DM SGLT2 knockout mice were much higher than normal (~300 mg/dL).

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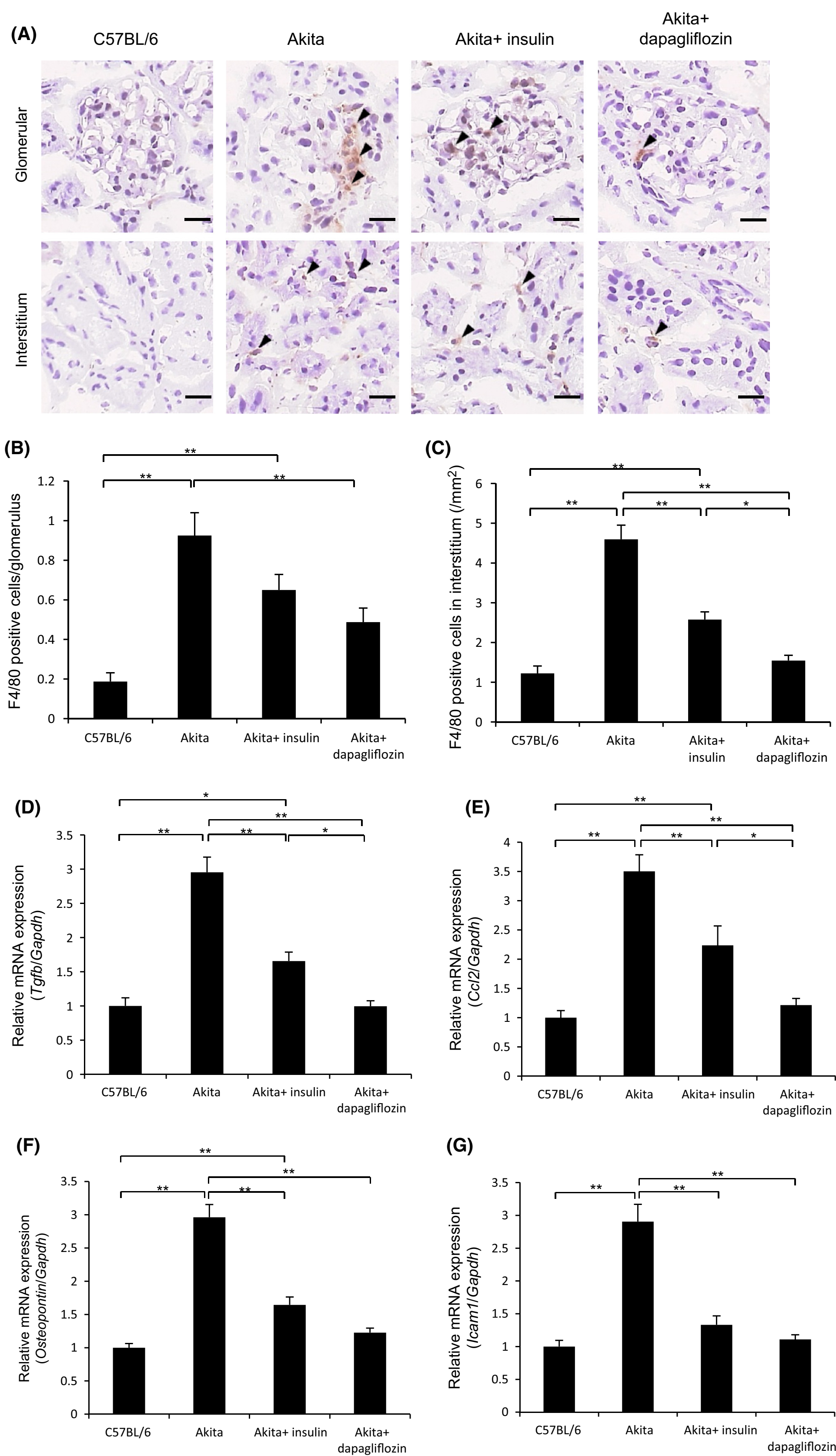



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**Figure 3.** Dapagliflozin inhibits infiltration of proinflammatory macrophages and expression of inflammatory genes in the renal cortex. (A) Immunohistochemistry of macrophages in the kidney. In the interstitium, dapagliflozin suppressed macrophage infiltration to a greater extent than insulin. Arrowheads indicate macrophages. Scale bar, 20 $\mu$m. (D) Number of intraglomerular macrophages. (E) Number of macrophages in the interstitium. Quantitative RT-PCR analyses of the expression of *Tgfb* (D), *Ccl2* (E), *osteopontin* (F), and *Icam1* (G) in the kidney. Data are the mean ± SEM. *$P < 0.05$; **$P < 0.01$.

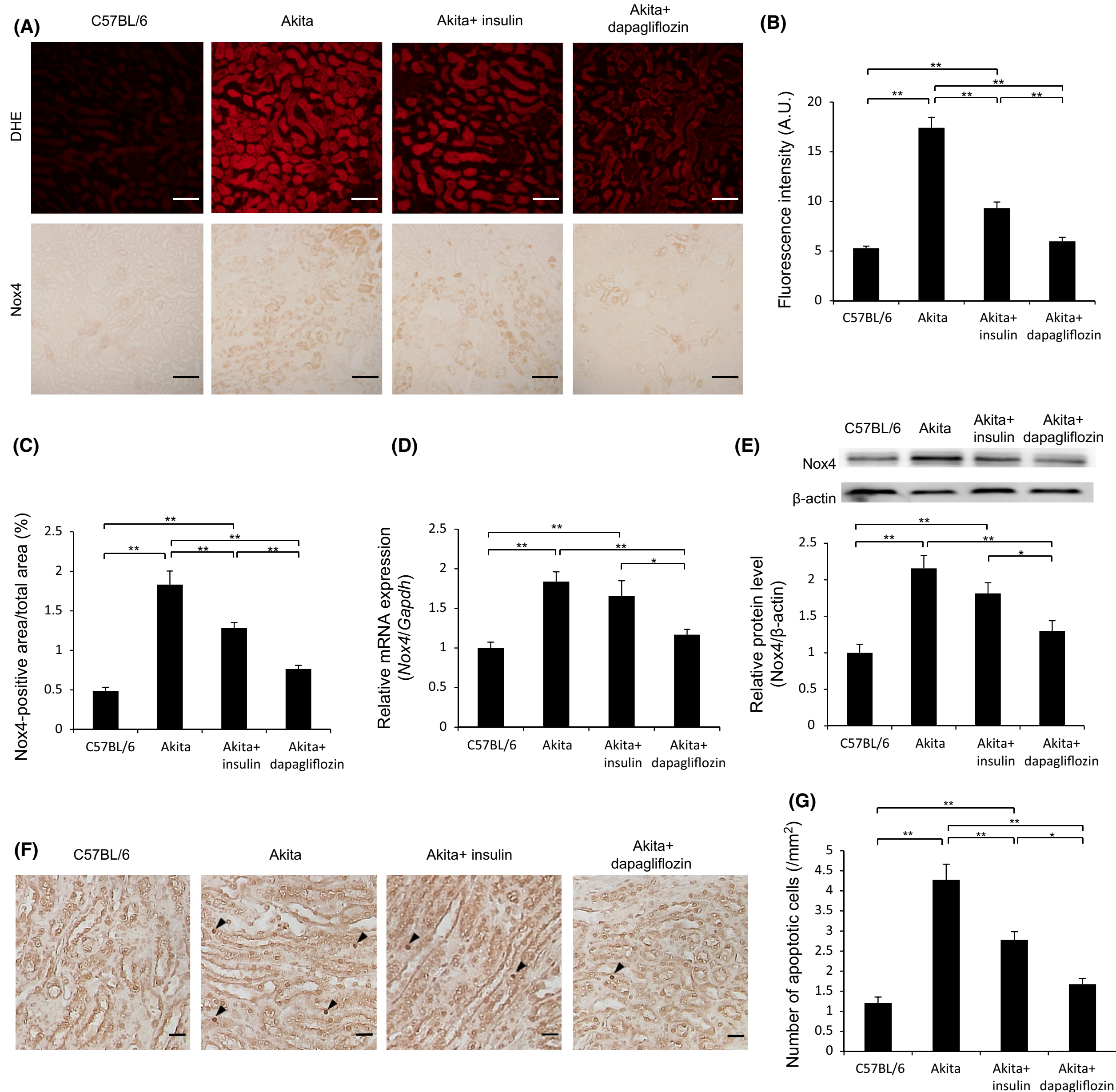


**Figure 4.** Dapagliflozin suppresses oxidative stress and apoptosis in the kidney. (A) Reactive oxygen species production was detected by fluorescence microscopy using dihydroethidium (DHE). Localization of Nox4 expression in the renal cortex was detected by immunohistochemistry. Scale bar, 100 $\mu$m. (B) Fluorescence intensity of DHE staining in the interstitium. (C) Nox4-positive area in the interstitium. (D) Quantitative RT-PCR analyses of *Nox4* expression in the kidney. (E) Western blotting of expression of Nox4 protein. (F, G) Apoptosis was detected by terminal transferase-mediated dUTP nick-end labeling (TUNEL) staining. Arrowheads indicate apoptotic nuclei. Scale bar, 25 $\mu$m. Data are the mean ± SEM. *$P < 0.05$; **$P < 0.01$.

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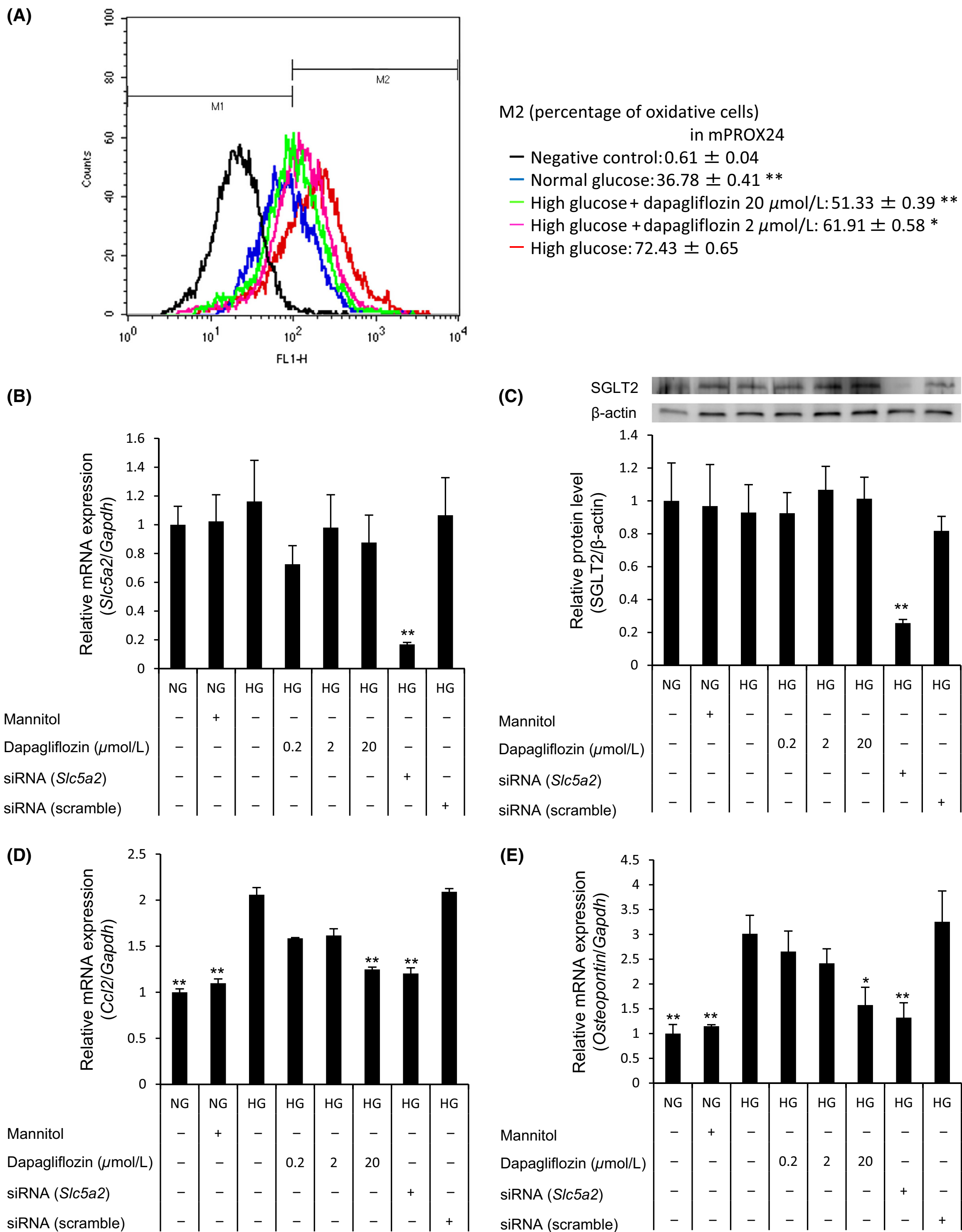


**Figure 5.** Dapagliflozin and *Slc5a2* knockdown suppress oxidative stress and expression of inflammatory genes in cultured proximal tubular epithelial (mProx24) cells. (A) Reactive oxygen species levels in mProx24 cells were upregulated by high glucose (25 mmol/L) compared with normal glucose (5.5 mmol/L). Cellular ROS levels were assessed by DCF-DA staining and subsequent FACS analyses. (B) Expression of *Slc5a2* mRNA in mProx24 cells. (C) Expression of SGLT2 protein in mProx24 cells. Quantitative RT-PCR analyses of expression of *Ccl2* (D) and *osteopontin* (E) in mProx24 cells. Data are the mean ± SEM. $^*P < 0.05$; $^{**}P < 0.01$ versus high glucose (A, D, E). $^{**}P < 0.01$ versus high glucose with scrambled siRNA (B, C).


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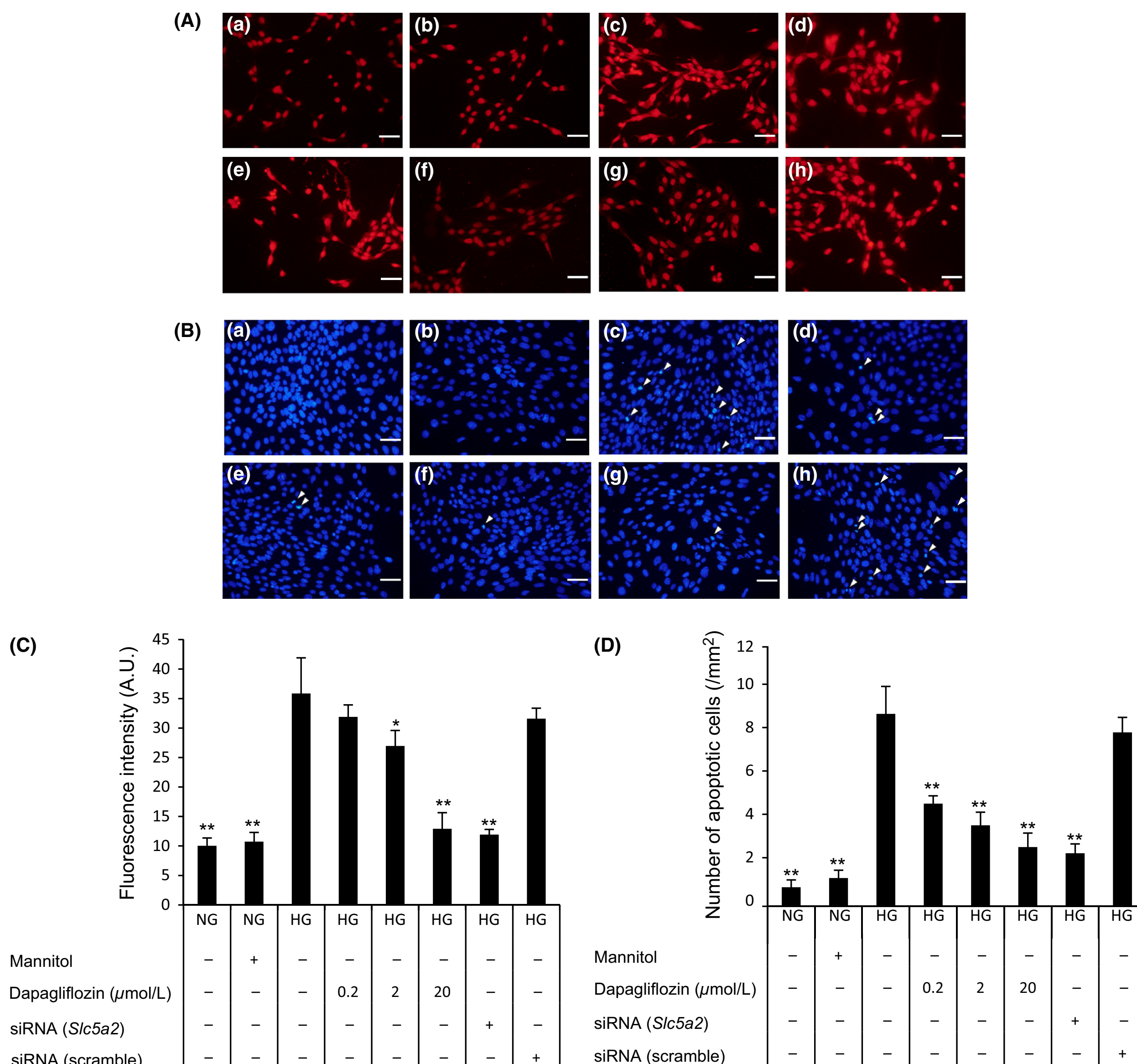


**Figure 6.** Dapagliflozin and *Slc5a2* knockdown suppress oxidative stress and apoptosis in cultured proximal tubular epithelial (mProx24) cells. (A, C) Reactive oxygen species (ROS) production was detected by fluorescence microscopy using dihydroethidium. ROS production did not increase with mannitol (b) compared with normal glucose (a), but did increase with high glucose (c). High glucose-induced ROS production was attenuated by dapagliflozin pretreatment in a dose-dependent manner (d: 0.2 nmol/L; e: 2.0 nmol/L; f: 20.0 nmol/L). ROS production was suppressed significantly in mProx24 cells grown in high-glucose medium and transfected with *Slc5a2* siRNA (g) compared with cells grown in high-glucose medium and transfected with scrambled siRNA (h). Scale bar, 100 $\mu$m. (B, D) Apoptosis was detected by fluorescence microscopy using Hoechst. Arrowheads indicate apoptotic nuclei. (E–H) Western blotting of protein expression for Nox4, TGF-$\beta$, and SOD2. Data are the mean ± SEM. *$P < 0.05$; **$P < 0.01$.

A recent study reported that 8-week administration of empagliflozin ameliorated glomerular hyperfiltration in patients with T1DM (Cherney et al. 2014). Vallon et al. (2014) also reported that empagliflozin attenuates DM-induced albuminuria and renal growth by improving hyperfiltration in DM Akita mice. In our study, there were no significant differences in blood pressure, BUN, serum creatinine, or expansion of the mesangial matrix in glomeruli. Furthermore, urinary concentrations of sodium ions and chloride ions were similar in dapagliflozin and insulin groups. These data suggest that dapagliflozin did not alter tubuloglomerular feedback or affect glomerular

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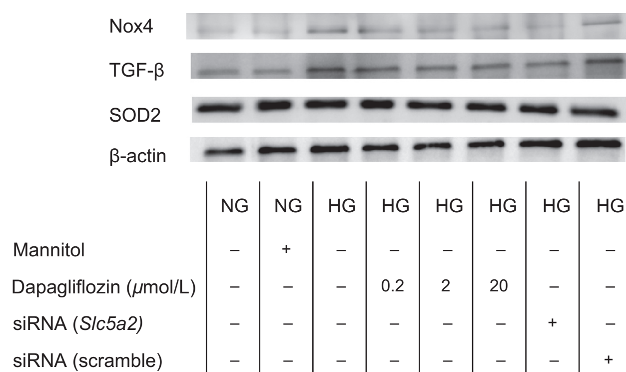


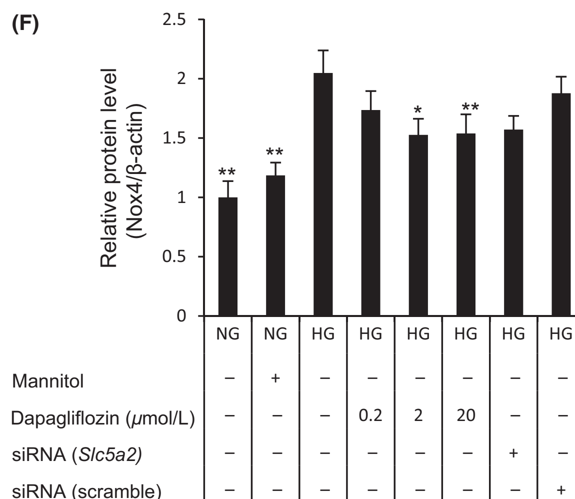


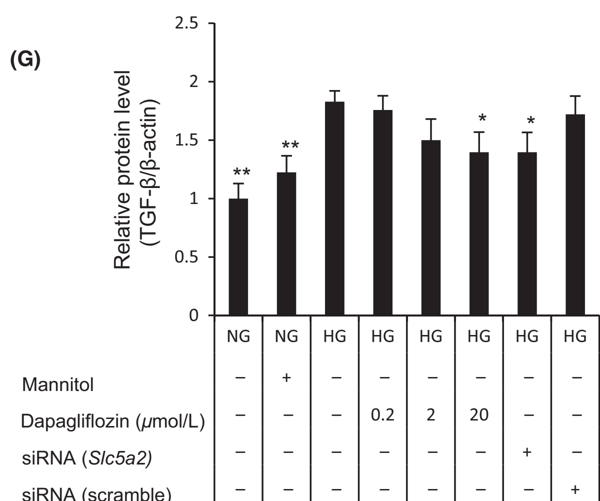


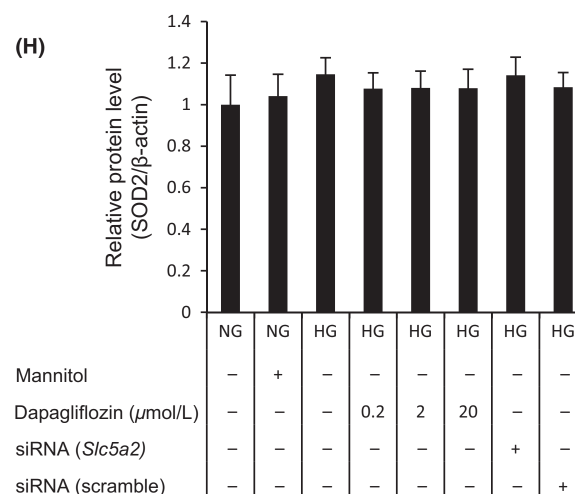


**Figure 6.** Continued

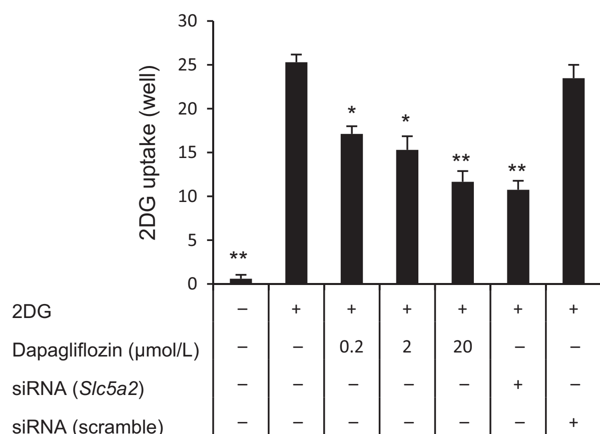


**Figure 7.** Dapagliflozin and knockdown of *Slc5a2* suppress glucose uptake into cultured proximal tubular epithelial (mProx24) cells. 2DG, 2-deoxy-D-glucose. Data are the mean ± SEM. *$P < 0.05$; **$P < 0.01$.

hyperfiltration in our study. On the contrary, macrophage infiltration, fibrosis, and oxidative stress in the interstitium were attenuated in the dapagliflozin group compared with those in the insulin group; these favorable effects might have contributed to the reduction of albuminuria in Akita mice.

To corroborate our in vivo findings, we investigated the effects of dapagliflozin on mProx24 cells. As revealed by flow cytometric analyses and microscopy observations, dapagliflozin suppressed the oxidative stress and apoptosis induced by high glucose levels in cultured mProx24 cells (Figs. 5A, 6A–D). Furthermore, *Slc5a2* knockdown also suppressed oxidative stress and apoptosis in these cells (Fig. 6A–D). In support of our in vitro study, SGLT2 RNAi in other proximal tubular cells (e.g., human kidney proximal tubular cell line and primary cultured cells from human kidneys) has also resulted in suppression of high


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glucose-induced ROS generation and inflammation (Maeda et al. 2013; Panchapakesan et al. 2013). Moreover, 2DG uptake experiments showed that pharmacological and genetic inhibition of SGLT2 resulted in reduction of glucose influx into the cells. These findings strongly suggest that the antioxidative effects of dapagliflozin can be attributed, at least partly, to direct inhibition of SGLT2. The present study is the first to demonstrate the protective effects of an SGLT2 inhibitor by in vivo and in vitro experiments carried out in parallel.

Severity of diabetic nephropathy in mice models varies depending on the mouse strain. C57BL/6-derived models represent a wide range of disease stages. Pathological features of Akita mice represent a relatively early stage of diabetic nephropathy (Fujita et al. 2009), but another C57BL/6-derived T1DM model has demonstrated severe oxidative stress induced by hyperglycemia (Giacco et al. 2014). In our study, dapagliflozin improved microalbuminuria and histological deteriorations in Akita mice, suggesting potential application of the drug for the early stages of diabetic nephropathy. SGLT2 inhibitors are not approved as treatment for ESRD. However, application of dapagliflozin at an early stage of diabetic nephropathy could prevent its progression and improve the prognosis of renal failure.

In conclusion, dapagliflozin ameliorates diabetic nephropathy by suppressing high glucose-induced oxidative stress in a manner independent of hyperglycemia improvement in Akita mice. Glucose control by insulin often causes hypoglycemic episodes and body weight increases, so our findings strongly suggest that dapagliflozin may be a novel therapeutic approach for the treatment of diabetic nephropathy.

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## Author Contributions

T. H. conducted the research and contributed to discussions. D. O. wrote the manuscript, conducted the research, and contributed to discussions. H. T., J. E., and T. I. conducted the research. H. Y., K. T., H. M., and J. W. contributed to discussions and reviewed/edited the manuscript. T. H. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

## Disclosures

D. O. and T. I. belong to the Department of Diabetic Nephropathy, which is endowed by Boehringer Ingelheim, has received research grant support from Eli Lilly and Sumitomo Dainippon Pharma, and has received speaker honoraria from AstraZeneca and Ono Pharmaceutical. J. W. is a consultant for Boehringer Ingelheim and received speaker honoraria from Novartis, Boehringer Ingelheim, and Novo Nordisk. H. M. is a consultant for AbbVie, Astellas, and Teijin, received speaker honoraria from Astellas, Boehringer Ingelheim, Chugai, Daiichi Sankyo, Sumitomo Dainippon Pharma, Kyowa Hakko Kirin, MSD, Pfizer, Takeda, and Mitsubishi Tanabe Pharma, and received grant support from Astellas, Boehringer Ingelheim, Daiichi Sankyo, Sumitomo Dainippon Pharma, Kyowa Hakko Kirin, Mochida, MSD, Novartis, Novo Nordisk, Pfizer, Takeda, and Mitsubishi Tanabe Pharma.

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