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Effect of sodium-glucose cotransporter 2 (SGLT2) inhibition on weight loss is partly mediated by liver-brain-adipose neurocircuitry

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ABSTRACT

Sodium-glucose cotransporter 2 (SGLT2) inhibitors have both anti-diabetic and anti-obesity effects. However, the precise mechanism of the anti-obesity effect remains unclear. We previously demonstrated that the glycogen depletion signal triggers lipolysis in adipose tissue via liver-brain-adipose neurocircuitry. In this study, therefore, we investigated whether the anti-obesity mechanism of SGLT2 inhibitor is mediated by this mechanism. Diet-induced obese mice were subjected to hepatic vagotomy (HVx) or sham operation and loaded with high fat diet containing 0.015% tofogliflozin (TOFO), a highly selective SGLT2 inhibitor, for 3 weeks. TOFO-treated mice showed a decrease in fat mass and the effect of TOFO was attenuated in HVx group. Although both HVx and sham mice showed a similar level of reduction in hepatic glycogen by TOFO treatment, HVx mice exhibited an attenuated response in protein phosphorylation by protein kinase A (PKA) in white adipose tissue compared with the sham group. As PKA pathway is known to act as an effector of the liver-brain-adipose axis and activate triglyceride lipases in adipocytes, these results indicated that SGLT2 inhibition triggered glycogen depletion signal and actuated liver-brain-adipose axis, resulting in PKA activation in adipocytes. Taken together, it was concluded that the effect of SGLT2 inhibition on weight loss is in part mediated via the liver-brain-adipose neurocircuitry.

1. Introduction

Type 2 diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia due to insulin resistance and relative insulin deficiency [1]. The prevalence of type 2 diabetes has been increasing dramatically worldwide in recent years, and more than 380 million people worldwide have diabetes mellitus [2]. Thus, diabetes is now a major threat to global public health.

Recently, sodium-glucose cotransporter 2 (SGLT2) inhibitors have attracted much attention as they exert both anti-diabetic and anti-obesity effects [3,4]. SGLT2 is a glucose transporter that was cloned in 1994 by Kanai et al. [5], 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. Recent clinical studies have indicated that oral administration of SGLT2 inhibitors induces urinary glucose excretion (UGE), improves hyperglycemia and reduces body weight of patients with type 2 diabetes [6–12]. In animal studies, SGLT2 inhibitors also improve glucose and lipid metabolism in diabetic animal model

Abbreviations: HVx, hepatic vagotomy; SGLT2, sodium-glucose cotransporter 2; TOFO, tofogliflozin; PKA, protein kinase A.

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[13–15]. However, mechanisms of anti-obesity effect of SGLT2 inhibitors were not fully understood.

Recently, a new paradigm of inter-organ networks via the autonomic nervous system has emerged as a potential regulatory mechanism of metabolic homeostasis [16,17]. In fact, a number of studies are being conducted on the roles of the neural signal from the liver [18–21]. Especially, we previously documented that liver glycogen shortage directly facilitates lipolysis in white adipose tissue by activating a liver-brain-adipose neurocircuitry independently of the blood glucose and insulin/glucagon levels, demonstrating the presence of "glycogen depletion signal" [20]. Because liver glycogen reduction is reported with tofogliflozin (TOFO) and dapagliflozin [22,23], it is possible that SGLT2 inhibition can activate this neural circuit by depleting hepatic glycogen.

These situations prompted us to hypothesize that SGLT2 inhibition triggers the glycogen depletion signal in the liver and activates lipolysis in adipocytes through a liver-brain-adipose neurocircuitry, leading to fat mass reduction and body weight loss. Therefore, we investigated the effect of SGLT2 inhibitor on the neural pathway using hepatic vagotomy (HVx) mice model in the current study.

2. Materials and methods

2.1. Materials

Tofogliflozin (TOFO) was provided by KOWA Co., Ltd. Anti-PKA substrate and anti-α-tubulin antibodies were purchased from Cell Signaling Technology. Mouse Insulin ELISA KIT was purchased from Shibayagi Co., Ltd. Ketone test B liquid was purchased from Sanwa Co., Ltd. Glucose CII test Wako, Triglycerides E-test Wako and Cholesterol E-test Wako were purchased from Wako chemicals.

2.2. Animals

C57BL/6 J mice (6-week-old) were purchased from CLEA. All animals were housed in a temperature-controlled environment with 14 h light/10 h dark cycle and given free access to high fat diet (HFD32 Oriental Yeast, 507.6 kcal/100 g) and water. 2 weeks after HFD feeding, mice were subjected to sham operation (Sham) or to selective hepatic vagotomy (HVx) as described below. Following 2 weeks of recovery period, 0.015% TOFO was administered with HFD for 3 weeks. For tissue sampling, mice were sacrificed after 8 h fasting and samples were strored at −80 °C. All animals studied were anesthetized and euthanized according to a protocol approved by the Tsukuba University Animal Care and Use Committee.

2.3. Selective hepatic vagotomy (HVx)

8-week-old male mice were subjected to dissection of hepatic branch of the vagus [20]. A laparotomy incision was made on the ventral midline and the abdominal muscle wall was opened with a second incision. The gastrohepatic ligament was severed using fine forceps, and the stomach was gently retracted, revealing the descending ventral oesophagus and the ventral subdiaphragmatic vagal trunk. The hepatic branch of this vagal trunk was then transected using fine forceps.

2.4. DEXA analysis

PIXImus2 DEXA (GE Medical Systems) was used to measure weight and composition of lean mass and fat mass.

2.5. mRNA quantification

mRNA measurement was performed as described previously [24,25]. Total RNA from tissues was prepared using Sepasol-RNA I (Nacalai Tesque) and was used for cDNA synthesis (Invitrogen). Real-time PCR was performed using the ABI Prism 7300 System (Applied Biosystems) with SYBR Green master mix (Roche Diagnostics).

The primer sequences are as follows:

Pck1 5′-TGTCATCCGCAAGCTGAAGA-3′ 5′-TTCGATCCTGGCCACATCTC-3′

G6pc 5′-CGGCGCAGCAGGTGTATACTAT-3′ 5′-CAGAATCCCAACCACAAGATGA-3′

Cpt1a 5′—CCTGGGCATGATTGCAAAG -3′ 5′-GGACGCCACTCACGATGTT -3′

Pparα 5′—CCTCAGGGTACCACTACGGAGT -3′ 5′-GCCGAATGTTCGCCGAA -3′

Fgf21 5′—CCTCTAGGTTTCTTTGCCAACAG -3′ 5′-AAGCTGCAGGCCTCAGGAT -3′

Rplp0 5′-GAAGACAGGGCGACCTGGAA -3′ 5′-TTGTCTGCTCCCACAATGAAGC -3′

Data were analyzed using the comparative cycle threshold method, and mRNA expression was normalized to *Rplp0* expression.

2.6. Immunoblot analysis

Immunoblotting was performed as described previously [26,27]. Briefly, tissue extracts were fractionated by SDS—PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skim milk in TBS-T (10 mM Tris at pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 30 min, the membrane was incubated with anti-PKA substrate and anti-α-tubulin antibodies at 4 °C for 12 h. Membranes were washed three times for 10 min and incubated with rabbit or mouse second antibody at 1:5000 dilution for 1 h. Signals were detected using the ECL western blotting Detection System (GE Healthcare) and exposed to ChemiDocTM XRS+ (Bio-Rad) and IMAGE LAB TM Software (BioRad).

2.7. Measurement of liver glycogen content

Liver glycogen content was measured as described previously [20]. Liver were dissolved in 30% KOH and 66% EtOH was added, then was placed at −20 °C for 1 h. Then liver were centrifuged at 8000 rpm and a supernatant was discarded. Pellet was dissolved with dH_2O, added acetate buffer and amyloglucosidase, and measured with Glucose CII test Wako (Wako).

2.8. Statistical analyses

The results were expressed as the mean ± standard error of the mean (SEM). Statistical differences between groups were analyzed by Mann-Whitney *U* test analysis. $P < 0.05$ was taken as statistical significance.

3. Results

3.1. HVx attenuated weight loss and fat reduction by TOFO

Diet-induced obese mice were subjected to selective HVx and loaded with high fat diet containing 0.015% TOFO for 3 weeks. As shown in Fig. 1A and B, TOFO suppressed body weight and fat mass gain. As expected, the effect of TOFO was significantly attenuated by HVx (Fig. 1A and B). Meanwhile, no change was observed in lean mass among groups (Fig. 1C), indicating that the body weight

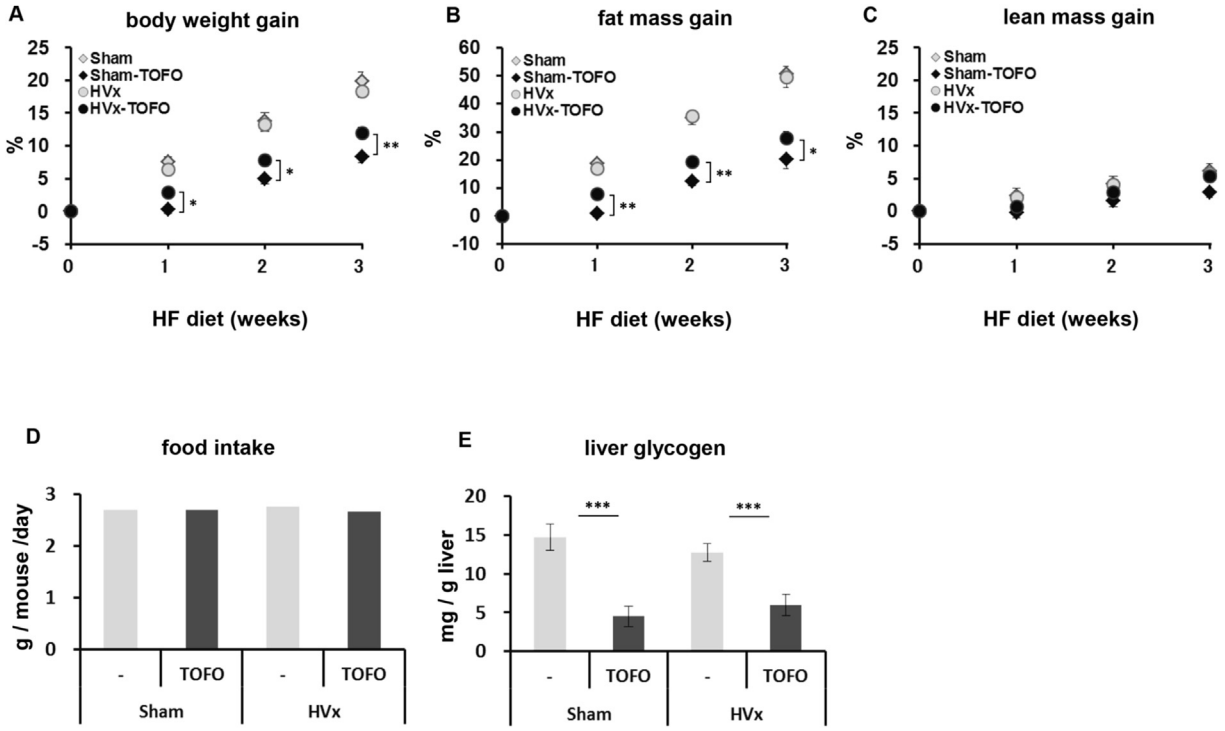

Fig. 1. HVx attenuated weight loss and fat reduction by TOFO despite similar levels of glycogen depletion. Mice were anesthetized and body weight (**A**), fat mass (**B**) and lean mass (**C**) were measured by DEXA after 3-week treatment. (**D**) Food intake. (**E**) Liver glycogen level. Data are shown as mean ± SE (n = 10–12). *P < 0.05 and **P < 0.01 by Mann-Whitney *U* test.

alternation might be mainly due to a decrease in fat mass.

As shown in Fig. 1D, food intake was unchanged among groups, suggesting that the difference of fat mass gain between HVx and sham groups with TOFO might not be caused by food consumption.

3.2. TOFO similarly reduced liver glycogen both in HVx and sham mice

Based on our previous study demonstrating that lipolysis via neural axis was triggered by liver glycogen depletion [20], we measured liver glycogen contents in diet-induced obese mice with or without TOFO/HVx. As shown in Fig. 1E, TOFO significantly reduced liver glycogen contents both in HVx and sham groups to a similar level. Despite similar levels of glycogen depletion, fat mass in HVx mice showed a diminished response to TOFO due to the blocking of neural signals from the liver.

3.3. Blood parameters were comparable between HVx and sham

Next, the effects on blood parameters were examined. Blood glucose and insulin levels were significantly reduced by TOFO, but there were no differences between HVx and sham groups (Fig. 2A and B). Ketone body was increased by TOFO, suggesting that fatty acid oxidation might be increased and energy source might be changed from sugar to fat (Fig. 2C). There were no differences in triglyceride (TG) and total cholesterol (TC) among groups (Fig. 2D and E). These results suggest that blood parameters might not be affected by HVx operation.

3.4. No differences in hepatic gene expression between HVx and sham

The effects of TOFO and/or HVx on hepatic gene expression were analyzed to investigate the influence of TOFO and/or HVx. Gluconeogenic genes such as *Pck1* and *G6pc* were slightly increased by TOFO, but no differences were observed between HVx and sham groups (Fig. 3A and B). Genes involved in lipid utilization including *Cpt1a*, *Pparα* and *Fgf21* also showed no differences between HVx and sham groups (Fig. 3C–E). These results indicate that hepatic gene expressions were not affected by HVx treatment.

3.5. HVx attenuated the increase in PKA-dependent phosphorylation by TOFO

We previously demonstrated that a decrease in liver glycogen facilitates lipolysis in white adipose tissue via a liver-brain-adipose neurocircuitry and that the phosphorylation of hormone sensitive lipase involved in lipolysis was attenuated by HVx [20]. Because it is well known that hormone sensitive lipase is phosphorylated via protein kinase A (PKA) pathway [28], we confirmed whether PKA pathway in adipocytes is involved in the TOFO-induced liver-brain-adipose axis activation. TOFO increased the phosphorylation of PKA-substrate protein at around 70 kDa in white adipose tissue, and the effect of TOFO was attenuated by HVx as expected (Fig. 4A). The same phosphorylated protein as shown in Fig. 4A was increased under a fasting condition (Fig. 4B). This result is consistent with the notion that the effect of TOFO on fat reduction is mediated via the liver-brain-adipose neurocircuitry.

4. Discussion

In the present study, we clearly demonstrated that SGLT2 inhibition triggered glycogen depletion signal in the liver and activated a liver-brain-adipose neurocircuitry, resulting in PKA activation in adipocytes and thereby fat reduction and weight loss.

The presence of liver-brain-adipose neurocircuitry was first reported by Katagiri et al., in 2006 [18]. They found a regulatory mechanism by which the hepatic branch of the vagus nerve sends

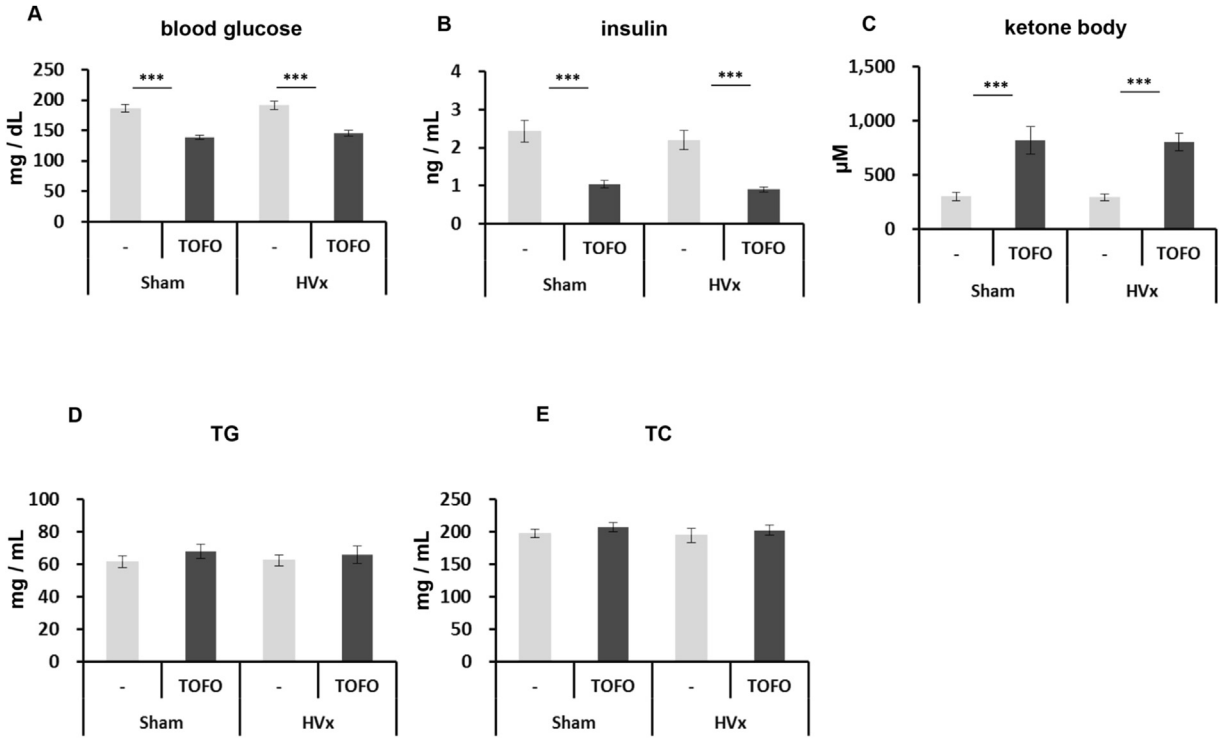

Fig. 2. Blood parameters were comparable between HVx and sham. Blood glucose (**A**) was measured in ad lib condition. Iinsulin (**B**), ketone body (**C**), TG (**D**) and TC (**E**) were measured in 8 h fasting condition. Data are shown as mean ± SE (n = 10–12). ***P < 0.001 by Mann-Whitney *U* test. TG, triglycerides; TC, total cholesterol.

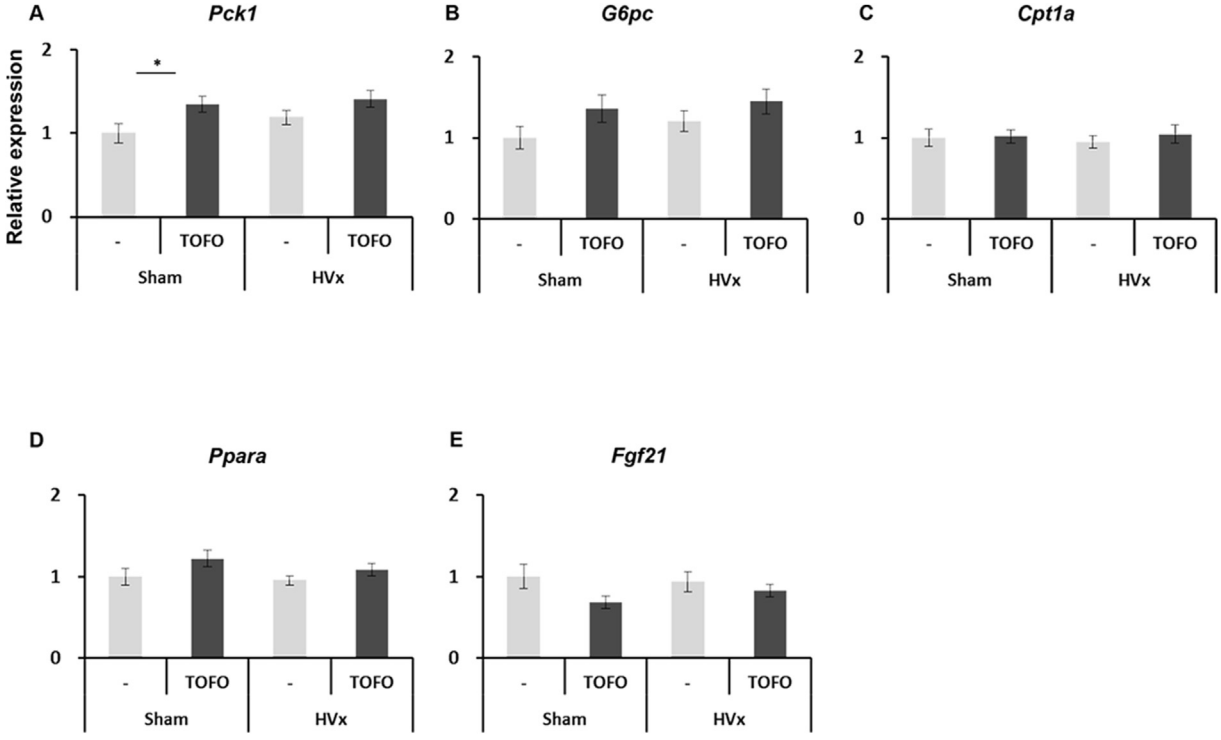

Fig. 3. Hepatic gene expression in HVx and sham mice. mRNA expressions of *Pck1* (**A**) and *G6pc* (**B**), related to gluconeogenesis, and *Cpt1a* (**C**), *Ppara* (**D**) and *Fgf21* (**E**), related to lipid utilization, are shown as mean ± SE (n = 10–12). *P < 0.05 by Mann-Whitney *U* test.

signals to break down fats when there is an excess fat accumulation in the liver; after passing through the liver-brain-adipose-neural axis, these signals finally activate the sympathetic nervous system to enhance lipolysis in white adipose tissue. This important discovery proved that with hyperalimentation, afferent nerve signals (vagal nerve signals) accelerate lipolysis and energy consumption,

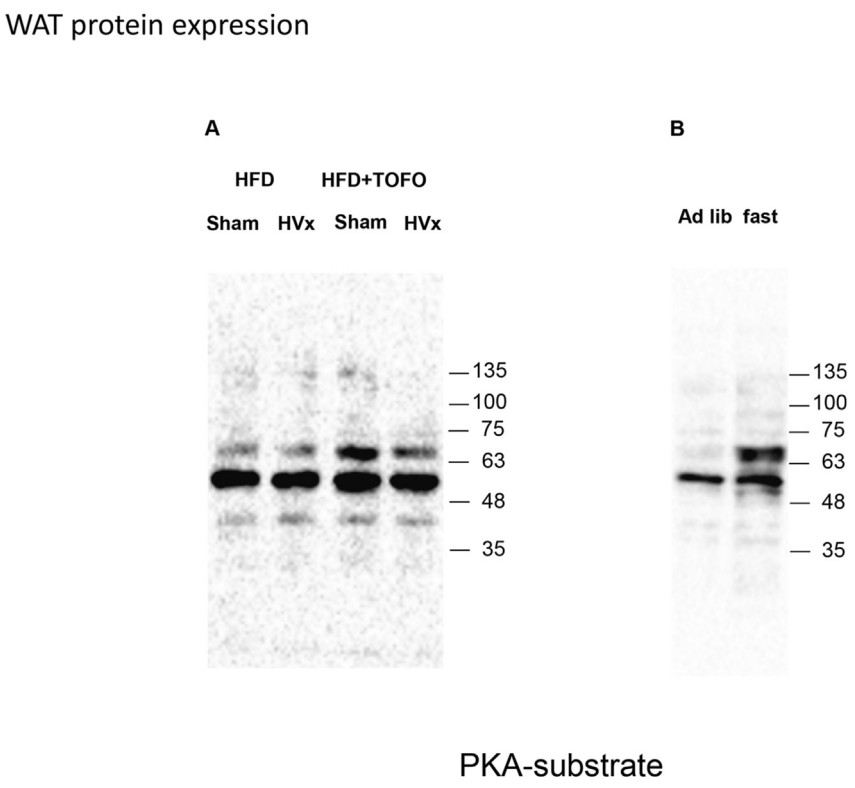

Fig. 4. HVx attenuated the increase in PKA-dependent phosphorylation by TOFO. White adipose tissue lysates from mice with HVx or sham operation with or without tofogliflozin (**A**) and overnight fasting (**B**) were immunoblotted with anti-PKA substrate antibody. HFD, high fat diet.

and contribute to the maintenance of body weight homeostasis. After the discovery of the liver-brain-adipose-neural axis, we sought to learn more about the related physiological functions of this pathway, and found that this neural axis plays an important role in fat mobilization during fasting [17,20]. As an application of this research, here we demonstrated that glycogen depletion signal caused by SGLT2 inhibition activate this neural pathway like in a fasting condition. Thus, it was further established that this neuronal network plays an important role in body weight homeostasis.

It is reported that administration of SGLT2 inhibitor increases food intake in some cases [22,29,30]. However, in the present study where the experiments were performed on a high fat diet, the food intake was not increased by TOFO administration, suggesting that increased food intake induced by SGLT2 inhibition may be limited to high carbohydrate diets. Although the mechanism of increases in food intake by SGLT2 inhibitors is generally understood as a compensation for increased urinary glucose excretion, precise mechanism is unknown, and the above finding is intriguing. Because the problem of increased food intake, hyperphagia, by SGLT2 inhibitors is also important from a clinical standpoint, further investigations are needed.

In the present study, the effect of TOFO on fat reduction was not completely canceled by HVx. One possible explanation is a contribution of insulin action. A decrease in insulin is known to activate cAMP-PKA pathway, leading to the induction of lipolysis [31]. Given that insulin was decreased by TOFO in this study, and that SGLT2 inhibitors other than TOFO generally decrease plasma insulin level [29,30,32], it was suggested that at least a part of fat reduction by TOFO might be attributable to decreased insulin levels, in addition to the neural pathway. As other candidate hormones that might be involved in the mechanisms of fat reduction by TOFO, FGF21 can be mentioned, because FGF21 can regulate lipolysis in adipocytes [33]. In the present study, *Fgf21* gene expression in the liver, the main source of FGF21 circulating in the blood [34], was not significantly changed by TOFO, denying the possible contribution of FGF21 to the fat reduction by TOFO.

In conclusion, it was revealed that the anti-obesity effect of SGLT2 inhibitor is partly mediated by liver-brain-adipose neurocircuitry.

Author contributions

N.Y. conceived the experiments. Y.S. and Y.I. performed the experiments and analyzed the data together with N.Y. Y.S. and N.Y. cowrote the paper. All authors discussed the results and commented on the manuscript.

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