Tofogliflozin Improves Insulin Resistance in Skeletal Muscle and Accelerates Lipolysis in Adipose Tissue in Male Mice

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Sodium glucose cotransporter 2 inhibitors have attracted attention as they exert antidiabetic and antiobesity effects. In this study, we investigated the effects of tofogliflozin on glucose homeostasis and its metabolic consequences and clarified the underlying molecular mechanisms. C57BL/6 mice were fed normal chow containing tofogliflozin (0.005%) for 20 weeks or a high-fat diet containing tofogliflozin (0.005%) for 8 weeks ad libitum. In addition, the animals were pair-fed in relation to controls to exclude the influence of increased food intake. Tofogliflozin reduced the body weight gain, mainly because of fat mass reduction associated with a diminished adipocyte size. Glucose tolerance and insulin sensitivity were ameliorated. The serum levels of nonesterified fatty acid and ketone bodies were increased and the respiratory quotient was decreased in the tofogliflozin-treated mice, suggesting the acceleration of lipolysis in the white adipose tissue and hepatic β-oxidation. In fact, the phosphorylation of hormone-sensitive lipase and the adipose triglyceride lipase protein levels in the white adipose tissue as well as the gene expressions related to β-oxidation, such as *Cpt1*α in the liver, were significantly increased. The hepatic triglyceride contents and the expression levels of lipogenic genes were decreased. Pair-fed mice exhibited almost the same results as mice fed an high-fat diet ad libitum. Moreover, a hyperinsulinemic-euglycemic clamp revealed that tofogliflozin improved insulin resistance by increasing glucose uptake, especially in the skeletal muscle, in pair-fed mice. Taken together, these results suggest tofogliflozin ameliorates insulin resistance and obesity by increasing glucose uptake in skeletal muscle and lipolysis in adipose tissue. **(*Endocrinology* 157: 1029–1042, 2016)**

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Abbreviations: ATGL, adipose triglyceride lipase; BMD, bone mineral density; CMC, carboxymethylcellulose; CT, computed tomography; GIR, glucose infusion rate; HFD, high-fat diet; HGP, hepatic glucose production; HOMA-R, homeostasis assessment of insulin resistance; HSL, hormone-sensitive lipase; NC, normal chow; NEFA, nonesterified fatty acid; OGTT, oral glucose tolerance test; Rd, rate of glucose disappearance; RQ, respiratory quotient; SGLT2, sodium glucose cotransporter 2; TG, triglyceride; UGE, urinary glucose excretion; WAT, white adipose tissue.

Type 2 diabetes is characterized by chronic hyperglycemia caused by a combination of insulin resistance and insufficient insulin secretion (1). The prevalence of type 2 diabetes has been increasing dramatically worldwide in recent years, and the development of strategies to minimize complications, such as micro- and macrovascular events, is now an urgent issue (2).

Recently, Sodium glucose cotransporter 2 (SGLT2) inhibitors have attracted attention as they exert both antidiabetic and antiobesity effects in an insulin-independent manner. SGLT2 is a glucose transporter that was cloned in 1994 by Kanai et al (3) and is known to play an important role in the renal reabsorption of glucose, which is dependent on the sodium concentration gradient. SGLT2 is mainly present in the apical aspect of the S1 segment of the proximal renal tubules and is responsible for approximately 90% of the total renal glucose reabsorption (4). Intriguingly, SGLT2 expression in the kidney was reportedly increased in both a diabetic animal model and diabetic patients, suggesting that renal glucose reabsorption is paradoxically increased in the diabetic state (5, 6). Thus, SGLT2 inhibition has been suggested to be a valid method of stopping this vicious cycle.

Rodents and human studies have attested to the efficacy of SGLT2 inhibition as a valid strategy for the treatment of type 2 diabetes (7–19). Although many reports have attested to the efficacy of SGLT2 inhibitors in improving glucose intolerance and ameliorating obesity, few studies have provided insight into the mechanisms underlying the antidiabetic and antiobesity effects of this class of drugs.

In this study, we administered tofogliflozin, a novel SGLT2 inhibitor, to a lean model and to a diet-induced obesity model of C57BL/6 mice to investigate the effects of this drug on glucose homeostasis and its metabolic consequences and to clarify the underlying molecular mechanisms.

Materials and Methods

Animals

C57BL/6 male mice were purchased from Japan CLEA. Normal chow (NC) (CE-2: 8.9% moisture, 24.9% crude protein, 4.6% crude fat, 4.1% crude fiber, 6.6% crude ash, 51% nitrogen free extract; 344.9 kcal/100 g; metabolizable amount of sugars/carbohydrates, 204 kcal/100 g) and a high-fat diet (HFD) (HF-32: 6.2% moisture, 25.5% crude protein, 32% crude fat, 2.9% crude fiber, 4.0% crude ash, 29.4% nitrogen free extract; 507.6 kcal/100 g; metabolizable amount of sugars/carbohydrates, 117.6 kcal/100 g) were also purchased from Japan CLEA (20). The mice were housed under a 12-hour light, 12-hour dark cycle. The animal care and experimental procedures were approved by the Animal Care Committee of the University of Tokyo.

Tofogliflozin treatment study

Tofogliflozin was kindly provided by Chugai Pharmaceutical Co, Ltd (21, 22). Nine-week-old C57BL/6 male mice were fed NC containing 0.005% [wt/wt%] tofogliflozin for 20 weeks ad libitum (Supplemental Table 1). Another group of 9-week-old C57BL/6 mice was fed an HFD containing tofogliflozin (0.005%) for 8 weeks ad libitum (Supplemental Table 2). In the pair-feeding experiment, 9-week-old mice were fed 2.7 g/d of HFD containing tofogliflozin (0.005%) for 8 weeks to exclude the influence of increased food intake (Supplemental Table 3 and Supplemental Materials and Methods). Ethanol was used as a solvent for the tofogliflozin. The ethanol volume corresponded to 2% of the total components (200 mL/10 kg), and the ethanol was assumed not to have had any influence on the experiment, as it would have evaporated during the creation of the pellets. During the study, the daily food intake, water intake, and body weight were monitored. The serum drug concentrations were measured at Chugai Pharmaceutical Co, Ltd. A detailed outline of the experiment is provided in Supplemental Figure 1.

Oral glucose tolerance test (OGTT)

For the OGTT performed after a single administration of tofogliflozin, animals were denied access to food for 16 hours and carboxymethylcellulose (CMC), CMC containing 1 mg/kg of tofogliflozin or CMC containing 10 mg/kg of tofogliflozin were administered 15 minutes before glucose administration. For OGTTs conducted after tofogliflozin treatment, the animals were denied access to food for 24 hours (NC) or for 48 hours (HFD) to allow for drug washout. Glucose (1.5 g/kg) was administered by oral gavage, and the blood glucose level was monitored at 0, 15, 30, 60, and 120 minutes after glucose administration. Blood glucose was measured using an automatic glucometer (Sanwa Kagaku Kenkyusho Co, Ltd). Serum insulin was determined using a mouse insulin ELISA kit (Morinaga Institute of Biological Science, Inc) (23).

Sixteen hours of fasting after tofogliflozin administration

Animals were denied access to food for 3 hours, and CMC or CMC containing 10 mg/kg of tofogliflozin was administered by oral gavage. The blood glucose levels were monitored at 1, 2, 4, 8, 12, and 16 hours after CMC or tofogliflozin administration. All the animals were denied access to food during the experiment.

Hyperinsulinemic-euglycemic clamp

An infusion catheter was inserted into the right jugular vein of the mice, as previously reported (23) with some modifications, to take urinary glucose excretion (UGE) into account. All the mice were subjected to a 5-hour fast and allowed to urinate under conscious and unstressed conditions before the clamp. All the urine was collected during the clamp to measure the urinary glucose concentration. Insulin (Humulin R; Lilly) was administered at a rate of 7.5 mU/kg·min, and the blood glucose concentration was maintained at approximately 80 mg/dL, which is lower than that in the regular protocol to minimize UGE, by the administration of glucose (5 g of glucose/10 mL, enriched to about 20% with [6,6–2H2]glucose [Sigma]) for 90 minutes. After the blood glucose concentration stabilized, blood samples were collected 3 times every 15 minutes. The glucose infusion rate (GIR) was calculated by averaging the rates of glucose in-

fusion for these 3 time points. Then, the glucose concentration of the urine collected during the clamp was measured (Wako Pure Chemical Industries), and the UGE (mg/kg·min) was calculated for each mouse. We determined the modified GIR as the GIR-UGE. Thereafter, the rate of glucose disappearance (Rd) was calculated according to nonsteady-state equations, and the hepatic glucose production (HGP) was calculated as the difference between Rd values and the modified GIR.

Statistics

The results were expressed as the mean ± SEM. Differences between groups were examined for statistical significance using the Student's *t* test and an ANOVA with the Fisher protected least significant difference test. $P < .05$ was considered to denote statistical significance. Further details of the materials and methods used in this study are provided in the Supplemental Materials and Methods.

Results

Body weight gain and the weight of white adipose tissue (WAT) were both reduced after 20 weeks of tofogliflozin treatment in mice fed NC

In an OGTT conducted 15 minutes after the oral administration of tofogliflozin, the blood glucose levels were significantly decreased in the groups treated with 1 or 10 mg/kg of tofogliflozin, compared with those in the controls, without any increase in the serum insulin levels (Figure 1, A and B). A dramatic increase in UGE that occurred in a dose-dependent manner was also observed in the tofogliflozin-treated mice (Figure 1, C and D).

To confirm the long-term effects of tofogliflozin on a lean model, C57BL/6 mice were fed NC containing 0.005% tofogliflozin for 20 weeks. Tofogliflozin significantly reduced the body weight gain (Figure 1E), whereas there was no significant change in the lean body mass (Figure 1F). Instead, food intake (Figure 1G) and water intake (Figure 1H) were increased in the tofogliflozin-treated mice, which agreed with the increases in the urine volume and UGE (Figure 1, I and J). No significant difference in the rectal temperature was observed between the tofogliflozin-treated mice and the mice that were not treated with tofogliflozin (data not shown). An analysis conducted after 20 weeks of treatment revealed a significant reduction in the weights of the epididymal WAT (Figure 1K). Although the liver weight and liver glycogen content were unchanged, the liver triglyceride (TG) content was significantly decreased in the tofogliflozin-treated mice (Figure 1, L–N). In addition, the average adipocyte size was also reduced in these mice (Figure 1O). These data suggest that tofogliflozin reduces body weight gain mainly by reducing the WAT mass in the lean model.

Glucose tolerance and insulin sensitivity improved after 20 weeks of tofogliflozin treatment in mice fed NC, accompanied by increased β-oxidation and decreased lipogenesis in the liver

To evaluate the effect of tofogliflozin on glucose tolerance and insulin sensitivity, the OGTT was conducted after the animals were denied access to food for 24 hours, which was a sufficiently long period to allow for drug washout (Supplemental Figure 2). Slight, but significant, decreases in the blood glucose levels were observed during the OGTT in the tofogliflozin-treated mice (Figure 2, A and B). The homeostasis assessment of insulin resistance (HOMA-R) was also significantly reduced in the tofogliflozin-treated mice, compared with the nontreated mice (Figure 2D). In the random-fed state, the blood glucose (Figure 2E) and the serum insulin levels (Figure 2F) were significantly reduced in the tofogliflozin-treated mice. In contrast, the *Pepck* mRNA expression level in the liver was significantly increased in these mice, suggesting increased gluconeogenesis (Figure 2H). The nonesterified fatty acid (NEFA) and ketone body levels were significantly increased in the tofogliflozin-treated mice, compared with the control mice (Figure 2, I and J). Respiratory quotient (RQ) was significantly decreased during the dark cycle in the tofogliflozin-treated mice (Figure 2K). These results suggest the increased efflux of free fatty acids from the adipose tissue, with these free fatty acids becoming substrates for β-oxidation in the liver. In fact, the *Cpt1α* mRNA expression level was significantly increased in the tofogliflozin-treated mice (Figure 2L). Although the expressions of genes related to lipogenesis tended to be lower, only the hepatic mRNA expression level of *Dgat2* was reduced significantly (Figure 2, M–Q).

Body weight gain and WAT and liver weights were reduced after 8 weeks of tofogliflozin treatment in mice fed an HFD

To confirm the effects of tofogliflozin in an obese, insulin-resistant model, C57BL/6 mice were fed an HFD containing 0.005% tofogliflozin for 8 weeks, beginning from 9 weeks of age. The body weight gain was reduced despite an observed increase in food intake in the tofogliflozin-treated group (Figure 3, A–C). Unlike the case of mice fed NC, a slight, but significant, decrease in the lean body mass was observed in the tofogliflozin-treated group (Figure 3B). Water intake was increased in the tofogliflozin-treated mice (Figure 3D), which agreed with the increases in the urine volume and UGE (Figure 3, E and F). No significant difference in the rectal temperature was observed between the tofogliflozin-treated mice and mice not treated with tofogliflozin (data not shown). The weights of the epididymal WAT were significantly reduced after 8 weeks of tofogliflozin treatment in these mice (Fig-

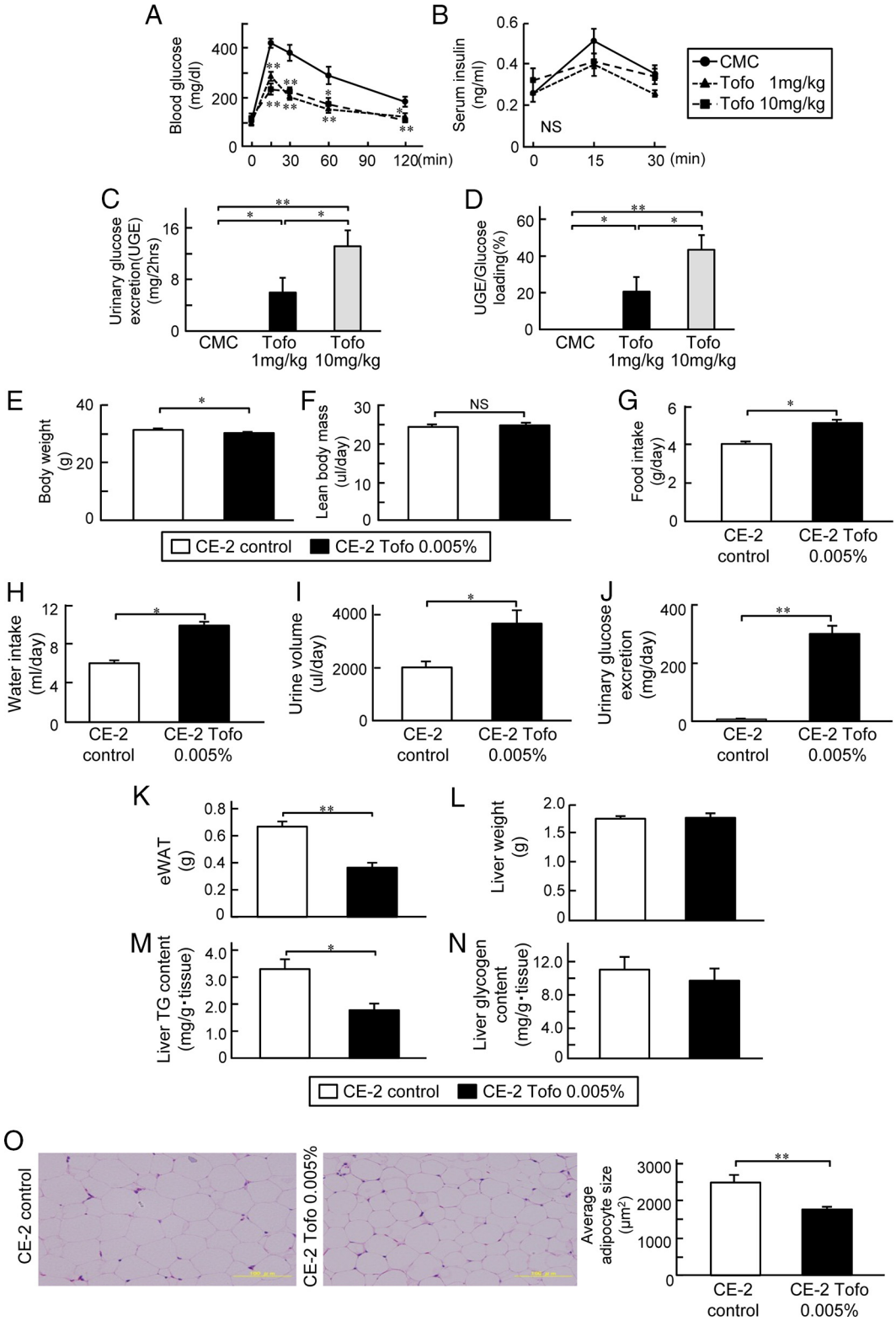

Figure 1. Body weight and WAT mass after tofogliflozin treatment in mice fed NC. A and B, Blood glucose and serum insulin during an OGTT in mice treated with CMC (filled circle), 1 mg/kg of tofogliflozin (filled triangle), or 10 mg/kg of tofogliflozin (filled square) (n = 7). C and D, UGE and UGE/glucose loading during an OGTT in mice treated with CMC (open bars), 1 mg/kg of tofogliflozin (filled bars), or 10 mg/kg of tofogliflozin (gray bars) (n = 5). E and F, Body weight and lean body mass of C57BL/6 mice not treated (open bars) or treated with 0.005% tofogliflozin (filled bars) (n = 14–20). G and H, Food and water intake (n = 7). I and J, Urinary volume and UGE during 24 hours (n = 6). K–N, Epididymal WAT mass, liver weight, liver TG content, and liver glycogen content (n = 8). O, H&E staining and average size of adipocyte from eWAT (n = 9). Values are the mean ± SEM of data obtained from each group. *, $P < .05$; **, $P < .01$.

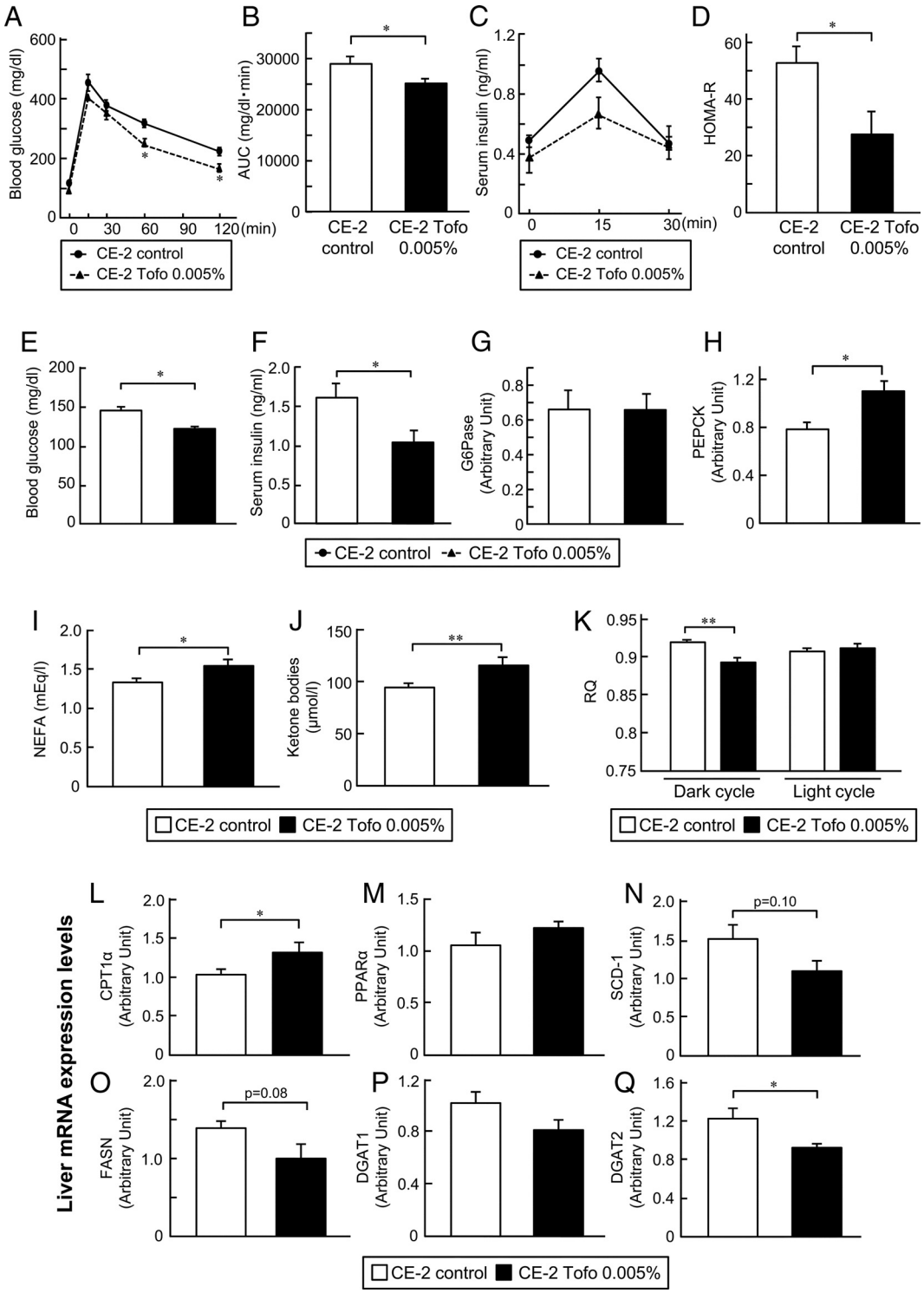

Figure 2. Glucose tolerance and insulin sensitivity after tofogliflozin treatment in mice fed NC. A–C, Blood glucose levels, AUC, and serum insulin levels during an OGTT in mice not treated (solid line, open bar) or treated with 0.005% tofogliflozin (dotted line, filled bar) (n = 8). D, HOMA-R in mice not treated (open bars) or treated with 0.005% tofogliflozin (filled bars) (n = 8). E and F, Blood glucose and serum insulin levels in mice with access to food and water ad libitum (n = 7–8). G and H, *G6Pase* and *Pepck* mRNA expression levels in the liver (n = 6–7). I and J, Serum NEFA and ketone body levels (n = 7). K, RQ during the dark and light cycle (n = 6). L–Q, Liver mRNA expression levels (n = 8). Values are the mean ± SEM of data obtained from the analysis of each group. *, $P < .05$; **, $P < .01$.

ure 3G). Moreover, in addition to the decrease in the hepatic TG content, as seen in the mice fed NC, the liver weight and hepatic glycogen content were also significantly reduced in the tofogliflozin-treated mice (Figure 3, H–J), unlike the observations in the lean model (Figure 1, L and N). In the histological analysis, the adipocyte size

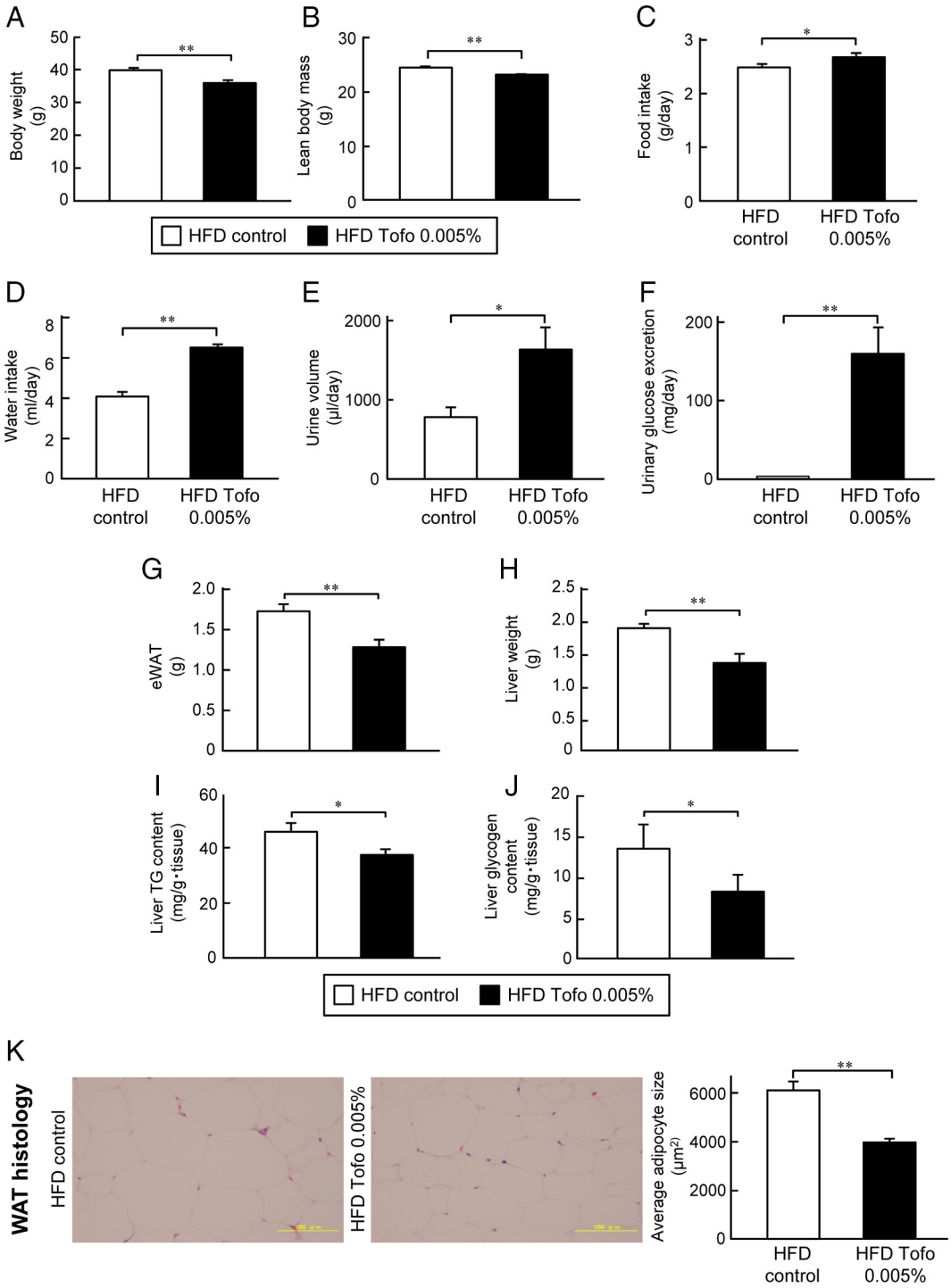

Figure 3. Body weight and the WAT mass after tofogliflozin treatment in mice fed an HFD. A and B, Body weight and lean body mass of C57BL/6 mice not treated (open bars) or treated with 0.005% tofogliflozin (filled bars) (n = 6–8). C and D, Food and water intake (n = 7). E and F, Urinary volume and UGE during 24 hours (n = 5). G–J, Epididymal WAT mass, liver weight, liver TG content, and liver glycogen content (n = 8). K, H&E staining and average size of adipocyte from eWAT (n = 5). Values are the mean ± SEM of data obtained from each group. *, $P < .05$; **, $P < .01$.

was significantly reduced in the tofogliflozin-treated mice (Figure 3K). As observed in mice fed NC, glucose intolerance and insulin resistance improved after 8 weeks of tofogliflozin treatment in mice fed an HFD, accompanied by an increase in β-oxidation and the suppression of lipogenesis in the liver (Supplemental Figure 3).

Body weight gain was suppressed and the WAT and liver weights were reduced after pair-feeding for 8 weeks in mice fed an HFD

To elucidate the influence of increased food intake, pair-feeding experiments were conducted. C57BL/6 mice were fed 2.7 g of the HFD containing 0.005% tofogliflozin

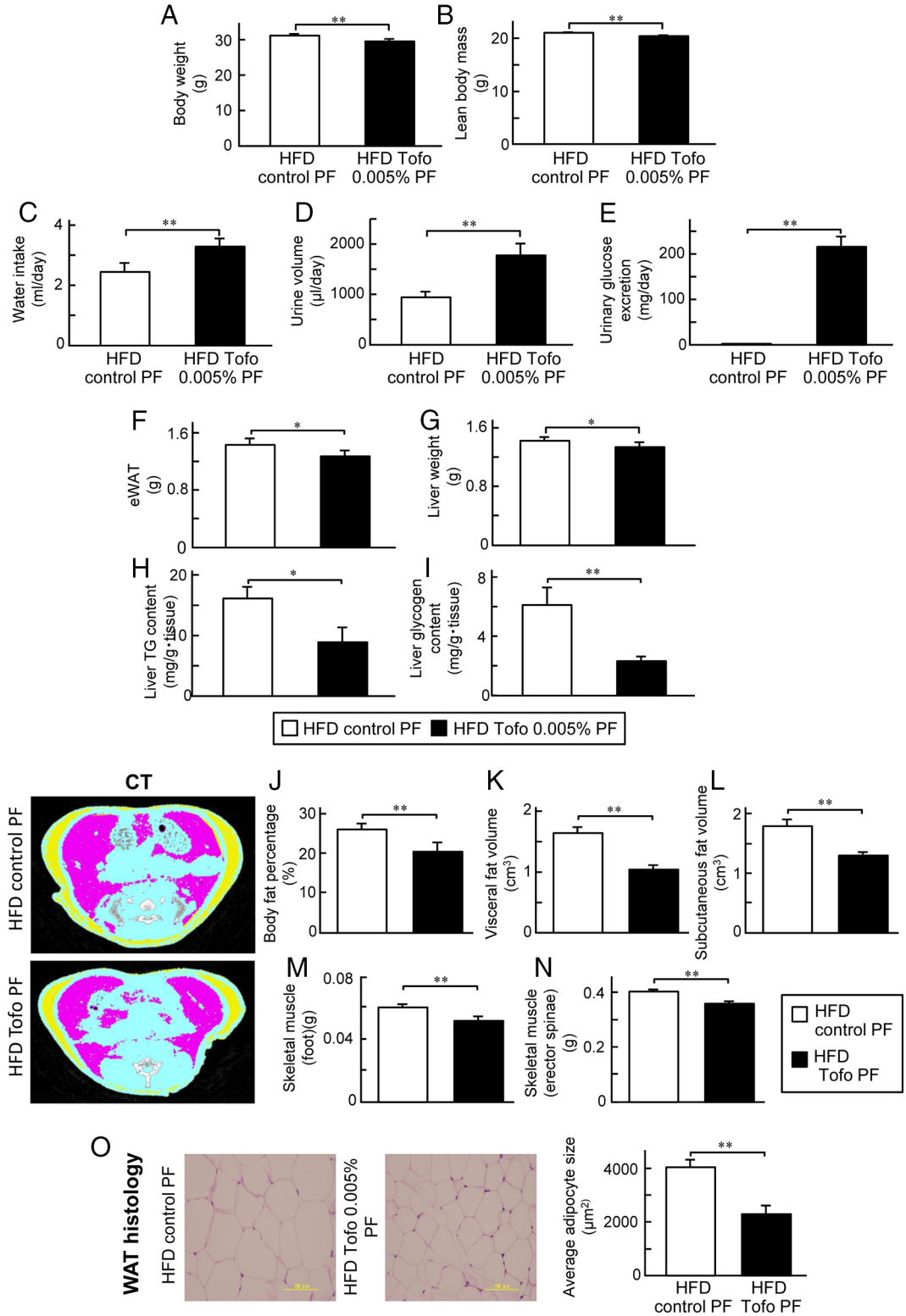

Figure 4. Body weight and WAT mass after pair feeding in mice fed an HFD. A and B, Body weight and lean body mass of C57BL/6 mice, pair-fed for 8 weeks, not treated (open bars) or treated with 0.005% tofogliflozin (filled bars) (n = 8–9). C, Water intake (n = 8). D and E, Urinary volume and UGE during 24 hours (n = 8). F–I, Epididymal WAT mass, liver weight, and liver TG and glycogen contents (n = 8). J–N, Fat and skeletal muscle mass measured using CT (n = 12–13). O, H&E staining and average adipocyte size from eWAT (n = 5). Values are the mean ± SEM of data obtained from each group. *, $P < .05$; **, $P < .01$

for 8 weeks, beginning from 9 weeks of age. Body weight gain was reduced in the tofogliflozin-treated mice, with a slight reduction in the lean body mass (Figure 4, A and B). Water intake was increased (Figure 4C), which was associated with an increased urine volume and UGE (Figure 4, D and E). No significant difference in the rectal temperature was observed between the tofogliflozin-treated mice and mice not treated with tofogliflozin (data not shown). The weights of the epididymal WAT were significantly reduced (Figure 4F). The liver weight was also significantly reduced, accompanied by a decrease in the liver TG and glycogen contents (Figure 4, G–I). In addition, tofogliflozin reduced the serum TG and tended to decrease the blood pressure in the pair-feeding experiment (Supplemental Figures 4 and 5). To elucidate the changes in body composition in further detail, we conducted computed tomography (CT) to measure the fat, skeletal muscle, and bone volumes in mice that were pair-fed for 6 weeks. The body fat percentage and the visceral fat and sc fat volumes were significantly decreased in the tofogliflozin-treated mice (Figure 4, J–L); these findings were consistent with the results of a dissection analysis (Figure 4F). Although no significant difference in the bone volume was observed between the tofogliflozin-treated mice and the mice that were not treated with tofogliflozin (Supplemental Figure 6), the skeletal muscle volume (in both the foot and erector spinae) was significantly decreased in the tofogliflozin-treated mice (Figure 4, M and N). Furthermore, the average adipocyte size was also significantly reduced in the treated mice (Figure 4O), consistent with the results of the experiments conducted in animals given ad libitum access to food (Figure 3K).

Glucose intolerance and insulin resistance were ameliorated after 8 weeks of pair feeding in mice fed an HFD, accompanied by an increase in β-oxidation and a reduction in lipogenesis in the liver

During the OGTT, a slight, but significant, decrease in the blood glucose levels and a significant decrease in the serum insulin levels were observed in the tofogliflozin-treated mice (Figure 5, A and B, and Supplemental Figure 7). The HOMA-R was significantly improved in the tofogliflozin-treated mice, compared with the control mice (Figure 5C). The blood glucose and serum insulin levels in the random-fed state were significantly decreased (Figure 5, D and E). In addition, the hyperinsulinemic-euglycemic clamp revealed a significantly increased GIR, HGP, and Rd in tofogliflozin-treated mice, indicating that tofogliflozin treatment improved insulin resistance by increasing glucose uptake, especially in the skeletal muscle, in pair-fed mice (Figure 5, F–H).

The elevation of the *Pepck* mRNA expression level in the tofogliflozin-treated mice was consistent with the increased HGP in the euglycemic clamp (Figure 5J). Other findings for the tofogliflozin-treated mice were as follows: the serum NEFA and ketone body levels were significantly increased and the RQ was decreased (Figure 5, K–M); the *Cpt-1α* mRNA expression level was significantly increased and the *Pparα* expression level tended to be increased in the liver, suggesting enhanced hepatic β-oxidation (Figure 5, N and O); and the expression levels of lipogenic genes, such as *Scd-1*, *Fasn*, *Dgat1*, and *Dgat2*, were significantly reduced in the liver (Figure 5, P–S). These results were consistent with the reduced liver TG content (Figure 4H). In epididymal WAT, the expression levels of *Mcp-1* and *Il-6* were significantly reduced, and the *Tnf-α* expression level tended to be lower in the tofogliflozin-treated mice (Figure 5, T–V).

Phosphorylation of hormone-sensitive lipase (HSL) and the adipose triglyceride lipase (ATGL) protein levels were increased, along with the decreased phosphorylation of Akt in the WAT, after tofogliflozin treatment

To further investigate the molecular mechanism underlying the enhancement of β-oxidation in the liver and of lipolysis in the WAT, we next examined the effect of the single oral administration of tofogliflozin. Blood glucose in the tofogliflozin-administered group decreased rapidly and was maintained at around 60 mg/dL during the 16-hour period of no access to food after tofogliflozin administration (Figure 6A). The serum insulin levels were significantly lower after tofogliflozin administration (Figure 6B), whereas the serum glucagon levels were 18.2 ± 7.2 pmol/L in the control group and 16.7 ± 3.0 pmol/L in the tofogliflozin-administered group, respectively (n = 10–11, no significant difference). The serum NEFA level was significantly increased, whereas no significant change in the serum glycerol level was observed. These results might be explained by the increased *Aqp9* mRNA expression level in the liver and the increased uptake of glycerol by the liver (Figure 6, C, D, and O). The serum levels of ketone bodies were significantly increased after tofogliflozin administration (Figure 6E). Insulin is known to suppress lipolysis by inactivating HSL through Akt activation. At 8 hours after tofogliflozin administration, the p-Akt level in the WAT was indeed significantly suppressed after tofogliflozin administration (Figure 6F). The amounts of phosphorylated HSL p-660 and p-563 were significantly increased, as was the ATGL protein expression level, at 16 hours after tofogliflozin administration (Figure 6G), providing strong evidence that the reduction in the fat mass was induced by the acceleration of lipolysis.

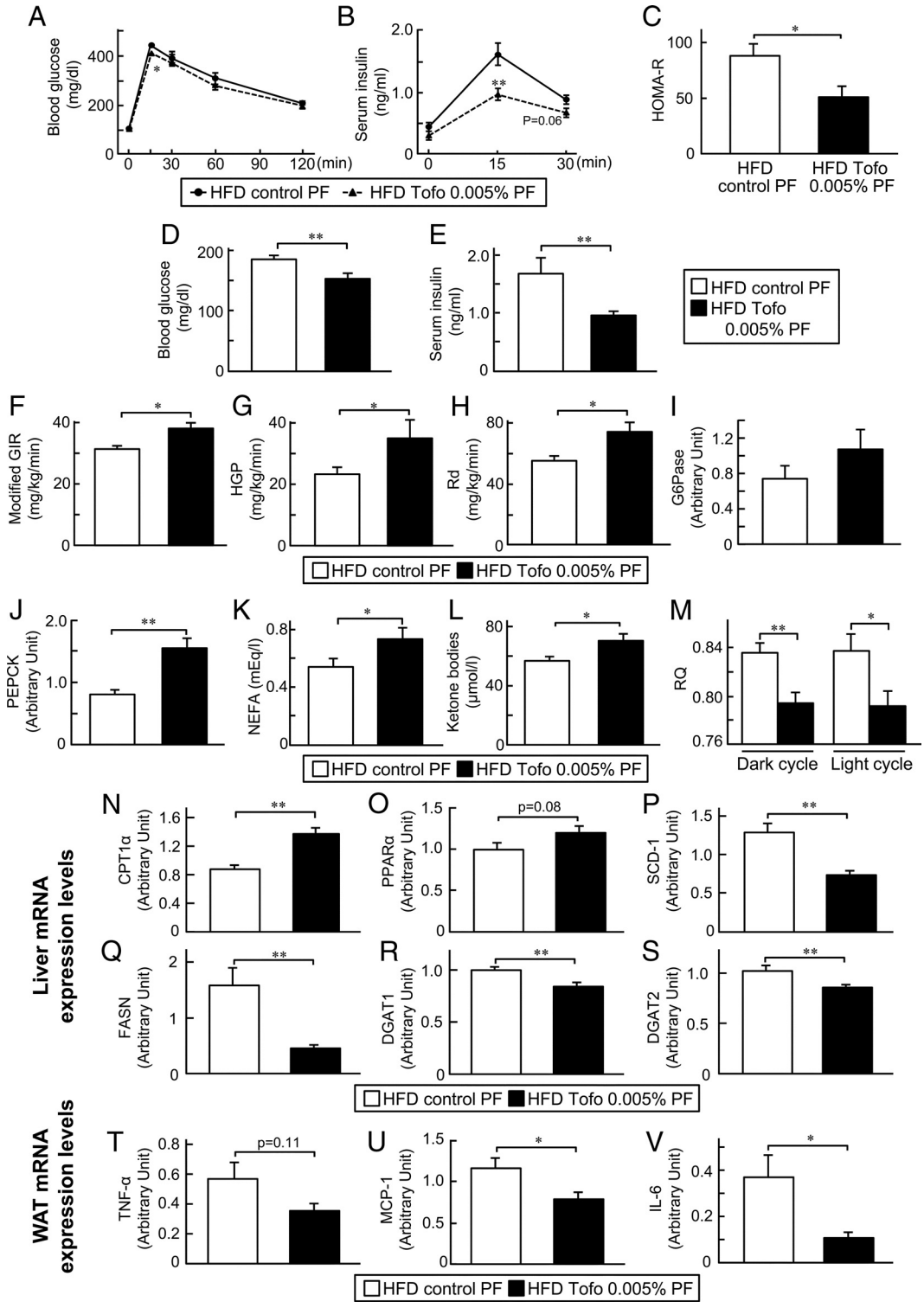

Figure 5. Glucose intolerance and insulin resistance after pair feeding in mice fed an HFD. A and B, Blood glucose levels and serum insulin levels during an OGTT in mice, pair-fed for 8 weeks, not treated (solid line) or treated with 0.005% tofogliflozin (dotted line) (n = 8–9). C, HOMA-R in mice not treated (open bars) or treated with 0.005% tofogliflozin (filled bars) (n = 8–9). D and E, Blood glucose and serum insulin levels (n = 8–9). F–H, Euglycemic clamp in mice not treated (open bars) or treated with 0.005% tofogliflozin (filled bars) (n = 9–12). I and J, *G6Pase* and *Pepck* mRNA expression levels in the liver (n = 7–8). K and L, Serum NEFA and ketone body levels (n = 8–9). M, RQ during the dark and light cycle (n = 6). N–S, mRNA expression levels in the liver (n = 8–9). T–V, mRNA expression levels in WAT in each group (n = 8–9). Values are the mean ± SEM of data obtained from the analysis of each group. *, $P < .05$; **, $P < .01$.

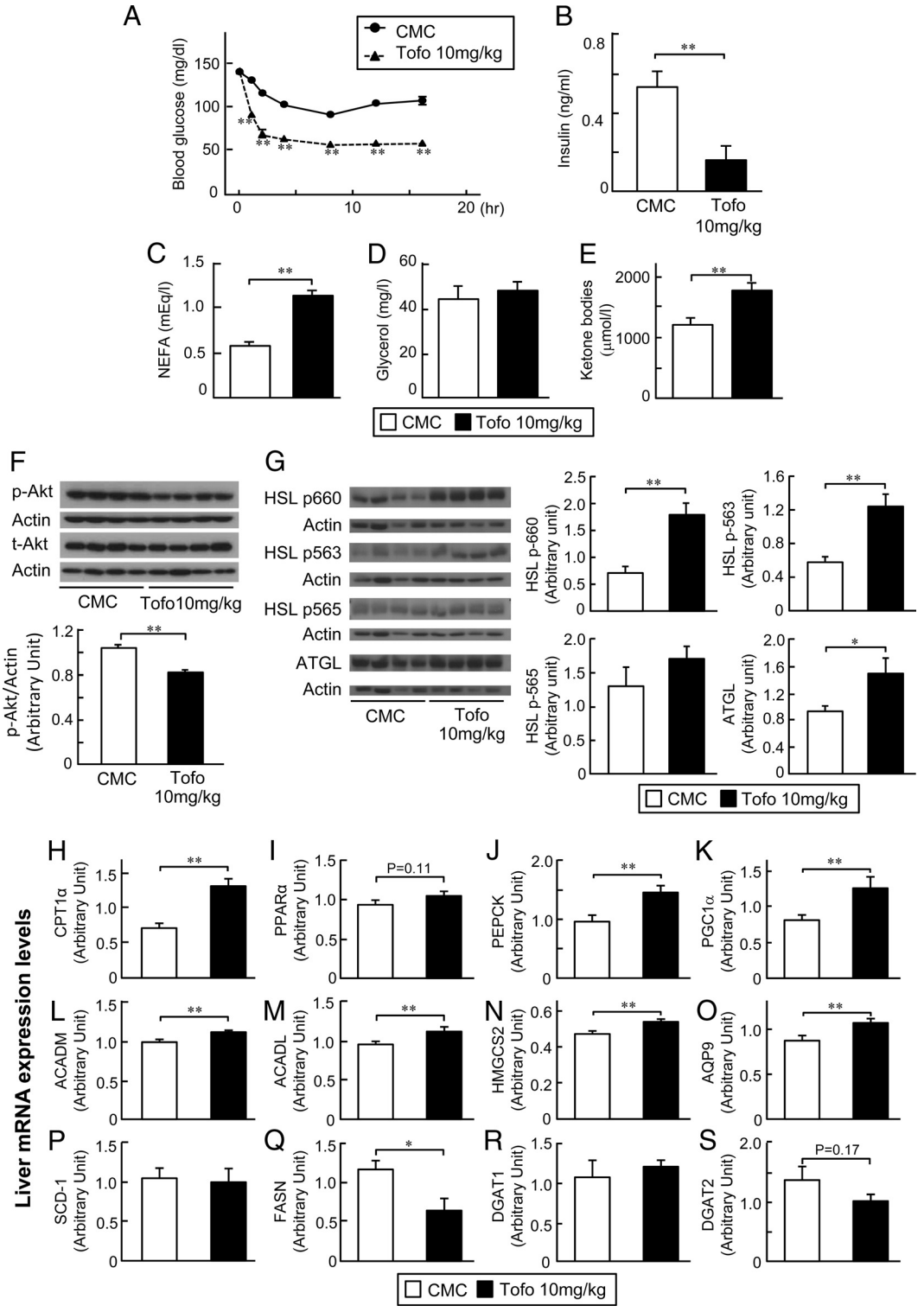

Figure 6. Tofogliflozin treatment increased the phosphorylation of HSL and the ATGL protein levels and decreased the phosphorylation of Akt in WAT. A and B, Blood glucose levels after a single administration of CMC (solid line) and tofogliflozin 10 mg/kg (dotted line), and serum insulin levels after the administration of CMC (open bar) and tofogliflozin 10 mg/kg (filled bar) (n = 8). C–E, Serum NEFA, glycerol, and ketone body levels at 16 hours after tofogliflozin administration (n = 8). F, Phosphorylated Akt and total Akt protein levels in the eWAT at 8 hours after tofogliflozin administration (n = 4). G, Phosphorylated HSL and ATGL protein expression levels in the eWAT at 16 hours after tofogliflozin administration (n = 4). H–S, mRNA expression levels in the liver (n = 8). Values are the mean ± SEM of data obtained from the analysis of mice treated with CMC as a control and 1 or 10 mg/kg of tofogliflozin. *, $P < .05$; **, $P < .01$.

Furthermore, these results suggest that low serum insulin levels reduced the phosphorylation of Akt, triggering the activation of HSL and lipolysis in the WAT (see antibody table, Table 1).

In the liver, mRNA expression levels related to β-oxidation, such as *Cpt-1α*, *Acadm*, and *Acadl*, were significantly increased in the tofogliflozin-administered group (Figure 6, H, L, and M), consistent with the increased ketone body levels. In fact, the mRNA expression level of *Hmgcs2*, which catalyzes the first reaction of ketogenesis, was significantly increased (Figure 6N). In contrast, the expression of *Fasn* was significantly decreased, and the expression of *Dgat2* tended to be reduced in the tofogliflozin-administered group (Figure 6, Q and S). The expressions of gluconeogenic genes, such as *Pepck* and *Pgc1α*, were significantly increased after tofogliflozin treatment (Figure 6, J and K).

Discussion

In this study, we presented novel insights into the mechanisms underlying the antidiabetic and antiobesity effects of tofogliflozin (Figure 7). Tofogliflozin exerts a direct glucose-lowering effect by accelerating UGE, thereby reducing body weight gain as a result of the calorie loss associated with the increased UGE rate. In addition to these effects, tofogliflozin reduces the serum insulin level along with blood glucose reduction, leading to the acceleration of lipolysis in adipose tissue and the release of NEFA into circulating blood. These actions increase lipolysis, leading to a reduction in fat mass and adipocyte size. In addition, the decreased serum insulin level induces the up-regulation of hepatic gluconeogenesis and reduces hepatic lipogenesis. Moreover, NEFA derived from the adipose tissue becomes the substrate for β-oxidation in the liver, enhancing hepatic β-oxidation and ketone body synthesis. Increased β-oxidation and reduced lipogenesis in the liver result in a decrease in the hepatic TG content. Furthermore, glucose uptake in the skeletal muscle is increased. Taken together, these results suggest that tofogliflozin ameliorates insulin resistance and obesity by increasing glucose uptake in the skeletal muscle and lipolysis in the adipose tissue.

Tofogliflozin accelerated lipolysis in the WAT at least partially by lowering the serum insulin level. In fact, we showed a decrease in the phosphorylated Akt level in WAT and an increase in the phosphorylated HSL level after tofogliflozin administration. However, in our data, the blood glucose levels reached around 60 mg/dL after the administration of tofogliflozin, suggesting not only a low insulin level, but also that β-adrenergic signaling might have activated lipolysis accompanied by a reduction in the blood glucose level. Although we confirmed that the heart rates did not differ between tofogliflozin-treated mice and mice not treated with tofogliflozin (Supplemental Figure 5), the possibility that β-adrenergic signaling may contribute to the acceleration of lipolysis cannot be excluded. In addition, Izumida et al (24) recently demonstrated that liver glycogen shortage directly facilitates lipolysis in the WAT by activating a liver-brain-adipose neural axis independently of the blood glucose and insulin/glucagon levels. In our data, tofogliflozin reduced the liver glycogen content in mice fed an HFD. Thus, this neural axis might partially contribute to fat mass reduction, especially in mice fed an HFD.

The acceleration of lipolysis in the WAT could activate hepatic β-oxidation by supplying NEFA, which becomes a substrate of β-oxidation. Moreover, ketone body synthesis is accelerated, as acetyl-CoA produced by β-oxidation is not metabolized in the TCA cycle, especially when hepatic gluconeogenesis is stimulated because of a low insulin level. Ketone body synthesis may continue to increase when low blood glucose and serum insulin levels are maintained through tofogliflozin treatment. However, lipolysis in the WAT and ketone body synthesis in the liver appear to be balanced. This is probably because tofogliflozin treatment improves insulin sensitivity, presumably because of an improvement in obesity and a reduction in

Table 1. Antibody Table

Peptide/Protein Target	Antigen Sequence (if Known)	Name of Antibody	Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody	Species Raised in; Monoclonal or Polyclonal	Dilution Used
p-Akt(S473)		Anti-p-Akt (S473)	CST, 9271	Rabbit; polyclonal	1:1000
t-Akt		Anti-t-Akt	CST, 9272	Rabbit; polyclonal	1:1000
Actin		Anti-actin	Santa Cruz Biotechnology, Inc, sc-1616-R	Rabbit; polyclonal	1:1000
HSL p-S660		Anti-HSL p-S660	CST, 4126	Rabbit; polyclonal	1:1000
HSL p-S563		Anti-HSL p-S563	CST, 4139	Rabbit; polyclonal	1:1000
HSL p-S565		Anti-HSL p-S565	CST, 4137	Rabbit; polyclonal	1:1000
ATGL		Atni-ATGL	CST, 2138	Rabbit; polyclonal	1:1000

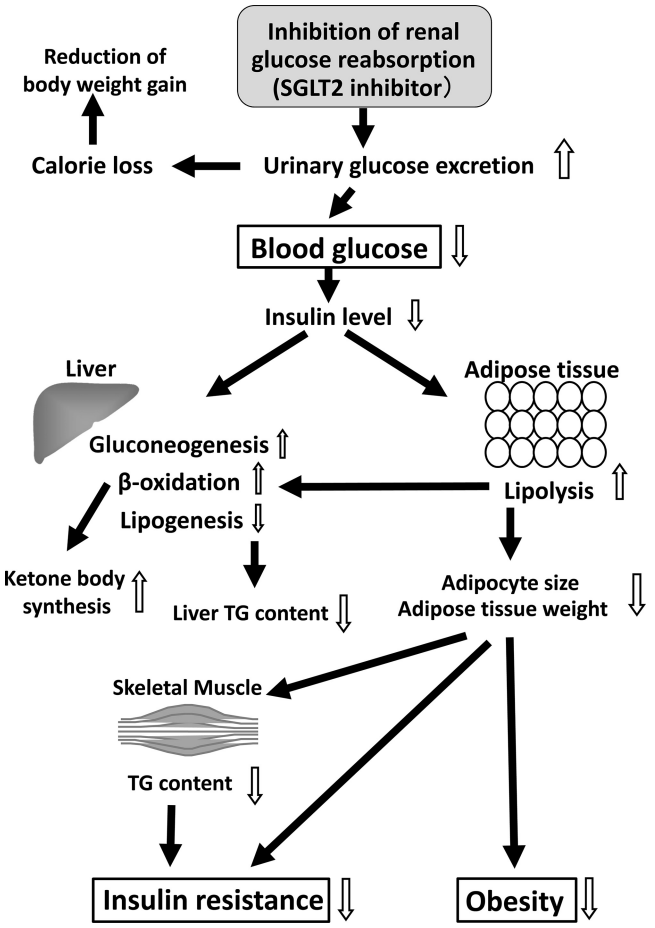

Figure 7. Mechanisms underlying the antidiabetic and antiobesity effects of tofogliflozin.

inflammation. In fact, animal and human clinical studies have revealed that the ketone body level and body weight were maintained at certain levels (25).

As previously reported, SGLT2 inhibitors improve not only glycemic control, but also obesity. It is well known that the amelioration of obesity decreases inflammation and ectopic fat accumulation, enhances insulin action in the liver and skeletal muscle, and improves insulin resistance. In fact, a hyperinsulinemic-euglycemic clamp showed that SGLT2 inhibitors improve whole body insulin sensitivity in humans (26) because of the increased glucose uptake in the skeletal muscle, which was consistent with the findings of our study (Figure 5H). In our data, the TG content in the skeletal muscle was significantly reduced in the tofogliflozin-treated mice (Supplemental Figure 8). In addition to the improvement in obesity, this reduction in the TG content in the skeletal muscle might lead to an improvement in insulin resistance in the skeletal muscle, thereby increasing glucose uptake. Recent intriguing studies, however, have demonstrated that SGLT2 inhibitors paradoxically increase endogenous HGP even when obesity and muscle insulin sensitivity are improved (26–28). This phenomenon was observed in our data as well. Tofogliflozin actually improved total body insulin resistance, although it increased the hepatic *Pepck* mRNA expression levels and actually increased HGP in the euglycemic clamp.

In our study, the hepatic *Pepck* levels were increased in all the experiments. However, the *G6Pase* levels did not increase in parallel with the *Pepck* levels. It cannot be denied that glucose might be accumulated in the hepatic cells as *G6Pase* was not increased as much as *Pepck* in the liver. In general, excessive glucose would be used for glycogen synthesis or fatty acid generation. In our experiments, hepatic glycogen levels were decreased or tended to be decreased in the liver. Moreover, hepatic TG contents were significantly decreased in all experiments. These results suggest that produced glucose was assumed to be released, not to be accumulated, from the liver. In fact, the hyperinsulinemic-euglycemic clamp directly revealed the increased HGP in the tofogliflozin-treated group. Hepatic glycogen and glycerol derived from free fatty acid and amino acids were assumed to be the major substrates for gluconeogenesis.

It is known that when the blood glucose levels are high, the mRNA expression levels of *Pepck* are decreased, whereas transcription of the *G6Pase* gene as well as the stability of its mRNA is increased in the liver (29–31). In our study, the insulin levels were decreased in the tofogiflozin-treated group, which would have increased both the *G6Pase* and *Pepck* mRNA levels. However, in the tofogliflozin-treated group, the blood glucose levels were lower than those in the control group, so that the transcription level of *G6Pase* and the stability of its mRNA might have been suppressed in tofogliflozin-treated group. We think that the discrepancy between the *G6Pase* and *Pepck* mRNA expression levels may be attributable to this difference in the transcriptional regulations of *G6Pase* and *Pepck*.

Bonner et al (32) reported that blood examination after 18 hours of starvation of C57BL/6 mice treated with dapagliflozin 5 mg/kg for 4 days revealed no significant differences in the blood glucose levels, but significantly increased plasma glucagon and insulin levels. In this model, both the hepatic *G6Pase* and *Pepck* levels were increased. In our experiment, single administration of tofogliflozin resulted in significantly lower blood glucose and serum insulin levels, and no significant change of the serum glucagon levels, leading to relatively suppressed *G6Pase* expression (data not shown) and increased *Pepck* expression. In Bonner's study, both *G6Pase* and *Pepck* expressions were increased, probably due to up-regulation of the glucagon expression levels and unaltered blood glucose levels. On the other hand, in our study, the suppressed *G6Pase* and increased *Pepck* expressions were possibly due to decreased blood glucose levels, because the glucagon levels remained unchanged. It is, of course, difficult to

rule out the possibility that the difference in the drug delivery method or the characteristics of each drug also affected the expression levels of *G6Pase* and *Pepck*. In the human study, the plasma glucagon/insulin ratio was increased, which led to increased hepatic gluconeogenesis (26). However, the serum glucagon level per se was not increased after single administration of tofogliflozin in our study. Therefore, at least, the decreased insulin level may have strongly affected the increase of gluconeogenesis in the liver rather than glucagon in our study. Further metabolic studies are needed and should provide additional clarification.

In our study, tofogliflozin treatment yielded a phenotype that was similar in many respects for mice fed NC and those fed an HFD, including the amelioration of glucose intolerance and the reduction of fat mass; however, there were also some differences, such as the lean body mass and liver glycogen content. CT examinations conducted in the pair-feeding experiment under the HFD condition revealed a reduced skeletal muscle volume without a reduction of bone volume and bone mineral density (BMD). This result suggests that the reduction in the lean body mass was mainly due to a reduction in skeletal muscle, but not bone volume. Consistent with these findings, a few human clinical reports have indicated that SGLT2 inhibition does not affect markers of bone turnover and BMD, although the effects of SGLT2 inhibitors on BMD and the risk of fracture are still unknown (33, 34). In our study, no significant difference in the lean body mass was observed between control and tofogliflozin-treated mice fed NC, whereas the lean body mass was significantly reduced in the mice fed an HFD. Compared with the mice fed NC, those fed an HFD gained more weight, suggesting that the cause of the lean body mass reduction was not a shortage of calories. However, the average daily caloric intake from carbohydrates in the tofogliflozin-treated mice was 4.03 kcal for mice fed the HFD, whereas that of mice fed NC was 10.2 kcal. This result suggests that glucose loss by accelerated UGE tends to be compensated for by the catabolism of skeletal muscle under an HFD condition, which contains a lower amount of carbohydrates compared with NC. Although some human studies have reported that SGLT2 inhibitors reduce the lean body mass as well as the fat mass, there are no reports referring to the differential effects of SGLT2 inhibitors on body composition, depending on the dietary composition (15, 16). The hepatic glycogen content was significantly decreased in the tofogliflozin-treated mice fed an HFD, but not in the mice fed NC; this difference might have arisen from the lower carbohydrate content of the HFD. These results suggest that SGLT2 inhibitors might tend to reduce the hepatic glycogen under a low-carbohydrate diet condition, which might be associated with a risk of hypoglycemia, especially in patients undergoing insulin treatment or taking agents stimulating insulin secretion, such as sulfonylurea. Taken together, these results suggest the importance of long-term clinical trials for determining whether a low-carbohydrate diet increases the risk of muscle mass reduction and hypoglycemia when SGLT2 inhibitors are used.

In this study, we demonstrated that tofogliflozin, a novel SGLT2 inhibitor, ameliorates not only diabetes, but also obesity and insulin resistance by improving insulin resistance in skeletal muscle and accelerating lipolysis in adipose tissue.

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