

RESEARCH PAPER

Tofogliflozin, a novel sodium–glucose co-transporter 2 inhibitor, improves renal and pancreatic function in *db/db* mice

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BACKGROUND AND PURPOSE

Although inhibition of renal sodium–glucose co-transporter 2 (SGLT2) has a stable glucose-lowering effect in patients with type 2 diabetes, the effect of SGLT2 inhibition on renal dysfunction in type 2 diabetes remains to be determined. To evaluate the renoprotective effect of SGLT2 inhibition more precisely, we compared the effects of tofogliflozin (a specific SGLT2 inhibitor) with those of losartan (an angiotensin II receptor antagonist) on renal function and beta-cell function in *db/db* mice.

EXPERIMENTAL APPROACH

The effects of 8-week tofogliflozin or losartan treatment on renal and beta-cell function were investigated in *db/db* mice by quantitative image analysis of glomerular size, mesangial matrix expansion and islet beta-cell mass. Blood glucose, glycated Hb and insulin levels, along with urinary albumin and creatinine were measured

KEY RESULTS

Tofogliflozin suppressed plasma glucose and glycated Hb and preserved pancreatic beta-cell mass and plasma insulin levels. No improvement of glycaemic conditions or insulin level was observed with losartan treatment. Although the urinary albumin/creatinine ratio of untreated *db/db* mice gradually increased from baseline, tofogliflozin or losartan treatment prevented this increase (by 50–70%). Tofogliflozin, but not losartan, attenuated glomerular hypertrophy. Neither tofogliflozin nor losartan altered matrix expansion.

CONCLUSIONS AND IMPLICATIONS

Long-term inhibition of renal SGLT2 by tofogliflozin not only preserved pancreatic beta-cell function, but also prevented kidney dysfunction in a mouse model of type 2 diabetes. These findings suggest that long-term use of tofogliflozin in patients with type 2 diabetes may prevent progression of diabetic nephropathy.

Abbreviations

ACR, albumin/creatinine ratio; DAB, diaminobenzidine; DN, diabetic nephropathy; HE, hematoxylin–eosin; IRI, immunoreactive insulin; PG, plasma glucose; SGLT2, sodium–glucose co-transporter 2; STZ, streptozotocin; TGF, tubuloglomerular feedback; UGE, urinary glucose excretion.

Introduction

Type 2 diabetes is characterized by impaired glycaemic control as a result of insulin resistance or disturbed insulin secretion. Chronic hyperglycaemia itself may impair insulin secretion and cause pancreatic beta-cell dysfunction. Therefore, the preservation of beta-cells through appropriate glycaemic control is important to prevent both the onset of diabetes and the progressive deterioration of the diabetic disease state (Kahn, 2003; Marchetti *et al.*, 2009).

The renal sodium–glucose co-transporter 2 (SGLT2) is specifically expressed in renal proximal tubules, and plays the dominant role in renal glucose reabsorption (Kanai *et al.*, 1994; Wright *et al.*, 2011). Studies with animal models of type 2 diabetes have shown SGLT2 inhibitors to have a blood glucose-lowering effect via increased urinary glucose excretion (UGE) and to have low safety concerns (Han *et al.*, 2008; Yamamoto *et al.*, 2011; Suzuki *et al.*, 2012). Consequently, several SGLT2 inhibitors are now being developed in clinical studies (Chao and Henry, 2010; Ferrannini and Solini, 2012).

Because SGLT2 functions predominantly in the kidney (Chen *et al.*, 2010; Wright *et al.*, 2011), it is important to know the effects of long-term SGLT2 inhibition on renal function, especially under diabetic conditions. As chronic hyperglycaemia is also considered to contribute to the progression of diabetic nephropathy (DN; The DCCT Research Group, 1993; UKPDS Group, 1998), the glucose-lowering effects of SGLT2 inhibition may prevent the progression of DN by ameliorating glucose toxicity. Although studies with mouse models of type 2 diabetes suggest that the long-term inhibition of SGLT2 by remogliflozin, an SGLT2 inhibitor (Fujimori *et al.*, 2008), or by SGLT2 genetic deletion (Jurczak *et al.*, 2011) preserves beta-cells with improved glycaemic conditions, no details on the effects of long-term SGLT2 inhibition on renal function have been reported, even in the preclinical studies mentioned earlier.

Long-term administration of T-1095, an SGLT inhibitor, suggested that it has renoprotective effects in *db/db* mice together with improved glycaemic conditions (Arakawa *et al.*, 2001). However, T-1095 and its active metabolite T-1095A may inhibit not only SGLT2, but also SGLT1, which also functions in the kidney, because both compounds have lower selectivity towards SGLT2 (Oku *et al.*, 1999). Unfortunately, that study showed no comparison of the renal function with any clinically validated drug, such as an angiotensin II receptor antagonist (Ruster and Wolf, 2006). Therefore, the actual effects of long-term SGLT2 inhibition on renal dysfunction in type 2 diabetes were still unclear.

Tofogliflozin is a potent and highly selective inhibitor of SGLT2 (Ohtake *et al.*, 2012) currently under clinical development (Kadowaki *et al.*, 2012). A single oral administration of this compound lowered blood glucose levels in Zucker diabetic rats with increased renal glucose clearance and treatment for 4 weeks with this compound improved glucose tolerance in *db/db* mice (Suzuki *et al.*, 2012).

In the present study, to evaluate the long-term effects of SGLT2 inhibition on renal and pancreatic function under diabetic conditions, we compared the effects of long-term (8 weeks) treatment with tofogliflozin on the renal and pancreatic functions in *db/db* mice, a mouse model of type 2

diabetes, with those of losartan, an angiotensin II receptor antagonist.

Methods

Animals

All animal care and experiments were performed in accordance with the guidelines for the care and use of laboratory animals at Chugai Pharmaceutical Co., Ltd, and the protocol was approved by the Institutional Animal Care and Use Committee at the company. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 69 animals was used in the experiments described here. Female *db/db* mice (BKS.Cg-Dock7^m+/+ Lepr^{db}/J; stock no. 000642) and their lean controls (*db/+m* mice) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan) at 6 weeks of age. These animals were housed under a 12 h/12 h light/dark cycle (lights on 07:00–19:00 h) with controlled room temperature (20–26°C) and humidity (35–75%), and were allowed *ad libitum* access to a diet of standard laboratory chow (CE-2 pellets; Clea Japan) and water. The animals were 8 weeks of age at the beginning of the experiments.

Long-term administration

The *db/db* mice were randomly allocated into four dietary treatment groups matched for both 24 h urinary albumin excretion and body weight at 8 weeks of age. The *db/db* mice were kept on the standard diet or on a diet containing 0.005 or 0.015% tofogliflozin or 0.045% losartan for 8 weeks. The tofogliflozin content was determined according to previous pharmacokinetic data (Suzuki *et al.*, 2012) and the estimated food consumption of *db/db* mice in order to inhibit SGLT2 completely, but not affect SGLT1. The *db/+m* mice were kept on the standard diet. Blood glucose, glycated Hb, plasma insulin, plasma creatinine, urinary glucose, urinary creatinine and urinary albumin levels were measured periodically. Blood samples were collected from the tail vein or inferior vena cava to measure blood glucose, glycated Hb, plasma insulin and plasma creatinine levels. Metabolic cages were used to collect urine to measure urinary glucose, urinary creatinine, and urinary albumin excretion. At the end of 8 weeks' treatment, animals were killed by whole blood collection from the abdominal aorta under anaesthesia with isoflurane. The kidneys and pancreas were isolated for the histological analysis described later. As part of these studies a separate group of *db/db* mice (16 weeks of age, $n = 9$) was kept on the diet containing 0.015% tofogliflozin for 4 days, then three mice each were killed at 10:00, 15:00 and 20:00 h on day 4 by whole blood collection from the abdominal aorta under anaesthesia and the plasma samples were obtained by centrifugation to determine plasma tofogliflozin concentrations. Urine and plasma samples were stored at -80°C until use.

Data collection

Plasma tofogliflozin concentrations were measured with an HPLC–MS/MS system (Shimadzu 20A; Shimadzu, Kyoto,

Japan; API-4000; AB SCIEX, Framingham, MA, USA). Blood glucose levels were determined using a plasma-glucose monitoring system (Accu-Chek Aviva; Roche Diagnostics, Tokyo, Japan). Urinary glucose concentrations were measured by the hexokinase G-6-PDH method (L-Type Glu 2; Wako Pure Chemical Industries, Ltd., Osaka, Japan) with an automated analyzer (TBA-120FR; Toshiba Medical Systems, Tochigi, Japan). Creatinine concentrations in plasma and urine were measured by the creatininase-HMMPs method (L-Type Creatinine M; Wako Pure Chemical Industries, Ltd.) with the automated analyzer. Glycated Hb levels were measured by turbidimetric inhibition immunoassay (Auto Waco HbA1c; Wako Pure Chemical Industries, Ltd.) with the automated analyzer. Urinary albumin concentrations were measured by turbidimetric immunoassay (Testant Mouse Urinary Microalbumin Assay; TAUNS Laboratories, Inc., Shizuoka, Japan) with the automated analyzer. Urinary albumin excretion was evaluated as the ratio of urinary albumin concentration to that of the urinary creatinine concentration [albumin/creatinine ratio (ACR)].

Renal creatinine and glucose clearance were determined by dividing the rate of urinary creatinine and glucose excretion for each urine collection period (24 h cumulative urine sample) by the plasma creatinine and glucose levels respectively. Plasma insulin levels were determined with an insulin ELISA kit (Morinaga Institute of Biological Science, Kanagawa, Japan).

Evaluation of glomerular size and mesangial expansion

The kidneys were fixed in methanol-Carnoy's solution and embedded in paraffin. The blocks were sliced into sections 3 μm thick and stained with an anti-type IV collagen antibody (20441; Novotec, Lyon, France) or with the hematoxylin and periodic acid Schiff (PAS), and the type IV collagen-stained sections were then visualized with biotinylated secondary antibody (K4003; Dako Japan Inc., Tokyo, Japan) and HRP-conjugated streptavidin with diaminobenzidine (DAB) chromogen (K3466; Dako), and finally stained with hematoxylin by Sapporo General Pathology Laboratory Co., Ltd. (Sapporo, Japan).

For the quantification of glomerular size and mesangial type IV collagen-positive area, sections were scanned with the ScanScope and ImageScope imaging and analysis systems (Aperio Technologies, Inc., Vista, CA, USA). Fifty glomeruli per mouse from two different sections were randomly selected in the cortex region. The average of glomerular size was calculated by dividing total glomerular area (mm^2) by the number of analysed glomeruli for each mouse. The mesangial matrix area was defined as the type IV collagen-positive area and is expressed as the average number of the total numbers of 'Positive and Strong Positive' pixels from among each of the analysed glomeruli for each mouse by using the Positive Pixel Count V9 algorithm on the default setting, and converted to the type IV collagen-positive area (mm^2) from the correlation formula between the total numbers of pixels and the area in analysed glomeruli as follows (the area = the number of pixels $\times 2.5139974 \times 10^{-7} + 2.814267 \times 10^{-8}$; $r^2 = 1$).

Mesangial expansion in the PAS-stained sections was evaluated by light microscopy, without knowledge of the treatments. A total of 72–100 glomeruli were randomly selected from each animal. The mesangial matrix expansion was graded on a semiquantitative scale from 0 to 4+, as described by other investigators (Qi *et al.*, 2005; Guo *et al.*, 2006), by scoring the PAS-positive area in the glomerular as follows: 0 indicates no expansion; 1+ indicates matrix expansion occupying up to 25% of a glomerulus; and 2+, 3+, and 4+ indicate matrix expansion occupying 25–50, 50–75 and more than 75% of a glomerulus respectively. The mesangial matrix score for each animal was shown as the average score of all the glomeruli that had been graded.

Measurement of islet beta-cell mass

Collected pancreases were embedded in OCT compound (Sakura Finetek, Tokyo, Japan) in cryomolds and frozen in *n*-hexane chilled with acetone and dry ice, then sliced into sections 5 μm thick and stained with hematoxylin-eosin (HE) by Sapporo General Pathology Laboratory. Pancreas sections adjacent to the HE-stained sections mentioned earlier were immunostained with anti-insulin antibody (sc-9168; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and visualized with biotinylated secondary antibody (BA-1000; Vector Laboratories Inc., Burlingame, CA, USA) and HRP-conjugated streptavidin with DAB chromogen (Discovery DAB Map Kit 760-124; Roche Diagnostics K.K., Tokyo, Japan). For four pancreas sections randomly selected from each mouse, the total beta-cell area was calculated as the ratio of the insulin-positive area to total pancreas area, with the imaging and analysis systems mentioned earlier. The insulin-positive area and total pancreas area were determined with the 'Strong Positive' and the total ('Positive + Negative') pixel numbers, respectively, using the Positive Pixel Count V9 algorithm with the intensity threshold settings as follows: weak, 220–150; medium, 150–100; strong, 100–0.

Data analysis

Data are presented as means \pm SEM. Statistical analysis was performed with the SAS System for Windows, Release 8.02 (SAS Institute Japan, Tokyo, Japan). Statistical significance was determined by the parametric Dunnett's multiple comparison test for metabolic parameters in treated and untreated *db/db* mice, by the non-parametric Dunnett's multiple comparison (joint-ranking) for histological analysis in treated and untreated *db/db* mice, or by Student's *t*-test in untreated *db/db* and *db/+m* mice.

Materials

Tofogliflozin ((1S,3'R,4'S,5'S,6'R)-6-[(4-ethylphenyl)methyl]-3',4',5',6'-tetrahydro-6'-(hydroxymethyl)-spiro[isobenzofuran-1(3H),2'-[2H]pyran]-3',4',5'-triole), was synthesized in our laboratories at Chugai Pharmaceutical Co., Ltd. Losartan was purchased from LKT Laboratories Inc. (Saint Paul, MN, USA). Laboratory chow (CE2-pellets) containing 0.005% (w/w) tofogliflozin, 0.015% tofogliflozin, or 0.045% losartan was prepared by Clea Japan, Inc. (Tokyo, Japan).

Results

The effect of long-term tofogliflozin administration on hyperglycemia

The fed-state plasma glucose (PG) and glycated Hb levels were both significantly higher in untreated diabetic mice than in *db/+m* mice throughout the experiment. The PG and glycated Hb levels after 4 and 8 weeks were significantly lower in the groups of *db/db* mice treated with 0.005 or 0.015% tofogliflozin than in the untreated control group

(Figure 1A, B). The plasma tofogliflozin concentrations in the mice given the diet containing 0.015% tofogliflozin for 4 days (16 weeks of age, $n = 3$) at 10:00, 15:00 and 20:00h were 364 ± 35 , 216 ± 44 and 606 ± 42 ng·mL⁻¹ respectively. No improvement in glycaemic condition was observed in the group treated with 0.045% losartan.

The urine volume and UGE were significantly higher in untreated *db/db* mice than in *db/+m* mice (Figure 1C, D). Tofogliflozin treatment lowered urine volume compared with the untreated control group at 8 weeks of treatment (Figure 1C). Tofogliflozin treatment tended to decrease the

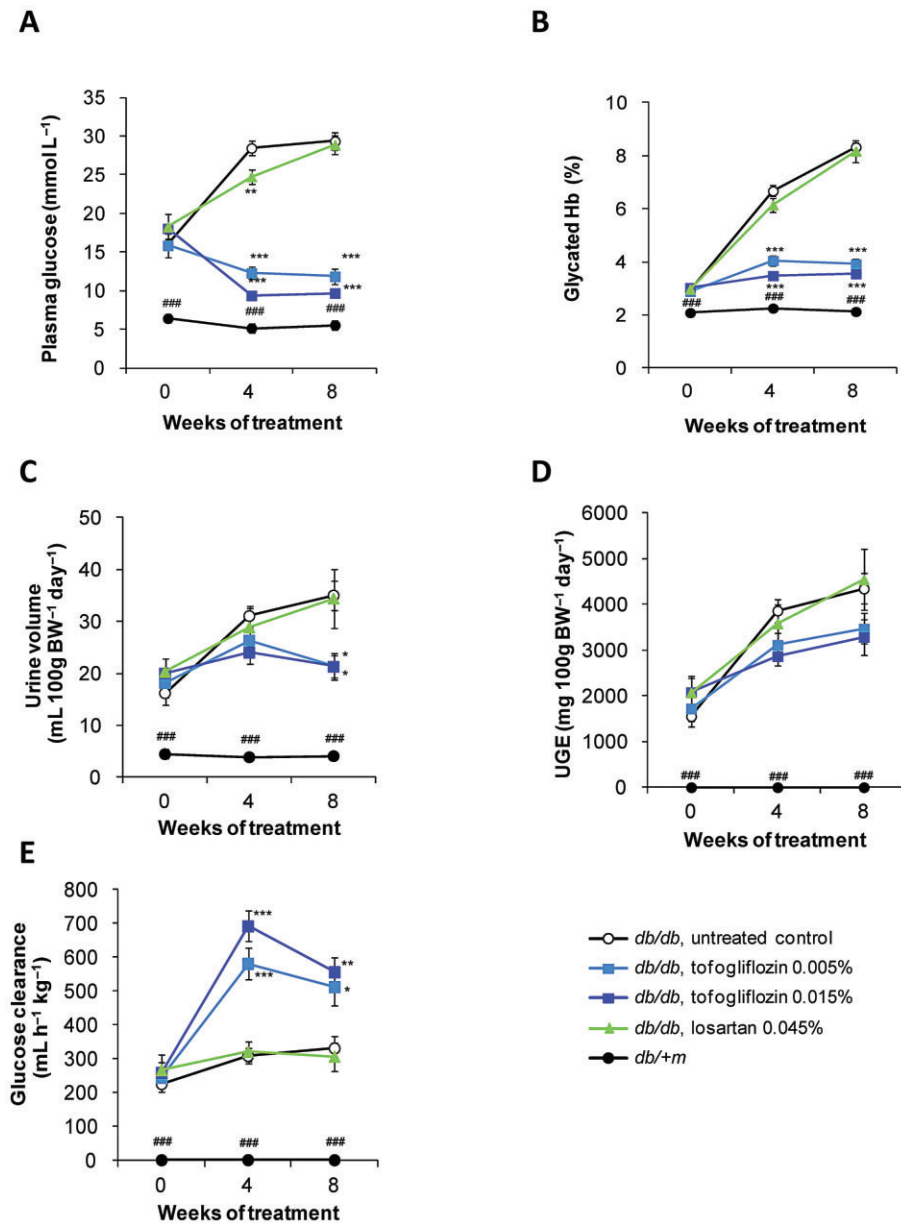


Figure 1

Blood glucose and glycated Hb in *db/db* mice. (A–B) Tofogliflozin lowered blood glucose concentration (A) and suppressed glycated Hb (B) in *db/db* mice. (C–E) Long-term tofogliflozin administration lowered the urine volume (C), lowered UGE (D), and elevated glucose clearance (E) in *db/db* mice. Data shown are means \pm SEM (*db/db* mice, $n = 8$; *db/+m* mice, $n = 10$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ significantly different from control group by Dunnett's multiple comparison test. **** $P < 0.0001$ significantly different from control group by *t*-test.

UGE compared with the untreated control group at 4 and 8 weeks of treatment, but the differences did not reach statistical significance (Figure 1D). Tofogliflozin treatment increased renal glucose clearance levels compared with untreated *db/db* mice, whereas losartan treatment had no effect on this parameter (Figure 1E).

At baseline (0 weeks of treatment), the UGE was detectable at PG above around $10 \text{ mmol}\cdot\text{L}^{-1}$ and increased depending on the PG levels, suggesting the glucose filtration levels around $10 \text{ mmol}\cdot\text{L}^{-1}$ exceeded the threshold of glucose reabsorption in *db/db* mice (Supporting Information Figure S1A). At 4 and 8 weeks, the UGE in control and losartan groups was also apparent with increases in PG to around $20\text{--}30 \text{ mmol}\cdot\text{L}^{-1}$. In contrast, although tofogliflozin treatment reduced the PG to around $5\text{--}15 \text{ mmol}\cdot\text{L}^{-1}$ at 4 and 8 weeks, their daily UGE levels were still higher than those at 0 weeks when the PG was around $10\text{--}15 \text{ mmol}\cdot\text{L}^{-1}$ (Supporting Information Figure S1B, C). These results indicate that tofogliflozin treatment reduced the threshold of glucose reabsorption in *db/db* mice and increased the UGE, and then reduced the PG.

The body weight of untreated *db/db* mice increased gradually till 10 weeks of age (2 weeks of treatment) and reached a maximum of about 50 g (Figure 2A). Tofogliflozin treatment increased the body weight and food consumption of *db/db* mice, whereas losartan treatment had no effect on these parameters (Figure 2B). At baseline, there was no clear relationship between the body weight and daily food consumption

(Supporting Information Figure S2A). Interestingly, at 4 weeks, when the difference in body weight between the tofogliflozin and the control groups began to be clear (Figure 2A, B), the daily food consumption in tofogliflozin groups was greater than that in the control group, even between animals with similar body weight (Supporting Information Figure S2B). Finally, at 8 weeks, there was a clear positive relationship between the body weight and food consumption, suggesting that the levels of food consumption at this stage were closely linked to the body weight of the animals (Supporting Information Figure S2C).

The effect of long-term tofogliflozin administration on renal function

At baseline, the ACR value ($\mu\text{g}\cdot\text{mg}^{-1}$) was higher in the untreated *db/db* mice than in the *db/+m* mice (Figure 3A). There were no significant differences in the ACR values at baseline among any of the treatment groups in the *db/db* mice. The ACR values in *db/+m* mice at 4 and 8 weeks of treatment were slightly lower than the ACR values at baseline (Figure 3A). In contrast, the ACR values in untreated *db/db* mice at 4 and 8 weeks of treatment increased markedly over the baseline value (Figure 3B), resulting in a steady increase of ACR values at 4 and 8 weeks of treatment, respectively (Figure 3A). Both losartan and tofogliflozin treatment significantly prevented the increase of the ACR value by about 50–70% (Figure 3A, B).

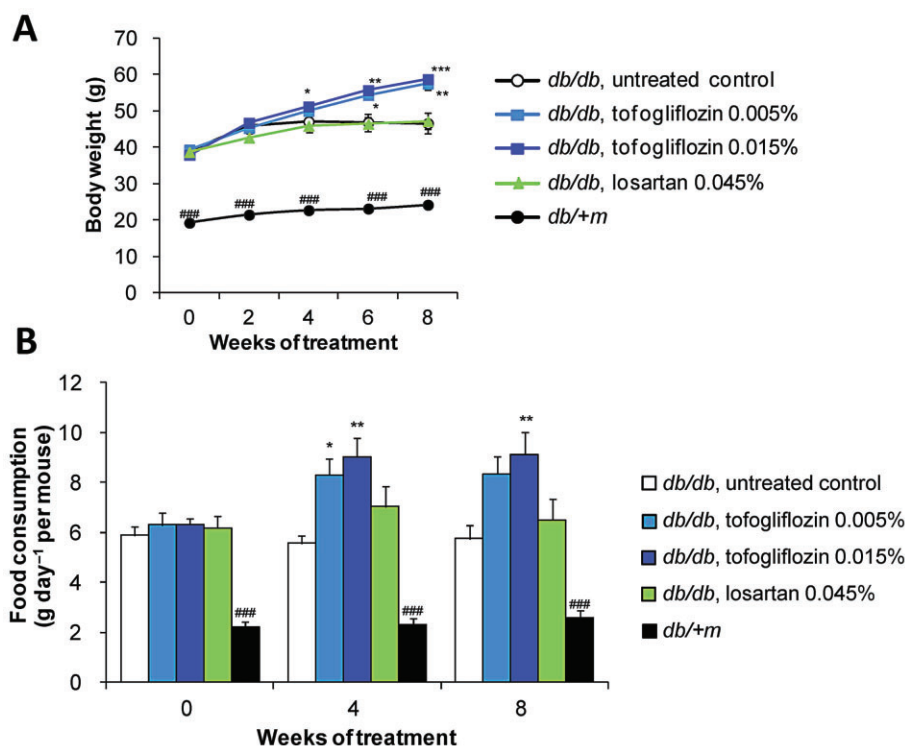


Figure 2

Body weight and food consumption in *db/db* mice. (A–B) Tofogliflozin treatment increased body weight (A) and food consumption (B) in *db/db* mice. Data shown are means \pm SEM (*db/db* mice, $n = 8$; *db/+m* mice, $n = 10$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ significantly different from control group by Dunnett's multiple comparison test. ### $P < 0.001$ significantly different from control group by *t*-test.

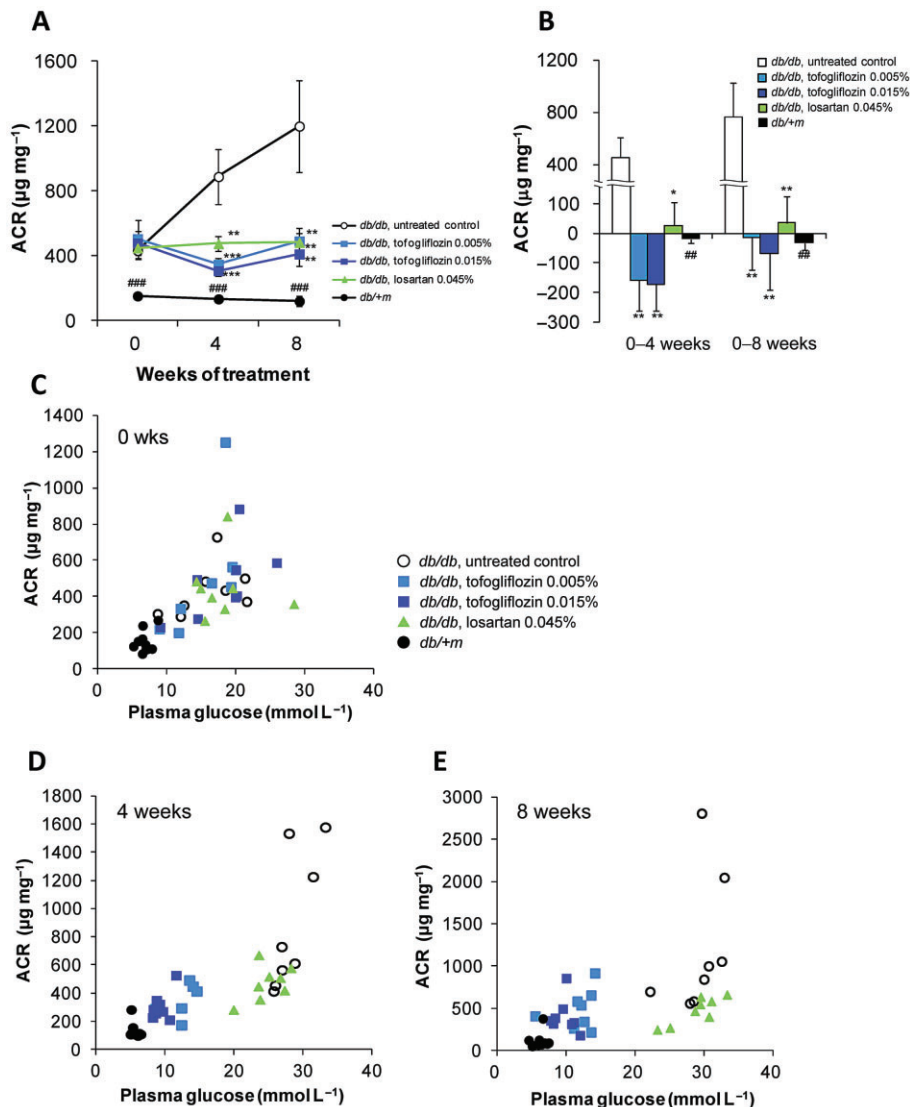


Figure 3

Urinary albumin excretion in *db/db* and *db/+m* mice. (A–B) Values shown are the ratio of urinary albumin excretion to urinary creatinine excretion (ACR). Tofogliflozin treatment suppressed the 24 h urinary ACR in *db/db* mice. ACR was significantly lower in tofogliflozin-treated mice than in control *db/db* mice (A). Changes in ACR values from the start of study in the tofogliflozin-treated groups were comparable with changes in ACR values in the losartan-treated group at 4 and 8 weeks of treatment (B left, 0–4 weeks; B right, 0–8 weeks). Data shown are means \pm SEM * P < 0.05, ** P < 0.01, *** P < 0.001 significantly different from control group by Dunnett’s multiple comparison test. ## P < 0.01, ### P < 0.001 significantly different from control group by *t*-test. (C–E) Scatter plot of plasma glucose and ACR values in *db/db* and *db/+m* mice. (C) 0 weeks (Baseline value), (D) 4 weeks of treatment, (E) 8 weeks of treatment.

At baseline, a trend towards a positive correlation between ACR values and PG levels was observed (Figure 3C). At 4 and 8 weeks, although the ACR values of several animals in the control groups exceeded 1000 and 2000 $\mu\text{g}\cdot\text{mg}^{-1}$, respectively, the ACR values in the tofogliflozin and losartan groups were still maintained at levels less than 1000 $\mu\text{g}\cdot\text{mg}^{-1}$, with improved glycaemic conditions in the tofogliflozin groups, but not in the losartan group (Figure 3D, E).

Creatinine clearance was significantly higher in the untreated *db/db* mice than in the *db/+m* mice (Figure 4A), which is consistent with the reported hyperfiltration in *db/db*

mice (Gartner, 1978; Sugaru *et al.*, 2006b) or streptozotocin-induced (STZ-induced) diabetic mice (Dunn *et al.*, 2004; Vallon *et al.*, 2013). Tofogliflozin and losartan treatment slightly ameliorated the increase in creatinine clearance. A tendency towards a positive correlation between the PG and the creatinine clearance levels was observed in the tofogliflozin and losartan groups (Figure 4B). The plasma creatinine concentrations at 8 weeks of treatment were $0.59 \pm 0.05 \text{ mg}\cdot\text{L}^{-1}$ in the untreated *db/db* mice, $0.64 \pm 0.03 \text{ mg}\cdot\text{L}^{-1}$ in the 0.005% tofogliflozin-treated mice, $0.64 \pm 0.03 \text{ mg}\cdot\text{L}^{-1}$ in the 0.015% tofogliflozin-treated mice, $0.64 \pm 0.05 \text{ mg}\cdot\text{L}^{-1}$

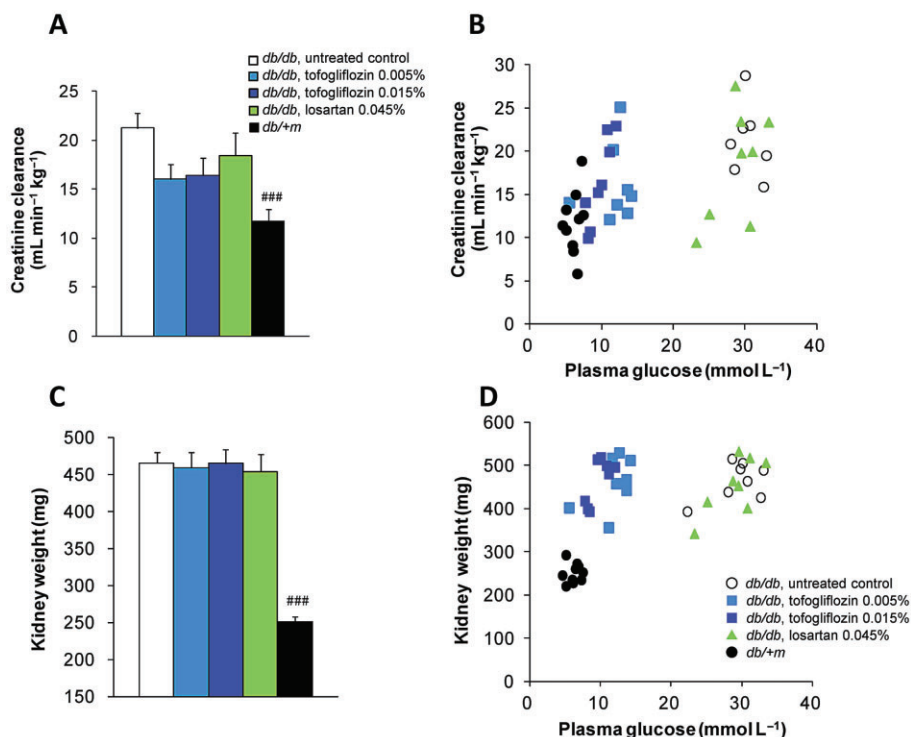


Figure 4

Creatinine clearance and kidney weight in *db/db* and *db/+m* mice. (A) Creatinine clearance in *db/db* mice was not affected by 8 weeks' treatment with tofogliflozin or losartan. Creatinine clearance was significantly higher in untreated *db/db* mice than in *db/+m* mice. Data shown are means \pm SEM (*db/db* mice, $n = 8$; *db/+m* mice, $n = 10$). $^{###}P < 0.001$ significantly different from control group by *t*-test. (B) Scatter plot of plasma glucose and creatinine clearance in *db/db* and *db/+m* mice. (C) Kidney weight in *db/db* mice was not affected by 8 weeks' treatment with tofogliflozin or losartan. Kidney weight was significantly greater in untreated *db/db* mice than in *db/+m* mice. Data shown are means \pm SEM (*db/db* mice, $n = 8$; *db/+m* mice, $n = 10$). $^{###}P < 0.001$ significantly different from control group by *t*-test. (D) Scatter plot of plasma glucose and kidney weight in *db/db* and *db/+m* mice.

in the 0.045% losartan-treated *db/db* mice, and $1.24 \pm 0.12 \text{ mg}\cdot\text{L}^{-1}$ in the *db/+m* mice (untreated *db/db* mice vs. *db/+m* mice, $P < 0.005$).

The kidney weight of the *db/db* mice (control group) was significantly greater than that of *db/+m* mice. Neither tofogliflozin nor losartan treatment reduced the kidney weight, and there was no clear relationship between the glucose levels and the kidney weight in *db/db* mice (Figure 4C, D).

The renoprotective effect of tofogliflozin was also assessed by histopathological analysis (Figures 5A, 6A). The mean glomerular area was significantly larger in the untreated *db/db* mice than in the *db/+m* mice. Tofogliflozin treatment significantly attenuated glomerular hypertrophy in a dose-dependent manner, whereas losartan treatment had no effect on the glomerular size (Figure 5B). Both in the PAS- and type IV collagen-stained sections, the mesangial matrix area was also significantly greater in the untreated *db/db* mice than in the *db/+m* mice. Neither tofogliflozin nor losartan significantly altered the mesangial expansion (Figures 5C, 6B).

The effect of long-term tofogliflozin administration on beta-cell function

The levels of plasma immunoreactive insulin (IRI) in the untreated *db/db* mice decreased from $9.9 \pm 2.4 \text{ ng}\cdot\text{mL}^{-1}$ at the

start of study to $2.0 \pm 0.3 \text{ ng}\cdot\text{mL}^{-1}$ at week 8 ($P < 0.05$). Tofogliflozin treatment prevented the decrease in IRI levels and resulted in a significant increase in IRI levels, compared with the untreated group at 8 weeks of treatment. Losartan treatment had no effect on IRI level (Figure 7A). To assess the potential for preventing beta-cell loss by improving hyperglycaemia, the total beta-cell mass was determined from the proportion of the total pancreatic area that stained positively for insulin (insulin-positive ratio). The total beta-cell mass was significantly larger in the untreated *db/db* mice than in the *db/+m* mice at the start of the study (Figure 7B, C). After 8 weeks, the beta-cells of untreated *db/db* mice were degraded and the total beta-cell mass was significantly reduced compared with that of control lean mice, implying beta-cell loss in the *db/db* mice. Tofogliflozin treatment significantly and dose-dependently elevated the total beta-cell mass, suggesting that beta-cell loss was prevented (Figure 7D, E).

Discussion and conclusion

Several SGLT inhibitors are being developed as a new class of anti-diabetic agents. Studies in *db/db*-SGLT2^{-/-} mice (Jurczak *et al.*, 2011) and studies of SGLT inhibitors such as T-1095 (Arakawa *et al.*, 2001) and remogliflozin (Fujimori *et al.*,

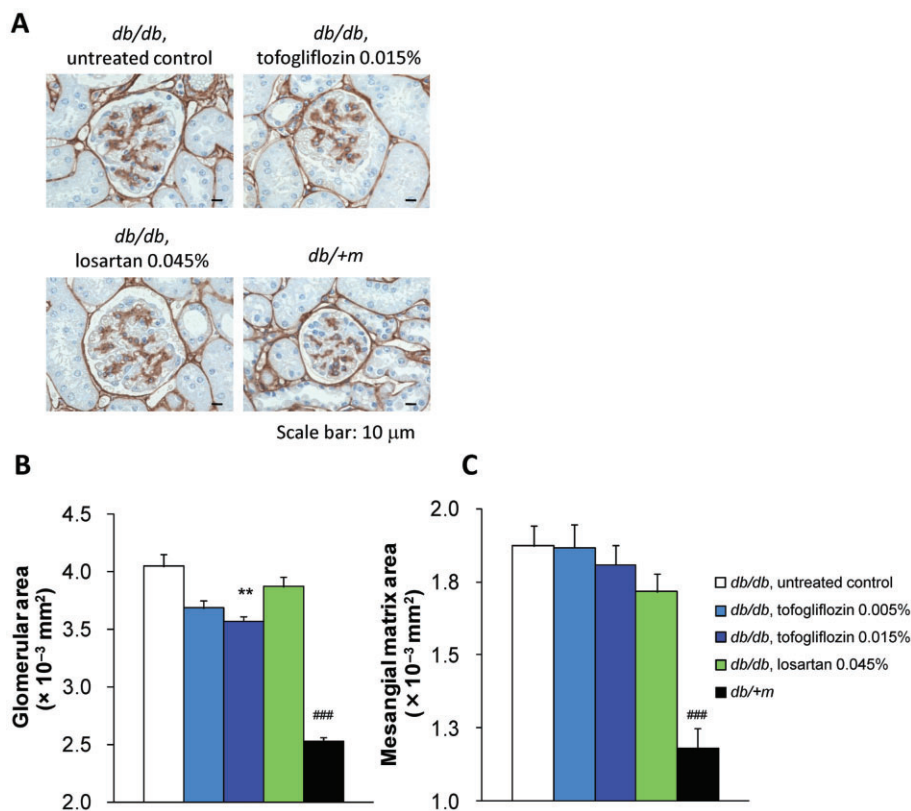


Figure 5

Histological analysis of glomerulus at 8 weeks of treatment (1). (A) Kidney sections were stained with anti-type IV collagen antibody. Representative images are shown (scale bar, 10 μm). (B–C) Kidney sections from each mouse were stained with anti-type IV collagen antibody. Glomerular size and mesangial matrix area were determined by imaging analysis. (B) Treatment with the diet containing tofogliflozin (0.015%) significantly prevented glomerular expansion in *db/db* mice. (C) Mesangial matrix area was significantly lower in *db/+m* mice than in untreated *db/db* mice. Tofogliflozin and losartan treatment had no effect on type IV collagen-positive area. Data shown are means \pm SEM (*db/db* mice, $n = 8$; *db/+m* mice, $n = 10$). ** $P < 0.01$ significantly different from control group by Dunnett's multiple comparison test. ### $P < 0.001$ significantly different from control group by *t*-test.

2008) in *db/db* mice have suggested that long-term inhibition of SGLT might exert a protective effect on kidneys and beta-cells in type 2 diabetes. However, the actual renoprotective effects exerted via SGLT2 inhibition are unclear owing to the lack of positive renoprotective effects in the study on the non-selective SGLT1/2 inhibitor T-1095 (Arakawa *et al.*, 2001) and the lack of parameters on renal function in the study on long-term SGLT2 inhibition (Jurczak *et al.*, 2011).

To evaluate the renoprotective effects of long-term SGLT2 inhibition more precisely, we compared the effects of tofogliflozin (a highly specific SGLT2 inhibitor) with the effects of losartan, (angiotensin II receptor antagonist), on renal and beta-cell functions, together with a quantitative analysis of glomerular and islet beta-cell mass. We demonstrated that long-term SGLT2 inhibition with tofogliflozin prevented not only loss of islet β -cells, but also the progression of renal impairment in *db/db* mice.

In this study, sustained blood glucose-lowering effects and stably reduced glycated Hb levels were observed over 4–8 weeks of treatment with tofogliflozin together with a significant increase in glucose clearance (Figure 1A, B, E), suggesting that stable long-term glycaemic control can be achieved by

tofogliflozin treatment. Based on the measured concentrations of tofogliflozin in plasma in the mice (0.015% tofogliflozin group) and the protein-binding properties of tofogliflozin, we estimated the unbound tofogliflozin concentrations to be between 120 and 350 nM. These concentrations are about 24–70 times the IC_{50} value of tofogliflozin against mouse SGLT2 (5.0 nM) and one-fifteenth to one-fifth of its IC_{50} value against mouse SGLT1 (1800 nM; Suzuki *et al.*, 2012). Therefore, the unbound concentrations of tofogliflozin mentioned earlier are sufficient to inhibit mouse SGLT2 almost completely, but not to inhibit mouse SGLT1.

DN is a major cause of chronic kidney failure and end-stage renal disease in diabetic patients. Renal failure caused by chronic hyperglycaemia via metabolic factors, such as increased oxidative stress, renal polyol formation and advanced glycosylated end-products (Cooper, 2001), is a major micro-vascular complication in type 2 diabetes patients, and intensive glycaemic control to prevent the progression of DN is recommended (The DCCT Research Group, 1993; UKPDS Group, 1998; National Kidney Foundation, 2007; American Diabetes Association, 2013). In addition to metabolic factors, the haemodynamic factors of increased systemic and intra-

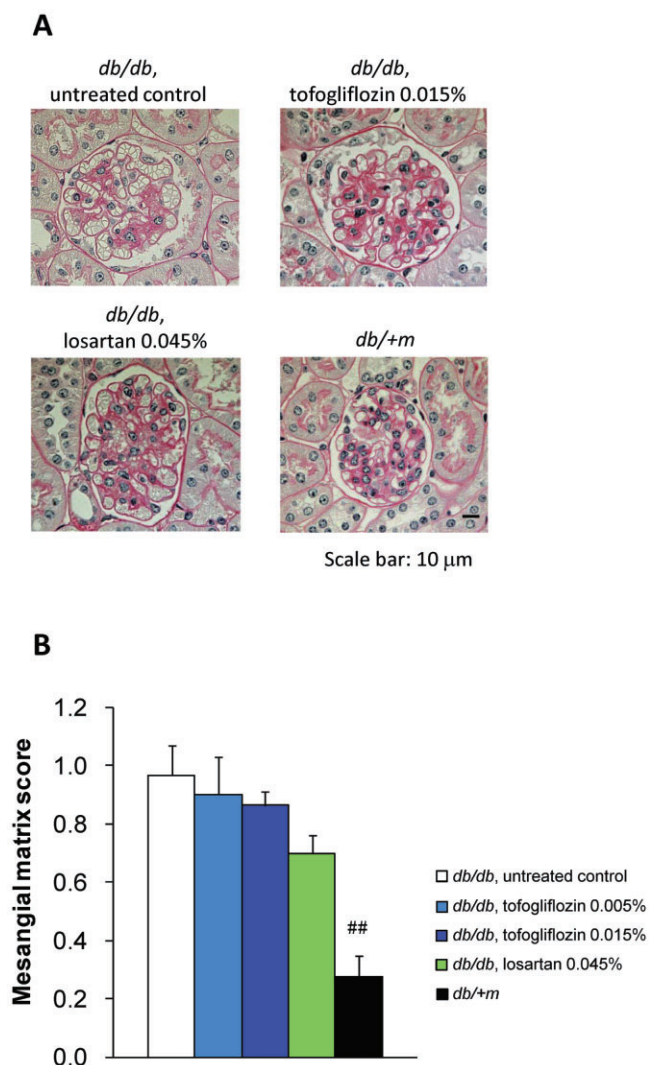


Figure 6

Histological analyses of glomeruli at 8 weeks of treatment (2). (A) Kidney sections were stained with PAS. Representative images are shown (scale bar, 10 μ m). (B) Kidney sections from each mouse were stained with PAS. Mesangial matrix area was determined by scoring. Mesangial matrix area was significantly lower in *db/+m* mice than in untreated *db/db* mice. Tofogliflozin and losartan treatment had no effect on the expansion of mesangial matrix area. Data shown are means \pm SEM (*db/db* mice, $n = 8$; *db/+m* mice, $n = 10$). ## $P < 0.01$ significantly different from control group by *t*-test.

glomerular pressure are also implicated in the pathogenesis of DN (Forbes *et al.*, 2007; Dronavalli *et al.*, 2008). The *db/db* mouse has been used as an animal model of type 2 diabetes that develops progressive kidney disease similar to human DN (Breyer *et al.*, 2005; Soler *et al.*, 2012). In *db/db* mice, glomerular hyperfiltration during the early stage of diabetes has been reported (Gartner, 1978; Sogaru *et al.*, 2006b), which was also suggested by the increased creatinine clearance in *db/db* mice in this study (Figure 4A). Although several discrepancies have been noted between the severity of nephropathy in this mouse strain and that in humans, a number of basic

and novel therapeutic agents have been investigated in *db/db* mice and been found to reduce the progression of renal failure with decreased albuminuria (Tesch and Lim, 2011).

Arakawa *et al.* (2001) reported that albuminuria and mesangial expansion were improved in *db/db* mice treated with another SGLT2 inhibitor, T-1095. However, their study did not compare any renal function parameters (including glomerular filtration rate) after treatment with clinically validated anti-nephropathy drugs, such as angiotensin II receptor antagonists. Moreover, the selectivity of T-1095 towards SGLT2 versus SGLT1 is low (Oku *et al.*, 1999). Therefore, the precise potential of SGLT2 inhibition for the treatment of DN was still unknown. To address this issue, we compared the renoprotective effects of tofogliflozin in *db/db* mice with those of losartan, which has been shown to suppress the increase in urinary albumin excretion until 17 weeks of age (Sogaru *et al.*, 2006a). We found that 8-week treatment with either tofogliflozin or losartan was equally effective in preventing the increase in creatinine-corrected urinary albumin excretion (ACR), an important marker of glomerular dysfunction. Considering that the actual plasma concentration of tofogliflozin in the *db/db* mice was sufficient to inhibit only mSGLT2, we suggest that specific SGLT2 inhibition has the potential to delay the progression of DN. Furthermore, given the concerns with inhibition of SGLT1, such as gastrointestinal side effects, the increased risk of hypoglycaemia and unknown effects with long-term inhibition of this ubiquitous transporter (Washburn and Poucher, 2013), we consider that specific SGLT2 inhibitors have potential as anti-diabetic agents, without safety concerns, for long-term administration.

We confirmed the presence of glomerular hyperfiltration in *db/db* mice, compared with *db/+m* mice, and observed that creatinine clearance tended to be suppressed in both tofogliflozin- and losartan-treated *db/db* mice. Therefore, we suggest that suppression of glomerular hyperfiltration may contribute to suppressing the increase in urinary albumin excretion. Interestingly, imaging analysis of renal glomeruli revealed that tofogliflozin suppressed glomerular hypertrophy, whereas losartan had no effect on glomerular size. These findings indicate that tofogliflozin and losartan may each suppress the deterioration of renal function via different mechanisms.

Long-term treatment with losartan delayed the progression of nephropathy in *db/db* mice (Sogaru *et al.*, 2006a). The renoprotective effects of ACE inhibitors and angiotensin II receptor antagonists are mainly considered to be due to the antihypertensive effects they achieve by inhibiting the renin-angiotensin system (Thomas and Atkins, 2006). The blood pressure of *db/db* mice was reported to be higher than that of *db/+m* mice with higher plasma ACE activity and angiotensin II concentration, and the treatment with losartan reduced the blood pressure of *db/db* mice to the normal range (Senador *et al.*, 2009). In addition, both enalapril, an ACE inhibitor (Moriyama *et al.*, 2004), and valsartan, an angiotensin II receptor antagonist (Dong *et al.*, 2010), exerted their renoprotective effects in *db/db* mice accompanied by lowering of blood pressure. Therefore, we assume that the renoprotective effect of losartan in our study is mainly due to the reduction of intraglomerular pressure. The mild antihypertensive effect that SGLT inhibitors have on systolic blood

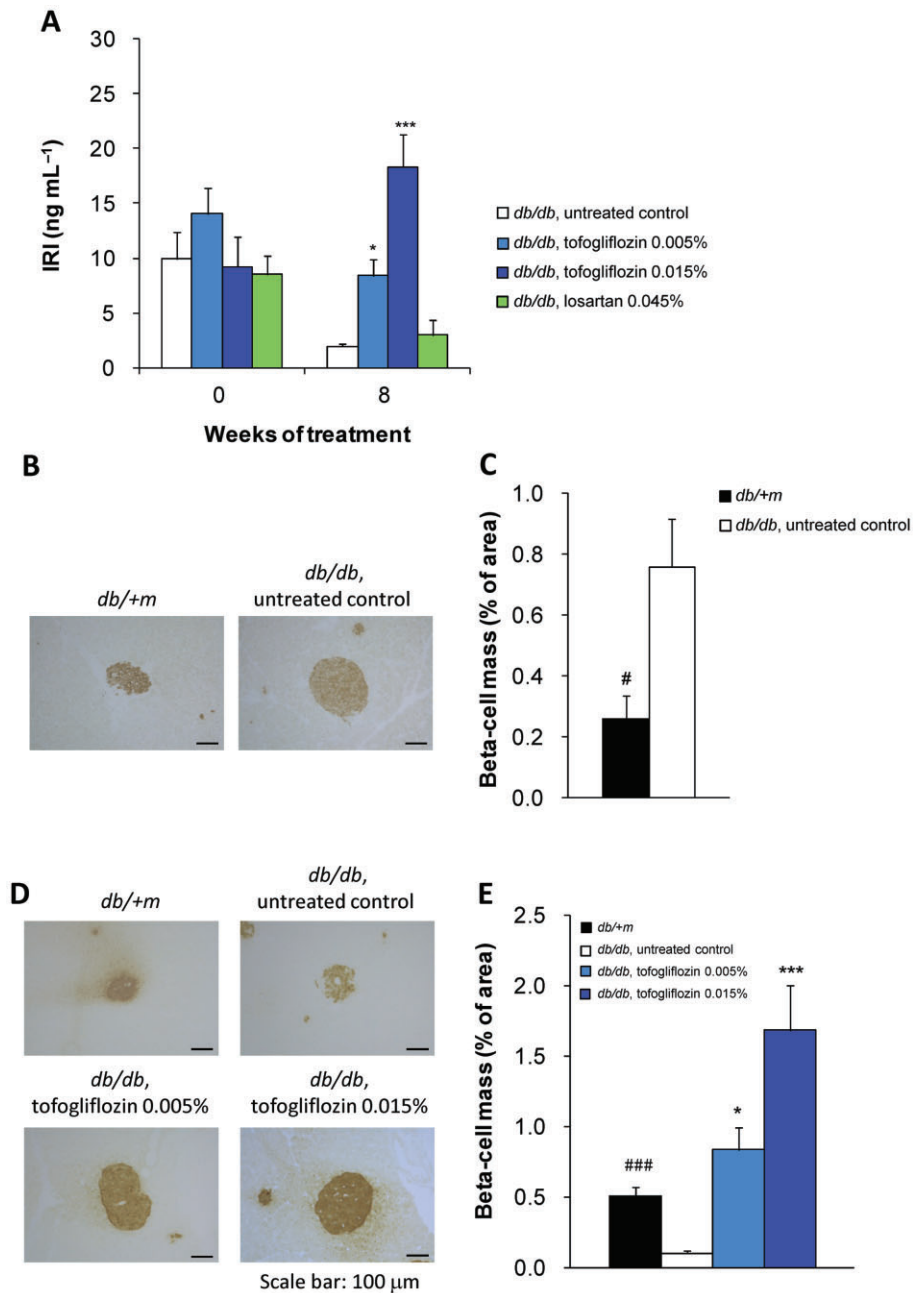


Figure 7

Mean plasma insulin concentration and histological analysis of pancreatic beta-cell mass. (A) Tofogliflozin treatment increased plasma insulin levels (IRI) in *db/db* mice. (B–E) Total beta-cell mass was calculated from the insulin-positive area of pancreatic sections. Total beta-cell mass (% of total pancreatic area) was significantly smaller in *db/+m* mice than in *db/db* mice at the start of the study (B, C). Total beta-cell mass was significantly higher in the tofogliflozin treatment groups than in the control group at the end of the study (D, E). Data shown are means \pm SEM (*db/db* mice, $n = 8$; *db/+m* mice, $n = 10$). * $P < 0.05$, *** $P < 0.001$ significantly different from control group by Dunnett's multiple comparison test. # $P < 0.05$, ### $P < 0.001$ significantly different from control group by *t*-test.

pressure has also been reported following phlorizin treatment in a STZ/high-salt diet model (Osorio *et al.*, 2010) and dapagliflozin treatment in patients with type 2 diabetes (Wilding *et al.*, 2009; Bailey *et al.*, 2010).

Tofogliflozin showed a sustained lowering of blood glucose in *db/db* mice, whereas losartan had no effect on glucose levels (Figure 1A, B). Therefore, reduction of glucose

toxicity by tofogliflozin may contribute to the differences in renoprotective effect between losartan and tofogliflozin mentioned earlier. Recently empagliflozin, an SGLT2 inhibitor, was reported to reduce the inflammatory and fibrotic markers in human kidney proximal tubular cells by blocking glucose entry (Panchapakesan *et al.*, 2013). The amelioration of hyperglycaemic conditions in the proximal tubular cells by

SGLT2 inhibition might explain the difference in the renoprotective effects between losartan and tofogliflozin.

The contribution of the tubuloglomerular feedback (TGF) system in early DN has been well investigated in STZ-induced diabetic models in rodents (Vallon *et al.*, 2003). The enhanced Na⁺ reabsorption coinciding with higher reabsorption of filtered glucose through SGLT1/2 that occurs in the diabetic state, compared with that in the normoglycaemic state, reduces the TGF signal at the macula densa, leading to an increase in single nephron glomerular filtration rate (Vallon and Thomson, 2012). More recently, Vallon *et al.* showed that the knockout of SGLT2 attenuated hyperglycaemia and glomerular hyperfiltration, thus preventing the increase in ACR of STZ diabetic mice, and they suggested that the TGF system contributed to these effects (Vallon *et al.*, 2013). However, in their study, the SGLT2 knockout showed no effect on kidney growth or on the marker of renal injury. In this study, we also observed that the increased ACR was attenuated by tofogliflozin with a long-term improvement of hyperglycaemic condition and tendency towards a decrease in hyperfiltration without reducing kidney weight. Considering the up-regulation of SGLT2 in the kidney of *db/db* mice (Vallon *et al.*, 2013), we assume that the TGF system may contribute to hyperfiltration of *db/db* mice.

Taken together, there are at least two mechanisms by which SGLT inhibition could reduce glomerular hyperfiltration and the increase of ACR. One is the improvement of TGF signalling and the other is the reduction of systolic blood pressure. Further detailed characterization, using, for instance, the micro-puncture technique, is required to address the precise mechanisms involved in the reduction of glomerular hypertrophy with long-term tofogliflozin treatment.

A reduction in body weight by SGLT2 inhibition through a negative energy balance was confirmed in a human clinical study (Zhang *et al.*, 2010) and in diet-induced and genetically obese rodents (Devenny *et al.*, 2012; Liang *et al.*, 2012). Paradoxically, in the present study, the body weight of tofogliflozin-treated *db/db* mice increased gradually throughout the study, with increased food consumption (Figure 2B), whereas the untreated *db/db* mice showed no weight gain after 10 weeks of age with no increase in food consumption. Thus there was a significant increase in body weight in the tofogliflozin groups compared with the untreated group after 4 weeks (Figure 2A). Hyperphagia has been induced in rodents by inhibiting SGLT2, both in diet-induced obese rats treated with an SGLT2 inhibitor (Devenny *et al.*, 2012) and in SGLT2 knock-out mice (Vallon *et al.*, 2013). In addition, similar weight gain and preserved plasma insulin levels were also reported for *db/db* mice treated with other SGLT inhibitors, such as T-1095 (Arakawa *et al.*, 2001) and remogliflozin (Fujimori *et al.*, 2008). Because the plasma IRI levels of the untreated *db/db* mice decreased markedly from 0 to 8 weeks of treatment, the lack of further body weight gain in untreated *db/db* mice may be due to the decreased insulin secretion, which is consistent with the previous reports on the disease progression of *db/db* mice (Gibbs *et al.*, 1995; Lenhard *et al.*, 1999). On the other hand, plasma insulin secretion was significantly preserved in tofogliflozin-treated *db/db* mice (Figure 7A). These results imply that the preserved insulin levels in the tofogliflozin groups would have maintained the

anabolic actions of insulin in *db/db* mice, leading to the increased food consumption and body weight (Figure 2A, B). Although modest but significant body weight reductions by SGLT2 inhibitors, including tofogliflozin, were observed both in type 2 diabetes patients (Kadowaki *et al.*, 2012; Wilding *et al.*, 2012; Fonseca *et al.*, 2013; Schernthaner *et al.*, 2013) and in diet-induced obese rats (Devenny *et al.*, 2012) and *KKAy* mice, a mouse model of type 2 diabetes without beta-cell loss with aging (Katsuno *et al.*, 2009), further evaluations will be needed to understand the interactions among UGE, appetite and insulin secretion of type 2 diabetes patients treated with SGLT2 inhibitors.

In an earlier study, we reported that tofogliflozin had no direct effect on glucose-stimulated insulin secretion by isolated pancreatic islets (Suzuki *et al.*, 2012). Imaging analysis revealed that beta-cell mass was significantly increased in tofogliflozin-treated *db/db* mice (Figure 7D, E), implying that preserved islet mass may contribute to maintaining the plasma insulin secretion. The preserved beta-cell function of *db/db*-SGLT2^{-/-} mice was associated with increased beta-cell mass and reduced incidence of beta-cell apoptosis (Jurczak *et al.*, 2011). The plasma tofogliflozin concentrations measured in the 0.015% tofogliflozin group were sufficient to specifically inhibit mSGLT2. Therefore, it is likely that increased beta-cell mass in tofogliflozin-treated *db/db* mice was caused by mechanisms similar to those in SGLT2^{-/-} *db/db* mice. Because no human clinical studies have directly addressed the effect of SGLT2 inhibitors on beta-cell loss, this beneficial effect remains to be determined in human type 2 diabetes.

Several study limitations should be considered in this study. First, the lack of haemodynamic data from the *db/db* mice means that the mechanisms of the renoprotective effects of losartan and tofogliflozin through their haemodynamic effects discussed earlier are speculative. Second, although the mechanisms underlying the reduction of ACR with tofogliflozin are considered to be closely related to its lowering of blood glucose, exactly how much of the action of tofogliflozin is dependent on the decrease of glucose toxicity and how much independent of glucose, remains to be elucidated.

In conclusion, we have provided evidence for prevention of kidney and pancreatic dysfunctions in a mouse model of type 2 diabetes by long-term SGLT2 inhibition with tofogliflozin. Further studies are required to evaluate the therapeutic usefulness of tofogliflozin for preservation of renal function and beta-cells in patients with type 2 diabetes.

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Conflict of interest

All authors are employees of Chugai Pharmaceutical Co., Ltd. or its affiliate company Chugai Research Institute for Medical Science, Inc.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Scatter plot of plasma glucose and UGE levels in *db/db* and *db/+m* mice. (A) 0 weeks (baseline value), (B) 4 weeks of treatment, (C) 8 weeks of treatment symbols represent individual values.

Figure S2. Scatter plot of body weight and food consumption in *db/db* mice. (A) 0 weeks (baseline value), (B) 4 weeks of treatment, (C) 8 weeks of treatment symbols represent individual values.